INHIBITION OF ENDOosomal-LYSOSOMAL SYSTEM ACIDIFICATION ENHANCES PORCINE CIRCOVIRUS 2 INFECTION OF PORCINE EPITHELIAL CELLS

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Shortened running title: blocking cell’s acidification enhances PCV2 infection

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The number of characters and spaces in running title is 53. The total number of words in the abstract is 250 and 4,596 in main text of the paper. The paper contains 5 figures and 3 tables.
Recently, Misinzo et al. (J. Gen. Virol. 86: 2057-68, 2005) reported that inhibiting endosomal-lysosomal system acidification reduced porcine circovirus 2 (PCV2) infection of monocytic 3D4/31 cells. The present study examined the effect of inhibiting endosomal-lysosomal system acidification in epithelial cells, since epithelial cells support PCV2 infection \textit{in vivo} and are used in culturing PCV2 \textit{in vitro}. Ammonium chloride (NH₄Cl), chloroquine diphosphate (CQ) and monensin were used to inhibit endosomal-lysosomal system acidification. NH₄Cl, CQ or monensin increased PCV2 (Stoon-1010) infection, respectively, by 726±110%, 1212±34% and 1100±179% in porcine kidney (PK-15) cells, by 128±7%, 158±3% and 142±11% in swine kidney (SK) cells, by 160±28%, 446±50% and 162±56% in swine testicle (ST) cells, and by 313±25%, 611±86% and 352±44% in primary kidney epithelial cells. Similarly, increased PCV2 infection was observed with six other PCV2 strains in PK-15 treated with endosomal-lysosomal system acidification inhibitors. The mechanism behind increased PCV2 infection was further investigated in PK-15 using CQ. PCV2 infection of PK-15 was increased only when CQ was added early during PCV2 infection. CQ did not affect PCV2 virus-like particles (VLP) attachment onto PK-15 but increased disassembly of internalized PCV2-VLP. In untreated PK-15, internalized PCV2-VLP localized within the endosomal-lysosomal system. PCV2 infection of untreated 3D4/31 and PK-15, and CQ-treated PK-15 was blocked by a serine protease inhibitor (AEBSF) but not by aspartyl- (peptatin A), cysteine- (E-64) and metallo- (phosphoramidon) protease inhibitors. These results suggest that serine protease-mediated PCV2 disassembly is
enhanced in porcine epithelial cells but inhibited in monocyctic cells after inhibiting endosomal-lysosomal system acidification.

INTRODUCTION

Porcine circovirus 2 (PCV2) is a member of the Circoviridae. PCV2 has a circular single stranded DNA genome of 1768 bases that encodes for replication-associated proteins (Rep and Rep’) on open reading frame (ORF) 1, a capsid protein on ORF2 and an 105 amino acid protein on ORF3 (9, 34, 52, 54). PCV2 infection is associated with postweaning multisystemic wasting syndrome (PMWS), a multifactorial disease in weaned pigs characterized by severe growth retardation, rapid weight loss and high mortality rates in affected pigs (13, 20, 52). High PCV2 replication has been found to be associated with the development of PMWS and has thus been included as a criterion in the case definition of PMWS (61). In PCV2 positive swine herds and experimentally PCV2 inoculated pigs, only a small proportion of pigs experience high PCV2 replication and subsequently develop severe clinical signs or PMWS (33, 55, 59). The identification of factors that predispose individual pigs towards a high PCV2 replication compared to other pigs within the same herd is important in understanding the pathogenesis of PMWS. Some host specific factors that influence the level of PCV2 replication in pigs have already been identified. The absence of PCV2 neutralizing antibodies (44), a general stimulation of the immune system arising from concurrent infections, vaccination or concanavalin A treatment (3, 31, 32, 43, 56) and/or immunosuppression (30, 47) may predispose pigs to persistent high PCV2 replication and development of PMWS.
The susceptibility of different cell types to PCV2 infection depends on the age of the foetus or the piglet (59). The differential susceptibility of cells has been linked to mitosis since PCV2 does not encode for its own DNA polymerase and requires active cellular polymerases for viral replication (59). Treatment of cells in vitro either with glucosamine or interferon-gamma increases PCV1 and PCV2 infection, respectively (46, 66). However, other factor(s) or mechanism(s) that explain the specific tropism and differential susceptibility of target cells to PCV2 infections may be involved. Misinzo et al. (51) showed that the susceptibility of 3D4/31 monocytic line to PCV2 infection was reduced following inhibition of endosomal-lysosomal system acidification, suggesting that an acidic environment is necessary for PCV2 infection. Generalization of this finding to other cell types is not possible. For instance, inhibition of endosomal-lysosomal system acidification inhibits human immunodeficiency virus type 1 (HIV-1) replication in primary T cells and monocytes as well as T cell and monocytic cell lines (62), whereas it increases HIV-1 infectivity in human 293T and HeLa Magi cells (16).

Epithelial cells support PCV2 replication in pigs experimentally infected with PCV2 as well as in pigs with naturally occurring PCV2 associated PMWS (23, 57). In addition, PCV2 replicates and is cultivated in vitro in porcine kidney (PK-15) (2, 42) and swine kidney (SK) epithelial cell lines (58). Up till now, no study has investigated the effect of inhibiting endosomal-lysosomal system acidification on PCV2 infection of PCV2 target cells other than the monocytic cell line 3D4/31. The aim of this study was to investigate the importance of endosomal-lysosomal system acidification on PCV2 infection of epithelial cells.
MATERIALS AND METHODS

Cells, virus and PCV2 virus-like particles

PK-15, SK and swine testicle (ST) epithelial cell lines and 3D4/31 monocytic cell line free from porcine circoviruses were used. Cells were seeded at 2 x 10^5 cells/ml and maintained in culture medium containing 10 % fetal bovine serum (FBS; Invitrogen, Grand Island, USA), 0.3 mg/ml L-glutamine (BDH Chemicals Ltd., Poole, England), 1 % non-essential amino acids (100x; Invitrogen), 1 mM sodium pyruvate (Invitrogen), 100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.1 mg/ml kanamycin in RPMI-1640 (Invitrogen).

Primary porcine kidney epithelial cells were obtained by trypsinization of the kidney cortices from three 3-weeks-old PCV2 negative conventional piglets. Kidney cortices were dissected from medulla after the removal of the kidney capsule, minced and incubated in phosphate-buffered saline (PBS) containing 2.5 mg/ml trypsin (Sigma, Bornem, Belgium) at 37 °C for 10 minutes. Trypsinization of minced kidney cortices was done twice. After each trypsinization, primary kidney cells were collected by sieving through three layered cotton gauze. Cells were pelleted by centrifugation and re-suspended at 2 x 10^5 cells/ml in Dulbecco’s modified Eagle medium (DMEM; Invitrogen) containing 10 % FBS, 0.3 mg/ml L-glutamine and antibiotics.

Seeded cells were maintained at 37 °C in a humidified 5 % CO₂ incubator. All experiments were carried out 24 hours post seeding when cells reached approximately 50 % confluency.

Different PCV2 strains passaged in PK-15 cells previously described by Meerts et al. (45) were enclosed in this study. Cells were inoculated with PCV2 strains Stoon-1010,
114 1121, 1103, 48285, VC2002, 1206 and 1147 at a multiplicity of infection (m.o.i) of 0.3 115 for 1 hour at 37 °C.
116 Recombinant PCV2 virus-like particles (VLP) were used for analysis of PCV2 117 binding, intracellular localization and disassembly in PK-15 cells as previously described 118 (50, 51).
119
120 **Effect of lysosomotropic agents on PCV2 infection of epithelial cells**
121 Endosomal-lysosomal system acidification was inhibited using lysosomotropic weak 122 bases (ammonium chloride (NH$_4$Cl) and chloroquine diphosphate (CQ)), and the 123 carboxylic ionophore monensin. NH$_4$Cl, CQ and monensin are lysosomotropic agents 124 because they selectively accumulate into lysosomes (12). Apart from inhibiting the 125 acidification of the endosomal-lysosomal system, NH$_4$Cl, CQ and monensin decrease 126 intralysosomal proteolysis and cause intracellular vesicular swelling (10, 14). Therefore, 127 other lysosomotropic agents without an effect on endosomal-lysosomal system 128 acidification but (i) that decrease intralysosomal proteolysis and cause intracellular 129 vesicular swelling (suramin) (1, 26, 27) or (ii) that have no effect on intralysosomal 130 proteolysis and cause intracellular vesicular swelling (polyvinylpyrrolidone (PVP)) (10, 131 17, 27) were included. All chemical compounds were purchased from Sigma.
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133 The highest concentrations for each of the lysosomotropic agents that did not affect 134 PK-15 cells viability after a 24 hours incubation period were used; 25 mM, 125 µM, 6 135 µM, 1 mg/ml and 1 mg/ml of NH$_4$Cl, CQ, monensin, suramin and PVP, respectively. PK- 136 15, ST, SK and primary porcine kidney epithelial cells were washed once and pretreated 137 with or without lysosomotropic agents for 1 hour. Cells were inoculated with an equal
dose of PCV2 in the presence or absence of the lysosomotropic agents. The viral
inoculum was washed-off and cells were further incubated in culture medium with or
without lysosomotropic agents (i) for 24 hours to assess the effect on PCV2 infection or
(ii) for different durations to determine the time point at which the lysosomotropic agents
had their effect. Subsequently, culture medium with or without lysosomotropic agents
was replaced with culture medium without lysosomotropic agents. After the first cycle of
PCV2 replication, at 36 hours post inoculation (hpi), cells were fixed with methanol at -20 °C for 10 minutes.

For epithelial cell lines, PCV2-infected cells were stained using an
immunoperoxidase monolayer assay (IPMA) as previously described (50). PCV2-
infected cells were counted by examination under an Olympus light microscope
(Olympus Optical Co., Hamburg, Germany). The number of infected cells per well in
untreated cells was used as a reference and all results were expressed as a percentage of
this reference.

For primary porcine kidney epithelial cells, a double immunofluorescence staining
was performed to identify epithelial cells and PCV2-infected cells in primary kidney cell
cultures. Kidney epithelial cells were identified with mouse anti-human cytokeratin
monoclonal antibody (clone MNF116; DakoCytomation, Glostrup, Denmark) followed
by incubation with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG
(Invitrogen). PCV2-infected cells in kidney cell cultures were identified by incubation
with biotinylated anti-PCV2 swine antibodies (59) followed by streptavidin-conjugated
Texas Red (Invitrogen). Each of the staining incubation was carried out for 1 hour at 37
°C. Cell nuclei were stained by incubating the cells for 10 minutes at room temperature
with Hoechst 33342 (Invitrogen) at a concentration of 10 µg/ml. After each of the incubations, cells were washed with PBS. Finally, stained cells were mounted and analysis of the number of PCV2-infected kidney epithelial cells per 10^4 cells was done by using a Leica DM/RBE fluorescence microscope. The number of infected cells in untreated cells was used as a reference and all results were expressed as a percentage of this reference.

All experiments were performed three times and each condition in an experiment was performed in quadruplicate.

Effect of inhibiting endosomal-lysosomal system acidification on PCV2-VLP attachment and disassembly of internalized PCV2-VLP

PK-15 cells were chilled on ice and washed with ice cold RPMI-1640. PCV2-VLP were then added at 4 °C for 1 hour in the presence or absence of 125 µM CQ to allow binding onto PK-15 cells. Unbound PCV2-VLP were washed-off and cells were refreshed either with pre-warmed medium without FBS or similar culture medium containing 125 µM CQ. PK-15 cells were then incubated at 37 °C in a humidified 5 % CO_2 incubator. At 0 and 3 hours after the 37 °C shift, cells were fixed in 3 % (w/v) paraformaldehyde in PBS containing Ca^{2+} and Mg^{2+} (PBS+). A double immunofluorescence staining was performed in order to distinguish bound and internalized PCV2-VLP. Bound PCV2-VLP were stained using anti-PCV2 capsid specific monoclonal antibody F190 (40) followed by FITC-conjugated goat anti-mouse IgG. Cells were subsequently washed with PBS+ and permeabilized with Triton X-100 (0.1 % in PBS+) for 2 minutes at room temperature. After permeabilization, all PCV2-
VLP were identified using the same monoclonal antibody F190 followed by Texas Red-conjugated goat anti-mouse IgG (Invitrogen). Finally, cells were mounted and analyzed using a Leica TCS SP2 laser-scanning spectral confocal system linked to a Leica DM/IRB inverted microscope. The total fluorescence area of attached PCV2-VLP in cells treated with or without CQ was estimated using the image analysis software SigmaScan Pro 5.0 as previously described (50, 51).

**Intracellular localization of internalized PCV2-VLP in PK-15 cells**

To identify the intracellular organelle(s) in which PCV2-VLP localizes, PK-15 cells were chilled on ice, washed with ice cold RPMI-1640 and incubated with PCV2-VLP for 1 hour at 4 °C. Unbound PCV2-VLP were washed-off and cells were refreshed with pre-warmed medium without FBS. PK-15 cells were then incubated at 37 °C in a humidified 5 % CO₂ incubator for 3 hours after which they were fixed in 3 % (w/v) paraformaldehyde in PBS+. Cells were subsequently washed and permeabilized with Triton X-100 (0.1 % in PBS+) for 2 minutes at room temperature. Intracellular localization of PCV2-VLP was determined by a double immunofluorescence labeling of internalized PCV2-VLP and early endosomes, or lysosomes, or Golgi apparatus and or the endoplasmatic reticulum. Early endosomes were labeled using goat polyclonal anti-early endosome antigen 1 (EEA-1) IgG (Santa Cruz Biotechnology, Santa Cruz, California, USA), lysosomes were labeled with goat polyclonal anti-cathepsin D IgG (Santa Cruz Biotechnology), Golgi apparatus was labeled using rabbit polyclonal anti-giantin IgG (Eurogentec, Seraing, Belgium), and the endoplasmatic reticulum was labeled using goat polyclonal anti-calnexin IgG (Santa Cruz Biotechnology). Cells were
then incubated with Alexa Fluor 594-conjugated rabbit anti-goat (Invitrogen) to recognize goat polyclonal IgG or Texas Red-conjugated goat anti-rabbit (Invitrogen) to recognize rabbit polyclonal IgG. PCV2-VLP were labeled using anti-PCV2 capsid-specific monoclonal antibody F190 followed by FITC-conjugated goat anti-mouse IgG. Cells were mounted and images were acquired by confocal microscopy.

The effect of protease inhibitors on PCV2 infection of CQ-treated PK-15 cells

Protease inhibitor 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF; inhibits serine proteases), pepstatin A (inhibits aspartyl proteases), trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane (E-64; inhibits cysteine proteases) and phosphoramidon (inhibits metalloproteases) were used to analyse the potential involvement of proteases during PCV2 infection and to identify the class of cellular protease potentially responsible for PCV2 capsid disassembly. All protease inhibitors were from Sigma. PK-15 and 3D4/31 cells were preincubated with or without 0.5 mM or 0.25 mM AEBSF, 1 µg/ml pepstatin A, 100 µM E-64 or 45 µM phosphoramidon for 1 hour at 37 °C. After 1 hour, PK-15 and 3D4/31 cells were inoculated with PCV2 (Stoon-1010) for 1 hour at 37 °C in the presence or absence of protease inhibitors. Cells were then washed and incubated for 24 hours in culture medium with or without protease inhibitors containing 125 µM CQ for PK-15 cells and 0 µM CQ for 3D4/31 cells. At 24 hpi, culture medium with or without protease inhibitors and CQ was replaced with fresh culture medium. Cells were fixed at 36 hpi and an IPMA staining was performed to identify PCV2-infected cells. The number of PCV2-infected PK-15 cells in cells treated with a combination of a protease inhibitor and CQ was expressed as a relative percentage.
to the number of PCV2-infected cells in cells treated with CQ alone. The number of
PCV2-infected 3D4/31 cells in cells treated with a protease inhibitors was expressed as a
relative percentage to the number of PCV2-infected cells in untreated cells.

The effect of protease inhibitors on disassembly of internalized PCV2-VLP in CQ-
treated PK-15 cells

PK-15 cells were preincubated with or without 0.5 mM AEBSF for 1 hour at 37 °C.
Afterwards, PK-15 cells were chilled on ice and washed with ice cold RPMI-1640.
PCV2-VLP were then added at 4 °C for 1 hour in the presence of 125 µM CQ to allow
binding onto PK-15 cells. Unbound PCV2-VLP were washed-off and cells were
refreshed either with pre-warmed medium without FBS or similar culture medium
containing 125 µM CQ with or without 0.5 mM AEBSF. PK-15 cells were then
incubated at 37 °C in a humidified 5 % CO₂ incubator. At 0 and 3 hours after the 37 °C
shift, cells were fixed in 3 % (w/v) paraformaldehyde in PBS+. The double
immunofluorescence staining to distinguish bound and internalized PCV2-VLP, confocal
microscopy and analysis was performed as described above for the investigation of the
effect of inhibiting endosomal-lysosomal system acidification on disassembly of
internalized PCV2-VLP.
RESULTS

Inhibiting endosomal-lysosomal system acidification enhances the number of PCV2-infected cells in porcine epithelial cells.

The endosomal-lysosomal system is characterized by gradual acidification of its vesicles as they mature from early endosomes (pH ~ 6.0 - 6.8) into lysosomes (pH ~ 5.0) (48). Endosomal-lysosomal system acidification can be inhibited by some lysosomotropic agents. Epithelial cell lines (PK-15, SK an ST) and primary porcine kidney epithelial cells were treated with lysosomotropic agents to investigate the effect of inhibiting endosomal-lysosomal system acidification on PCV2 infection. The effect of different lysosomotropic agents on PCV2 infection of epithelial cells inoculated with the prototype PCV2 strain Stoon-1010 is shown in Fig. 1 and 2, and Table 1 and 2. Higher increase in the number of PCV2-infected cells was mainly observed in PK-15 and primary porcine kidney epithelial cells compared to SK and ST cells treated with NH₄Cl, CQ, monensin and suramin (Table 2). Treatment of cells with PVP did not increase the number of PCV2-infected cells in any of the epithelial cell type.

Different PCV2 strains were used in order to investigate if the enhancement of PCV2 infection by inhibitors of endosomal-lysosomal system acidification is general to other PCV2 strains. PCV2 infection was increased in PK-15 cells with all strains following treatment with NH₄Cl, CQ, monensin and suramin (Table 3). The magnitude of increase of PCV2 infection following inhibition of endosomal-lysosomal system acidification was different in between strains, with PCV2 strain 1103 showing the least increase in the number of infected cells. No significant increase on the number of PCV2-infected PK-15 cells were found in cells treated with PVP.
CQ treatment of PK-15 cells increases the disassembly of internalized PCV2-VLP

In order to examine the stage during PCV2 replication cycle in which lysosomotropic agents exert their effect, CQ was added at different time points before, during or after PCV2 inoculation as shown in Fig. 3. Addition of CQ for 1 hour before inoculation, for 1 hour during inoculation or starting from 12 hpi onwards did not increase the number of PCV2 infected PK-15 cells significantly. However, addition of CQ during the first six hours of PCV2 increased the number of PCV2-infected PK-15 cells significantly (Fig. 3). Thus, inhibiting endosomal-lysosomal system acidification increases PCV2 infection by affecting early stage(s) of PCV2 infection.

The effect of CQ on early stages of PCV2 entry such as attachment and disassembly was further investigated. To examine attachment, PCV2-VLP were allowed to bind to PK-15 cells for 1 hour at 4°C in the absence or presence of CQ. The relative total fluorescence of bound PCV2-VLP in PK-15 cells treated with or without CQ was 107 ± 22 and 100 ± 20, respectively. In order to examine the effect of endosomal-lysosomal system acidification inhibition on PCV2 internalization and disassembly, cells were incubated with PCV2-VLP for 3 hours at 37 °C in the absence or presence of CQ. PCV2-VLP were visible within the cells after 3 hours of incubation in untreated cells but not in CQ-treated cells (Fig. 4). This observation indicates that, under normal circumstances PCV2 is slowly disassembled within the cell and that inhibiting endosomal-lysosomal system acidification provides an optimal environment that enhances PCV2 disassembly.
Internalized PCV2-VLP localize within endosomal-lysosomal system compartments of PK-15 cells.

To allow a better understanding of the mechanism by which CQ enhances PCV2 infection, identification of the intracellular compartment in which PCV2 is transported following internalization was performed. Intracellular localization of PCV2-VLP was determined using double immunofluorescent labeling for PCV2-VLP and markers of early endosomes, lysosomes, Golgi apparatus, and endoplasmatic reticulum. Immunofluorescent labeling of PCV2-VLP prior to shifting the cells to 37 °C demonstrated no co-localization of PCV2-VLP with markers for early endosome, lysosome, Golgi apparatus and endoplasmatic reticulum. PCV2-VLP within the cell, 3 hours after internalization (shift to 37 °C), co-localized with early endosomes and lysosomes but not with Golgi apparatus and the endoplasmatic reticulum (Fig. 5).

Proteolytic cleavage of PCV2 capsid is mediated by a cellular serine protease

Internalized PCV2-VLP disassembled within endosomal-lysosomal system compartments in CQ-treated PK-15 cells. Because disassembly of viruses within the endosomal-lysosomal system can be mediated by proteases, the role of cellular proteases on PCV2 infection was investigated. Cellular proteases are classified into serine, cysteine, aspartic and metalloproteases (4). PCV2 infection was completely blocked when CQ-treated PK-15 cells were incubated with 0.5 mM AEBSF. Treatment of CQ-untreated PK-15 cells with 0.5 mM also completely blocked PCV2 infection. The relative number of PCV2-infected cells in CQ-treated PK-15 cells was 30 ± 14%, 82 ± 2%, 84 ± 3% and 82 ± 14% when they were incubated with 0.25 mM AEBSF, 1 µg/ml pepstatin A,
100 µM E-64 and 45 µM phosphoramidon, respectively. To investigate if the reductions of infection resulted from an effect on PCV2 disassembly, the effect of AEBSF on uncoating of PCV2-VLP was analysed. The disassembly of PCV2-VLP observed in CQ-treated PK-15 cells was inhibited by AEBSF. The accumulation of internalized PCV2 VLP in CQ-treated PK-15 cells treated with AEBSF indicated that the absence of PCV2 VLP within the cell in CQ-treated cells was not a result of CQ interference with internalization but rather on accelerated disassembly of internalized PCV2 VLP. The effect of inhibiting cellular protease on PCV2 infection was also investigated in 3D4/31 cells, in which inhibiting endosomal-lysosomal system acidification in monocytic cells reduces PCV2 infection, in contrast to epithelial cells. PCV2 infection of 3D4/31 cells was also completely blocked by treatment of cells with 0.5 mM AEBSF. The relative number of PCV2-infected cells in 3D4/31 cells was 36 ± 14%, 128 ± 26%, 70 ± 10% and 93 ± 10% when 3D4/31 cells were incubated with 0.25 mM AEBSF, 1 µg/ml pepstatin A, 100 µM E-64 and 45 µM phosphoramidon, respectively.

**DISCUSSION**

In the present study, inhibition of endosomal-lysosomal system acidification enhanced PCV2 infection of porcine epithelial cell lines as well as primary porcine kidney epithelial cells. Increased PCV2 infection occurred when endosomal-lysosomal system acidification was inhibited during early stages of PCV2 infection. The effect of inhibitors of endosomal-lysosomal system acidification was not at the level of PCV2 capsid attachment but at the level of PCV2 capsid disassembly of internalized PCV2. The disassembly of PCV2 capsid was mediated by a serine protease.
To our knowledge, the present study is the first to show enhanced infection of a single stranded DNA nonenveloped virus as a result of elevating the pH of endosomal-lysosomal system. The fact that neutralizing the acidic endosomal-lysosomal system enhances PCV2 infection of epithelial cells is in contrast with ample studies which have shown that neutralizing the acidic endosomal-lysosomal system inhibits the infection of pH-dependent nonenveloped viruses (7, 8, 18, 35, 36, 63) and enveloped viruses (21, 38, 64, 65, 70). Inhibiting endosomal-lysosomal system acidification also inhibits PCV2 infection of 3D4/31 monocytic cells (51), further contrasting the results presented in this study for epithelial cells. *In vitro*, inhibition of endosomal-lysosomal system acidification has been shown to increase the infection of enveloped HIV-1 (16, 69). Most HIV-1 particles are endocytosed (37, 60) and do not result into productive infection because an acidic environment within the endosomal-lysosomal system leads to virus degradation in lysosomes (15, 37, 60). Inhibition of endosomal-lysosomal system acidification has been suggested to slow down the proteolytic degradation of endocytosed infectious HIV-1 particles thus providing sufficient time for HIV-1 to fuse with endocytic membranes and deliver virion cores to the cytoplasm (16). PCV2 is a nonenveloped virus and does not uncoat by fusion with endocytic membranes. Instead, this study shows that a serine protease-mediated PCV2 capsid disassembly is essential for PCV2 uncoating. Several possible scenarios may explain the enhanced PCV2 infection of epithelial cells resulting from elevated pH within the endosomal-lysosomal system. Firstly, inhibiting endosomal-lysosomal system acidification may provide sufficient time and optimum pH for a serine protease to disassemble the PCV2 capsid. Inhibiting endosomal-lysosomal system acidification causes all compartments of the endosomal-lysosomal system to have an
“endosome-like” pH. This “endosome-like” pH is assumed to be optimal to serine proteases that cleave the PCV2 capsid. The inhibitors of endosomal-lysosomal system acidification also inhibit the maturation of endosomes to lysosomes, further restricting the localization of internalized PCV2 within endosomes, where optimum PCV2 capsid cleavage seems to occur. Secondly, inhibitors of endosomal-lysosomal system acidification may directly activate certain proteases within the endosomal-lysosomal system (5, 28, 29), which may be responsible for the PCV2 capsid dissassembly. In this way, PCV2 capsid disassembly may be increased. Thirdly, inhibition of endosomal-lysosomal system acidification alters the sorting of certain proteins (including proteases) that are normally destined to be transported to the plasma membrane, to be directed towards endosomal-lysosomal system compartments (24, 25). If these proteins happen to be proteases that cleave PCV2 capsid, an enhanced PCV2 disassembly may occur, explaining the observed increase in PCV2 infection.Fourthly, lysosomotropic agents that inhibit endosomal-lysosomal system acidification can also cause intracellular vesicular swelling (10, 14, 49), which may result into increased release of endocytosed cargo into the cytoplasm. This is unlikely for endocytosed PCV2 since PVP, a lysosomotropic agent with no effect on endosomal-lysosomal system acidification but which cause osmotic related intracellular vesicular swelling (10, 17, 27), did not affect PCV2 infection. Surprisingly, suramin which causes intracellular vesicular swelling and decreases intralysosomal proteolysis (7, 8, 26, 27, 29), slightly increased PCV2 infection of epithelial cells. Another suramin activity may be responsible for the enhanced PCV2 infection such as the activation of DNA synthesis (53, 71), since PCV2 replication is cell
cycle dependent and the stimulation of cellular proliferation by suramin may help PCV2 to replicate.

PCV2 infects PK-15 and SK porcine epithelial cell lines (2, 40, 42, 58). In this study, a testis-derived epithelial cell line (ST) was susceptible to PCV2 infection, showing that epithelial cells from other organs may also be susceptible to PCV2 infection. PCV2 infects epithelial cells in pigs experimentally infected with PCV2 (23), in PMWS pigs (13, 57) and in primary kidney cultures (22), based on histological and morphological identification. This study confirms the susceptibility of primary kidney epithelial cells to PCV2 infection using double immunofluorescence labeling of PCV2 antigens and cytokeratin, an epithelial-specific intermediate filament. The susceptibility of primary kidney epithelial cells was equal to that observed in PK-15 cells, suggesting that the latter provides a good in vitro model for studying PCV2 interactions with epithelial cells. This observation is supported by previous studies which have reported that PK-15 cells display similar cellular growth characteristics to those of primary epithelial cells in culture (11).

This study investigated the effect of inhibiting endosomal-lysosomal system acidification on the infection of different PCV2 strains isolated from cases of PMWS (13, 41, 47), porcine dermatitis and nephropathy syndrome (PDNS) (41) or reproductive failure (41). All PCV2 strains (with the exception of the 1103 strain) showed increased infection upon inhibition of endosomal-lysosomal system acidification, however, differences in magnitude were observed. This indicates that, although a common PCV2 infection mechanism at the level of disassembly in epithelial cells is shared by PMWS-, PDNS- and reproductive failure-associated PCV2 strains, there are subtle differences that differentiate them. These might arise from differences in amino acid sequence at protease
cleavage site(s) that may cause the different susceptibility of PCV2 capsids to cleavage by a cellular serine protease(s). At this point these differences cannot be determined because the serine protease(s) responsible for PCV2 capsid disassembly is (are) unknown. Other studies have also reported differences in replication kinetics of different PCV2 strains isolated from distinct PCV2 related diseases (45). Why the replication of strain 1103 could not be influenced by the inhibitors of endosomal-lysosomal system acidification is not clear. It is possible that the serine protease cleavage site is absent with this specific strain.

PCV2 is internalized via clathrin-mediated endocytosis in monocytic (51) and dendritic cells (67). Although the mechanism of PCV2 entry in epithelial cells was not investigated, the localization of PCV2 in early endosomes in epithelial cells suggests that PCV2 becomes internalized via a pathway whose vesicles bud onto the early endosome. Proteins endocytosed into early endosomes can be recycled to the cell surface, transported to the trans-Golgi network or routed to late endosomes and lysosomes (19). Internalized PCV2 were localized in lysosomes but not in trans-Golgi network indicating that PCV2 was transported from endosomes onto lysosomes. The exact internalization pathway for PCV2 in epithelial cells remains to be determined.

The present study shows that a serine protease is important also in the disassembly of PCV2 in monocytic 3D4/31 cells. Because PCV2 disassembly requires a low pH in 3D4/31 cells and a neutral pH in epithelial cells, it is reasonable to suggest that two different serine proteases in these cell types are involved in PCV2 disassembly. Further studies are required to identify these serine proteases.
PCV2 had been circulating in the pig population for years before the emergence of PMWS (61). The reason why PMWS emerged suddenly is still not clear. One may speculate that drugs which inhibit endosomal-lysosomal system acidification may predispose pigs to a high PCV2 replication and PMWS. The carboxylic ionophore monensin has been used for the prevention or treatment of certain porcine diseases and another carboxylic ionophore, salinomycin, has been used as a growth promoter in pigs (6). Whether PCV2 infection can be enhanced in pigs, which may result into PMWS, following administration of endosomal-lysosomal system acidification inhibitors in PCV2-infected pigs remains to be investigated. Further, different human pathophysiological conditions characterized by failure of endosomal-lysosomal system acidification such as hereditary and acquired forms of the Fanconi syndrome have been reported (39, 68). It is not known if such conditions exist in pigs and if they exist if they predispose pigs to PCV2-induced PMWS.

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FIGURE LEGENDS

Fig. 1: Effect of lysosomotropic weak bases on PCV2 Stoon-1010 infection of PK-15.
PK-15 cells were treated with RPMI-1640 (control), 25 mM NH₄Cl or 125 µM CQ starting from 1 hour before virus inoculation until 24 hours after virus inoculation. Cells were inoculated with the same dose of PCV2 (Stoon-1010) for 1 hour. At 36 hours after virus inoculation cells were fixed and stained for PCV2 antigens by IPMA. Magnification = 100x.

Fig. 2: PCV2 infection of primary kidney epithelial cells. Primary kidney cell cultures were obtained by trypsinization of kidney cortices. After 24 hours in culture, kidney cells were inoculated with PCV2 (Stoon-1010) and further cultured in (A) cell culture medium or (B) cell culture medium containing 125 µM CQ. Cells were fixed at 36 hours after virus inoculation and analyzed by fluorescent confocal microscopy after performing a double immunofluorescence staining to visualize epithelial cells (cytokeratin, green fluorescence) and PCV2 antigens (red fluorescence). Susceptibility of primary kidney epithelial cells to PCV2 infection increased following treatment with CQ (B). Each panel represents an overlay of confocal images taken from the apex to the base of cells. Bar, 20 µm.

Fig. 3: Effect of CQ at different time points and durations throughout the PCV2 infection cycle. PK-15 cells were inoculated with the same dose of PCV2 Stoon-1010 for 1 hour (0 to 1 hpi) after which the viral inoculum was washed-off. 0 or 125 µM CQ were added at different hours before (hbi) or after (hpi) virus inoculation and left with the cells for
different durations as indicated. After 36 hpi cells were fixed and stained for PCV2 antigens. The percentages of PK-15 PCV2-infected cells following CQ treatment are expressed relative to the number of PK-15 PCV2-infected cells in untreated cells. Data represent means ± standard deviations of results from three experiments with each experimental condition performed in quadruplicate.

Fig. 4: Effect of CQ treatment on PCV2 attachment and internalization. PCV2-VLP were either allowed to bind to PK-15 cells for 1 hour at 4 °C or further incubated for 3 hours at 37 °C in the absence or presence of 125 µM CQ, and then fixed with 3 % paraformaldehyde. PCV2-VLP were stained with F190 monoclonal antibody and FITC-conjugated goat anti-mouse followed by permeabilization of the cells. PCV2-VLP were stained again with F190 and Texas Red-conjugated goat anti-mouse antibodies. Bound PCV2-VLP showed both green and red fluorescence (yellow) while internalized PCV2-VLP only showed red fluorescence in merged confocal images of single z-sections showed in panels A - D. Internalized PCV2-VLP were visible in untreated cells (C) while no PCV2-VLP were visible within the cell in CQ treated cells (D), most probably due to increased disassembly of internalized PCV2-VLP. Bar, 20 μm.

Fig. 5: Co-localization of internalized PCV2-VLP with cellular organelles. PCV2-VLP were allowed to bind to PK-15 cells at 4 °C for 1 hour before cells were shifted to 37 °C to initiate PCV2-VLP internalization. After 3 hours of internalization, cells were fixed and stained for PCV2-VLP (green fluorescence) and one of the cellular organelles (red fluorescence). Representative merged images of PCV2-VLP and early endosomes (A),
lysosomes (B), the Golgi apparatus (C) and the endoplasmatic reticulum (D) after 3 hours of internalization are shown. Bar, 20 μm.
Table 1: Effect of lysosomotropic agents on PCV2 (Stoon 1010) infection of PK-15 cells.

The percentages of PCV2-infected PK-15 cells following treatment with lysosomotropic agents are expressed relative to the number of PCV2-infected cells in untreated PK-15 cells. Data represent means ± standard deviations from three experiments with each experiment performed in quadruplicate.

<table>
<thead>
<tr>
<th>Lysosomotropic agent</th>
<th>Concentration</th>
<th>Relative % of PCV2-infected cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH₄Cl</td>
<td>25.0 mM</td>
<td>726 ± 110</td>
</tr>
<tr>
<td></td>
<td>6.25 mM</td>
<td>540 ± 96</td>
</tr>
<tr>
<td></td>
<td>1.56 mM</td>
<td>252 ± 79</td>
</tr>
<tr>
<td>CQ</td>
<td>125.0 µM</td>
<td>1212 ± 34</td>
</tr>
<tr>
<td></td>
<td>31.25 µM</td>
<td>834 ± 169</td>
</tr>
<tr>
<td></td>
<td>7.81 µM</td>
<td>501 ± 121</td>
</tr>
<tr>
<td>Monensin</td>
<td>6 µM</td>
<td>1100 ± 179</td>
</tr>
<tr>
<td></td>
<td>1.5 µM</td>
<td>859 ± 134</td>
</tr>
<tr>
<td></td>
<td>0.375 µM</td>
<td>574 ± 24</td>
</tr>
<tr>
<td>Suramin</td>
<td>1000 µg/ml</td>
<td>237 ± 29</td>
</tr>
<tr>
<td></td>
<td>250 µg/ml</td>
<td>184 ± 23</td>
</tr>
<tr>
<td></td>
<td>62.5 µg/ml</td>
<td>133 ± 28</td>
</tr>
<tr>
<td>PVP</td>
<td>1000 µg/ml</td>
<td>73 ± 6</td>
</tr>
<tr>
<td></td>
<td>250 µg/ml</td>
<td>91 ± 3</td>
</tr>
<tr>
<td></td>
<td>62.5 µg/ml</td>
<td>95 ± 10</td>
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</table>
Table 2: Effect of lysosomotropic agents on infection of the prototype PCV2 strain Stoon-1010 in PK-15, SK, ST and primary porcine kidney epithelial cells (PPKE). The number of PCV2-infected cells following treatment with lysosomotropic agents are expressed as a relative percentage to the number of PCV2-infected cells in respective untreated cells. Data represent means ± standard deviations of three experiments with each experiment performed in quadruplicate.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>NH₄Cl</th>
<th>CQ</th>
<th>Monensin</th>
<th>Suramin</th>
<th>PVP</th>
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<tbody>
<tr>
<td>PK-15</td>
<td>726 ± 110</td>
<td>1212 ± 34</td>
<td>1100 ± 179</td>
<td>237 ± 29</td>
<td>73 ± 6</td>
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<tr>
<td>SK</td>
<td>128 ± 7</td>
<td>158 ± 3</td>
<td>142 ± 11</td>
<td>118 ± 3</td>
<td>80 ± 27</td>
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<tr>
<td>ST</td>
<td>160 ± 28</td>
<td>446 ± 50</td>
<td>162 ± 56</td>
<td>117 ± 5</td>
<td>86 ± 17</td>
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<tr>
<td>PPKE</td>
<td>313 ± 25</td>
<td>611 ± 86</td>
<td>352 ± 44</td>
<td>109 ± 15</td>
<td>90 ± 19</td>
</tr>
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</table>
Table 3: Effect of lysosomotropic agents on infection of different PCV2 strains of PK-15 cells. The relative percentages of PK-15 PCV2-infected cells following treatment with lysosomotropic agents are expressed relative to the number of PK-15 PCV2-infected cells in untreated cells. Data represent means ± standard deviations of results from three experiments with each experimental condition performed in quadruplicate.

<table>
<thead>
<tr>
<th>PCV2 strain</th>
<th>NH₄Cl</th>
<th>CQ</th>
<th>Monensin</th>
<th>Suramin</th>
<th>PVP</th>
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<tbody>
<tr>
<td>Stoon-1010</td>
<td>726 ± 110</td>
<td>1212 ± 34</td>
<td>1100 ± 179</td>
<td>237 ± 29</td>
<td>73 ± 6</td>
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<tr>
<td>1121</td>
<td>1041 ± 277</td>
<td>1452 ± 62</td>
<td>752 ± 148</td>
<td>206 ± 24</td>
<td>60 ± 10</td>
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<tr>
<td>1103</td>
<td>138 ± 86</td>
<td>183 ± 151</td>
<td>115 ± 12</td>
<td>128 ± 10</td>
<td>115 ± 9</td>
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<tr>
<td>48285</td>
<td>345 ± 147</td>
<td>856 ± 76</td>
<td>994 ± 119</td>
<td>434 ± 71</td>
<td>102 ± 9</td>
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<tr>
<td>VC2002</td>
<td>340 ± 92</td>
<td>905 ± 150</td>
<td>963 ± 246</td>
<td>271 ± 67</td>
<td>94 ± 20</td>
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<tr>
<td>1206</td>
<td>235 ± 88</td>
<td>446 ± 87</td>
<td>792 ± 206</td>
<td>344 ± 109</td>
<td>95 ± 2</td>
</tr>
<tr>
<td>1147</td>
<td>234 ± 88</td>
<td>530 ± 185</td>
<td>457 ± 42</td>
<td>201 ± 46</td>
<td>108 ± 2</td>
</tr>
</tbody>
</table>
FIGURES

Fig. 1

Control

NH₄Cl

CQ

Fig. 2

Control

CQ

A

B

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Fig. 3

Relative percentage of PCV2-infected cells

- 1 hbi to 0 hpi
- 0 hpi to 1 hpi
- 1 hbi to 1 hpi
- 1 hbi to 24 hpi
- 3 hpi to 24 hpi
- 6 hpi to 24 hpi
- 12 hpi to 24 hpi
- 18 hpi to 24 hpi
Fig. 4

Control

Chloroquine diphosphate

A

B

C

D

1 h at 4 °C

1 h at 4 °C

+ 3 h at 37 °C