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Acid-Resistant Bovine Pestivirus Requires Activation for pH-Triggered Fusion during Entry

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The route of internalization of the pestivirus bovine viral diarrhea virus (BVDV) was studied by using different chemical and biophysical inhibitors of endocytosis. Expression of the dominant-negative mutant Dyn^{K44A} of the GTPase dynamin in MDBK cells, as well as the treatment of the cells with chlorpromazine and β-methyl-cyclodextrin inhibited BVDV entry. BVDV infection was also abolished by potassium (K^+) depletion, hyperosmolarity, and different inhibitors of endosomal acidification. We conclude that BVDV likely enters the cell by clathrin-dependent endocytosis and that acidification initiates fusion with the endosomal membrane. Further studies revealed that BVDV was unable to undergo "fusion from without" at low pH. The finding that low pH is not sufficient to force adsorbed BVDV into fusion with the plasma membrane is compatible with the remarkable resistance of pestiviruses to inactivation by low pH. The importance of the abundant intra- and intermolecular disulfide bonds in BVDV glycoproteins for virus stability was studied by the use of reducing agents. The combination of dithiothreitol and acidic pH led to partial inactivation of BVDV and allowed fusion from without at low efficiency. Evidence is provided here that acid-resistant BVDV is destabilized during endocytosis to become fusogenic at an endosomal acidic pH. We suggest that destabilization of the virion occurs by breakage of disulfide bonds in the glycoproteins by an unknown mechanism.

The genus *Pestivirus* consists of four species that are important pathogens in farm animals: classical swine fever virus (CSFV), border disease virus, and bovine viral diarrhea virus type 1 (BVDV-1) and BVDV-2. Pestiviruses are small (40 to 60 nm) enveloped RNA viruses, which, together with the genera *Flavivirus* and *Hepacivirus*, constitute the family *Flaviviridae*. The virion consists of a message sense single-stranded RNA of at least ca. 12,300 nucleotides and four structural proteins, namely, the capsid protein and the three glycoproteins E^{rns}, E1, and E2. The glycoproteins are present in the envelope as disulfide-linked homo- and heterodimers (15, 39, 65, 68). The host range of pestiviruses is restricted to cloven-hoofed animals (*Artiodactyla*, e.g., ruminants and pigs), and susceptible cell culture systems mainly comprise cells from this order. Recently, we reported that bovine CD46 acts as a cellular receptor for BVDV (45). Binding of virions to this molecule has been clearly demonstrated, and also an increase in susceptibility to BVDV is correlated with the expression of $CD46_{bov}$ in porcine PK15 cells. Heparan sulfate has been postulated as a receptor for tissue culture-adapted BVDV and CSFV (33, 34). Although no further details concerning the route of pestivirus entry have been reported, it is believed that pestiviruses invade host cells by receptor-mediated endocytosis (40).

Enveloped viruses use two general mechanisms of penetration: receptor-mediated endocytosis and fusion at the cell surface (36, 43). Different pathways of receptor-mediated endocytosis have been characterized in the last decade. Most frequently, clathrin-dependent endocytosis is used by viruses, as reported for enveloped viruses, e.g., Sindbis virus (SinV) (14), vesicular stomatitis virus (64), Hantaan virus (35), and nonnenveloped viruses, e.g., JC virus (52), canine parvovirus (49), and foot-and-mouth disease virus (47). An alternative endocytic pathway is the internalization via caveolae, which is used by several viruses, e.g., simian virus 40 and respiratory syncytial virus (16, 50). An important difference between these pathways concerns the fate of the internalized organelles. In clathrin-coated vesicles, vacuolar H^+-ATPases induce luminal acidification during internalization, whereby the luminal pH has dropped to 5.6 in the late endosome (46). In contrast, caveolae are supposed to predominantly cycle between the plasma membrane and secretory organelles and fuse with caveosomes which remain at neutral pH (50).

During entry of enveloped viruses, fusion of the viral envelope with cellular membranes occurs either in the endosome or at the cell surface. The cell surface fusion, which is used by viruses such as herpes simplex virus and human immunodeficiency virus (HIV) (38, 59), is mediated by viral fusion proteins and leads to a close proximity of the virus to the plasma membrane after receptor binding at neutral pH. In contrast, enveloped viruses that invade the host cell via receptor-mediated endocytosis, i.e., members of the genus *Flavivirus* (53) and the alphavirus Semliki Forest virus (31), require an acidic pH for fusion. The endosomal acidification triggers membrane fusion by inducing irreversible conformational changes in the viral fusion protein. Fusion proteins can be divided into at least two different classes (29). Class I comprises the fusion proteins of orthomyxo-, retro-, paramyxo-, and filoviruses that are primarily activated by a proteolytic cleavage during virus assembly. They occur as trimers, harbor an N-terminal fusion peptide (12, 44, 69), and form a characteristic α-helical coiled-coil core structure after fusion (58, 69). Class II fusion proteins are found in alpha- and flaviviruses and are activated by a proteo-

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lytic cleavage during virion release, which leads to the detachment of an accessory protein. Flavivirus E protein is activated by cleavage of the glycoprotein prM, which covers the fusion peptide in immature virus particles (30). For alphaviruses the furin-like cleavage of the p62 glycoprotein precursor during late secretory pathway was shown to induce the formation of activated virions (41).

During endocytosis the low-pH-triggered conformational changes lead to insertion of the fusion peptide into the target membrane (7), upon which class II fusion proteins trimerize (2, 37, 61). Because the postfusion state of viral fusion proteins is an irreversible energetically favorable state and thus considered "stable" the proteins in the prefusion conformation are locked in a higher energetic state and are thus "metastable" (8, 23, 60). Although for some class I fusion proteins transition to the postfusion state could be induced by elevated temperatures and other protein-destabilizing conditions as well (i.e., as seen with influenza virus and Sendai virus) (8, 70), this was not possible for class II fusion proteins (23, 60). The dimer-to-trimer transition of TBEV E protein resulting in the stable postfusion confirmation was reported to be a two-step process in which only the dissociation of the dimer depends on acidification by the required protonation of the E protein (60).

The conformational changes due to acidic pH lead to exposition of the fusion peptide (7, 24, 48) and consequent structural changes supply the energy required for membrane fusion. With the exception of rhabdovirus glycoprotein G, whose pH-induced conformational change is reversible (22), pH-induced conformational changes result in the loss of receptor binding. Thus, most enveloped viruses, which use endocytosis, are highly sensitive to exposure to low pH. Interestingly, a remarkable resistance to acidic environments as they occur during the maturation of meat products has been reported for pestiviruses (13).

We describe here the determination of the entry pathway of a bovine pestivirus by systematic inhibitor studies, and we propose an unusual mechanism that triggers fusogenicity of BVDV.

MATERIALS AND METHODS

Cells, viruses, and antibodies. Madin-Darby bovine kidney (MDBK) cells (ATCC no. CCL-22) were grown in Dulbecco modified Eagle medium (DMEM) plus nonessential amino acids containing 10% fetal calf serum at 37°C in 5% CO_2. BVDV strain NADL (ATCC no. VR-534) and bovine herpesvirus 1 "M" (BHV-1; W. Eichhorn, Munich, Germany) were propagated on MDBK cells and stored at −70°C. SinV and a polyclonal α-SinV serum were kindly provided by G. Wengler, Giessen, Germany. Anti-hemagglutinin-conjugated monoclonal antibody (α-HA-MAb; clone 12CA5; Boehringer) was used at a 1:2,500 dilution of a 0.4-mg/ml stock solution. Hybridoma cell lines producing MAbs D5 and 121/3/3 (α-BHV-1; E. Weiland) were grown in DMEM plus nonessential amino acids plus 15% fetal calf serum.

Immunohistochemistry. MAbs D5 (α-BVDV-E2) or 121/3/3 (α-BHV-1) were used in a 1:5 dilution, and polyclonal α-SinV serum was used at a 1:300 dilution. Peroxidase-conjugated anti-mouse immunoglobulin G (IgG; Dianova) was used at a 1:10,000 dilution. Cells were washed once with phosphate-buffered saline (PBS) and fixed with 1 ml of methanol-acetone (1:1) for 20 min at −20°C. Antigen was detected with 200 μl of antibody for 1 h at room temperature and washed three times with PBS containing 0.1% Tween 20 for 5 min each time; this was followed by incubation with 200 μl of peroxidase-conjugated anti-mouse IgG for 1 h at room temperature. After being washed, cells were exposed to 3-amino-9-ethylcarbazole reagent.

Construction of inducible MDBK cells expressing Dyn^{wt} or Dyn^{K44A}. Dynamin wild-type (Dyn^{wt}) cDNA was cloned by reverse transcription-PCR from a Dyn^{wt}-expressing cell line (11) by using oligonucleotides BVTK21 (5′-AAGAATTCATGGGCAACCGCGGCATGGAAG) and BVTK22 (5′-TTTCTAGAGTTTAGAGGTCGAAGGGGGGCCTG). The K44A mutation and an N-terminal HA tag were introduced by QuikChange mutagenesis and blunt-end ligation into the cloned gene. Both cDNAs were ligated into the pTRE plasmid (Clontech) and cotransfected with pEF-Pac (54) into MDBK Tet on cells, which stably express the rtTa (for reverse Tet-responsive transcriptional activator) (25). MDBK Tet on cell lines stably transfected with Dyn^{wt} or Dyn^{K44A} were selected by using G418 and puromycin; single cell clones were picked and expanded, and the expression of either form of dynamin was monitored by immunoblotting.

Immunoblotting of MDBK cells expressing dynamin. Crude cell lysates from equal numbers of cells grown in the presence or absence of 10 mg of doxycycline/ml for 24 h were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis under nonreducing conditions, transferred on a nitrocellulose membrane, and blocked with 5% nonfat dried milk. After incubation with α-HA-MAb 12CA5 and peroxidase-conjugated anti-mouse IgG, signals were revealed by chemiluminescence.

Plaquing efficiency on dynamin-expressing MDBK cells. A total of 5×10^5 MDBK cells or MDBK cells expressing either form of dynamin were grown in the absence or presence of 10 μg of doxycycline/ml for 24 h. A total of 2×10^5 PFU of BVDV strain NADL or SinV was adsorbed for 1 h at 4°C and washed with DMEM, followed by incubation at 37°C. The numbers of infected cells were determined by immunohistochemical detection with an MAb to viral E2 protein (D5) or to BHV-1 (121/3/3), respectively, or a polyclonal α-SinV serum at 12 to 16 h postinfection (p.i.). The number of infected cells on MDBK cells was set to 100%, and susceptibility was then calculated as the percentage of control cells.

Inhibition of BVDV infection. (i) Endocytosis and endosomal acidification. A total of 5×10^5 MDBK cells were inoculated with 2×10^5 PFU of BVDV strain NADL for 1 h at 4°C and then washed, and DMEM with different concentrations of chlorpromazine (Sigma), β-methyl-cyclodextrin (β-MCD; Sigma), bafilomycin A1 (Alexis), chloroquine (Fluka), or ammonium chloride (Fluka) was added.

(ii) Hyperosmolarity. A total of 5×10^5 MDBK cells were inoculated with 2×10^5 PFU of BVDV strain NADL for 90 min at 37°C in the presence or absence of 0.45 M sucrose, followed by the addition of DMEM containing 0.2 μM bafilomycin A1.

(iii) Potassium depletion. A total of 5×10^5 MDBK cells were washed with hypotonic medium (H_2O-DMEM [1:1]), which was replaced after 5 min by K^+-free buffer or K^+ buffer (control), respectively. K^+-free buffer contained 140 mM NaCl, 20 mM HEPES-NaOH (pH 7.4), 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mg of glucose/ml, and 0.5% bovine serum albumin; the control buffer also contained 10 mM KCl and 130 mM NaCl instead of 140 mM NaCl. Cells were inoculated with 2×10^5 of PFU BVDV strain NADL in the respective buffer for 50 min at 37°C, followed by the addition of DMEM containing 0.2 μM bafilomycin A1.

After 12 to 16 h, the numbers of infected cells were determined by immunohistochemical detection with an MAb to viral E2 protein (D5). The number of infected cells without inhibitor was set to 100%, and the susceptibility was calculated as a percentage of the control value.

pH stability of BVDV. A total of 2×10^3 PFU of BVDV strain NADL were incubated for 15 min in a citrate-phosphate buffer (McIlvaine buffers were prepared with 0.1 M citric acid and 0.2 M dibasic sodium phosphate as described previously [62]) of the indicated pH in the presence or absence of 10 mM dithiothreitol (DTT). MDBK cells were infected and, 12 to 16 h p.i., the numbers of infected cells were determined by immunohistochemistry. The number of infected cells at pH 7.0 without DTT was set to 100%, and infectivity was calculated as a percentage of the control value.

"Fusion from without" of SinV and BVDV strain NADL on MDBK cells. A total of 5×10^5 MDBK cells were inoculated with 2×10^2 PFU of SinV or BVDV strain NADL for 1 h at 4°C. Cells were washed with prewarmed citrate-phosphate buffers at the indicated pH for 2 min at 37°C, followed by replacement of the buffer with DMEM containing 0.2 μM bafilomycin A1. Cells were fixed 12 to 16 h p.i., and the numbers of infected cells were determined by immunohistochemistry. The number of infected cells at pH 7.0 without bafilomycin A1 was set to 100%, and the fusion activity was calculated as a percentage of the control value.

Fusion from without of BVDV strain NADL in the presence or absence of DTT. A total of 5×10^5 MDBK cells were inoculated with 2×10^2 PFU of BVDV strain NADL for 1 h at 4°C and washed with prewarmed citrate-phosphate buffer at the indicated pH in the presence or absence of 10 mM DTT for 2 min at 37°C. Afterward, the buffer was replaced by DMEM containing 0.2 μM bafilomycin A1. Cells were fixed 12 to 16 h p.i., and the numbers of infected cells were determined by immunohistochemistry. The number of infected cells at pH 7.0 without DTT and bafilomycin A1 was set to 100%, and the fusion activity was calculated as a percentage of the control value.

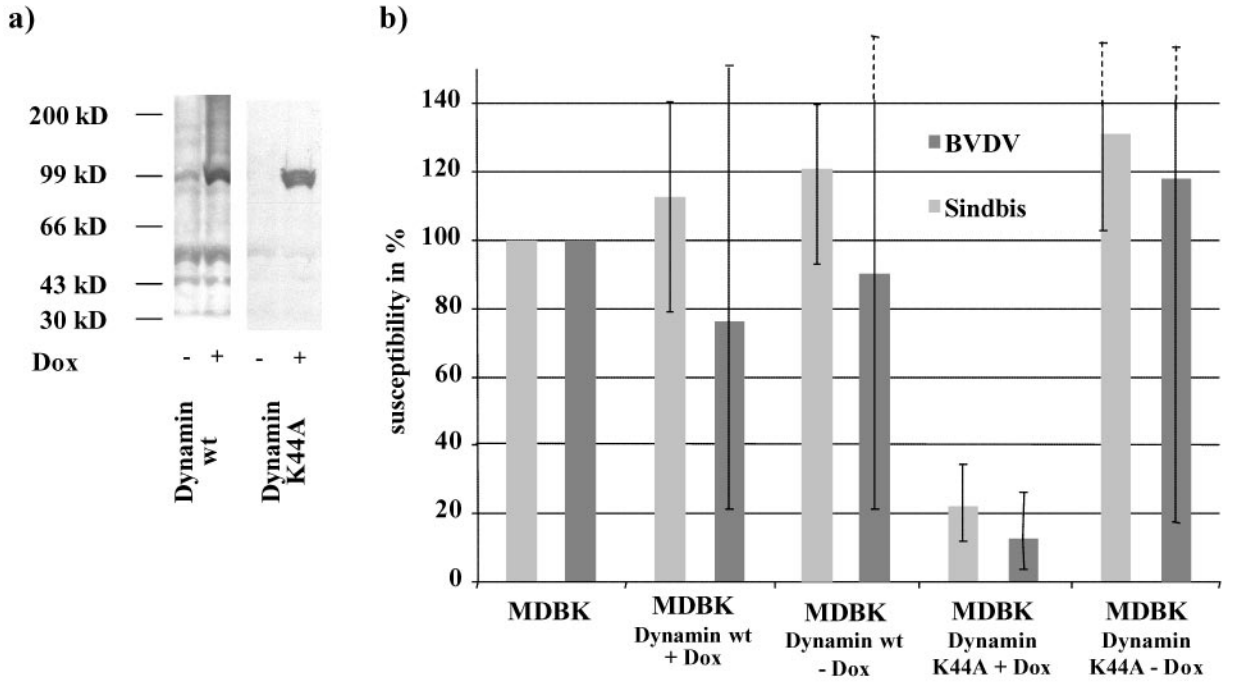

FIG. 1. Expression of Dyn^{K44A} reduces susceptibility to BVDV infection. (a) Immunoblot of MDBK Tet on dynamin-overexpressing cell lines. Crude cell lysates from equal numbers of cells grown in the presence or absence of 10 μg of doxycycline/ml were separated. After induction, a 99-kDa band of each HA-tagged protein is visible. (b) Inhibition of BVDV NADL/SinV infection by overexpression of dominant-negative Dyn^{K44A}. Each indicated cell line was tested for its susceptibility to BVDV or SinV infection by inoculation with 2×10^5 PFU of BVDV strain NADL or SinV, respectively. MDBK cells overexpressing mutant dynamin after induction with doxycycline exhibited a 10-fold- reduced susceptibility compared to MDBK cells. The columns represent mean values of triplicate experiments; bars indicate maximum and minimum values.

RESULTS

Entry of BVDV involves dynamin. Different methods were applied to determine the route of entry used by BVDV strain NADL after adsorption to its cellular receptor $CD46_{bov.}$ First, we attempted to differentiate between cell surface fusion and endocytosis. Cell surface fusion is difficult to inhibit, but there are a number of experimental procedures that specifically block endocytosis. A pivotal role in endocytosis is played by dynamin, a cell membrane-associated GTPase, which mediates the scission event of different vesicles from the plasma membrane (5). To analyze the effect of a dominant-negative dynamin mutant (Dyn^{K44A} [11]) on BVDV invasion, tetracycline-inducible MDBK cell lines were generated that express Dyn^{K44A} or Dyn^{wt}. Both genes were modified during cloning by addition of an HA tag (MEYDVPDYAH) to facilitate serological detection. Western blot analysis with an α-HA-MAb confirmed that each MDBK cell line inducibly expressed dynamin (Fig. 1a).

The effect of the expression of Dyn^{wt} and Dyn^{K44A} was analyzed by determining of the plaquing efficiency of BVDV strain NADL. Infected cells were revealed by immunohistochemical detection of BDVD E2 at 16 h p.i. before significant spread of virus occurred. The plaquing efficiency of BVDV on uninduced MDBK Dyn^{K44A} cells (−Dox) was equal to parental MDBK or MDBK Dyn^{wt} cells, but a 10-fold reduction was observed on MDBK Dyn^{K44A} cells when doxycycline was present (Fig. 1b).

Evaluation of Dyn^{wt}- and Dyn^{K44A}-expressing cell lines was performed by determination of the plaquing efficiency of SinV, which frequently served as model for viral uptake via endocytosis. Susceptibility of MDBK Dyn^{K44A} cells (+Dox) to SinV was reduced by 84% compared to uninduced cells (Fig. 1b). BHV-1 infection, which occurs by cell surface fusion, was unaffected by Dyn^{wt} and Dyn^{K44A} expression (data not shown).

BVDV entry is blocked by inhibitors of clathrin-dependent endocytosis. The effect of Dyn^{K44A} expression on BVDV infection suggested that endocytosis is involved in BVDV entry. This was further studied by using chemical and biophysical inhibitors of endosome formation. Chlorpromazine has been shown to disturb the assembly of clathrin lattices at the plasma membrane (67). BVDV strain NADL was adsorbed to MDBK cells for 1 h at 4°C, followed by the addition of different concentrations of chlorpromazine. Cells were fixed at 12 h p.i., and infected cells were revealed by immunohistochemistry. The presence of 50 μg of chlorpromazine/ml resulted in a complete inhibition of the BVDV infection, but 10 μg of chlorpromazine/ml reduced the susceptibility of MDBK cells by a factor of 400 (Fig. 2). β-MCD has recently been shown to affect clathrin-mediated endocytosis by perturbing the formation of clathrin-coated vesicles at the plasma membrane (55). MDBK cells were inoculated with BVDV strain NADL for 1 h at 4°C, followed by the addition of different concentrations of β-MCD. At a concentration of 0.5 mM β-MCD the plaquing efficiency was reduced eightfold, and at 5 mM β-MCD BVDV entry was almost completely blocked (>1,000-fold) (Fig. 2). In contrast, 5 mM β-MCD decreased susceptibility of MDBK cells to

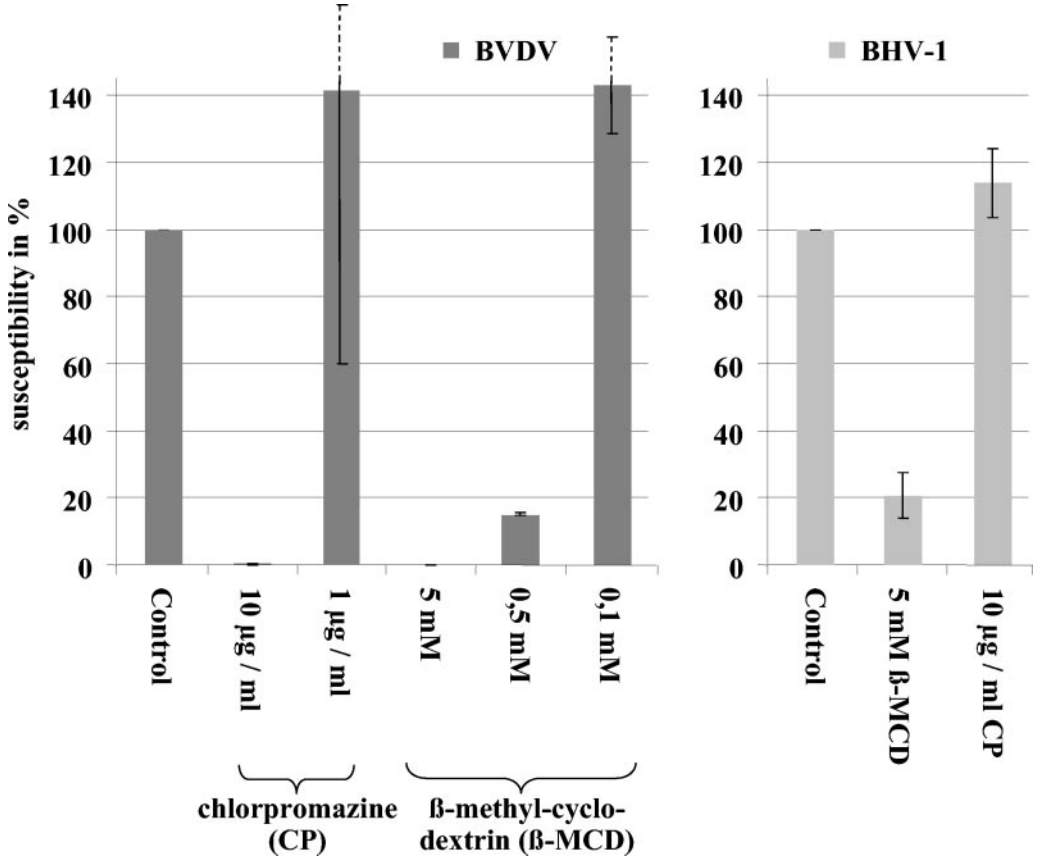

FIG. 2. Effect of chlorpromazine and β-MCD on BVDV and BHV-1 infection. BVDV NADL (dark gray bars) or BHV-1 (light gray bars) was adsorbed to MDBK cells, and the effects of chlorpromazine and β-MCD on infection were investigated. Susceptibility to BVDV infection was decreased up to 1,000-fold, whereas BHV-1 infection was inhibited five-fold by β-MCD but not by chlorpromazine. The columns represent mean values of duplicate experiments; bars indicate maximum and minimum values.

BHV-1 only fivefold, whereas 10 μg of chlorpromazine had no inhibitory effect on a BHV-1 infection (Fig. 2).

The susceptibility of MDBK cells to BVDV was also decreased by rather crude biophysical methods, namely, K^+ depletion (4) and hyperosmolarity (28). Either method has been reported to affect clathrin-dependent endocytosis. The influence of K^+ depletion on BVDV entry was assayed by incubating MDBK cells in hypotonic medium (DMEM-H_2O [1:1]), which was replaced after 5 min by a K^+-free buffer (28) or a control buffer containing 10 mM KCl. K^+ depletion reduced the susceptibility of MDBK cells to 14% (data not shown). A 1,000-fold inhibition of BVDV entry was observed when MDBK cells were infected in the presence of 0.45 M sucrose (data not shown). The treatment of BVDV with 0.45 M sucrose or K^+-free buffers did not lead to any reduction of infectivity (data not shown).

Internalization of BVDV requires endosomal acidification. Most enveloped viruses that invade the host cell via endocytosis require a low-pH step to initiate fusion of viral envelope with the endosomal membrane. To determine whether this mechanism also applies to BVDV, the effect of different inhibitors of endosomal acidification was assayed. MDBK cells were inoculated with BVDV NADL in the absence or presence of increasing concentrations of bafilomycin A1, ammonium chloride, or chloroquine, respectively. Bafilomycin A1 is an irreversible inhibitor of vacuolar H^+ ATPases, and ammonium chloride and chloroquine are lysosomotropic weak bases and reversibly neutralize acidification (9, 51). BVDV infection of MDBK cells was completely blocked at minimal concentrations of 0.05 μM bafilomycin A1, 50 mM ammonium chloride, and 50 μM chloroquine, whereas the susceptibility to BHV-1 was unchanged. Lower concentrations of the drugs resulted in partial inhibition (Fig. 3).

Low-pH treatment is not sufficient to initiate BVDV fusion. The pH triggered events that lead to fusion of endocytosed viruses can be mimicked at the cell surface by lowering the environmental pH. This reaction, referred to here as "fusion from without," is the acid-induced fusion of adsorbed virions with the plasma membrane and is a common property of enveloped viruses whose invasion depends on endosomal acidification, as shown for flaviviruses (63) and alphaviruses (71). Two effects can be observed after the exposure of surface-bound virions to acidic pH: formation of syncytia by virus-mediated fusion of neighboring cells and/or virus invasion independent of endocytosis. To determine whether MDBK cells permit fusion from without, SinV and BVDV were adsorbed to the cells, washed with prewarmed buffers at different pH values for 2 min, and replaced with prewarmed culture medium. To differentiate between infection events from fusion from without and endocytic uptake, bafilomycin A1 was added at a

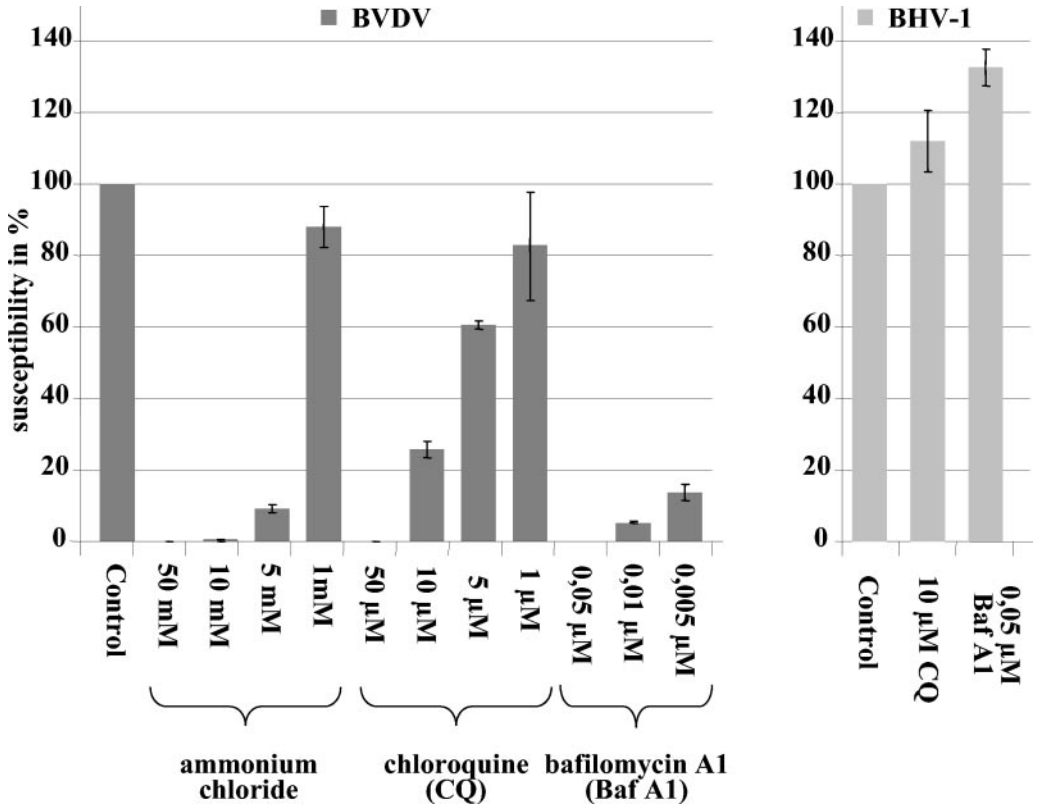

FIG. 3. Effect of different inhibitors of endosomal acidification on BVDV and BHV-1 infection. Directly after adsorption of BVDV NADL (dark gray bars) or BHV-1 (light gray bars) to MDBK cells, different inhibitors of endosomal acidification (bafilomycin A1, chloroquine, or ammonium chloride) were applied to determine the pH dependence of viral entry. Each inhibitor of endosomal acidification blocks BVDV infection in a concentration-dependent manner, whereas BHV-1 infection is not affected. The columns represent mean values of duplicate experiments; bars indicate maximum and minimum values.

concentration of 0.1 μM after the low-pH step. For SinV, fusion from without facilitated a rate of infection of 5% at pH 6.0 and of 80% at pH 5.0 (Fig. 4). In contrast, exposure of adsorbed BVDV to low pH did not result in any detectable infection of MDBK cells.

BVDV stability is decreased by reducing agents. The inability of BVDV to enter MDBK cells by fusion from without is compatible with the acid resistance that has been earlier reported for pestiviruses (13) and is highly unusual for an enveloped virus that requires low pH in the endosome. The acid resistance may be due to the fact that the glycoproteins form complex intra- and intermolecular disulfide bonds, which is a unique property of pestiviruses within the *Flaviviridae*. To analyze whether disulfide bonds contribute to pestivirus stability and, hence, acid resistance, 2×10^6 PFU of BVDV strain NADL were exposed to acidic pH (i.e., pH 3.0 to 7.0) for 15 min at 25°C in the presence or absence of a reducing agent (10 mM DTT). The virus was buffered to pH 7.0, and the titer was determined by serial dilution and infection of MDBK cells. Although exposure of BVDV to low pH led only to a minor loss of infectivity (35%, Fig. 5), the simultaneous presence of DTT resulted in 90% inactivation. At neutral pH the presence of DTT had no effect on virus stability (Fig. 5). Pretreatment of BVDV with DTT and a secondary shift to low pH led to a similar loss of infectivity.

To determine whether stabilization by disulfide bonds might also account for the inability of BVDV to undergo fusion at the cell surface, modified fusion-from-without experiments were performed in the presence or absence of DTT (10 mM). The combined treatment of adsorbed BVDV with 10 mM DTT at a pH of ≤6.0 led to infection of MDBK cells by BVDV (Fig. 6). The efficiency of infection by fusion from without was considerably lower (0.5%) compared to the values obtained with SinV (60 to 80%), but the singular treatment with either low pH or DTT led also in repeated experiments to no detectable infection.

DISCUSSION

The effects of chemical and biophysical inhibitors of endocytosis together with the requirement for endosomal acidification strongly suggest that BVDV invades the host cell via receptor-mediated endocytosis. This entry pathway had been postulated in the past, but the experimental evidence was only preliminary (1, 19). An elegant method to determine the involvement of endosomes is the expression of a dominant-negative dynamin mutant (Dyn^{K44A}) that competitively displaces the functional GTPase and thereby inhibits the release of vesicles from the plasma membrane. Expression of Dyn^{K44A} reduced susceptibility of MDBK cells to BVDV and SinV infection by 84 to 90%. These values are consistent with reports from the literature (14). Dynamin was initially reported to be

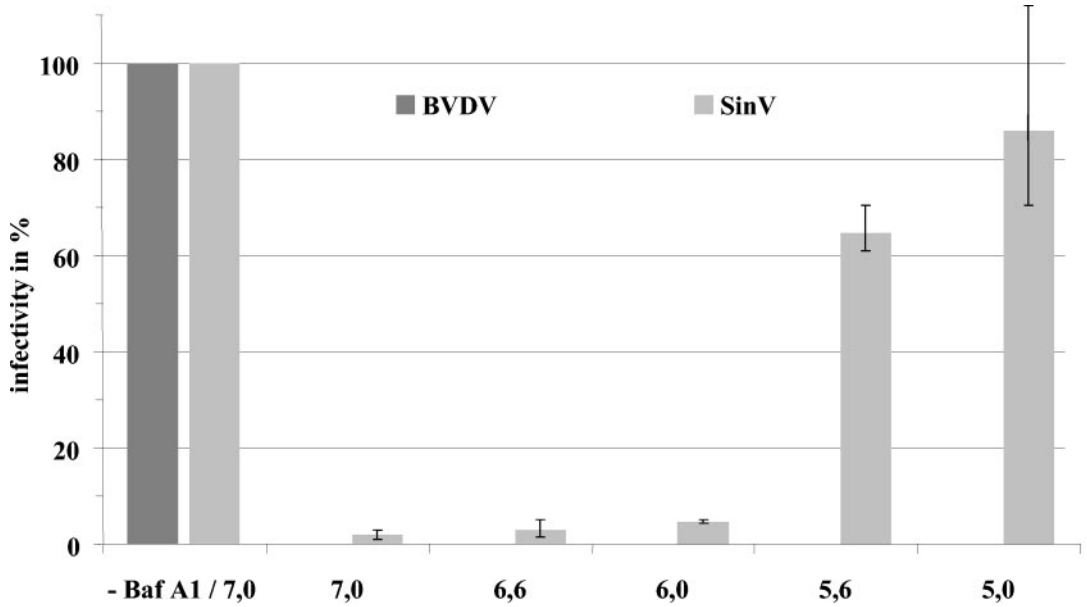

FIG. 4. Fusion from without of BVDV and SinV. MDBK cells were inoculated with BVDV strain NADL or SinV, respectively, for 1 h at 4°C. Medium was replaced by prewarmed buffers of the indicated pH, followed by incubation for 2 min at 37°C. Viral uptake via endocytosis was blocked by replacing buffer with DMEM containing bafilomycin A1. Since fusion from without is cell type specific, SinV was used as control. The columns represent mean values of triplicate experiments; bars indicate maximum and minimum values.

specific for clathrin-dependent endocytosis but has been shown later to affect other endocytic pathways as well. Formation of clathrin lattices at the plasma membrane is disturbed by chlorpromazine and also by K^+ depletion and hyperosmolarity. The exact molecular mechanism for the inhibitory effect of the latter two methods is not known, but it can be expected that also other cellular functions are affected. β-MCD has been shown to block endocytosis by depleting cholesterol from the

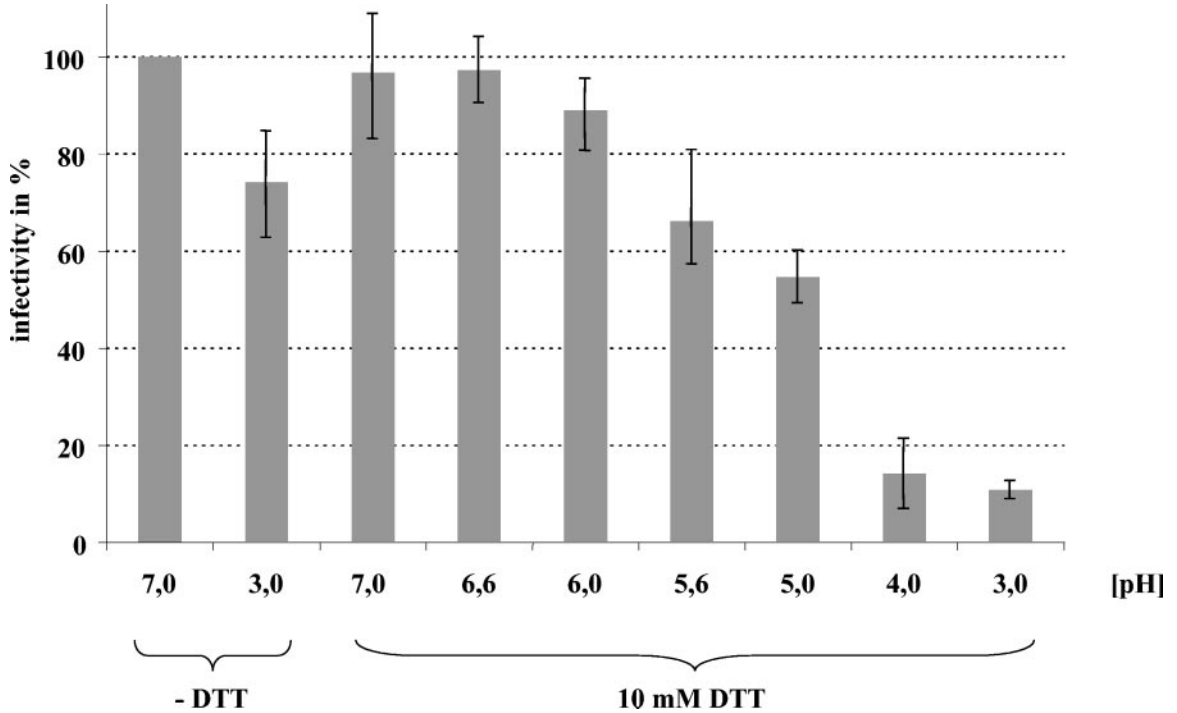

FIG. 5. pH stability of BVDV. A total of 2×10^6 PFU of BVDV strain NADL were incubated in citrate-phosphate buffers of a defined pH (pH 3.0 to 7.0) in the presence of 10 mM DTT for 15 min at 25°C and titrated on MDBK cells. The same experiment was performed in the absence of DTT, but only the infectivity after treatment at pH 3.0 and 7.0 is indicated. The columns represent mean values of triplicate experiments; bars indicate maximum and minimum values.

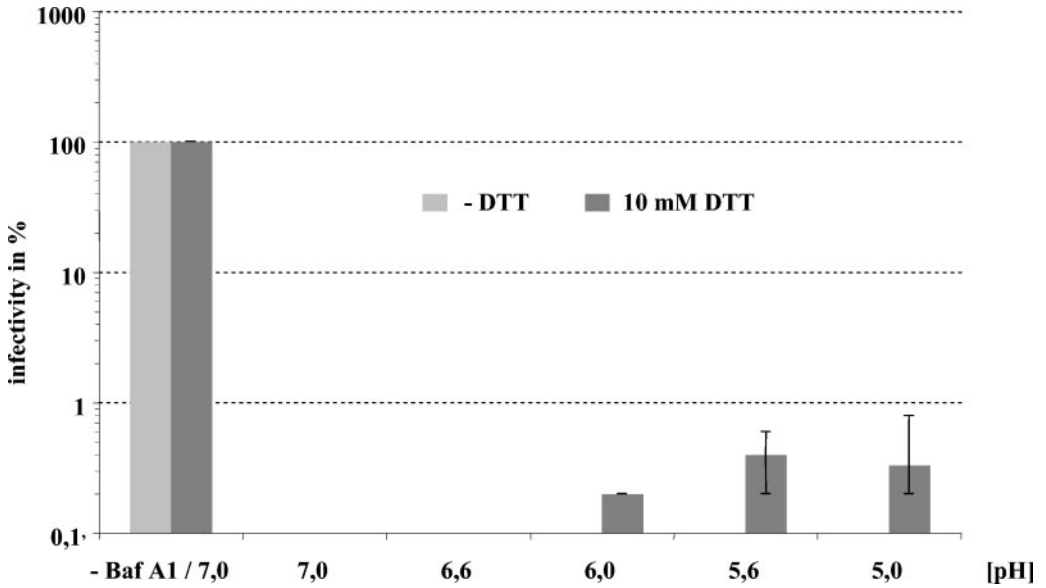

FIG. 6. Fusion from without of BVDV in the presence or absence of DTT. MDBK cells were inoculated with BVDV strain NADL and briefly shifted to 37°C at the indicated pH in the presence or absence of 10 mM DTT; virus uptake via endocytosis was blocked by replacing buffer with DMEM containing bafilomycin A1. Higher concentrations of DTT could not be used due to high cell toxicity. The numbers of infectious centers were determined 12 to 16 h p.i. The columns represent mean values of triplicate experiments; bars indicate maximum and minimum values.

plasma membrane; it likely also causes many side effects. However, all of the methods used lead to inhibition of BVDV entry, and thus endocytosis as route of entry can be safely assumed. Moreover, all of the inhibitors have been shown to interfere with the clathrin-dependent pathway (4, 11, 28, 55, 67), although none acts with absolute specificity concerning the route of endocytosis. The involvement of caveolae cannot be excluded by the inhibitors of endocytosis used here. However, the requirement of BVDV infection for endosomal acidification renders this endocytotic pathway unlikely because in caveosomes a neutral pH prevails (50). In conclusion, we suggest that BVDV enters the cell via clathrin-coated vesicles and undergoes fusion with the endosomal membrane upon acidification below a certain threshold.

Apparently, BVDV uses the same internalization route as well-studied members of the genera *Flavivirus* and *Alphavirus* (14, 32, 53). However, pestiviruses have one property that does not fit into this scheme. BVDV and CSFV are highly stable in acidic environments, whereas flavi- and alphaviruses are rapidly inactivated during exposure to low pH due to a structural reorganization of viral glycoproteins. For both flavi- and alphaviruses, this conformational change has been demonstrated to result in trimerization of the fusion protein (2, 6) and insertion of the fusion loop into the target membrane (24, 48). The metastable viral envelope proteins shift due to a minimal energetic trigger from the "receptor binding" state to the fusogenic and subsequently to the postfusion state, which is energetically much more favorable than the metastable state. pH sensitivity can be taken as an indicator for the metastable state of extracellular virions because most enveloped viruses, which enter via an pH-dependent endosomal pathway, are highly sensitive to an acidic environment. As a result, particles of the immature and fusion-incompetent flavivirus Murray Valley virus were shown to exhibit a considerable greater acid resistance than mature fusion-competent virions (27). Resistance to low pH can also result from reversibility of the conformational change in viral glycoproteins, i.e., rhabdovirus G protein. In the case of vesicular stomatitis virus, the G protein was shown to occur in a pH-dependent equilibrium of three different states (21). Exposure of vesicular stomatitis virus-infected cells to low pH leads to the formation of syncytia (72), which indicates that acidic conditions force the G protein to fuse with neighboring membranes. In contrast to rhabdoviruses, pestivirus glycoproteins do not accumulate at the cell surface; thus, fusion-from-without experiments had to be performed with virions of BVDV. BVDV adsorbed to the cell surface was not able to infect cells by an exogenous pH shift, which was sufficient to allow an infection with SinV by fusion from without at an efficiency of 80%. The pH resistance of BVDV and the inability to undergo fusion from without indicate that the term "metastability" does not apply for extracellular infectious particles of BVDV. Apparently, the metastability (or fusogenicity) of BVDV is not gained during virus release as described for alphaviruses and flaviviruses (30, 41). A maturation event in addition to low pH probably occurs during internalization of BVDV that allows the virus to become fusogenic.

Although it is tempting to postulate analogous invasion mechanisms for all members of the *Flaviviridae*, pestiviruses differ by the route of virus transmission. Transmissions by blood-sucking arthropods (as for most flaviviruses) or by parenteral exposure to blood (as for hepacivirus) do not require protective shields against environmental hazards. Pestiviruses, in contrast, are primarily acquired via direct or indirect contact. This implies the need for enhanced stability in the extracorporeal environment in order to ensure virus dissemination. It is likely that the increased environmental stability of extra-

cellular virions also comprises the acid resistance of pestiviruses.

A unique feature of pestiviruses is the extensive linkage of the three envelope glycoproteins by complex intermolecular disulfide bridges. Virions of BVDV and CSFV contain both covalently linked homodimers (E^{rns}-E^{rns} and E2-E2) and heterodimers (E^{rns}-E2 and E1-E2) (15, 39, 65, 68). Preliminary evidence that these disulfide bridges may contribute to acid resistance and hence account for virus stability came from the treatment of BVDV with a reducing agent. Inactivation of BVDV was only observed when virions were treated with a reducing agent in combination with low pH. Since a redox potential equivalent to 10 mM DTT is hardly achieved in the endosome enzymatic shuffling of disulfide bridges by disulfide isomerases could be considered. Protein disulfide isomerases (PDIs) were described as secreted proteins but are also present on the cell surface (10, 17) or located in the lysosome (3). Interestingly, PDIs have been claimed to be involved in the entry of several viruses, e.g., HIV (56), human T-cell lymphotropic virus (HTLV) (66), Moloney murine leukemia virus (Mo-MuLV) (57, 66), and murine hepatitis virus (20). For HIV, evidence for the reduction of disulfide bonds during virus entry came mainly from the use of membrane-impermeable thiol active reagents (DTNB and qBBr) and bacitracin, a specific inhibitor of PDIs (56). Analysis of HIV infection in treated target cells was carried out after 4 to 7 days of culture, at which point no cytotoxicity was observed. With these drugs, inhibition of BVDV infection was also detected; however, the strong cytotoxicity of these inhibitors raises doubt regarding the specificity of the inhibition.

Direct evidence for involvement of disulfide shuffling in the process of BVDV destabilization during invasion is provided by the fusion-from-without experiments wherein BVDV was treated with low pH and DTT. Although an infection of MDBK cells by fusion from without could not be observed in the presence of either low pH or DTT, the combined treatment of adsorbed BVDV led to successful infections, albeit at a low level. Given that DTT reduces the disulfide bonds in the glycoproteins stochastically, the efficiencies may be greatly increased by selective enzymatic processes.

Pestiviruses have apparently developed an unusual way to increase their physicochemical stability without sacrificing the highly efficient clathrin-dependent endocytosis and pH-dependent fusion mechanism. The experimental evidence presented here suggests that disulfide bond shuffling participates in partial destabilization of virions in order to cause them to become metastable and fusogenic at low pH.

In this context it is interesting that for Mo-MuLV and HTLV Env glycoproteins a cryptic disulfide isomerase activity was demonstrated (66). All thiol-disulfide exchange enzymes contain a typical CxxC motif as the active site (18). This motif has been found in the peripheral subunit (SU) of Mo-MuLV and HTLV envelope protein (57) and the E1 protein of the togavirus rubella virus (26); for both retroviruses it was shown that receptor binding induces disulfide isomerase activity. This activity is essentially required for virus entry into host cells and results in dissociation of the SU and the transmembrane subunit (TM) and the subsequent fusion function of TM (66). Among pestiviruses a CxxC motif is highly conserved in the glycoprotein E2. Interestingly, an alignment of different CxxC motifs of viral origin indicates the presence of a large aromatic amino acid within the CxxC motif in Mo-MuLV env and rubella virus E1, as well as in pestivirus E2 (Fig. 7). Whether the CxxC motif is in fact involved in BVDV entry will be the subject of further studies.

				C	x	x	C				
202	I	E	S	C	K	W	C	G	Y	Q	BVDV NADL E2
201	V	K	Q	C	R	W	C	G	F	E	CSFV Alfort E2
346	V	T	G	C	Y	Q	C	G	T	P	Rubella Virus E1
333	T	Q	E	C	W	L	C	L	V	A	MoMuLV env

FIG. 7. Comparison of CxxC motifs from different viruses. A decapeptide of BVDV strain NADL and CSFV Alfort E2 containing the CxxC motif was aligned to the corresponding sequence in rubella virus E1 (GI:33415288) and Mo-MuLV gPr80 glycosylated envelope polyprotein (GI:18448745). The numbers denote the position of the decapeptide in the respective protein; the conserved CxxC motif is indicated above the sequence. Conserved cysteine residues are boxed.

A particularly interesting finding of the present study concerns the mechanistic connection between endosomal entry on the one side and the putative cellular receptor of BVDV, bovine CD46, on the other. Human CD46 has been described to be excluded from endocytosis due to a sorting signal (Phe-Thr-Ser-Leu) in the C-terminal cytosolic tail (42). In preliminary studies with fluorescently labeled anti-CD46 antibodies, internalization of bovine CD46 was also not detectable (not shown). Based also on previously reported data (45), our model therefore postulates that after adsorption to bovine CD46, BVDV interacts with one or more additional cellular molecule(s) that initiate endocytosis. Characterization of these putative coreceptors and elucidation of mechanisms how endocytosis is initiated will be the focus of future studies.

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