Spring viraemia of carp virus enters grass carp ovary cells via clathrin-mediated endocytosis and macropinocytosis

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INTRODUCTION

Spring viraemia of carp virus (SVCV) is the causative pathogen of the outbreaks of an acute haemorrhagic and contagious viraemia responsible for the significant mortality in several cyprinid species. However, the endocytic pathway(s) and their regulatory molecules have not been characterized for SVCV. Here, using a combination of specific pharmacological inhibitors, transmission electron microscopy, immunofluorescence microscopy and real-time quantitative PCR, we found that SVCV entered grass carp ovary cells via clathrin-mediated endocytosis and macropinocytosis in a low-pH-dependent manner. We also discovered that dynamin II, actin microfilaments and microtubules were essential for SVCV internalization. Moreover, we found that the P21-activated kinase 1 inhibitor IPA-3 and the protein kinase C inhibitor rottlerin could block SVCV cell entry and replication, while phosphatidylinositol 3-kinase inhibitors wortmannin and LY294002 could promote SVCV infection. Results presented in this study provide helpful insight into revealing the initial steps of SVCV infection, and they may facilitate the development of therapeutic interventions.
triggers the activation of receptor tyrosine kinases to initiate macroinocytosis. Then, the activated signalling cascades induce plasma membrane ruffling, forming lamellipodial ruffles, circular ruffles or collapsing blebs. Notably, the cell-wide plasma membrane ruffling requires extensive cytoskeletal re-organization of actin microfilaments. Many cellular proteins have been demonstrated to be indispensable, like Rho GTPases (Rac1 or Cdc42), Na⁺/H⁺ exchangers, kinases [P21-activated kinase 1 (Pak1), phosphatidylinositol 3-kinase (PI3K) and protein kinase C (PKC)], myosin II, microtubules and Arf6 (Amstutz et al., 2008; Mercer & Helenius, 2009). However, reports also indicated that differential signalling molecules would be activated with different stimuli (Yoshida et al., 2015).

In the present study, we discovered that SVCV entered CO (grass carp ovary) cells via CME and macropinocytosis. We found that SVCV infection required dynamin II, actin microfilaments, microtubules and endosomal acidification. We also demonstrated that Pak1 and PKC were also indispensable for SVCV cell entry and replication, while PI3K inhibition could promote SVCV infection. Our findings will facilitate the revealing of the cell entry mechanism of SVCV and the development of anti-SVCV drugs. Our results also will be helpful for the elucidation of the initial infection steps of members in the family Rhabdoviridae.

RESULTS

SVCV enters CO cells via CME and macroinocytosis

To identify the SVCV endocytic pathway(s), we firstly blocked CME with chlorpromazine (CPZ) and macroinocytosis with 5-(N-ethyl-N-isopropyl)amiloride (EIPA). CPZ could specifically inhibit the assembly of clathrin-coated pits on the plasma membrane and could induce the assembly of clathrin lattice on endosomes (Liu et al., 2011). EIPA is an inhibitor of the Na⁺/H⁺ exchangers, which could prevent the formation of membrane ruffles in macroinocytosis (Mercer & Helenius, 2009). First of all, working concentrations of inhibitors were determined by cell viability testing with CCK-8 assays (Huang et al., 2015), and the highest concentrations that would not induce significant cytotoxic effects were used as the highest working concentrations (Table 1). Then, Alexa Fluor 488-labelled transferrin and Alexa Fluor 594-labelled dextran were used as control cargos for evaluation of the efficacies of CPZ and EIPA on CME and macroinocytosis (Piccinotti et al., 2013; Han et al., 2016). As shown in Fig. 1(a), flow cytometry assays indicated 10 µM CPZ and its 1 : 2 and 1 : 10 dilutions all could inhibit the uptake of transferrin, while 2.5 µM EIPA and its 1 : 2 and 1 : 10 dilutions could inhibit the uptake of dextran in CO cells. Next, to examine whether the inhibitors were effective for SVCV entry and to discriminate the effects between entry and post-entry steps of viral infection, we separately added CPZ and EIPA to CO cells 2 h prior to or post-infection (p. i.) with SVCV at an m.o.i. of 10. Release and synthesis of the internal virion matrix (M) protein following successful permeabilization were detected by immunofluorescence microscopy (IF) at 18 h p.i. As indicated in Fig. 1(b), pre-inoculation treatments with both CPZ and EIPA inhibited SVCV infection to CO cells in a dose-dependent manner. Besides, real-time quantitative PCR (RT-qPCR) quantification of the N gene at 24 h pi (Fig. 1c) also resulted in gradually improving inhibitory efficiency for pre-treatments with the increase of drug concentration. Similarly, Western blot analysis of the M protein at 24 h pi also confirmed the dose-dependent inhibition result (Fig. 1d). Meanwhile, all post-treatments induced no significant changes of SVCV infection compared with the controls, and statistical analysis revealed that all the differences between pre-treatments and post-treatments were significant.

To further investigate the endocytic process of SVCV, we visualized the internalization of SVCV by transmission electron microscopy (TEM). SVCV virions were identified as bullet-shaped particles with the diameter of approximately 100 nm. To observe the internalization of SVCV, primary CO cells were infected with SVCV at an m.o.i. of 10. Release and synthesis of the M protein following successful permeabilization were detected by immunofluorescence microscopy (IF) at 18 h p.i. As indicated in Fig. 1(b), pre-inoculation treatments with both CPZ and EIPA inhibited SVCV infection to CO cells in a dose-dependent manner. Besides, real-time quantitative PCR (RT-qPCR) quantification of the N gene at 24 h pi (Fig. 1c) also resulted in gradually improving inhibitory efficiency for pre-treatments with the increase of drug concentration. Similarly, Western blot analysis of the M protein at 24 h pi also confirmed the dose-dependent inhibition result (Fig. 1d). Meanwhile, all post-treatments induced no significant changes of SVCV infection compared with the controls, and statistical analysis revealed that all the differences between pre-treatments and post-treatments were significant.

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Table 1. Pharmacological inhibitors used in this study

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Target</th>
<th>Highest concentration used</th>
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<tr>
<td>CPZ</td>
<td>CME</td>
<td>10 µM</td>
</tr>
<tr>
<td>Pitstop2</td>
<td>CME</td>
<td>5 µM</td>
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<tr>
<td>EIPA</td>
<td>Macropinocytosis</td>
<td>2.5 µM</td>
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<tr>
<td>Nystatin</td>
<td>Caveola-dependent endocytosis</td>
<td>25 µg ml⁻¹</td>
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<tr>
<td>Methyl-β-cyclodextrin</td>
<td>Caveola-dependent endocytosis</td>
<td>2 µM</td>
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<tr>
<td>Dynasore</td>
<td>Dynamin</td>
<td>100 µM</td>
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<td>Bafilomycin A1</td>
<td>Vacular ATPase inhibition</td>
<td>2 nM</td>
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<td>Latrunculin B</td>
<td>Actin microfilaments</td>
<td>0.5 µM</td>
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<td>Nocodazole</td>
<td>Microtubules</td>
<td>10 µM</td>
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<td>Wortmannin</td>
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<td>LY294002</td>
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<td>IPA-3</td>
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<td>Rottlerin</td>
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100 nm containing an envelope structure (Fig. 2a). CO cells were then inoculated with SVCV at an m.o.i. of 100 at 4°C for 30 min. At low temperature, viruses can bind to their receptors on the cell membrane but cannot be internalized into cells. Attachment of SVCV particles to the plasma membrane was observed after absorption, but no internalized viral particle was detected (Fig. 2b). Then, cultures were quickly warmed up to 20°C and incubated for 15 min to allow viral internalization. As shown in Fig. 2(c), clathrin-coated pits were identified as invaginations of the

![Graphs and Images]

**Fig. 1.** CPZ and EIPA inhibit SVCV infection. (a) Effects of CPZ and EIPA on the uptake of control cargos. Alexa Fluor 488-labelled transferrin was used as the control cargo for clathrin-mediated endocytosis and Alexa Fluor 594-labelled dextran was used as the control cargo for macropinocytosis. Cells were pre-treated with inhibitors for 15 min, and 20 μg ml⁻¹ transferrin or 0.5 mg ml⁻¹ dextran was added. Dextran was washed out after 10 min of incubation, and transferrin was washed out after 20 min of incubation. Cells were then proceeded to flow cytometry assay. **P<0.01 and ***P<0.001. (b–d) CO cells were treated with double-distilled water (ddH₂O), DMSO, CPZ or EIPA 2 h prior to (pre-inoculation) or post-inoculation with SVCV (m.o.i.=10). (b) IF statistical result of SVCV infection. Cells were fixed at 18 h pi. Release and expression of the M protein were monitored as the indication of viral infection. Total and fluorescent cells were counted in five random fields at ×100 magnifications under a microscope. Relative infectivity was calculated by dividing total cells by fluorescent cells. Mean number of the five micrographs was taken as the percentage of infected cells. Control treatments were normalized to 100%. (c) Quantitative analysis of the N gene by RT-qPCR method. Cells were harvested at 24 h pi. Five independent experiments were performed. (d) Western blot analysis of the M protein expression at 24 h pi. Tubulin was taken as the endogenous reference.
plasma membrane surrounded by an electron-dense coat with an average interior diameter of 60–100 nm (Piccinotti et al., 2013). Virion-like particles were found in the caveole-like invaginations on the cell surface (Fig. 2c). In addition, macropinosomes were identified as large vacuoles of the plasma membrane with a diameter of 0.5–10 µm (Mercer & Helenius, 2009). At 15 min p.i., SVCV infection also induced the protrusion of plasma membrane and the formation of lamellipodia ruffles. SVCV particles also were found to be engulfed in the ruffles (Fig. 2d). Taking the results in Fig. 1 together, we speculated that the endocytosis of SVCV was CME and macropinocytosis dependent in CO cells.

**SVCV cell entry is caveola independent**

Existing researches indicate that caveola-dependent endocytosis strictly depends on cholesterol, which is the main component of lipid rafts in caveolae. To check whether caveola-dependent endocytosis also served as a route for SVCV internalization, we examined the effects of the cholesterol-depletion reagent methyl-b-cyclodextrin (MβCD). As indicated in Fig. 3(a), both nystatin and MβCD inhibited the uptake of cholera toxin subunit B (CTxB), a marker for caveola-dependent endocytosis (Hernaez et al., 2010). Despite this, IF results indicated that cells subjected to nystatin or MβCD treatments showed no difference in SVCV infection compared with control treatments (data not shown). Quantification of the N gene copies with RT-qPCR also revealed that there was no significant difference between nystatin, MβCD and control treatments (Fig. 3b). Furthermore, Western blot analysis proved that there was negligible difference of the expression of the M protein between nystatin, MβCD, DMSO and double-distilled water (ddH₂O) treated cells (Fig. 3c). All these results indicated that the entry of SVCV into CO cells was not via caveola-dependent endocytosis.

**SVCV entry requires dynamin II and endosomal acidification and depends on only CME and macropinocytosis in CO cells**

Dynamin II, a 100 kDa large GTPase, is reported to be essential for the scission step of CME. For macropinocytosis, many reports indicated that dynamin II was not required (Mercer & Helenius, 2009). However, as reported by Han et al. (2016), dynamin II was crucial for the macropinocytosis of FMDV. Moreover, there were also reports illustrated that dynamin II was involved in the regulation of Rac1 localization and function. Activated Rac1 could further regulate the polymerization of actin and the contractibility of myosin, which induced the formation of membrane ruffles and the closure of macropinosomes (Ridley et al., 1992). To ascertain whether SVCV infection was dynamin II dependent, we evaluated the effect of dynasore, a cell-permeable inhibitor of the GTPase activity of dynamin II (Macia et al., 2006). Compared with DMSO treated controls, IF, RT-qPCR and Western blot assays all demonstrated pre-additions of dynasore significantly inhibited SVCV infection (Fig. 4a).

After transporting in cytosol, macropinosomes or uncoated clathrin pit vesicles generally will rapidly be delivered to early endosomes, which further mature to late endosomes. pH values for early endosomes are about 6.8–6.1, while pH values are about 6.0–4.8 for late endosomes (Huotari & Helenius, 2011). The acidification in endosomal maturation plays a fundamental role in the infection of many viruses, which provides a low-pH environment that could induce conformational rearrangements of viral fusion proteins to expose the hydrophobic fusion peptides and then to release the virions. To assess the role of endosomal acidification in SVCV infection, we examined the effect of Bafilomycin A1 (BAF A1), an inhibitor of the H⁺ ATPase pump, on CO cells. IF statistical results showed that all BAF A1 pre-treatments significantly inhibited SVCV infection and 0.2 nM BAF A1 pre-treatment was enough to block SVCV infection.

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**Fig. 2.** Ultrastructural visualization of the uptake of SVCV into CO cells by transmission electron microscope. (a) Electron micrographs of negatively stained purified SVCV particles. (b–d) CO cells were incubated with SVCV at an m.o.i. of 100 for 30 min at 4 °C and then shifted to 20 °C for 15 min. Then cells were fixed and processed as outlined in Methods. The arrows indicate SVCV virions. (b) Binding of SVCV particles after absorption on the plasma membrane. (c) Internalization of an SVCV particle by a clathrin-coated pit. (d) Internalization of an SVCV particle by a macropinosome.
by more than 50% (Fig. 4b, left). Consistently, RT-qPCR quantification of the N gene and Western blotting analysis of the M protein also revealed a dose-dependent inhibition of the entry of SVCV by BAF A1 (Fig. 4b). Meanwhile, all BAF A1 post-treatments showed no obvious disruption of the expression of the M and N gene. Hence, SVCV infection was low pH dependent.

It has been reported that lymphocytic choriomeningitis virus, influenza virus and human papillomavirus type 16 can use clathrin- and caveola-independent endocytic pathway to enter host cells (Quirin et al., 2008; Schelhaas et al., 2012; Sieczkarski & Whittaker, 2002). To exclude other potential pathways that SVCV could utilize, we treated CO cells with combinations of 1 µM CPZ and 0.25 µM EIPA, 1 µM CPZ and 10 µM

![Fig. 3. SVCV infection is caveola independent. (a) Effects of nystatin and MβCD on the uptake of the control cargo Alexa Fluor 488-labelled CTxB. Cells were pre-treated with inhibitors for 15 min, and 20 µg ml⁻¹ CTxB was added. After 20 min of incubation, CTxB was washed out. Cells were then proceeded to flow cytometry assays. ***P<0.001. (b, c) CO cells were treated with nystatin or MβCD 2 h prior to (pre-inoculation) or post-inoculation with SVCV (m.o.i.=10). (b) Quantitative analysis of the N gene by RT-qPCR method. Cells were harvested at 24 h pi. Five independent experiments were performed. (c) Western blot analysis of the M protein expression. Cells were harvested at 24 h pi and α-tubulin was taken as the endogenous reference.](image-url)
dynasore, 0.2 nM BAF A1 and 0.25 µM EIPA and 0.2 nM BAF A1 and 1 µM CPZ. As indicated in Fig. 4(c), both IF and Western blot assays indicated all combinations induced additional inhibitory effects than single treatments. Meanwhile, we also treated CO cells with 5 µM CPZ and 1.25 µM EIPA, 5 µM CPZ and 100 µM dynasore, 1 nM BAF A1 and 1.25 µM EIPA and 1 nM BAF A1 and 5 µM CPZ, combinations 2 h prior to SVCV infection at an m.o.i. of 40, resulting in nearly no positive infection with IF and Western blot detections (data not shown). All these features were consistent with the CME and macropinocytosis-mediated SVCV uptake and eliminated other possible SVCV endocytic pathways in CO cells.

**SVCV cell entry depends on actin microfilaments and microtubules**

The cytoskeleton is an important transportation network responsible for the uptake of extracellular materials. Documents also indicated the sequential involvement of actin microfilaments and microtubule networks in CME and macropinocytosis (Cureton et al., 2009; Yarar et al., 2005).
To explore the roles of cytoskeletal proteins in SVCV infection, we tested the actin microfilaments depolymerization drug Latrunculin B (Lat B) and the microtubules depolymerization drug nocodazole. If results indicated that all Lat B and nocodazole pre-treatments significantly inhibited the internalization of SVCV, and the inhibition efficiencies displayed a dose-dependent manner (Fig. 5a). RT-qPCR analysis of the N gene and Western blot detection of the M protein also indicated that all pre-treatments significantly inhibited the entry of SVCV compared with the controls and the corresponding post-treatments (Fig. 5b, c). Consequently, the entry of SVCV required cytoskeletal actin microfilaments and microtubules. However, post-administration of Lat B or nocodazole at high dose also moderately depressed the N and M gene expression (Fig. 5b, c). Moreover, cells seemed to lose of contractibility and went to round at 16 h post-treatment with high concentrations of Lat B or nocodazole (data not shown).

PI3K is not required for SVCV endocytosis

Reports indicated that PI3K was indispensable for the macropinocytic cups closure, ruffle formation and micropinocytosis (Mercer et al., 2010b). Studies in vaccinia virus, African swine fever virus and respiratory syncytial virus also revealed that PI3K was pivotal for their macropinocytosis (Mercer & Helenius, 2008, 2009; Sandgren et al., 2010). In the present study, PI3K inhibitors wortmannin (Wort) and LY294002 were used to define the role of PI3K in SVCV macropinocytosis. At first, CO cells were treated with 5 or 2 µM Wort 2 h pre-SVCV inoculation or post-SVCV inoculation at an m.o.i. of 10. However, IF results indicated that all treatments did not induce any inhibitory effect on SVCV infection (data not shown). Then, we lowered the inoculation m.o.i. to 1. As shown in Fig. 6(a), IF analysis revealed that Wort and LY294002 treatments did not block SVCV infection. Instead, both pre-treatments promoted SVCV infection (compared with DMSO treatment: 1.42-fold for 5 µM Wort pre-treatment, 2.76-fold for 2 µM Wort pre-treatment, 1.58-fold for 10 µM LY294002 pre-treatment and 1.27-fold for 5 µM LY294002 pre-treatment, all P<0.05). Moreover, compared with corresponding post-treatments, RT-qPCR quantification of the N gene also displayed a 2.28-fold increase for 2 µM Wort pre-treatment, a 1.81-fold increase for 10 µM LY294002 pre-treatment and a 2.20-fold increase for 5 µM LY294002 pre-treatment (all P<0.05) (Fig. 6b). Likewise, Western blot analysis confirmed that Wort and LY294002 pre-treatments could promote SVCV infection (Fig. 6c). Additionally, compared with DMSO post-treatment, all Wort post-treatments brought no statistically significant changes of the entry of SVCV, while LY294002 post-treatments induced some enhancement. Meanwhile, dextran uptake assay indicated that both compounds inhibited the cellular macropinocytosis. Taken together, inhibition of PI3K did not prevent SVCV endocytosis but promoted viral infection, implying that SVCV entry did not require PI3K activity.

Pak1 activation and PKC are required for SVCV entry

Pak1 is a Rho GTPase Cdc42/Rac1 activated serine/threonine kinase, which can regulate the PI3K/Akt, Ras/ERK, Src/Fak and various other signalling pathways. Pak1 is also an effector of the actin cytoskeleton, macropinocytic ruffle formation and macropinosome closure. Requirement of Pak1 has been reported to be essential during all stages of macropinocytosis (Mercer & Helenius, 2009). Meanwhile, PKC is another serine/threonine kinase frequently involved in regulating membrane-proximal cellular processes, cytoskeletal regulation and macropinocytosis. Activation of PKC also has been reported to promote the formation of membrane ruffles and macropinosomes. Here, we employed IPA-3, an allosteric inhibitor of Pak1, to define the role of Pak1 in the endocytosis of SVCV. We also used rottlerin to explore of the role of PKC in SVCV infection. As indicated in Fig. 7(a), both IPA-3 and rottlerin could block dextran uptake in CO cells. Meanwhile, results of IF, RT-qPCR and Western blotting all showed that IPA-3 and rottlerin pre-treatments strongly decreased viral multiplication in a dose-dependent manner (Fig. 7b–d). Nonetheless, post-treatments with both drugs at high dose also suppressed the replication of SVCV, indicating that Pak1 and PKC are indispensable not only for the entry step but also for the post-entry steps of SVCV infection.

DISCUSSION

SVCV is a worldwide spread OIE-listed notifiable pathogen, which has brought huge economic loss to the aquaculture industry. In this study, a variety of specific inhibitors were utilized to investigate the exact endocytic pathway(s) taken by SVCV. We found that CPZ and EIPA pre-treatments both inhibited SVCV infection. We also observed engulfs SVCV particles in clathrin-coated pits and in macropinosome-like structures, implying that SVCV internalization was CME and macropinocytosis dependent. Meanwhile, IF, Western blotting and RT-qPCR assays all proved that nystatin and MβCD treatments did not impair SVCV infection, suggesting that SVCV entry was caveola independent in CO cells. Moreover, 5 µM CPZ and 1.25 µM EIPA combination treatment totally abolished SVCV infection at an m.o.i. as high as 40. Hence, it is unlikely that SVCV could use other pathways to enter CO cells. Noteworthy, some viruses, like enterovirus 71 and Japanese encephalitis virus, can exploit different pathways to enter cells from different tissues. For SVCV, it shows a broad tissue tropism and can exploit systemic haemorrhagic symptoms, while the CO cell line is a culture from grass carp ovary. Therefore, the results in this study cannot be deduced unlimitedly, and whether there are alternative endocytic pathways for the infection of SVCV to other tissues remains to be verified.

In this study, 10 µM CPZ or 2.5 µM EIPA single treatment could block SVCV entry by more than 90 %, suggesting that one or both inhibitors exerted certain off-target, unspecific effects. As reported by Vercauteren et al. (2010), the CPZ...
inhibitory effect was highly cell line dependent and relatively poorly specific. Thus, we also tested Pitstop2, an inhibitor of the interaction of amphiphysin with the N-terminal domain of clathrin heavy chain (Pelassa et al., 2014). However, it did not show any inhibitory effect on the uptake of transferrin in CO cells and exhibited a negligible effect on the endocytosis of SVCV with the dose of 5 µM or below (data not shown). Meanwhile, treatments with concentrations higher than 5 µM induced high cytotoxicity to CO cells, and nearly all cells died after 2 h of incubation. Besides, although macropinocytosis is uniquely sensitive to EIPA and its analogues, Koivusalo et al. (2010) reported

![Graphs and images]

**Fig. 5.** Cytoskeletal actin microfilaments and microtubules are required for SVCV infection. CO cells were treated with DMSO, Lat B or nocodazole 2 h prior to (pre-inoculation) or post-inoculation with SVCV (m.o.i.=10). (a) IF statistical result of SVCV infection. Release and expression of the M protein were monitored at 18 h pi as the indication of viral infection. Total and fluorescent cells were counted in five random fields at ×100 magnifications. Relative infectivity was calculated by dividing total cells by fluorescent cells. Mean number of the five micrographs was taken as the percentage of infected cells. Control treatments were normalized to 100 %. ***P<0.001. (b) Quantitative analysis of the N gene by RT-qPCR method. Cells were harvested at 24 h pi. Five independent experiments were performed. *P<0.05 and **P<0.01. (c) Western blot analysis of the M protein expression. Cells were harvested at 24 h pi and α-tubulin was taken as the endogenous reference.
that EIPA was neither a direct nor a specific inhibitor of macropinocytosis in A431 cells. They also indicated that the inhibitory effect of EIPA was the consequence of submembranous acidification caused by metabolic $H^+$ generation, and this acidification also affected transferrin uptake by CME. EIPA also has been reported to inhibit conductive $Na^+$ channels and $Na^+$/Ca$^{2+}$ exchangers (Alvarez de la Rosa et al., 2000; Masereel et al., 2003). Thus, for further

**Fig. 6.** PI3K inhibition promotes SVCV infection. CO cells were treated with DMSO, Wort or LY294002 2 h prior to (pre-inoculation) or post-inoculation with SVCV (m.o.i.=1). (a) IF statistical result of SVCV infection. Release and expression of the M protein were monitored at 18 h pi as an indication of viral infection. Total and fluorescent cells were counted in five random fields at $\times 100$ magnifications. Relative infectivity was calculated by dividing total cells by fluorescent cells. Mean number of the five micrographs was taken as the percentage of infected cells. Each experiment was carried out in triplicate. Control treatments were normalized to 100 %. *$P<0.05$, **$P<0.001$. (b) Quantitative analysis of the N gene by RT-qPCR method at 24 h pi. Five independent experiments were performed. **$P<0.01$. (c) Western blotting evaluation of the M protein expression. Cells were harvested at 24 h pi and $\alpha$-tubulin was taken as the endogenous reference. (d) Effects of Wort and LY294002 on the uptake of dextran. Cells were pre-treated with inhibitors for 15 min, and 0.5 mg ml$^{-1}$ Alexa Fluor 594-labelled dextran was added. After 10 min of incubation, dextran was washed out and cells were then proceeded to flow cytometry assay.
Fig. 7. SVCV infection requires Pak1 and PKC. (a) Effects of IPA-3 and rottlerin on the uptake of dextran. Cells were pretreated with inhibitors for 15 min, and 0.5 mg ml$^{-1}$ Alexa Fluor 594-labelled dextran was added. After 10 min of incubation, dextran was washed out, and cells were then proceeded to flow cytometry assays. **$P<0.01$ and ***$P<0.001$. (b–d) CO cells were treated with DMSO, IPA-3 or rottlerin 2 h prior to (pre-inoculation) or post-inoculation with SVCV (m.o.i.=10). (b) IF statistical result of SVCV infection. Cells were fixed at 18 h pi. Release and expression of the M protein were monitored as an indication of viral infection. Total and fluorescent cells were counted in five random fields at $\times 100$ magnifications under
confirmation, we examined roles of other key participators in CME and macropinocytosis. We found that dynamin II, actin microfilaments, microtubules and endosomal acidification were all essential for SVCV infection. However, to verify the SVCV endocytic pathways more concretely and to discriminate which pathway is the predominate one, further works are still needed.

Although PI3K activity was frequently reported to be crucial for macropinocytosis, in the present study, treatments with PI3K inhibitors Wort and LY294002 did not suppress SVCV infection. Recently, similar observations have been reported in vaccinia virus, coxsackievirus A9 and FMDV (Huttunen et al., 2014; Mercer et al., 2010a; Han et al., 2016), while the regulatory mechanism remains unrevealed. Araki et al. (1996) reported that Wort and LY294002 could block macropinocytic cups closure but not ruffle closure. Additionally, in contrast to circular ruffles, the membrane scission step that separates lamellipodial macropinosomes from the plasma membrane requires CtBP-1 instead of dynamin (Mercer et al., 2010a). Besides, CtBP-1 has been reported to be activated by Pak1. In this study, we also observed the formation of lamellipodial ruffles under TEM after SVCV stimulation. Moreover, we discovered that the Pak1 inhibitor IPA-3 could block SVCV infection. Furthermore, pre-treatment with PKC inhibitor rottlerin also suppressed SVCV entry and replication. Thus, we speculate that SVCV infection may trigger the activation of PKC and then induces the formation of lamellipodial ruffles. Meanwhile, similar to FMDV, Wort and LY294002 treatments promoted SVCV infection, whereas the molecular mechanisms are also still unknown. Kwon et al. (2000) reported that there was a putative phosphorylation site in Rac1, which could be phosphorylated by the activated Akt kinase in the PI3K signalling pathway, and the phosphorylation of Rac1 would inhibit its GTP-binding activity. Therefore, it is possible that inhibition of PI3K up-regulates Rac1 activity and then further promotes the viral macropinocytosis.

To our knowledge, this is the first study investigating the endocytic pathways utilized by SVCV. Our results demonstrate that SVCV enters CO cells via CME and macropinocytosis in a low-pH-dependent manner. The endocytic process also requires the participation of dynamin II, actin microfilaments and microtubules. Meanwhile, the macropinocytosis of SVCV does not require PI3K activity, but it needs the participation of Pak1 and PKC. Our findings provide preliminary insights into the mechanism of SVCV internalization and will be helpful for the development of anti-SVCV drugs.

**METHODS**

**Cells and viruses.** CO (grass carp ovary) cells were obtained from the Chinese Academy of Inspection and Quarantine and maintained in monolayers in M199 medium (GIBCO) supplemented with 10% FBS at 25 °C. The SVCV-265 strain was isolated and propagated in our laboratory (Xiao et al., 2014). Viral titres were determined by plaque assay.

**Inhibitor cytotoxicity test.** Stock solutions of inhibitors were prepared as follows: 10 mM CPZ (Sigma), 10 mM Pitstop2 (Abcam), 10 mM EIPA (Sigma), 5 mg ml⁻¹ nystatin (Sigma), 20 mM MJCD (Sigma), 40 mM dynasore (Sigma), 40 µM BAF A1 (Calbiochem), 2 mM Lat B (Sigma), 4 mM nocodazole (Sigma), 20 mM IPA-3 (Selleck), 8 mM rottlerin (Sigma), 20 mM LY294002 (Selleck) and 10 mM Wort (Selleck). As specified by the manufacturers, CPZ and MJCD were dissolved in ddH₂O, while the others were prepared in DMSO (Sigma).

Cytotoxic effects of all inhibitors were examined by the CCK-8 assay. Briefly, CO cells were seeded in a 96-well plate at 10⁵ per well density 12 h before treatment. Then, serially 10-fold diluted inhibitors were added into the culture, and after 6 h of incubation, cultures were replaced with fresh medium and subjected to cell viability analysis with CCK-8 kit (Dojindo). Optical density was read at 450 nm using a universal microplate reader (Bio-Tek). Three independent experiments were carried out for each set of drugs, and differences among dilutions were compared with t-tests. The highest working concentration was further determined by twofold serial dilutions of the lowest concentration that induced significant cytotoxicity (Table 1).

**Effect assay of inhibitor on SVCV infection.** CO cells were seeded in a 24-well plate the day before infection and grown to 80% confluency. Next day, cells were washed twice by serum-free M199 medium and pre-treated with freshly prepared inhibitors at 20 °C for 2 h, followed by inoculation with SVCV at an m.o.i. of 10. This time point was assigned as 0 h pi. At 2 h pi, cells were washed twice by M199 medium and fresh medium containing inhibitors and 2% FBS were added. Meanwhile, identical concentrations of inhibitors were added to parallel infected cells. After 2 h of incubation, FBS was added to the medium of post-treated groups to reach 2% concentration. Cells were then proceeded to IF assay at 18 h pi, as well as to Western blot and RT-qPCR analyses at 24 h pi. Cells treated with corresponding solvents were used as the controls.

**Transferrin, CTxB and dextran uptake.** CO cells were seeded in a 24-well plate the day before the experiment and grown to 80% confluency. Next day, cells were washed twice by serum-free M199 medium and were pre-treated with inhibitors or corresponding solvents at 20 °C for 15 min. Then, 20 µg ml⁻¹ Alexa Fluor 488-labelled transferrin (Invitrogen), 20 µg ml⁻¹ Alexa Fluor 488-labelled CTxB (Invitrogen) or 0.5 mg ml⁻¹ Alexa Fluor 594-labelled dextran (Invitrogen) was added. After 10 min of incubation, plates were placed on ice and dextran was washed out with the pre-cooled washing buffer (0.1 M sodium acetate and 0.05 M NaCl, pH 5.5) two times. After 20 min of incubation, transferrin was washed out with pre-cooled acid solution (PBS, 0.5 M NaCl and 0.2 M acetic acid) and then three times with pre-cooled PBS, while CTxB was washed out with pre-cooled PBS three times. Further, cells were harvested by digestion with 0.25% trypsin and were carefully inverted up and down for full dispersal in 500 µl PBS. After

**Transferrin, CTxB and dextran uptake.** CO cells were seeded in a 24-well plate the day before the experiment and grown to 80% confluency. Next day, cells were washed twice by serum-free M199 medium and were pre-treated with inhibitors or corresponding solvents at 20 °C for 15 min. Then, 20 µg ml⁻¹ Alexa Fluor 488-labelled transferrin (Invitrogen), 20 µg ml⁻¹ Alexa Fluor 488-labelled CTxB (Invitrogen) or 0.5 mg ml⁻¹ Alexa Fluor 594-labelled dextran (Invitrogen) was added. After 10 min of incubation, plates were placed on ice and dextran was washed out with the pre-cooled washing buffer (0.1 M sodium acetate and 0.05 M NaCl, pH 5.5) two times. After 20 min of incubation, transferrin was washed out with pre-cooled acid solution (PBS, 0.5 M NaCl and 0.2 M acetic acid) and then three times with pre-cooled PBS, while CTxB was washed out with pre-cooled PBS three times. Further, cells were harvested by digestion with 0.25% trypsin and were carefully inverted up and down for full dispersal in 500 µl PBS. After
centrifugation at 10,000 r.p.m. (FA-45-24-11 rotor, Eppendorf 5424R centrifuge) for 10 min, the supernatant was removed and cells were adjusted to 10⁶ cells ml⁻¹ concentration with PBS. Finally, samples were loaded on a flow cytometer (BD, FACS Aria III), and data were analysed with FlowJo 7.6 software. The experiment was carried out in triplicate.

Transmission electron microscopy. For visualization of SVCV, viral particles were pelleted from infected cell culture supernatant through a 30 % sucrose cushion at 28,000 r.p.m. for 4 h, 4 °C. The pellet was then re-suspended in PBS and loaded onto a 15–60 % sucrose gradient and centrifuged in an SW41Ti rotor (Beckman) at 40,000 r.p.m. for 1 h, 4 °C. The sharp visible band was collected and re-suspended in PBS and pelleted by centrifugation at 35,000 r.p.m. for 2 h, 4 °C. The pellet was re-suspended in PBS. The purified viral particles were deposited onto carbon-coated copper grids and negatively stained with 2 % (w/v) phosphophotungstic acid (pH 6.8). For TEM examination of ultrathin sections, CO cells were seeded in a six-well plate the day before infection and grown for 24 h at 25 °C to 90 % confluence. Next day, cells were washed twice by 4 °C pre-cooled, serum-free M199 medium and inoculated with SVCV at an m.o.i. of 100. After 30 min of absorption at 4 °C, plates were transferred to 20 °C. At 15 min post-temperature shift, cells were washed three times with PBS and harvested with cell scrapers. After centrifugation at 2500 r.p.m. for 10 min, cells were fixed with 2.5 % glutaraldehyde and 1 % osmium tetroxide and then embedded in Araldite epoxy resin. Ultrathin sections of 70 nm thick were obtained with an ultramicrotome (Leica), stained with 3 % uranyl acetate and lead citrate and examined under a transmission electron microscope (JEM-1230, 80 KV).

Immunofluorescence microscopy. Polyclonal rabbit anti-M antibody was prepared in our laboratory, briefly as the following: the M gene was constructed to the pRSET A vector (Invitrogen); the recombinant plasmid was transformed into Escherichia coli BL21 and induced with IPTG; the over-expressed M protein was purified with the ProBond Purification System (Invitrogen); New Zealand white rabbits were immunized with the purified M protein for four times; anti-serum IgG was purified with Protein A Sepharose and antibody titres were determined by ELISA.

For IF assay, cells were washed twice with PBS and fixed for 30 min at room temperature with 4 % paraformaldehyde. Fixed cells were washed three times with PBS and permeabilized for 15 min with 0.2 % Triton X-100, followed by three times PBS washing and blocking for 1 h at 37 °C with 5 % BSA. Then, cells were incubated with rabbit anti-M antibody for 2 h at 37 °C. After that, three times washing was conducted and Alexa Fluor 488-labelled donkey anti-rabbit IgG (Invitrogen) was added. After incubation for another 1 h, cells were washed three times with PBS and stained with DAPI (Roche) for 10 min to visualize the nuclei. Finally, cells were viewed under an Olympus IX73 inverted fluorescence microscope and images were obtained and processed with cellSens Standard v2.0.6. Total and fluorescent cells were scored with Image-Pro Plus 6.0 software in five random selected fields at ×100 magnifications. Relative infectivity was calculated by dividing total cells by fluorescent cells. Mean number of the five micrographs was taken as the percentage of infected cells. Three independent experiments were carried out.

Western blotting. For Western blot analysis, cells were lysed with cell lysis buffer (Beyotime), and protein concentrations were determined by the Bradford method. Equal amounts of proteins were resolved by 10 % SDS polyacrylamide gels and transferred to PVDF membranes (Pall) by a semi-dry transfer cell (Bio-Rad) for 40 min at 20 V. Membranes were then blocked by 3 % BSA and further incubated with rabbit anti-M antibody or mouse monoclonal anti-α-tubulin antibody (Sigma). The HRP-coupled goat anti-rabbit IgG or goat anti-mouse IgG (Sigma) was used as the secondary antibody. Protein bands were detected by using the ECL kiti (Tiangen Biotech) according to the manufacturer’s instructions.

Real-time quantitative PCR. For RT-qPCR, cells were washed three times with PBS. Total RNA was extracted using the RNasy Mini Kit (Qiagen) according to the manufacturer’s instructions. cDNA synthesis was carried out using the RevertAid First Strand cDNA Synthesis Kit (Fermentas). Quantitative PCR was performed on the ABI 7500 Fast Real-Time PCR system (Applied Biosystems) as described previously (Shao et al., 2016).

Statistical analysis. Difference was evaluated by using one-way ANOVA and t-tests for two-group comparison, while two-way ANOVA was used for multiple-group comparison. All statistical analyses were performed by using SPSS 14.0 and GraphPad Prism 6 software packages. The probability of P<0.05 was considered to be statistically significant.

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