The knowledge of the mechanisms involved in the control of NO synthesis by different cell types is a subject of current interest because of the multiple physiological and pathological effects elicited by this molecule on different cell targets (1–3). At least three distinct but functionally and structurally related genes involved in the biosynthesis of NO have been identified. Transfection of RAW 264.7 cells with plasmids harboring protein kinase C (PKC)-epsilon type isotype but not with PKC-alpha, -beta-1, -delta, or constitutively active -alpha and -beta-1 isotypes resulted in the expression of nitric oxide synthase type II (iNOS), as reflected by the synthesis of nitric oxide measured in the culture medium of transfected cells. Cotransfection of RAW 264.7 cells with the -1592 to +121-base pair promoter region of the murine iNOS gene and PKC isotypes specifically induced the transactivation of this promoter in the case of the plasmids containing the PKC-epsilon isotype. The mechanism by which PKC-epsilon induced iNOS expression involved the activation of nuclear factor binding to kappaB (NF-kappaB) as deduced by the suppressive effect of pyrrolidine dithiocarbamate on nitric oxide synthesis, an inhibitor of NF-kappaB activation, and by the activation of kappaB sites in cells transfected with a vector containing a kappaB motif linked to a chloramphenicol acetyltransferase reporter gene. These results suggest that PKC-epsilon can regulate a pathway that promotes iNOS expression in macrophages in response to phorbol ester activation.

Stimulation of the murine macrophage RAW 264.7 cell line with phorbol esters fails to promote nitric oxide synthesis as occurs in rat hepatocytes or peritoneal macrophages. Transfection of RAW 264.7 cells with plasmids harboring protein kinase C (PKC) -epsilon isotype but not with PKC-alpha, -beta-1, -delta, or constitutively active -alpha and -beta-1 isotypes resulted in the expression of nitric oxide synthase type II (iNOS), as reflected by the synthesis of nitric oxide measured in the culture medium of transfected cells. Cotransfection of RAW 264.7 cells with the -1592 to +121-base pair promoter region of the murine iNOS gene and PKC isotypes specifically induced the transactivation of this promoter in the case of the plasmids containing the PKC-epsilon isotype. The mechanism by which PKC-epsilon induced iNOS expression involved the activation of nuclear factor binding to kappaB (NF-kappaB) as deduced by the suppressive effect of pyrrolidine dithiocarbamate on nitric oxide synthesis, an inhibitor of NF-kappaB activation, and by the activation of kappaB sites in cells transfected with a vector containing a kappaB motif linked to a chloramphenicol acetyltransferase reporter gene. These results suggest that PKC-epsilon can regulate a pathway that promotes iNOS expression in macrophages in response to phorbol ester activation.

The knowledge of the mechanisms involved in the control of NO synthesis by different cell types is a subject of current interest because of the multiple physiological and pathological effects elicited by this molecule on different cell targets (1–3). At least three distinct but functionally and structurally related genes involved in the biosynthesis of NO have been identified. Transfection of RAW 264.7 cells with plasmids harboring protein kinase C (PKC) -epsilon type isotype but not with PKC-alpha, -beta-1, -delta, or constitutively active -alpha and -beta-1 isotypes resulted in the expression of nitric oxide synthase type II (iNOS), as reflected by the synthesis of nitric oxide measured in the culture medium of transfected cells. Cotransfection of RAW 264.7 cells with the -1592 to +121-base pair promoter region of the murine iNOS gene and PKC isotypes specifically induced the transactivation of this promoter in the case of the plasmids containing the PKC-epsilon isotype. The mechanism by which PKC-epsilon induced iNOS expression involved the activation of nuclear factor binding to kappaB (NF-kappaB) as deduced by the suppressive effect of pyrrolidine dithiocarbamate on nitric oxide synthesis, an inhibitor of NF-kappaB activation, and by the activation of kappaB sites in cells transfected with a vector containing a kappaB motif linked to a chloramphenicol acetyltransferase reporter gene. These results suggest that PKC-epsilon can regulate a pathway that promotes iNOS expression in macrophages in response to phorbol ester activation.

The knowledge of the mechanisms involved in the control of NO synthesis by different cell types is a subject of current interest because of the multiple physiological and pathological effects elicited by this molecule on different cell targets (1–3). At least three distinct but functionally and structurally related genes involved in the biosynthesis of NO have been identified. Transfection of RAW 264.7 cells with plasmids harboring protein kinase C (PKC) -epsilon type isotype but not with PKC-alpha, -beta-1, -delta, or constitutively active -alpha and -beta-1 isotypes resulted in the expression of nitric oxide synthase type II (iNOS), as reflected by the synthesis of nitric oxide measured in the culture medium of transfected cells. Cotransfection of RAW 264.7 cells with the -1592 to +121-base pair promoter region of the murine iNOS gene and PKC isotypes specifically induced the transactivation of this promoter in the case of the plasmids containing the PKC-epsilon isotype. The mechanism by which PKC-epsilon induced iNOS expression involved the activation of nuclear factor binding to kappaB (NF-kappaB) as deduced by the suppressive effect of pyrrolidine dithiocarbamate on nitric oxide synthesis, an inhibitor of NF-kappaB activation, and by the activation of kappaB sites in cells transfected with a vector containing a kappaB motif linked to a chloramphenicol acetyltransferase reporter gene. These results suggest that PKC-epsilon can regulate a pathway that promotes iNOS expression in macrophages in response to phorbol ester activation.

The knowledge of the mechanisms involved in the control of NO synthesis by different cell types is a subject of current interest because of the multiple physiological and pathological effects elicited by this molecule on different cell targets (1–3). At least three distinct but functionally and structurally related genes involved in the biosynthesis of NO have been identified. Transfection of RAW 264.7 cells with plasmids harboring protein kinase C (PKC) -epsilon type isotype but not with PKC-alpha, -beta-1, -delta, or constitutively active -alpha and -beta-1 isotypes resulted in the expression of nitric oxide synthase type II (iNOS), as reflected by the synthesis of nitric oxide measured in the culture medium of transfected cells. Cotransfection of RAW 264.7 cells with the -1592 to +121-base pair promoter region of the murine iNOS gene and PKC isotypes specifically induced the transactivation of this promoter in the case of the plasmids containing the PKC-epsilon isotype. The mechanism by which PKC-epsilon induced iNOS expression involved the activation of nuclear factor binding to kappaB (NF-kappaB) as deduced by the suppressive effect of pyrrolidine dithiocarbamate on nitric oxide synthesis, an inhibitor of NF-kappaB activation, and by the activation of kappaB sites in cells transfected with a vector containing a kappaB motif linked to a chloramphenicol acetyltransferase reporter gene. These results suggest that PKC-epsilon can regulate a pathway that promotes iNOS expression in macrophages in response to phorbol ester activation.

The knowledge of the mechanisms involved in the control of NO synthesis by different cell types is a subject of current interest because of the multiple physiological and pathological effects elicited by this molecule on different cell targets (1–3). At least three distinct but functionally and structurally related genes involved in the biosynthesis of NO have been identified. Transfection of RAW 264.7 cells with plasmids harboring protein kinase C (PKC) -epsilon type isotype but not with PKC-alpha, -beta-1, -delta, or constitutively active -alpha and -beta-1 isotypes resulted in the expression of nitric oxide synthase type II (iNOS), as reflected by the synthesis of nitric oxide measured in the culture medium of transfected cells. Cotransfection of RAW 264.7 cells with the -1592 to +121-base pair promoter region of the murine iNOS gene and PKC isotypes specifically induced the transactivation of this promoter in the case of the plasmids containing the PKC-epsilon isotype. The mechanism by which PKC-epsilon induced iNOS expression involved the activation of nuclear factor binding to kappaB (NF-kappaB) as deduced by the suppressive effect of pyrrolidine dithiocarbamate on nitric oxide synthesis, an inhibitor of NF-kappaB activation, and by the activation of kappaB sites in cells transfected with a vector containing a kappaB motif linked to a chloramphenicol acetyltransferase reporter gene. These results suggest that PKC-epsilon can regulate a pathway that promotes iNOS expression in macrophages in response to phorbol ester activation.

The knowledge of the mechanisms involved in the control of NO synthesis by different cell types is a subject of current interest because of the multiple physiological and pathological effects elicited by this molecule on different cell targets (1–3). At least three distinct but functionally and structurally related genes involved in the biosynthesis of NO have been identified. Transfection of RAW 264.7 cells with plasmids harboring protein kinase C (PKC) -epsilon type isotype but not with PKC-alpha, -beta-1, -delta, or constitutively active -alpha and -beta-1 isotypes resulted in the expression of nitric oxide synthase type II (iNOS), as reflected by the synthesis of nitric oxide measured in the culture medium of transfected cells. Cotransfection of RAW 264.7 cells with the -1592 to +121-base pair promoter region of the murine iNOS gene and PKC isotypes specifically induced the transactivation of this promoter in the case of the plasmids containing the PKC-epsilon isotype. The mechanism by which PKC-epsilon induced iNOS expression involved the activation of nuclear factor binding to kappaB (NF-kappaB) as deduced by the suppressive effect of pyrrolidine dithiocarbamate on nitric oxide synthesis, an inhibitor of NF-kappaB activation, and by the activation of kappaB sites in cells transfected with a vector containing a kappaB motif linked to a chloramphenicol acetyltransferase reporter gene. These results suggest that PKC-epsilon can regulate a pathway that promotes iNOS expression in macrophages in response to phorbol ester activation.
PKC-ε-dependent Expression of iNOS

**Materials and Methods**

**Chemicals**—Materials and chemicals for electrophoresis were from Bio-Rad or from Amersham Corp. Metabolites and biochemicals were from Sigma or from Boehringer Mannheim. Serum and media were from BioWhittaker, Inc. (Walkersville, MD).

**Cell Culture**—RAW 264.7 cells were obtained from ATCC and correspond to a murine macrophage cell line. The cells were cultured in RPMI 1640 medium supplemented with 2 mM glutamine, 10% FCS, and antibiotics (50 μg/ml of penicillin, streptomycin, and gentamicin). Peritoneal macrophages were obtained from Swiss mice following a published method (13). Rat hepatocytes, murine splenocytes and B cells, and human neutrophils were isolated and purified as described previously (14, 26).

**Plasmid Constructs and Preparation**—The 1,749-bp HinclII fragment, corresponding to the 5′-flanking region of iNOS (21, 22), fused to a promoterless CAT reporter gene (p1-NOS-CAT), was a generous gift from Dr. Q.-w. Xie and C. Nathan (Cornell University, NY). A (εB)3-CAT plasmid construct that contains three copies of the εB motif from the human immunodeficiency virus long terminal repeat enhancer with the conalbumin promoter was used as a reference for maximal efficiency of the transfection (28). The pCO2 vector (Dr. S. Goodbourne, St. Georges Hospital, London) was used to express the PKC isotypes (24), and they were refered to as pPKC-α, β1, β2, and γ. Plasmids directing expression of constitutively active isotypes were referred to as pPKC-α*, β1*, β2*, and γ* respectively (see Table I). Plasmids were purified using Qiagen columns (Hilden, FRG), and only those preparations that once passed the quality test were used. Additionally, to evaluate the stimulatory capacity of the plasmid preparations to mediate iNOS expression due to bacterial product contamination, cells were electroporated in the absence of plasmids and challenged with an equivalent amount of plasmid DNA under culture. Under these conditions PKC plasmids never induced an NH3 release higher than 10% over the basal value.

**Transfection of RAW 264.7 Cells**—The cell layer was trypsinized, and macrophages were collected by centrifugation at 200 × g for 5 min. The cell pellet was resuspended in ice-cold PBS (3 × 106 cells/ml) and kept at 4 °C. Plasmids were added to the cells at the indicated concentration and incubated at 4 °C for at least 5 min prior to electroporation (0.4 V/cm, 500 microfarad of capacitance) in a BTX electroporator. Cells were then incubated for a further 5 min at 4 °C and then transferred to RPMI 1640 medium containing 5% FCS. After two h of seeding to select adherent cells, the dishes were washed twice with PBS and maintained for 24 h in culture medium (phenol red-free medium plus 5% FCS). To follow NO release, the medium was changed, and stimuli (usually 50 ng/ml PDBu) were added. Cell incubation was continued for up to 48 h. When cells were transected by lipofection (DOTAP reagent from Boehringer Mannheim), it was done following the recommendations of the supplier manufacturer.

**Characterization of iNOS Expression by Northern Blot**—Total RNA (2–4 × 106 cells) was extracted using the guanidinium thiocyanate method (29). After electrophoresis in a 0.9% agarose gel containing 2% formaldehyde, the RNA was transferred to a Nytran membrane (NY Southern, FRG), and the levels of iNOS mRNA were determined using an EcoRI-HindII fragment from the iNOS cDNA (1, 5) labeled with α-32PdCTP using the Readyprime labeling kit (Amerham). The membranes were exposed to x-ray films (Hyperfilm, Amerham), and the intensity of the bands was measured by laser densitometry (Molecular Dynamics). Hybridization with an 18 S ribosomal probe was used as an internal standard.

**Tables and Figures**

**Table I**

| Plasmid Constructs | References |
|--------------------|------------|
| pCO2 (PKC isotypes) | (24)       |
| pPKC-α*             | (21, 22)   |
| pPKC-β1*            |           |
| pPKC-β2*            |           |
| pPKC-γ*             |           |
| p1-NOS-CAT          | (27)       |
| (εB)3-CAT           | (27)       |
| kSV2-CAT            | (28)       |

*The β1* pseudosubstrate mutant construct was prepared as the α construct (not previously characterized).

**Determination of NO2− Synthesis**—NO was measured as the accumulation of nitrite and nitrate in the incubation medium. Nitrate was reduced to nitrite with nitrate reductase (30). Nitrite was determined spectrophotometrically with Griess reagent (30) by adding 100 μl of 1 mM sulfanilic acid and 100 mM HCl (final concentration) to 850 μl of culture medium. After incubation for 5 min, the absorbance at 548 nm was measured, and 50 μl of naphthylenediamine (1 mM in the assay) were added. The reaction was completed after 15 min of incubation, and the absorbance at 548 nm was compared with a standard of NaNO2. The amount of nitrate produced from NO release was below 20% of the nitrite measured.

**Determination of PKC Activity**—The cell layers were washed twice with ice-cold PBS and homogenized in a Dounce homogenizer using 20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 5 mM EGTA, 10 mM β-mercaptoethanol, 200 μM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, and 0.5% Nonidet P-40 (lysis buffer). The extract was centrifuged in an Eppendorf centrifuge for 15 min, and the supernatant was applied to a DEAE column (500–μl gel) to partially purify the enzyme (31). The activity was assayed in a final volume of 250 μl using myelin basic protein peptide (MBP) or the peptide ERMRRPRQGSVRRRV (β-PS) as substrates following previously described methods (31, 32).

**Characterization of PKC and iNOS by Western Blot**—Cultured RAW 264.7 cells (3–4 × 106) were washed twice with PBS and homogenized in lysis buffer (see above), and after centrifugation in a microcentrifuge for 15 min, the proteins present in the supernatant were size separated in 10% SDS-polyacrylamide gel electrophoresis. The gels were blotted onto a polyvinylidene difluoride membrane (Millipore) and incubated with several anti-PKC-specific antibodies (33 and references herein) or with an iNOS antibody (Transduction Laboratories). PKC isotypes or iNOS were revealed by ECL following manufacturers instructions (Amersham).

**Characterization of CAT Activity**—In experiments investigating the effect of PKC isotypes on the expression of the transfected p1-NOS-CAT promoter (21) or (εB)3-CAT construct (27), cells were cotransfected with both plasmids and incubated with the indicated stimuli for 24–48 h. After two washes of the cell layer with ice-cold PBS, the plates were treated with 0.5 ml of 0.25 mM Tris-HCl, pH 7.8, at 4 °C, and the cells were scraped off the dishes. The cell extract was submitted to three cycles of freezing and thawing followed by centrifugation at 12,000 × g for 10 min, and the soluble protein was measured. Aliquots of the supernatant (150–200 μl) were normalized for protein (200 μg/ml) and heated at 65 °C for 10 min. CAT activity was measured in a final volume of 250 μl by the synthesis of acetylated [14C]chloramphenicol following the thin layer chromatography method (28). To quantify the amount of acetylated substrate, the silica spots were scraped off the matrix, and the radioactivity was determined by liquid scintillation counting.

**Data Analysis**—Statistical differences (p < 0.05) between mean values were determined by one-way analysis of the variance followed by Student’s t test. In experiments using x-ray films (hyperfilm), different exposure times were used to ensure that bands were not saturated.

**Results**

**Transfection of RAW 264.7 Cells with pPKC-ε Promotes the Expression of iNOS**—Incubation of cultured RAW 264.7 cells with LPS promoted a large release of NO to the medium,
phorbol ester responsive wild-type transfected by electroporation with plasmids encoding the boring distinct PKC isoenzyme constructs might restore a PKC expression of PKC isotypes after transfection with plasmids harboring iNOS in samples collected at 18 and 24 h after transfection shows, transfection with pPKC- absence or in the presence of 50 ng/ml of PDBu. As Fig. 2 pendent NOx inability of RAW 264.7 cells to display a significant PDBu-de- downsteam of the PKC network. Taking advantage of this because of a distinct distribution of the PKC isotypes prevailing in each cell type or because of a divergent signaling mechanism downstream of the PKC network. Taking advantage of this inability of RAW 264.7 cells to display a significant PDBu-dependent NOx synthesis, we studied whether a transient expression of PKC isoatypes after transfection with plasmids harboring distinct PKC isoenzyme constructs might restore a PKC-dependent induction of iNOS. To do this, RAW 264.7 cells were transfected by electroporation with plasmids encoding the phorbol ester responsive wild-type α, β1, δ, and ε isoforms of PKC, and the release of NOx was followed from 24 to 48 h after transfection (Fig. 2). Also, cells were transfected with plasmids encoding constitutively active forms of PKC (αc and βc isoatypes), and the release of NOx was followed either in the absence or in the presence of 50 ng/ml of PDBu. As Fig. 2 shows, transfection with pPKC-ε resulted in a substantial NOx synthesis that was barely affected by addition of PDBu. To ensure that this NOx synthesis effectively corresponded to the expression of iNOS, cells were collected at the end of the incubation period, and the presence of iNOS was assessed by Western blot (Fig. 2, inset). Also, the nature of the NOS present in PKC-transfected cells was determined following the mRNA levels corresponding to iNOS. Only cells transfected with PKC-ε exhibited a substantial increase in the mRNA levels of iNOS in samples collected at 18 and 24 h after transfection (Fig. 3). Similar results in NOx synthesis were obtained when cells were transfected by lipofection (DOTAP reagent) with pPKC-α, -αc, and -ε (Table II).

As Fig. 2 shows, the extent of NOx release from cells transfected with pPKC-ε was not further stimulated by the addition of phorbol esters. One explanation of this result is that there may be a high over-expression of the enzyme in the transfected cells. To test this idea, cells were transfected with increasing amounts of pPKC-ε plasmid followed by the addition of PDBu. As Fig. 4 shows, when cells were transfected with low amounts of plasmid, a gain in sensitivity to PDBu stimulation was observed; however, this was not the case when cells were trans-
PKC-ε-dependent Expression of iNOS

Table II

| Plasmid | NOx | -PDBu | +PDBu |
|---------|-----|-------|-------|
| pCO2    | 49 ± 5 | 38 ± 4 |
| pPKC-α  | 46 ± 4 | 52 ± 5 |
| pPKC-ε  | 52 ± 6 | 44 ± 5 |
| pPKC-ε | 129 ± 12 | 139 ± 11 |

Because the activation of the κB sequences located in the 1.8-kb fragment of the iNOS promoter plays an important role in the transactivation of the gene (21–23), we investigated whether this was the case in cells cotransfected with PKC isotypes and an expression vector carrying a tandem of 3 κB consensus sequences linked to a CAT reporter gene (27). As Fig. 7 shows, 24 h after transfection with vectors directing the expression of PKC-α and -ε isotypes, a clear increase in CAT activity was observed in cells expressing PKC-ε (10.2-fold increase), whereas CAT activity was minimal in cells transfected with PKC-α. When cells transfected with PKC expression vectors were incubated with PDTC, an inhibitor of κB degradation (34), CAT activity and NOx synthesis were abrogated in agreement with the expected results using this NF-κB activation inhibitor. As a control, stimulation with LPS of cells transfected with the (κB)3-CAT vector induced a large CAT activity confirming the effect of LPS on κB activation in these cells (21–23).

DISCUSSION

The study of the induction of iNOS in murine peritoneal macrophages and in macrophage cell lines has constituted the...
reference model for the characterization of the mechanisms responsible for the transcriptional control of this enzyme (1, 5, 7, 10–11, 21–23). The murine promoter region of iNOS has proved to be quite complex, containing at least 24 consensus sequences for the binding of transcription factors; among them, proteins of the NF-κB family appear to be essential components for the transactivation of iNOS (21–23). Protein kinase C stimulation by phorbol esters is able to activate NF-κB and AP-1, two transcription factors that bind to the iNOS promoter (25, 35, 36). However, treatment of RAW 264.7 cells with different phorbol esters and using a broad range of concentrations of these molecules to prevent side-effects in the course of PKC activation (such as the down-regulation of some PKC isotypes) failed to promote iNOS expression and, therefore, NO synthesis. For this reason, RAW 264.7 cells were transfected with expression vectors encoding different phorbol ester-responsive PKC isotypes as an approach to assess whether any of these enzymes might provide an appropriate signal to trigger the expression of iNOS. Our results clearly show that among the PKC isotypes tested, PKC-ε is a likely candidate to mediate the stimulatory effect on NO synthesis observed in other cell types in response to PKC activation (13–18). The complete absence of PKC-ε in RAW 264.7 cells (undetectable by Western blot when compared with 3T3 fibroblasts as a murine reference) was unexpected; however, it provided a plausible explanation for the lack of response of NO synthesis after incubation of these cells with phorbol esters. Indeed, overexpression of the β1 isotype barely induced NO release to the medium, whereas expression of the α and δ isotypes failed to induce iNOS. An additional conclusion from these results is that expression of iNOS in response to LPS or proinflammatory cytokines does not require PKC-ε engagement, at least in these cells. However, it is of interest to note that in this cell model, several authors have described an inhibitory effect in NO synthesis using more or less specific PKC inhibitors (19, 37, 38).

The identification of biological effects dependent on the specific activation of a particular PKC isotype is a matter of current research interest since differences among the PKC isotypes exists in the requirements of cofactors for activation, phospholipid-dependence, and subcellular distribution (39). To provide a few examples, it has been reported that overexpression of PKC-α in Chinese hamster ovary cells specifically inhibited the signaling of several members of the insulin receptor family (40). Regarding PKC-ε, this enzyme, and to a lesser extent the α isotype, has been reported as a regulator of the transcription factors AP-1 and nuclear factor of activated T cells (NF-AT-1) in human T cell lymphoma, whereas only PKC-ε transactivated κB constructs (44). In this system, activation via overexpression of PKC-ε required the use of constitutively active mutants, whereas wild-type PKC-ε was ineffective. These data contrast with our results where a gain of function (NO synthesis) was exclusively mediated by overexpression of this isotype and in the absence of PKC-ε activators. However, incubation of the transfected cells with phorbol esters produced a leftward shift in the dose dependence of PKC-ε cDNA required to induce iNOS (see Fig. 4). Moreover, it is possible that specific subcellular locations of PKC isotypes play an important role in the regulation of the activation pathway (e.g. PKC-ε has been reported to be localized to the Golgi via its Zn-finger domain, see Ref. 41). This interaction with subcellular structures has been reported for other PKC isotypes; for example, changes in the subcellular localization of PKC-α, but not in the total amount of enzyme, are closely associated with the transformation of rat embryo fibroblasts (42). Recently, a role for PKC-ε has been reported in the signaling mechanism elicited by platelet-derived growth factor involving two independent pathways, phospholipase C-γ and phosphatidylinositol-3-kinase activation (43). In this case, PKC-ε would appear to integrate signals released by redundant, but independent, input stimuli.

Deletion analysis of the promoter region of iNOS upon transfection of RAW 264.7 cells with a 1.8-kb region revealed the requirement for the integrity of the κB sequences for the transactivation after triggering with LPS or when LPS was acting synergistically with IFN-γ (5, 21–23). Indeed, treatment with PDTC of RAW 264.7 cells transfected with PKC-ε blocked iNOS expression, revealing the necessity of NF-κB activation in this process. Cotransfection with the −1592 to +121 promoter region of iNOS and distinct PKC isotypes also showed a specific response in the reporter activity when PKC-ε was expressed, confirming an important degree of PKC specificity in the process of iNOS induction. In agreement with these results, NF-κB was activated in cells transfected with a pPKC-ε vector. These data agree with results obtained in nontransfected cells stimulated with PDBu in which a lack was observed in the binding of nuclear proteins of the ref family to oligonucleotide se-
quences corresponding to the κB sites of the iNOS promoter (nucleotides extending from position −85 to −76 and −971 to −962; see Refs. 21–23) when assaying following the electrophoretic mobility shift of these oligonucleotides (results not shown). Moreover, the occurrence in RAW 264.7 cells of autocrine mechanisms switched on through PKC-ε activation (i.e. release of proinflammatory cytokotyes) that could contribute to the process of iNOS expression cannot be excluded. Indeed, this is the case for LPS that promotes the synthesis of cytokines such as TNF-α in activated macrophages in such a way that the control of iNOS expression relies on the signaling of more than one cytokine (1, 7, 44). In addition to these potential regulatory mechanisms, a cell-specific modulation of the promoter activity of iNOS can be proposed. This is the case for vascular smooth muscle cells in which the activity of the 1.8-kb murine iNOS promoter appears to be differentially regulated when compared with the macrophage counterparts (45). This situation has been interpreted in terms of a local and beneficial response that facilitates the delivery of immunomodulatory effectors intended to restore the normal function of the injured area.

In summary, evidence is presented for the selective and specific effect of PKC-ε in inducing iNOS in RAW 264.7 cells. All the PKC isotypes tested are phorbol ester responsive and can induce biological effects in other contexts (see Ref. 24). The specific ability of PKC-ε to induce iNOS and to confer PDBu responsiveness to the RAW 264.7 cells indicates a particular role for PKC-ε that cannot be fulfilled by these other phorbol ester responsive PKC isotypes. Consistent with the iNOS promoter analysis, it is only the PKC-ε isotype that is capable of inducing NF-κB. Thus the specificity of this response suggests an important role for the PKC-ε/NF-κB/iNOS pathway in host defense, inflammation and cytotoxic responses. While apparently not involved in the LPS response, it will be of future interest to establish those agonists that trigger iNOS via PKC-ε.

Acknowledgements—The authors thank Drs. Q.-w. Xie and C. Nathan for the generous gift of the inducible NOS cDNA probe and the p1NOS vector.

REFERENCES
1. Nathan, C., and Xie, Q.-w. (1994) FASEB J. 8, 3051–3064
2. Moncada, S., Palmer, R. M. J., and Higgs, E. A. (1991) Pharmacol. Rev. 43, 109–142
3. Bredt, D. S., and Snyder, S. H. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 682–685
4. Lowenstein, C. J., and Snyder, S. H. (1992) Cell 70, 705–707
5. Nathan, C., and Xie, Q.-w. (1994) J. Biol. Chem. 269, 15725–15728
6. Knowles, R. G., and Moncada, S. (1992) Trends Biochem. Sci. 17, 399–402
7. Nathan, C., and Xie, Q.-w. (1994) Cell 78, 915–918
8. Cho, H. J., Xie, Q.-w., Calaycay, J., Mumford, R. A., Swidersk, K. M., Lee, T. D., and Nathan, C. (1992) J. Exp. Med. 176, 599–604
9. Marletta, M. A. (1985) J. Biol. Chem. 260, 12231–12234
10. Geller, D. A., Nussler, A. K., Di Silvio, M., Lowenstein, C. J., Shapiiro, R. A., Wang, S. C., Simmons, R. L., and Biliar, T. R. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 522–526
11. Geng, Y., Maier, R., and Lotz, M. (1996) J. Cell. Physiol. 163, 545–554
12. Chu, S. C., Wu, H.-P., Banke, T. C., Eissa, N. T., and Moss, J. (1995) J. Biol. Chem. 270, 10625–10630
13. Hortalano, S., Genaro, A. M., and Boscá, L. (1993) FEBS Lett. 320, 135–139
14. Hortalano, S., Genaro, A. M., and Boscá, L. (1992) J. Biol. Chem. 267, 24937–24940
15. Jun, C. D., Choi, B. M., Hoon, R., Um, J. Y., Kwak, H. J., Lee, B. S., Paik, S. G., Kim, H. M., and Chung, H. T. (1994) J. Immunol. 153, 3684–3690
16. Sandos, A., Bulat, V., Severn, A., Xu, D., and Liew, F. Y. (1994) Eur. J. Immunol. 24, 2345–2350
17. Simmons, M. L., and Murphy, S. (1984) Glia 11, 227–234
18. Yoon, H. J., Jun, C. D., Kim, J. M., Rim, G. N., Kim, H. M., and Chung, H. T. (1994) Neuroimmunomodulation 1, 377–382
19. Jun, C. D., Choi, B. M., Kim, H. M., and Chung, H. T. (1995) J. Immunol. 154, 6541–6547
20. Nishizuka, Y. (1995) FASEB J. 9, 484–496
21. Xie, Q.-w., Whisnant, R., and Nathan, C. (1993) J. Exp. Med. 177, 1779–1784
22. Xie, Q., Kashivabara, Y., and Nathan, C. (1994) J. Biol. Chem. 269, 4705–4708
23. Lowenstein, C. J., Alley, E. W., Raval, P., Snowman, A. M., Snyder, S. H., Russell, S. W., and Murphy, W. J. (1995) Proc. Natl. Acad. Sci. U. S. A. 90, 9730–9734
24. Genot, E. M., Parker, P. J., and Cantrell, D. A. (1995) J. Biol. Chem. 270, 9833–9839
25. Hirano, M., Hirai, S., Mizuno, K., Osada, S., Hosaka, M., and Ohno, S. (1995) Biochem. Biophys. Res. Commun. 206, 429–436
26. Genaro A. M., Hortalano S., Alvarez A. Martinez-A. C., and Boscá, L. (1995) J. Clin. Invest. 95, 1884–1890
27. Lozano, J., Berra, E., Munio, M. M., Diaz-Meco, M. T., Dominguez, I., Sanz, L., and Moscat, J. (1994) J. Biol. Chem. 269, 19200–19205
28. Gorman, C., Moffat, L., and Howard, B. (1982) Mol. Cell. Biol. 2, 1044–1051
29. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156–159
30. Terenzi, F., Diaz-Guerra, M. J. M., Casado, M., Hortalano, L., Leoni, S., and Boscá, L. (1995) J. Biol. Chem. 270, 6017–6021
31. Diaz-Guerra M. J. M., Sanchez-Prieto, J., Boscá, L., Pocock, J., Barrie, A., and Nicholls, D. (1988) Biochim. Biophys. Acta 970, 157–165
32. Marais, R. M., and Parker, P. J. (1998) Eur. J. Biochem. 182, 129–137
33. Oliver, A. R., and Parker, P. J. (1992) J. Cell. Physiol. 152, 244–244
34. Thanos, D., and Maniatis, T. (1995) Cell 80, 529–532
35. Angel, P., and Karin, M. (1991) Science 253, 1263–1269
36. Ghosh, S., and Baltimore, D. (1990) Nature 344, 678–682
37. Paul, A., Pendreigh, R. H., and Plevir, R. (1995) Br. J. Pharmacol. 114, 482–488
38. McKenna, T. M., Clegg, J. M., and Williams, T. J. (1994) Shock 2, 84–89
39. Dekker, L. V., and Parker, P. J. (1994) Trends Biochem. Sci. 19, 73–77
40. Dinaelsen, A. G., Liu, F., Hosomi, Y., Shii, K., and Roth, R. A. (1995) J. Biol. Chem. 270, 21600–21605
41. Lehel, C., Olah, Z., Jakab, G., and Anderson, W. B. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 1406–1410
42. Liao, L., Ramsay, K., and Jaken, S. (1994) Cell Growth & Differentiation 5, 1185–1194
43. Moriya, S., Kazlauskas, A., Akimoto, K., Hirai, S., Mizuno, K., Takenawa, T., Fukui, Y., Watanabe, Y., Ozaki, S., and Ohno, S. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 151–155
44. West, M. A., Seatter, S. C., Bellingham, J., and Clair, L. (1995) Surgery 118, 220–228
45. Spink, J., Cohen, J., and Evans, T. J. (1995) J. Biol. Chem. 270, 29541–29547