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FIP-*fve* Stimulates Interferon-Gamma Production via Modulation of Calcium Release and PKC-α Activation

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Fungal immunomodulatory protein, FIP-*fve*, has been isolated from *Flammulina velutipes*, and its immunomodulatory effects are believed to be associated with the enhanced activation of IFN-γ-releasing Th1 cells. However, the mechanisms of FIP-*fve*-mediated signal transduction in the regulation of interferon-gamma (IFN-γ) gene expression in human peripheral blood mononuclear cells (PBMCs) are still poorly understood. Using fluo-3 AM, we found that FIP-*fve* induces a rapid elevation in calcium concentration. ELISA, RT-PCR and Western blot assays demonstrated significant increases in the production and mRNA expression of IFN-γ and protein kinase C-alpha (PKC-α) activation in activated PBMCs, which were abolished by EGTA, nifedipine and GÖ6976. In conclusion, Ca^{2+} release and PKC-α activation are required for IFN-γ production induced by FIP-*fve* in PBMCs.

KEYWORDS: FIP; PKC-α; IFN-γ; PBMC; calcium

INTRODUCTION

Fungal immunomodulatory protein (FIP-*fve*) has been isolated and purified from the edible golden needle mushroom (*Flammulina velutipes*). FIP-*fve* has no direct cytotoxic effect on normal cells such as thymocytes but possesses hemagglutinate activity in human red blood cells. The immunomodulatory activity of FIP-*fve* has been demonstrated by its stimulatory activity in human peripheral blood lymphocytes, and its suppression of systemic anaphylactic reactions and localized swelling of mouse footpads (*1*). FIP-*fve* has also been found to stimulate the production of IFN-γ in PBMCs (*2*). Th1 cells produce IFN-γ, which is essential for the eradication of intracellular pathogens, whereas Th2 cells secrete IL-4, IL-5, and IL-13, which are crucial for the elimination of extracellular organisms and for sustained allergic reactions (*3*). IFN-γ is a pleiotropic cytokine that is essential for both innate and adaptive immunities (*4*). An animal study revealed that oral administration of FIP-*fve* has an immunoprophylactic effect on the development of the allergen-specific immune response, and may serve as a potent preventive strategy for food allergies and other allergy-related diseases (*5*).

Some mechanisms have been proposed to explain the capacity of FIPs to enhance immune activity *in vitro*. For instance, FIP-*fve* induces IFN-γ production by p38 mitogen-activated protein kinase signaling pathway (*2*). The other immunomodulatory protein, FIP-*gts*, can also act as a potent activator in PBMCs through phosphatidylinositol 3-kinase signal pathway (*6*). However, other molecular mechanisms are involved in the cytokine production (*7*, *8*). It has been established that divalent cation calcium (Ca^{2+}) is a secondary messenger and signal transducer in both excitable and nonexcitable cells. It is involved in many cellular responses including muscle contraction, metabolism, enzyme activation, gene expression, secretion, cell proliferation, and cell death (*9*, *10*). Ca^{2+} also plays a pivotal role in T cell activation (*11*). A decrease in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) results in the impairment of IL-2 production in T lymphocytes and PBMCs (*12*). It is well-known that IL-12 and IFN-γ are major Th1-inducing cytokines (*13*). However, to the best of our knowledge the extent of Ca^{2+} involvement in IFN-γ secretion by FIPs has not been investigated.

Immune cells respond to numerous stimuli, including microbial antigens, mitogens and cytokines, through several signal transduction pathways to mediate gene expression and immune

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function. Activation of T lymphocytes is crucial for the potent response of the immune system. Activated T cells initiate signal cascades which are transduced from the cell membrane to the nucleus through different specific signaling pathways in order to modulate cytokine gene transcription (*14*). One of these signal transduction events initiates phosphorylation and activation of PLC-γ, which cleaves phosphatidylinositol 4,5-bisphosphate to generate diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). These respectively activate various protein kinase Cs (PKCs) and trigger a release of Ca^{2+} from the endoplasmic reticulum (*14*, *15*). PKCs are protein serine/threonine kinases with activities dependent on Ca^{2+} and phospholipids. They are particularly important mediators of immune intracellular signaling (*16*). It has been reported that PKC-α is activated by different stimuli such as growth factors and mitogen and acts as an active secondary messenger of TCR-induced proliferation and IL-2 production (*16*, *17*).

Ca^{2+} and PKCs mediate a pivotal positive signal necessary for IL-2 secretion (*8*). However, little is known about the involvement of Ca^{2+} and/or PKC signal transduction pathways in the regulation of IFN-γ production in PBMCs by FIP-*fve*. Here, our results demonstrate that Ca^{2+} increase and activation of PKC-α play positive regulatory roles in IFN-γ production by FIP-*fve* in PBMCs, which may modulate Th1 immune response in vivo.

MATERIALS AND METHODS

Chemicals and Reagents. FIP-*fve* was purified as previously described (*1*). It resolved to a single band on SDS−15% PAGE with Coomassie brilliant blue R staining. Fluo-3-acetoxymethyl (fluo-3 AM) and pluronic acid F-127 were purchased from Molecular Probes (Eugene, OR). Nifedipine, GÖ6976 and EGTA were obtained from Sigma (Sigma Chemical Co., St. Louis, MO). Phospho-PKC-α and antirabbit IgG horseradish peroxidase- (HRP-) linked Ab were obtained from New England Biolabs (Beverly, MA). All other chemicals were of analytical grade.

Determination of Cytokine Production in PBMCs. To determine the optimum concentration of FIP-*fve*, PBMCs were cultured with varying concentrations of FIP-*fve*. In another set of experiments, PBMCs (2×10^6 cells/mL) were pretreated with different doses of nifedipine (L-type Ca^{2+} channel blocker), GÖ6976 (an inhibitor of PKC-α, β) or 1 mM EGTA (extracellular Ca^{2+} chelator) for 1 h and incubated with FIP-*fve* (7.69 μM). After 48 h at 37 °C, the cell supernatants were collected and assayed for IFN-γ concentrations with enzyme immunoassays (ELISA).

Measurement of $[Ca^{2+}]_i$ in PBMCs. Analysis of Ca^{2+} in PBMCs was carried out as described previously with some modifications (*18*, *19*). The PBMCs were loaded with HBSS containing 0.5% BSA at final concentration of 10 μM fluo-3 AM for 30 min at 37 °C. Cells were washed twice and then resuspended in Ca^{2+}-free HBSS to a density of 4×10^6 cells/mL. One milliliter of PBMC suspension was equilibrated with an equal volume of 2 mM Ca^{2+}-containing buffer and transferred to individual cuvettes followed by gentle mixing with micromagnetic stirrer at 37 °C for 2 min before the addition of PBS or FIP-*fve* (1.92 and 7.69 μM). For experiments conducted in the absence of external Ca^{2+}, 1 mL of PBMC suspension was incubated with 1 mL of Ca^{2+}-containing or Ca^{2+}-free buffer at 37 °C for 2 min before the addition of FIP-*fve* (7.69 μM). In another set of experiments, 1 mL of PBMC suspension was pretreated with EGTA or nifedipine. After pretreatment for 20 min, the PBMC suspension was equilibrated with an equal volume of 2 mM Ca^{2+}-containing buffer and transferred to individual cuvettes followed by gentle mixing with micromagnetic stirrer at 37 °C for 2 min before the addition of FIP-*fve* (7.69 μM). The fluorescence was measured by a fluorescence spectrophotometer (F-4500, Hitachi, Tokyo, Japan) with a multiwavelength time-scan program. The fluorescence activity was recorded at excitation wavelength of 506 nm and emission wavelength of 526 nm. Calibration procedures were performed as described by Grynkiewicz and co-workers (1985)(*20*), using 10% Triton x-100 to obtain the maximal fluorescence (F_{max}) with fluo-3 AM saturation with Ca^{2+}, followed by 50 mM EGTA to obtain minimum fluorescence (F_{min}) in the absence of any fluo-3 AM/Ca^{2+} complex. F depicts the fluorescence of the experimental sample. Values of $[Ca^{2+}]_i$ was calculated using the equation (*20*)

$$[Ca^{2+}]_i = K_d(F - F_{min})/(F_{max} - F)$$

The dissociation constant (K_d) for the fluo-3 AM/Ca^{2+} complex was assumed to be 800 nM at 37 °C (*21*).

Protein Kinase C Assay. After treatment with the indicated agents, the cells were washed twice in ice-cold phosphate-buffered saline and then solubilized in extraction buffer containing 20 mM Tris-HCl, 0.5 mM EGTA, 2 mM EDTA, 2 mM DTT, 1 mM PMSF, and 10 μg/mL leupeptin at pH 7.5. After 10 min, the lysates were sonicated 6 times with 10 s bursts and centrifuged at 12000*g* for 20 min. The supernatant was collected and then immunoprecipitated with polyclonal antibody against PKC-α. The protein concentration was assayed using Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA).

Western Blotting. PKC-α and β-actin were quantified by Western blot analysis. Equal amounts of proteins (20 μg) were subjected to gel electrophoresis on a 10% gel and then transferred to Polyvinilydene difluoride (PVDF) membranes by electroblotting. Blots were incubated in a Tris-buffered saline solution at pH 7.6, containing 5% nonfat dry milk and 0.1% (v/v) Tween 20 (TBS-T). Western blots were performed using polyclonal antibodies against PKC α (Cell Signaling Technology, Beverly, MA; 1:500) and monoclonal anti-β-actin (AC-40, Sigma, St. Louis, MO). After washing with TBS-T buffer, a 1:5000 dilution of horseradish peroxidase-labeled antirabbit or antimouse IgG (Cell Signaling Technology, Beverly, MA) was added at room temperature for 1 h. ECL Western blotting detection reagents (Amersham Milan, Italy) were used to visualize specific hybridization signals. Blots were then developed using an enhanced luminol chemiluminescence (ECL) reagent (NEN, Boston, MA).

Extraction of Total Cellular RNA and Transcriptase Polymerase Chain Reaction. The total cellular RNA was extracted from PBMCs by a method described previously (*1*). Following stimulation, cells were lysed by RareRNA reagent. Aliquots of 2 mg of RNA were reverse-transcribed using the Advantage TM RT for-PCR kit according to the manufacturer's instructions and a previously described method. To detect IFN-γ mRNA, the following PCR primers were used: N-terminal primers were nucleotides 101−122, 5′-TCTTTGGCTTAATTCTCTCGGAA and C-terminal primers were 500−478, 5′-TCAGTTACCGAATAATTAGTCAG. Two microliters of cDNA were amplified in a reaction volume of 50 μL containing 0.5 unit of Taq polymerase (Ex taq, TaKaRa), 200 mM dNTPS, 10 mM Tris-HCl (pH8.0), 1.5 mM $MgCl_2$, 75 mM KCl and 10 pmol of each primer. The PCR reaction involved denaturation (94 °C, 5 min) followed by 32 cycles for IFN-γ, each consisting of denaturation (94 °C, 1 min), annealing (55 °C, 1 min) and extension (72 °C, 2 min), with a final extension phase (10 min). The PCR reaction was performed on a programmable thermal controller instrument-thermal cycler model 2400. Meanwhile, the same amount of cDNA was amplified using specific β-actin including sense and antisense primers (CAGGGAGTGATGGTGGGCA, CAAACATCATCTGGT CATCTTCTC), according to the manufacturer's instructions (Life Technologies). The samples underwent 28 cycles of 5 min at 94 °C followed by denaturation (94 °C, 5 min), annealing (55 °C, 1 min) and extension (72 °C, 2 min). The final cycle was modified to allow for a 10 min extension at 72 °C. The products were visualized using electrophoresis on 1.5% agarose gel and ethidium bromide staining. We confirmed the quality of cellular mRNA by measuring the intensity of β-actin.

Statistical Analysis. One-way ANOVA was used to compare the IFN-γ production among the subgroups in **Figures 1** and **4**. Results are expressed as means ± standard deviation, and error bars indicate SD.

RESULTS AND DISCUSSION

Our previous study demonstrated that FIP-*fve* is a potent activator of PBMCs and through the p38 MAP kinase pathway regulates the expression of IFN-γ in Th1 cells (*2*). In the present study, we have shown that FIP-*fve* increases production and mRNA expression of IFN-γ in activated PBMCs. In addition, we observed that FIP-*fve* stimulates IFN-γ production via the modulation of Ca^{2+} release and, as a consequence, induces PKC phosphorylation and its activation. Hence, both the elevation of

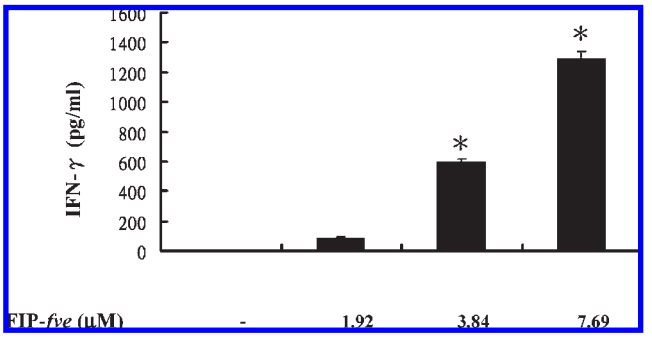

Figure 1. Effects of FIP-*fve* on IFN-γ secretion in PBMCs. Cultured peripheral T cells (2×10^6 cells/mL, 1 mL/well) were treated with the indicated concentrations of FIP-*fve* in RPMI 1640 supplemented with 5% FBS for 48 h. Conditioned media were subjected to ELISA to measure amounts of secreted IFN-γ (mean value from three independent experiments). Asterisks (*) represent a significant difference from the control group ($P < 0.05$).

Ca^{2+} and PKC activation play a vital role in FIP-*fve* stimulation of IFN-γ production in PBMCs.

FIP-*fve* induces IFN-γ production in PBMCs. IFN-γ was not detected in nonstimulated cultures. The production of IFN-γ in activated PBMCs increased significantly by FIP-*fve* in a concentration-dependent manner (**Figure 1**). In this study, 7.69 μM which achieves maximal production of IFN-γ was chosen for subsequent experiments.

Effects of FIP-*fve* on Ca^{2+} release. Ca^{2+} release is an initial event in the activation of T cells and PBMCs, and is necessary for a wide variety of cellular processes (*22*). To study whether the IFN-γ production in PBMCs by FIP-*fve* is associated with Ca^{2+} release, PBMCs were incubated with FIP-*fve* and Ca^{2+} concentrations were determined by a fluorescence spectrophotometer. The results are shown in **Figure 2**. When FIP-*fve* was added to PBMCs at 70 s, Ca^{2+} began to rapidly and transiently increase about 30 s later and reached a maximum value at about 100 s. However, an increase in Ca^{2+} was not observed in PBS-treated PBMCs. As shown in **Figure 2A**, FIP-*fve* caused an elevation of $[Ca^{2+}]_i$ in a dose-dependent manner. It has been long considered that an elevation in intracellular Ca^{2+} can be induced from two different sources; Ca^{2+} release from an intracellular Ca^{2+} stores such as the endoplasmic reticulum (ER) and the Ca^{2+} influx from the extracellular medium (*23*). The absence of Ca^{2+} in the extracellular buffer considerably weakened the FIP-*fve*-induced Ca^{2+} extent of Ca^{2+} peak compared with that in the presence of Ca^{2+} in the extracellular buffer (**Figure 2B**). In comparison to FIP-*fve*-evoked $[Ca^{2+}]_i$, the amplitude of the Ca^{2+} peak was evidently lower in the presence of EGTA. L-type Ca^{2+} channel blocker (nifedipine) could not fully block the action of FIP-*fve*-evoked Ca^{2+} mobilization (**Figure 2C**). These results seemed to show that the increase in $[Ca^{2+}]_i$ induced by FIP-*fve* was mostly influxed from extracellular store and only a small part from intracellular store. Our findings are similar to those of another study. After depletion of the T cell receptor-mediated store, the activated Ca^{2+} influx channel is considered to be a major mechanism for sustained elevation in cytosolic Ca^{2+} concentration necessary for T cell activation, whereas the ability of intracellular Ca^{2+} release channels is believed to be minor (*24*).

As far as we know, at least two Ca^{2+} release channel families for the Ca^{2+} mobilization from intracellular compartments have been reported (*24*, *25*). One is the IP_3 receptor (IP3R) family which is sensitive to inositol-trisphosphate (IP_3). The other is the ryanodine receptor (RyR) family which was originally found in the sarcoplasmic reticulum of skeletal muscle and cardiac muscle.

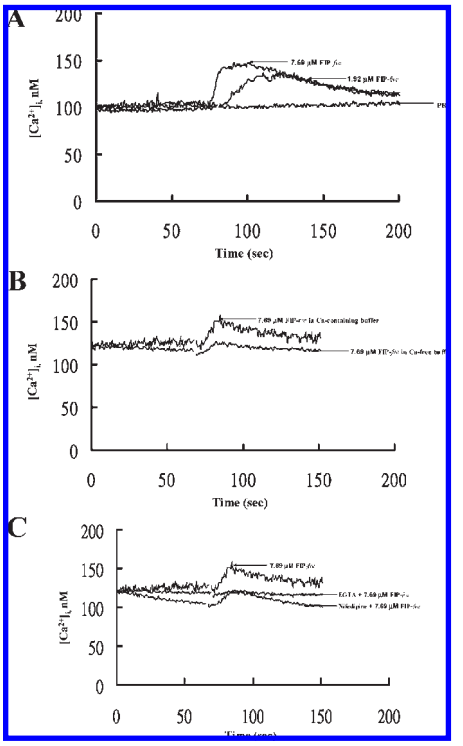

Figure 2. Effects of FIP-*fve* on $[Ca^{2+}]_i$ release in PBMCs. PBMCs were loaded with 10 μM fluo-3 AM at 37 °C for 30 min. The cells were then resuspended in HBSS solution to a concentration of 4×10^6 cells/mL. (**A**) Increasing concentrations of FIP-*fve* (1.92 and 7.69 μM) were added to fluo-3 AM loaded cells. (**B**) Curve representing the experiments performed in Ca^{2+}-containing or Ca^{2+}-free buffer. (**C**) Fluo-3 AM loaded cells were preincubated with extracellular Ca^{2+} chelator (EGTA, 1 mM) or L-type Ca^{2+} channel blocker (nifedipine, 100 μM) at 37 °C for 20 min before the addition of FIP-*fve* (7.69 μM). All experiments were performed in Ca^{2+}-containing buffer. The data are representative of three independent experiments.

IP3R isoforms are expressed in immune cells (*24*), whereas PBMCs express two RyR isoforms (RyR1 and RyR2) (*25*). It has been reported that increased $[Ca^{2+}]_i$ may stimulate Ca^{2+} release from the IP_3R and/or RyR either via direct interactions or through Ca^{2+}-induced Ca^{2+} release (CICR) mechanism (*26*). However, the specific intracellular channel has not been elucidated that supports the CICR and the functional significance of the CICR in nonexcitable cells (*24*). An early signal pathway of mitogen- or cell surface receptor-stimulated immune cell activation is a biphasic increase in $[Ca^{2+}]_i$. The increased $[Ca^{2+}]_i$ occurs first as Ca^{2+} mobilization from the intracellular stores via the activation of different Ca^{2+} release channels and second as Ca^{2+} influx through various plasma membrane Ca^{2+} channels. The Ca^{2+} release and Ca^{2+} influx act synergistically to sustain Ca^{2+} elevation that is necessary for the biochemical cascade of signal transduction pathways which include the activation of PKCs, that of specific transcription factors and cytokine production (*15*, *24*, *27*). Hence, we strongly suspect that FIP-*fve* mediates Ca^{2+} release from endoplasmic reticulum stores via IP_3R and/or RyR.

In the present study, L-type Ca^{2+} channel blocker could not fully block the action of FIP-*fve*-evoked Ca^{2+} release; thus, we cannot rule out the possibility of other unidentified types of Ca^{2+} channels. Voltage-gated channels are most typically expressed in excitable cells (*18*). But some reports suggested L-type and T-type channels did present in nonexcitable cells such as PBMCs (*28*−*30*). In fact, Ca^{2+} mobilizing messengers, Ca^{2+} release channels

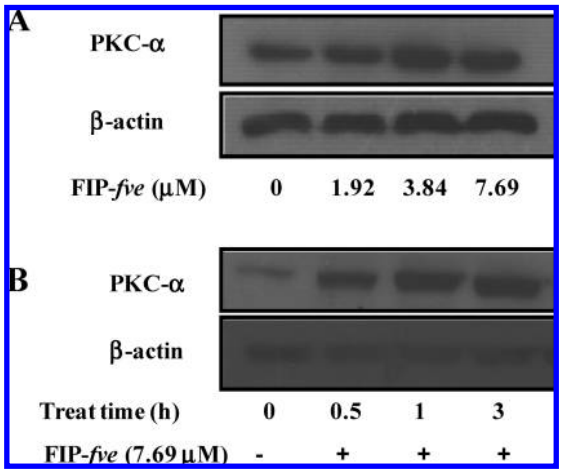

Figure 3. FIP-*fve* induces phosphorylation of PKC-α. PBMCs were incubated with FIP-*fve* at varying concentrations (1.92, 3.84, and 7.69 μM, respectively for 3 h). PBMCs were incubated with FIP-*fve* (7.69 μM) for 3 h at different times (0.5, 1, and 3 h). The phosphorylation levels of PKC-α were detected by Western blotting, as described in Materials and Methods.

and Ca^{2+} store are multiple and complex (*26*). We hypothesized that the increase in $[Ca^{2+}]_i$ induced by FIP-*fve* was mostly influxed from extracellular store and only a small part from endoplasmic reticulum (ER). Whether $[Ca^{2+}]_i$ can be increased by opening plasma membrane Ca^{2+} channels, to generate an influx of Ca^{2+} from extracellular fluids, remains to be determined.

Ca^{2+} is a well-established regulator of transcriptional change in gene expression, which is important for cellular function, differentiation, and proliferation (*31*−*33*). The sustained elevation of Ca^{2+} is critical for activation of NF-AT (*27*). NF-AT and NF-κB, two major downstream effectors of Ca^{2+} influx and PKC activation (*27*, *34*), have been reported to regulate many cytokine gene expressions including those of IL-2 and IFN-γ (*35*). Increased Ca^{2+} has been shown to participate in secretion of several molecules, including cytokines (*7*, *36*). In the present study, we demonstrated that FIP-*fve* evokes an initial rise in Ca^{2+} in a dose-dependent manner. Our findings are similar to those of another study in which Ca^{2+} was demonstrated to play important roles in reLZ-8-induced signal transduction of IL-2 gene expression within T cells (*8*).

Effects of FIP-*fve* on PKC-α Activities in PBMCs. Most PKCs are fully phosphorylated immediately after translation (*16*). The PKC signaling pathway often converges with other signaling pathways and triggers a cascade of events at the point of nuclear protein factors (NF-AT and NF-κB) that are indispensable for gene transcription such as cytokine genes (*37*). The FIP-*fve* stimulated IFN-γ transcription required about 3−4 h, and the subsequent translation took about 48 h to express the IFN-γ protein (*1*). Therefore, 3 and 48 h were chosen to indicate the required time for determining PKC-α activation and IFN-γ production, respectively. FIP-*fve* treatment of PBMCs resulted in the activation of PKC-α in a dose dependent manner (**Figure 3A**). In addition, expression of PKC-α was induced by the treatment of FIP-*fve* at various time intervals. Time kinetics revealed that PKC-α activation by FIP-*fve* was in a time-dependent manner. Maximum activation of PKC-α was observed at 3 h of incubation (**Figure 3B**). PKC activation makes significant effects in the control of a wide range of cellular signaling processes, including membrane receptor function, cell differentiation, cell death, and cytokine secretion (*8*, *15*, *38*). In resting cells, most PKCs are localized primarily to the cytosol, and translocated to the cell membrane surface after stimulation, including

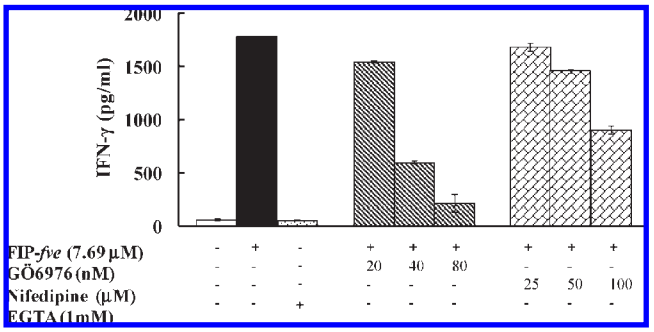

Figure 4. Effects of EGTA, nifedipine and G06976 on IFN-γ production in FIP-*fve*-stimulated PBMCs. Cultured PBMCs were pretreated with different doses of nifedipine, GÖ6976 or 1 mM EGTA for 1 h and then in combination with 7.69 μM FIP-*fve* for 48 h. Conditioned media were subjected to ELISA to measure amounts of secreted IFN-γ (mean value from three independent experiments).

PMA and PHA stimulation (*39*, *40*). In this study, we observed that PKC-α activation dramatically increases in the presence of FIP-*fve* in dose- and time-dependent manners in whole cell lysate. These results indicated that FIP-*fve* stimulates IFN-γ production by increasing PKC-α activation.

The Inhibitor or Chelator Affects IFN-γ Production and mRNA Expression in PBMCs. Recent in vitro studies have revealed that PKC activation and Ca^{2+} release are required for regulation of IL-2 and IFN-γ gene expressions in PHA-stimulated PBMCs (*39*). To study whether IFN-γ production induced by FIP-*fve* is regulated by Ca^{2+}, PBMCs were pretreated with EGTA (extracellular Ca^{2+} chelator) or nifedipine (L-type Ca^{2+} channel blocker) for 1 h. As shown in **Figure 4**, IFN-γ production decreased with pretreatment with nifedipine, in a dose-dependent manner. FIP-*fve*-induced IFN-γ production was abolished in the presence EGTA, which suggests that Ca^{2+} is critically influential in IFN-γ secretion. In addition, to study whether FIP-*fve*-induced IFN-γ production is regulated by PKC activation, PBMCs were pretreated with GÖ6976 (a PKC-α inhibitor). The stimulated production of cytokine by FIP-*fve* was abolished by GÖ6976, in a concentration-dependent manner (**Figure 4**). PKC-α inhibitor, Ca^{2+} chelator and Ca^{2+} channel blocker markedly reduced IFN-γ production by FIP-*fve*, raising the possibility that FIP-*fve*-induced Ca^{2+} release and activation of PKC-α both play pivotal roles in IFN-γ production. In a previous study, we found that FIP-*fve* enhances gene expression of IL-2 and IFN-γ in mouse spleen cells. However, in PHA-activated PBMCs, IFN-γ production has been found to have relation to mRNA expression (*39*). Hence, in our study total cellular RNA was extracted from FIP-*fve*-activated PBMCs for 3 h and subjected to reverse transcriptase PCR (RT-PCR). As shown in **Figure 5A**, nonstimulated cultures did not show any effect on IFN-γ mRNA expression in PBMCs. In comparison with the nonstimulated cultures, the expression of IFN-γ mRNA increased significantly in the presence of FIP-*fve* in a concentration-dependent manner. Therefore, FIP-*fve* enhances the production of IFN-γ in PBMCs through an increase in IFN-γ mRNA expression (**Figure 5A**). In addition, pretreatment with EGTA, nifedipine, or GÖ6976 also significantly reduced the IFN-γ mRNA expression in PBMCs (**Figure 5B**). These results clearly indicated that both Ca^{2+} release and PKC-α activation are involved in IFN-γ production and mRNA expression in PBMCs by FIP-*fve*.

The Inhibitor or Chelator Affects PKC-α Activities by PBMCs. To determine whether PKC-α activation is directly affected by Ca^{2+} release made from FIP-*fve* inside the cells, we pretreated cells with EGTA or nifedipine. These cells then underwent FIP-*fve*

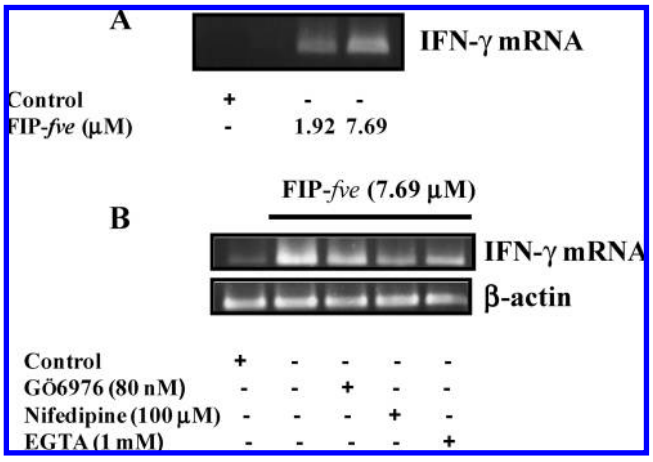

Figure 5. Effects of FIP-*fve* on IFN-γ mRNA expression in PBMCs. (**A**) PBMCs were incubated with FIP-*fve* at varying concentrations (1.92 or 7.69 μM) for 3 h, and then the IFN-γ mRNA expression was analyzed by RT-PCR of total RNA extracts from PBMCs. (**B**) PBMCs were pretreated with nifedipine (100 μM), GÖ6976 (80 nM), or 1 mM EGTA for 1 h, and then stimulated with FIP-*fve* (7.69 μM) for 3 h. Reverse transcriptase PCR was performed to analyze IFN-γ mRNA expression.

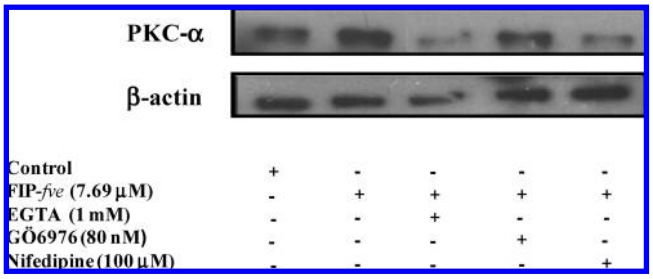

Figure 6. Effects of inhibitor on FIP-*fve*-induced phosphorylation of PKC-α. Before measuring PKC activity, PBMC cells (1.5×10^7 cells/mL) were pretreated with nifedipine (100 μM), GÖ6976 (80 nM), or 1 mM EGTA for 1 h and incubated with FIP-*fve* (7.69 μM) for 3 h. Western blotting was carried out to analyze phosphorylation levels of PKC-α, as described in Materials and Methods.

treatment for 3 h. As shown in **Figure 6**, FIP-*fve* is able to activate PKC-α, but pretreatment of the cells with EGTA or nifedipine abolished activation of PKC-α. This finding indicates that FIP-*fve*-induced activation of PKC-α is calcium-dependent. In this study, we did find a slight mobilization of FIP-*fve*-induced Ca^{2+} in presence of EGTA, (**Figure 2C**), and it subsequently abolishes PKC activation and IFN-γ production induced by FIP-*fve* (**Figure 6** and **Figure 4**). Furthermore, our experiments also demonstrated that inhibition of PKC-α by GÖ6976 (an inhibitor of PKC-α, β) leads to a significant decrease in the level of FIP-*fve*-induced PKC-α activation in PBMCs (**Figure 6**). The result suggests that FIP-*fve*-stimulated IFN-γ production is regulated by PKC-α signal pathway.

IFN-γ, a homodimeric cytokine produced by T lymphocytes and natural killer cells, belongs to a family of glycoproteins that has antiviral activity and antiproliferative and immunomodulatory properties in response to a viral infection or other enzyme inducers (*41*). Therefore, IFN-γ has shown some promise in the treatment of immunological, viral and neoplastic diseases, and for its antitumor effects (*41*, *42*). However, in the clinical application of IFN-γ, injections are necessary for frequent administration due to the short biologic half-life of cytokine proteins. Yet, high concentrations of IFN-γ in serum yield significant side effects and toxicities (*42*). FIP-*fve* is a natural compound that exerts no toxic effects on normal cells (*43*) and possesses high stability in thermal/freezing, acidic conditions and under dehydration (*44*). It has been reported that FIP-*fve* treatment inhibits systemic anaphylactic reactions and localized swelling of footpads in mice, as well as exhibits antiallergic effects *in vivo* (*1*, *5*). Therefore, FIP-*fve* from natural sources is an efficient, stable, and safe protein to achieve optimal health benefit or therapeutic purpose. FIP-*fve* is likely to be a preferable immunomodulatory agent, as not only is there a long history of mushroom consumption in Asian countries which proves its safety, but also FIP-*fve* has been reported to have health-promoting properties making it desirable for the production of various health and functional foods.

The results drawn from this study show that FIP-*fve* at least initially induces Ca^{2+} release which results in facilitating the activation of Ca^{2+}-dependent PKC-α. According to our previous study, p38 also plays a critical signal transduction role or acts downstream of PKC-α in FIP-*fve*-induced IFN-γ production in cultured PBMCs (*2*). Hence, FIP-*fve*-mediated signal transduction pathways in the regulation of IFN-γ gene expression in PBMCs appear to be complex and multiple. We conclude that proper Ca^{2+} signaling is one of the events to FIP-*fve*-stimulated IFN-γ.

ABBREVIATIONS USED

FIP-*fve*, fungal immunomodulatory protein; IFN-γ, interferon-gamma; PBMCs, human peripheral blood mononuclear cells; fluo-3AM, fluo-3 acetoxymethylester; RT-PCR, reverse transcription-polymerase chain reaction; PKC-α, kinase C-alpha; DAG, diacylglycerol; IP3, inositol 1,4,5-trisphosphate; PVDF, polyvinilydene difluoride; CICR, Ca^{2+}-induced Ca^{2+} release.

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