Activation and Up-regulation of Phospholipase D Expression by Lipopolysaccharide in Human Peripheral T Cells

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Abstract

In a previous study, we showed that bacterial LPS activates protein kinase C (PKC) and causes an intracellular pH (pH_i) increase, but does not elevate intracellular calcium ($[Ca^{2+}]_i$) in human peripherial T cells. Hence this study aimed to investigate whether the activation of PKC was resulted from phospholipase D (PLD) catalysis by LPS. The activity of PLD was measured by the production of ^{3}H-phosphatidylethanol from phosphatidic acid (PA), and the expression of PLD or IL-2 Rα was determined by reverse transcription-polymerase chain reaction (RT-PCR) analysis. Enzyme-linked immunosorbent assay (ELISA) was used to analyze IL-2 and IL-4. Phytohemagglutinin (PHA), and phorbol 12-myristate 13-acetate (PMA) were used as controls. Our results indicated that (1) LPS-stimulated pH_i elevation was PKC dependent; (2) After 30 min stimulation, LPS increased PLD activity via a measured production of ^{3}H-phosphatidylethanol from phosphatidic acid and the initiation of PLD1a mRNA expression started; (2) LPS stimulated IL-2 R expression but not IL-2 and IL-4 secretion. Our findings suggested that the stimulation of PLD activity and its mRNA expression by LPS might be required for IL-2 R expression and a sustained PKC dependent pH_i elevation but not for the secretion of IL-2 or IL-4 in human T cells. This indicated that LPS might enhance T cell adaptive immunity to resist Gram-negative bacterial infection.

Key Words: LPS, pH_i, phospholipase D, T cells

Introduction

Bacterial lipopolysaccharide (LPS) has been observed to be released into blood streams during severe Gram-negative bacterial infections and hence causes a range of pathophysiological conditions, for example, multi-organ failure and septic shock. However, low doses of LPS are known to be useful to the host; for instance, they cause immunostimulation and improved resistance to infections and malignancy. LPS stimulates the proliferation and antibody production in B cells (8). However, LPS is known as nonmitogenic in human T cells. LPS and its lipid A component are recognized as potent inducers of human T cell proliferation and of Th1-like lymphokines (24). LPS-primed monocytes are required for stimulating the proliferation of T cells by costimulatory signals *via* CD28 (25). Thus, the stimulation of T cell proliferation by LPS is dependent on signals induced by direct cell-to-cell contact between T cells and accessory monocytes. LPS has been found to stimulate human cells *via* a toll-like receptor (TLR) causing expression of genes encoding inflammatory mediators (26). The innate immune response of Toll has been preserved from fruit fly to vertebrates and it not only helps resistance to infection before adaptive immunity is induced, but also induces signals that inform the adaptive system of the presence

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of pathogens (26).

Signal transduction following T cell activation and proliferation is characterized by the activation of various biochemical processes of which the two most important are the sustained increases of intracellular Ca^{2+} ($[Ca^{2+}]_i$) and protein kinase C (PKC) activity (1, 37). Therefore, stimulation of T cell proliferation by lectins can be mimicked by the combination of PKC activation, induced by phorbol esters, and Ca^{2+} influx, induced by ionophores (7, 20, 36). Moreover, the PKC inhibitor, H7 can inhibit interleukin-2 receptor (IL-2 R) expression and T cell proliferation (16). Blocking the increase of intracellular Ca^{2+} by the removal of extracellular calcium with chelators or calcium channel blockers is associated with an inhibition of IL-2 secretion and T cell proliferation (13, 28, 29).

The proliferation by LPS has not been observed in human T cells (14, 27, 34), and our previous study also demonstrates that LPS activates PKC and causes an increase in intracellular pH (pH_i) but not intracellular calcium elevation and proliferation in T cells. However, the combination of the calcium ionophore A23187 with LPS stimulates T cell proliferation (5). The failure of LPS to $[Ca^{2+}]_i$ elevation indicates that the origin of DAG is probably not derived from phosphatidylinositol hydrolysis. The alternative pathway in obtaining sufficient DAG on long-term activation of PKC is from phosphatidylcholine (PC) hydrolysis by phospholipase D (PLD) (10, 33). The PLD catalysis is reported to mediate in many receptor-mediated cellular signaling events (11, 15, 17). PMA or anti-CD3 antibodies can activate phospholipase D in T cells (4). In our previous findings, the response of alkalinization by phytohemagglutinin (PHA) is dependent on PKC activation, whereas, after down-regulation of PKC, acidification by PHA is observed in T cells (6). Thus, the response of alkalinization by LPS was suspected to be resulted from PKC activation in stimulated T cells. As mentioned above, the increase activity of PKC is associated with IL-2 R expression (37) and the increase of $[Ca^{2+}]_i$ is associated with IL-2 secretion (13, 28). Thus, in the present study, we tried to further defined whether the activation of PKC was through phospholipase D (PLD) catalysis by LPS. The observation of IL-2 receptor expression and cytokines, IL-2 or IL-4 secretion were also observed in T cells by LPS. In this study, pH_i was measured using the fluorescent dyes, BCECF, in human peripheral T cells. The activity of PLD was measured by the production of ^{3}H-phosphatidylethanol from phosphatidic acid (PA). PLD or IL-2 Rα mRNA expression was determined by reverse transcription-polymerase chain reaction (RT-PCR). Enzyme-linked immunosorbent assay (ELISA) was used to analyze IL-2 and IL-4. Proliferation was detected by [^{3}H]-thymidine incorporation into T cells. Mitogen, phytohemagglutinin (PHA) and co-mitogen, phorbol 12-myristate 13-acetate (PMA) were used as controls of LPS.

Materials and Methods

Chemicals

Fura-2/AM and BCECF/AM, nigericin and valinomycin were purchased from Molecular Probes (Eugene, OR, USA). Phytohemagglutinin (PHA), RPMI 1640 medium (RPMI), Hank's balanced salt solution (HBSS) and fetal calf serum (FCS) were obtained from Gibco (Grand Island, NY, USA). Lipopolysaccharide (*E coli* 026:B6, 30000 endotoxin units/mg), phorbol 12-myristate 13-acetate (PMA), 4-α-phorbol, bovine serum albumin (BSA), dimethylsulphoxide (DMSO), EGTA and Ficoll/Hypaque were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Phosphatidylethanol (PEt) was obtained from Biomol Research Laboratories Inc. (Plymouth Meeting, PA, USA). PHA was dissolved in distilled water. The culture media were supplemented with 10% heat-inactivated FCS (v/v). Radioactively labeled [9, 10(n)-^{3}H]palmitic acid (specific activity 51.0 Ci/mmol) was purchased from Amersham Life Science (Buckinghamshire, UK). HPTLC plates (Kieselgel 60, 10 × 10 cm) and the organic solvents were obtained from E. Merck (Darmstadt, Germany). Anti-CD25-PE, anti-CTLA-4-PE, and anti-CD28-FITC were purchased from Beckman Coulter Co. (Miami, FL, USA).

Preparation of T Cells

Heparinized peripheral blood samples were taken from healthy male volunteers (aged 20 to 25) and the blood mononuclear cells (MNCs) isolated using the Ficoll-Hypaque gradient-density method. The MNC suspension (15 ml) was added to a 100 × 15-mm plastic Petri dish and the cells incubated for 50 min in a humidified incubator at 37°C, 5% CO_2. The adhering cells were harvested using a rubber policeman and washed. The entire process was repeated three times. The non-adhering T cells were prepared by E-rosetting and the rosetted erythrocytes lysed using cold distilled water. To verify the effectiveness of the separation procedure, the isolated T cells were incubated for 30 min at 4°C with phycoerythrin-labeled monoclonal anti-CD3 antibodies (Ortho Pharmaceuticals, Raritan, NJ, USA), and the antibody-coated T cells were analyzed on a fluorescence-activated cell sorter (EPICS C, Hialeah, FL, USA). The results showed that the T cell suspension contained almost 100% CD3-positive cells (22). LPS (10 μ/ml), PHA (10 μg/ml), and PMA (100 nM) were used as stimulants.

Measurement of the pH_i

T cell suspensions (2×10^7 cells/ml) were incubated at 37°C for 30 min with BCECF/AM (3 μM) in HBSS containing 5 mM glucose and 0.2% BSA. Then the cells were washed three times with HBSS and resuspended in RPMI 1640 containing 10% FCS. For pH_i measurements, 1×10^6 cells were washed twice with HBSS, resuspended in 2.5 ml of the same solution, transferred to a plastic cuvette at 37°C and allowed to stabilize for 15 min before stimulation. Upon excitation at wavelengths of 435 nm and 500 nm, the BCECF fluorescence emission at 525 nm was measured using a dual-wavelength spectrofluorimeter (Spex Industries, model CM1T11I, Edison, NJ, USA) and the emission ratio was calculated. To prepare the calibration curve, a mixture of 1×10^6 cells and 3 μM nigericin was added to K^+ HBSS at pH values of 2–10, then valinomycin (3 μM) was added and allowed to react for 5 min before the fluorescence signals were measured. The pH of the K^+ HBSS was measured to the nearest 0.001 units using a pH meter (Radiometer Copenhagen, model PHM 93, Denmark). The calibration values were fitted to a standard sigmoid curve that was then used to calculate the unknown pH_i values.

PLD Assay

PLD activity was measured according to the original method by analyzing the accumulation of phosphatidylethanol (PEt) in the presence of 300 mM ethanol (20, 23). Isolated T cells (10^8 cells/ml) were incubated with 1 μCi/ml [9, 10(n)-^{3}H]palmitic acid in RPMI 1640 containing 10% FCS (v /v) for 24 h. At the end of incubation, cells were washed twice with RPMI 1640 and resuspended. 2×10^6 cells were stimulated with PHA (10 μg/ml), PMA (100 pM), or LPS (10 μg/ml) for 30 min and 300 mM ethanol was added 5 min before the end of incubation. The reactions were stopped by aspiration, followed by addition of 1 ml of ice-cold methanol. The cells were transferred to a borosilicate glass test tube (1.3×100 mm). The lipids were extracted by the addition of 2 ml of ice-cold chloroform, after which the sample was mixed by vortexing and centrifuging at 400 g for 5 min to allow phase separation. The lower organic phases were transferred to new test tubes, where they were then evaporated to dryness (35). The lipids were then redissolved in 200 ml of chloroform and applied to 1% potassium oxalate-preimpregnated 10×10 cm Kiesegel 60 HPTLC plate. Additional unlabeled PEt was also applied to each sample, and the plates were separated by a one-dimensional solvent system using the upper layer of mixture of ethyl acetate/isooctane/acetic acid/H_2O (65:10:10:50, by volume). After development, and the plates were dried, and the lipid bands were visualized by exposure to iodine vapor. The PEt and phospholipid (PL) bands were scraped into scintillation vials for counting by scintillation spectrometry. The level of radioactivity in the PEt was standardized as dpm PEt/100,000 dpm in PL (9).

Reverse Transcription-polymerase Chain Reaction

The RT-PCR was performed using the programmable thermal controller, Mastercycler personal (Eppendorf, USA). Total RNA was isolated from human T cell using Trizol reagent and reverse transcribed with M-MLV reverse transcriptase using random hexamer-mixed oligonucleotides. The cDNA was then amplified by PCR in a final volume of 50 μl containing 100 ng of cDNA, 5 μl of 10X PCR buffer, each deoxynucleotide 5'-triphsphate at 0.25 mM, each primer at 0.2 μM, and 2 U of Taq DNA polymerase (Biomen Co., Taiwan, ROC). Specific primers for the amplification of PLD1 transcripts were designed on the basis of published human sequences to discriminate between PLD1a, PLD1b and PLD2 (19, 21). The sense and antisense primers were: 5'-CTGTGGGCTCATCACGAGAA-3' and 5'-GTGAAGTTCCAGCGCTGAT-3', respectively. Specific primers for the amplification transcripts were designed on the basis of published human sequences. The sense and antisense primers of IL-2 Rα were: 5'-GGT CCC AAG GCT CAG GAA GAT G-3' and 5'-CTG TTC CCG GCT TCT TAC CA-3', respectively (MBBio Inc, Tw). Amplification conditions for PLD orIL-2 Rα were 95°C for 2 min (one cycle); 95°C for 45s, 57°C for 60s, and 72°C for 90s (30 cycles); and 72°C for 5 min (one cycle). The mRNA signals for PLD or IL-2 Rα (884 bp) and GAPDH, within the linear range, were used for band volume quantification using a Personal Densitometer SI (Amersham Biosciences, Buckinghamshire, UK). The relative level of PLD or IL-2 Rα mRNA for each sample was normalized by comparison with the internal GAPDH control.

Enzyme-linked Immunosorbent Assay

Cell culture supernatants were collected after treatment with PHA (10 μg/ml), PMA (100 pM), or LPS (10 μg/ml) for 24 h, and the levels of human IL-2 and IL-4 were determined using commercial ELISA kits (R&D Systems, Minneapolis, MN, USA), according to the vendor's instructions. The values were obtained using a DYNEX microplate reader (Microtiter Co, VA, USA) and were fitted to a standard curve that was then used to calculate the unknown IL-2 or IL-4 values. The sensitivities for IL-2 and IL-4 were 7 pg/ml and 10 pg/ml, respectively.

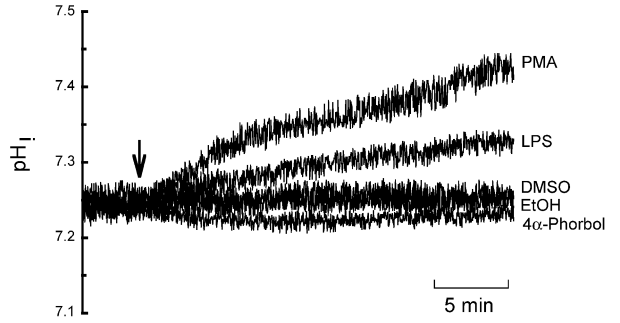

Fig. 1. The pH_i responses to LPS in human peripheral T cells. BCECF/AM-loaded T cells were suspended in Na^+ Hanks solution with 10 μg/ml LPS, 100 pM PMA, 100 pM 4-α-phorbol against vehicles (EtOH for 4-α-phorbol and DMSO for PMA). Arrows indicate the addition of the ligands. Tracings are from one representative of six similar experiments.

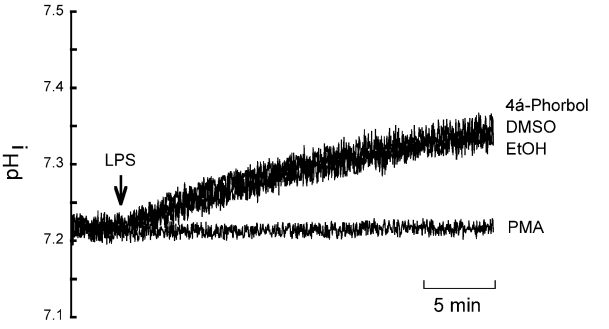

Fig. 2. The pH_i responses to LPS in PKC down-regulated T cells. BCECF/AM-loaded T cells were preincubated with PMA (1 μM), the vehicle (DMSO) or PKC insensitive, phorbol ester, 4-α-phorbol (1 μM), the vehicle (EtOH) for 18 h before stimulation with LPS (10 μg/ml), indicated by the arrow. The traces are representative of 6 experiments.

Statistical Analysis

The PLD, IL-2, IL-4 and cell surface marker expression data were analyzed by Student's *t* test with a significance level set at $P < 0.05$. All values are quoted as the mean ± SEM.

Results

LPS-induced pH_i Changes in PKC Down-regulated T Cells

The responses of the isolated T cells to PHA in experiments have been reported (5). The rise of pH_i by LPS was blocked by pretreatment of the T cells with the Na^+/H^+ exchange inhibitor, 5-(N,N-dimethyl)-amiloride (DMA, 10 μM), for 20 min (data not shown). The effects of LPS (10 μg/ml) and PMA (100 pM) on pH_i are shown in Fig. 1. Both LPS and PMA stimulated the rise of pH_i in T cells, whereas no effects were observed in vehicles (DMSO, alcohol) and PKC insensitive phorbol ester, 4-α-phorbol (Fig. 1).

The PKC activity has been reported to be down-regulated in T cells by PMA (1 μM, 18 h) (6). In addition to down-regulate PKC activity by PMA, cells were also treated with vehicles (DMSO or alcohol) or the PKC insensitive phorbol ester, 4-α-phorbol (1 μM), for 18 h as controls. The LPS-induced rise in pH_i was not shown in PKC down-regulated T cells, but it was present in vehicle treated cells (Fig. 2).

Effects of LPS, PMA and PHA on Phospholipase D (PLD) Activity in Human Peripheral T Cells

T cells pre-incubated with [9, 10(n)-^{3}H]-palmitic acid were used to study the change of PLD activity.

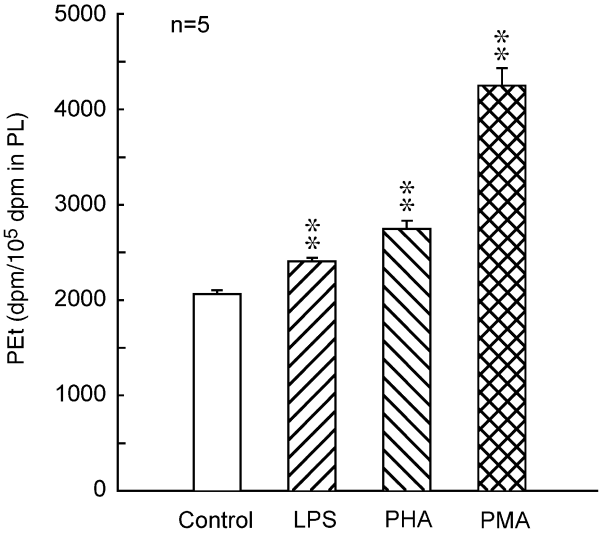

Fig. 3. Effects of LPS, PMA or PHA on phospholipase D (PLD) activity in human peripheral T cells. ^{3}H -palmitic acid-loaded T cells were stimulated with LPS (10 μg/ml), PMA (100 pM) or PHA (10 μg/ml) for 0.5 h. The activity of PLD was analyzed by a thin layer of chromatography in ^{3}H-palmitic acid-incubated cells and measured by the production of ^{3}H-phosphatidylethanol from phosphatidic acid (PA). The level of radioactivity in phosphatidylethanol (PEt) was standardized as dpm PEt/100,000 dpm in phospholipids. **, $P < 0.01$ as compared with the vehicle control. Each value represents mean ± SEM, $n = 5$.

As mentioned above, the activity of PLD was standardized as dpm PEt/100,000 dpm in the phospholipid. Administration of LPS (10 μg/ml), PMA (100 pM), or PHA (10 μg/ml) for 0.5 h resulted in a significant increase ($n = 5$, $P < 0.01$) in PLD activity to 2408 ± 37, 4248 ± 184, 2746 ± 81, respectively, as compared with 2064 ± 38 for the unstimulated T cells (Fig. 3).

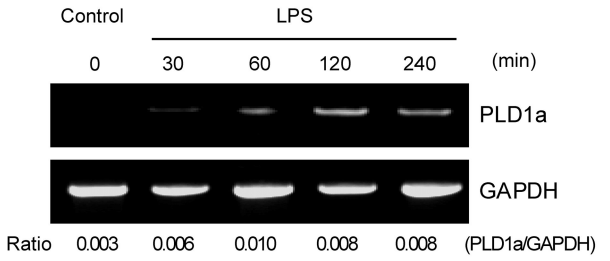

Fig. 4. Time course of LPS stimulation of PLD mRNA expression in T cells. T cells were exposed to 10 μg/ml LPS. Total RNA was isolated after 0, 30, 60, 120 or 240 min and mRNA levels were analyzed by RT-PCR using human PLD primers. The agarose gel was a representative of 3 similar experiments. The relative level induction of PLD1a mRNA (733 bp) for each sample was normalized by comparison with the internal GAPDH control.

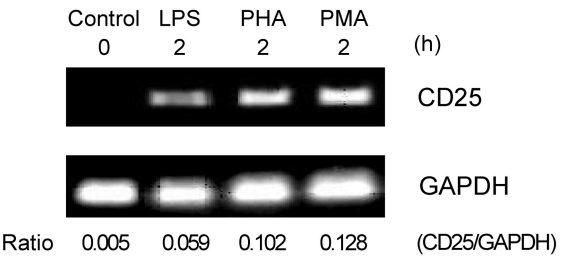

Fig. 5. The effects of CD25 (IL-2 Rα) mRNA expression in LPS-stimulated T cells. T cells were exposed to LPS (10 μg/ml), PMA (100 pM) or PHA (10 μg/ml) for 2 h. Total RNA was isolated and mRNA levels were analyzed by RT-PCR using human CD25 primers. The agarose gel was a representative of 3 similar experiments. The relative level induction of CD25 mRNA (884 bp) for each sample was normalized by comparison with the internal GAPDH control.

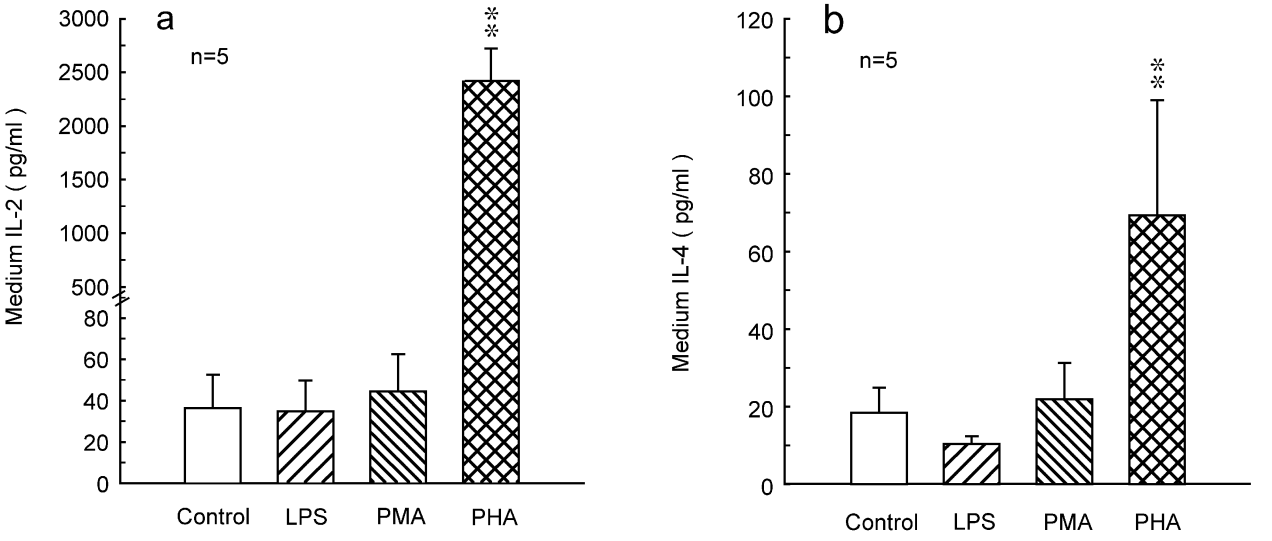

Fig. 6. Effects of LPS, PMA or PHA on IL-2 and IL-4 secretion in human peripheral T cells. T cells were treated with LPS (10 μg/ml), PMA (100 pM) or PHA (10 μg/ml) for 24 h and supernatants were collected to determine the levels of human IL-2 (a) and IL-4 (b), which were determined by ELISA. **, $P < 0.01$ as compared with the vehicle control. Each value represents mean ± SEM, $n = 5$.

Effects of LPS on Expression of Phospholipase D1a (PLD1a) mRNA in Human Peripheral T Cells

As shown in Fig. 4, very little PLD1a mRNA expression was observed in resting T cells. The relative level of PLD1a mRNA (733bp) for each sample was normalized by comparison with the internal GAPDH control. After 30 min stimulation, LPS significantly increased by 2 fold the expression of PLD1a mRNA (733 bp) and reached a 3 fold plateau at 60 min. This condition was maintained for at least 4 h.

Effects of LPS on Expression of CD25 (IL-2 Rα) mRNA in Human Peripheral T Cells

As shown in Fig. 5, very little CD25 mRNA expression was observed in resting T cells. The relative level of CD25 mRNA (884 bp) for each sample was normalized by comparison with the internal GAPDH control. After 2 h stimulation, LPS significantly increased by 12 fold the expression of CD25 mRNA and PHA or PMA increased by 20 or 26 fold the expression.

Effects of LPS, PMA and PHA on IL-2 and IL-4 Production in Human Peripheral T Cells

These experiments further define the lack of increase of $[Ca^{2+}]_i$ to be associated with the lack of cytokine secretion in T cells by LPS. After T cells were treated with LPS (10 μg/ml), PHA (10 μg/ml), or PMA (100 pM) for 24 h, the IL-2 or IL-4 production in medium was assessed. Compared with the controls,

PHA significantly stimulated the production of IL-2 and IL-4 in treated T cells (Fig. 6); in contrast, neither LPS nor PMA stimulated the specific cytokines production.

Discussion

The bacterial lipopolysaccharide (LPS) has become a popular microbial activator in many studies. In the present study, LPS stimulated an increase in pH_i (alkalinization) in T cells (Fig. 1), and these results were consistent with our previous findings (6). The Na^+/H^+ exchange (NHE) inhibitor, DMA, blocks the alkalinization by lectin or by antibodies to the T cell surface receptors (30, 31). The NHE inhibitor, DMA, also blocked the increase in pH_i by LPS. This indicated that the NHE activation might be the cause of alkalinization (data not shown). In addition, if the activity of PKC is down-regulated by PMA (1 μM, 18 h), the PMA-induced or lectin-induced alkalinization will be blocked in T cells (6). Similar results in the blockage of LPS-induced alkalinization were also observed in PKC down-regulated T cells (Fig. 2). To confirm that LPS stimulation of PKC-related alkalinization was not caused by LPS contamination, the PKC insensitive, phorbol ester, 4-α-phorbol, was used as a control. We found that pretreatment T cells with 4-α-phorbol (1 μM, 18 h) did not affect LPS-induced alkalinization (Fig. 2). This finding suggested that the alkalinization by LPS was a PKC-related signaling event, rather than an artifact caused by LPS contamination.

The alternative pathway whereby DAG is formed by phosphatidylcholine (PC) hydrolysis is recognized as a requirement for long-term activation of PKC (10, 33). PMA or anti-CD3 antibodies have been reported to activate phospholipase D in T cells (4); therefore, LPS, PMA or PHA were used to stimulate T cells to examine the activity of phospholipase D. The administration of LPS, PHA, or PMA to the T cells resulted in a significant increase in PLD activity (Fig. 3). To further confirm the phospholipase D induced by LPS, the mRNA expression of PLD by LPS in T cells was investigated. In these experiments, the expression of both PLD1a and PLD2 mRNA was observed in T cells. LPS was found not only to increase the PLD activity, but also to stimulate the expression of PLD1a mRNA in T cells (Fig. 4). In contrast, LPS did not induce the expression of PLD2 mRNA (386 bp), compared to the control T cells (data not shown).

In granulocytes, the activities of phospholipase D can be stimulated by two different mechanisms: by phorbol esters that activate the PKC, or by calcium ionophores that elevate $[Ca^{2+}]_i$ (2). In our previous findings, LPS activates PKC but does not elevate $[Ca^{2+}]_i$ in T cells (5). After 30 min stimulation, LPS increased 17% of the PLD activity (Fig. 3) and 2 fold in PLD1a expression (Fig. 4). According to the levels of signal amplification, the increase of PLD1a expression was apparently a down stream response via PKC activation by PLD in LPS-stimulated T cells. The stimulation of PLD plays an important role in T cell activation and the regulation of activation of the transcription factor AP-1 (32).

The sustained elevation in $[Ca^{2+}]_i$ and PKC activity are recognized as two major early signals in T cells and are associated with responses in the form of cytokines secretion and receptors expression, respectively (1, 37). However, lectin or anti-CD3 monoclonal antibodies can stimulate T cells to secrete IL-2 and IL-4 (3, 12). In this study, LPS stimulated CD25 expression (Fig. 5). This expression might be the result from PKC activation by LPS. We found that the productions of IL-2 and IL-4 were only stimulated by PHA, but not by LPS, or PMA (Fig.6). In contrast to PHA, the lack of the of $[Ca^{2+}]_i$, elevation, LPS failed to stimulate IL-2 and IL-4 secretion (5).

Thus, these results demonstrated that endotoxins from bacteria played a potentially important role in immune surveillance. Further experiments are needed to explore whether the PLD activation is associated with the expression of accessory molecules on T cells by LPS. Recently, it has been shown that lipid A, a toxic center of LPS, can stimulate, possibly through Toll-like receptors, the rise of intracellular calcium in human peripheral T cell (18). The innate immune responses of T cells might be able to respond to bacterial components differentially, and this may be relevant to the design of vaccine adjuvants to selectively induce the desired type of acquired immunity (Th1/Th2). The resulting increase in PLD activity and the up-regulation of PLD and CD25 molecules expression on T cells by LPS might be important innate immune responses that influence acquired immunity. Thus, LPS affected differentiation and might affect future responses in T cell activation, and therefore enhanced the acquired immunity of the host defense against Gram-negative bacterial infection.

Acknowledgments

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