British Journal of Pharmacology (2001) 134, 1055–1065

www.nature.com/bjp

PKC- and ERK-dependent activation of IκB kinase by lipopolysaccharide in macrophages: enhancement by P2Y receptor-mediated CaMK activation

[1]Bing-C. Chen & *,[1]Wan-W. Lin

[1]Department of Pharmacology, College of Medicine, National Taiwan University, Taipei, Taiwan

1 Although accumulating studies have identified IκB kinase (IKK) to be essential for controlling NF-κB activity in response to several cytokines, the upstream kinases that control IKK activity are still not completely known. We have previously reported that G protein-coupled $P2Y_6$ receptor activation by UTP potentiates lipopolysaccharide (LPS)-induced IκB phosphorylation and degradation, and NF-κB activation in J774 macrophages. In this study, we investigated the upstream kinases for IKK activation by UTP and LPS.

2 In murine J774 macrophages, LPS-induced NF-κB activation was inhibited by the presence of PDTC, D609, Ro 31-8220, PD 098059 and SB 203580.

3 Accompanying NF-κB activation, LPS induced IκB degradation and IKK activation were reduced by PDTC, D609, Ro 31-8220 and PD 098059, but not by SB 203580.

4 Although UTP itself slightly induced IKK activation, this response was synergistic with LPS. BAPTA/AM and KN-93 (a calcium/calmodulin-dependent protein kinase (CaMK) inhibitor) attenuated UTP- but not LPS-stimulated IKK activity. Synergistic IKK activation between LPS and thapsigargin was further demonstrated in peritoneal macrophages.

5 LPS and UTP co-stimulation additively increased p65 NF-κB phosphorylation. *In vitro* kinase assays revealed that LPS and UTP induced extracellular signal-regulated protein kinase (ERK) and p38 mitogen-activated protein kinase activation were respectively inhibited by PD098059 and SB 203580.

6 Taken together, we demonstration that Gq protein-coupled $P2Y_6$ receptor activation can potentiate LPS-stimulated IKK activity. While PKC and ERK participate in IKK activation by LPS and UTP, the phosphatidylinositide-phospholipase C-dependent activation of CaMK plays a major role in UTP potentiation of the LPS response.
British Journal of Pharmacology (2001) **134,** 1055–1065

Keywords: P2Y receptor; CaMK; IKK; NF-κB; PKC; ERK; p38 MAPK; macrophages

Abbreviations: CaMK, calcium/calmodulin-dependent protein kinase; COX-2, cyclooxygenase-2; DMEM, Dulbecco's modified Eagle's medium; ECL, enhanced chemiluminescence; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated protein kinase; IKK, IκB kinase; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; MEK, mitogen-activated protein kinase kinase; NIK, NF-κB-inducing kinase; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PDTC, pyrrolidine dithiocarbamate; PGE_2, prostaglandin E_2; PI, phosphatidylinositide; PKC, protein kinase C; PLC, phospholipase C; PMA, phorbol 12-myristate 13-acetate; TG, thapsigargin; TNFα, tumor necrosis factor-α

Introduction

The transcription factor, NF-κB, plays a key role in the transcriptional regulation of many proteins involved in chronic inflammatory diseases (Barnes & Karin, 1997). NF-κB is a dimer of transcription factors primarily consisting of p65 (RelA) or p50; p65 is the transcriptional activation component of the most common form of the NF-κB heterodimer (Baldwin, 1996). In the resting state, this dimer is anchored by IκBα and IκBβ, which function to retain NF-κB in the cytosol (Thompson *et al*., 1995). Recently, two closely related IκB kinases (IKKs), IKKα and IKKβ, were identified which directly phosphorylate IκBα at Ser^{32} and Ser^{36}, and phosphorylate IκBβ at Ser^{19} and Ser^{23} (DiDonato *et al*., 1997; Maniatis, 1997; Woronicz *et al*., 1997). These phosphorylations lead to ubiquitination of IκBs at specific lysine residues, and their degradation by the 26S proteasome (Chen *et al*., 1995). This process releases active NF-κB, which then translocates to the nucleus, binds to specific DNA enhancer sequences (κB binding sites), and activates gene transcription. Based on approaches using transient transfection overexpression, the best-characterized upstream kinase candidate involved in the activation of the IKK complex in response to specific stimuli through phosphorylation of the two IKK subunits has been identified as NF-κB-inducing kinase (NIK) (Malinin *et al*., 1997). All NF-κB activation either by cytokines, such as interleukin-1 and tumour necrosis factor-α (TNFα), or by bacterial endotoxin lipopolysaccharide (LPS) requires NIK activation (Israel, 2000).

*Author for correspondence; E-mail: wwl@ha.mc.ntu.edu.tw

Transcriptional activity of NF-κB can be regulated by mechanisms other than cytosolic degradation of IκB. Several reports have demonstrated that the DNA binding (Hayashi *et al*., 1993) and transactivating capacity (Zhong *et al*., 1997; Wang & Baldwin, 1998) of NF-κB are up-regulated by inducible phosphorylation of p65. In this context, protein kinases, such as IKKs (Sakurai *et al*., 1999), protein kinase A (PKA) (Zhong *et al*., 1998), p38 mitogen-activated protein kinase (MAPK) (Vanden Berghe *et al*., 1998; Jefferies & O'Neill, 2000), extracellular signal-regulated protein kinase (ERK) (Vanden Berghe *et al*., 1998; Jefferies & O'Neill, 2000), and PKC (Lozano *et al*., 1994) have been shown to phosphorylate p65, for example at Ser^{276} by PKA or at Ser^{536} by IKKs. This p65 phosphorylation in turn increases the transcriptional activity of NF-κB, and represents another mechanism, independent of IκB degradation, for enhancing NF-κB activation.

Although there has been a tremendous increase in our understanding of the mechanisms leading to NF-κB activation during the past 3 years, it is still unclear whether other kinases, with the exception of NIK, mediate the regulation of IKK complex activity. One of the reasons this unanswered question has been raised is that recent data have implicated mitogen-activated protein kinase/ERK kinase kinase 1 (MEKK1) (Lee *et al*., 1997), PKC, ERK, and p38 MAPK in NF-κB activation. With respect to PKC, PKCζ was shown to phosphorylate IκBα *in vitro* (Diaz Meco *et al*., 1994), and transfection of a dominant negative mutant of PKCζ severely impairs NF-κB activation by sphingomyelinase (Lozano *et al*., 1994). *In vivo* overexpression studies also demonstrated PKCε and PKCα to be inducers of NF-κB activation (Genot *et al*., 1995; Trushin *et al*., 1999). Some studies, including ours, have also demonstrated that p38 MAPK is a crucial signal kinase mediating LPS-elicited NF-κB activation (Chen *et al*., 1999; Nick *et al*., 1999). At present, the roles of PKC, ERK, and p38 MAPK in LPS-induced IKK activity have yet to be investigated.

Extracellular nucleotides such as ATP and UTP exert diverse effects on cellular function by acting on P_2 receptors. In murine J774 macrophages, we have demonstrated that the expressed $P2Y_6$ receptors mediate UTP and UDP actions in increasing phosphoinositide (PI) turnover, PKC activation, and intracellular Ca^{2+} ($[Ca^{2+}]_i$) mobilization (Lin & Chen, 1997). Subsequently, we showed the potentiation effects of UTP on LPS-elicited inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and interleukin-6 (IL-6) gene induction. UTP potentiating effects are ascribed to a signalling pathway controlled by a calcium/calmodulin-dependent protein kinase (CaMK), which involves the enhancement of IκB phosphorylation and degradation as well as NF-κB activation (Chen *et al*., 1998; Chen & Lin, 1999; 2000). Apart from these findings, the molecular mechanisms by which UTP potentiates IκB phosphorylation are still unclear. Thus, in this study, we address the effects of UTP on IKK activity and the roles of CaMK, PKC, ERK, and p38 MAPK in LPS plus UTP-induced NF-κB activation.

Methods

Cell culture

The murine J774 macrophages obtained from American Type Culture Collection were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal bovine serum and antibiotics (100 U ml^{-1} of penicillin and 100 μg ml^{-1} streptomycin). The peritoneal macrophages were prepared from Balb/c mice which had been i.p. injected with 1.5 ml of 3% thioglycolate 3 days before macrophage isolation. The peritoneal cavities were flushed with ice-cold 0.9% NaCl to remove the elicited peritoneal macrophages which were then cultured at 37°C in RPMI 1640, supplemented with 10% foetal bovine serum and antibiotics (100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin). Cells were cultured at 37°C in a humidified atmosphere of 95% air/5% CO_2.

Immunoblot analysis of IκBα and IκBβ

To quantify IκBα and IκBβ proteins in cells following LPS treatment for different periods, cells were washed twice in ice-cold phosphate-buffered saline (PBS), and then solubilized in buffer containing (mM): Tris-HCl 20 (pH 7.5), EGTA 0.5, EDTA 2, dithiothreitol 2, p-methylsulphonyl fluoride 0.5, and 10 μg ml^{-1} leupeptin. Samples of equal amounts of protein (100 μg) were subjected to SDS–PAGE on 12% polyacrylamide gels, then transferred onto a nitrocellulose membrane, which was then incubated in TBST buffer (150 mM NaCl, 20 mM Tris-HCl, 0.02% Tween 20, pH 7.4) containing 1% nonfat milk. The IκBα or IκBβ band was visualized by immunoblotting with a specific antibody. Immunoreactivity was detected by enhanced chemiluminescence (ECL) following the manufacturer's instructions. All the protein levels were expressed as percentages of control group.

Immunoprecipitation and protein kinase assays

Cells were washed twice with ice-cold PBS, lysed in 1 ml of lysis buffer containing: 20 mM Tris, pH 7.5, 1 mM $MgCl_2$, 125 mM NaCl, 1% Triton X-100, 1 mM p-methylsulphonyl fluoride, 10 μg ml^{-1} leupeptin, 10 μg ml^{-1} aprotinin, 25 mM β-glycerophosphate, 50 mM NaF, and 100 μM sodium orthovanadate, and centrifuged. The supernatant was then immunoprecipitated with respective polyclonal antibodies against IKKα, IKKβ, ERK2, or p38 MAPK in the presence of A/G-agarose beads overnight. The beads were washed three times with lysis buffer and two times with kinase buffer (20 mM HEPES, pH 7.4, 20 mM $MgCl_2$, 2 mM dithiothreitol). Then the beads were equally divided into two parts respectively for kinase assay and immunoblotting. The kinase reactions were performed by incubating immunoprecipitated beads with 20 μl of kinase buffer supplemented with 20 μM ATP and 3 μCi of [γ-^{32}P]ATP at 30°C for 30 min. For IKK assay, both IKKα and IKKβ antibodies were used to immunoprecipitate both kinases, and 2.5 μg of bacterially expressed GST-IκBα (amino acids 5–55), GST-p65 (amino acids 354–551), or GST-p65 S536A (Ser^{536} substituted by Ala^{536}) was added as a substrate. For ERK and p38 MAPK assays, 50 μg ml^{-1} of myelin basic protein (MBP) was added as a substrate. The reaction mixtures were analysed by SDS–PAGE followed by autoradiography. All the kinase activity were expressed as percentages of the control groups. The precipitated protein levels of IKKα, ERK or p38 MAPK were assayed by immunoblotting as an internal control.

In vivo *p65 phosphorylation*

J774 cells were starved in medium without serum for 24 h. Then the medium was removed, replaced with phosphate-free DMEM containing 0.1 mCi ml^{-1} of [^{32}P] orthophosphate, and incubated overnight. Cells were treated with LPS, UTP, or both for different periods, followed by cell harvest in 1 ml lysis buffer and centrifugation. The supernatant was then immunoprecipitated with p65 polyclonal antibody overnight. The immunoprecipitates were then analysed by SDS–PAGE followed by autoradiography.

Preparation of nuclear extracts and electrophoretic mobility shift assays (EMSA)

Nuclear extracts were prepared as described previously (Chen *et al*., 1998). To detect NF-κB activation using the EMSA method, binding reaction mixtures (15 μl) contained 0.25 μg of poly (dI-dC) and 20,000 d.p.m. of ^{32}P-labelled DNA probe in binding buffer consisting of (mM): Tris-HCl 10, pH 7.5, EDTA 1, 4% Ficoll, dithiothreitol 1, and KCl 75. The binding reaction was initiated by the addition of cell extracts, and it was allowed to continue for 30 min. Samples were analysed on native 5% polyacrylamide gels. For super-shift experiments, 4 μg of anti-p65 or anti-p50 antibody was mixed with the nuclear extract proteins.

CaMK activity assay

Confluent cells on 35 mm dishes were washed with physiological saline solution (PSS, composition in mM): NaCl 118, KCl 4.7, $CaCl_2$ 1.8, $MgCl_2$ 1.2, KH_2PO_4 1.2, glucose 118, and HEPES 20, pH 7.4, and then the indicated drugs were added. Cells were collected on ice and disrupted by sonication at 4°C in lysis buffer. Each homogenate was immediately used for CaMK assay (Hanson & Schulman, 1992). Reaction assay mixture contained (final concentration in 50 μl): 50 mM PIPES (pH 7.0), 10 mM $MgCl_2$, 0.1 mg ml^{-1} BSA, 0.3 mM $CaCl_2$, 20 μM [γ-^{32}P]ATP, 0.3 μM calmodulin, and 15 μM autocamide-3. Reactions were carried out for 30 min in 30°C, and incorporation of ^{32}P into autocamide-3 was determined. Phosphorylation of autocamide-3 in the absence of Ca^{2+} (by removing 0.3 mM $CaCl_2$ and addition of 0.5 mM EDTA) and calmodulin relative to its phosphorylation in the presence of Ca^{2+} and calmodulin is defined as the autonomous activity.

Chemicals

Oligonucleotides were synthesized on a PS 250 CRUACHEM DNA synthesizer using the cyanoethyl phosphoroamidate method, and purified by gel filtration. The sequence of the double-stranded oligonucleotide used to detect the DNA-binding activities of NF-κB is shown with the binding site underlined: 5′-GATCAGTTGAGGGGACTTTCCCAGGC-3′. DMEM, RPMI 1640, foetal bovine serum, penicillin/streptomycin and autocamide-3 were obtained from Gibco BRL (Grand Island, NY, U.S.A.). [α-^{32}P]dATP (3000 Ci $mmol^{-1}$), [^{32}P]-orthophosphate, horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies, and the ECL detection agent were purchased from Amersham Pharmacia Biotech (Piscataway, NJ, U.S.A.). Ro31-8220 and KN-93 were purchased from Calbiochem (La Jolla, CA, U.S.A.). D609 was from Biomol (Plymouth Meeting, PA, U.S.A.). Rabbit polyclonal antibodies specific for p65 NF-κB, p50 NF-κB, IκBα, IκBβ, IKKα, IKKβ, ERK2, and p38 MAPK, and protein A/G beads were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Plasmid of pGEX-IκBα (amino acids 5–55) was provided by Dr Frank S. Lee (Pennsylvania Medical Center, PA, U.S.A.). Plasmids of pGEX-p65 (354–551) and pGEX-p65 (point mutant of amino acid Ser^{536} substitution by Ala^{536}) were a kind gift from Dr H. Sakurai (Tanabe Seiyaku, Osaka, Japan). All materials for SDS–PAGE were obtained from Bio-Rad Laboratories (Hercules, CA, U.S.A.). All other chemicals were obtained from Sigma (St. Louis, MO, U.S.A.).

Statistical analysis

Values are expressed as the mean ± standard error of the mean (s.e.mean) of at least three experiments. Analysis of variance (ANOVA) was used to assess the statistical significance of the differences, and a P value less than 0.05 was considered statistically significant.

Results

Inhibition of LPS-induced NF-κB activation by D609, Ro 31-8220, PD 098059, and SB 203580

Our previous results showed that LPS can induce NF-κB activation as well as IκBα phosphorylation and degradation, and in turn cause iNOS, COX-2, and IL-6 gene expression in murine J774 macrophages. Moreover, activation of NF-κB is indispensable for these LPS actions (Chen *et al*., 1998; Chen & Lin, 1999; 2000).

To investigate the regulatory roles of phosphatidylcholine-phospholipase C (PC–PLC), PKC, ERK, and p38 MAPK in LPS-induced NF-κB activation, D609 (a selective PC-PLC inhibitor) (Muller-Decker, 1989), Ro 31-8220 (a selective PKC inhibitor) (Keller & Niggli, 1993), PD 098059 (a selective mitogen-activated protein kinase kinase (MEK) inhibitor) (Dudley *et al*., 1995), and SB 203580 (a selective p38 MAPK inhibitor) (Lee *et al*., 1994) were examined. When J774 macrophages were treated for 30 min with LPS, the nuclear translocation of NF-κB was evidenced by *in vitro* binding to a specific DNA sequence. First, we performed antibody gel super-shift assays to determine whether the LPS-induced DNA-protein complex contained p50 (NFκB1) and p65 (RelA) subunits. Upon addition to nuclear extracts prepared from LPS-treated cells, p50-specific and p65-specific antibodies caused super-shifts of the DNA-protein complexes, p50/p65 heterodimer, and p50/p50 homodimer (Figure 1, arrowheads), suggesting the existence of two types of NF-κB complexes. When J774 cells were pretreated for 20 min with (μM): pyrrolidine dithiocarbamate (PDTC) 50, D609 30, Ro 31-8220 1, PD 098059 30, or SB 203580 3, LPS-induced NF-κB activation was inhibited by 40 ± 7%, 64 ± 5%, 69 ± 11%, 66 ± 6%, and 72 ± 5% (n = 3), respectively (Figure 1). At the concentrations used, none of these inhibitors significantly affected the basal level of NF-κB. These results suggest that PKC, ERK, and p38 MAPK are all involved in the upstream signal pathways which mediate LPS-elicited

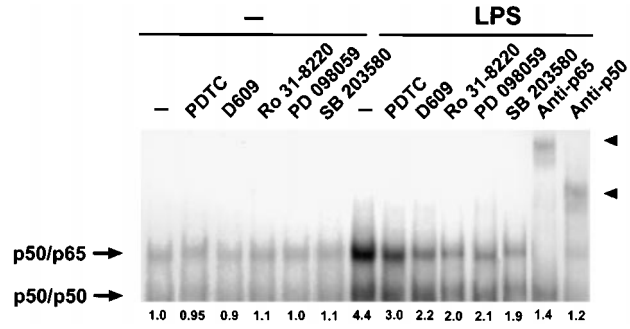

Figure 1 Effects of pharmacological agents on LPS-induced NF-κB activation. J774 macrophages were preincubated with a vehicle (in μM): PDTC 50, D609 30, Ro 31-8220 1, PD 098059 30, or SB 203580 3, for 20 min followed by stimulation with LPS (1 μg ml^{-1}) for another 30 min. Following incubation, nuclear extracts were prepared and subjected to EMSA as described in the Methods section. The NF-κB species in the DNA-protein complex was determined by a super-shift with inclusion of 4 μg of the anti-p65 or anti-p50 antibody in the nuclear extract of LPS-stimulated cells as indicated by arrowheads. The results are representative of three independent experiments.

NF-κB activation. Furthermore, this suggests that PC-PLC-derived diacylglycerol is a major component for LPS-induced PKC activation.

Effects of pharmacological inhibitors on IκBs degradation

NF-κB is generally retained in the cytoplasm of unstimulated cells by interaction with IκBα and IκBβ. Upon response to most NF-κB-inducing signals, both IκBs are targeted for IKK-specific phosphorylation, followed by proteasome-dependent degradation, resulting in NF-κB dissociation from IκBs, as well as its nuclear translocation and DNA binding. To investigate the underlying mechanism for NF-κB inhibition caused by kinase inhibitors, we next studied whether the PKC-, ERK-, and p38 MAPK-dependent pathways are essential for LPS-mediated IκB degradation. As shown in Figure 2a, treatment with LPS for 10 min resulted in IκBα degradation, which reached the maximum extent at 30 min and began to recover after that because of re-synthesis of IκBα. LPS also induced IκBβ degradation, albeit with a slower onset at 30 min and a delayed maximal effect at 60 min (Figure 2a). These results are consistent with previous findings showing that LPS exhibits a delayed kinetic on the degradation of IκBβ compared to that on IκBα (Velasco *et al.*, 1997). We next examined the effects of these inhibitors on LPS-induced IκBα degradation. As shown in Figure 2b, PDTC (50 μM), D609 (30 μM), Ro 31-8220 (1 μM), and PD 098059 (30 μM), but not SB 203580 (3 μM), markedly reduced LPS induction of IκBα degradation. At the concentrations used, none of these inhibitors affected the basal level of IκBα protein. These results suggest the crucial roles played by PKC and ERK, but not p38 MAPK, in the upstream signal cascades required for LPS induction of IκBα degradation.

Time-dependent IKK activation by LPS and UTP

Our previous study demonstrated that $P2Y_6$ receptor stimulation by UTP triggers a Ca^{2+}-dependent pathway

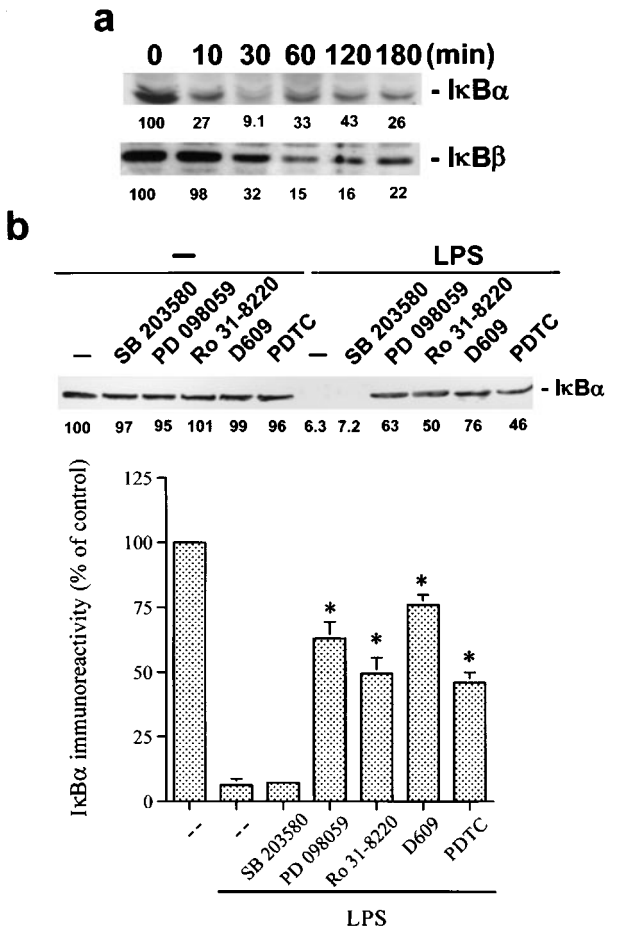

Figure 2 Effects of protein kinase inhibitors on LPS-induced IκB degradation. (a) Time course analysis of IκBα and IκBβ degradation induced by LPS. Following incubation for different periods with LPS (1 μg ml^{-1}), IκBα and IκBβ degradations were determined by immunoblotting with IκBα- or IκBβ-specific antibody. (b) Cells were preincubated with vehicle (in μM): PDTC 50, D609 30, Ro 31-8220 1, PD 098059 30, or SB 203580 3, for 20 min and then incubated with LPS (1 μg ml^{-1}) for an additional 30 min. Measurement of IκBα degradation was as described above. The traces are representative of three experiments with similar results, which are shown as the mean$\pm$s.e.mean. *$P<0.05$ as compared to the control LPS response without inhibitor pretreatment.

that synergizes LPS-induced NF-κB activation by increasing phosphorylation and degradation of IκBα (Chen *et al.*, 1998). To determine whether this enhanced IκB phosphorylation by UTP results from upstream IKK activation, we directly measured IKK complex kinase activity by immunoprecipitating IKKα and IKKβ from J774 cells that were either activated or not by UTP and/or LPS. As shown in Figure 3, treatment of J774 cells with LPS (1 μg ml^{-1}) elicited a marked (about a 15 fold activity increase with 20 min of incubation) and sustained (lasting for at least 3 h) activation of the IKK complex (Figure 3a). Compared to the LPS response, UTP (100 μM) elicited a slight and transient IKK activation (Figure 3a).

To explore the mechanism responsible for UTP-induced IKK activation, two pharmacological agents, thapsigargin and phorbol 12-myristate 13-acetate (PMA), which respectively mimic PI-PLC-transduced Ca^{2+}- and PKC-dependent

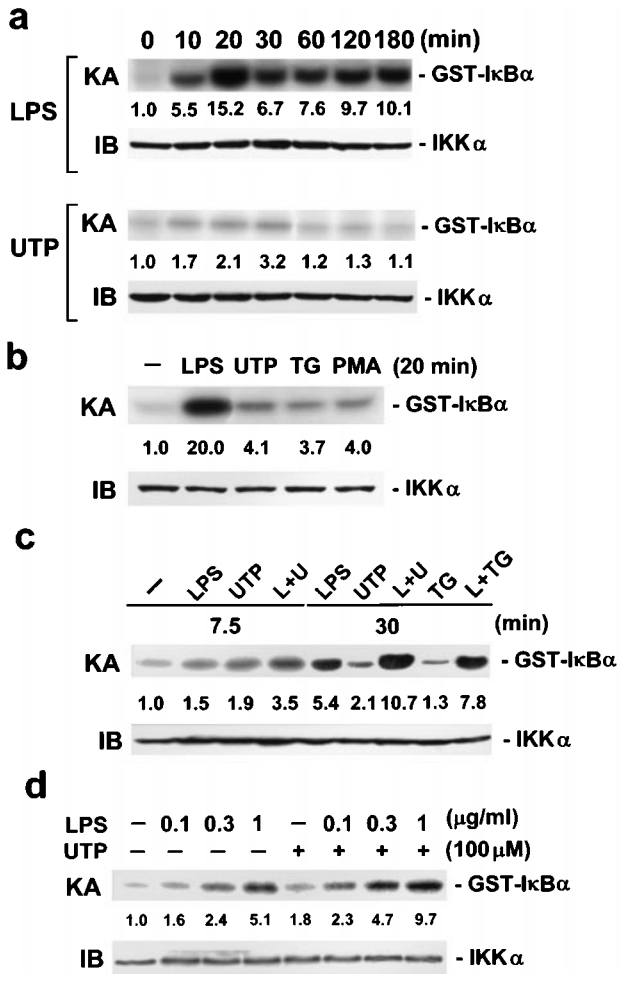

Figure 3 IKK activation by LPS and UTP. Cells were, as indicated, treated with 1 μg ml^{-1} LPS, 100 μM UTP, 30 nM thapsigargin (TG), 1 μM PMA (a–c) or 0.1–1 μg ml^{-1} LPS (d) for different intervals, and the cell lysates were then immunoprecipitated with antibodies specific for IKKs. One set of immunoprecipitates was subjected to kinase assay (KA) as described in the Methods section using GST-IκBα (5–55) as a substrate (a, b, c and d, top). The multiples of induction of IKK activity under each stimulation condition are shown. The other set of immunoprecipitates was subjected to SDS–PAGE and analysed by immunoblotting (IB) with anti-IKKα antibody (a, b, c and d, bottom). Equal amounts of the immunoprecipitated kinase complex present in each kinase assay were confirmed by immunoblotting for IKKα. Shown are representative results from three independent experiments.

signalling cascades, were investigated. Treatment with thapsigargin (30 nM) or PMA (1 μM) for 20 min moderately increased IKK activity to a similar extent as did UTP (Figure 3b), suggesting that both bifurcating signal pathways of PI-PLC, Ca^{2+} and PKC, participate in UTP activation of IKK. The combined effects of UTP and LPS on IKK activity, as shown in Figure 3c, also indicate their synergistic action, with a more marked response seen at 30 min than at 7.5 min. Consistent with UTP effect, thapsigargin potentiation of LPS-induced IKK activity was seen (Figure 3c). The concentration-dependent IKK activation by LPS and potentiation by UTP was shown in Figure 3d. UTP at 100 μM elicited dramatic potentiation of LPS response at 0.3 and 1 μg ml^{-1}.

Roles of PKC, ERK, and CaMK in LPS- and UTP-induced IKK activation

To support our suggestions that PKC and ERK are involved in LPS induction of IκB degradation and that CaMK participates in UTP potentiation of IκB degradation, we examined the effects of pharmacological inhibitors on IKK activation. As shown in Figure 4a, PDTC (50 μM), Ro 31-8220 (1 μM), and PD 098059 (30 μM) respectively reduced LPS-stimulated IKK activities by 65±6%, 77±4%, and 42±5% ($n=3$), and UTP-stimulated IKK activities by 70±4%, 68±3%, and 56±2% ($n=3$). Consistent with its inhibition of LPS-induced NF-κB activation and IκB degradation, D609 (30 μM) decreased LPS-induced IKK activity by 54±7% ($n=3$), while it did not change IKK activation by UTP. On the contrary, upon cell treatment with 10 μM KN-93 (a selective CaMK inhibitor) (Sumi *et al.*, 1991), UTP-induced, but not LPS-induced, IKK activity was inhibited by 51±5% ($n=3$) (Figure 4a). BAPTA/AM (30 μM), an intracellular calcium chelator, completely inhibited UTP-induced IKK activity, but did not affect LPS-induced IKK activity. SB 203580 (3 μM), on the other hand, did not affect IKK activation induced by either stimulus. Consistent with the individual attenuation of inhibitors, the synergistic IKK stimulation by LPS and UTP was sensitive to inhibition by Ro 31-8220, KN-93, PD 098059, Go 6976 (an inhibitor of classical PKC) and BAPTA/AM (Figure 4b). These results suggest that PKC

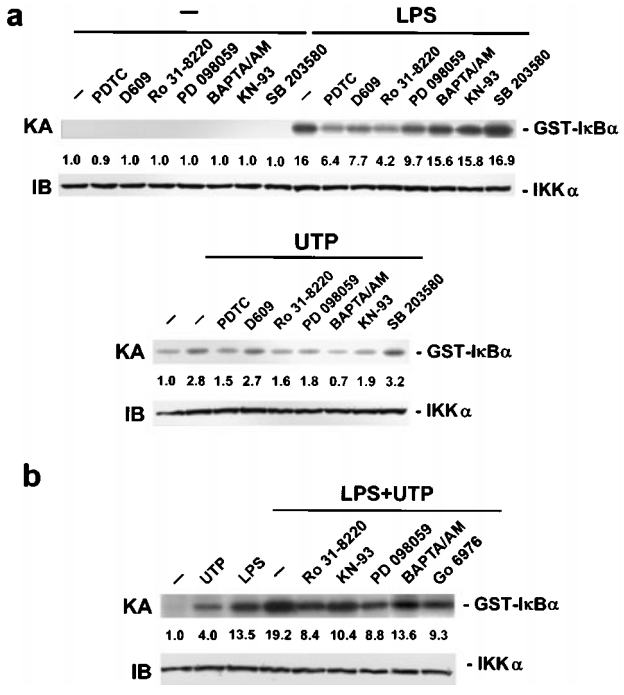

Figure 4 Effects of protein kinase inhibitors on LPS- and UTP-induced IKK activation. Cells were preincubated with vehicle (in μM): PDTC 50, D609 30, Ro 31-8220 1, PD 098059 30, BAPTA/AM 30, KN-93 10, SB 203580 3 or Go 6976 1, for 20 min before the addition of LPS (1 μg ml^{-1}), UTP (100 μM), or both for another 20 min. Cell lysates were then immunoprecipitated with IKKs antibodies. Both kinase assays and IKKα immunoblotting were analysed as described in Figure 3. The multiples of induction of IKK activity under each stimulation condition are shown. The typical traces shown are representative of three experiments.

and ERK converge the signal cascades triggered by LPS and UTP to activate IKK, and that the Ca^{2+}-dependent CaMK pathway also participates in the UTP response.

LPS and UTP activation of ERK and p38 MAPK

To directly confirm the crucial roles of ERK and p38 MAPK in NF-κB activation, we determined both kinase activities in response to LPS and UTP stimulation. The results using MBP as a kinase substrate indicated that LPS (1 μg ml^{-1}) and UTP (100 μM) stimulated ERK and p38 MAPK in a time-dependent manner, which occurred as early as 5–10 min of incubation and reached their peak responses at 30 min (Figure 5a,b). The ERK and p38 MAPK activity caused by LPS and UTP was respectively attenuated by the presence of 30 μM PD 098059 (Figure 5c) and 3 μM SB 203580 (Figure 5d).

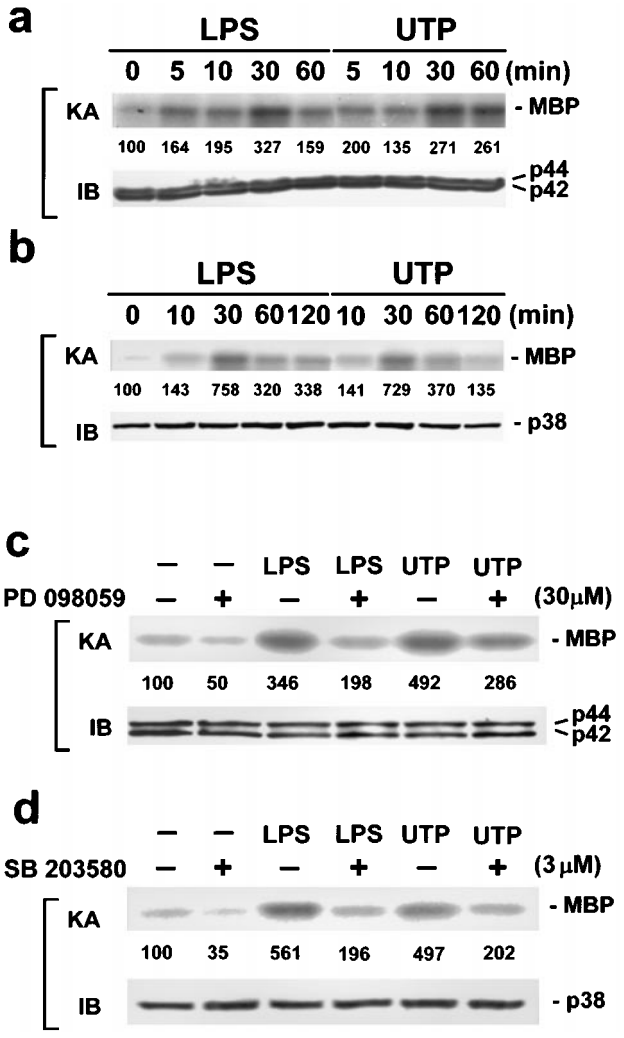

Figure 5 LPS- and UTP-induced ERK and p38 MAPK activation. After cells were treated with 1 μg ml^{-1} LPS or 100 μM UTP for different intervals, cell lysates were immunoprecipitated with an antibody specific for ERK (a) or p38 MAPK (b). Both kinase assays and MAPK immunoblotting were analysed as described. In some experiments, cells were preincubated with vehicle, 30 μM PD 098059 (c) or 3 μM SB 203580 (d) for 20 min before the addition of LPS (1 μg ml^{-1}) or UTP (100 μM) for another 30 min. Both kinase assays and MAPK immunoblotting were analysed as described. Shown are representative results from three to four independent experiments.

KN-93 inhibits UTP-induced CaMK activity

To confirm CaMK playing a major role in IKK potentiation by UTP, we determined CaMK activity following UTP treatment. The results indicated that the autonomous activity of CaMK was rapidly increased by 100 μM UTP treatment for 10 s (from 16.9±2.7% of total CaMK activity in control cells to 25.8±0.5% in UTP-treated cells). The CaMK activity stimulated by UTP was abrogated by the presence of 10 μM KN-93 (Figure 6).

LPS and UTP induction of p65 phosphorylation

In addition to IκBα/β degradation which causes nuclear translocation of NF-κB, post-translational modification of p65 NF-κB subunit, such as phosphorylation, represents another mechanism for controlling NF-κB activity. A recent study has shown that the endogenous IKK complex can phosphorylate IκBs as well as p65 NF-κB subunit in TNF-α-stimulated HeLa cells (Sakurai *et al*., 1999). The serine residue at amino acid 536 was demonstrated to be the phosphorylation target of IKK (Sakurai *et al*., 1999). To establish whether the UTP increase of LPS-induced IKK activation contributes to p65 phosphorylation, we performed two experiments. First, direct detection of p65 phosphorylation by *in vivo* [^{32}P] metabolic labeling plus immunoprecipitation analysis showed that both LPS (1 μg ml^{-1}) and UTP (100 μM) elicited p65 phosphorylation within 30 min and that both responded in an additive manner at 30 min (Figure 7a). Second, we found that the above results showing the synergistic IKK stimulation by LPS and UTP (Figures 3c and 4b) could also be reflected in p65 phosphorylation. Using GST-p65 (354–551) recombinant protein as the *in vitro* kinase substrate, LPS plus UTP showed synergistic effects on IKK activities as compared to the extent caused by each individually. Moreover, the recombinant protein with the point substitution of Ser^{536} with Ala was determined not to be

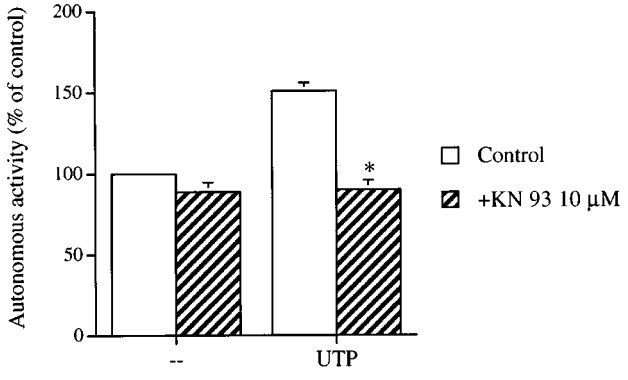

Figure 6 KN-93 inhibits UTP-induced CaMK activity in J774 cells. Cells were preincubated with vehicle, or 10 μM KN-93 for 20 min before the addition of UTP (100 μM) for another 10 s. The CaMK activity was assayed as described in the Methods section. Basal levels of autonomous activity was 44.1±1.4 pmol min^{-1} mg^{-1} (n=3). The data represent the mean±s.e.mean of three experiments performed in duplicate.

the IKK target, confirming the IKK phosphorylation site at Ser^{536} (Figure 7b).

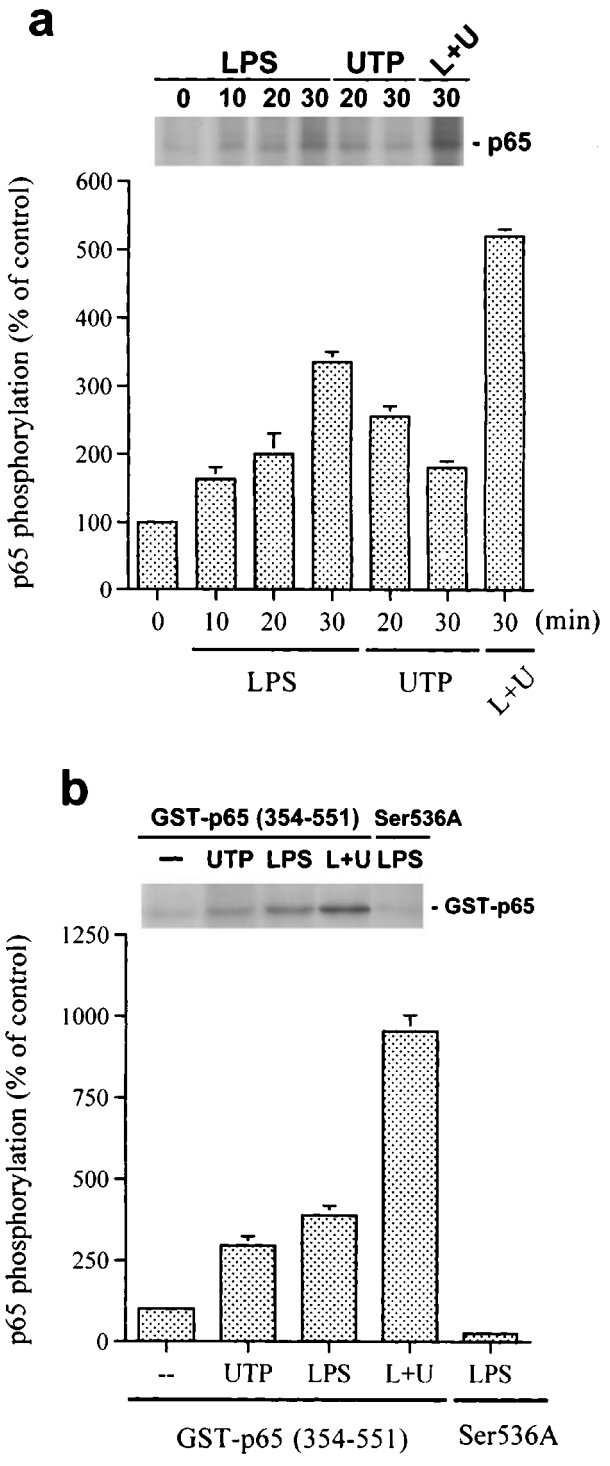

Figure 7 LPS and UTP mediation of p65 phosphorylation. (a) Cells loaded with [^{32}P]-orthophosphate were treated with LPS (1 μg ml^{-1}), UTP (100 μM), or both for the indicated time periods, and p65 was then immunoprecipitated and analysed by SDS–PAGE and autoradiography. (b) Cells were treated with LPS with or without UTP for 30 min, and cell lysates were then immunoprecipitated with antibodies specific for IKKs. The enzyme activity of IKK immunoprecipitates was assayed by using GST-p65 (354–551) or GST-p65 (Ser^{536} to Ala^{536}) as a substrate. Traces are representative results from three independent experiments, which are shown as mean ± s.e.mean.

LPS and TG induction of IKK activity in peritoneal macrophages

We next confirm that the calcium-dependent IKK potentiation observed in the J774 cell line is not cell type-dependent. Thapsigargin was previously shown to enhance LPS-elicited NO, prostaglandin E_2 (PGE_2), IL-6, and TNF-α release from peritoneal macrophages *via* a CaMK-dependent mechanism (Chen *et al*., 2001). Using recombinant IκBα as a substrate, we found that LPS (1 μg ml^{-1}) and thapsigargin (30 nM) increased peritoneal macrophage IKK activity with similar kinetics. Each stimulus elicited its maximal IKK activity at 20 min (Figure 8a). Although the thapsigargin response was less than that of LPS, thapsigargin also exerted a synergistic effect with LPS. This synergistic IKK activation was further evidenced by using GST-p65 (354–551) as a kinase substrate (Figure 8b). Likewise, the IKK activity caused by LPS plus thapsigargin was respectively attenuated by the presence of BAPTA/AM (30 μM), KN-93 (10 μM), Ro 31-8220 (1 μM), or PD 098059 (30 μM) (Figure 8c).

Discussion

NF-κB is a ubiquitous transcription factor and plays a crucial role in regulating many genes in a variety of cell types. Although NF-κB activation has been widely studied in many kinds of cytokine signalling, only a little is known about its activation by G protein-coupled receptors. Histamine (Hu *et al*., 1999), platelet-activating factor (Kravchenko *et al*., 1995), lysophosphatidic acid (Shahrestanifar *et al*., 1999), thrombin (Mari *et al*., 1994; Rahman *et al*., 1999), bradykinin (Pan *et al*., 1996; Xie, *et al*., 2000), leukotriene B_4, fMet-Leu-Phe (Browning *et al*., 1997), substance P (Lieb *et al*., 1997), endothelin-1 (Gallois *et al*., 1998), and carbachol (Li *et al*., 1996) were reported to induce NF-κB activation, while the upstream signalling molecules required for NF-κB activation by these G protein-coupled receptors have not been clearly explored.

In J774 macrophages, UTP is known to bind the $P2Y_6$ receptors to stimulate PI breakdown, increase $[Ca^{2+}]_i$, and activate PKC isoforms (Lin & Chen, 1997; Chen & Lin, 2000). In addition, UTP elicits the potentiation of LPS-induced iNOS (Chen *et al*., 1998), COX-2 (Chen & Lin, 2000), IL-6 (Chen & Lin, 1999), and TNF-α (Chen *et al*., 2001) gene expression. Notably our previous studies further indicated that CaMK-dependent NF-κB potentiation primarily accounts for these potentiation effects of UTP (Chen *et al*., 1998; 2001; Chen & Lin, 1999; 2000). To better understand the upstream mechanism for NF-κB activation, in this study, we primarily focused on the biochemical assay of IKK, which is well known as a key element in the signal transduction cascade that leads to phosphorylation and degradation of IκBα/β, and thereby subsequently induces NF-κB nuclear translocation and transactivation. We have examined not only the effect of UTP on IKK activity but also the possible involvement of the PKC, ERK, p38 MAPK, and CaMK pathways in this respect. The present study shows the ability of the Gq-coupled receptor to activate the IKK complex, and the involvement of CaMK, PKC, and ERK in this action.

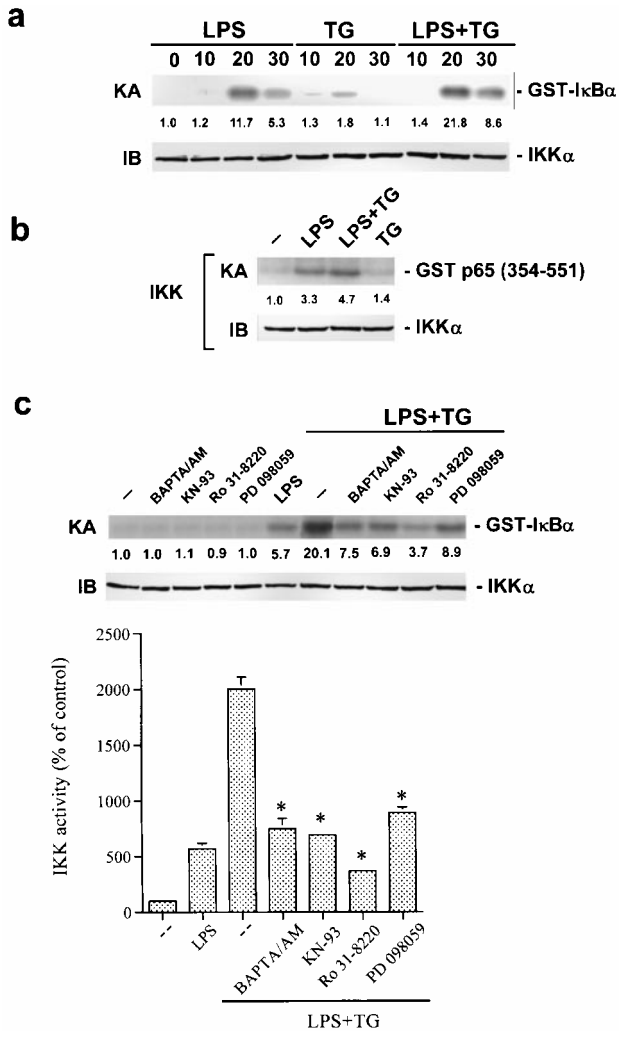

Figure 8 IKK activation by LPS and thapsigargin in peritoneal macrophages. (a) Cells were treated with 1 μg ml^{-1} LPS, 30 nM thapsigargin (TG), or both for different intervals, then cell lysates were immunoprecipitated with IKKs antibodies. Both kinase assay, as an index to GST-IκBα phosphorylation, and IKK immunoblotting were used to analyse IKK immunoprecipitates. (b) Cells were treated with LPS with or without thapsigargin for 20 min, then cell lysates were immunoprecipitated with IKK antibodies and assayed for kinase activity toward GST-p65 (354–551) phosphorylation. (c) Cells were preincubated with (μM): BAPTA/AM 30, KN-93 10, Ro 31-8220 1, or PD 098059 30, for 20 min before the addition of LPS with or without thapsigargin for another 20 min. Cell lysates were then immunoprecipitated as described. The typical traces are representative of three experiments, which are shown as mean$\pm$s.e.mean. *$P<0.05$ as compared to the control response of LPS plus thapsigargin without inhibitor pretreatment.

Several studies have demonstrated the indispensability of the generation of reactive oxygen intermediates for IKK-dependent NF-κB activation. Thus PDTC, a scavenger of reactive oxygen intermediates, has been widely used as an inhibitor of NF-κB. In our experimental system, we show that PDTC is able to inhibit LPS-induced IKK activity, IκB degradation, and NF-κB activation as well as UTP-induced IKK activity. All these results further confirm the involvement of reactive oxygen intermediates in IKK activation.

Our data provide evidence to show that the two downstream signal pathways of PI turnover, Ca^{2+} increase and PKC activation, mediate UTP activation of IKK. BAPTA/AM (an intracellular Ca^{2+} chelator), KN-93 (a selective CaMK inhibitor), Ro 31-8220 and Go 6976 (two selective PKC inhibitors) all effectively reduce the UTP response. Our previous study has shown that the increased intracellular Ca^{2+} concentration by UTP in macrophages is neutralized by BAPTA/AM (Chen *et al*., 2001). Extending this finding, we showed the ability of UTP to induce a KN-93 sensitive CaMK activity in this study. The results that IKK activation by thapsigargin in peritoneal macrophages is also attenuated by BAPTA/AM, KN-93 and Ro 31-8220 further support the key roles of Ca/CaMK and PKC in mediating IKK activation. Although thapsigargin is not a stimulator of PI turnover, it can somewhat mimic downstream signals of PI turnover *via* its inhibition of endoplasmic reticulum Ca^{2+}-ATPase, its increase in $[Ca^{2+}]_i$, and its activation of conventional PKC isoforms (Thastrup, 1990). The involvement of PKC is supported by the moderate IKK activation caused by the potent PKC activator, PMA, and the inhibitory abilities of the two selective PKC inhibitors, Ro 31-8220 and Go 6976, in reducing IKK activation. Although IKK activity is increased by UTP as shown in this study, UTP in the same cell system cannot cause any significant changes in IκB degradation or NF-κB activation, which has previously been reported (Chen *et al*., 1998). We think this inconsistency might be explained by the insufficiency of the weak and transient IKK activity to trigger IκB phosphorylation.

NF-κB activation is one of the most important and phylogenetically conserved cellular signalling transduction pathways implicated in the innate immune response and pathology of microbial pathogen LPS (Hoffmann *et al*., 1999). LPS-induced IKK activation pathways have been intensively studied, and recent genetic and biochemical experiments have highlighted the critical roles of several signal mediators in LPS signalling and have directed their sequential signalling towards NF-κB activation as the CD14/TLR $\rightarrow$ MyD88 $\rightarrow$ IRAK $\rightarrow$ TRAF6 $\rightarrow$ TAK1 $\rightarrow$ NIK $\rightarrow$ IKK complex (Kirschning *et al*., 1998). However, besides these well-characterized signal pathways, LPS is suggested to act through different signal pathways on the same integrator IKK. This suggestion is primarily based on the finding that LPS is still able to elicit NF-κB activity in MyD88$^{-/-}$ macrophages (Kawai *et al*., 1999).

Several pieces of evidence obtained from recent reports have shown the role of PKC in IKK regulation. GF 109203X, an inhibitor of conventional and novel PKC isoforms, abrogates PMA and ionomycin activation of the IKK complex in Jurkat and primary CD3$^+$ T lymphocytes (Trushin *et al*., 1999). Transient overexpression studies have shown that PKCε (Genot *et al*., 1995) and PKCα (Trushin *et al*., 1999) induce, while the dominant negative mutant of PKCζ (Lozano *et al*., 1994) impairs, NF-κB-dependent reporter gene activation. In J774 macrophages, we have shown that PKC activation is required for LPS induction of iNOS, COX-2, and IL-6 gene expressions (Chen *et al*., 1998; Chen & Lin, 1999; 2000). In the present study, the selective PKC inhibitor, Ro 31-8220, inhibits LPS-induced NF-κB activation, IκB degradation, and IKK activity, strongly

indicating that PKC is a supplementary signalling pathway for LPS-mediated NF-κB activation.

PKC is usually activated by the physiological activator, diacylglycerol, which can be generated by the enzymatic action of PI-PLC and PC-PLC. Recent studies have demonstrated that LPS-mediated diacylglycerol formation and PKC activation result from PC-PLC pathway and is inhibited by D609 (a PC-PLC inhibitor) (Tschaikowsky *et al*., 1994; Monick *et al*., 1999). In this study we found that D609 is able to inhibit LPS-induced NF-κB activation, IκB degradation, and IKK activation, but has no effects on UTP. This result supports our previous report indicating that LPS-induced NO, PGE_2, and IL-6 releases are sensitive to treatment with D609 but not with U73122 (a PI-PLC inhibitor). On the contrary, UTP potentiation of LPS action is resistant to D609 but sensitive to U73122 (Chen *et al*., 1998). Thus, although coupling to different PLC systems and catalysing different phospholipid substrates, both LPS and UTP generate diacylglycerol and activate PKC. The fact that LPS is unable to activate PI turnover or increase $[Ca^{2+}]_i$ (data not shown) together with the finding that its signalling (Figure 4a) and functional responses (Chen *et al*., 1998; Chen & Lin, 2000) are unaffected by BAPTA/AM and KN-93 all confirm its activation of PC-PLC but not PI-PLC.

Pharmacological approaches have demonstrated the requirement of p38 MAPK and/or ERK for NF-κB transactivation in response to LPS (Chen *et al*., 1999), TNF-α (Bergmann *et al*., 1998; Vanden Berghe *et al*., 1998), and IL-1 (Bergmann *et al*., 1998). Although both MAPKs have been suggested to act as necessary and cooperative machinery for NF-κB transactivation, the mechanism is not clear yet. In the present study, we found that PD 098059 (a MEK inhibitor) attenuates not only LPS-mediated IKK activation, IκB degradation, and NF-κB activation, but also UTP-induced IKK activation. These results suggest that ERK also plays a role in IKK activation in response to LPS and UTP. In this study, although the p38 MAPK inhibitor, SB 203580, inhibited NF-κB activation, it had no effects on IKK-dependent IκB degradation. Thus, as previously suggested (Bergmann *et al*., 1998), an IκB degradation-independent mechanism may contribute to p38 MAPK-linked NF-κB activation.

In addition to the NIK/IKK/IκB cascade, recent data have shown that different NF-κB-inducing stimuli such as TNF-α and IL-1 appear to cause p65 RelA phosphorylation at unique sites prior to its nuclear translocation, and to positively regulate NF-κB-dependent transcription (Vanden Berghe *et al*., 1998; Sakurai *et al*., 1999; Jefferies & O'Neill, 2000). Protein kinases such as IKK (Sakurai *et al*., 1999), PKA (Zhong *et al*., 1998), p38 MAPK (Vanden Berghe *et al*., 1998; Jefferies & O'Neill, 2000), ERK (Vanden Berghe *et al*., 1998; Jefferies & O'Neill, 2000), and PKC (Lozano *et al*., 1994) have all been shown to phosphorylate p65. In this study, we show that IKK activation by LPS and UTP can target p65 phosphorylation at Ser^{536}, and p65 phosphorylation induced by both stimuli *in vivo* might contribute to their coordinated increase in κB gene transcription.

Taken together, this report demonstrates that Gq protein-coupled receptor activation can activate IKK and potentiate LPS-stimulated NF-κB activation. Not only do PKC and ERK play regulatory roles in LPS activation of IKK/IκBs/NF-κB signalling, but the increase in intracellular calcium and subsequent CaMK activation also play a major role in UTP potentiation of the LPS response.

This work was supported by a grant from the National Science Council, Taiwan (NSC 89-2320-B002-260)

References

BALDWIN, JR. A.S. (1996). The NF-κB and IκB proteins: new discoveries and insights. *Annu. Rev. Immunol.,* **14,** 649–683.

BARNES, P.J. & KARIN, M. (1997). Nuclear factor-κ B: a pivotal transcription factor in chronic inflammatory diseases. *N. Eng. J. Med.,* **336,** 1066–1071.

BERGMANN, M., HART, L., LINDSAY, M., BARNES, P.J. & NEWTON, R. (1998). IκBα degradation and nuclear factor-κB DNA binding are insufficient for interleukin-1β and tumor necrosis factor-α-induced κB-dependent transcription. *J. Biol. Chem.,* **273,** 6607–6610.

BROWNING, D.D., PAN, Z.K., PROSSNITZ, E.R. & YE, R.D. (1997). Cell type and developmental stage-specific activation of NF-κB by fMet-Leu-Phe in myeloid cells. *J. Biol. Chem.,* **272,** 7995–8001.

CHEN, B.C. & LIN, W.W. (1999). Potentiation of lipopolysaccharide-induced IL-6 release by uridine triphosphate in macrophages: cross-interaction with cyclooxygenase-2-dependent prostaglandin E_2 production. *J. Biol. Sci.,* **6,** 425–432.

CHEN, B.C. & LIN, W.W. (2000). Pyrimidinoceptor potentiation of macrophage PGE_2 release involved in the induction of nitric oxide synthase. *Br. J. Pharmacol.,* **130,** 777–786.

CHEN, B.C., CHEN, Y.H. & LIN, W.W. (1999). Involvement of p38 mitogen-activated protein kinase in lipopolysaccharide-induced iNOS and COX-2 expression in J774 macrophages. *Immunology,* **97,** 124–129.

CHEN, B.C., CHOU, C.F. & LIN, W.W. (1998). Pyrimidinoceptor-mediated potentiation of inducible nitric-oxide synthase induction in J774 macrophages: role of intracellular calcium. *J. Biol. Chem.,* **273,** 29754–29763.

CHEN, B.C., HSIEH, S.L. & LIN, W.W. (2001). Involvement of protein kinases in the potentiation of LPS-induced inflammatory mediators formation by thapsigargin in peritoneal macrophages. *J. Leukoc. Biol.,* **69,** 280–288.

CHEN, Z., HAGLER, J., PALOMBELLA, V.J., MELANDRI, F., SCHERER, D., BALLARD, D. & MANIATIS, T. (1995). Signal-induced site-specific phosphorylation targets IκBα to the ubiquitin-proteasome pathway. *Genes. Dev.,* **9,** 1586–1597.

DIAZ MECO, M.T., DOMINGUEZ, I., SANA, L., DENT, P., LOZANO, J., MUNICIO, M.M., BERRA, E., HAY, R.T., STURGILL, T.W. & MOSCAT, J. (1994). PKCζ induces phosphorylation and inactivation of I κB-α in vitro. *EMBO J.,* **13,** 2842–2848.

DIDONATO, J.A., HAYZKAWA, M., ROTHWARF, D.M., ZANDI, E. & KARIN, M. (1997). A cytokine-responsive IκB kinase that activates the transcription factor NF-κB. *Nature,* **388,** 548–554.

DUDLEY, D.T., PANG, L., DECKER, S.J., BRIDGES, A.J. & SALTIEL, A.R. (1995). A synthetic inhibitor of the mitogen-activated protein kinase cascade. *Proc. Natl. Acad. Sci. USA.,* **92,** 7686–7689.

GALLOIS, C., HABIB, A., TAO, J., MOULIN, S., MACLOUF, J., MALLAT, A. & LOTERSZTAJN, S. (1998). Role of NF-κB in the antiproliferative effect of endothelin-1 and tumor necrosis factor-α in human hepatic stellate cells. Involvement of cyclooxygenase-2. *J. Biol. Chem.,* **273,** 23183–23190.

GENOT, E.M., PARKER, P.J. & CANTRELL, D.A. (1995). Analysis of the role of protein kinase C-α, -ε, and -ζ in T cell activation. *J. Biol. Chem.,* **270,** 9833–9839.

HANSON, P.I. & SCHULMAN, H. (1992). Inhibitory autophosphorylation of multifunctional Ca^{2+}/calmodulin-dependent protein kinase analyzed by site-directed mutagenesis. *J. Biol. Chem.,* **267,** 17216 – 17224.

HAYASHI, T., SEKINE, T. & OKAMOTO, T. (1993). Identification of a new serine kinase that activates NF-κB by direct phosphorylation. *J. Biol. Chem.,* **268,** 26790 – 26795.

HOFFMANN, J.A., KAFATOS, F.C., JANEWAY, JR., C.A. & EZEKOWITZ, R.A.B. (1999). Phylogenetic perspectives in innate immunity. *Science,* **284,** 1313 – 1318.

HU, Q., DESHPANDE, S., IRANE, K. & ZIEGELSTEIN, R.C. (1999). $[Ca^{2+}]_i$ oscillation frequency regulates agonist-stimulated NF-κB transcriptional activity. *J. Biol. Chem.,* **274,** 33995 – 33998.

ISRAEL, A. (2000). The IKK complex: an integrator of all signals that activate NF-κB? *Trends Cell. Biol.,* **10,** 129 – 133.

JEFFERIES, C.A. & O'NEILL, L.A.J. (2000). Rac1 regulates interleukin 1-induced nuclear factor κB activation in an inhibitory protein κBα-independent manner by enhancing the ability of the p65 subunit to transactivate gene expression. *J. Biol. Chem.,* **275,** 3114 – 3120.

KAWAI, T., ADACHI, O., OGAWA, T., TAKEDA, K. & AKIRA, S. (1999). Unresposiveness of MyD88-deficient mice to endotoxin. *Immunity,* **11,** 115 – 122.

KELLER, H.U. & NIGGLI, V. (1993). The PKC inhibitor Ro 31-8220 selectively suppress PMA- and diacylglycerol-induced fluid pinocytosis and polymerization in PMNs. *Biochem. Biophys. Res. Commun.,* **194,** 1111 – 1116.

KIRSCHNING, C.J., WESCHE, H., MERRILL, AYRES, T. & ROTHE, M. (1998). Human toll-like receptor 2 confers responsiveness to bacterial lipopolysaccharide. *J. Exp. Med.,* **188,** 2091 – 2097.

KRAVCHENKO, V.V., PAN, Z., HAN, J., HERBERT, J.-M., ULEVITCH, R.J. & YE, R.D. (1995). Platelet-activating factor induces NF-κB activation through a G protein-coupled pathway. *J. Biol. Chem.,* **270,** 14928 – 14934.

LEE, F.S., HAGLER, J., CHEN, Z.J. & MANNIATIS, T. (1997). Activation of the IκBα complex by MEKK1, a kinase of the JNK pathway. *Cell,* **88,** 213 – 222.

LEE, J.C., LAYTON, J.T., MCDONNELL, P.C., GALLAGHER, T.F., KUMAR, S., GREEN, D., MCNULTY, D., BLUMENTHAL, M.J., HEYS, J.R., LANDVATTER, S.W., STRICKLER, J.E., MCLANGHLIN, M.M., SIEMERNS, I.R., FISHER, S.M., LIVI, G.P., WHITE, J.R., ADAMS, J.L. & YOUNG, P.R. (1994). A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature,* **372,** 739 – 746.

LI, X., SONG, L. & JOPE, R.S. (1996). Cholinergic stimulation of AP-1 and NF-κB transcription factors is differentially sensitive to oxidative stress in SH-SY5Y neuroblastoma: relationship to phosphoinositide hydrolysis. *J. Neurosci.,* **16,** 5914 – 5922.

LIEB, K., FIEBICH, B.L., BERGER, M., BAUER, J. & SCHULZE-OSTHOFF, K. (1997). The neuropeptide substance P activates transcription factor NF-κB and κB-dependent gene expression in human astrocytoma cells. *J. Immunol.,* **159,** 4952 – 4958.

LIN, W.W. & CHEN, B.C. (1997). Involvement of protein kinase C in the UTP-mediated potentiation of cyclic AMP accumulation in mouse J774 macrophages. *Br. J. Pharmacol.,* **121,** 1749 – 1757.

LOZANO, J., BERRA, E., MUNICIO, M.M., DIAZ, MECO, M.T., DOMINGUEZ, I., SANZ, L. & MOSCAT, J. (1994). Protein kinase C zeta isoform is critical for κB-dependent promoter activation by sphingomyelinase. *J. Biol. Chem.,* **269,** 19200 – 19202.

MALININ, N.L., BOLDIN, M.P., MOVALENKO, A.V. & WALLACH, D. (1997). MAP3K-related kinase involved in NF-κB induction by TNF, CD95 and IL-1. *Nature,* **385,** 540 – 544.

MANIATIS, T. (1997). Catalysis by a multiprotein IκB kinase complex. *Science,* **278,** 818 – 819.

MARI, B., IMBERT, V., BELHACENE, N., FAR, D.F., PEYRON, J.F., POUYSSEGUR, J., VAN OBBERGHEN-SCHILLING, E., ROSSI, B. & AUBERGER, P. (1994). Thrombin and thrombin receptor agonist peptide induce early events of T cell activation and synergize with PCR cross-linking for CD69 expression and interleukin 2 production. *J. Biol. Chem.,* **269,** 8517 – 8523.

MONICK, M.M., CARTER, A.B., GUDMUNDSSON, G., MALLAMPALLI, R., POWERS, L.S. & HUNNINGHAKE, G.W. (1999). A phosphatidylcholine-specific phospholipase C regulates activation of p42/44 mitogen-activated protein kinases in lipopolysaccharide-stimulated human alveolar macrophages. *J. Immunol.,* **162,** 3005 – 3012.

MULLER-DECKER, K. (1989). Interruption of TPA-induced signals by an antiviral and antitumoral xanthate compound: inhibition of a phospholipase C-type reaction. *Biochem. Biophys. Res. Commun.,* **162,** 198 – 205.

NICK, J.A., AVDI, N.J., YOUNG, S.K., LEHMAN, L.A. & MCDONALD, P.P. (1999). Selective activation and functional significance of p38α mitogen-activated protein kinase in lipopolysaccharide-stimulated neutrophils. *J. Clin. Invest.,* **103,** 851 – 858.

PAN, Z.K., ZURAW, B.L., LUNG, C.C., PROSSNITZ, E.R., BROWNING, D.D. & YE, R.D. (1996). Bradykinin stimulates NF-κB activation and interleukin 1β gene expression in cultured human fibroblasts. *J. Clin. Invest.,* **98,** 2042 – 2049.

RAHMAN, A., ANWAR, K.N., TRUE, A.L. & MALIK, A.B. (1999). Thrombin-induced p65 homodimer binding to downstream NF-κB site of the promoter mediates endothelial ICAM-1 expression and neutrophil adhesion. *J. Immunol.,* **162,** 5466 – 5476.

SAKURAI, H., CHIBA, H., MIYOSHI, H., SUGITA, T. & TORIUMI, W. (1999). IκB kinases phosphorylate NF-κB p65 subunit on Serine 536 in the transactivation domain. *J. Biol. Chem.,* **274,** 30353 – 30356.

SHAHRESTANIFAR, M., FAN, X. & MANNING, D.R. (1999). Lysophosphatidic acid activates NF-κB in fibroblasts. *J. Biol. Chem.,* **274,** 3828 – 3833.

SUMI, M., KIUCHI, K., ISHIKAWA, T., ISHII, A., HAGIWARA, M., NAGATSU, T. & HIDAKA, G. (1991). The newly synthesized selective Ca^{2+}/calmodulin dependent protein kinase II inhibitor KN-93 reduces dopamine contents in PC12 h cells. *Biochem. Biophys. Res. Commun.,* **181,** 968 – 975.

THASTRUP, O. (1990). Role of Ca^{2+}-ATPase in regulation of cellular Ca^{2+} signalling, as studied with selective microsomal Ca^{2+}-ATPase inhibitor, thapsigargin. *Agents Actions,* **29,** 8 – 15.

THOMPSON, J.E., PHILLIPS, R.J., BROMAGE, H.E., TEMPST, P. & GHOSH, S. (1995). IκB-β regulates the persistent response in a biphasic activation of NF-κB. *Cell,* **80,** 573 – 582.

TRUSHIN, S.A., PENNINGTON, K.N., ALGECIRAS-SCHIMNICH, A. & PAYA, C.V. (1999). Protein kinase C and calcineurin synergize to activate IκB kinase and NF-κB in T lymphocytes. *J. Biol. Chem.,* **274,** 22923 – 22931.

TSCHAIKOWSKY, K., MEISNER, M., SCHONHUBER, F. & RUGHEIMER, E. (1994). Induction of nitric oxide synthase activity in phagocytic cells inhibited by tricyclodecan-9-yl-xanthogenate (D609). *Br. J. Pharmacol.,* **113,** 664 – 668.

VANDEN BERGHE, W., PLAISANCE, S., BOONE, E., DE BOSSCHER, K., SCHMITZ, M.L., FIERS, W. & HAEGEMAN, G. (1998). p38 and extracellular signal-regulated kinase mitogen-activated protein kinase pathways are required for nuclear factor-κB p65 transactivation mediated by tumor necrosis factor. *J. Biol. Chem.,* **172,** 3285 – 3290.

VELASCO, M., DIAZ-GUERRA, M.J.M., MARTIN-SANZ, P., ALVAREZ, A. & BOSCA, L. (1997). Rapid up-regulation of IκBβ and abrogation of NF-κB activity in peritoneal macrophages stimulated with lipopolysaccharide. *J. Biol. Chem.,* **272,** 23025 – 23030.

WANG, D. & BALDWIN, JR. A.S. (1998). Activation of nuclear factor-κB-dependent transcription by tumor necrosis factor-α is mediated through phosphorylation of RelA/p65 on serine 529. *J. Biol. Chem.,* **273,** 29411 – 29416.

WORONICZ, J.D., GAO, X., CAO, Z., ROTHE, M. & GEODDEL, D. (1997). IκB kinase-β: NF-κB activation and complex formation with IκB kinase-α and NIK. *Science,* **278,** 866 – 869.

XIE, P., BROWNING, D.D., HAY, N., MACKMAN, N. & YE, R.D. (2000). Activation of NF-κB by bradykinin through a Gαq- and Gβγ-dependent pathway that involves phosphoinositide 3-kinase and Akt. *J. Biol. Chem.,* **275,** 24907 – 24914.

ZHONG, H., SUYANG, H., ERDJUMENT-BROMAGE, H., TEMPST, P. & GHOSH, S. (1997). The transcriptional activity of NF-κB is regulated by the IκB-associated PKAc subunit through a cyclic AMP-independent mechanism. *Cell,* **89,** 413–424.

ZHONG, H., VOLL, R.E. & GHOSH, S. (1998). Phosphorylation of NF-κB p65 by PKA stimulates transcriptional activity by promoting a novel bivalent interaction with the coactivator CBP/p300. *Mol. Cell.,* **1,** 661–671.

(Received May 31, 2001
Accepted August 9, 2001)