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PKCδ is required for porcine reproductive and respiratory syndrome virus replication

Haiyan Zhao [a,b], Xue-kun Guo [a,b], Yanmin Bi [a,b], Yihui Zhu [c], Wen-hai Feng [a,b,*]

[a] State Key Laboratories of Agrobiotechnology, China
[b] Department of Microbiology and Immunology, College of Biological Science, China Agricultural University, Beijing 100193, China
[c] College of Biological Science, Hunan Normal University, Changsha 410006, China

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ABSTRACT

Protein kinase C (PKC) that transduces signals to modulate a wide range of cellular functions has been shown to regulate a number of viral infections. Herein, we showed that inhibition of PKC with the PKC inhibitor GF109203X significantly impaired porcine reproductive and respiratory syndrome virus (PRRSV) replication. Inhibition of PKC led to virus yield reduction, which was associated with decreased viral RNA synthesis and lowered virus protein expression. And this inhibitory effect by PKC inhibitor was shown to occur at the early stage of PRRSV infection. Subsequently, we found that PRRSV infection activated PKCδ in PAMs and knockdown of PKCδ by small interfering RNA (siRNA) suppressed PRRSV replication, suggesting that novel PKCδ may play an important factor in PRRSV replication. Taken together, these data imply that PKC is involved in PRRSV infection and beneficial to PRRSV replication, extending our understanding of PRRSV replication.

Introduction

Porcine reproductive and respiratory syndrome (PRRS) is the cause of a complex systemic disease in pigs, most notably affecting the respiratory and reproductive systems of infected hosts, and leading to significant economic losses in the swine industry worldwide (Collins et al., 1992; Meulenberg, 2000; Wensvoort et al., 1991). The causative agent, PRRS virus (PRRSV), is an enveloped single stranded positive-sense RNA virus belonging to the family *Arteriviridae* in the order *Nidovirales* (Thiel et al., 1993). Recently, a highly pathogenic PRRSV (HP-PRRSV) has emerged in China and affected more than 20 million pigs (Tian et al., 2007).

PRRSV genome is approximately 15.4 kb, containing at least 10 open reading frames (ORF1a, ORF1b, ORF2a, ORF2b, and ORFs 3 through 7, including ORF5a). ORF1a and ORF1b encode polyproteins that are processed to at least 14 non-structural proteins (nsps) by viral proteases, whereas the remaining ORFs in the 3′ terminal region code for 8 structural proteins including GP2, small envelope (E), GP3, GP4, 5a, GP5, membrane (M), and nucleocapsid (N) (Conzelmann et al., 1993; Johnson et al., 2011).

Viruses utilize host signaling pathways to help their replication. Bovine foamy virus activates NF-κB pathway to enhance viral transcription (Wang et al., 2010), and mouse hepatitis virus, porcine circovirus type 2, enterovirus 71, junín virus and dengue virus have been shown to manipulate the host ERK signaling pathway to regulate viral replication and gene expression (Cai et al., 2007; Rodriguez et al., 2014; Smith et al., 2014; Wang et al., 2012; Wei and Liu, 2009). JNK and p38 MAPK pathways contribute to coxsackie virus B3, porcine circovirus type 2 (PCV2) and infectious bursal disease virus infections (Si et al., 2005; Wei et al., 2011; Wei et al., 2009). It has been reported that PRRSV infection induces ERK, JNK and p38 MAPK activations in cultured cells and these signaling pathways play important roles in PRRSV replication (Lee and Lee, 2010, 2012). However, whether other signaling pathways contribute to PRRSV replication remains to be investigated.

Protein kinase C (PKC) comprises a family of serine/threonine kinases with at least 12 members divided into three subgroups based on their structures and requirements for activation. Classical PKCs (cPKC: PKCα, PKCβI, PKCβII and PKCγ) require calcium and diacylglycerol (DAG) for activation. Novel PKCs (nPKC: PKCδ, PKCε, PKCη and PKCθ) depend only on DAG, and atypical PKCs (aPKC: PKCζ, PKCλ, PKCμ and PKCι) are independent of DAG and calcium. PKC plays a central role in diverse cellular processes and signal transduction pathways that control cell proliferation, migration, differentiation and apoptosis (Griner and Kazanietz, 2007; Mochly-Rosen et al., 2012). For virus infections, PKC are involved

* Corresponding author at: Department of Microbiology and Immunology, College of Biological Science, China Agricultural University, Beijing 100193, China. Tel.: +86 10 62733335; fax: +86 10 62732012.
E-mail address: whfeng@cau.edu.cn (W.-h. Feng).

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in virus replication cycle, including entry, replication, and release (Contreras et al., 2012; Filone et al., 2010; Hoffmann et al., 2008; Kudoh et al., 2014; Sieczkarski et al., 2003). Of these, novel PKCs (nPKC) isoform PKCδ is a very attractive cellular cofactor for virus infection. For example, PKCδ has been reported to regulate HIV-1 replication at an early post-entry step by altering the actin cytoskeleton in macrophages. In this report, the authors showed that inhibition of PKCδ altered the redistribution of actin which is required at the early reverse transcription, right after the initiation of viral cDNA synthesis (Contreras et al., 2012). Moreover, PKCδ can regulate replication and budding of respiratory syncytial virus through PKCδ/hypoxia-inducible factor-1α/NF-κB signaling pathway (Masaki et al., 2011). Apoptosis has been shown to be regulated by PKCδ during infection with Sindbis Virus (SV) and avian reovirus (ARV). Although the relationship between viral replication and apoptotic responses regulated by PKCδ has not been studied, these reports suggest that PKCδ-mediated anti- or pro-apoptotic responses may influence viral replication (Lin et al., 2009; Zrachia et al., 2002).

In the present study, we investigated the roles of protein kinase C (PKC) in PRRSV replication. Our results demonstrated that PKC was essential for efficient PRRSV replication and played a critical role at early steps of infection. Moreover, we showed that PKCδ isoform was involved in PRRSV replication.

Results

PRRSV replication is impaired by PKC inhibitor GF109203X

In order to investigate the biological importance of PKC in the replication of PRRSV, a highly specific inhibitor of all the PKC isoforms GF109203X was used and viral production was analyzed. As shown in Fig. 1A (upper panel) and Fig. 1B, about 100-fold reductions in HV (a HP-PRRSV strain) growth was observed in the presence of 10 μM GF109203X when compared with the cells treated with DMSO control. To further investigate whether this inhibitory effect is strain-dependent, a similar experiment was performed with the CH-1a strain in Marc-145 cells. As shown in Fig. 1A (lower panel) and Fig. 1C, GF109203X showed a stronger inhibitory effect against CH-1a replication in Marc-145 cells, and about 10^4-fold suppression was observed at the concentration of 10 μM, indicating that GF109203X can inhibit PRRSV replication in both PAMs and Marc-145 cells. PKC inhibitor GF109203X impaired PRRSV replication with 50% effective concentration (EC_{50}) values of 4.59 μM for HV strain in PAMs and 2.89 μM for CH-1a strain in Marc-145 cells. These data suggest that PKC might play an important role in PRRSV replication.

To rule out the possibility that the reduced viral growth is due to the cytotoxic effect of the inhibitor, cell viability for PAMs and Marc-145 cells in the presence of various concentrations of GF109203X or DMSO was analyzed using MTT assay. As shown in Fig. 1D, no obvious cellular toxicity was observed at 48 h after treatment with GF109203X at the indicated doses (5 and 10 μM). The 50% cytotoxic concentrations (CC_{50}) of GF109203X for PAMs and Marc-145 cells were 29.79 μM and 22.24 μM, respectively, which greatly exceeds its EC_{50}.

Inhibition of PKC reduces viral RNA synthesis, protein expression, and viral progeny production

We next investigated the kinetics of GF109203X-mediated inhibition of PRRSV RNA synthesis and virus yield. PAMs were

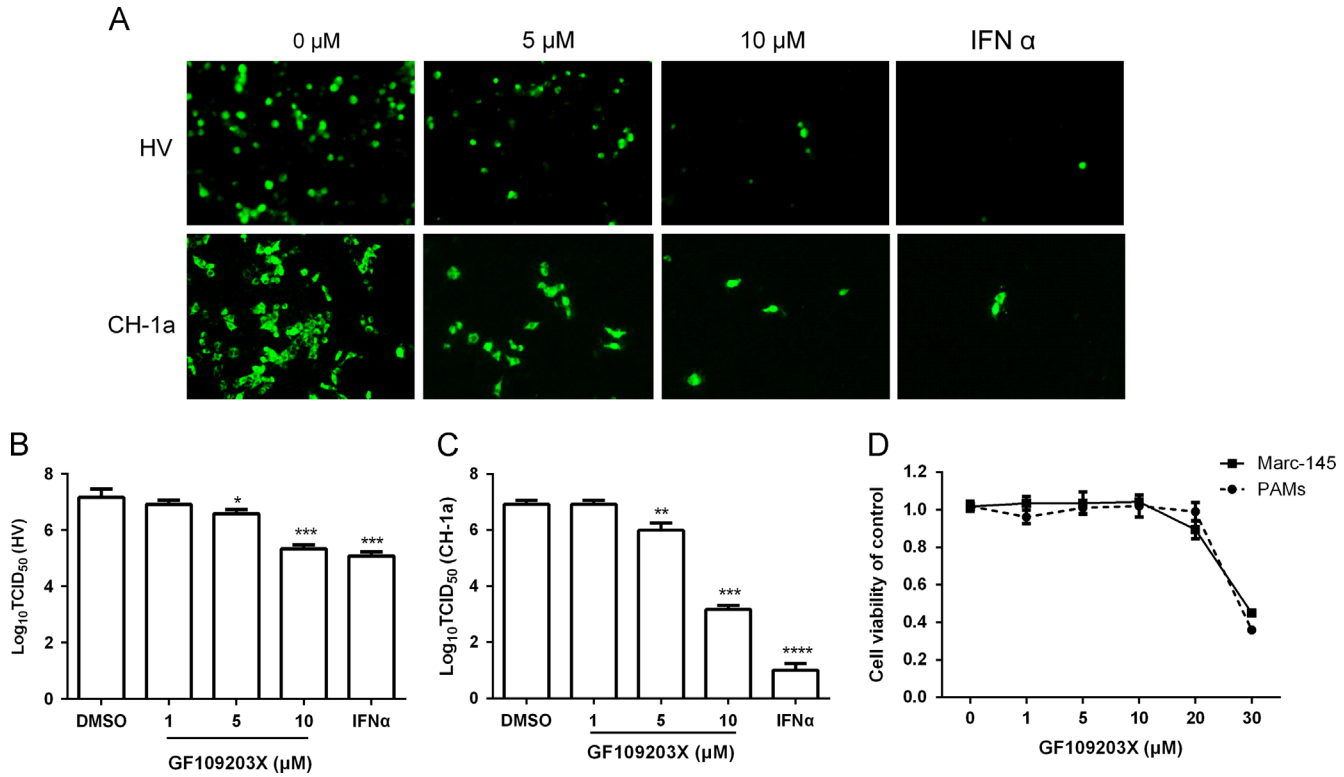

Fig. 1. PRRSV propagation is impaired by treatment with GF109203X. The inhibitory effect of GF109203X (an inhibitor of all PKCs) was examined against the HP-PRRSV strain HV and traditional PRRSV strain CH-1a in PAMs and Marc-145 cells, respectively. Cells were pretreated with GF109203X at indicated concentrations or DMSO for 1 h prior to infection and were then infected with PRRSV at an MOI of 0.3. GF109203X or DMSO was present in the medium throughout the infection. Cells infected with PRRSV and treated with porcine IFNα (10 units/ml) were served as a positive control. (A) Indirect immunofluorescence assay (IFA) was performed to detect PRRSV N protein at 24 hpi for HV and 36 hpi for CH-1a. Upper panels: HV in PAMs; lower panels: CH-1a in Marc-145 cells. (B and C) The experiments were performed as in panel A and culture supernatants were collected for virus titration. (D) Effect of GF109203X on PAMs and Marc-145 cells viability compared to the viability of DMSO-treated cells (set up as 1) was determined by MTT assay. The data represent the mean ± standard deviation from three independent experiments. Statistical significance was analyzed by *t*-test; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ and $^{****}P<0.0001$.

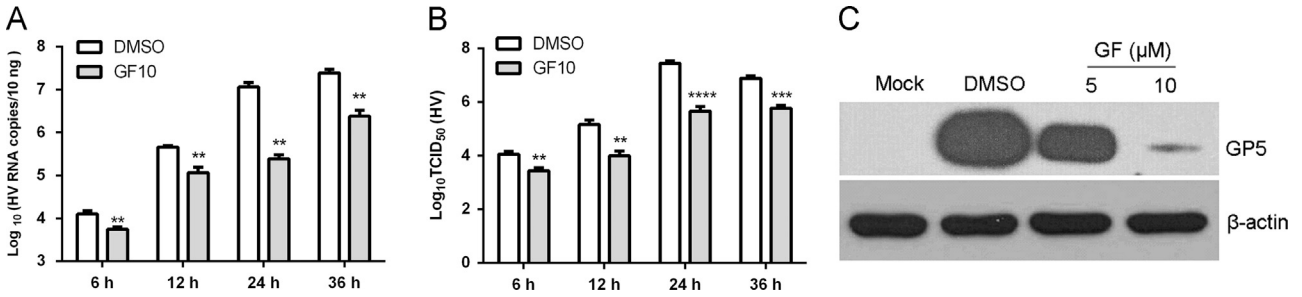

Fig. 2. PKC inhibitor decreases viral RNA synthesis, protein expression and viral progeny titers. (A) Effect of GF109203X on PRRSV viral RNA synthesis. HP-PRRSV strain HV-infected PAMs were treated from 0 hpi on with GF109203X (10 μM), and total cellular RNA was extracted and viral ORF7 RNA copy number was analyzed by quantitative real-time PCR assay (qPCR). (B) Viral growth curves in PAMs. PAMs were pretreated with DMSO or GF109203X for 1 h and were infected with HV at an MOI of 0.3. At the indicated time points, virus titers in supernatants were determined. (C) Effect of GF109203X on PRRSV protein expression. HV-infected PAMs were treated from 0 hpi on with GF109203X and viral GP5 protein expression was analyzed at 24 hpi by Western blot. β-actin was used as a loading control. GF109203X (10 μM) were abbreviated as GF10. The data represent the mean ± standard deviation from three independent experiments. Statistical significance was analyzed by *t*-test; $^{**}P<0.01$, $^{***}P<0.001$ and $^{****}P<0.0001$.

cultured either in the presence or absence of GF109203X and infected with HV. Compared with DMSO-treated cells, the addition of 10 μM GF109203X resulted in a significant reduction of viral RNA copies and this reduction reached a peak of more than 40-fold at 24 h following GF109203X addition (Fig. 2A), and the virus titer was decreased to 4.57×10^5 $TCID_{50}$/ml from 2.78×10^7 $TCID_{50}$/ml (DMSO-treated control) in the presence of 10 μM GF109203X at 24 hpi (Fig. 2B).

We also examined PRRSV GP5 expression in PAMs infected with PRRSV in the presence or absence of GF109203X. As shown in Fig. 2C, GP5 protein expression was significantly repressed in the presence of 10 μM GF109203X, while a moderate reduction in protein expression was observed at 5 μM GF109203X. These findings suggest that PKC is required for PRRSV replication.

Inhibition of PKC reduces PRRSV replication at early steps of the virus infection

To further assess which stage GF109203X acts on during PRRSV infection, PAMs were treated with GF109203X at various time points post-infection. As shown in Fig. 3A, cells treated with GF109203X up to 2 h after the time of infection resulted in about 94–85% decrease in HV replication compared to the DMSO-treated control. Addition of the inhibitor at 6 hpi and 12 hpi also reduced the production of PRRSV by 70% and 30%, respectively (Fig. 3A). We also examined the kinetics of GF109203X activity against CH-1a replication in Marc-145 cells using time-of-addition assays (Fig. 3B). Similarly, a significant reduction in CH-1a production was also observed when GF109203X was added at −1, 0, and 2 hpi. Addition of GF109203X at 6 hpi and 12 hpi, virus replication was also suppressed, but to lesser extents. In contrast, no significant reduction of viral production was observed when GF109203X was added at 24 hpi in Marc-145 cells. These results indicate that the inhibitory effect of GF109203X on PRRSV propagation is mainly at early stages of the virus replication cycle.

Inhibition of PKC partially inhibits PRRSV entry

Next, we sought to investigate whether inhibition of PKC affects the earliest step such as PRRSV entry. PRRSV entry is broadly divided into two major steps: (i) virus attachment and receptor binding, followed by (ii) receptor-mediated endocytosis and internalization. To address this issue, the binding and internalization assays were performed as described previously on Marc-145 cells (Yang et al., 2013). Our results showed that treatment with GF109203X had slight effects on virus replication when added during the period of virus attachment, suggesting that GF109203X has little effect on PRRSV attachment to Marc-145 cells (Fig. 3C and D).

To test whether GF109203X inhibits PRRSV internalization, we examined the inhibitory effect of GF109203X against PRRSV replication using virus internalization assay. As shown in Fig. 3E, treatment with GF109203X during the first 5 h of the virus internalization process resulted in a 17-fold reduction of viral RNA copies while a 3.5-fold decrease was observed when GF109203X was added 3–5 h following temperature switch. We also analyzed the production of PRRSV infectious progeny in culture supernatants of GF109203X-treated Marc-145 cells. As shown in Fig. 3F, when GF109203X was added 0–5 h following temperature switch, the virus titer was decreased about 2 log 10, and when GF109203X was added at 3 hpi the reduction in CH-1a titer was less than 1 log 10 (about 4-fold decrease). These results indicate that GF109203X partially blocks the events in PRRSV entry that is downstream of cell attachment.

PKCδ is required for PRRSV replication

GF109203X is a pan PKC kinase inhibitor. To investigate which specific PKC subgroups or isoforms are involved in PRRSV replication, we investigated the effects of selective PKC inhibitors. As shown in Fig. 4A, rottlerin (5 μM), a specific PKCδ inhibitor (Gschwendt et al., 1994), strongly blocked HV replication, whereas Gö6976, a classical PKC inhibitor, which inhibits PKC-α, β and γ, had limited effects on HV replication. The cell viability in the presence of various concentrations of rottlerin or DMSO was analyzed using MTT assay (Fig. 4C), showing that there was no observable cellular toxicity after treatment with the inhibitor rottlerin at the indicated dose. Similar results were got with CH-1a on Marc-145 cells (data not shown). We observed a slight inhibition of HV replication by Gö6976 at a concentration of 5 μM or 10 μM, while cell viability was strongly affected by Gö6976 at the concentration of 5 μM (data not shown). Given that rottlerin inhibits PRRSV replication as effectively as GF109203X, we speculate that PKCδ might be the main PKC isoform required for PRRSV replication.

To further confirm this, we next used small interfering RNA (siRNA) to see if knockdown of PKCδ could affect PRRSV replication. Transfection with PKCδ-specific siRNA significantly decreased PKCδ protein expression, while transfection with non-targeting control siRNA (Crtl siRNA) did not affect PKCδ expression (Fig. 4D). PAMs transfected with PKCδ-specific siRNA were infected with HV at 36 h post transfection, and HV replication was analyzed at 24 h after infection. As shown in Fig. 4E and F, treatment with control siRNA (Ctrl siRNA) had no effect on PRRSV replication. However, silencing of PKCδ with PKCδ-specific siRNA affected both viral RNA replication (57% reduction) (Fig. 4E) and the virus progeny titers

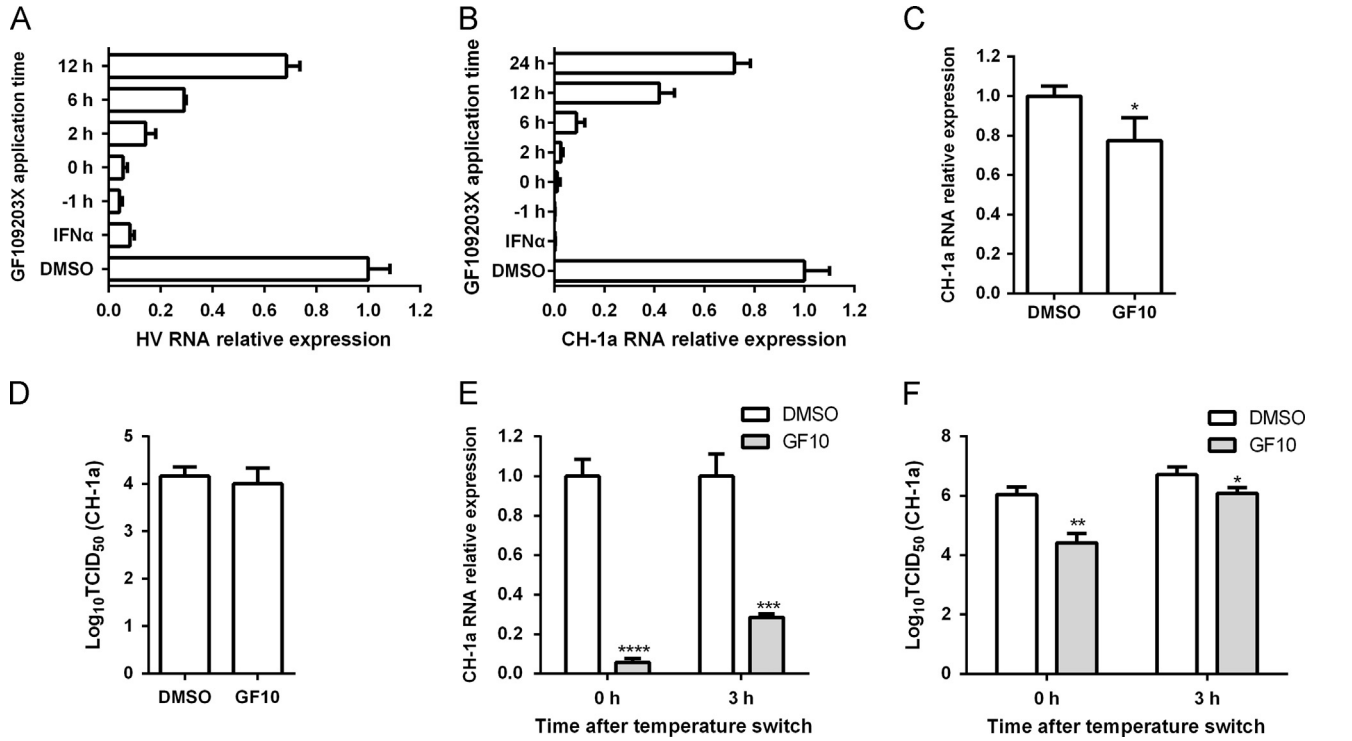

Fig. 3. PKC inhibitor reduces PRRSV replication at the early stage of infection. (A and B) PRRSV-infected cells were incubated with GF109203X (10 μM) from −1, 0, 2, 6, 12 hpi or 24 hpi onwards. PRRSV-infected cells treated with porcine IFNα (10 units/ml) from 0 hpi were served as a positive control. Total cellular RNA was extracted and PRRSV ORF7 RNA level in cells was quantified and normalized to the control cells (set up as 1) that were treated with DMSO. The relative PRRSV expression level in each sample was calibrated with the GAPDH copy number. (A) HV in PAMs at 24 hpi. (B) CH-1a in Marc-145 cells at 36 hpi. (C–F) GF109203X inhibits PRRSV entry into Marc-145 cells. (C and D) Viral binding assay. Marc-145 cells were incubated with CH-1a strain at an MOI of 1 at 4 °C for 2 h in the presence of GF109203X (10 μM) or DMSO. The cells were washed and then switched to 37 °C to continue culture. The PRRSV ORF7 RNA level in cells (C) and the virus titer in the medium (D) were determined at 36 hpi. (E and F) GF109203X blocks virus internalization. Marc-145 cells were precooled to 4 °C and incubated with CH-1a (MOI=1) for 2 h. Unbound virus was removed, and the cells were shifted to 37 °C (this time point was set up as 0 h). GF109203X (10 μM) or DMSO was added at the indicated time following the temperature shift. Five hours after temperature switch, virus and inhibitor were removed, and the cells were refreshed with medium. The intracellular viral RNA (E) and the virus titer in the culture medium (F) were determined at 36 hpi. The data represent the mean ± standard deviation from three independent experiments. Statistical significance was analyzed by *t*-test; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ and $^{****}P<0.0001$.

(0.7 log 10 decrease, about 5-fold reduction) (Fig. 4F) compared to that in the control cells (no siRNA). These results suggest that PKCδ might be activated during PRRSV infection.

One of the hallmarks for PKC activation is the translocation of these enzymes from the cytoplasm to the membrane or the cytoskeleton (Mochly-Rosen, 1995). To investigate whether PKCδ is activated during PRRSV infection, we harvested cytosol and membrane protein fractions from PAMs infected with PRRSV at 24 hpi or stimulated with phorbol-12-myristate-13-acetate (PMA) for 10 min, which is known as a specific activator of cPKC and nPKC in the PKC family. Western blot analysis showed that upon PRRSV infection, PKCδ exhibited a significant translocation from the cytosol to the plasma membrane fraction (Fig. 4G), indicating that PKCδ is activated by PRRSV infection.

Alteration of cytokine gene expression by inhibition of PKC in PAMs

Cytokines play an important role in the modulation of immune responses and interfere with viral infection. To examine whether inhibition of PKC affects the transcription of immune-response genes in PAMs upon HV infection, we analyzed their expression patterns in the presence or absence of GF109203X (10 μM). As shown in Fig. 5, inflammatory cytokines including IL-1α, IL-1β, TNF-α and IFN levels were significantly reduced in the presence of GF109203X at 24 hpi. The mRNA levels of toll-like receptor (TLR) 3 and 7 genes were not affected in the presence of GF109203X upon HV infection (Fig. 5). Only IL-10 level was slightly up-regulated by inhibition of PKC in the early time (12 h) after HV infection and then decreased in the presence of GF109203X at 24 hpi. These data suggest that PKC regulates the cytokine genes for immune-response during PRRSV infection.

Discussion

Protein kinases C plays an essential role in the replication cycle of many viruses including HIV, influenza virus, and Kaposi's sarcoma-associated herpesvirus (Contreras et al., 2012; Naranatt et al., 2003; Sieczkarski et al., 2003). In the present study, we showed that PRRSV replication could be impaired by the broad PKC inhibitor GF109203X. Subsequently, we found that the inhibitor of the novel isoform PKCδ, rottlerin, significantly inhibited PRRSV replication. Finally, we demonstrated that PKCδ was activated during PRRSV infection and knockdown of PKCδ with specific siRNA could impair PRRSV replication, suggesting that PKCδ might play an important role in PRRSV replication.

The effect of the PKC inhibitor GF109203X on PRRSV propagation was examined in two different cell types and with two different PRRSV strains (Fig. 1A–C). Our results demonstrated that GF109203X had a general inhibitory effect on PRRSV propagation, which is independent of cell type and virus strain. Previous study has shown that 1 μM GF109203X completely inhibits the activation of conventional PKCs, whereas up to 10 μM GF109203X is needed to completely block all classes of PKCs (Gunaratne et al., 2012; Valledor et al., 2000). In this study, we incubated cells with three different concentrations of GF109203X, and our data showed that the reduction in PRRSV replication was much more prominent with 10 μM GF109203X, suggesting that nPKC subgroup is

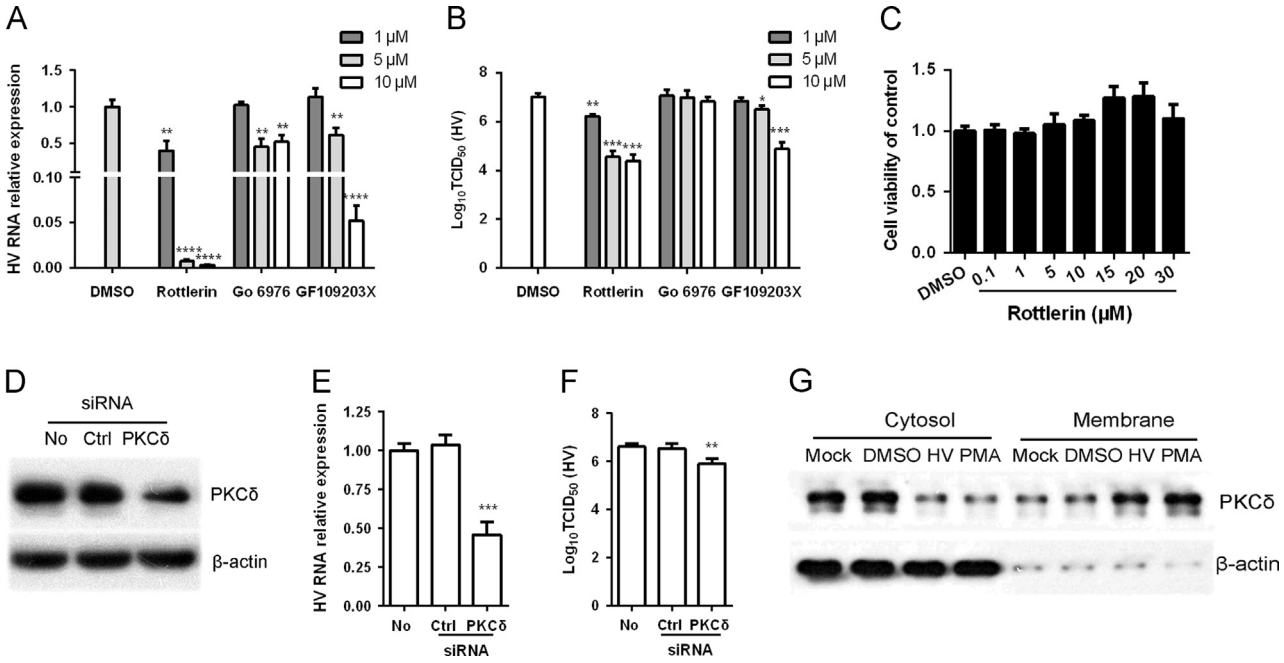

Fig. 4. PKCδ is required for PRRSV replication in PAMs. (A and B) PAMs were pretreated for 1 h with GF109203X, rottlerin (PKCδ inhibitor), Gö6976 (cPKC inhibitor) or DMSO at indicated concentrations and then infected with HP-PRRSV strain HV. (A) PRRSV ORF7 RNA level at 24 hpi in cells was determined by qPCR and (B) the virus titers in the supernatants were measured. (C) Effect of rottlerin on PAMs viability compared to DMSO-treated cells (set up as 1) was determined using MTT assay. (D–F) RNAi-mediated knockdown of PKCδ reduces PRRSV replication. PAMs were transfected with a nontargeting control siRNA (Ctrl) or siRNA targeting PKCδ. (D) PKCδ level was examined by Western blot using antibody against PKCδ. (E) PRRSV ORF7 RNA level at 24 hpi in cells transfected with the siRNAs, which were infected with HV at an MOI of 0.3 at 36 h post-transfection with the siRNAs. (F) The experiments were performed as described for panel D and virus titers in the supernatant were determined at 24 hpi. (G) PRRSV infection activates PKCδ is determined by analyzing PKCδ translocate from cytoplasm to plasma membrane. PAMs were mock-infected or infected with HV at an MOI of 0.3 for 24 h. Cells treated with PMA (100 nM) for 15 min were used as a positive control. Cell cytoplasm and plasma membrane fractions were isolated and detected by Western blot with PKCδ and β-actin antibodies. The data represent the mean ± standard deviation from three independent experiments. Statistical significance was analyzed by *t*-test; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ and $^{****}P<0.0001$.

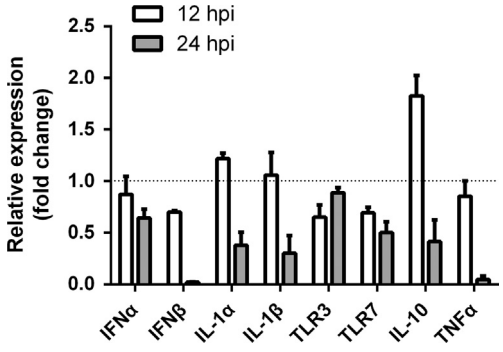

Fig. 5. Inhibition of PKC regulates transcript levels of immune-related genes during PRRSV infection. PAMs were pretreated with GF109203X (10 μM) or DMSO for 1 h and infected with HP-PRRSV strain HV (MOI=0.3). Total RNA was extracted at 12 hpi (white bar) and 24 hpi (gray bar) from lysates of the infected cells and the mRNA level of each cytokine gene was assessed by qPCR and normalized to GAPDH. Relative expression (fold changes) was compared with HV-infected cells without GF109203X (set up as 1). The data represent the mean ± standard deviation from three independent experiments.

important for PRRSV growth. A similar inhibitory effect of PRRSV replication was observed upon treatment with 5 μM PKCδ specific inhibitor rottlerin (Fig. 4A and B). We therefore analyzed the effect of siRNA-mediated knockdown of PKCδ expression on PRRSV infectivity (Fig. 4E and F). We found that PKCδ was required for efficient PRRSV replication, as siRNA-mediated knockdown of PKCδ reduced PRRSV replication, although we could not exclude the importance of PKC isoforms other than PKCδ involved in the replication of PRRSV. Finally, we demonstrated that PRRSV was able to activate PKCδ in PAMs (Fig. 4G), indicating that activation of this isoform may be beneficial to PRRSV replication.

PKC regulation of viral replication can act at specific steps of the virus replication cycle (Cirone et al., 1990; Contreras et al., 2012; Park and Baines, 2006; Root et al., 2000; San-Juan-Vergara et al., 2004). Previous study has identified that HIV can activate PKC, and this activation facilitates HIV-1 replication at different steps of its replicative cycle including entry, early post-entry step, integration and gene expression (Contreras et al., 2012; Harmon and Ratner, 2008). It has also been reported that influenza virus requires the activity of PKC for entry and PKC inhibitors induce an accumulation of virus in late endosomes (Root et al., 2000; Sieczkarski et al., 2003). However, Mahmoudian et al. reported that PKC inhibitor Gö6976 was found to not only block viral entry, but also have a post-entry anti-influenza viral effect (Mahmoudian et al., 2009). In agreement with these observations, we showed that PKC was important at early steps of the viral replication, which may exert its effect at both the entry and an early post-entry stage of the virus replication cycle (Fig. 3).

Conventional PKCα and PKCβ were found to be important negative regulators of the RIG-I-mediated type I IFN response (Maharaj et al., 2012). Kontny et al. reported that PKC was involved in the synthesis of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) in human monocytes (Kontny et al., 2000, 1999). We also assessed whether inhibition of PKC activation affected the induction of cytokine genes by PRRSV in PAMs. Our results indicated that treatment with GF109203X significantly reduced the expression of infection-induced antiviral cytokine including IFN-α, IFN-β, IL-1α, IL-1β and TNF-α. In contrast, inhibition of PKC resulted in a slightly elevation of IL-10 at the early time after HV infection. Therefore, based on our results, GF109203X-impaired PRRSV replication is not due to the alteration of antiviral cytokine expressions.

The replication of PRRSV was blocked by 10 μM GF109203X or 5 μM rottlerin, but there was no obvious cellular toxicity observed

at the doses used in our experiments. Thus, we assume the reason that inhibition of PKC impairs PRRSV replication is not due to the cytotoxic effect of the inhibitors on cells. PKC has been demonstrated to play a pivotal role in cell survival and proliferation (Griner and Kazanietz, 2007), and PRRSV infection triggers apoptosis in vitro and in vivo (Ma et al., 2013; Sur et al., 1998). In most cell types, PKCδ is pro-apoptotic and negatively regulates proliferation (Zhao et al., 2012), although in a few cases, it can mediate mitogenic responses (Jackson and Foster, 2004). Successful viral replication is critically dependent on the maintenance of pro-survival and anti-apoptotic signals in host cells. Moreover, PRRSV N protein is modified by phosphorylation and the protein kinase motif for protein kinase C (PKC) (S-X-R) is found in N protein amino acid sequence (Wootton et al., 2002) even though the functional roles for their phosphorylation is poorly understood (Kimman et al., 2009). Therefore, a detailed understanding of the inhibition of PKC on PRRSV replication remains an important area for further investigation.

In summary, our study demonstrates that PKC plays an important role in PRRSV replication. Inhibition of PKC significantly impairs PRRSV replication by exerting its effect at the early stage of infection. Moreover, we also identify that novel PKCδ isoform is required for PRRSV replication. The role of PKC in PRRSV replication will help us to understand the molecular mechanism of PRRSV infection.

Materials and methods

Cells and viruses

Porcine alveolar macrophages (PAMs) were obtained by post-mortem lung lavage of 8-week-old specific pathogen free pigs, and maintained in RPMI 1640 supplemented with 10% FBS and penicillin/streptomycin. Marc-145 cells, a PRRSV-permissive cell line derived from MA-104 cells (Kim et al., 1993), were maintained in FBS-DMEM supplemented with penicillin/streptomycin.

PRRSV strains, HV (GenBank accession no. JX317648), a HP-PRRSV strain isolated from a pig farm with an atypical PRRS outbreak in Jiangxi Province, China, and CH-1a (GenBank accession, AY032626), the first type 2 PRRSV strain isolated in China, were propagated and titrated on PAMs or Marc-145 cells. Briefly, PRRSV was serially diluted 10-fold in RPMI 1640 or DMEM to infect 5×10^4 PAMs or Marc-145 cells in 96-well plates. The virus titer of PRRSV infection was determined 36 h postinfection (hpi) using immunofluorescent staining for the PRRSV N protein. Virus titer was calculated using Reed–Muench method, and expressed as tissue culture infective dose 50% ($TCID_{50}$).

MTT assay

Cell viability in the presence of inhibitor was determined by the MTT (3-(4,5)-dimethylthiahiazo(-z-y1)-3,5-di-phenytetrazolium-romide) (Sigma) assay. PAMs or Marc-145 cells in 96-well plates were incubated with 100 μL growth medium containing dimethyl sulfoxide (DMSO) or different concentrations of the inhibitor for 48 h at 37 °C. Next, 20 μL MTT solution (5 mg/ml) was added to each well, and the cells were incubated at 37 °C for another 4 h until the medium was replaced with 200 μL DMSO to dissolve the crystals. And then, the absorbance of 495 nm was measured by an enzyme-linked immunosorbent assay reader. The 50% cytotoxic concentration (CC_{50}) was analyzed by GraphPad Prism (Graph-Pad Software, San Diego, CA).

Indirect immunofluorescence assay (IFA)

The cells were washed with phosphate-buffered saline (PBS) and fixed with cold methanol–acetone (1:1) at 4 °C for 10 min, and then blocked with 5% normal goat serum for 30 min at room temperature. After blocking, cells were stained with anti-PRRSV N protein monoclonal antibody SDOW17 (1:10,000; Rural Technologies) at 37 °C for 1 h. After three washes with PBS, cells were incubated with FITC-conjugated anti-mouse immunoglobulin G (H+L) (1:200) at 37 °C for 1 h and examined by fluorescence microscopy using Leica Microsystems CMS GmbH.

Quantitative real-time PCR (qPCR)

Total RNA was extracted from PAMs or Marc-145 cells with TRIzol (Invitrogen) following manufacturer's instructions and reverse-transcribed to cDNA using M-MLV Reverse Transcriptase (Promega). Quantitative real-time PCR was performed to determine the PRRSV loads and the levels of cytokines in cells collected from PRRSV-infected PAMs or Marc-145 cells using ViiA™ 7 real-time PCR System (Applied Biosystems) and FastSYBR Mixture (CWBIO). For the absolute quantification, a plasmid containing PRRSV ORF7 sequence (Han et al., 2009) was used to generate a standard curve, and then RNA copies in all samples were calculated by comparing with the standard curve. Relative expression levels were analyzed using the ΔΔCt method (Bookout et al., 2006) and GAPDH was set up as endogenous control. The primers used for qPCR amplification are listed in Table 1.

Extraction of membrane and cytoplasmic proteins

Cell plasma membrane and cytoplasmic fractions were isolated from PAMs using Cell Fractionation kit (#9038, CST) according to the manufacturer's instructions with minor modification. Briefly, following stimulation, cells were washed and resuspended in Cytoplasm Isolation Buffer (CIB). The cell suspensions were vortexed for 10 s, incubated on ice for 10 min, and then centrifuged (500*g* for 5 min at 4 °C). The supernatants were collected as cytoplasmic fraction. Pellets were resuspended in Membrane Isolation Buffer (MIB) and vortexed for 15 s. Following incubation on ice for 10 min, cell debris was removed by centrifugation, and

Table 1
Quantitative RT-PCR primers.

Name	Sequence (5′-3′)
ORF7-F	AATAACAACGGCAAGCAGCA
ORF7-R	GCACAGTATGATGCGTCGGC
IL-10-F	CGGCGCTGTCATCAATTTCTG
IL-10-R	CCCCTCTCTTGGAGCTTGCTA
IFN-α-F	CTGCTGCCTGGAATGAGAGCC
IFN-α-R	TGACACAGGCTTCCAGGTCCC
IFN-β-F	AGCACTGGCTGGAATGAAACCG
IFN-β-R	CTCCAGGTCATCCATCTGCCCA
Il-1α-F	TTGAAGACCTGAAGAACTGCTACA
Il-1α-R	GCCATCACCACACTGTCCTT
TLR3-F	GGACTTTGAGGCAGGTGCCCTT
TLR3-R	TGGTGCACCTTGAATCTTTTGCAC
TLR7-F	TGCGACGCTGTGTGGCTTGT
TLR7-R	TGGCCCTTGTGTGCTCCTGG
IL-1β-F	TCTGCCCTGTACCCCAACTG
IL-1β-R	CCCAGGAAGACGGGCTTT
TNF-α-F	ACCACGCTCTTCTGCCTACTGC
TNF-α-R	TCCCTCGGCTTTGACATTGGCTAC
PKCδ-F	GCAGGGATTAAAGTGTGAAG
PKCδ-R	AACCTCCGACTCTGACTTTC
GAPDH-F	CCTTCCGTGTCCCTACTGCCAAC
GAPDH-R	GACGCCTGCTTCACCACCTTCT

the supernatants containing membrane-associated proteins were kept at −80 °C.

Western blotting

Equal amounts of proteins were separated on SDS-PAGE gel and then transferred to PVDF membranes (Millipore, USA). The membranes were blocked for 1 h at room temperature in blocking buffer PBST (PBS containing 0.5% Tween-20) containing 5% skim milk powder to prevent nonspecific binding. After blocking, the membranes were incubated with specific primary antibodies against GP5 (1:4000; made in our lab) or β-actin (1:5000; Sigma) for 1 h at room temperature or with PKC-delta (PKCδ) (1:500; Santa Cruz Biotechnology) overnight at 4 °C. The membranes were washed three times with PBST and then incubated for 1 h at room temperature with the appropriate HRP-conjugated secondary antibodies diluted in blocking buffer. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL) reagent according to the manufacturer's instructions.

PRRSV binding assay

For binding assay, Marc-145 cells were inoculated with medium containing CH-1a (MOI=1) and DMSO or GF109203X at 4 °C for 2 h. Then, unbound virus/inhibitor was removed by washing three times with cold PBS and the medium was replaced with 2% FBS–DMEM. The cells were then switched to 37 °C to culture for another 34 h. The total cellular RNA was extracted to analyze PRRSV ORF7 RNA level and supernatants were harvested for virus titration.

PRRSV internalization assay

For internalization assay, Marc-145 cells were incubated with CH-1a (MOI=1) at 4 °C for 2 h to permit binding. Next, the medium was removed and cells were washed 3 times with cold PBS to remove unattached virus particles. Then, fresh medium was added and cells were switched to 37 °C (this time point was set up as 0 h) to allow virus entry. GF109203X (10 μM) was then added at 0 or 3 h following temperature shift. At 5 h following temperature switch, the cells were carefully washed with PBS and then continued to be cultured with fresh medium at 37 °C. At 36 hpi, PRRSV ORF7 RNA level in cells and the virus titer in the medium were determined.

siRNA and transfection

siRNA duplexes (siRNAs) were synthesized and purified by Genepharma. siRNA sequences are as follows: for PKCδ (PKCδ siRNA), 5′-CCACGAGUUUAUCGCCACCTT-3′ (Choi et al., 2010); for nontargeting control siRNA (Ctrl siRNA), 5′-UUCUCCGAACGUGUCACGUTT-3′. Transient transfection with siRNA was carried out using HiPerFect (Qiagen) according to the manufacturer's recommendations at a final concentration of 50 nM.

Statistical analysis

All experiments were performed at least three independent replicates. Results were analyzed using Graph-Pad Prism Software, and differences were evaluated by Student's *t* test. A *P* value of less than 0.05 was considered to be significant.

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