Binding and entry characteristics of porcine circovirus 2 in cells of the porcine monocytic line 3D4/31

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Porcine circovirus 2 (PCV2) is associated with post-weaning multisystemic wasting syndrome and reproductive problems in pigs. Cells of the monocyte/macrophage lineage are important target cells in PCV2-infected pigs, but the method of binding and entry of PCV2 into these cells is unknown. Therefore, binding and entry of PCV2 to the porcine monocytic cell line 3D4/31 were studied by visualization of binding and internalization of PCV2 virus-like particles (VLPs) by confocal microscopy and chemical inhibition of endocytic pathways (clathrin- and caveolae-mediated endocytosis and macropinocytosis), followed by evaluation of the level of PCV2 infection.

It was shown that PCV2 VLPs bound to all cells, with maximal binding starting from 30 min post-incubation. Bound PCV2 VLPs were internalized in 47 ± 5.0 % of cells. Internalization was continuous, with 70 ± 5 ± 9.7 % of bound PCV2 VLPs internalized at 360 min post-incubation. Internalizing PCV2 VLPs co-localized with clathrin. PCV2 infection was decreased significantly by chemical inhibitors that specifically blocked (i) actin-dependent processes, including cytochalasin D (75 ± 5 ± 7.0 % reduction) and latrunculin B (71 ∓ 0 ± 0 % reduction), and (ii) clathrin-mediated endocytosis, including potassium depletion combined with hypotonic shock (50 ± 2 ± 6 ± 3 % reduction), hypertonic medium (56 ± 4 ± 5 ± 7 % reduction), cytosol acidification (59 ± 1 ± 7 ± 1 % reduction) and amantadine (52 ± 6 ± 7 ± 7 % reduction). Inhibiting macropinocytosis with amiloride and caveolae-dependent endocytosis with nystatin did not decrease PCV2 infection significantly. PCV2 infection was reduced by the lysosomotropic weak bases ammonium chloride (47 ± 0 ± 7 ± 9 % reduction) and chloroquine diphosphate (49 ± 0 ± 5 ± 6 % reduction).

Together, these data demonstrate that PCV2 enters 3D4/31 cells predominantly via clathrin-mediated endocytosis and requires an acidic environment for infection.

INTRODUCTION

Porcine circoviruses (PCVs) are small, non-enveloped viruses with a single-stranded, closed-circular DNA (Todd et al., 2000), classified in the genus Circovirus (Pringle, 1999) of the family Circoviridae. The first porcine circovirus (PCV1) was detected as a contaminant of the continuous porcine kidney (PK-15) cell line (Tischer et al., 1974) and was found to be non-pathogenic for pigs (Tischer et al., 1986). In 1997, an agent morphologically similar to but genetically and antigenically distinct from PCV1 was isolated from pigs with post-weaning multisystemic wasting syndrome (PMWS) (Ellis et al., 1998; Mankertz et al., 1998; Nawagitgul et al., 2000). The PCV2 virion has an icosahedral T=1 structure containing 60 capsid protein molecules arranged in 12 pentamer-clustered units (Crowther et al., 2003). PCV2 has been associated with different diseases and syndromes, two of which have been reproduced experimentally: PMWS (Allan et al., 1999) and reproductive problems (Sanchez et al., 2001). Recently, PCV2 target cells have been shown to be cardiomyocytes, hepatocytes and macrophages (mφ) during fetal life and mainly monocytes/mφ in early post-natal life (Sanchez et al., 2003). In experimentally inoculated pigs, the
majority of PCV2-infected pigs showed low or moderate levels of PCV2 replication, whilst a few showed high-level PCV2 replication (Sanchez et al., 2003). In those pigs with low or moderate levels of virus replication, the majority of PCV2-infected cells appeared to be differentiated mφ. In addition to differentiated mφ, infiltrating monocytes and lymphocytes were also infected in pigs with high levels of virus replication (Sanchez et al., 2004).

Although PCV2 target cells have been well characterized, the mechanistic details of the early stages of PCV2 infection, involving the attachment of virions to the cell surface by binding to their cellular receptors followed by entry into these target cells, are still poorly understood. The binding and entry of viruses determines the first phase of viral infection and it is important to characterize these early events for PCV2.

Initiation of viral infection requires entry of the virus into the host cell by direct penetration of the plasma membrane or, more often, through one or more of the endocytic pathways following interaction with cell-surface receptors. The endocytic pathways include clathrin- and caveolae-mediated endocytosis, macropinocytosis and clathrin- and caveolae-independent endocytosis (Conner & Schmid, 2003; Nichols & Lippincott-Schwartz, 2001). Non-enveloped viruses are internalized mainly via either clathrin- or caveolae-mediated endocytosis. Among the non-enveloped viruses, adeno-virus (Meier & Greber, 2003), adeno-associated virus (Bartlett et al., 2000), human polyomavirus JC (Pho et al., 2000) and canine parvovirus (Parker & Parrish, 2000) enter via clathrin-mediated endocytosis, whereas SV40 and mouse polyomavirus (Pelkmans & Helenius, 2002) enter via caveolae-mediated endocytosis.

Actin is involved in all forms of endocytosis (Engkvist-Goldstein & Drubin, 2003). Actin filaments facilitate uptake and delivery to the degradative compartments of ligands internalized via clathrin-mediated endocytosis (Durrbach et al., 1996). Actin polymerization is also required for the formation of the membrane protrusions at the site of internalization during macropinocytosis (Grimmer et al., 2002; Lee & Knecht, 2002), and cortical actin filaments confine and organize caveolae near the cell surface (Mundy et al., 2002). Polymerization of filamentous actin also occurs at endocytic sites in caveolae-mediated endocytosis (Pelkmans et al., 2002).

The endocytic pathway(s) offers a low pH-dependent conformational change that triggers fusion and/or uncoating of certain viruses. As such, some non-enveloped viruses, e.g. adenovirus type 2 (Varga et al., 1991), rhinovirus (Prchla et al., 1994), reovirus (Martinez et al., 1996) and canine parvovirus (Basak & Turner, 1992), are affected by lysosomotropic weak-base treatments that prevent endosomal acidification.

Several techniques have been used to determine the entry pathways of viruses, including the demonstration of co-localization of entering viruses with components of the cellular endocytosis machinery and the use of chemical inhibitors that affect different pathways of endocytosis (Sieczkarski & Whittaker, 2002). The aim of the present study was to determine the entry route of PCV2 into the porcine monocyte cell line 3D4/31 (Weingartl et al., 2002) by assessing the effect of different chemical entry inhibitors on PCV2 infection and by co-localization studies of recombinant PCV2 virus-like particles (VLPs) with components of the endocytic pathway(s) mediating PCV2 internalization by fluorescent confocal microscopy.

**METHODS**

**Virus.** PCV2 strain Stoon-1010 isolated from a PMWS-affected piglet in Canada (Ellis et al., 1998) was used in this study. A virus stock propagated in PK-15 cells with a titre of $10^{7.5}$ TCID$_{50}$ ml$^{-1}$ was used in all PCV2 infection experiments. Cells were inoculated with PCV2 at an m.o.i. of 0.5.

**Recombinant PCV2 VLPs.** A clarified lysate of *Spodoptera frugiperda* (Sf9) insect cells infected with a baculovirus recombinant P054, expressing ORF2 of PCV2, was used as a source of PCV2 VLPs. PCV2 VLPs were purified in a cesium chloride gradient as described previously (Nawagitgul et al., 2000). PCV2 VLPs were used in binding and internalization studies instead of PCV2 virions because of the difficulty in obtaining sufficient preparative amounts of the latter, due to low PCV2 titres. The correct conformation of PCV2 capsid proteins and PCV2 VLPs was demonstrated by the reactivity of PCV2-specific monoclonal antibodies F217 and F190 (McNeilly et al., 2001) and monospecific porcine polyclonal antibodies against PCV2 (Sanchez et al., 2003), and by electron microscopy. Negative-staining electron microscopy was performed according to the method of Nawagitgul et al. (2000) with 1 % phosphotungstic acid. Electron microscopy revealed the presence of single PCV2 VLPs (65 %), the rest being aggregates of two or more PCV2 VLPs (Fig. 1f and g). In parallel, different dilutions of PCV2 VLPs were smeared onto microscope slides (Menzel-Gläser), air-dried and fixed with 3 % (w/v) paraffomaldehyde in PBS with calcium and magnesium (PBS$+$) at room temperature for 10 min. PCV2 VLPs were then stained by using antibiotin-conjugated anti-PCV2 swine polyclonal antibodies (Sanchez et al., 2003) followed by fluorescein isothiocyanate (FITC)-conjugated streptavidin (Molecular Probes) for 1 h at each room temperature. For comparison purposes, $4.5 \times 10^{11}$ particles ml$^{-1}$ of 20 nm yellow-green-fluorescent (wavelength 505/515 nm) and $3.6 \times 10^{12}$ particles ml$^{-1}$ of 100 nm red-fluorescent (wavelength 580/605 nm) carbonylated-modified microspheres (Fluospheres; Molecular Probes) were used. Stained PCV2 VLPs and microspheres were mounted with a glycerol solution containing 1,4-diazabicyclo[2.2.2]octane (DABCO) anti-fading agent. Digital images of stained PCV2 VLPs and microspheres were acquired at the same magnification, using a Leica TCS SP2 laser-scanning spectral confocal system linked to a Leica DM/IRB inverted microscope (Fig. 1a–c). The fluorescence area of individual fluorescent spots in images of stained PCV2 VLPs and of 20 and 100 nm microspheres was calculated by using image-analysis software (SigmaScan Pro 5.0; Jandel Scientific) and their distribution is shown in Fig. 1(d). The number of PCV2 VLP fluorescence spots varied as a function of dilution (data not shown). The majority (55 %) of PCV2 VLP fluorescence spots had an area corresponding to that of 20 nm microspheres (Fig. 1d). However, some PCV2 VLP fluorescence spots had sizes larger than that of 20 nm microspheres, indicating the presence of small and large PCV2 VLP aggregates. Both fluorescent confocal and electron microscopy gave a comparable PCV2 VLP distribution (Fig. 1d and e).
Cells. The continuous porcine monocytic cell line 3D4/31, developed from porcine alveolar mφ (Weingartl et al., 2002), was grown in monocyte/mø medium comprising a 1:1 mixture of RPMI 1640 (Gibco-BRL) and minimal essential medium containing Earle’s salts (Gibco-BRL) supplemented with 10% fetal bovine serum (FBS), 0.3 mg glutamine ml⁻¹, 100 U penicillin ml⁻¹, 0.1 mg streptomycin ml⁻¹, 0.1 mg kanamycin ml⁻¹ and 1% non-essential amino acids (100 x; Gibco-BRL) in a humidified incubator at 37°C in the presence of 5% CO₂. Cells were seeded at 2 × 10⁵ cells ml⁻¹ in monocyte/mø medium onto microscope slides mounted with an eight-well cell-culture silicone chamber (Vivascience AG) for all PCV2 infection experiments. For experiments involving the use of PCV2 VLPs, cells were seeded at 1.5 × 10⁵ cells ml⁻¹ in monocyte/mø medium onto microscope slides mounted with a 12-well cell-culture silicone chamber (Vivascience AG). In both cases, cells were cultivated for 24 h before the experiments were performed.

Fig. 1. Visualization and analysis of PCV2 VLPs by fluorescent confocal and electron microscopy. (a–c) Representative fluorescent confocal images of immunostained PCV2 VLPs (a) and 20 nm yellow–green-fluorescent (b) and 100 nm red-fluorescent (c) carboxylate-modified microspheres acquired at the same magnification. Bar, 8 μm. (d) Fluorescence area distribution of PCV2 VLPs (□) and 20 nm (thin line) and 100 nm (thick line) fluorescent carboxylate-modified microspheres calculated from fluorescent confocal microscopy images by using SigmaScan Pro 5.0. (e) Distribution of PCV2 VLPs based on images from electron microscopy. (f, g) Electron microscopy images of PCV2 VLPs. Bar, 500 nm.
Binding kinetics of PCV2 VLPs on 3D4/31 cells. In order to establish the binding kinetics of PCV2 VLPs in 3D4/31 cells, cells were chilled on ice and washed with cold RPMI 1640 before the addition of PCV2 VLPs to the cells. PCV2 VLPs were added at a concentration of 2.7 × 10^{10} particles in 100 µl RPMI 1640 onto 3D4/31 cells and incubated for 0, 1, 5, 10, 15, 30 and 60 min at 4 °C. Cells were then washed to remove unbound PCV2 VLPs before they were fixed with 3 % (w/v) paraformaldehyde in PBS + at room temperature for 10 min. In order to stain the PCV2 VLPs, cells were incubated with biotin-conjugated anti-PCV2 swine polyclonal antibodies (Sanchez et al., 2003) for 1 h at room temperature before they were washed and incubated with FITC-conjugated streptavidin (diluted 1 : 100 in PBS; Molecular Probes) for 1 h at room temperature. The cells were mounted and analysed by acquisition of digital images of stained PCV2 VLPs by fluorescent confocal microscopy. Successive images from the apex to the base of a single cell were taken and merged. The total fluorescence area of PCV2 VLPs attached per cell was calculated by using SigmaScan Pro 5.0 for 20 cells at each time point to establish their binding kinetics onto 3D4/31 cells.

Internalization of PCV2 VLPs into 3D4/31 cells. To study the internalization of PCV2 VLPs, 3D4/31 cells were washed with RPMI 1640 at 37 °C and incubated with PCV2 VLPs at either 4 or 37 °C for 15 min, followed by washing of unbound PCV2 VLPs with RPMI 1640. Cells were then further incubated at 37 °C in monocyte/mésh medium without FBS. At 15, 60, 120, 180 and 360 min post-incubation of cells with PCV2 VLPs, cells were fixed with 3 % (w/v) paraformaldehyde in PBS + for 10 min at room temperature. Cells were subsequently washed with PBS + and permeabilized with Triton X-100 (0.1 % in PBS +) for 2 min at room temperature. After washing, PCV2 VLPs were stained by incubating the cells for 1 h at room temperature with polyclonal biotin-conjugated anti-PCV2 swine antibodies (Sanchez et al., 2003) followed by 1 h incubation at room temperature with FITC-conjugated streptavidin (1 : 100 in PBS; Molecular Probes). In order to visualize the cell border, actin filaments were stained by incubating the cells for 1 h at 37 °C with phalloidin-Texas red (1 : 100 in PBS; Molecular Probes). Stained cells were mounted and analysed by fluorescent confocal microscopy. PCV2 VLPs were scored as internalized once they crossed the cortical actin rim, based on merged confocal images. The fluorescence area of internalized and non-internalized PCV2 VLPs was taken and merged. The total fluorescence area of PCV2 VLPs as a function of time of incubation was calculated by using SigmaScan Pro 5.0 for 40 cells at each time point to establish their internalization kinetics onto 3D4/31 cells.

Effect of different entry inhibitors on PCV2 infection. 3D4/31 cells were susceptible to PCV2 infection and supported PCV2 replication with kinetics comparable to those observed in PK-15 cells (Meers et al., 2005). In order to investigate the entry route of PCV2 and a possible role of endosomal acidification in PCV2 infection of 3D4/31 cells, various chemicals that selectively disrupt cellular internalization pathways were used as shown in Table 1. Semi-confluent cells were washed and pre-incubated with twofold serial dilutions in monocyte/mésh medium of one of the following chemicals: ammonium chloride, amantadine, chloroquine diphosphate, cytochalasin D, latrunculin B, nystatin and/or amiloride for 1 h at 37 °C. Control cells were incubated in monocyte/mésh medium for 1 h at 37 °C. Control cells and treated cells were then inoculated with PCV2 at an m.o.i. of 0.5 at 37 °C for 1 h, followed by washing away of the inoculum with RPMI 1640. Thereafter, cells were further incubated for 24 h in monocyte/mésh medium before they were fixed. Some of the inhibitors were maintained in the monocyte/mésh medium during the course of infection (see Table 1). The concentrations of the inhibitors that were used were based on concentrations described in previous studies (see references in Table 1), provided that they were not toxic for the 3D4/31 cells. In order to determine cell viability, cells were incubated with different concentrations of inhibitors and washed with RPMI 1640, followed by incubation with 20 µg propidium iodide ml^{-1} for 10 min to stain dead cells.

Potassium depletion combined with hypotonic shock, cytosol acidification and hypertonic medium together with the appropriate controls were carried out as described by Hansen et al. (1993). Briefly, potassium depletion combined with hypotonic shock was done by rinsing and pre-incubating the cells for 15 min with potassium-depletion buffer (0-14 M NaCl, 20 mM HEPS, 1 mM CaCl_{2}, 1 mM MgCl_{2}, 1 mg d-glucose ml^{-1}, pH 7.4) before incubating the cells with a hypotonic buffer (potassium-depletion buffer diluted 1 : 1 with water to make it hypotonic) for 5 min (Hansen et al., 1993; Koval et al., 1998). Control cells were incubated in the same buffer supplemented with 10 mM KCl. Cells were treated with a hypertonic medium that consisted of Dulbecco’s modified Eagle’s medium (DMEM) without sodium bicarbonate (Gibco-BRL) supplemented with 2 mM l-glutamine and 20 mM HEPS and 0.45 M sucrose. Control cells were incubated in hypertonic medium lacking sucrose. For cytosol acidification, 1 M acetic acid, pH 5.0 (NaOH), diluted 1 : 100 in DMEM without sodium bicarbonate supplemented with 2 mM l-glutamine and 20 mM HEPS, pH 5.0 (HCl) was added, whereas control cells were incubated in DMEM without sodium bicarbonate supplemented with 2 mM l-glutamine, 20 mM HEPS, pH 5.0. After the treatments, cells were inoculated with PCV2. At 1 h post-inoculation, the viral inoculum was removed and cells were washed and further incubated in monocyte/mésh medium for 24 h before fixation with methanol (10 min at −20 °C). PCV2-infected cells were detected by immunofluorescence staining using a biotin-conjugated anti-PCV2 swine polyclonal antibody (Sanchez et al., 2003) and FITC-conjugated streptavidin (diluted 1 : 100 in PBS; Molecular Probes). Incubations were done at room temperature for 1 h. Cell nuclei were detected by incubating the cells for 10 min at room temperature with Hoechst 33342 (Molecular Probes) at a concentration of 10 µg ml^{-1}. After each of the incubations, cells were washed with PBS. Finally, stained cells were mounted and analysis of the percentage of PCV2-infected cells was done by using a Leica DM/RBE fluorescence microscope (see Fig. 4a). Approximately 10 000 cells were evaluated under each experimental condition and the data presented in this work are the results of independent triplicates. Differences between mean inhibitions with the different chemical inhibitors were analysed by using Student’s two-tailed t-test with si ss (version 6.1) software. A value of P<0.05 was considered significant.

Visualization of clathrin-mediated endocytosis of PCV2. Cells were washed with RPMI 1640 and incubated with either 2.7 × 10^{10} PCV2 VLPs in 100 µl monocyte/mésh medium without FBS containing biotinylated transferrin at 100 µg ml^{-1} or monocyte/mésh medium without FBS alone for 5, 15, 30 and 60 min. Cells were then washed with RPMI 1640, fixed with 3 % (w/v) paraformaldehyde in PBS + for 20 min at room temperature, washed again first with RPMI 1640 followed by Tris-buffered saline (20 mM Tris/HCl, 150 mM NaCl, pH 7.5) with 4.5 % sucrose and 2 % inactivated goat serum (TBS-GS) and permeabilized with methanol for 30 s at −20 °C (Racoosin & Swanson, 1994). Clathrin was stained by incubating the cells for 1 h at 37 °C with anti-clathrin heavy-chain IgM antibodies (ICN Biomedicals) diluted 1:50 in PBS supplemented with 0.3 % gelatin (PBS-G). Afterwards, cells were washed in TBS-GS and incubated for 1 h at 37 °C with biotin-labelled goat anti-mouse IgM (Santa Cruz Biotechnology) diluted 1:100 in PBS-G. After washing with TBS-GS, cells were incubated for 1 h at 37 °C with streptavidin–Texas red (Molecular Probes) diluted 1:50 in PBS-G and washed in TBS-GS (Van de Walle et al., 2001). PCV2 VLPs were stained using anti-PCV2 swine polyclonal antibodies.
### Table 1. Inhibitors of endocytosis and their modes of action

<table>
<thead>
<tr>
<th>Chemical inhibitor or method</th>
<th>Presence of inhibitor</th>
<th>Concentration range</th>
<th>Stage of inhibition</th>
<th>Mode of action</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>Hypertonic medium (sucrose)</td>
<td>+</td>
<td>0.45 mM</td>
<td>Clathrin-mediated endocytosis</td>
<td>Blocks clathrin-coated pit formation and interferes with interaction between clathrin and adaptors</td>
<td>Hansen <em>et al.</em> (1993); Heuser &amp; Anderson (1989)</td>
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<tr>
<td>Potassium depletion combined with hypotonic shock</td>
<td>+</td>
<td>25–50 mM</td>
<td>Clathrin-mediated endocytosis</td>
<td>Arrests coated pit formation and interferes with interaction between clathrin and adaptors</td>
<td>Hansen <em>et al.</em> (1993); Larkin <em>et al.</em> (1983)</td>
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<tr>
<td>Cytosol acidification</td>
<td>+</td>
<td>23.4–750 μM</td>
<td>Clathrin-mediated endocytosis</td>
<td>Stabilizes clathrin-coated vesicles, thereby limiting their fusion with other membranes and the formation of new ones</td>
<td>González-Dunia <em>et al.</em> (1998); Phonphok &amp; Rosenthal (1991); Van de Walle <em>et al.</em> (2001)</td>
</tr>
<tr>
<td>Amantadine</td>
<td>++</td>
<td>1.56–50 μM</td>
<td>Actin-dependent processes</td>
<td>Caps high-affinity ends of filamentous actin (F-actin), thereby retarding actin polymerization</td>
<td>Brown &amp; Spudich (1979); Cooper (1987); Flanagan &amp; Lin (1980); Fujimoto <em>et al.</em> (2000); Van de Walle <em>et al.</em> (2001)</td>
</tr>
<tr>
<td>Latrunculin B</td>
<td>+</td>
<td>2.96–95 μM</td>
<td>Actin-dependent processes</td>
<td>Specific sequestration of monomeric actin and binds to globular actin (G-actin) <em>in vitro</em>, inhibiting polymerization of these actin building blocks, and promotes depolymerization of F-actin</td>
<td>Fujimoto <em>et al.</em> (2000); Spector <em>et al.</em> (1983); Van de Walle <em>et al.</em> (2001)</td>
</tr>
<tr>
<td>Amiloride</td>
<td>+</td>
<td>0.375–6 mM</td>
<td>Macropinocytosis</td>
<td>A selective inhibitor of membrane protein Na⁺/H⁺ antiporters, described to selectively inhibit macropinocytosis and membrane ruffling without affecting clathrin-mediated endocytosis</td>
<td>Muro <em>et al.</em> (2003); West <em>et al.</em> (1989); Yan <em>et al.</em> (2004)</td>
</tr>
<tr>
<td>Nystatin</td>
<td>+</td>
<td>6.25–50 μM</td>
<td>Caveolae-mediated endocytosis</td>
<td>A sterol-binding agent that depletes membrane cholesterol, which is important for both maintenance of caveolae and the ability of caveolae to seal off from the plasma membrane</td>
<td>Akula <em>et al.</em> (2003); Rothberg <em>et al.</em> (1992); Sćiezkar &amp; Whittaker (2002)</td>
</tr>
<tr>
<td>Ammonium chloride</td>
<td>+</td>
<td>3.75–30 μM</td>
<td>Endosomal acidification</td>
<td>A weak lysosomotropic base that diffuses into acidic endosomes, where it becomes protonated. Once protonated, it is unable to diffuse out, thereby increasing the pH in these compartments</td>
<td>Akula <em>et al.</em> (2003); Gonzalez-Dunia <em>et al.</em> (1998); Maxfield (1982); Ohkuma &amp; Poole (1978); Rodríguez &amp; Everitt (1996)</td>
</tr>
<tr>
<td>Chloroquine diphosphate</td>
<td>+</td>
<td>3.12–50 μM</td>
<td>Endosomal acidification</td>
<td>A weak base that becomes protonated once in the endosomal vesicles, raising the pH in these compartments</td>
<td>Gonzalez-Dunia <em>et al.</em> (1998); Maxfield (1982); Ohkuma &amp; Poole (1978); Rodríguez &amp; Everitt (1996)</td>
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(diluted 1:100 in PBS-G) and FITC-labelled goat anti-swine antibodies (Jackson ImmunoResearch Laboratories) diluted 1:100 in PBS-G. Finally, stained cells were mounted and analysed by fluorescent confocal microscopy.

RESULTS

Kinetics of PCV2 VLP binding to 3D4/31 cells

The time course of binding of PCV2 VLPs to 3D4/31 cells was examined by incubating the cells with PCV2 VLPs at 4 °C for different durations, followed by staining for the PCV2 VLPs and acquisition of serial images across the cell by fluorescent confocal microscopy. The results of the interaction between PCV2 VLPs and 3D4/31 cells showed that single and aggregate forms of PCV2 VLPs bound to all of the 3D4/31 cells. The kinetics of binding of PCV2 VLPs to 3D4/31 cells were similar and time-dependent in all cells. The binding of PCV2 VLPs to 3D4/31 cells increased quickly within 5 min and reached a plateau starting from 15 min (Fig. 2a). These results showed that PCV2 VLPs bound to a saturable number of receptors on the surface of 3D4/31 cells. PCV2 VLPs bound to 3D4/31 cells with a random distribution over the entire cell surface (Fig. 2b).

Internalization of PCV2 VLPs into 3D4/31 cells

Many viruses are able to bind non-specifically to cells, which does not usually result in virus uptake and infection of a cell (Tardieu et al., 1982). To determine whether PCV2 VLP binding was followed by internalization, PCV2 VLPs were bound to 3D4/31 cells at 37 °C for 15 min; unbound PCV2 VLPs were then washed off and the cells were incubated at 37 °C for up to 360 min (Fig. 3). Internalization assays were carried out, starting with the binding of PCV2 VLPs at 37 °C instead of 4 °C, as it was shown that the internalization machinery of 3D4/31 cells was damaged after incubating them at 4 °C, as judged by transferrin internalization. PCV2 VLPs that crossed the cortical actin were scored as internalized (Fig. 3c). PCV2 VLPs were internalized slowly into 3D4/31 cells, with 47.3 ± 5.0 % of the 3D4/31 cells internalizing PCV2 VLPs during the course of the experiment (360 min) (Fig. 3a). In cells that showed internalization, the number of internalized PCV2 VLPs per cell increased gradually from 4.7 ± 3.6 % at 15 min to 70.5 ± 9.7 % at 360 min post-incubation (Fig. 3b). Single and aggregate forms of PCV2 VLPs crossed the cortical actin, indicating that both forms were internalized. Some cells showed complete internalization of the PCV2 VLPs at 120, 180 and 360 min post-incubation.

Disruption of actin inhibits PCV2 infection

3D4/31 cells were treated with cytochalasin D and latrunculin B to investigate the effect of actin polymerization on PCV2 infection. Cytochalasin D and latrunculin B treatments of 3D4/31 cells reduced PCV2 infection in a dose-dependent manner, as indicated in Fig. 4(b).

Inhibition of caveolae-mediated endocytosis and macropinocytosis does not decrease PCV2 infection significantly

3D4/31 cells were treated with nystatin and amiloride to investigate the involvement of caveolae-mediated endocytosis and macropinocytosis in PCV2 infection, respectively. Amiloride and nystatin did not reduce PCV2 infection significantly. Treatment of the cells at concentrations of 12.5
and 25 μM nystatin resulted in $4.4 \pm 7.2$ and $5.9 \pm 9.4\%$ reduction of PCV2 infection, respectively (Fig. 5). The inhibitory effect of nystatin on caveolae-mediated endocytosis in 3D4/31 cells was verified by using cholera toxin. The percentage of cholera toxin that was internalized into 3D4/31 cells after 30 min incubation at 37°C was $67.5 \pm 22.6\%$. Internalized cholera toxin was reduced to $11.4 \pm 5.4\%$ when 3D4/31 cells were treated with 25 μM nystatin. The maximum inhibition of PCV2 infection from all tested concentrations of amiloride was 2-4 ± 4-6% at 1-5 mM (Fig. 5).

**Inhibition of clathrin-mediated endocytosis reduces PCV2 infection**

Cells were treated with hypertonic medium, potassium depletion combined with hypotonic shock, cytosol acidification and amantadine in order to investigate the involvement of clathrin-mediated endocytosis during the initial stages of PCV2 infection. Potassium depletion combined with hypotonic shock decreased PCV2 infection by 50-2 ± 6-3%, whereas treatment of the cells with a hypertonic medium resulted in 56-4 ± 5-7% reduction. Acidification of the cytosol reduced PCV2 infection by 59-1 ± 7-1%. When the 3D4/31 cells were treated with different concentrations of amantadine, PCV2 infection was reduced in a dose-dependent manner. The percentage inhibition of PCV2 infection ranged from 35-7 ± 11-2 to 52-6 ± 6-7% when the cells were treated with concentrations ranging from 93-75 to 750 μM amantadine (Fig. 5).

**PCV2 VLPs co-localize with clathrin**

Visualization of the association of PCV2 VLPs and the cellular (clathrin-mediated) endocytic machinery was done by using fluorescent confocal microscopy. Clathrin

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**Fig. 3.** Analysis of PCV2 VLP internalization. 3D4/31 cells were incubated with PCV2 VLPs at 37°C for 15, 60, 120, 180 or 360 min. Cells were then fixed with 3% (w/v) paraformaldehyde in PBS+ and permeabilized. Cells were analysed by fluorescent confocal microscopy after performing a double immunofluorescence staining to visualize PCV2 VLPs (FITC signal) and actin (Texas red signal). In (a), the percentage of cells that showed PCV2 VLP internalization at each time point is shown, whilst (b) represents the percentage of PCV2 VLPs internalized per cell showing internalization. The images in (c) represent single sections through a cell at 15, 60, 180 and 360 min. PCV2 VLPs (left panels) were scored as internalized (arrowheads) once they were inside the cortical actin rim (middle panel) based on merged images (right panel). Bar, 8 μm.
co-localized with PCV2 VLPs in 3D4/31 cells at 30 and 60 min post-incubation, as indicated in Fig. 6. At earlier times before 30 min, few or no PCV2 VLPs were seen to co-localize with clathrin. Clathrin, but not PCV2 VLP, staining was observed in 3D4/31 cells not incubated with PCV2 VLPs and stained for clathrin and for PCV2 VLPs (Fig. 6). Similarly, cells incubated with PCV2 VLPs and stained for clathrin and PCV2 VLPs using negative swine serum instead of anti-PCV2 swine polyclonal antibodies also showed clathrin, but not PCV2 VLP, staining. As a control,

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**Fig. 4.** (a) Detection of PCV2-infected porcine monocytic 3D4/31 cells. 3D4/31 cells were inoculated with PCV2 strain Stoon-1010 at an m.o.i. of 0.5 for 1 h at 37 °C. The viral inoculum was then washed off and cells were incubated in monocyte/mφ medium before they were fixed at 24 h post-inoculation. The left panel shows 3D4/31 cells stained with anti-PCV2 swine polyclonal antibodies to detect PCV2-infected cells. The middle panel shows 3D4/31 cell nuclei following Hoechst 33342 staining, acquired at the same microscopic field as in the left panel. The merged image of both stainings in order to obtain the percentage of PCV2-infected cells is shown in the right panel. (b) Effect of actin disruption by cytochalasin D and latrunculin B on PCV2 infection. Semi-confluent monolayers of 3D4/31 cells were incubated with monocyte/mφ medium or monocyte/mφ medium containing various concentrations of cytochalasin D (■) and latrunculin B (□) for 1 h at 37 °C. Cells were then infected with PCV2 in the presence or absence of the inhibitors at 37 °C for 1 h. The viral inoculum was then washed away and cells were further incubated with monocyte/mφ medium for 24 h at 37 °C. Infection was evaluated by counting viral antigen-positive cells. Data are presented as percentage inhibition of virus infection compared with control cells (incubated with monocyte/mφ medium in the absence of inhibitors). Each point represents the mean ± SD of three experiments.

**Fig. 5.** Effect of inhibition of clathrin- and caveola-mediated endocytosis and macropinocytosis by amantadine, nystatin and amiloride, respectively, on PCV2 infection. Semi-confluent monolayers of 3D4/31 cells were incubated with monocyte/mφ medium or monocyte/mφ medium containing various concentrations of amantadine (○), nystatin (□) or amiloride (■) for 1 h at 37 °C. Cells were then infected with PCV2 in the presence or absence of the inhibitors at 37 °C for 1 h. Subsequently, the viral inoculum was washed away and cells were further incubated with monocyte/mφ medium for 24 h at 37 °C. Infection was evaluated by counting viral antigen-positive cells. Data are presented as percentage inhibition of virus infectivity observed when the cells were incubated with the virus in monocyte/mφ medium in the absence of inhibitors. Each point represents the mean ± SD of three experiments.
co-localization of transferrin with clathrin was studied. Transferrin was internalized and co-localized with clathrin at 5, 15 and 30 and 60 min post-incubation.

**Inhibition of acidification of endosomes reduces PCV2 infection**

Cells were treated with the lysosomotropic weak bases chloroquine diphosphate and ammonium chloride in order to establish the role of endosomal acidification on PCV2 infection. The degree of PCV2 infection inhibition by both inhibitors was dose-dependent, as shown in Fig. 7. Treatment of the cells with ammonium chloride reduced PCV2 infection maximally by 47.0 ± 7.9% at 30 μM and chloroquine diphosphate by 49.0 ± 5.6% at 50 μM.

**DISCUSSION**

The present study was undertaken to determine the entry pathway of PCV2 VLPs and virions into the porcine monocyctic cell line 3D4/31. By using fluorescent confocal microscopy, it was demonstrated in this study that PCV2 VLPs: (i) attached to the plasma membrane of all 3D4/31 cells in a rapid, time-dependent manner, giving rise to a random distribution pattern on the cell surface; (ii) internalized in 47.3 ± 5.0% of the 3D4/31 cells; and (iii) co-localized with clathrin during entry. Furthermore, it was observed that PCV2 infection of 3D4/31 cells was inhibited by: (i) chemical inhibition methods that perturbed clathrin-mediated endocytosis (including hypertonic medium, potassium depletion combined with hypertonic

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**Fig. 6.** PCV2 VLP co-localization with clathrin. 3D4/31 cells were incubated with PCV2 VLPs in monocyte/μφ medium without FBS at 37 °C for 30 or 60 min before they were fixed with 3% (w/v) paraformaldehyde in PBS+ for 20 min at room temperature, washed and permeabilized with methanol for 30 s at −20 °C. Control cells were incubated in monocyte/μφ medium without FBS and PCV2 VLPs for 30 min before they were fixed and permeabilized. Double immunofluorescence staining to detect PCV2 VLPs (left panels) and clathrin (middle panels) was performed in cells incubated with and without PCV2 VLPs. Cells were washed, mounted and analysed by fluorescent confocal microscopy. Arrowheads indicate co-localization between PCV2 VLP and clathrin in merged images (right panels). Bar, 8 μm.

**Fig. 7.** Effect of inhibition of endosomal acidification by ammonium chloride and chloroquine diphosphate on PCV2 infection. Semi-confluent monolayers of 3D4/31 cells were incubated with monocyte/μφ medium or monocyte/μφ medium containing various concentrations of ammonium chloride (■) or chloroquine diphosphate (◇) for 1 h at 37 °C. Cells were then infected with PCV2 in the presence or absence of inhibitor at 37 °C for 1 h. The viral inoculum was then washed away and cells were further incubated with monocyte/μφ medium for 24 h at 37 °C. Infection was evaluated by counting the number of viral antigen-positive cells. Data are presented as percentage inhibition of virus infectivity observed when the cells were incubated with the virus in monocyte/μφ medium in the absence of inhibitors. Each point represents the mean ± SD of three experiments.
shock, cytosol acidification and amantadine); (ii) actin disruption using cytochalasin D and latrunculin B; and (iii) lysosomotropic weak bases (ammonium chloride and chloroquine diphosphate) that prevent endosomal acidification.

To characterize the entry pathway of PCV2 VLPs into 3D4/31 cells, the attachment of PCV2 VLPs to the plasma membrane, an initial step in the virus infectious cycle, was first studied. PCV2 VLPs bound to the plasma membrane of all cells in a time-dependent manner, saturating the receptors within the first 15 min of incubation. The saturation of binding of PCV2 VLPs to 3D4/31 cells indicated that the PCV2 VLPs were interacting with cell-surface receptors. One of the criteria for viral recognition sites as receptors is saturability, the other criterion being specificity (Tardieu et al., 1982). The binding of PCV2 VLPs to all cells indicated that all cells expressed the attachment receptors for PCV2 at the time that the binding study was performed (24 h post-seeding). Furthermore, it was observed that PCV2 VLPs were distributed randomly throughout the surface of the plasma membrane of 3D4/31 cells, indicating that PCV2 attachment receptors have a similar distribution pattern. A specific, saturable binding of virions to the cell surface does not necessarily result in virus uptake, as some viruses require interaction with more than one cell-surface molecule to be internalized (Li et al., 1995; Roden et al., 1994). It was examined whether PCV2 VLPs that bound to the surface of 3D4/31 cells could subsequently be internalized by 3D4/31 cells. The results obtained in this study showed that, after 360 min incubation, only 47.3 ± 5.0% of the 3D4/31 cells internalized PCV2 VLPs. Although internalization of the PCV2 VLPs into cells showed a high variation, a time-dependent course was found. The binding and internalization of purified PCV2 virions in 3D4/31 cells closely resembled that of PCV2 VLPs (data not shown). It remains to be examined whether other cells known to be susceptible to PCV2 infection internalize PCV2 VLPs with characteristics similar to those described in this study with 3D4/31 cells. Together with the need for cellular polymerase, the restricted number of cells that allowed complete internalization of PCV2 VLPs may be an important cause of the very low percentage of target cells that are infected with PCV2 in vitro. Furthermore, the slow internalization may be the basis for the long replication cycle (24–36 h) of PCV2 in susceptible cells (Meerts et al., 2005). Whether the internalization of PCV2 VLPs observed in 47.3 ± 5.0% of the cells is sufficient to lead to infection in all of these cells could not be examined in this study. The proportion of PCV2 antigen-positive cells observed after in vitro inoculation of 3D4/31 cells with PCV2 at an m.o.i. of 5 was approximately 6%, implying that other factors, in addition to the internalization step, probably also govern the outcome of PCV2 infection.

The experimental results presented here demonstrate that clathrin-mediated endocytosis is involved in the entry process of PCV2 into 3D4/31 cells based on: (i) colocalization of clathrin with PCV2 VLPs, shown by fluorescent confocal microscopy; and (ii) inhibition of PCV2 infection by inhibitors of clathrin-mediated endocytosis. The blocking of PCV2 infection following the disruption of actin in 3D4/31 cells suggests that actin reorganization is involved in PCV2 entry. A role for the actin cytoskeleton in clathrin-mediated endocytosis has been shown by numerous studies (Durrbach et al., 1996; Jeng & Welch, 2001; Merrifield et al., 2002). However, disruption of the actin cytoskeleton gave greater inhibition of PCV2 infection than inhibitors for clathrin-mediated endocytosis. This suggests the involvement of other actin-dependent processes, in addition to clathrin-mediated endocytosis, in PCV2 entry. Inhibition of macropinocytosis with amiloride and caveolae-mediated endocytosis with nystatin did not affect PCV2 infection significantly, suggesting that these endocytic pathways are not involved in PCV2 internalization into 3D4/31 cells.

Early endosomes arise following the ATP-driven uncoating of clathrin-coated vesicles, and these vesicles mature into late endosomes and lysosomes. This is accompanied by a gradual pH drop that aids uncoating and escape into the cytoplasm for some viruses (Bomsel & Alfsen, 2003; Kirchhausen, 2000). The lysosomotropic weak bases ammonium chloride and chloroquine diphosphate clearly reduced PCV2 infection, indicating that endosomal acidification is necessary for successful PCV2 infection.

It can be concluded from these results that PCV2 enters monocytic cells predominantly via clathrin-mediated endocytosis and that endosomal acidification is an important requirement in PCV2 infection. Understanding the entry pathway of PCV2 can serve as an important basis for the screening of antiviral agents and the future development of antiviral strategies.

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REFERENCES


