Acta virologica **61**: 487 – 491, 2017 doi:10.4149/av_2017_412

Calcium signaling involved in bovine herpesvirus 1 replication in MDBK cells

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Received October 24, 2016; revised February 10, 2017; accepted October 13, 2017

Summary. – Calcium is one of the most prominent second messengers in eukaryotic cells. The involvement of calcium signaling in bovine herpesvirus 1 (BoHV-1) replication was not yet reported. In this study, we revealed that the L-type Ca^{2+} calcium channel blocker, Verapamil and store-operated calcium channel blocker, 2-aminoethyl diphenylborinate (2-APB) inhibited BoHV-1 replication in MDBK cells at the post-entry stages, and the Na^{+}/Ca^{2+} exchanger inhibitor, N-arachidonoyl glycine exchanger (NAGly) interfered with the viral entry process. NAGly also effected the phosphorylation of PLCγ-1 at Ser1248, which corroborated our previous findings, that PLCγ-1 is important for BoHV-1 entry. Collectively, these results suggest that diverse calcium channels are employed by BoHV-1 for efficient replication.

Keywords: BoHV-1; calcium; calcium channel blocker

Bovine herpesvirus 1 (BoHV-1), an enveloped virus belonging to the *Alphaherpesvirus* subfamily, infects cattle of all ages and breeds worldwide and causes great economical losses to cattle farms, due to the virus infection induced respiratory disease, abortions, and severe neonatal diseases (Muylkens *et al*., 2007; Tikoo *et al*., 1995). BoHV-1 together with the other pathogens, such as bovine viral diarrhea viruses, bovine respiratory syncytial virus, parainfluenza virus type 3 and bovine coronaviruses, as well as the bacteria including *Mannheimia haemolytica, Pasteurella multocida, Histophilus somni* and *Mycoplasma spp* are the causative agents of life-threatening pneumonia known as bovine respiratory disease complex (BRDC) (Fulton *et al*., 2016; Jones, 2009; Jones and Chowdhury, 2007).

Ca^{2+} is one of the most important signaling molecules involved in vast majority of cellular processes via modulating the activity of a repertoire of signaling components, such as the ion channels, Ca^{2+} buffers, Ca^{2+} effectors, Ca^{2+}-sensitive enzymes and transcriptional factors (Berridge *et al*., 2003; Zhou *et al*., 2009). It is well known that the virus depends on the cellular machinery for efficient entry of the host cells and subsequent replication and survival. It is not surprising that the viruses could utilize Ca^{2+} signals to create a favorable cellular environment benefiting for their infection, e.g., the Ca^{2+} is strictly required for rubella virus liposome association, membrane fusion, and virus infection (Dube *et al*., 2014); the endosomal calcium channels called two pore channels (TPCs) are required for Ebola virus entry into host cells (Sakurai *et al*., 2015); stromal interaction molecule 1 (STIM1)- and Orai-mediated Ca^{2+} entry is critical for Ebola virus, Marburg, Lassa and Junin virus infections (Han *et al*., 2015); and calcium signaling is a key regulator of influenza virus internalization (Fujioka *et al*., 2013). The involvement of calcium signaling in herpes simplex virus (HSV) infection has also been reported (Cheshenko *et al*., 2003). BoHV-1 and HSV are genetically closed, but little is known about the involvement of Ca^{2+} signaling in BoHV-1 infection.

Phospholipases C (PLC) with totally of 6 families (β, γ, δ, ε, η and ζ) are subdivided into 13 isoforms, which regulate numerous pathways, such as protein kinase C (PKC) and calcium spike (Vines, 2012). We have previously reported

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Abbreviations: BoHV-1 = bovine herpes virus 1; 2-APB = 2-aminoethyl diphenylborinate; NAGly = N-arachidonoyl glycine; PLC = phospholipase C; VOC = voltage-operated channel; SOC = store-operated channel; NCX = Na^{+}/Ca^{2+}

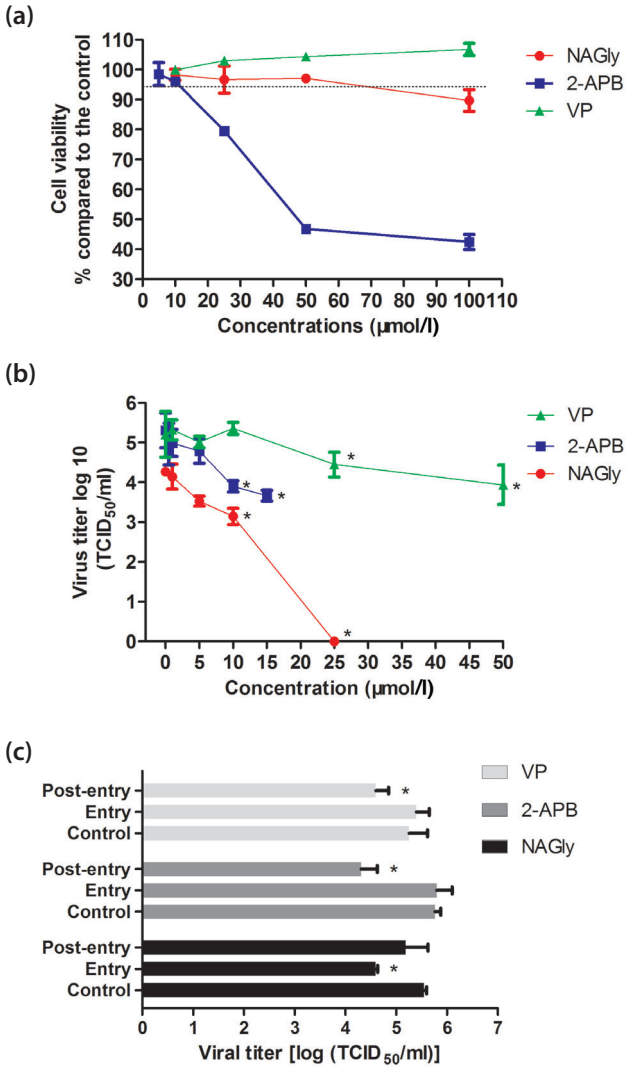

Fig. 1

The effects of Verapamil, 2-APB, and NAGly on BoHV-1 replication in MDBK cells

(**a**) The cytotoxicity assay for Verapamil, 2-APB and NAGly at indicated concentrations in MDBK cells. (**b**) Antiviral effect of Verapamil, 2-APB and NAGly on BoHV-1 infection. (**c**) The identification of virus entry stage(s) affected by these inhibitors. These studies were repeated 3 times and asterisks denote significant differences between the DMSO control and cultures treated with indicated chemical (*: P <0.05) as determined by the Student' t test.

that PLCγ-1 inhibitor U73122 and edelfosine possess strong antiviral activity against BoHV-1 infection, and are likely to interfere with viral early entry stages (Zhu *et al.*, 2017). This result is reminiscent of the speculation that some calcium channels may be involved in BoHV-1 infection.

Various Ca^{2+} channels, such as voltage-operated channel (VOC), receptor-operated channel (ROC) or store-operated channel (SOC) are responsible for feeding extracellular Ca^{2+} to the cytosol. These channels are extensively studied due to the availability of specific channel blockers, *e.g.*, Verapamil for VOC and 2-aminoethyl diphenylborinate (2-PAB) for SOC. Na^{+}/Ca^{2+} exchanger (NCX) is considered as one of the most important cellular mechanisms for extruding Ca^{2+} to the extracellular space. In this study, VOC blocker Verapamil (#V4629; Sigma-Aldrich), SOC blocker 2-APB (#D9754; Sigma-Aldrich), and NCX inhibitor N-arachidonoyl glycine (NAGly) (#CAS 179113-91-8; Santa Cruz Biotechnology) were employed to investigate the role of the calcium channels in BoHV-1 infection.

To identify proper concentrations for this study, the cytotoxicity of each chemical was assessed with WST-1 cell proliferation and cytotoxicity assay kit (Beyotime Biotechnology, China) following the manufacture's specification. As a result, the treatment of MDBK cells with Verapamil at a concentration of 100 µmol/l, with 2-APB at a concentration of 15 µmol/l, and with NAGly at a concentration of 50 µmol/l showed minor or no cytotoxicity to the cells, with reduced cell survival to a level less than 5% compared to the control (Fig. 1a). To test the effect of these chemicals on BoHV-1 infection, MDBK cells were treated with Verapamil (at a concentration of 50, 25, 10, 5 and 1 µmol/l), 2-APB (at a concentration of 15, 10, 5, 1 and 0.5 µmol/l) and NAGly (at a concentration of 25, 10, 5 and 1 µmol/l), respectively, during virus infection with a pretreatment for 1 h prior to viral infection. The infected cells treated with DMSO were used as a control. At 24 h post-infection the viral titer was determined and expressed as $TCID_{50}$/ml. Compared to DMSO control, Verapamil reduced the virus titer by ~1 log at concentrations of 50 and 25 µmol/l, 2-APB reduced the virus yield by ~1.5 log at concentrations of 50 and 25 µmol/l, and NAGly at a concentration of 25 µmol completely blocked the virus replication (Fig. 2b). These results indicate that VOC, SOC, and NCX exchanger mediated calcium signaling are important for BoHV-1 infection.

To test whether these inhibitors affected the viral entry stage of infection, confluent MDBK cells in a 24-well plate were incubated with BoHV-1(MOI = 1) for 1 h at 4°C. After extensive washing with ice-cold PBS, fresh medium with or without compounds were added, and the cells were cultivated in 37°C for 1 h. Fresh medium without inhibitor was replaced and continuously incubated for 24 h at 37°C. The virus yield was determined and expressed as $TCID_{50}$/ml. Addition of NAGly (10 µmol/l) during virus entry significantly reduced virus titer (~1 log) when compared to control. While, no effect on the virus replication was observed when either Verapamil (50 µmol/l) or 2-APB (10 µmol/l) was added (Fig. 1c). To test whether these inhibitors affect the post-entry stage of BoHV-1 infection, confluent MDBK cells in 24-well plates were infected with BoHV-1 (MOI = 1) for 1 h at 37°C. After washing with PBS, fresh medium with

or without inhibitors was replaced for further incubation. At 24 h post-infection, the virus yield was determined as $TCID_{50}$/ml. As a result, the treatment of cells with both Verapamil (50 μmol/l) and 2-APB (10 μmol/l), but not NAGly (10 μmol/l) significantly interfered with the virus production. These results indicate that the VOC blocker Verapamil and SOC blocker 2-APB mainly affected the virus post-entry stages, while the NCX inhibitor NAGly mainly interfered with viral entry process.

We have recently identified that BoHV-1 infection stimulated PLCγ-1 signaling to facilitate the viral entry (Zhu *et al.,* 2017). However, the mechanism underlying virus infection-stimulated PLCγ-1 signaling is poorly understood. Here, serum- starved MDBK cells were treated with NAGly at indicated concentrations for 1 h, then infected with BoHV-1 at MOI of 10 for 0.5 h, along with the treatment of NAGly. The cell lysates were prepared for western blot to detect phospho-PLCγ-1 (Ser1248) (Cell Signaling Technology), PLCγ-1 (Cell Signaling Technology) and GAPDH (Cell Signaling Technology). As a result, the activation of PLCγ-1 in response to BoHV-1 infection was reduced in a dose-dependent manner (Fig. 2). These results suggest that the NCX-mediated calcium signaling may be involved in the activation of PLCγ-1 by BoHV-1 infection. It has been reported that the activation of PLCγ-1 by calcium is required for calcium-induced human keratinocyte differentiation (Xie *et al*., 2005), which corroborated our results that calcium signaling is involved in PLCγ-1activation in response to BoHV-1 infection.

2-APB is a reliable blocker of store-operated Ca^{2+}entry (Bootman *et al*., 2002; Peppiatt *et al*., 2003). The inhibition of viral entry by 2-APB has been documented by several viruses, e.g., 2-APB inhibited HSV-1/2 penetration (Cheshenko *et al*., 2003), and pretreatment of cells with 2-APB led to a significant reduction in coxsackie virus B infection (Bozym *et al*., 2010). No inhibitory effect on West Nile virus infection was observed at the viral entry stages (Scherbik and Brinton, 2010). Here, we found that 2-APB affected the BoHV-1 replication mainly at the post-entry stage, but not at the entry stages. These data suggest that the store-operated Ca^{2+} entry may have diverse effect on virus replication in virus type-specific manner.

The voltage-operated channel antagonist Verapamil is a drug approved by the U.S. Food and Drug Administration (FDA) to treat cardiovascular diseases. This drug has also diverse effects on various virus infections. Verapamil enhanced some virus infections, *e.g.*, like HIV-1 expression in acute infection of lymphoid CEM cells (Harbison *et al*., 1991), and promotes oncolytic adenovirus release from the infected A549 cells (Gros *et al*., 2010). Whilst, the antiviral effect of Verapamil targeting various process of viral replication has also been documented, *e. g*., it inhibits filovirus entry to host cells (Gehring *et al*., 2014), inhibits budding of Sindbis and vesicular stomatitis viruses from infected chicken embryo fibroblasts (Schlesinger and Cahill, 1989), and blocks human rhinovirus 2 infection and release (Gazina *et al*., 2005; Schlesinger and Cahill, 1989). Here, we revealed that Verapamil interferes with BoHV-1 infection at the post-entry stage(s).

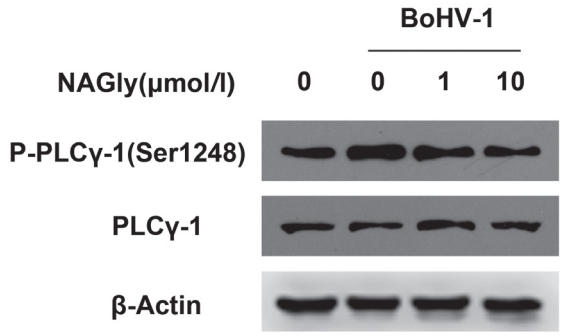

Fig. 2

NAGly interferes with PLCγ-1 phosphorylation (at Ser1248) in BoHV-1-infected MDBK cells

Western blot of phospho-PLCγ-1 (Ser1248), PLCγ-1 and GAPDH. MDBK cells subjected to serum starvation for 12 h were treated with NAGly at indicated concentrations for 1 h, followed by infection with BoHV-1 at MOI of 10 for 0.5 h, along with the treatment of NAGly. These results are representative of three independent experiments.

There is a complicated interaction between phospholipase C and calcium signaling. Upon activation PLC activates protein kinase C (PKC) and Ca^{2+} release from the endoplasmic reticulum to the cytoplasm, and in turn activates downstream effectors to mediate various cellular changes and activities (Bagley *et al*., 2004; Vines, 2012). The NCX is responsible for extrusion of Ca^{2+} to the extracellular space and import of sodium ions. There is evidence of the activation of PLC by a Na^{+}-dependent mechanism in MIN6 cells (Li *et al*., 2016). Here we showed that NCX also affects PLCγ-1 signaling stimulated by BoHV-1 infection. Maybe the activation of PLCγ-1 by the virus infection is orchestrated by NCX mediated calcium signaling, which needs further investigation.

In conclusion, we provide the first evidence that BoHV-1 infection in MDBK cells could be inhibited by diverse calcium channel blockers, suggesting that host Ca^{2+} signaling is involved in the virus infection. Moreover, we have showed that NCX-mediated calcium signaling mediated BoHV-1 entry is regulated with a PLCγ-1-dependent mechanism.

Acknowledgements. We thank Dr. Leonard J Bello, University of Pennsylvania, USA for providing the BoHV-1 Colorado 1 stain and MDBK cells. This research was supported by Chinese National Science Foundation Grant (No.31472172), and partially supported

by Grant No. 2016YFD0500900 and 2016YFD0500704 from the National Key Research and Development Program of China, the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD), the grant No. 14KJA230001 from the Jiangsu High Education Basic Research, the grant No. BE2014358 from the Science and Technology Department of Jiangsu province.

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