British Journal of Pharmacology (1997) 120, 940–946 © 1997 Stockton Press All rights reserved 0007–1188/97 $12.00

Differential regulation by protein kinase C isoforms of nitric oxide synthase induction in RAW 264.7 macrophages and rat aortic smooth muscle cells

[1]Andrew Paul, Kirsten Doherty & Robin Plevin

Department of Physiology and Pharmacology, University of Strathclyde, 204 George Street, Glasgow, G1 1XW Scotland

1 In RAW 264.7 murine macrophages and rat aortic smooth muscle (RASM) cells lipopolysaccharide (LPS) alone or in combination with interferon γ (IFNγ) or forskolin, respectively, stimulated the expression of the 130 kDa inducible isoform of nitric oxide synthase (iNOS) in both a time- and concentration-dependent manner.

2 Incubation with the direct activator of protein kinase C (PKC), phorbol 12-myristate 13-acetate (PMA) alone, did not result in detectable iNOS expression in either cell type.

3 Chronic PMA pretreatment resulted in significant down-regulation of α, β and ε isoforms of PKC in RAW 264.7 macrophages and corresponded to a 20–30% reduction in LPS-induced iNOS expression. In contrast, IFNγ alone or in combination with LPS stimulated an approximate 20% and 50% potentiation, respectively.

4 Pre-incubation with PKC inhibitors (calphostin C and H-7) showed similar effects upon stimulated induction of iNOS.

5 In RASM cells chronic PMA pretreatment resulted in down-regulation of α and ε PKC isoforms and corresponded to potentiation of iNOS expression in response to LPS alone or in combination with forskolin.

6 Co-incubation of RASM cells in the presence of PMA, angiotensin II (AII) or foetal calf serum (FCS) resulted in the inhibition of iNOS expression in response to LPS alone or in combination with forskolin.

7 Differential sensitivity to PKC inhibitors (calphostin C and H-7) was observed in RASM cells and exhibited both negative and positive modulation of stimulated induction.

8 In addition the PKC inhibitor compound Ro-31-8220 abolished stimulated induction in both cell types in response to all treatments.

9 These results suggest that PKC activation is required for induction of the 130 kDa isoform of NOS in both RAW 264.7 macrophages and RASM cells. However, individual PKC isoforms regulate iNOS expression in both a positive and negative manner.

Keywords: Nitric oxide synthase; lipopolysaccharide; interferon γ; protein kinase C

Introduction

The generation of the short lived radical nitric oxide (NO) has been proposed to function as the mediator of a number of diverse physiological responses in the neural, vascular and immune systems (Moncada *et al*., 1991; Schmidt & Walter, 1994). These include vasodilatation, platelet aggregation and varied immunological cytostatic or cytotoxic actions (Schmidt & Walter, 1994).

The generation of NO from the oxidation of L-arginine is catalysed by the members of the diverse enzyme family of nitric oxide synthases (Nathan and Xie, 1994). These have been characterized as the constitutively expressed neural and endothelial isoforms, dependent upon calcium/calmodulin (Ca^{2+}/CaM) for activity and the Ca^{2+}/CaM-independent 130 kDa inducible macrophage-type isoform (Nathan & Xie, 1994). A common response to both cytokines and bacterial lipopolysaccharide (LPS) is an increase in apparent nitric oxide synthase (NOS) activity. This corresponds to the induction of the 130 kDa isoform of the enzyme (Mitchell *et al*., 1992) and has been described in macrophages, smooth muscle cells, renal mesangial cells and hepatocytes (Hortelano *et al*., 1992; 1993; Mitchell *et al*., 1992; Kunz *et al*., 1994).

LPS-dependent activation of myeloid cells, mediated by the membrane bound surface differentiation antigen CD14, requires the participation of a 58–60 kDa serum derived LPS-binding protein (LBP) (Schumann *et al*., 1990; Wright *et al*., 1990; Schumann, 1992). Formation of a LPS/LBP complex allows binding and activation of CD14 (Schumann, 1992). LBP effectively enhances the ability of LPS to activate myeloid cells and consequently a number of intracellular signalling pathways (Martin *et al*., 1992). In contrast, non-myeloid cells such as vascular smooth muscle cells, do not possess or express an identified receptor for LPS. Therefore, alternative receptor-independent mechanisms may be required to initiate signal transduction events leading to induction of iNOS in these cell types.

The signalling pathways utilized in the induction process have remained contentious, though it has been proposed to include activation of tyrosine kinases (Marczin *et al*., 1993; Paul *et al*., 1995), protein kinase C (PKC) (Severn *et al*., 1993; Sodhi & Kumar, 1994; Paul *et al*., 1995), phosphatidylcholine-specific phospholipase C (PtdCho-PLC) (Sands *et al*., 1994; Tschaikowsky *et al*., 1994) and sphingomyelinase pathways (Tschaikowsky *et al*., 1994). We and others have previously demonstrated that one of the early responses to LPS in macrophages is the activation of PKC (Paul *et al*., 1995) and is obligatory for the induction of NOS activity (Severn *et al*., 1993; Sodhi & Kumar, 1994; Paul *et al*., 1995). However, in smooth muscle cells the involvement of PKC in the induction process has remained unclear (Marczin *et al*., 1993). Additionally, it has been shown that direct activation of PKC by phorbol esters stimulates the expression of iNOS in rat per5-

[1] Author for correspondence.

toneal macrophages and hepatocytes (Hortelano *et al*., 1992; 1993). Therefore, in this study we have examined the role of PKC activation in the induction of NOS in both murine macrophages and rat aortic smooth muscle cells. Here we show that while PKC activation is involved in the induction process, individual subclasses of PKC isoform may play differential roles in the regulation of expression. Under certain conditions this is manifest as both inhibition and potentiation of stimulated induction.

A preliminary account of some of these findings has been presented to the British Pharmacological Society (Paul & Plevin, 1995).

Methods

Cell culture

RAW 264.7 murine macrophages were obtained from the European Cell Culture Collection. Primary cultures of vascular smooth muscle cells were established from thoracic aortae excised from Wistar rats (180–240g) and enzymically dispersed by collagenase (1 mg ml^{-1}) and elastase (0.1 mg ml^{-1}) in the presence of soya bean trypsin inhibitor (1 mg ml^{-1}). Both RAW 264.7 macrophages and RASM cells were cultured in Dulbecco's Modified Eagle's Medium containing 10% foetal calf serum (FCS), 2 mM glutamine, 250 iu ml^{-1} penicillin and 250 μg ml^{-1} streptomycin at 37°C in a humidified atmosphere of air/CO_2 (19 : 1).

Cell stimulation

RAW 264.7 macrophages and vascular smooth muscle cells were grown as monolayer cultures on 6-well plates. Macrophages were treated at near confluence and incubated for 0–12 h in full culture medium with LPS and IFNγ as appropriate before termination of reactions. Vascular smooth muscle cells were grown to near confluence and rendered quiescent by serum deprevation for 48 h. After quiescence, cells were incubated with vehicle or agonist in serum free media for 24 h before termination of reactions.

Western blotting

For the detection of iNOS and PKC isoform protein levels, in both RAW 264.7 macrophages and vascular smooth muscle cultures, cells were washed twice with ice-cold 20 mM HEPES buffer, pH 7.4 (containing in mM: NaCl 150, NaF 50, $Na_4P_2O_7$ 10, EDTA 4, EGTA 2 and Na_3VO_4 2 (HPFEV)), then solubilized in hot (70°C) SDS–PAGE sample buffer. The samples were dispersed by repeated passage through a 21G needle and then transferred to Eppendorf tubes. The samples were boiled for 5 min and then stored at −20°C until analysis. Aliquots (15–100 μg protein) were subjected to SDS–PAGE on 7.5% polyacrylamide slab gels and then blotted onto nitrocellulose. The nitrocellulose membranes were incubated for 3 h in 150 mM NaCl, 20 mM Tris, 0.02% (v/v) Tween-20 pH 7.4 (NaTT), containing 3% BSA then incubated overnight in NaTT containing 0.2% BSA and 1 μg ml^{-1} of anti-NOS or 1 μg ml^{-1} of anti-PKC antibody. Following six washes in NaTT, the membranes were incubated with anti-mouse Ig-HRP for 90 min and then washed a further 6 times in NaTT. The immunoblots were developed by enhanced chemiluminescence (ECL) detection (Amersham). Western blots were quantitated by scanning densitometry.

Assessment of NOS activity

NOS activity was estimated by a modification of the method described by Mitchell *et al*. (1992). Cells were harvested from six well plates and centrifuged for 2 min at 1000 *g*. The supernatant was removed and the pellet resuspended in 25 mM HEPES buffer pH 7.5, containing 25 mM β-mercaptoethanol, 10 μM pepstatin, 10 μM leupeptin, 1% (v/v) Triton X-100 and incubated on ice for 30 min. The cell extracts were incubated in 10 mM HEPES buffer pH 7.4 containing 1 mM NADPH, 10 μM biopterin, 10 μM FAD, 10 μM FMN, and 10 μM L-arginine/[^{3}H]-L-arginine (specific activity = 37 MBq ml^{-1}, 0.1 μCi per tube) for 15 min at 37°C. [^{3}H]-L-citrulline was separated by Dowex cation-exchange chromatography and measured by liquid scintillation counting.

Materials

Mouse monoclonal antibodies to the 130 kDa inducible macrophage-type isoform of NOS and PKC isoforms were obtained from Affiniti Research Products Ltd. (Exeter, U.K.). [^{3}H]-L-arginine (specific activity 1.3 TMBq $mmol^{-1}$) was purchased from N.E.N. (Stevenage, U.K.). PKC inhibitors, [1-(5-isoquinolinesulphonyl)-2-methylpiperazine] (H-7) and calphostin C from *Cladosporium cladosporioides* were purchased from Calbiochem Novabiochem (Nottingham, U.K.). ECL detection reagents were purchased from Amersham International (Bucks., U.K.) and all cell culture reagents were supplied by Gibco (Paisley, Scotland). 1-[3-(amidinothio)propyl-1H-indoyl-3-yl]-3-(1-methyl-1H-indoyl-3-yl)-maleimide-methanesulphate (Ro-31-8220) (a kind gift from Dr. G. Lawton, Roche, U.K). All other chemicals were of the highest commercial grade available.

Statistical analysis

Statistical analysis of the data was performed by use of an unpaired *t* test.

Results

Time- and concentration-dependent induction of 130 kDa NOS in RAW 264.7 macrophages and rat aortic smooth muscle cells

Exposure of RAW 264.7 macrophages to LPS (1 μg ml^{-1}) stimulated the increase in the expression of the 130 kDa isoform of NOS, maximal at 8–12 h (Figure 1a) and in a concentration-dependent manner (Figure 1b). Similarly time- and concentration-dependent induction was also observed in response to IFNγ alone or in combination with LPS (data not shown).

In RASM cells, time- and concentration-dependent NOS induction was observed in response to LPS (100 μg ml^{-1}) alone or in combination with forskolin (10 μM). Maximal induction was observed at 100 μg ml^{-1} over 24 h of incubation and was potentiated in the presence of forskolin (Figure 1c and d). However, forskolin alone did not induce immunologically detectable expression of iNOS.

Effect of chronic phorbol ester pretreatment upon LPS-stimulated induction of NOS activity and protein expression

The activation of PKC has been shown to be involved in the induction response stimulated by LPS and cytokines (Severn *et al*., 1992; Sodhi & Kumar, 1994; Paul *et al*., 1995). Immunoblotting of cell lysates prepared from RAW 264.7 macrophages and RASM cells with PKC isotype specific antibodies allowed the detection of individual isoforms (Figure 2).

The profile of isoform expression in both cell types was examined subsequent to chronic phorbol ester pretreatment. RAW 264.7 macrophages were exposed to 30 nM PMA for 8 h and RASM cells to 100 nM for the last 24 h of their quiescing period and the levels of isoform expression were assessed by immunoblotting (Figure 2). In the presence of PMA, significant down regulation (>90%) of α, β and ε isoform was observed in RAW 264.7 macrophages and α and ε isoforms in RASM cells. The expression of the phorbol ester insensitive ζ isoform (Ways *et al*., 1993) remained unchanged (Figure 2).

Further, incubation of each cell type in the presence of the inactive phorbol ester, 4α-phorbol, showed no effect upon cellular PKC expression (data not shown).

Under these conditions, treatment of RAW 264.7 macrophages with 30 nM PMA for 8 h before exposure to LPS and IFNγ resulted in both inhibition and potentiation of iNOS protein expression (Figure 3a). The response to LPS alone was inhibited some 20–30% (23.1±3.6%, $n=4$) suggesting that diacylglycerol (DAG) sensitive isoforms are positively involved in the activation and initiation of the induction process. In contrast, exposure to both IFNγ alone or in combination with LPS subsequent to 8 h PMA pretreatment resulted in the potentiation of iNOS expression (approximately 20 and 50%, respectively). Use of a similar pretreatment protocol before the estimation of NOS activity recovered after stimulated induction was similar to the effects upon iNOS expression. PMA pretreatment resulted in a 39±11.3% reduction, a 20±6.3% and 66±4.3% increase in LPS-, IFNγ- and LPS and IFNγ-induced NOS activity respectively (Figure 4).

Under similar conditions, down regulation of PKC α and ε isoforms in RASM cells resulted in the potentiation of NOS expression in response to both LPS alone or in combination with forskolin (Figure 3b).

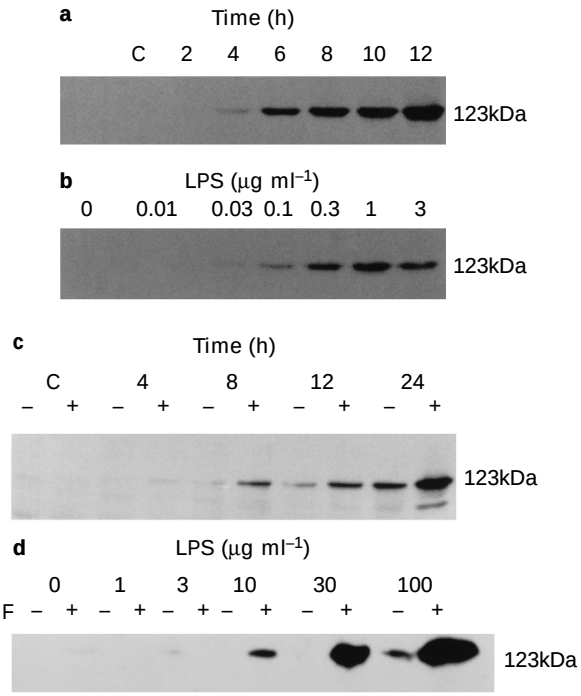

Figure 1 Time- and concentration-dependent induction of NOS in LPS-stimulated RAW 264.7 macrophages and vascular smooth muscle cells. In (a) and (b) RAW 264.7 macrophages were treated with vehicle control (C) or 1 μg ml^{-1} LPS for the times indicated or with increasing concentrations of LPS for 12 h as indicated. In (c) and (d) quiescent RASM cells were incubated with 100 μg ml^{-1} LPS alone (−) or 10 μg ml^{-1} LPS + 10 μM forskolin (F)(+) for the times indicated or with increasing concentrations of LPS alone (−) or in combination with 10 μM forskolin (F)(+) for 24 h as indicated. Subsequent to termination of incubations, prepared cell lysates were immunoblotted for expression of iNOS as outlined in the Methods section. Each experiment is representative of at least three others.

Effect of acute PKC activation upon LPS-stimulated induction of iNOS protein expression

Upon co-incubation of RASM cells with PMA, marked inhibition of iNOS expression in response to LPS alone or in

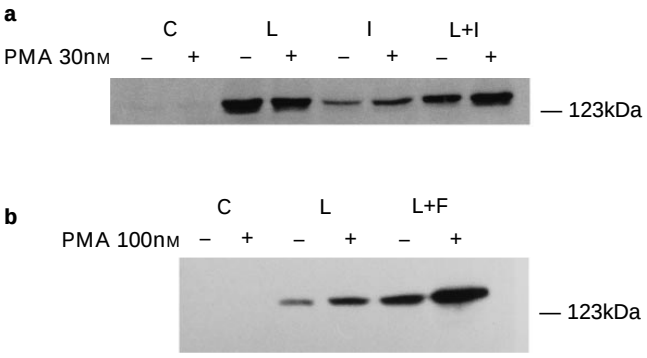

Figure 3 Effect of chronic phorbol ester pretreatment upon stimulated iNOS expression in RAW 264.7 macrophages and vascular smooth muscle cells. In (a), RAW 264.7 macrophages were pretreated in the presence (+) or absence (−) of 30 nM PMA (PMA) for 8 h before incubation with vehicle (C), LPS 1 μg ml^{-1} (L), IFNγ 30 iu ml^{-1} (I) or LPS 1 ng ml^{-1} + IFNγ 10 iu ml^{-1} (L + I) as indicated for 12 h. In (b) RASM cells were pretreated in the presence (+) or absence (−) of 100 nM PMA (PMA) during the last 24 h of the 48 h quiescing period before incubation with vehicle (C), LPS 100 μg ml^{-1} (L) alone or LPS 10 μg ml^{-1} + forskolin 10 μM (L + F). Subsequent to termination of incubations, prepared samples were immunoblotted for expression of iNOS as outlined in the Methods section. Each experiment is representative of three others.

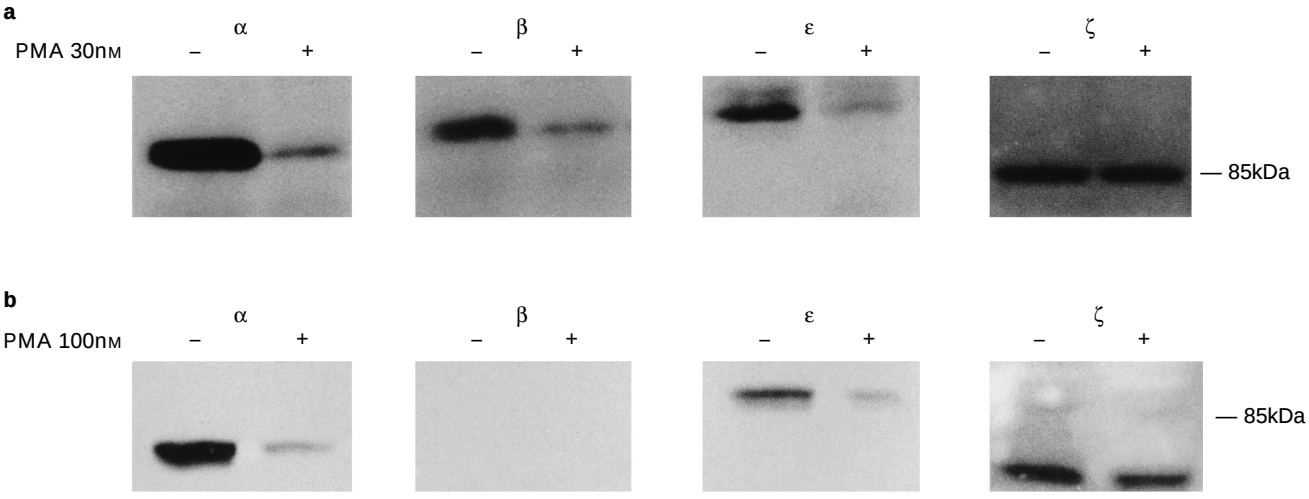

Figure 2 Expression of PKC isoforms in RAW 264.7 macrophages and vascular smooth muscle cells following chronic phorbol ester pretreatment. RAW 264.7 macrophages (a) and RASM cells (b) were pretreated in the presence (+) or absence (−) of 30 or 100 nM phorbol ester (PMA) as indicated for 8 or 24 h respectively. Subsequent to preparation of cell lysates, the expression of individual PKC isoforms was assessed by immunoblotting with isotype specific antibodies as outlined in the Methods section. Each experiment is representative of three others.

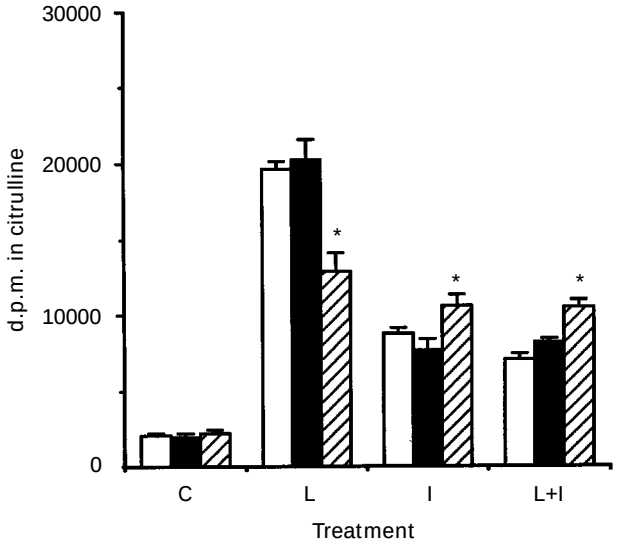

Figure 4 Effect of chronic phorbol ester pretreatment upon stimulated induction of NOS activity in RAW 264.7 macrophages. RAW 264.7 macrophages were pretreated in the absence (open columns) or presence of 4α-PMA 30 nM (solid columns) or PMA 30 nM (hatched columns) for 8 h before incubation with vehicle (C), LPS 1 μg ml^{-1} (L), IFNγ 30 iu ml^{-1} (I) or LPS 1 ng ml^{-1} + IFNγ 10 iu ml^{-1} (L + I) for 12 h. NOS activity recovered was assessed as described in the Methods section. Each value represents the mean ± s.e.mean of three experiments performed in duplicate (*significantly different from control, $P<0.05$).

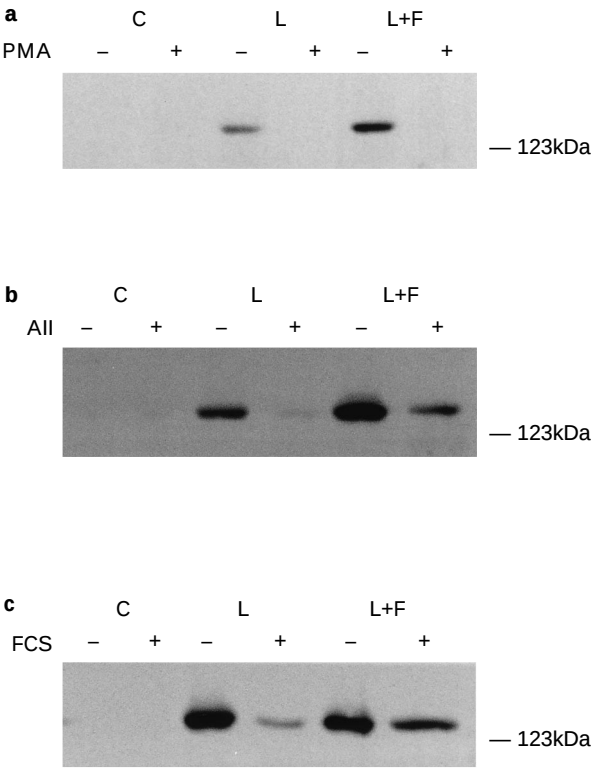

Figure 5 Effect of PMA, angiotensin II (AII) and serum co-incubation upon LPS-stimulated NOS induction in vascular smooth muscle cells. Quiescent RASM cells were incubated with vehicle (C), LPS 100 μg ml^{-1} (L), LPS 10 μg ml^{-1} + forskolin 10 μM (L + F) in the presence (+) or absence (−) of PMA 100 nM (PMA) (a), AII 100 nM (b) or 10% (v/v) foetal calf serum (FCS) (c) as indicated for 24 h. Subsequent to termination of co-incubations, prepared samples were immunoblotted for expression of iNOS as outlined in the Methods section. Each experiment is representative of three others.

combination with forskolin was observed over the 24 h period (Figure 5a). Similar inhibition of LPS-stimulated induction over 24 h was also observed upon co-incubation with angiotensin II (AII) or serum, known activators of PKC (Figure 5b and c).

Effect of PKC-inhibitor compounds Ro-31-8220, calphostin C and H-7 upon stimulated NOS induction

The involvement of PKC activity in the induction process in both cell types was further examined by the use of PKC inhibitor compounds, namely Ro-31-8220 (Davis *et al*., 1989), calphostin C (Kobayashi *et al*., 1989) and H-7 (Hidaka & Hagiwara, 1987). Preincubation of both cell types with a maximal concentration of Ro-31-8220 resulted in abrogation of induced NOS expression in response to all treatments (Figure 6). As Ro-31-8220 acts as a competitive inhibitor at the ATP binding site of PKC isoforms, its action may represent the inhibition of a number of PKC isoforms including those insensitive to phorbol ester pretreatment. To address this problem further, and assess the role of DAG-sensitive PKC isoforms in these signalling events, the sensitivity of stimulated NOS induction to calphostin C, a competitive inhibitor, active at the DAG binding site of DAG-sensitive PKC isoforms, was assessed.

In RAW 264.7 macrophages pretreatment with calphostin C resulted in the inhibition of LPS-stimulated induction. However, in response to INFγ alone or in combination with LPS the expression of iNOS was potentiated (Figure 7a). This profile of both positive and negative modulation of iNOS expression in the macrophages was similar to the effects of chronic phorbol ester pretreatment and was comparable to the modulation of iNOS activity able to be recovered subsequent to stimulated induction (Table 1). Pretreatment of RASM cells with Calphostin C in a similar manner, resulted in inhibition and potentiation of stimulated NOS induction in response to LPS alone and in combination with forskolin respectively (Figure 7b).

H-7 represents another PKC inhibitor active at the ATP binding site of PKC isoforms. This compound although active as a competitive inhibitor in this manner has been described as a more selective inhibitor of Ca^{2+}-dependent PKC isoforms (McGlynn *et al*., 1992). H-7 pretreatment in RAW 264.7 macrophages resulted in partial inhibition of LPS-stimulated NOS induction yet potentiated the response to IFNγ alone or in combination with LPS (Figure 8a). This was comparable to that observed for both PMA and calphostin C pretreatment.

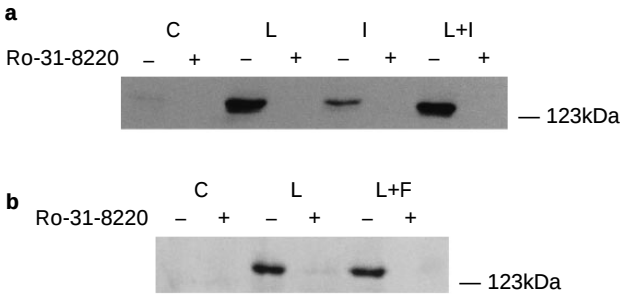

Figure 6 Effect of Ro-31-8220 upon stimulated NOS induction in RAW 264.7 macrophages and vascular smooth muscle cells. In (a), RAW 264.7 macrophages were pretreated in the presence (+) or absence (−) of Ro-31-8220 10 μM (Ro-31-8220) for 30 min before incubation with vehicle (C), LPS 1 μg ml^{-1} (L), IFNγ 30 iu ml^{-1} (I) or LPS 1 ng ml^{-1} + IFNγ 10 iu ml^{-1} (L + I) as indicated for 12 h. In (b) RASM cells were pretreated in the presence (+) or absence (−) of 10 μM Ro-31-8220 (Ro-31-8220) for 30 min before incubation with vehicle (C), LPS 100 μg ml^{-1} (L) alone or LPS 10 μg ml^{-1} + forskolin 10 μM (L + F) as indicated for 24 h. Subsequent to termination of incubations, prepared samples were immunoblotted for expression of iNOS as outlined in the Methods section. Each experiment is representative of three others.

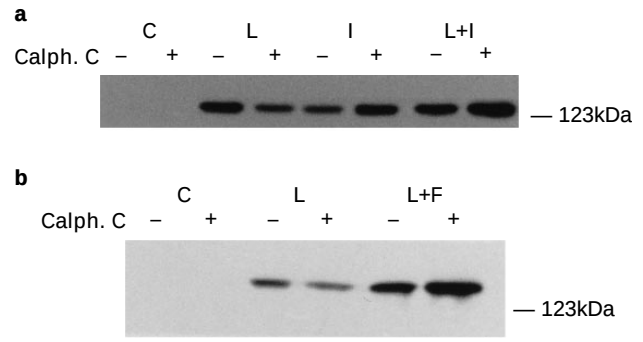

Figure 7 Effect of calphostin C on stimulated NOS induction in RAW 264.7 macrophages and vascular smooth muscle cells. In (a), RAW 264.7 macrophages were pretreated in the presence (+) or absence (−) of calphostin C 1 μM (Calph. C) for 1 h before incubation with vehicle (C), LPS 1 μg ml^{-1} (L), IFNγ 30 iu ml^{-1} (I) or LPS 1 ng ml^{-1} + IFNγ 10 iu ml^{-1} (L + I) as indicated for 12 h. In (b), RASM cells were pretreated in the presence (+) or absence (−) of calphostin C 1 μM (Calph. C) for 1 h before incubation with vehicle (C), LPS 100 μg ml^{-1} (L) alone or LPS 10 μg ml^{-1} + forskolin 10 μM (L + F) as indicated for 24 h. Subsequent to termination of incubations, prepared samples were immunoblotted for expression of iNOS as outlined in the Methods section. Each experiment is representative of three others.

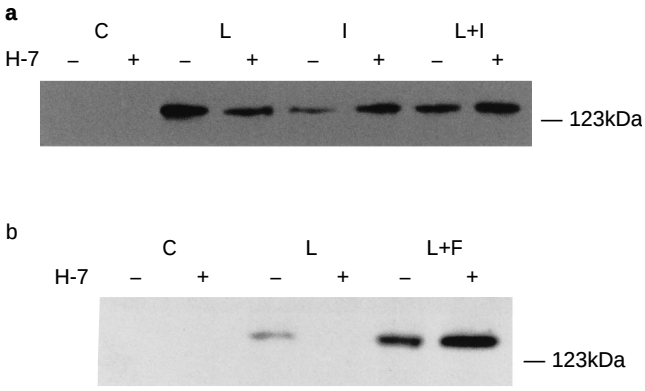

Figure 8 Effect of H-7 upon stimulated NOS induction in RAW 264.7 macrophages and vascular smooth muscle cells. In (a), RAW 264.7 macrophages were pretreated in the presence (+) or absence (−) of H-7 20 μM (H-7) for 30 min before incubation with vehicle (C), LPS 1 μg ml^{-1} (L), IFNγ 30 iu ml^{-1} (I) or LPS 1 ng ml^{-1} + IFNγ 10 iu ml^{-1} (L + I) as indicated for 12 h. In (b) RASM cells were pretreated in the presence (+) or absence (−) of 20 μM H-7 (H-7) for 30 min before incubation with vehicle (C), LPS 100 μg ml^{-1} (L) alone or LPS 10 μg ml^{-1} + forskolin 10 μM (L + F) as indicated for 24 h. Subsequent to termination of incubations, prepared samples were immunoblotted for expression of iNOS levels as outlined in the Methods section. Each experiment is representative of three others.

Table 1 Effect of PKC inhibition upon the induction of NOS activity in RAW 264.7 murine macrophages

	NOS activity (% of maximum activity)			
Pretreatment	*Control*	*LPS*	*INFγ*	*LPS + IFNγ*
PMA	ND	39 ± 11.3%	120 ± 6.3%	166 ± 4.3%
Ro-31-8220	ND	ND	ND	ND
Calphostin C	ND	68 ± 3.2%	142 ± 6.9%	134 ± 10.7%
H-7	ND	86 ± 1.7%	145 ± 15.3%	143 ± 1.1%

Cells were pretreated with either PMA (30 nM, 8 h), Ro-31-8220 (10 μM, 30 min), calphostin C (1 μM, 1 h) or H-7 (20 μM, 30 min) before incubation with vehicle, LPS 1 μg ml^{-1}, IFNγ 30 iu ml^{-1} LPS, 1 ng ml^{-1} IFNγ 10 iu ml^{-1} for 12 h and then assayed for iNOS activity as outlined in the methods section. Each value is expressed as a percentage of total activity recovered after stimulated induction calculated as the mean ± s.e.mean from 3 experiments performed in duplicate (ND-none detected).

Additionally, the recovery of NOS activity subsequent to identical inhibitor pretreatment and stimulation of RAW 264.7 macrophages (Table 1) was similar to the observed modulation of iNOS protein expression. Pretreatment of RASM cells with H-7 resulted in complete inhibition of iNOS expression in response to LPS alone. However, H-7 potentiated induction in response to LPS and forskolin added in combination (Figure 8b). This was similar to calphostin C pretreatment and again highlighted both positive and negative modulation of stimulated iNOS induction.

Discussion

In RAW 264.7 macrophages, LPS and IFNγ alone or in combination stimulated the expression of the 130 kDa isoform of NOS in both a time- and concentration-dependent manner. LPS also induced the expression of a similar immunologically detectable protein in rat aortic smooth muscle cells. This was synergistically enhanced in the presence of forskolin, a direct activator of adenylate cyclase. These results are consistent with studies conducted in the same or other cell types stimulated with LPS, cytokines and agents that raise intracellular cyclicAMP concentration (Wright *et al*., 1990; Hortelano *et al*., 1992; 1993; Mitchell *et al*., 1992; Koide *et al*., 1993; Kunz *et al*., 1994).

Evidence that PKC is involved in mediating stimulated NOS induction

We have previously found that LPS stimulates PKC activation and NOS induction in RAW 264.7 macrophages (Paul *et al*., 1995). PKC activation was apparently obligatory for induction and was consistent with similar studies conducted in murine peritoneal macrophages and the J744.2 murine macrophage cell line (Severn *et al*., 1993; Sodhi & Kumar, 1994). The PKC enzyme family now consists of at least eleven isoforms differing in structure, lipid activation, phorbol ester sensitivity, cellular distribution and tissue expression (Hug & Sarre, 1993). Immunoblot analysis of both cell types revealed the expression of classical, novel and atypical isoforms; α, β, ε and ζ in both RAW 264.7 macrophages and RASM cells respectively. The observed significant loss of α, β and ε isoforms in RAW 264.7 macrophages and parallel 20–30% reduction in stimulated iNOS induction following chronic phorbol ester pretreatment may correspond to DAG-sensitive PKC isoforms being required for LPS-stimulated induction. However, complete inhibition of LPS-stimulated induction was not observed. Therefore, involvement of DAG-insensitive PKC isoforms or distinct PKC-independent mechanisms may be relevant to stimulated induction.

Evidence that PKC is involved in inhibiting stimulated NOS induction

In RAW 264.7 macrophages, NOS induction in response to IFNγ alone or in combination with LPS was potentiated following chronic phorbol ester pretreatment (Figure 3a) suggesting that PMA-sensitive isoforms (α, β or ε) may play a role in the inhibition of NOS induction. In a similar manner, the significant potentiation of stimulated NOS induction in RASM cells pretreated with phorbol ester (Figure 3b) further suggests that PMA-sensitive PKC isoforms (α and ε) function in such a negative regulatory role. This is consistent with the observed inhibitory effect of PKCε upon IL-1β-stimulated NOS induction in rat renal mesangial cells, identified by differential PKC isoform down-regulation (Muhl & Pfielschifter, 1994). As such differential, phorbol ester-mediated down regulation protocols could not be utilized for cellular depletion of individual PKC isoforms in either cell type examined here. Hence, the identity of the down-regulated PKC isoform(s), in both RAW 264.7 macrophages (α, β, ε) and RASM (α, ε), responsible for negative regulation of stimulated induction

remains unclear. Therefore, it remains to be confirmed whether PKCε-mediated tonic inhibition of stimulated induction, as observed in rat mesangial cells (Muhl & Pfielschifter, 1994), represents a regulatory mechanism common to a number of cell types.

Additional support for negative regulation of stimulated induction by PKC isoforms was further illustrated by the observed inhibition of iNOS expression in response to LPS alone or in combination with forskolin upon co-incubation with PMA, AII or serum (Figure 5). Co-incubation with these agents routinely resulted in a marked reduction or complete inhibition of stimulated induction. Therefore, direct activation of PKC at the onset of LPS-stimulated induction highlights the negative modulatory regulation of iNOS expression by DAG-sensitive PKC isoforms in RASM cells.

Effect of PKC-inhibitor compounds

Following pretreatment of RAW 264.7 macrophages with either calphostin C or H-7, it was observed that LPS-stimulated induction of iNOS was inhibited whilst that in response to IFNγ alone or in combination with LPS was potentiated (Figures 7a and 8a). This occurred in a manner similar to that observed following chronic PMA pretreatment and may suggest that each of these pretreatment regimes results in the inhibition of the activation of common PKC isoforms, namely PMA/DAG sensitive ones. Similar experiments with calphostin C or H-7, conducted in RASM cells, resulted in inhibition of stimulated NOS induction in response to LPS alone and potentiation in response to LPS and forskolin added in combination (Figures 7b and 8b). Therefore, the similar differential actions of these two inhibitor compounds further highlighted both positive and negative PKC-mediated regulation of the induction process in RASM cells.

However, it must be noted, despite the fact that the profile of H-7-mediated inhibition of stimulated iNOS induction strongly mirrored the observed effects of other interventions utilised to either inhibit or modify PKC activity, these effects may not necessarily be confined to inhibition of PKC alone. H-7 is known additionally to inhibit both adenosine 3′:5′-cyclic monophosphate (cyclic AMP) and guanosine 3′:5′-cyclic monophosphate (cyclic GMP)-dependent kinases (Quick *et al*., 1992) and therefore the possibility that these effects may be ascribed to other cellular kinases distinct from PKC cannot be discounted. This remains to be examined further.

Complete abrogation of stimulated NOS induction in both cell types following pretreatment with Ro-31-8220 (Figure 6), irrespective of stimulus, may suggest that PKC activation is indeed obligatory for NOS induction. Moreover, as Ro-31-8220 is active at the ATP-binding site of PKC isoforms, complete abrogation of stimulated induction may additionally implicate an atypical PKC isoform in this role. This may represent PKC ζ, expressed in both cell types (Figure 2). However, the nature and specificity of inhibition by Ro-31-8220 remains questionable. The full isoform selectivity of Ro-31-8220 is not known though it is apparent that a structurally related bisindoylmaleimide (GF109203X/Go 6850) shows high selectivity for PKCα over PKCζ (Martiny-Baron *et al*., 1993). Therefore, Ro-31-8220, like calphostin C, may also show selectivity for PKCα over PKCζ and PKCα may be the main isoform involved in the regulation of stimulated iNOS induction.

Overall, the relative positive and negative contributions of individual PKC isoforms to the regulation of stimulated NOS induction remains to be clearly elucidated in both RAW 264.7 macrophages and vascular smooth muscle cells. It is apparent that particular isoforms are not involved *per se* in the signalling events that mediate the induction process directly but in the inhibition of stimulated expression of iNOS.

Involvement of both PKC-dependent and PKC-independent events in stimulated NOS induction

Involvement of PKC isoforms in both positive and negative modulation of NOS induction would predict receptor-stimulated diacylglycerol production as a requisite to PKC activation. This was not addressed in either RAW 264.7 macrophages or RASM cells. However, other studies in J744.2 macrophages have illustrated the potential involvement of a PtdCho-PLC pathway as a regulatory mechanism of stimulated induction (Sands *et al*., 1994; Tschaikowsky *et al*., 1994); diacylglycerol derived from such a hydrolytic event may fulfil this role. In preliminary studies, addition of exogenous bacterial PtdCho-PLC from *Bacillus cereus* to RAW 264.7 macrophages resulted in both time- and concentration-dependent induction of NOS protein expression (A.P. & R.P., unpublished results) and may further suggest that this phospholipid signalling pathway is indeed proximal to transcription of the iNOS gene.

The mechanism by which LPS initiates iNOS induction in RASMC also remains unclear as no defined receptor molecule for LPS is expressed by these cells. Therefore, receptor-independent activation of PKC isoforms and/or other signal transduction cascades that ultimately result in NOS induction in these cells also remain to be clearly elucidated. Previously, direct activation of Ca^{2+}-dependent PKC isoforms *in vitro* by LPS has been demonstrated (Wightman & Raetz, 1984) and may represent one possible component of the induction process. However, another possible mechanism may be the proposed action of LPS as a mimetic for membrane-derived ceramides (Joseph *et al*., 1994). In this role LPS has been suggested to function as an alternative activator of ceramide-activated kinases and phosphatase. These transduction pathways have been proposed to function upstream of the activation of the transcription factor NFκB (Schutze *et al*., 1993), previously implicated in mediating cytokine-stimulated iNOS expression in rat alveolar macrophages (Sherman *et al*., 1993) and murine bone marrow-derived macrophages (Mulsch *et al*., 1993). Therefore, LPS-mediated activation of an analogous sphingomyelinase/ceramide pathway may be apparent. However, the contribution of direct ceramide-stimulated NFκB activation to the induction process remains to be clarified. Further, addition of cell permeable ceramide analogues n-acetylsphingosine (C2) and n-hexanoylsphingosine (C6) (nM–μM) alone or in combination with PMA do not stimulate iNOS expression in RAW 264.7 macrophages (A.P & R.P., unpublished results).

Therefore, PKC activation contributes to the regulation of the stimulated induction of the 130 kDa isoform of NOS in response to LPS. In RAW 264.7 macrophages, it would appear that DAG-sensitive isoforms play a minor role in the activation of induction in response to LPS, though these isoforms also contribute to the negative regulation of IFNγ-stimulated induction. However, in RASM, DAG-sensitive PKC isoforms function primarily as negative regulators of LPS-stimulated iNOS expression. Complete inhibition of stimulated induction by Ro-31-8220 may implicate that DAG-insensitive PKC isoforms additionally contribute to the induction process, though LPS or cytokine stimulated activation of PKCζ remains to be examined. Thus, direct elucidation of the individual PKC isoforms activated by LPS and IFNγ may allow assessment of their relative contributions to the induction process in both cell types.

We would like to thank Dr I. Varndell for the kind gift of anti-iNOS and PKC antibodies used in the early part of this study, Dr P. Parker for the anti-PKCζ antibody, Dr G. Lawton (Roche) for the PKC inhibitor Ro-31-8220 used in this study and K. Malarkey for assistance in preparation of RASM PKC immunoblots. This work was funded by a Wellcome Trust grant to R.P.

References

DAVIS, P., HILL, C.H., KEECH, E., LAWSON, G., NIXON, T.S., SEDGEWICK, A.D., WADSWORTH, S.E., WESTMACOTT, D. & WILKINSON, S.E. (1989). Potent selective inhibitors of protein kinase C. *FEBS Lett*., **259,** 61–63.

HIDAKA, H. & HAGIWARA, M. (1987). Pharmacology of the isoquinoline sulfonamide protein kinase C inhibitors. *Trends Pharmacol. Sci*., **8,** 162–164.

HORTELANO, S., GENARO, A.M. & BOSCA, L. (1992). Phorbol esters induce nitric oxide synthase activity in rat hepatocytes. *J. Biol. Chem*., **267,** 24937–24940.

HORTELANO, S., GENARO, A.M. & BOSCA, L. (1993). Phorbol esters induce nitric oxide synthase and increase arginine influx in cultured peritoneal macrophages. *FEBS Lett*., **320,** 135–139.

HUG, H. & SARRE, T.F. (1993). Protein kinase C isoenzymes: divergence in signal transduction. *Biochem. J*., **291,** 329–343.

JOSEPH, C.K., WRIGHT, S.D., BORNMANN, W.G., RANDOLPH, J.T., KUMAR, E.R., BITTMAN, R., LIU, J. & KOLESNICK, R.N. (1994). Bacterial lipopolysaccharide has structural similarity to ceramide and stimulates ceramide-activated protein kinase in myeloid cells. *J. Biol. Chem*., **269,** 17606–17610.

KOBAYASHI, E., NAKANO, H., MORIMOTO, M. & TAMAOKI, T. (1989). Calphostin C (UCN-1028C): A novel microbial compound, is a highly potent and specific inhibitor of protein kinase C. *Biochem. Biophys. Res. Commun*., **159,** 548–553.

KOIDE, M., KAWAHARA, Y., NAKAYAMA, I, TSUDA, T. & YOKAYAMA, M. (1993). Cyclic AMP-elevating agents induce an inducible type of nitric oxide synthase in cultured vascular smooth muscle cells. *J. Biol. Chem*., **268,** 24959–24966.

KUNZ, D., MUHL, H., WALKER, G. & PFIELSCHIFTER, J. (1994). Two distinct signalling pathways trigger the expression of inducible nitric oxide synthase in rat renal mesangial cells. *Proc. Natl. Acad. Sci. U.S.A*., **91,** 5387–5391.

MARCZIN, N., PAPAPETROPOULOUS, A. & CATRAVAS, J.D. (1993). Tyrosine kinase inhibitors suppress endotoxin and Il-1β-induced NO synthase in aortic smooth muscle cells. *Am. J. Physiol*., **265,** H1014–1018.

MARTIN, T.R., MATHISON, J.C., TOBIAS, P.S., LETURCQ, D.J., MORIARTY, A.M., MAUNDER, R.J. & ULEVITCH, R.J. (1992). Lipopolysaccharide binding protein enhances the responsiveness for alveolar macrophages to bacterial lipopolysaccharide. *J. Clin. Invest*., **90,** 2209–2219.

MARTINY-BARON, G., KAZANIETZ, M.G., MISCHAK, H., BLUMBERG, P.M., KOCHS, G., HUG, H., MARME, D. & SCHACHTELE, C. (1993). Selective inhibition of protein kinase C isozymes by the indolocarbazole Go 6976. *J. Biol. Chem*., **268,** 9194–9197.

MCGLYNN, E., LIEBETANZ, J., REUTENER, S., WOOD, J., LYDON, J.B., HOFSTETTER, H., VANEK, M., MEYER, T. & FABBRO, D. (1992). Expression and partial characterisation of rat protein kinase C-δ and protein kinase C-ζ in insect cells using recombinant baculovirus. *J. Cell. Biochem*., **49,** 239–250.

MITCHELL, J.A., KOLHAAS, K.L., MATSUMOTO, T., POLLOCK, J.S., FORSTERMANN, U., WARNER, T.D., SCHMIDT, H.H.W. & MURAD, F. (1992). Induction of NADPH-dependent diaphorase and nitric oxide synthase activity in aortic smooth muscle and cultured macrophages. *Mol. Pharmacol*., **41,** 1163–1168.

MONCADA, S.R., PALMER, M.J. & HIGGS, E.A. (1991). Nitric oxide: physiology, pathophysiology and pharmacology. *Pharmacol. Rev*., **43,** 109–142.

MUHL, H. & PFEILSCHIFTER, J. (1994). Possible role of protein kinase C-ε isoenzyme in inhibition of interleukin 1β induction of nitric oxide synthase in rat renal mesangial cells. *Biochem. J*., **303,** 607–612.

MULSCH, A., SCHRAY-UTZ, B., MORDINTVEC, P.I., HAUSCHILDT, S. & BUSSE, R. (1993). Diethyldithiocarbamate inhibits induction of macrophage NO synthase. *FEBS Lett*., **321,** 215–218.

NATHAN, C. & XIE, Q.-W. (1994). Nitric oxide synthase: roles, tolls and controls. *Cell*, **78,** 915–918.

PAUL, A., PENDREIGH, R.H. & PLEVIN, R. (1995). Protein kinase C and tyrosine kinase pathways regulate lipopolysaccharide-induced nitric oxide synthase activity in RAW 264.7 macrophages. *Br. J. Pharmacol*., **114,** 482–488.

PAUL, A. & PLEVIN, R. (1995). Protein kinase C regulation of lipopolysaccharide-induced nitric oxide synthase activity in RAW 264.7 murine macrophages. *Br. J. Pharmacol*., **115,** 10P.

QUICK, J., WARE, J.A. & DRIEDGER, P.E. (1992). The structure and biological activity of the widely used protein kinase inhibitor, H7, differ depending on the commercial source. *Biochem. Biophys. Res. Commun*., **187,** 657–663.

SANDS, W.A., CLARK, J.S. & LIEW, F.Y. (1994). The role of a phosphatidylcholine-specific phospholipase C in the production of diacylglycerol for nitric oxide synthesis in macrophages activated by IFNγ and LPS. *Biochem. Biophys. Res. Commun*., **199,** 461–466.

SCHMIDT, H.H.H.W. & WALTER, U. (1994). NO at work. *Cell*, **78,** 919–925.

SCHUMANN, R.R. (1992). Function of lipolysaccharide (LPS)-binding protein (LBP) and CD14, the receptor for LPS/LBP complexes: a short review. *Res. Immunol*., **143,** 11–15.

SCHUMANN, R.R., LEONG, S.R., FLAGGS, G.W., GRAY, P.W., WRIGHT, S.D., MATHISON, J.C., TOBIAS, P.S. & ULEVITCH, R.J. (1990). Structure and function of lipopolysaccharide binding protein. *Science*, **249,** 1429–1431.

SCHUTZE, S., POTTHOFF, K., MACHLEIDT, T., BERKOVIC, D., WEIGMANN, K. & KRONKE, M. (1993). TNF activates NFkB by phosphatidylcholine-specific phospholipase C-induced 'acidic' sphingomyelin breakdown. *Cell*, **71,** 765–776.

SEVERN, A., WAKELAM, M.J.O. & LIEW, F.Y. (1992). The role of protein kinase C in the induction of nitric oxide synthase by murine macrophages. *Biochem. Biophys. Res. Commun*., **188,** 997–1002.

SHERMAN, M.P., AEBERHARD, E.E., WONG, V.Z., GRISCAVAGE, J.M. & IGNARRO, L.J. (1993). Pyrrolidine dithiocarbamate inhibits induction of nitric oxide synthase activity in rat alveolar macrophages. *Biochem. Biophys. Res. Commun*., **191,** 1301–1308.

SODHI, A. & KUMAR, R. (1994). Production of nitric oxide and its regulation in murine peritoneal macrophages treated with cisplatin and lipopolysaccharide. *Int. J. Immunopathol. Pharmacol*., **7,** 65–77.

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

WAYS, D.K., COOK, P.P., WEBSTER, C. & PARKER, P.J. (1993). Effects of phorbol esters on protein kinase C-ζ. *J. Biol. Chem*., **267,** 4799–4805.

WIGHTMAN, P.D. & RAETZ, C.R.H. (1984). The activation of protein kinase C by biologically active lipid moieties of lipopolysaccharide. *J. Biol. Chem*., **259,** 10048–10052.

WRIGHT, S.D., RAMOS, R.A., TOBIAS, P.S., ULEVITCH, R.J. & MATHISON, J.C. (1990). CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science*, **249,** 1431–1433.

(Received September 25, 1996
Accepted November 15, 1996)