PCV2 trigger apoptosis of PK-15 cells through the PLC-IP3R-Ca^{2+} signaling pathway

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Abstract：

Phospholipase C (PLC) is a key enzyme in the cell membrane. PLC hydrolyses phosphatidylinositol 4, 5-bisphosphate (PIP2) to generateinositol 1,4, 5-triphosphate (IP3) and diacylglycerol (DAG) that regulates a variety of cellular processes. Evidence indicates the pivotal role of PLC and inositol 1,4,5-trisphosphate receptor(IP3R) in influencing Ca^{2+} release from the endoplasmic reticulum(ER).At the same time, the imbalance of Ca^{2+} will stimulate endoplasmic reticulum stress(ERS), leading to cell apoptosis. Viral infection could triggers host defense through apoptosis of the infected cells.However, it is not clear how porcine circovirus type 2 (PCV2) induces apoptosis by affecting Ca^{2+} homeostasis. We show here that PCV2 infection induces the increased cytoplasmic Ca^{2+} level and apoptosis.We also found that the ER swelling of PK-15 cells after viral infection by transmission electron microscopy. Furthemore, the activation of PLC-IP3R-Ca^{2+} signaling enhanced apoptosis in infected PK-15 cells. Taken together,our findings suggest that PCV2 infection trigger ERS of PK-15 cells via the PLC-IP3R-Ca^{2+} signaling pathway to promoted the release of intracellular Ca^{2+}, and led to cell apoptosis.

KEYWORDS: Apoptosis; Porcine circovirus 2; PLC; IP3R; Ca^{2+}; Endoplasmic reticulum stress

1 INTRODUCTIONS

Porcine circovirus 2 (PCV2) is an economically swine pathogen that has the ability to cause many diseases in the pig herd, including Porcine dermatitis and nephrotic syndrome（PDNS）, A2 congenital tremor（A2CT）, Porcineproliferative and necrotizing pneumonia（PNP） and Postweaning multisystemic wasting syndrome（PMWS） etc (Dupont, Nielsen, Bækbo, & Larsen,2008);(Magar, Larochelle, Thibault,

& Lamontagne ,2000);(Thomson, Henderson, Meikle, & Macintyre,2001). High level of PCV2 viremia and viral load in tissues, granulomatous inflammation, immunosuppression were characterized as the symbols of severe PCV2 infection. To date, the exact mechanisms of porcine circovirus diseases and porcine circovirus-associated diseases (PCVD/PCVAD) are currently unknown. However, many studies have reported co-infection with other swine pathogens, such as Haemophilus parasuis, Mycoplasma pneumoniae and porcine parvovirus, are important cofactors that may enhance PCV2 infection and the severity of PCVD/PDVAD.(Nielsen et al.2003); (Darwich, Segalés, Domingo, & Mateu,2002);(Darwich, Segalés, & Mateu,2004)

Previous study found that Phospholipase C (PLC) activity could increase the concentration of free Ca^{2+} thus activating the apoptotic signaling pathway(Malli, Frieden, Hunkova, Trenker, & Graier,2007). PLC is an enzyme located in the nuclear membrane, and extracellular stimulated receptors promote PLC activation, which leads to the hydrolysis of membrane phospholipids phosphatidylinositol 4, 5-bisphosphate (PIP2) to inositol 1,4, 5-triphosphate (IP3) and diacylglycerol (DAG), and IP3 releases Ca^{2+} from intracellular stores.Then, the release of Ca^{2+} through inositol 1,4,5-trisphosphate receptor(IP3R) leads to Ca^{2+} depletion in the endoplasmic reticulum (ER) and increases the concentration of Ca^{2+} in the cytoplasm. ER is the main intracellular storage site for Ca^{2+} , which has a powerful ability to uptake and release Ca^{2+}. The IP3R was an IP3 binding protein and was a Ca^{2+} channel localized on the ER(Zhu et al,2017);(Zhou, Frey, & Yang,2009). Diverse cellular stresses such as lack of cellular nutrients, hypoxia, acid-base imbalance, or reactive oxygen species (ROS) accumulation cause unfold or misfold proteins to accumulation inside the ER lumen, a condition known as ER stress (ERS)(Mou, Yuan, Zhang, Song, & Chen ,2020) and activate cell damage(Wei, Davies, & Harper,2020). As an adaptive action, the unfolded protein response (UPR)is triggered to decrease the ER protein load(Zhou et al.,2017). When cells cannot recover from ERS, UPR will terminate this adaptive response and trigger cell apoptosis.

Many viruses can cause cell apoptosis when infecting host cells. Apoptosis,or programmed cell death, of an infected host cell has been regarded as a powerful defense mechanism against virus survival, infection and control of virus proliferation. Therefore, in host cells, viruses target apoptotic checkpoints to prevent host immune clearance and promote viral replication.At the same time, the virus can accelerate or induce apoptosis to promote the release and spread of virions (Galluzzi et al.,2010).

For PCV2, studies have shown that viral infection could induce apoptosis in cultured PK-15 cells by required the activation of caspase-8 and effector caspase-3 pathways(Liu, Chen, & Kwang,2005). In addition, compared with wild-type strain, the pathogenicity of PCV2 deficient ORF3 to pigs was reduced(Juhan, Leroith, Opriessnig, & Meng, 2010).Studies have shown that PCV2 is related to lymphocytosis and histopathological infiltration in histopathology, and causes apoptosis in mouse and pig models. Apoptosis is closely related to the changes and flow of Ca^{2+} in the endoplasmic reticulum (ER)and mitochondria (Dvorak, Puvanendiran, & Murtaugh,2013);(Lv, Guo, & Zhang,2014). At present, there are few studies on the mechanism of PCV2 inducing apoptosis and the kinetics of Ca^{2+} in PK-15 cells.Therefore, studying the relationship between viral infection and

apoptosis is of great significance to further explore and reveal the pathogenic mechanism of virus.

In this study, we aimed to explored the interplay between PCV2 and host cell apoptosis signalling pathways. And we found that PCV2 infection trigger ERS of PK-15 cells activates the PLC-IP3R-Ca^{2+}signaling pathway to promoted the release of intracellular Ca^{2+}, and induce the cell apoptosis. By exploring the relationship between viruses and apoptosis, it is of great significance to further explore and reveal the pathogenic mechanism of virus, and to provide a new theoretical basis for finding antiviral targets of host cells.

2 MATERIALS AND METHODS

2.1 Cells and viruses

Cell line PK-15 free of PCV1 contamination were cultured at 37 °C and 5% CO_2 in DMED medium (Gibco) supplemented with 8% fetal bovine serum (BIOIND,04-001-1ACS).

PCV2 virus strain used in this study was stored in ShandongProvincial Key Laboratory of Animal DiseaseControl & Breeding, Institute of AnimalScience and Veterinary Medicine ShandongAcademy of Agricultural Sciences.

2.2 Virus infection and drug treatment

PK-15 cells were infected with PCV2 at a multiplicity of infection (MOI,pfu number/cell) of 1. After 72 hours, the virus was detected according to the indirect immunofluorescence assay (IFA) procedure, which proved that the virus had proliferated in the cells. When performing inhibitor effects experiments, add 2-APB (Sigma-Aldrich, 100065), 10mM 4-PBA (Sigma-Aldrich, Y0000808), a final concentration of 40 μM configured with DMSO (Sigma-Aldrich, D2650), 50 μM U73122 (Sigma-Aldrich, U6756) fresh medium was cultured in an incubator.

2.3 Determination of cytoplasmic Ca^{2+} concentration in PK-15 cells

Set up the control group, virus infection group and inhibitor treatment group. Fluo-4AM kit (Invitrogen, F-14201), 5μM Fluo-4AM working solution needs to take 4 μL of 2mM stock solution and dilute with PBS; add 50 μL 5 μM Fluo-4AM working solution to each well, The plate was placed in the incubator for 30 min, and the fluorescence signal intensity was measured with a fluorescence microplate reader with 490 nm excitation light and 520 nm emission light.

2.4 Apoptosis detection

PK-15 cells were infected with PCV2 in the absence or presence of 4-PBA. The samples were collected at 24 h, 48 h, and 72 h after the virus infection. The TUNEL assay kit (One Step TUNEL Apoptosis Assay Kit, Beyotime, C1086) was used to visualize apoptotic cells following the manufacturer's instruction.

2.5 Cell transmission electron microscopy technique

After the cells were cultured for 24 hours, washed away the floating dead cells with PBS, collected the cells in a 1.5 mL centrifuge tube, centrifuged at 3000g for 5 min, added the electron microscope fixative, fixed at room temperature for 1h. Observed through a transmission electron microscope.

2.6 Western blot

Western blotting was performed as described previously. The membranes were blocked for 1 h and then probed for 1 h with the following primary antibodies: β-actin (Zhongshan Jinqiao TA-09), PCV2 Cap protein monoclonal antibody, Anti-rabbit IgG, HRP-linked Antibody (GeneTex), Anti-IP3 receptor antibody (abcam, ab5804), Anti-Phospholipase C beta1/PLC antibody (abcam, ab233157). Washed the membrane again and then incubated for another hour with the goat anti-mouse secondary antibody (Alexa Fluor® 488) diluted 1:5000. Used Image J software to perform grayscale analysis of western blot bands, calculate the ratio of target protein molecules (IP3R, PLC, PCV2 Cap to β-actin), set the ratio of the blank group to 1.0, and compare the other groups to the control group. Calculation).

2.7 REAL-TIME fluorescence quantitative PCR of PLC and IP3R

Total RNA extracted samples, used retroviruses Aidlab company, Kit (TUREscript 1 st Stand cDNA short Kit) reverse transcription operations, respectively to join primers PLC-F:AGAACGCTGGCTGTGGCTACG,PLCR:GGAGGGTCTGGGCTTTGGAAGG;IP3R-F:CTTGTGGGCTACGTGTCTGG,IP3R-R:CTCTTGGGTGTCTTCTTCTCTGG.AceQ® qPCR SYBR Green Master Mix(Vazyme, Q121) was used for qPCR experiment.

2.8 Statistical analysis

Statistical significance was calculated using multiple comparisons in GraphPad Prism software.“ns”represented no significant difference($P > 0.05$), “ * ”representeddifference($0.01<P<0.05$);**representeda very significant difference($P<0.01$).

3 Results

3.1 PCV2 proliferated successfully in PK-15 cells

To verifyPCV2 could successfully replicatesin PK-15 cells, we used the indirect immunofluorescence assays. Our results showed thata strong positive signal can be detected in the cells since infected with the PCV2 for 24h.(Figure 1). And with the prolonged effect of infection time, the stronger the fluorescent signal, indicating that the PCV2 was successfully proliferatein the PK-15 cells.

3.2 PCV2 infection of PK-15 cells results in up-regulation of Ca^{2+} concentration

To determine whether PCV2 could induce the Ca^{2+}release,PK-15 cells were infected with the multiplicity of infection (MOI) (MOI of 1).Fluo-4AM kit was used to detect the concentration of Ca^{2+} in PCV2-infected cells. The results of the luciferase marker showed that the levels of cytoplasmic free Ca^{2+} at 24h, 48h and 72h after PCV2 infection were significantly higher was significantly increased in virus-infected cells, compared with uninfected controls, suggesting that PCV2 infection induced the up-regulation of cytoplasmic free Ca^{2+}(Figure 2) (24h ： 117.5%VS100% ， $0.01<P<0.05$ ； 48h ： 119.6%VS100% ， $0.01<P<0.05$ ； 72 h ： 125.7%VS100% ， $0.01<P<0.05$) .

3.3 PCV2 infecion induced apoptosis and endoplasmic reticulum swelling

To detect whether PCV2 was capable of induce apoptosis, PK-15 cells were used to infected with PCV2 2W3 strains. The resultsshowed that PCV2led to increased numbers of apoptosis cells at a given MOI at 24 h postinfection (hpi), which increased with increasing time up to 72 hpi (Figure 3), suggesting that PCV2 infection induced cell apoptosis.

Early studies showed that PCV2 infection could lead to ER stress. We anticipated that the virus infection induces the swelling of the ER to assist in protection against cytosolic Ca^{2+} overload in cellular stress situations. We observed that there were significant differences in morphology between the virus-infected cells and uninfected controls. The cells in theuninfected controlshad dense cytoplasm, uniform nucleolus, normal organelles and normal ER. However, in the virus infected group, the cytoplasm becomes thinner, the ER is abnormally swollen (Figure 4), the attached ribosomes are reduced, and the perinuclear space is swollen, which has not been found in the uninfected controls. These results demonstrated that PCV2 could cause abnormal swelling of ER structure and morphologicalchanges of PK-15 cells, leading to the phenomenon of ERS. Taken together, these results indicating that PCV2 infection opened the calcium channels in the ER, and Ca^{2+} is released into the cytoplasm, leading to an increase in the concentration of free Ca^{2+} in the cytoplasm, which in turn triggers apoptotic pathways to induce cell apoptosis.

3.4Co-localization of PLC protein and IP3R protein in cells

Phospholipase C (PLC) is a critical signaling enzyme that hydrolyzes PIP2to generate IP3, which binds to IP3R and stimulates increases in intracellular Ca^{2+}.In order to determine the relationship between PLC and IP3R in PK-15 cells, PCV2 was used to infect PK-15 cells and observed the positional relationship of two proteins in the cell with a confocal laser scanning microscope:The IP3R protein was labeled as red, the PLC protein was labeled as green, and the co-location area of them were labeled as yellow.When PCV2 infected PK-15 cells, PLC protein and IP3R protein were observed to co-locate in the cells.(Figure 5).

3.5 PCV2 infection with PK-15 cells can activate the PCL-IP3R-Ca^{2+} signaling pathway and induce cell apoptosis

PLC is activated by various signalling molecules, including Ca^{2+}. In order to prove whether PCV2 induces endoplasmic reticulum stress by activating the PLC-IP3R-Ca^{2+} signaling pathway after infecting PK-15 cells, and releases Ca^{2+} into the cytoplasm to promote cell apoptosis.We added endoplasmic reticulum stress reliever 4-PBA, the PLC inhibitor (U73122) and IP3R inhibitor (2-APB)to the cells to detect apoptosis, Ca^{2+} concentration and protein expression in different experimental groups.The results showed that after theERS reliever4-PBA added into the PCV2 infected cells, resulting in a decrease in the expression of PCV2 Cap protein (Figure 6)and the concentration of $Ca^{2+}$was significantly lower with the 4-PBA (Figure 8), suggesting that the 4-PBA affected the opening of Ca^{2+} channels on the ER, thereby inhibiting the flow of Ca^{2+} to the cytoplasm,and finally alleviated the apoptosis of PK-15 cells (Figure 7). Besides, after PCV2 infection, the mRNA expression of IP3R and PLC was significantly up-regulated compared with the uninfected groupby 3.72- and 2.83-fold,respectively (Figure 9), this result strongly suggested that PCV2 could lead to ER stress through IP3R-PLC pathway.

Furthermore, after adding PLC and IP3R inhibitors, the mRNA expression of IP3R and PLC were decreased (Figure 9), and the PK-15 cells exhibits an apprent apoptosis disadvantage (Figure 7), and the concentration of Ca^{2+} increased with the prolongation of the virus infection time, but it was significantly lower than that of without inhibitor treatment virus infected cells (Figure 8), suggesting that the concentration of Ca^{2+} and the expression of PLC and IP3R in the cell related. Taken together, our result showed that PCV2 can induce ERS and cause cell apoptosis through the PLC-IP3R signaling pathway to promote viralreplication.

4 Discussion

As a DNA virus, PCV2 does not have the ability to complete the entire life cycle independently, and it must rely on the life cycle of host cells to complete its own proliferation. After the virus enters the host cell, it replicates itself with the help and threat of the host.(Hao, Wang, Xing, Liu, & Zhang,2019)Viruses seek advantages and avoid disadvantages, and also affect the internal environment of host cells.It has been shown that increased Ca^{2+} in the cytoplasm results from the release of ER.(Bano & Nicotera,2007)Current research generally believes that Ca^{2+} is the initial signal of the apoptosis pathway, and that high intracellular calcium can trigger cell apoptosis.In this study, we observed the endoplasmic reticulum swelling of PK-15 cells after viral infection by transmission electron microscopy, and found that PCV2 infection triggered ERS of PK-15 cells through PLC-IP3R-Ca^{2+} signaling pathway,and cause apoptosis.

When cells were stimulated by the external environment, the ES released Ca^{2+} into the cytoplasm.When the concentration of Ca^{2+} reached a certain threshold and discontinuously decreases, the calcium-dependent endogenous endonuclease was activated and the DNA is decomposed into 180-200 bp oligos, leading to apoptosis.(Xiang, Wang, Zhang, & Han, 2017)Ca^{2+} acts as a messenger molecule in cell proliferation, the transmission of external molecular stimuli, and the activation of antiviral invasion molecules.(Zhou, Frey, & Yang, 2009)Current studies have shown that endoplasmic reticulum Ca^{2+} homeostasis affects ERS , and induces apoptosis through activation of autophagy and inflammation groups. (Mou, Yuan, Zhang, Song, & Chen,2020)Virus infection can interfere with endoplasmic reticulum homeostasis and cause endoplasmic reticulum stress. In order to cope with the harmful effects of virus-induced endoplasmic reticulum stress, cells activate key signal transduction pathways, including UPR and inherent mitochondrial cell apoptosis. Up to now, about 36 viruses have been found to trigger ERS and differentially activate ERS related signaling pathways.(Li, Kong, & Yu ,2015); (Benali-Furet et al.2005)Our research also found that the apoptosis caused by PCV2 infection of PK-15 cells is related to ERS caused by the increase of intracellular Ca^{2+} concentration. 4-PBA can inhibit cells from entering S phase. Because PCV2 replication requires S and G2/M phases, when the cell cycle is blocked, PCV2 replication cannot be achieved.(Xue et al.2017);(Hung et al. 2015)We verified in PK-15 cells that 4-PBA can alleviate the stress of PCV2 on the endoplasmic reticulum of PK-15 cells and the expression of PCV2 Cap protein. The use of drugs to interfere with ERS caused by the virus can reduce virus replication and alleviate cell apoptosis.

IP3R and PLC are Ca^{2+} dependent cellular proteins. PLC stimulates IP3 to bind to IP3R, opensCa^{2+} channels in ER, and makes Ca^{2+} flow into the cytoplasm. This was confirmed in PK-15 cells infected with PCV2 in this study.The finding confirms that

PCV2 attacks host cells by affecting the dynamics of intracellular Ca^{2+}. As mentioned in previous reports, Ca^{2+} is a limiting cytokine for virus budding.Increasing the concentration of Ca^{2+} in the cytoplasm can enhance virus release. (Aliyu, Ling, Md Hashim, & Chee，2019)

In this study, we first discovered the co-localization of PLC and IP3R in the cytoplasm of PK-15, which preliminarily verified that the PLC-IP3R pathway in PK-15 cells regulates the flow of Ca^{2+}, which leads to the occurrence of cell apoptosis. In future research, we can provide new targets for the study of antiviral strategies by exploring the molecules in the PLC-IP3R-Ca^{2+} pathway.These findings provide a theoretical basis for understanding the pathogenic mechanism of PCV2 and exploring new virus prevention and control strategies. On the other hand, the knowledge gained from this field will help reveal the mechanism of virus replication and pathogenesis, and provide insights and development for future research and antiviral drugs.

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ETHICAL STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTION

Jun Li and PanPanSun conceived the project; PanPanSun designed the experiments, Shuo Wang, Xiaoyan Wu, Jianli Shi, Zhe Peng, Chang Liu, Hong Han, Hongbin He performed most of the experiments; Chen Li, Yao Tian, Jiaxin Li, Shaojian Xu contributed materials and participated in discussion; Shuo Wang wrote the manuscript; Jun Li supervised the work and edited the final version of the manuscript which was read and approved by all authors.

Data Availability Statement

The data used to support the findings of this study are available from the corresponding author upon request.

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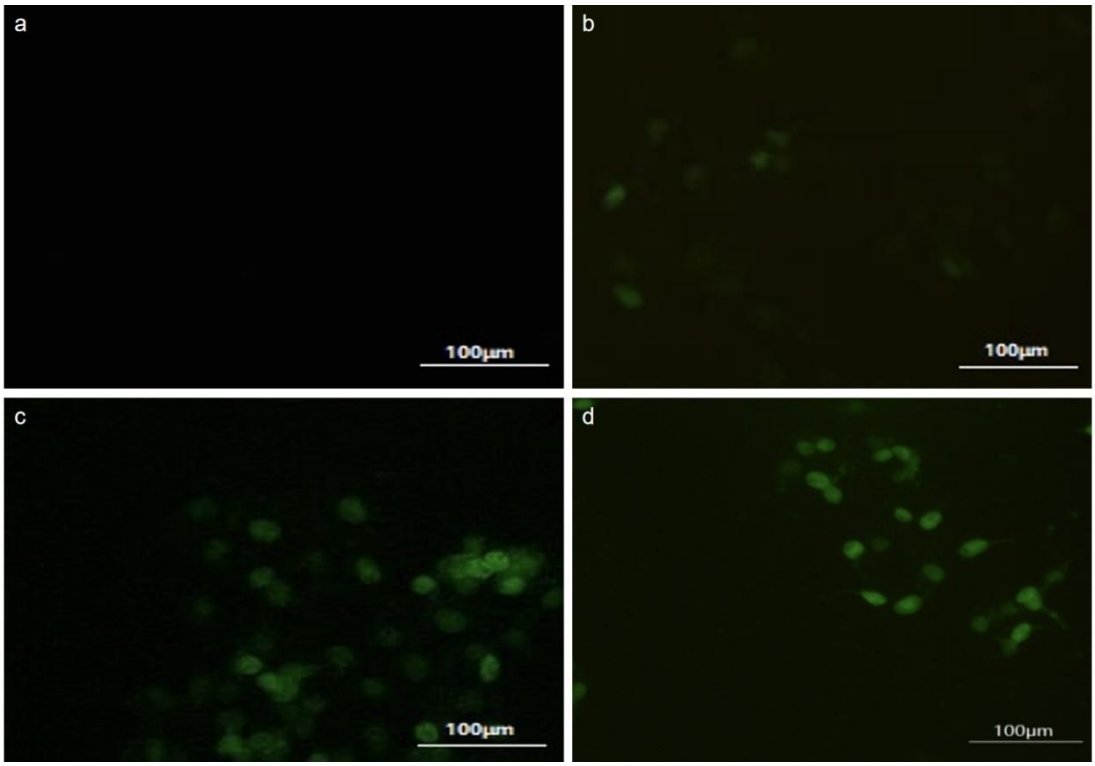

Figure.1 The result of IFA (200×)

a. Negative control; b. Group 24 h of exposure;c. Group 48 h infected; d. Group 72 h of exposure

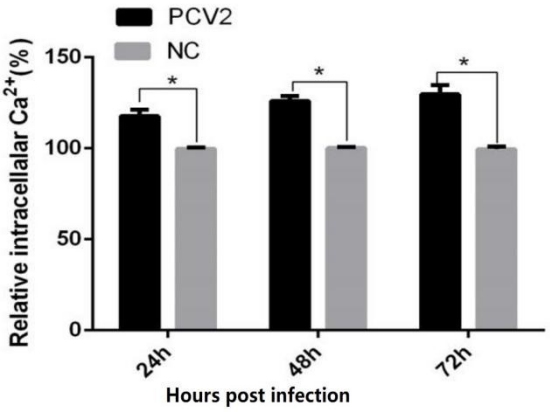

Figure.2 Results of intracellular calcium concentration in cell

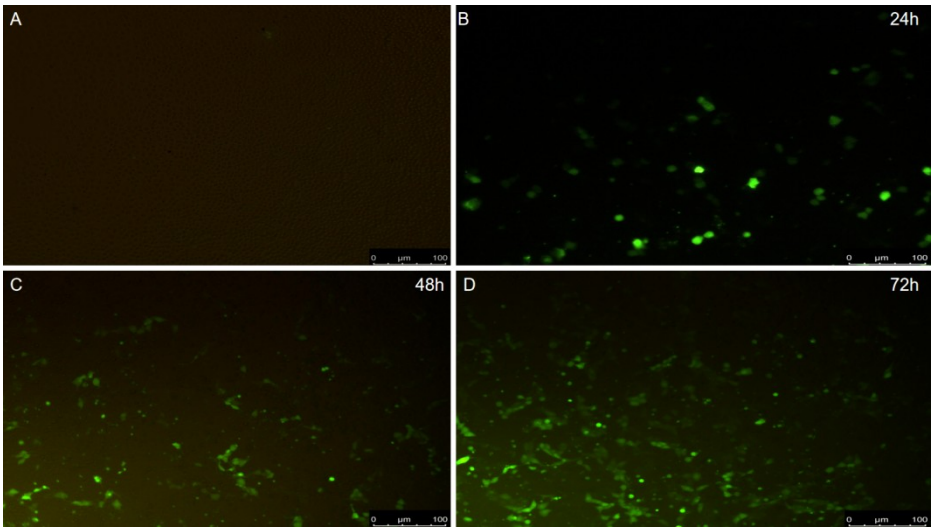

Figure.3 The results of apoptosis

A. Negative control; B. Virus infection group 24 h; C. Virus infection group 48 h; D. Group 72 h of virus Infection

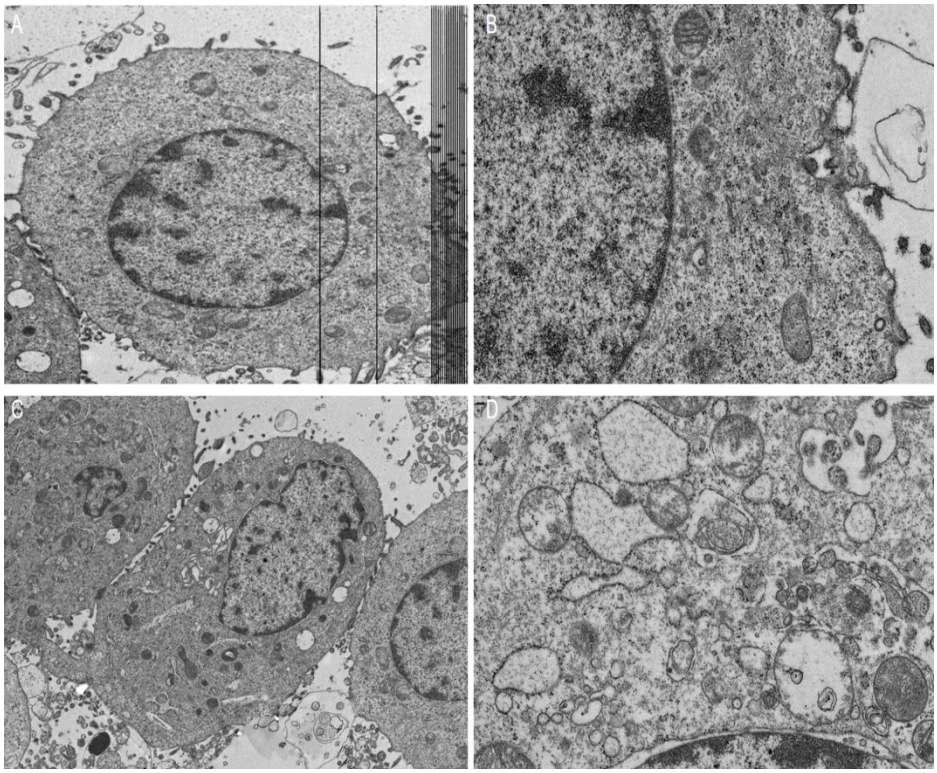

Figure.4 Results of cell electron microscopy

A: Control cells at 5000 x magnification; B. Challenge group cells amplified at 5000 times; C. Control cells at 10000 magnification, with the arrow pointing to the endoplasmic reticulum; D. Cells in the challenge group at 10000 magnification, the arrow indicates the endoplasmic reticulum with abnormal enlargement

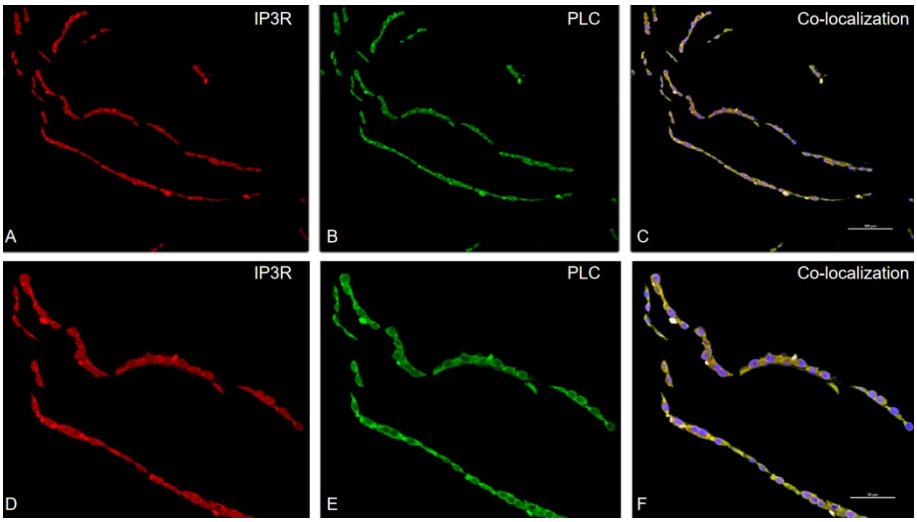

Figure.5 PLC co-localizes with IP3R in PCV2 infect PK 15 cells

Laser confocal immunofluorescence was used to detect the expressions of PLC and IP3R in PK15 cells infected with PCV2 (The red is IP3R protein, the green is PLC protein, and the yellow part is the co-location of the two proteins. A，B,C is the protein expression image at 200 times magnificationand D,E,F is the protein expression image at 400 timesamplification.)

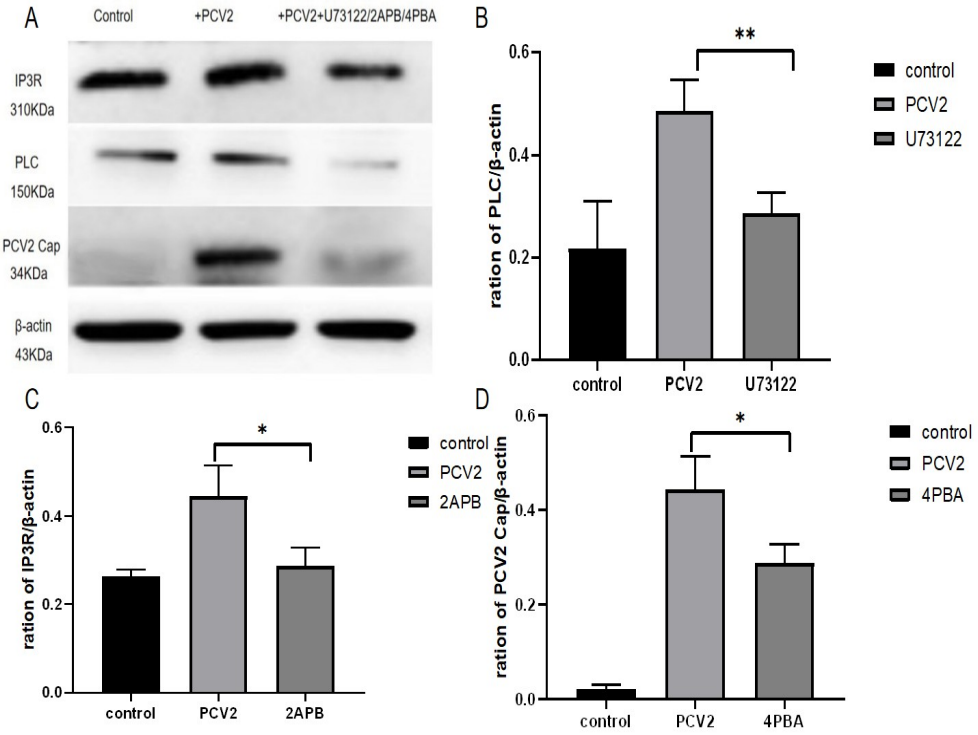

Figure. 6 Expression of different proteins after inhibitor addition changes of the control group

A: Western blot results; B: Histogram of PLC protein expression in different experimental groups after the addition of U73122 showed that the PLC expression in the virus infection group after the addition of inhibitor was significantly different from that in the inhibitor group. C: Histogram of IP3R protein expression in different experimental groups after the addition of 2APB; D: The histogram of PCV2 Cap protein expression in different experimental groups after the

addition of 4PBA showed that the expression level of PCV2 Cap protein in host cells was significantly decreased after the addition of 4PBA.

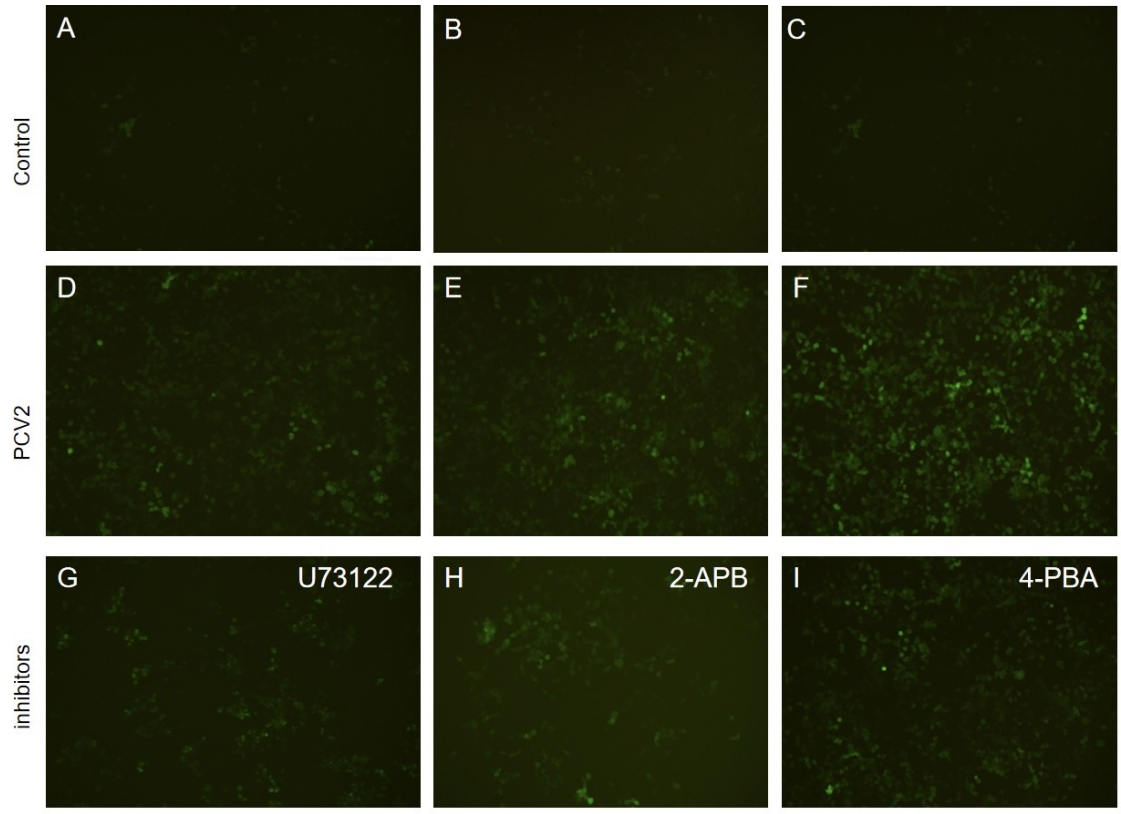

Figure. 7 Fluorescence results of apoptosis detection (×200)

A,B,C: Apoptosis in the control group; D,E,F: Apoptosis of cells infected with PCV2 virus after 72 hours; G: Apoptosis after adding PLC inhibitor (U73122)H: apoptosis after adding IP3R receptor inhibitor 2APB I: apoptosis after adding endoplasmic reticulum stress inhibitor

4PBA.

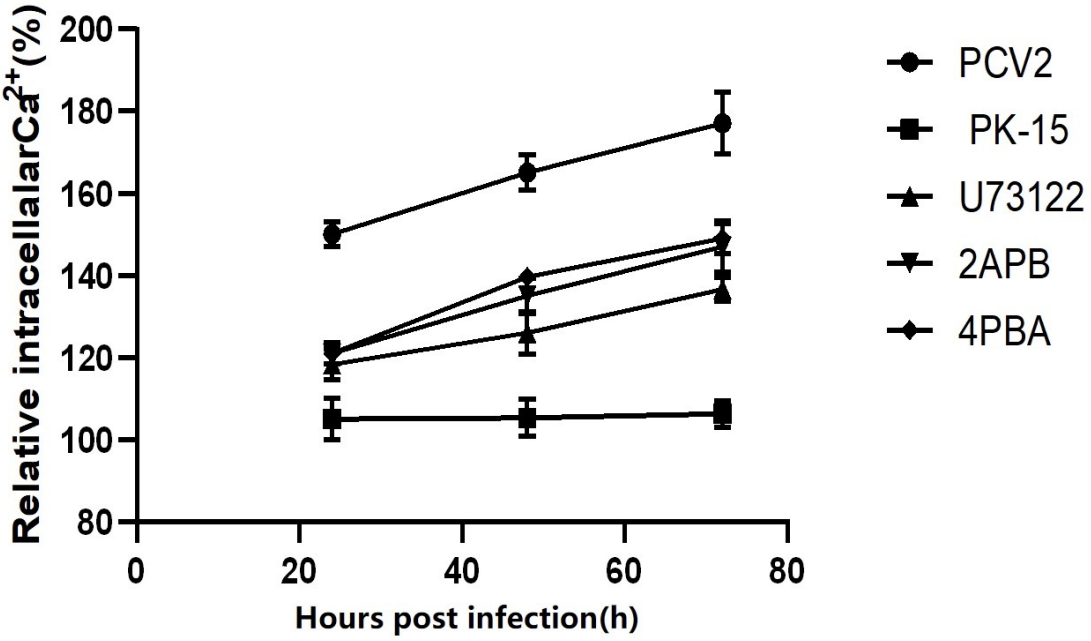

Figure. 8 Changes of Ca^{2+} after virusinfection

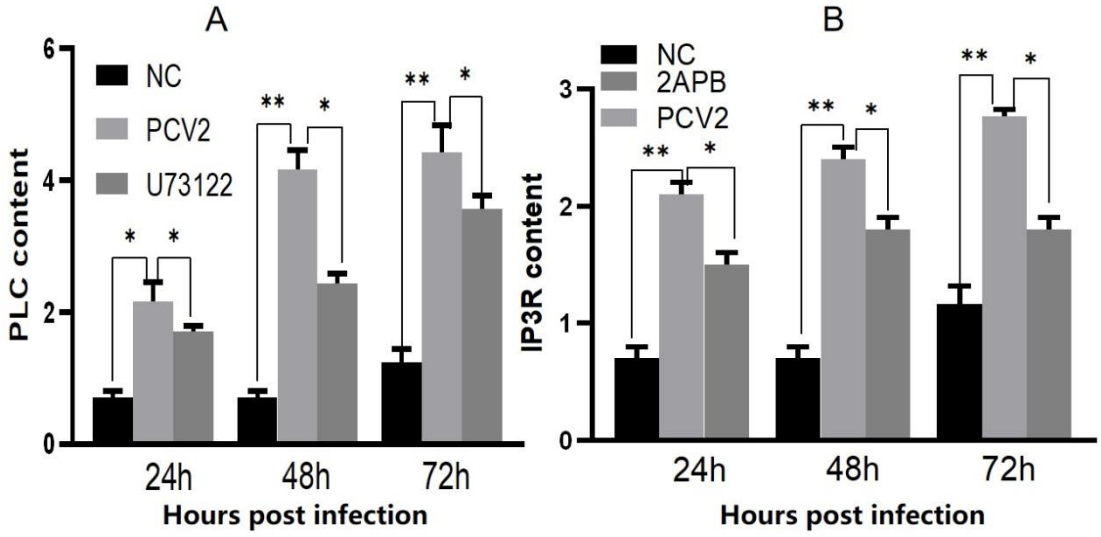

Figure.9 Changes of IP3R and PLC mRNA expression levels after PCV2 infection in PK15 cells