Protein Kinase Cα Is Involved in Interferon Regulatory Factor 3 Activation and Type I Interferon-β Synthesis

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Protein kinase C (PKC) isoforms are critically involved in the regulation of innate immune responses. Herein, we investigated the role of conventional PKCα in the regulation of IFN-β gene expression mediated by the Toll-like receptor 3 (TLR3) signaling pathway. Inhibition of conventional PKC (cPKC) activity in monocyte-derived dendritic cells or TLR3-expressing cells by an isoform-specific inhibitor, Gö6976, selectively inhibited IFN-β synthesis induced by double-stranded RNA polynosine-polycytidyl acid. Furthermore, reporter gene assays confirmed that PKCα regulates IFN-β promoter activity, since overexpression of dominant negative PKCα but not PKCB, repressed interferon regulatory factor 3 (IRF-3)-dependent but not NF-κB-mediated promoter activity upon TLR3 engagement in HEK 293 cells. Dominant negative PKCα inhibited IRF-3 transcriptional activity mediated by overexpression of TIR domain-containing adaptor inducing IFN-β (STING) and Tank-binding kinase 1. Additional biochemical analysis demonstrated that Gö6976-treated dendritic cells exhibited IRF-3 phosphorylation, dimerization, nuclear translocation, and DNA binding activity analogous to their control counterparts in response to polynosine-polycytidylic acid. In contrast, co-immunoprecipitation experiments revealed that TLR3-induced cPKC activity is essential for mediating the interaction of IRF-3 but not p65/RelA with the co-activator CREB-binding protein. Furthermore, PKCα knockdown with specific small interfering RNA inhibited IFN-β expression and down-regulated IRF-3-dependent promoter activity, establishing PKCα as a component of TLR3 signaling that regulates IFN-β gene expression by targeting IRF-3-CREB-binding protein interaction. Finally, we analyzed the involvement of cPKCs in other signaling pathways leading to IFN-β synthesis. These experiments revealed that cPKCs play a role in the synthesis of IFN-β induced via both TLR-dependent and -independent pathways.

Type I interferons (IFNs), comprising IFN-β and the IFN-α family are central to the innate immunity of mammals and the development of effective adaptive immune responses against viruses and tumors (1, 2). Toll-like receptors (TLRs) expressed by innate immune cells, including dendritic cells (DCs), recognize distinct pathogen-associated molecular patterns, leading to the activation of innate immunity, which shapes the subsequent adaptive immune response (3, 4). TLR3 triggering by double-stranded RNA generated during viral infection with synthetic double-stranded RNA analog poly(I:C), endogenous cellular mRNA structures, and small interfering RNAs results in DC activation to promote type I IFNs (5–7). Recent studies have demonstrated the involvement of TLR3 in antiviral defense and its prominent role in the cross-priming mechanism, since DCs from TLR3-deficient mice exhibit reduced inflammatory responses mediated by reovirus genomic double-stranded RNA and poly(I:C) and are compromised in CTL cross-priming (5, 8–10).

The signaling pathway downstream of TLR3 requires the adaptor molecule TRIF that recruits tumor necrosis factor-associated factor-6 to channel NF-κB activation and mediate the phosphorylation of the two noncanonical 1κB kinases, 1κB kinase-ε and Tank-binding kinase 1 (TBK1) (9, 11–13). Uncoupling at the level of TRIF, TBK1, and IKKε kinases induces events leading to IRF-3 phosphorylation-dependent activation (12, 14). Importantly, both of these phosphorylate distinct C-terminal Ser residues on IRF-3, which results in IRF-3 homodimerization, nuclear translocation, and enhanced ability to bind to the IFN-β promoter (6, 15). Upon activation, IRF-3 homodimers associate with the transcriptional co-activators C/EBP-binding protein (CBP) and p300 in the nucleus, an event that enables the optimal induction of the IFN-β promoter (16–19).

The enhancer region of the IFN-β promoter has been extensively characterized as containing four overlapping regulatory elements that are designated as positive regulatory domain (PRD)-I, -II, -III, and -IV (17, 19, 20). Promoter region analysis of the Ifnβ gene revealed that PRDII and PRDIV are bound by NF-κB and ATF-2/c-Jun, respectively, and that these elements

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cooperate with PRDI and PRDIII in the induction of the IFN-β promoter (21). The PRDI and PRDIII elements were shown to be bound by distinct members of the IRF family with much attention focused on IRF-3, which binds to the PRDIII-I composite site on the IFN-β promoter and is required for IFN-β gene transcription (22–24).

PKCs mediate an evolutionarily conserved function in host defense against fungal and bacterial infections from primitive organisms up to mammals (25, 26). The PKC family subdivides into three main groups based on the presence or absence of distinct motifs determining co-factor requirements for their activity. Conventional PKCs (α, βI, βII, and γ) require Ca²⁺ binding and are activated by diacylglycerol and phorbol esters, whereas novel PKCs (δ, ε, and η) do not require Ca²⁺ but are also diacylglycerol/phorbol ester-sensitive. The last subgroup, atypical PKC (ζ and λ/ι) require neither Ca²⁺ nor diacylglycerol/phorbol esters (27, 28). Previous studies demonstrated that PKC isoforms regulate several signaling pathways, including innate immune responses induced by microbial products (26). Indeed, Gram-negative LPS was shown to activate distinct PKC isoforms in DCs and macrophages (Mφs) (29–33). Specifically, PKCe is an essential integrator of LPS-mediated inflammatory cytokine production through activation of NF-κB and mitogen-activated protein kinases (29, 34). Importantly, PKCe-deficient Mφs display defects in cleaving both Gram-negative and positive bacterial infections (34). Although PKC involvement in poly(I:C)-mediated innate responses was previously observed using broad range pharmacological inhibitors (35, 36), participation of PKC isoforms in poly(I:C)-mediated activation of TLR3 pathway still remains unknown.

In this work, we investigated the role of cPKC isoforms in poly(I:C)-induced cytokines, particularly IFN-β and the molecular targets downstream of the TLR3 signaling pathway. Cellular inhibition assays, using a conventional PKC inhibitor, GÖ6976, performed in monocyte-derived DCs, HEK 293, and HEK 293 cells stably expressing TLR3 demonstrated that conventional PKC activity is involved in IFN-β gene expression. In support of this observation, we showed that overexpression of dominant negative (DN) PKCα as well as GÖ6976 inhibits IRF-3-dependent but not NF-κB-mediated reporter gene activities, correlating with repression of IFN-β promoter activity. Further biochemical analysis revealed an unexpected role for cPKC activity that is essential for mediating IRF-3 association with CBP. Particularly, PKCα knockdown by siRNA in TLR3-293 cells established that PKCα participates in IRF-3-mediated IFN-β gene expression. Finally, our data show that cPKCs play a general role in IFN-β synthesis induced either by TLR-dependent or TLR-independent pathways.

EXPERIMENTAL PROCEDURES

Generation of Monocyte-derived DC—DCs were generated from peripheral blood mononuclear cells, as described by Romani et al. (37). Briefly, peripheral blood mononuclear cells were harvested from healthy volunteers by density centrifugation on Lymphoprep (Nycomed, Oslo, Norway), resuspended in culture medium, and allowed to adhere onto 75-cm² flasks. After 2 h at 37 °C, nonadherent cells were removed, and adherent cells were cultured in 20 ml of medium containing granulocyte-macrophage colony-stimulating factor (800 units/ml) and IL-4 (100 units/ml). Every 2 days, 800 units of granulocyte-macrophage colony-stimulating factor and 100 units of IL-4 were added. After 6 days, nonadherent cells that corresponded to the DC-enriched fraction routinely contained more than 95% DC as assessed by morphologic and fluorescence-activated cell sorter analysis as described previously (38).

Stable Cell Lines and Reagents—Human embryonic kidney (HEK) 293 parental cells and cells stably expressing FLAG-TLR3 (TLR3-293) were kindly provided by S. Akira (Osaka University, Osaka, Japan). The multimerized NF-κB was kindly provided by W. Vanden Berghe (University of Gent); PRDIII-I (12), IFN-β promoter (IFN-β pGL-3) (39), Gal4-luciferase reporter construct, and Gal4-IRF-3 expression vector were described earlier (16). E-tag-TRIF was a gift from R. Beyaert (University of Gent). pHACE-PKC-KR (DN) expression plasmids were generated by ligating full-length open reading frames of PKC isoforms with a K368R point mutation at the ATP binding site into pHACE digested with EcoRI (40). Poly(I:C) was purchased from Amersham Biosciences, ds-β-DNA was purchased from InvivoGen (Toulouse, France), and GÖ6976 was from Boehringer Biochem (Leuven, Belgium). Granulocyte-macrophage colony-stimulating factor was obtained from R&D Systems (Abingdon, United Kingdom).

Transfection Assays—HEK 293 or TLR3-293 cells were seeded in 24-well plates at a density of 5 × 10⁵/ml. The following day, cells were transfected with 1 μg of the indicated luciferase reporter plasmids, using FuGENETM-6 (Roche Applied Science) according to the manufacturer’s specifications. All transfections included 40 ng of Renilla luciferase DNA in the pRL-TK vector (Promega, Leiden, The Netherlands) as an internal control. Where indicated, the cells were stimulated with poly(I:C) (50 μg/ml) for 18 h, and subsequently the cells were harvested and the whole cell extracts (WCEs) were obtained, followed by analysis of promoter activities using the dual luciferase reporter assay system (Promega). Promoter activities were normalized to Renilla luciferase activities. Data are expressed as the mean relative stimulation ± S.D.

siRNA Transfection—Duplexed siRNAs targeting human PKCα and GFP, each labeled with 3’ Alexafluor 647, were purchased from Qiagen (K) Venlo, The Netherlands). HEK 293 cells stably expressing TLR3 were seeded in 24-well plates at a density of 1.25 × 10⁶. Twenty-four hours later, cells were transfected with the indicated concentrations of PKCα siRNA or control, GFP siRNA using X-treme GENE siRNA Transfection reagents (Roche Applied Science) according to the manufacturer’s protocol. After 48 h of incubation, the supernatant was removed and replaced with fresh medium and subsequently stimulated with poly(I:C) (10 μg/ml). The cells were collected after 18 h of stimulation, and the efficiency of PKCα knockdown was assessed by Western blotting. The cell supernatants were analyzed for IFN-α and IL-8 production, as described below. For DNA/RNA co-transfection studies, TLR3-293 cells (1.25 × 10⁵/ml) were transfected with 1 μg of IRF-3-Gal4 reporter plasmid using X-treme gene (Roche Applied Science). 24 h later, PKCα- or GFP-siRNA was added. After 48 h of incubation, the supernatant was removed and replaced with fresh medium. Where indicated,
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**FIGURE 1. Inhibition of conventional PKC activity down-regulates poly(I:C)-mediated IFN-β synthesis.** Immature DCs were incubated in vehicle (Me2SO) or the indicated concentrations of G66976 for 2 h and then activated by poly(I:C) (10 μg/ml) or left unstimulated. A, poly(I:C) induces activation of novel PKC and conventional PKC isoforms in DCs. At the indicated time intervals, cells were harvested and lysed, and the protein extracts were analyzed by direct Western blotting using an anti-phospho-pan-PKC antibody. Protein loading was controlled by probing with an anti-PKCα antibody. One representative of three independent experiments is shown. B, conventional PKC inhibitor diminishes IFN-β production. IFN-β concentrations in culture supernatants were analyzed by ELISA. Data represent means ± S.E. of five independent experiments. ns, statistically not significant; *, p < 0.05 as compared with cytokine levels between vehicle-treated DCs activated by poly(I:C).

Conventional PKC inhibitor represses IFN-β mRNA transcription. IFN-β mRNA accumulation at 4 h after poly(I:C) stimulation was quantified by quantitative reverse transcription-PCR. The IFN-β mRNA levels were normalized to β-actin mRNA levels and depicted as -fold index compared with unstimulated samples. Data represent means ± S.E. of six independent experiments performed from different donors. *, p < 0.05 as compared with cytokine levels between vehicle-treated DCs activated by poly(I:C). C, conventional PKC inhibitor represses IFN-β mRNA transcription. IFN-β mRNA accumulation at 4 h after poly(I:C) stimulation was quantified by quantitative reverse transcription-PCR. The IFN-β mRNA levels were normalized to β-actin mRNA levels and depicted as -fold index compared with unstimulated samples. Data represent means ± S.E. of five independent experiments. ns, statistically not significant; *, p < 0.05 as compared with cytokine levels between vehicle-treated DCs activated by poly(I:C).

**Determination of Cytokine Levels**—DCs or HEK 293 cells (5 × 10⁵/ml) were stimulated with poly(I:C) (10 and 50 μg/ml, respectively) or LPS (1 μg/ml) for 20 h following quantification of cytokines in culture supernatants. HEK 293 cells were seeded in 24-well plates at a density of 5 × 10⁵/ml. The following day, cells were transfected with 2 μg of synthetic ds-β-DNA, using FuGENETM.6 (Roche Applied Science) according to the manufacturer’s specification. Reverse transcription and quantitative reverse transcription-PCR were performed using Light-Cycler® Master Hybridization probes (one-step procedure) on a Lightcycler® apparatus (Roche Applied Science). The conditions were previously described (38). The oligonucleotide sequences used for amplification of the IFN-β were as follows: sense primer, 5'-GGATGCA-GGAAGGAGATCAGT-3'; antisense primer, 5'-CGATCC-ACACGAGATCTTC-3'; probe, 5'-6-carboxyfluorescein succinimidyl ester-CCCTGGAACGCAACATG(6-carboxytetramethylrhodamine succinimidyl ester) (phosphate)-3'. The oligonucleotides used to amplify β-actin were described earlier (38).

**Immunoblotting**—DC or TLR3-293 cells (1 × 10⁵/ml) were collected and directly lysed in Laemml buffer. Equal volumes of WCEs from each condition were resolved by 8% SDS-PAGE and analyzed by Western blotting. Immuneblotts were probed with polyclonal antibodies directed against phosphopan-PKC (Cell Signaling, Leusden, the Netherlands), phospho-IFR-3 (Cell Signaling, Leusden, the Netherlands), phospho-ATF-2 (Cell Signaling), and phospho-c-Jun (Cell Signaling). Equal loading was verified either by anti-PKCα (Santa Cruz Biotechnology, Inc.), PKCβ (Santa Cruz Biotechnology), anti-HA (Euroscreen, Rixensart, Belgium), phospho-ATF-2 (Cell Signaling), or phospho-c-Jun (Cell Signaling). The immunoreactive bands were revealed using the ECL® detection kit (Amersham Biosciences). For co-immunoprecipitation studies, WCEs (400 μl) of DCs or TLR3-293 cells were lysed in buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, pH 8.0, 30 mM NaF, 1 mM Na₃VO₄, 40 mM β-glycerophosphate, 0.1 mM phenylmethylsulfonyl fluoride in EtOH, protease inhibitor mixture (Roche Applied Science), 1% glycerol, and 1% Nonidet-P40 (Nonidet P-40) and then incubated overnight with 1 μg of anti-CBP antibody (C-1; Santa Cruz Biotechnology). WCEs were incubated with 30 μl of a 1:1 slurry of protein A/protein G-Sepharose beads (Amersham Bio-
firmed TLRs by their specific ligands induces activation of several PKC isoforms in various cell types (26). Therefore, we first assessed whether poly(I:C) induced the activation of PKC isoforms in monocyte-derived DCs. As shown in Fig. 1A, DCs exhibited phosphorylated forms of conventional PKCα/β and novel PKCδ isoforms within 30 min following the poly(I:C) encounter. Gö6976, a potent and selective conventional PKC inhibitor targeting Ca2+-dependent PKC isoforms (42), completely abolished the phosphorylation-dependent activation of conventional PKC isoforms without effecting PKCδ activation.

The following experiments were designed to evaluate the functional outcomes of blocking poly(I:C)-mediated conventional PKC activation on cytokine production in DCs. As shown in Fig. 1B, Gö6976 dose-dependently inhibited poly(I:C)-induced IFN-β secretion from DCs. Next, we analyzed whether the inhibitory effect of Gö6976, observed on IFN-β protein secretion, was due to an effect on IFN-β mRNA transcription. Using quantitative reverse transcription-PCR analysis, we observed that the accumulation of IFN-β mRNA transcripts occurred as early as 2 h, peaking maximally at 4 h following poly(I:C) stimulation (data not shown). At 4 h, Gö6976-treated poly(I:C)-activated DCs exhibited significantly reduced IFN-β mRNA levels in comparison with poly(I:C)-activated control counterparts (Fig. 1C). In contrast, poly(I:C) exposure of DCs resulted in IL-8 and tumor necrosis factor-α production that was comparable between the Gö6976-treated cells and their control counterparts (Fig. 1, D and E).

**PKCα Is Involved in TLR3-mediated Induction of IFN-β-dependent Promoter Activities**—In the next set of experiments, we investigated the effects of inhibiting poly(I:C)-induced conventional PKC activity on human IFN-β promoter activity in TLR3-expressing 293 cells. As shown in Fig. 2, poly(I:C) stimulation of TLR3-293 cells resulted in a strong induction of IFN-β promoter activity, which was repressed 3-fold in Gö6976-treated counterparts.

Our data pointed out that cPKCs were involved in IFN-β gene expression through regulation of IFN-β promoter activity.
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Furthermore, in two distinct cell types, we found that cPKC signaling is selectively involved in the regulation of IFN-β but not inflammatory cytokines upon poly(I:C) encounter. Since IFN-β gene transcription requires the cooperation of NF-κB (12, 43, 44) and IRF-3 transcription factors (22–24), we investigated the involvement of conventional PKC isoforms in the regulation of IRF-3- and/or NF-κB-mediated transcriptional activities using either IRF-3- or NF-κB-dependent promoters in TLR3-293 cells, which also express PKCα and PKCβ, conventional isoforms endogenously (Fig. 3A). We transfected TLR3-293 cells with either Gal4-IRF-3, the PRDIII-I site from the IFN-β promoter, or a multimerized NF-κB site from the IL-8 promoter in the presence of increasing concentrations of Gö6976 and subsequently stimulated it with poly(I:C). In Fig. 3B (top and middle), we demonstrated that the Gö6976 treatment resulted in a dose-dependent repression of poly(I:C)-mediated Gal4-IRF-3 reporter activity as well as transcription mediated at the PRDIII-I site in TLR3-293 cells. In order to assess which of the conventional PKC isoforms are involved in TLR3 signaling, we overexpressed DN PKCα and PKCβ, isoforms in TLR3-293 cells. As shown in Fig. 3C (top), IRF-3 reporter activity was dose-dependently inhibited (~6-fold at the highest dose) by DN PKCα but not by DN PKCβ, overexpression. In a similar setting, poly(I:C)-induced reporter gene transcription at the PRDIII-I site from the IFN-β promoter was similarly inhibited (Fig. 3C, middle). Strikingly, poly(I:C)-induced NF-κB reporter gene activity from the IL-8 promoter was altered by neither DN PKCα nor PKCβ, expression vector nor Gö6976 treatment (Fig. 3, B and C, bottom). In the reporter assay settings we tested, DN PKCβ had no effect while being optimally expressed in HEK 293 cells (Fig. 3A, right). Hence, we concluded that PKCα selectively regulates transcriptional activity of IRF-3-dependent promoters induced by poly(I:C) triggering of TLR3.

PKCα Is Involved in TRIF-mediated IRF-3 Activation Downstream of TBK1—Transcriptional activity at the PRDIII-I site from the IFN-β promoter requires TRIF interaction with TBK1, which channels phosphorylation-dependent activation of IRF-3 (12, 45, 46). Next we investigated whether TRIF-mediated and TBK1-mediated signaling to IRF-3 are targeted by conventional PKC isoforms. To assess this question, we analyzed the effects of DN PKCα and DN PKCβ, isoforms on IRF-3-mediated reporter gene activity induced by TRIF or TBK1 overexpression in HEK 293 parental cells. As shown in Fig. 4A (top), TRIF overexpression resulted in the increase of IRF-3-dependent reporter gene activity. Co-expression of DN PKCα but not DN PKCβ, dose-dependently inhibited (~3-fold at the highest dose) TRIF-mediated IRF-3 transcriptional activity. In Fig. 4A (bottom), TRIF-mediated induction of reporter gene activity at the PRDIII-I site in 293 cells was inhibited (~6-fold at the highest dose) by DN PKCα, whereas DN PKCβ, had no effect. Furthermore, DN PKCα (but not DN PKCβ,) overexpression dose-dependently repressed IRF-3-mediated reporter gene activity.
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- PKCα regulates IRF-3-mediated IFN-β synthesis.

**Inhibition of Conventional PKC Activity Hinders IRF-3 Binding to Co-activator CBP**—Thus far, we have shown that PKCa participates in TLR3-mediated transcriptional activity of IRF-3-dependent promoters. Given that conventional PKC inhibitor, G66976, diminishes IFN-β expression without influencing the initial events important for IRF-3 phosphorylation, homodimerization, nuclear translocation, and DNA binding, we investigated whether conventional PKCs are important for IRF-3 association with the co-activator CBP. In co-immunoprecipitation experiments, we observed a robust physical interaction between the endogenous forms of IRF-3 and CBP in poly(I:C)-stimulated DCs that was severely decreased in G66976-treated counterparts (Fig. 6A, left). Similar results, showing strongly reduced IRF-3 interaction with CBP, were obtained from TLR3-293 cells exposed to G66976 (Fig. 6B, left). Previously, the co-activator CBP was shown to interact with other transcription factors, including the p65/RelA member of NF-κB (50). To find out whether the effects of G66976 resulting in the hindrance of IRF-3 interaction with CBP prevent CBP from binding to NF-κB members, we performed co-immunoprecipitation assays to visualize p65 interaction with CBP.

**Conventional PKCs Are Not Involved in Poly(I:C)-mediated IRF-3 Phosphorylation**—Our data showing diminished IFN-β gene expression and IRF-3-dependent transcriptional activity led us to investigate the functional outcomes of inhibiting conventional PKCs on early signaling events important for IRF-3 activation. Poly(I:C)-mediated site-specific IRF-3 Ser396 phosphorylation is critical for IRF-3 nuclear translocation and DNA binding on IFN-stimulated response element sites on IRF-3-responsive promoters (39, 47). Therefore, we first examined the phosphorylation status of IRF-3 at Ser396 in the presence or absence of G66976 in poly(I:C)-activated DCs. Interestingly, Western blotting experiments demonstrated that poly(I:C)-mediated phosphorylation of IRF-3 at Ser396 residue was comparable whether or not DCs were exposed to G66976, shown by a slower migrating band (Fig. 5A). Following phosphorylation, IRF-3 forms a homodimer, an important process required for its transcriptional activity (39, 48, 49). We therefore analyzed the effects of inhibiting conventional PKCs on IRF-3 homodimer formation in DC extracts by native PAGE analysis. Our data demonstrated that conventional PKC inhibitor G66976 does not influence the formation of IRF-3 homodimers that occur following poly(I:C) encounter by DCs (Fig. 5B).

Next, using confocal microscopy, we showed that inhibition of conventional PKC activity did not affect poly(I:C)-mediated nuclear localization of IRF-3 in DCs, since poly(I:C)-induced IRF-3 nuclear translocation was comparable between G66976-treated DCs and the vehicle-treated control counterparts (Fig. 5C, top). As displayed in Fig. 5C (middle), G66976-treated DCs exhibited similar levels of poly(I:C)-induced p65/RelA translocation in comparison with their control counterparts. Finally, we assessed poly(I:C)-induced IRF-3 DNA binding activity in DCs exposed to G66976. IRF-3 DNA binding was increased in nuclear extracts from DCs, detected at 90 min following poly(I:C) stimulation (Fig. 5D, left). The level of IRF-3 DNA binding in G66976-treated DCs was not significantly altered. Likewise, poly(I:C)-induced NF-κB DNA binding was also not affected by G66976 treatment, indicating that neither NF-κB nuclear translocation nor its DNA binding is influenced by G66976 (Fig. 5D, right). Hence, our data indicated that the modifications rendered by inhibiting conventional PKC activity in TLR3 signaling do not involve previously documented events leading to IRF-3 activation, specifically dimerization, nuclear translocation, and DNA binding ability.

**Conventional PKCs Are Not Involved in Poly(I:C)-mediated IRF-3 Phosphorylation, Dimerization, Nuclear Translocation, and DNA Binding**—Our data showing diminished IFN-β gene expression and IRF-3-dependent transcriptional activity led us to investigate the functional outcomes of inhibiting conventional PKCs on early signaling events important for IRF-3 activation. Poly(I:C)-mediated site-specific IRF-3 Ser396 phosphorylation is critical for IRF-3 nuclear translocation and DNA binding on IFN-stimulated response element sites on IRF-3-responsive promoters (39, 47). Therefore, we first examined the phosphorylation status of IRF-3 at Ser396 in the presence or absence of G66976 in poly(I:C)-activated DCs. Interestingly, Western blotting experiments demonstrated that poly(I:C)-mediated phosphorylation of IRF-3 at Ser396 residue was comparable whether or not DCs were exposed to G66976, shown by a slower migrating band (Fig. 5A). Following phosphorylation, IRF-3 forms a homodimer, an important process required for its transcriptional activity (39, 48, 49). We therefore analyzed the effects of inhibiting conventional PKCs on IRF-3 homodimer formation in DC extracts by native PAGE analysis. Our data demonstrated that conventional PKC inhibitor G66976 does not influence the formation of IRF-3 homodimers that occur following poly(I:C) encounter by DCs (Fig. 5B).

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FIGURE 5. Inhibition of conventional PKCs does not prevent IRF-3 activation. Immature DCs were treated with vehicle (Me2SO) or G66976 (1 μM) for 2 h and then were activated by poly(I:C) (10 μg/ml) or were left unstimulated. A, G66976 effects on IRF-3 phosphorylation. At the indicated time intervals, cells were harvested and lysed in Laemmli buffer, followed by direct Western blotting using anti-phospho-IRF-3 Ser396 antibody. One representative experiment of five is shown. B, IRF-3 homodimerization in poly(I:C)-activated DCs. 90 min after poly(I:C) activation, cells were harvested, and WCEs were prepared and subjected to native PAGE followed by immunoblotting as described under “Experimental Procedures.” One representative of four independent experiments is shown. C, nuclear translocation of IRF-3 and NF-κB in DCs. 90 min after poly(I:C) activation, cells were collected, washed, and fixed on coverslips, followed by staining using antibodies specific for IRF-3 (top) or p65/RelA (middle). Nuclei were stained with TOTO®-3 iodide (bottom). Nuclear translocation was visualized using confocal microscopy. The images shown are representative of nonoverlapping fields. One representative of three independent experiments is shown. D, DCs were harvested 90 and 120 min after cellular activation, and nuclear extracts were prepared and analyzed for IRF-3 (left) or p65/RelA (right) DNA binding activies, respectively, using the TransAM transcription factor assay kits. Data are means ± S.E. of 5–8 independent experiments. ns, statistically not significant.

Strikingly, poly(I:C)-mediated interaction between p65 and CBP remained intact whether or not DCs or TLR3-293 cells were exposed to G66976 (Fig. 6, A and B, right). Overall, these observations indicated that inhibition of conventional PKC activity abrogates IRF-3 but not NF-κB interaction with the co-activator CBP.

Inhibition of Conventional PKCs Does Not Interfere with ATF-2 and c-Jun Activation—We then evaluated the role of cPKCs in TLR3-mediated activation of ATF-2/c-Jun, which is the third transcription factor required for IFN-β induction. As shown in Fig. 7A, TLR3-293 cells exhibited a phosphorylated form of ATF-2 within 30 min following poly(I:C) exposure, which persisted up to 60 min after stimulation and was not affected by G66976 treatment. Similarly, the poly(I:C)-induced phosphorylation of c-Jun occurred in an identical kinetic and was maintained even in the presence of G66976 (Fig. 7B). Next, using co-immunoprecipitation experiments, we examined the role of cPKCs on ATF-2 or c-Jun-CBP interactions in the same system. TLR3-293 cells exhibited basal ATF-2 or c-Jun-CBP interaction but we were not able to detect an increase of such interaction after poly(I:C) stimulation. Nevertheless, the basal interaction between ATF-2 or c-Jun and CBP was not disrupted by G66976 (Fig. 7C). We then performed additional experiments in human DCs; in this setting, the increase of ATF-2 or c-Jun and CBP interaction was still barely detectable even upon poly(I:C) stimulation. However, in a single experiment, where the induction was clearly apparent, the addition of the drug did not affect the ATF-2 or c-Jun-CBP interaction (data not shown).

PKα Is Essential for IFN-β Expression in Response to Poly(I:C)—To further confirm the role of PKα in the regulation of IFN-β gene expression, we transiently transfected TLR3-293 cells with siRNA targeting PKα. TLR3-293 cells were transfected with PKα-siRNA ranging from 0 to 200 μM. As shown in Fig. 8A (left), PKα knockdown by its target-specific siRNA occurred in a dose-dependent manner, whereas the control GFP-siRNA
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Conventional PKCs Participate in the Control of LPS and ds-β-DNA-induced IFN-β Synthesis—Our data showing the critical role of conventional PKCs in TLR3-mediated IFN-β expression led us to investigate the possible involvement of cPKCs on other signaling pathways leading to IFN-β synthesis. Therefore, we first assessed the effect of cPKC inhibition on TLR4-mediated IFN-β synthesis in DCs. As shown in Fig. 9A, LPS-induced IFN-β secretion is significantly inhibited in G66976-treated DCs. We next evaluated the effect of inhibiting cPKCs on IFN-β synthesis induced by a synthetic ds-β-DNA, previously described for its ability to trigger a TLR-independent cytosolic pathogen recognition receptor (51). For that purpose, we used HEK 293 cells, which do not express any TLRs. HEK 293 cells were transfected with ds-β-DNA in the presence of increasing concentrations of G66976. As shown in Fig. 9B, ds-β-DNA-induced IFN-β was strongly inhibited in a dose-dependent manner, 5-fold at the highest dose. These results revealed that cPKCs are involved in the synthesis of IFN-β induced via both TLR4- and TLR-independent pathways.

DISCUSSION

PKC isoforms are essential signaling components that contribute to innate immune defense against microbial and viral infections and regulate adaptive immune responses (25, 26). LPS triggering of TLR4 activates PKCe, which is involved in inflammatory cytokine expression by modulating NF-κB activation in human DCs and murine MΦs downstream of TRAM. PKCe was shown to regulate TLR4 signaling by phosphorylating TRAM, a key process required for its function (29, 33, 34). Furthermore, poly(I:C)-activated PKCs were shown to be involved in IFN-β expression, since PKC inhibitors were shown to effectively block type I IFN production in human, simian, and mouse fibroblasts (35, 36).

In this study, we have shown that conventional PKCs, particularly the PKCα isoform, participates in poly(I:C)-mediated IFN-β gene expression through modulation of IRF-3-depend-
PKCα Regulates IRF-3-mediated IFN-β Synthesis

A.

![Graph A](image)

B.

![Graph B](image)

C.

![Graph C](image)

FIGURE 8. PKCα participates in IRF-3 transcriptional activity and IFN-β expression. A, TLR3-293 cells were transiently transfected with the indicated concentrations of PKCα-siRNA (left and right) or with 150 μM PKCα and GFP-siRNA (right). After 48 h, the cells were harvested and lysed, and the protein extracts were analyzed by direct Western blotting using an anti-PKCα antibody. Protein loading was controlled by probing with an anti-p65 antibody. One representative of three independent experiments is shown. B, TLR3-293 cells were transiently transfected with 1 μg of Gal4-IRF-3 reporter plasmids. The following day, cells were transiently transfected with PKCα- or GFP-siRNA (50, 100, and 150 μM). After 48 h, the cells were stimulated with poly(I:C) (10 μg/ml) or left unstimulated for 18 h. WCEs were harvested, and luciferase reporter gene activity was measured and normalized using Renilla luciferase activities. Data represent means ± S.D. of one experiment performed in triplicate. C, TLR3-293 cells were transiently transfected for 48 h with PKCα-siRNA (150 μM) or GFP-siRNA (150 μM). They were then stimulated with poly(I:C) (10 μg/ml) or left unstimulated for 18 h. IFN-β (left) or IL-8 (right) levels in culture supernatants were analyzed by ELISA. Data represent means ± S.D. of three independent experiments performed in triplicate.

ent transcription in two distinct cell types, human DCs and HEK 293 cells. Inhibition of conventional PKCs activity downstream regulated IFN-β mRNA transcription and repressed IFN-β promoter activation in poly(I:C)-activated DCs and TLR3-293 cells, respectively. Moreover, G66976-mediated inhibitory effects on IFN-β synthesis cannot be attributed to defects in IFNRI-mediated signaling, because inhibition of conventional PKC activity did not modify recombinant type I IFN-mediated phosphorylation of signal transducer and activator of transcription Tyr701 residue (data not shown).

Collectively, our data obtained from reporter assays with DN PKCα overexpression as well as PKCα-siRNA knockdown establish that TLR3-induced conventional PKCα isoform is involved in the regulation/activation of early signaling events that induce activation of the IFN-β promoter. Since the cooperation of IFR-3 and NF-κB transcription factors for poly(I:C)-mediated induction of IFN-β promoter activity has been well documented (21, 22, 24, 52), we investigated the involvement of conventional PKCα and β isoforms in IRF-3- and NF-κB-dependent reporter gene transcription. Our data confirm that DN PKCα (but not DN PKCβ1) effectively repressed IRF-3-dependent but not NF-κB-mediated reporter gene activity. Therefore, PKCα mediates IRF-3 transcriptional activity but not NF-κB-dependent transcription downstream of the TLR3 signaling pathway. In addition, the results from reporter gene assays performed using HEK 293 parental cells demonstrate that DN PKCα down-regulates IRF-3 transcriptional activity induced by TRIF or TBK1 overexpression. These findings suggest that PKCα operates downstream of TLR3-TRIF-TBK1 and participates in IRF-3 transcriptional activity.

The signaling events controlling poly(I:C)-mediated IRF-3 transcriptional activity are complex and multifaceted. Shortly after poly(I:C) or virus exposure, IRF-3 undergoes a conformational change following phosphorylation of specific serine residues in the C-terminal serine-rich region of IRF-3 that allows its homodimerization (53). Following homodimer formation, IRF-3 translocates to the nucleus, where it binds to the PRDIII-I site on IFN-β promoter as well as IFN-stimulated response element sites found on IFN-responsive genes (24, 52). The minimal phosphoacceptor site at Ser396 on IRF-3 was shown to be targeted by poly(I:C) signaling, important for IRF-3 translocation and DNA binding ability upon poly(I:C) or viral activation (39). In addition, Fujita and co-workers (16) have demonstrated that point mutations of Ala of Ser385 or Ser386 (referred to as the 2S site), which is part of the serine-rich region, abolishes the phosphorylation and dimerization of IRF-3 by TBK1. Through biochemical analysis, we have assessed the contribution of conventional PKCs in the early signaling events critical for IRF-3 activation, such as (a) IRF-3 phosphorylation, (b) homodimerization, (c) nuclear translocation, (d) DNA-binding ability, and (e) IRF-3 association with CBP/p300. Our data from Western blotting experiments show that poly(I:C)-activated DCs in the presence of G66976 exhibited comparable levels of IRF-3 Ser386 and also Ser385/386 phosphorylation (data not shown). In accordance with the lack of effect of conventional PKC inhibition on these critical phospho-Ser residues, native PAGE experiments and confocal microscopy analysis demonstrated that conventional PKCs are not involved in IRF-3 phosphorylation,
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IRF-3 with CBP, which is essential for TLR3-mediated IFN-β promoter induction and expression.

Similar to TLR3, TLR4 also mediates type I IFN, using MyD88-independent and TRIF-dependent pathways to induce IRF-3 activation (13, 56). In our system, LPS-induced IFN-β production by DCs was likewise sensitive to cPKC inhibition. In conjunction with our data on the TLR3 pathway, this demonstrates a broader role of cPKCs in TLR-dependent signaling. Another important pathway leading to type I IFN synthesis involves the activation of the cytosolic pathway by ds-β-DNA. Viruses as well as intracellular bacteria that have cytosolic phases in their life cycles are recognized by TLR-independent cytosolic receptors, such as the helicase retinoic acid-inducible gene 1 and melanoma differentiation-associated gene 5, that recognize viral RNAs and synthetic double-stranded RNA (57, 58). These two receptors use the adaptor molecule interferon-β promoter stimulator 1 to induce IFN-β and cytokines. Subsequently, we investigated the possible role of cPKCs in the induction of IFN-β through this TLR-dependent pathway. These results revealed that cPKCs are involved in IFN-β synthesis induced by both TLR-dependent and TLR-independent pathways.

Although the role of TLR3 in direct priming of antiviral response is controversial, viral evasion of TLR-dependent and -independent pathways argues for the contribution of these signaling pathways in optimal host defense against viruses (9, 59, 60). It is proposed that TLR signaling and the resulting cytokine network plays an active role in the pathogenesis of autoimmunity (2, 61, 62). Type I IFNs, induced by viral infections and/or by ligand-dependent TLR activation, are principal factors that contribute to the breakdown of peripheral tolerance and autoimmune disease generation or progression (2, 62). In this perspective, our study and others (29, 34) demonstrating that distinct PKC isoforms take diverging roles to control TLR signaling pathways highlight the significance of isoform-selective PKC inhibition not only as potential therapy to control inflammation-induced pathologies but also as a target for viral evasion of host immunity. Hence, control of PKC activity may represent a potential strategy to treat autoimmune pathologies originating from TLR-dependent or -independent type I IFN action. Finally, since PKC inhibitors are part of current regimens used for anti-tumor therapy, we suggest that the inhibitory effects of conventional PKC inhibitors on IRF-3 transcriptional activity and IFN-β expression should be more cautiously addressed in vivo.

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