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Priming effects of lipopolysaccharide on UTP-induced arachidonic acid release in RAW 264.7 macrophages

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Abstract

Stimulation of mouse RAW 264.7 macrophages with UTP activates both the inositol phosphate signal transduction pathway and the phospholipase A_2 pathway. In the present study, we investigated the interactions between bacterial lipopolysaccharide and UTP in these two systems and the underlying mechanisms involved. While the UTP-induced release of arachidonic acid was only 2.9-fold that in controls, priming the cells with 1 μg/ml lipopolysaccharide for 1 h before UTP treatment resulted in 9.2-fold arachidonic acid release upon stimulation with UTP. Lipopolysaccharide priming was both concentration- and time-dependent with a peak effect after 1 h treatment at a concentration of 1 μg/ml. Lipopolysaccharide treatment affected neither the basal nor the UTP-stimulated inositol phosphate formation and $[Ca^{2+}]_i$ rise. Pretreatment of the cells with staurosporine, calphostin, *N*-(2-aminoethyl)-5-isoquinolinesulfonamide (H-7), genistein or K-252a led to marked inhibition of the priming effect, suggesting that both protein kinase C and tyrosine kinase are involved in the lipopolysaccharide effect. Buffering intracellular Ca^{2+} levels using [1,2-bis-(*o*-aminophenoxyl)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetra(acetoxymethyl)ester] (BAPTA/AM) or pretreatment with either *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89), 2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one (PD098059) or {1-[*N*,*O*-bis-(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenyl-piperazine (KN-62) did not affect the lipopolysaccharide-induced priming effect. Primed UTP stimulation was inhibited by actinomycin D and cycloheximide, indicating a requirement for both gene expression and protein translation. To further examine whether the stimulatory effects of lipopolysaccharide on phospholipase A_2 activity were independent of $[Ca^{2+}]_i$ levels but dependent on protein phosphorylation, a fixed Ca^{2+} concentration and inhibitors of protein phosphatases were used in primed permeabilized cells. Arachidonic acid release from permeabilized cells containing 100 nM Ca^{2+} was high in lipopolysaccharide-primed cells and potentiated by addition of microcystin, orthovanadate or FK 506. These results suggest that the Ser/Thr and tyrosine phosphorylation cascades induced by protein kinase C and tyrosine kinase, respectively, are required for the arachidonic acid potentiation effect of lipopolysaccharide, which was independent of modulation of the upper stream signaling pathways of UTP.

Keywords: Lipopolysaccharide; Arachidonic acid; Phospholipase A_2; Protein kinase; UTP; RAW 264.7 macrophage

1. Introduction

Arachidonic acid release from phospholipids by phospholipase A_2 is of major importance in inflammatory responses. A number of pathways have recently been implicated in the regulation of phospholipase A_2 activation. Phospholipase A_2 activity in various cells, especially inflammatory cells, has been shown to be regulated by cytokines and the active component of endotoxin, lipopolysaccharide. Induction of the expression of cytosolic phospholipase A_2 or type II phospholipase A_2 protein or enzymatic activity by lipopolysaccharide has been demonstrated in a wide variety of cells, such as monocytes (Roshak et al., 1994), leukocytes (Rodewald et al., 1994), astrocytes (Oka and Arita, 1991), vascular smooth muscle cells (Nakano et al., 1990) and choriodecidua (Nguyen et al., 1994), suggesting that phospholipase A_2 plays an important role in the inflammatory response. In in vitro systems, lipopolysaccharide primes the agonist-induced release of arachidonic acid or prostaglandin E_2 in neutrophils (Forehand et al., 1993; Doerfler et al., 1994), $P388D_1$ macrophages (Glaser et al., 1990; Asmis et al., 1994) and Mac 6 monocytes (Aepfelbacher et al., 1992).

A number of pathways have recently been implicated in cytosolic phospholipase A_2 activation, including increased levels of cytosolic Ca^{2+} (Clark et al., 1991), activation of

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G protein (Kim et al., 1989; Winitz et al., 1994), protein kinase C and mitogen-activated protein kinase (MAP kinase) (Chahraborti et al., 1992; Lin et al., 1993; Nemenoff et al., 1993; Qiu et al., 1993) and tyrosine phosphorylation (Glaser et al., 1993). However, the intracellular signaling pathway by which lipopolysaccharide causes activation of phospholipase A_2 is, as yet, unresolved. A protein kinase C-dependent pathway involved in lipopolysaccharide-induced phospholipase A_2-II expression has been demonstrated in rat astrocytes (Oka and Arita, 1991), while, in mouse peritoneal macrophages, bacterial activation of cytosolic phospholipase A_2, leading to the release of arachidonic acid via protein kinase C-independent phosphorylation of cytosolic phospholipase A_2, has also been reported (Svensson et al., 1991, 1993). Moreover, the mechanisms involved in the synergistic effects of platelet activating factor and lipopolysaccharide appear to be different. Up-regulation of platelet activating factor-stimulated $[Ca^{2+}]_i$ levels by lipopolysaccharide was seen in human monocytic Mac 6 monocytes (Aepfelbacher et al., 1992), but not in murine P388 D_1 macrophages (Asmis et al., 1994).

In a previous study, we have shown that UTP and UDP are more effective than ATP in initiating phosphoinositide turnover, phospholipase A_2 activation and $[Ca^{2+}]_i$ increases in RAW 264.7 macrophages (Lin and Lee, 1996). In the present study, we have used this cell line with the phenotypic and functional characteristics of mature macrophages (Raschke et al., 1978) to study the interaction between lipopolysaccharide and UTP, and the underlying mechanisms involved. By assessing the effects of protein kinase C, tyrosine kinase and protein phosphatase inhibitors, we have shown that activation of protein kinase C and tyrosine kinase are absolute requirements for the induction of lipopolysaccharide-stimulated phospholipase A_2 activity.

2. Materials and methods

2.1. Materials

Cell culture medium and supplements were purchased from Gibco BRL (Grand Island, NY, USA). [^{3}H]Arachidonic acid (100 Ci/mmol) and [^{3}H]*myo*-inositol (20 Ci/mmol) were purchased from New England Nuclear (Boston, MA, USA). *E. coli* lipopolysaccharide, UTP, fura-II/AM, sodium orthovanadate, staurosporine, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7), actinomycin D, cycloheximide and microcystin-LR were obtained from Sigma (St. Louis, MO, USA). {1-[*N*,*O*-bis-(5-Isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenyl-piperazine (KN 62), 2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one (PD098059) and genistein were from RBI (Natick, MA, USA). Calphostin was obtained from Biomol (Plymouth Meeting, PA, USA). *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89), K-252a and [1,2-bis-(*o*-aminophenoxyl)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetra(acetoxymethyl)ester] (BAPTA/AM) were purchased from Calbiochem. FK 506 was obtained from Fujisawa Pharmaceuticals (Osaka, Japan).

2.2. Cell culture

RAW 264.7 cells, generously provided by Dr. Yen-Jen Sung (Department of Anatomy, National Yang-Ming University School of Medicine, Taiwan), were grown in 35 mm Petri dishes at 37°C in Dubecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin, in a humidified atmosphere of 95% air/5% CO_2.

2.3. [^{3}H]Arachidonic acid release from intact cells

Cells in 35 mm dishes were incubated for 24 h with 0.3 μCi/ml [^{3}H]arachidonic acid in DMEM. In lipopolysaccharide priming experiments, cells in DMEM were pretreated with different concentrations of lipopolysaccharide for various periods as indicated. When stated, inhibitors were added to the cells 20 min before the lipopolysaccharide treatment. After lipopolysaccharide priming, the cells were washed three times with physiological saline solution (PSS, 118 mM NaCl, 4.7 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM KH_2PO_4, 11 mM glucose and 20 mM Hepes, pH 7.4) and incubated in PSS containing 0.5% fatty acid-free bovine serum albumin, before stimulation with UTP at 37°C for 30 min. At the end of the incubation, the medium was removed and centrifuged at $250 \times g$ for 5 min to remove floating cells, and the radioactivity in the supernatant measured.

2.4. [^{3}H]Arachidonic acid release from permeabilized cells

To measure arachidonic acid release from permeabilized cells, the prelabeled cells were washed twice with phosphate-buffered saline solution, then permeabilized for 5 min at 37°C with 10 μM digitonin in an intracellular potassium glutamate (KG) buffer, containing 139 mM potassium glutamate, 20 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), 1 mM $MgCl_2$, 2 mM MgATP, 5 mM ethylene glycol-bis(β-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid (pH 7.4) and the indicated protein phosphatase inhibitors. The dishes were then washed with KG buffer, without digitonin but containing 1 mM $CaCl_2$, then incubated for 30 min. The free $[Ca^{2+}]$ in the buffer was calculated by a computer program to be 100 nM (Fabiato and Fabiato, 1979).

2.5. Measurement of $[Ca^{2+}]_i$

Cells grown on glass slides were loaded with 3 μM fura-II/AM and pluronic F-127 (0.02% v/v) in DMEM at 37°C for 45 min. Fluorescence was monitored on a PTI

M-series spectrofluorometer with dual excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. $[Ca^{2+}]_i$ was calculated using the equation described by Grynkiewicz et al. (1985).

2.6. Measurement of phosphoinositide turnover

Phosphoinositide hydrolysis was measured by the accumulation of inositol phosphates in the presence of 10 mM LiCl as described previously (Lin and Lee, 1996). Confluent cells on 35 mm Petri dishes were labeled with [^{3}H]*myo*-inositol (2.5 μCi/dish) in growth medium for 24 h. The cells were then washed with PSS containing 10 mM LiCl and incubated at 37°C for 20 min. After this preincubation step, the indicated drugs were added and incubation continued for a further 30 min; the reaction was then terminated by aspiration of the reaction solution and addition of ice-cold methanol. The cells were scraped off and the [^{3}H]inositol phosphate isolated using an AG-1X8 (formate form, 100–200 mesh, Bio-Rad, Richmond, CA, USA) column and elution with 0.2 M ammonium formate/0.1 M formic acid.

2.7. Statistical analysis

Each experiment was performed in duplicate and repeated several times. The data are the means ± S.E.M. The significance of the differences between the means was evaluated by using Student's *t*-test and value of $P < 0.05$ considered significant. The error bar was omitted when it fell within the limits of representing the mean value.

3. Results

In RAW 264.7 cells, UTP, over the concentration range of 0.3–100 μM, caused a dose-dependent increase in [^{3}H]arachidonic acid release, with an EC_{50} value of 3 μM and with the maximal response (obtained at 30 μM) being about 2.9-fold of basal release. Treatment of the cells with 1 μg/ml lipopolysaccharide alone for 1 h caused a 2.8-fold increase in [^{3}H]arachidonic acid release compared with controls and, as a pretreatment, markedly potentiated the UTP-induced response up to 9.2-fold of the basal release seen in non-lipopolysaccharide-primed and UTP unstimulated cells. This potentiation effect reached a maximum at a UTP concentration as low as 1 μM (Fig. 1).

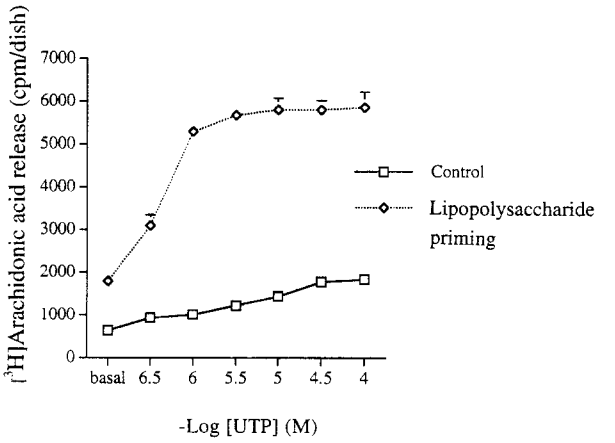

Fig. 1. Priming effects of lipopolysaccharide on the concentration-response curve of UTP-mediated [^{3}H]arachidonic acid release. Cells were preincubated with vehicle or lipopolysaccharide (1 μg/ml) for 1 h, then various concentrations of UTP added and incubated for 30 min. [^{3}H]Arachidonic acid released into the medium was collected and measured on a β-counter. Results are expressed as the means ± S.E.M. for three independent experiments.

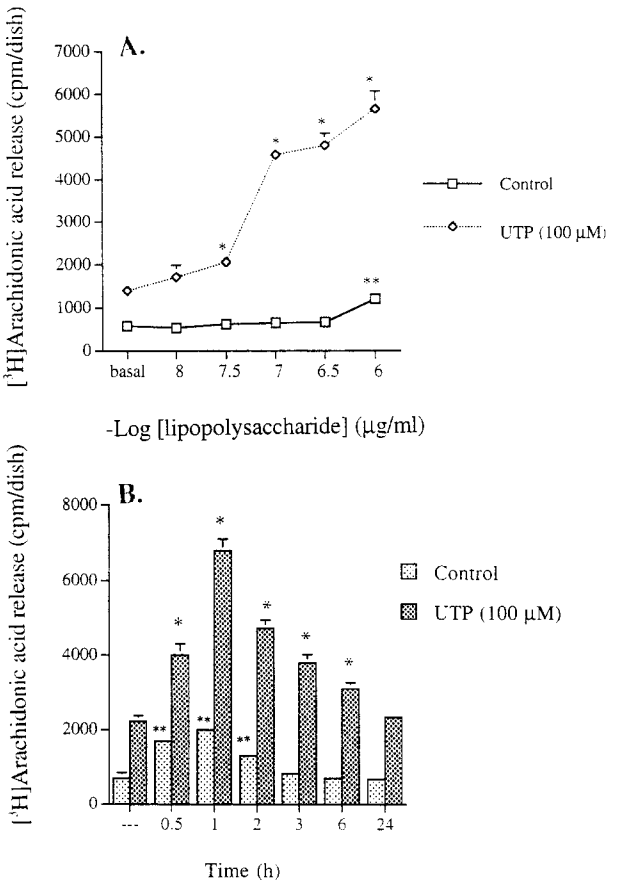

Fig. 2. Concentration- and time-dependent effects of lipopolysaccharide-priming on the stimulus-induced increase in [^{3}H]arachidonic acid release in RAW 264.7 cells. (A) Various concentrations of lipopolysaccharide were added to the cells 1 h prior to addition of 100 μM UTP. (B) Cells were pretreated with lipopolysaccharide (1 μg/ml) for different period before stimulation with 100 μM UTP. After 30 min, the medium was collected and [^{3}H]arachidonic acid release determined. Results are expressed as the means ± S.E.M. of three independent experiments, performed in duplicate. * $P < 0.05$ compared to the UTP response in lipopolysaccharide-unprimed cells. ** $P < 0.05$ compared to the control response in lipopolysaccharide-unprimed and UTP non-stimulated cells.

As shown in Fig. 2A, the effect of the 1 h priming step with lipopolysaccharide was dose-dependent. Under conditions in which the basal arachidonic acid release was unaffected by 10–300 ng/ml lipopolysaccharide, the response to UTP (100 μM) was significantly and dose dependently increased by lipopolysaccharide. At a lipopolysaccharide concentration of 1 μg/ml, the degree of

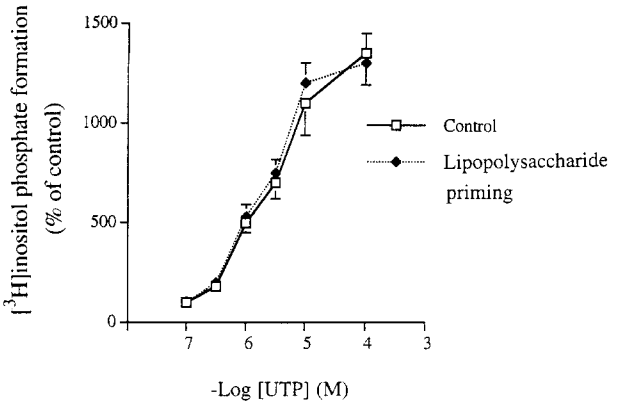

Fig. 3. Lack of effect of lipopolysaccharide on UTP-induced inositol phosphate formation. Cells labeled with [^{3}H]*myo*-inositol were stimulated with UTP for 30 min after incubation with lipopolysaccharide (1 μg/ml) for 1 h, then the [^{3}H]inositol phosphate formation was measured. Results are expressed as the means ± S.E.M. of three independent experiments.

potentiation was also dependent on the incubation time (Fig. 2B). Although a significant increase in basal arachidonic acid release was only seen for the first 2 h lipopolysaccharide (1 μg/ml) exposure, the lipopolysaccharide-priming effect appeared as early as 30 min after exposure to lipopolysaccharide, and lasted for at least 6 h. The maximal response was seen after 1 h and the effect was lost after 24 h exposure.

To determine if the lipopolysaccharide priming effect was due to changes in the signaling cascades by which UTP has been reported to initiate phospholipase A_2 activation (Lin and Lee, 1996), we studied the effects of lipopolysaccharide on UTP-induced phosphoinositide turnover and $[Ca^{2+}]_i$ increases. Lipopolysaccharide itself does not increase the basal inositol phosphate formation nor $[Ca^{2+}]_i$ (data not shown). As shown in Fig. 3, UTP markedly increased inositol phosphate accumulation with the same similar dose dependency seen for arachidonic acid release, but this response was unchanged in lipopolysaccharide-pretreated cells. Similar results were seen for the UTP-induced $[Ca^{2+}]_i$ increase (data not shown).

Various protein kinase inhibitors were used to determine the contribution of protein phosphorylation to the lipopolysaccharide priming effect. Pretreatment of cells with staurosporine (10 nM to 1 μM), calphostin (10 nM to 1 μM), H-7 (10, 50 μM), K-252a (0.3 μg/ml) or genistein (10, 50 μM) for 20 min prior to the addition of lipopolysaccharide concentration dependently inhibited the effect, while H-89 (1 μM), KN-62 (30 μM), PD098059 (30 μM) or BAPTA/AM (30 μM) had no effect (Fig. 4). The way by which these drugs were applied, i.e., incubation for 1 h, followed by washout, does not itself affect the cell viability and the subsequent UTP response. The cell viability was assayed by using MTT test. After 1 h incubation with each inhibitor then washout, UTP-induced arachidonic acid release was approximately remained within 90–110% of the control response without inhibitor preincubation.

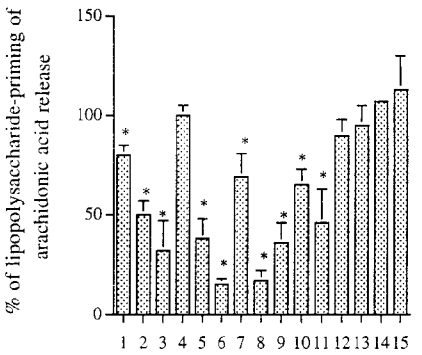

Fig. 4. Effect of inhibitors of protein kinases and BAPTA/AM on the priming effect of lipopolysaccharide. Cells were pretreated with vehicle, staurosporine (10 nM, 100 nM or 1 μM, as indicated by columns 1–3), calphostin (10 nM, 100 nM or 1 μM, as indicated by columns 4–6), H-7 (10 or 50 μM, as indicated by columns 7 and 8), K-252a (0.3 μg/ml, column 9), genistein (10 or 50 μM, columns 10 and 11), H-89 (1 μM, column 12), PD098059 (30 μM, column 13), KN-62 (30 μM, column 14) or BAPTA/AM (30 μM, column 15) for 20 min, then primed with lipopolysaccharide (1 μg/ml) for 1 h. The cells were washed with PSS, then stimulated with UTP (100 μM). The increased arachidonic acid release by lipopolysaccharide priming was expressed as percentage of the value without drug pretreatment. Results are expressed as the means ± S.E.M. of three independent experiments. * $P < 0.05$ compared to the 100% value representing the priming effect of lipopolysaccharide without any drug pretreatment.

To confirm the involvement of protein phosphorylation (Fig. 4), its independence of $[Ca^{2+}]_i$, and the involvement of protein phosphatase in the action of lipopolysaccharide, we performed the following permeabilization studies. KG buffer containing protein phosphatase inhibitors, microcystin (an inhibitor of protein phosphatase 1 and protein phosphatase 2A), FK 506 (protein phosphatase 2B inhibitor) or orthovanadate (tyrosine phosphatase inhibitor),

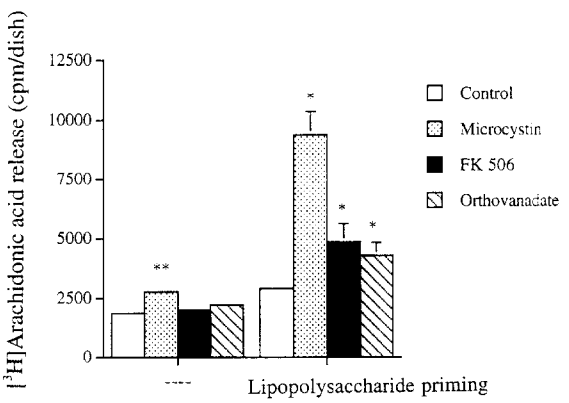

Fig. 5. Protein phosphatase inhibitors potentiate the priming effects of lipopolysaccharide on arachidonic acid release from permeabilized cells. Cells incubated with vehicle or lipopolysaccharide (1 μg/ml) for 1 h were permeabilized in KG buffer containing microcystin (1 μM), FK 506 (0.2 μg/ml) or orthovanadate (100 μM), and then incubated in KG buffer containing 100 nM free Ca^{2+} and the appropriate inhibitor. Results are expressed as the means ± S.E.M. of three independent experiments. * $P < 0.05$ compared to control arachidonic acid release in lipopolysaccharide-primed cells. ** $P < 0.05$ compared to control arachidonic acid release in lipopolysaccharide-unprimed cells.

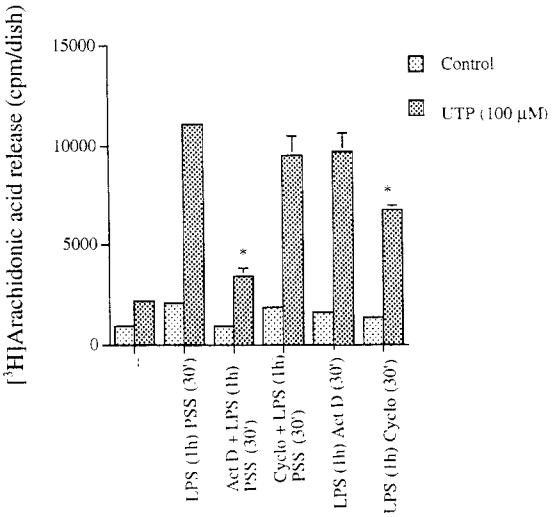

Fig. 6. Effects of actinomycin D or cycloheximide on the priming effects of lipopolysaccharide. Actinomycin D (1 μM) or cycloheximide (10 μM) were present during the 1 h period of lipopolysaccharide priming or were added for 30 min after lipopolysaccharide priming for 1 h. After lipopolysaccharide priming, cells were stimulated with 100 μM UTP for 30 min and the [^{3}H]arachidonic acid release measured. Results are expressed as the means ± S.E.M. of three independent experiments. $P < 0.05$ compared to the control UTP response in lipopolysaccharide-primed cells without actinomycin D or cycloheximide coincubation.

was added to untreated cells and lipopolysaccharide-treated cells and the effects on arachidonic acid release measured. As shown in Fig. 5, in 100 nM Ca^{2+}-containing KG buffer, the basal arachidonic acid release was slightly but significantly enhanced by KG buffer containing 1 μM microcystin, but not by KG buffer containing 0.2 μg/ml FK 506 or 100 μM orthovanadate. In agreement with the results seen for the intact cells, lipopolysaccharide effects were evident in the permeabilized cells, with the arachidonic acid release being increased 60%. In addition, microcystin, FK 506 and orthovanadate all increased the lipopolysaccharide-stimulated arachidonic acid release by 5.0-, 2.6- and 2.3-fold, respectively.

In the P388 D_1 macrophage, lipopolysaccharide priming of prostaglandin E_2 production in response to platelet activating factor has been shown to be dependent on gene transcription and protein synthesis (Glaser et al., 1990). As shown in Fig. 6, we obtained similar results for arachidonic acid release in lipopolysaccharide-primed RAW 264.7 cells. When actinomycin D (1 μM) was added together with lipopolysaccharide during the priming period, the lipopolysaccharide effect was inhibited by 86 ± 7% (the net arachidonic acid release caused by UTP was from 1245 ± 56 cpm/dish in lipopolysaccharide-unprimed cells to 9006 ± 108 cpm/dish in lipopolysaccharide-primed cells and to 2331 ± 416 cpm/dish in actinomycin D-pretreated and lipopolysaccharide-primed cells). If actinomycin D was added for 30 min but after lipopolysaccharide priming, no significant inhibition was observed. In contrast, the effects of cycloheximide (10 μM) were more obvious when it was added for 30 min after lipopolysaccharide priming, with 51 ± 4% inhibition, than during priming (The net arachidonic acid release caused by UTP was from 1245 ± 56 cpm/dish in lipopolysaccharide-unprimed cells to 9006 ± 108 cpm/dish in lipopolysaccharide-primed cells and to 5047 ± 245 cpm/dish in cycloheximide-pretreated and lipopolysaccharide-primed cells). At the concentrations tested, actinomycin D and cycloheximide alone had no significant effects on basal arachidonic acid release and did not affect cell viability, as shown by vital dye exclusion and MTT test.

4. Discussion

Macrophages play key roles, both as phagocytic cells and accessory cells in the immune system. One of the key events in macrophage activation is the generation of large amounts of oxygenated metabolites of arachidonic acid and the rate-limiting step in eicosanoid biosynthesis is thought to be the liberation of arachidonic acid from membrane phospholipids by activation of the key enzyme, phospholipase A_2. In the mouse macrophage cell line, RAW 264.7, cytosolic phospholipase A_2 has been identified and characterized (Channon and Leslie, 1990). In previous studies, we have demonstrated, by assessing the formation of inositol phosphate and arachidonic acid, that UTP is much more potent than ATP in activating phosphoinositide-phospholipase C and phospholipase A_2 in RAW 264.7 cells (Lin and Lee, 1996). We now show that exposure of RAW 264.7 cells to lipopolysaccharide causes potentiation of the arachidonic acid, but not inositol phosphate, response to UTP.

The concentration-dependent inhibition seen with calphostin, staurosporine and H-7 at concentrations inhibiting protein kinase C suggests that activation of protein kinase C is an obligatory event in lipopolysaccharide-mediated regulation of phospholipase A_2 activation. Indeed, the inhibition by calphostin, a rather specific inhibitor of protein kinase C as compared to other protein kinases, would strengthen the contribution of protein kinae C. The K_i values (μM) of calphostin are 0.05, > 50, > 25 and > 5 for protein kinase C, protein kinase A, protein kinase G and myosin light chain kinase, respectively; while those of staurosporine were 0.0007, 0.007, 0.0085 and 0.0013, and those of H-7 were 6, 3, 5.8 and 97, respectively. This conclusion is supported by the activation and/or translocation of protein kinase C induced by lipopolysaccharide in RAW 264.7 macrophages (Paul et al., 1995) and other cells, such as THP-1 and U937 cells (Natarajan and Iwamoto, 1994), cardiac myocytes (Heard et al., 1994; McKenna et al., 1995), aorta (McKenna et al., 1994) and human monocytes (Liu et al., 1994). However, short-term treatment with phorbol 12-myristate 13-acetate alone only slightly increased arachidonic acid release by about 50 ± 20% ($n = 14$), suggesting that protein kinase C

activation itself is not sufficient for full activation of phospholipase A_2 in RAW 264.7 cells and that other lipopolysaccharide-triggered protein kinase C-independent signal pathways are required for phospholipase A_2 activation. The source of the protein kinase C activator, diacylglycerol, increased by lipopolysaccharide, has also been investigated. Sands et al. (1994) reported that lipopolysaccharide could activate phosphatidylcholine-specific phospholipase C and produce diacylglycerol in J774 murine macrophages (Sands et al., 1994). However, the diacylglycerol formation via stimulation of phosphoinositide-specific phospholipase C appears not to be the case for lipopolysaccharide, as [^{3}H]inositol phosphate accumulation was not seen after lipopolysaccharide treatment in RAW 264.7 cells (our present results).

Pretreatment of the cells with the tyrosine kinase inhibitor, genistein, also significantly inhibited the lipopolysaccharide priming effect. The concentration we used (10, 50 μM) is quite correlated to its specific action on tyrosine kinase ($IC_{50} = 2.6$ μM) (Akiyama et al., 1987). These results are supported by the rapid tyrosine phosphorylation of several proteins known to occur in RAW 264.7 cells stimulated by lipopolysaccharide (Weinstein et al., 1991). Similar inhibition of lipopolysaccharide priming by genistein has been seen in P388D_1 macrophages (Glaser et al., 1990, 1993), while, in B cells, lipopolysaccharide priming was insensitive to treatment with genistein, thus tyrosine phosphorylation does not appear to be involved in lipopolysaccharide action in B cells (Campbell and Sefton, 1990).

Lipopolysaccharide itself, at concentrations as high as 1 μg/ml, did not induce any changes in $[Ca^{2+}]_i$. In addition, lipopolysaccharide priming had no effect on either the phosphoinositide turnover or the $[Ca^{2+}]_i$ increase induced by UTP. The lack of effects of H-89 (a selective inhibitor of protein kinase A) (Chijiwa et al., 1990), KN-62 (an inhibitor of Ca/Calmodulin-dependent protein kinase II) (Tokumitsu et al., 1990) and PD098059 (a selective inhibitor of mitogen-activated protein kinase kinase) (Pang et al., 1995), suggest that these three protein kinases are not involved in the lipopolysaccharide priming effect.

To completely exclude any effect of $[Ca^{2+}]_i$ and to ascertain the involvement of protein phosphorylation on phospholipase A_2 activation, permeabilized cells were used. Permeabilization of cells with digitonin allows the introduction of membrane-impermeant compounds (e.g., microcystin) and the manipulation of fixed Ca^{2+} concentrations, while retaining the membrane stimulus-response coupling necessary for cell function. The data from experiments using microcystin (a more selective inhibitor of protein phosphatases 1 and 2A), FK506 (an inhibitor of protein phosphatase 2B) and orthovanadate (an inhibitor of tyrosine phosphatase) suggest that both Ser/Thr and Tyr phosphorylation contribute to phospholipase A_2 activation, with the former playing a more major role than the latter. Although the present data do not directly implicate phospholipase A_2 as the phosphorylation target of lipopolysaccharide, this has been shown to be the case for cytosolic phospholipase A_2 in neutrophils (Doerfler et al., 1994).

The abilities of actinomycin D, an inhibitor of gene transcription, and cycloheximide, a protein synthesis inhibitor, to inhibit the lipopolysaccharide priming response, indicate that the lipopolysaccharide priming required for UTP stimulation of arachidonic acid release involves transcriptional events, which occurs during lipopolysaccharide priming for 1 h, and de novo protein synthesis, which usually occurs after 1 h lipopolysaccharide priming. This is consistent with previous findings in P388 D_1 cells (Glaser et al., 1990). Moreover, the possibility that the lipopolysaccharide effect is mediated by the generation of inflammatory cytokines released from macrophages appears unlikely, since mouse recombinant tumor necrosis factor α (TNFα) and interleukin-1β are unable to mimic the priming effect (data not shown). Although lipopolysaccharide (1 μg/ml) could induce TNFα release from 10 ng/ml to about 40 ng/ml within 1 h, TNFα (30 and 100 ng/ml) itself could not potentiate the basal and UTP-induced arachidonic acid release at the same condition as lipopolysaccharide did (data not shown). Moreover, TNFα release within 1 h was only increased by 1 μg/ml lipopolysaccharide, while the priming effect on arachidonic acid release was already manifest after 0.1 μg/ml lipopolysaccharide treatment (Fig. 2).

In conclusion, lipopolysaccharide causes priming of the UTP-stimulated phospholipae A_2 activation in RAW 264.7 macrophages. Lipopolysaccharide-induced protein kinase C and tyrosine kinase activation may be necessary intermediates in the potentiation of UTP-induced arachidonic acid release. UTP-induced inositol phospholipid hydrolysis and Ca^{2+} signaling are unchanged and thus are not the priming mechanisms.

Acknowledgements

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