Interleukin-1β Activates Protein Kinase Cζ in Renal Mesangial Cells

POTENTIAL ROLE IN PROSTAGLANDIN E2 UP-REGULATION*

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Protein kinase C (PKC) plays a role in signal transduction mediated by interleukin-1β (IL-1β) leading to the increase in prostaglandin E2 (PGE2) production. In the present study we suggest that there are at least two distinct PKC isotypes involved in the signaling mechanism. Staurosporine potentiated the effect of IL-1β on coxl mRNA expression while calphostin C totally inhibited mRNA expression. The down-regulation of PKC by growing mesangial cells in the presence of phorbol 12-myristate 13-acetate for 24 h failed to modify the up-regulated response in PGE2 formation by IL-1β. Furthermore, incubation of mesangial cells with IL-1β causes translocation of PKCζ from cytosol to a presumed membrane compartment, and this translocation phenomenon was not inhibited by incubating the cells with staurosporine but was inhibited with calphostin C. Gel retardation assays also demonstrated that staurosporine did not inhibit the IL-1β-stimulated binding of nuclear extracts to the NF-κB motif. In contrast, calphostin C inhibited binding to the κB motif in a dose-dependent manner. Finally, antisense oligonucleotides to PKCζ partially inhibited the IL-1β-induced PGE2 formation while control sense oligonucleotides were without effect. Taken together, these data suggest that PKCζ is involved in the IL-1β signaling responses.

Activation of protein kinase C (PKC) plays a major role in agonist-stimulated function in a variety of cell types (1–3). To date there are at least 12 isotypes of PKC described and identified as α, β, β1, γ, δ, ε, z, η, θ, τ, ρ, and μ. These PKC isotypes are unique with respect to their primary structure, expression patterns, subcellular localization, and responsiveness to extracellular ligands. Recent reviews have highlighted the evidence that the isotypes might have separate and unique functions in the cell (2). Activation of PKC is associated with its translocation from the cytosolic (soluble) fraction to the particulate (membrane) fraction (4, 5). Furthermore, prolonged activation of some cells with phorbol esters is associated with the binding of phorbol esters to the kinase and resultant down-regulation.

PKCζ does not appear to bind phorbol esters (6) and is not associated with the down-regulation of PKCζ (7). The Cys-rich region within the C1 domain of PKC consists of two zinc finger motifs with six cysteine residues each and a homologous DNA-binding motif found in transcription factors like GAL4 (8). The use of deletion mutants of different PKC isotypes has revealed that the Cys-rich region is necessary for diacylglycerol and phorbol ester binding (9, 10). PKCζ contains only one zinc finger and does not bind diacylglycerol or phorbol ester (6).

The observations that PKCζ is resistant to staurosporine (11, 12), appears to be critical for mitogenic signal transduction (13), and is expressed in the rat mesangial cell (14) led us to evaluate its role in the signal transduction process resulting in an up-regulation of PGE2 in this cell type and whether this pathway of signaling is relevant to transcriptional activation of the coxl gene by IL-1β.

EXPERIMENTAL PROCEDURES

Materials—NaF, sodium orthovanadate, pepstatin, leupeptin, and aprotinin were from Sigma. GeneScreen was from DuPont, and the murine cDNA probes ligated in BlueScript SK− for coxI and coxl were a generous gift of Dr. Karen Seibert, Monsanto Co. (St. Louis, MO). The coxl cDNA was excised from the plasmid as a BamHI-HindIII digest. The coxl cDNA was isolated by a KpnI-BamHI digest. The DNA was purified from 1% agarose gels with GeneClean (BIO 101, Inc.). Anti-PKCζ antibody and PKCζ carboxyl-terminal peptide were purchased from Santa Cruz Biotechnology. H7, staurosporine and calphostin C were from Calbiochem. IL-1β was from Boehringer Mannheim.

Cell Culture—Primary mesangial cell cultures were prepared from male Sprague-Dawley rats as described previously (15). Cells were grown in RPMI 1640 medium supplemented with 15% (v/v) heat-inactivated fetal calf serum, 0.6% (v/v) insulin, 100 units/ml penicillin, 100 μg/ml streptomycin, 250 μg/ml Fungizone, and 15 μM HEPES. All experiments were performed with confluent cells grown in 75-cm2 flasks (about 0.5–1.0 × 106 cells) and used at passages 3–6.

Northern Blot Analysis—Total RNA was isolated using RNA-staq™ (Tel-Test “B” Inc.). Total cellular RNA was fractionated on 1% formaldehyde-agarose gels and transferred onto nylon filters (GeneScreen, DuPont) in 0.25 M Na+ phosphate buffer, pH 6.5. 20 μg of total RNA was loaded per lane. Membrane filters were dried at 80°C for 15 min, and RNA was fixed by cross-linking in a UV Stratalinker-1800 (Stratagene) with 1800 J. Membrane filters were then prehybridized for 6 h at 42°C in 50% deionized formamide (v/v), 0.04% polyvinylpyrrolidone (w/v), 0.04% bovine serum albumin (w/v), 0.04% Ficoll (w/v), 5 × SSC, 1% SDS (w/v), and denatured salmon sperm DNA (100 μg/ml). Full-length murine probes for coxI and coxl were radiolabeled with 32P by the random priming method. Hybridization was performed at 42°C for 18–20 h in a solution containing 50% deionized formamide (w/v), 0.02% polyvinylpyrrolidone (w/v), 0.02% bovine serum albumin (w/v), 0.02% Ficoll (w/v), 5 × SSC, 1% SDS (w/v), and denatured salmon sperm DNA at 100 μg/ml. Filters were washed twice at room temperature for 5 min in 2 × SSC and twice at 60°C for 30 min in 2 × SSC and with 0.1% SDS (w/v) SDS and were then exposed overnight to Kodak X-omat AR film at −70°C with intensifying screens. To control for variability in the loaded quantity of RNA, all membranes were probed with a 550-base pair HindII–Xba1 restriction fragment of pRcRat 15 to determine the steady state levels of glyceraldehyde-3-phosphate dehydrogenase gene-related se-
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quencies and were used to normalize mRNA for coxl and coxl. In all of the Northern analyses, RNA was harvested 3 h after the addition of drugs.

PGE2 Determination—PGE2 in the media was determined by stable isotope gas chromatography-mass spectrometry as described previously (16). At the end of predetermined times, media were removed and spiked with 25–50 ng of tetradecuterated PGE2(d14, d-PE2). The medium was then acidified to pH 3.5, and PGE2 was extracted by 1 ml octadecyl columns (J. T. Baker, Inc.). Extracts were derivatized for gas chromatography-mass spectrometry analysis. The samples were analyzed as the pentafluorobenzyl methoxime trimethylsilyl ether by negative ion chemical ionization using methane as the reagent gas. Ions monitored were m/z 524 (d14, PE2) and m/z 528 (d-PE2). Mass spectrometry was performed on a Hewlett-Packard 5989B spectrometer using a 25-μm Ultra 1 capillary column (Hewlett-Packard Co.), and data collection and analysis were performed using Vector 2 software (Teknivent, St. Louis, MO).

Preparation of Cell Supernatants for Western Blotting—Cells were stimulated with interleukin-1β (IL-1β) for 20–30 min, washed twice with ice-cold phosphate-buffered saline, and lysed in 1 ml of ice-cold extraction buffer containing 2 mM EDTA, 10 mM NaF, 1 mM Na4P2O7, 0.1 mM phenylmethylsulfonyl fluoride, 2 mM sodium orthovanadate, 10 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 5 mM 1 μg/ml aprotinin, 200 μM leupeptin, and 200 μM pepstatin. The nuclei were collected by centrifugation at 14,000 rpm for 5 min, washed once with ice-cold extraction buffer, and incubated for 20 min on ice with shaking. The nuclear extract was centrifuged at 14,000 rpm for 5 min and the supernatant was collected for further analysis.

Electrophoretic Mobility Shift Assay—Confluent mesangial cells in 75-cm2 flasks were treated with IL-1β and/or pharmacological agents for 0–30 min. Cells were gently washed twice with ice-cold phosphate-buffered saline and scraped into Eppendorf tubes and centrifuged at 14,000 rpm for 5 min. Supernatants were collected by centrifugation at 14,000 rpm for 10 min and vortexed vigorously for 10 s. The nuclei were collected by centrifugation at 14,000 × g for 10 s. The nuclear pellets were resuspended in 100 μl of high salt extraction buffer (20 mM HEPES-KOH, pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 5 μg/ml pepstatin, 5 μg/ml leupeptin, 0.6% Nonidet P-40). The cells were allowed to swell on ice for 10 min and vortexed vigorously for 10 s. The nuclei were collected by centrifugation at 14,000 × g for 10 s. The nuclear pellets were resuspended in 100 μl of high salt extraction buffer (20 mM HEPES-KOH, pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 5 μg/ml pepstatin, 5 μg/ml leupeptin) and incubated for 20 min on ice with shaking. The nuclear extract was centrifuged at 14,000 × g for 5 min at 4 °C, and the supernatant was aliquoted and stored at −80 °C. Protein concentration was determined by microbicinchoninic acid assay (Sigma).

A double-stranded oligonucleotide probe containing NFκB sequences was end-labeled with [32P]ATP. The xB sequences used were forward (5′-AGTTGAGGGAGGTCTCCAGGCGC-3′) and complementary (3′-TCACACTCCCTGAAAGGGCTC-5′). 10 μg of nuclear protein was incubated for 20 min on ice with radiolabeled oligonucleotide probes (25,000–50,000 cpm) in a 25-μl reaction buffer containing 2 μg of poly(dI-dC), 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 10 mM dithiothreitol, 1 μg/ml bovine serum albumin, 10% glycerol (v/v). Nucleoprotein-oligonucleotide complexes were resolved by electrophoresis on a 5% nondenaturing polyacrylamide gel (acrylamide/bisacrylamide at 20:1) in 0.3 mM Tris borate/EDTA buffer at 150 V for 2 h at 4 °C. The gel was dried and autoradiographed with intensifying screen at ~80 °C for 1–5 h.

Transfection of Mesangial Cells with Oligonucleotides—A 20-mer antisense oligonucleotide (5′-GGCCACACATGGTCGTCGACT-3′) complementary to the cDNA for rat PKCζ (6) starting 128 nucleotides from the translational start site was utilized for these experiments. The sense oligonucleotide (5′-AGTGGGACAGTGTGGGC-3′) was used as a control. The oligonucleotides were synthesized in the Protein and Nucleic Acid Laboratory at Washington University School of Medicine.

Mesangial cells were grown in 6-well plates to 70% confluence in RPMI 1640 medium supplemented with 15% fetal calf serum. On the day before the transfection, the medium was replaced overnight with fresh medium containing 0.5% fetal calf serum. For the transfections, the method of Reddy and Hershman was used (18). Briefly, 100 μl of serum-free RPMI 1640 medium containing 25 μg of Lipofectin reagent (Life Technologies, Inc.) was mixed with 100 μl of serum-free medium containing antisense or sense oligonucleotides and incubated for 30 min at room temperature. This oligonucleotide-Lipofectin complex solution was added to 0.8 ml of serum-free medium, and the final oligonucleotide concentration was 15 μM. The cells were washed with serum-free medium and overlaid with 1 ml of the diluted complex solution. After 24 h of incubation, the cells were washed with serum-free medium and replaced with fresh RPMI 1640 medium containing 5% fetal calf serum. At the same time IL-1β (100 units/ml) was added and incubated for an additional 24 h. The conditioned medium was collected and assayed for PGE2 content.

RESULTS

To assess the effects of protein kinase C inhibition on IL-1β-induced expression of coxl, we incubated mesangial cells with and without IL-1β and the protein kinase C inhibitors H7 and staurosporine. Fig. 1 demonstrates that IL-1β induces the message for coxl but not for coxl. In addition, staurosporine at both 10 and 100 μM produced a marked potentiation of coxl induction by IL-1β. Furthermore, staurosporine alone at a concentration of 100 μM induced coxl. Interestingly, H7 alone had no effect on coxl expression; however, it seemed to potentiate the effect of IL-1β on coxl expression although to a lesser extent than staurosporine. Because of these results, we carried out another series of experiments comparing the effects of staurosporine and calphostin C, and in addition, we added a PKC activator, PMA, as a positive control. Fig. 2 shows the results of a Northern analysis of such an experiment. As shown in Fig. 1, IL-1β-stimulated coxl expression (lanes 2 and 3), which was potentiated by 100 nM staurosporine. PMA alone also enhanced expression of coxl (lane 4), but somewhat surprisingly, this effect was potentiated by staurosporine (lane 6). In contrast to the effects of staurosporine, calphostin C inhibited both the effects of IL-1β and PMA on coxl expression (lanes 8 and 9). We considered two possibilities to explain these observations. First, we hypothesized that IL-1β-stimulated coxl expression involved the participation of protein kinase C since it was calphostin C-inhibitable. We further hypothesized that this protein kinase C was staurosporine-insensitive. Another possibility was that staurosporine was exerting effects on the mesangial cells independent of its protein kinase C inhibitory activity. In an attempt to test whether protein kinase C was involved in IL-1β signaling, we assessed the effects of protein kinase C down-regulation by PMA on IL-1β induction of PGE2.

Fig. 3 shows such an experiment. Mesangial cells were first incubated with 500 nM PMA for 24 h followed by IL-1β for an additional 24 h and then compared with untreated cells for their ability to increase PGE2 formation. This experiment dem-
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FIG. 2. Northern analysis of total mesangial cell RNA probed with coxII (COX 2) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). 50 units/ml IL-1β, 100 nM PMA, 100 nM staurosporine (Stauro), and 5 μM calphostin C (Cal C) were used.

FIG. 3. PGE2 production by mesangial cells in response to IL-1β. Control (■) cells that were and cells PKC down-regulated by incubation with 100 nM PMA for 24 h prior to stimulation with IL-1β are shown.

Demonstrates that down-regulation of PKC does not modify the cellular response to IL-1β. The results thus far were consistent with the involvement of a staurosporine-resistant (12), calphostin C-sensitive PKC that was not down-regulated by PMA (7). PKCζ fits these requirements and is expressed in renal mesangial cells (14). Therefore, we evaluated the effect of IL-1β on PKCζ translocation from the cytosol in renal mesangial cells by immunoblot analysis. Fig. 4 shows such an experiment. Fig. 4B shows a time course of immunologically detectable PKCζ in the cytosol from cells treated with IL-1β for 0 (control), 5, 15, and 30 min. Fig. 4A shows a similar Western blot obtained from cells treated with IL-1β but that had been treated with 100 nM staurosporine 10 min prior to the addition of IL-1β. Fig. 4C shows a Western blot obtained from cells treated with IL-1β in the presence of 2 μM calphostin C 15 min prior to the addition of IL-1β. Fig. 4D shows the densitometric quantitation of the 68-kDa PKCζ bands from Fig. 4, A–C (arrow). It can be seen that PKCζ was translocated from the cytosol in response to IL-1β, and this translocation was unaffected by staurosporine. In contrast with pretreatment of cells with calphostin C, IL-1β failed to initiate translocation of PKCζ. These experiments represent a mean of two in duplicate. Control experiments carried out with a 10-fold molar excess of PKCζ carboxyl-terminal peptide completely inhibited antibody detection of the 67-kDa protein (data not shown).

Since IL-1β induces the translocation of a factor to the nucleus that binds the NFκB motif in the proximal promoter of pghs2 (coxII), we determined the effect of staurosporine and calphostin C on the IL-1-stimulated binding by electromobility shift assays. Fig. 5A shows that IL-1β induces binding of a factor to the oligonucleotide NFκB motif, and this binding event was not influenced by staurosporine. In contrast, Fig. 5B shows that calphostin C produced a dose-dependent inhibition of binding, again suggesting the involvement of a staurosporine-insensitive but calphostin C-sensitive activation of NFκB in the renal mesangial cell stimulated with IL-1β.

To determine whether the activation of PKCζ by IL-1β was involved in the up-regulation of PGE2 formation in the renal mesangial cell, we utilized antisense oligonucleotides to PKCζ and transfected mesangial cells and assayed the PGE2 released into the media. Experiments using sense oligonucleotides transfected into mesangial cells were used as controls. Fig. 5 shows that the PKCζ antisense oligonucleotide inhibited PGE2 formation by 37% (p < 0.05) while the sense control oligonucleotide did not inhibit PGE2 formation in response to IL-1β. Fig. 6 represents four experiments, and the data are expressed as the mean ± S.E. Fig. 7 shows a representative Western blot of cellular extracts obtained from control cells (nontransfected) and cells transfected with sense and with antisense oligonucleotides. It demonstrates that the antisense oligonucleotide inhibited CoxII protein expression by 40%.

To determine whether the antisense oligonucleotide interrupted the synthesis of PKCζ, we performed Western blots on cytosolic extracts of mesangial cells and compared the expression of PKCζ in control cells, cells treated with IL-1β for 30 min, and cells transfected with antisense oligonucleotides to PKCζ as described previously. Fig. 8 shows such an experiment. Lane 1 shows the expression of PKCζ in 10 μg of cytosolic protein in control cells. Lane 2 shows the levels detected in cytosol from similar amounts of protein from IL-1β-treated cells, and lane 3 represents cytosol from antisense transfected cells. These experiments demonstrate that the antisense oligonucleotide reduced the level of expression of PKCζ in cell cytosol.

DISCUSSION

In an attempt to assess the functional contribution of PKCζ as a signaling mechanism recruited by IL-1β in the activation of coxII gene transcription, we have utilized a pharmacological approach using PKC inhibitors. These experiments demonstrate that staurosporine alone at 100 nM induced mRNA for coxII and markedly potentiated the effects of IL-1β and PMA on inducing coxII mRNA. In contrast, calphostin C alone had no effect on coxII mRNA expression but completely inhibited the ability of IL-1β and PMA to increase coxII mRNA. These surprising results could be explained by suggesting that a PKC isotype that was both calphostin C inhibitable and staurosporine-resistant was involved in IL-1β-stimulated coxII expression.

Since the renal mesangial cell expresses multiple isotypes including α, γ, ε, and ζ (14, 19, 20) and since PKCζ is both staurosporine-insensitive (12) and calphostin C-sensitive (21) and is not down regulated by PMA (7), we postulated that if PKCζ was involved in the IL-1β signaling pathway leading to coxII expression, then down-regulation of PKC with PMA should not influence the response of the mesangial cell to IL-1β. Fig. 3 supports this thesis and demonstrates that exposure of mesangial cells to 500 nM PMA for 24 h does not influence the cellular response to IL-1β with respect to PGE2 formation. It should be stated that this experimental maneuver
inhibited both nitrite and inducible nitric oxide synthase expression by 50% (data not shown). This experiment confirms that down-regulation of PKC by PMA does not influence the cellular response to IL-1β and again suggests the involvement of a PKC isotype that is not down-regulated by PMA. Fig. 4 indeed demonstrates that IL-1β initiates a translocation of NFκB from cytosol, presumably to some membrane component, and that incubating the cells with staurosporine does not prevent this translocation phenomenon while translocation was inhibited by calphostin C.

Furthermore, staurosporine at 10 and 100 nm did not inhibit the IL-1β-stimulated binding of a nuclear protein to the κB motif as determined by electromobility shift assays (Fig. 5A). In contrast, calphostin C inhibited the binding event in a dose-dependent manner, again confirming that IL-1β stimulates a staurosporine-insensitive, but calphostin C-sensitive PKC isotype that is not down-regulated by PMA.

The experiments described thus far have demonstrated that IL-1β induces the mRNA for CoxII and that this inductive phenomenon is dependent on a staurosporine-insensitive, calphostin C-sensitive PKC isotype. We have demonstrated that PKCζ is translocated from the cytosol in response to IL-1β. Therefore, in an attempt to determine whether or not this
activation of PKCζ is relevant to CoxII induction of PGE2 formation, we reasoned that if we were able to decrease the activity of PKCζ selectively with antisense oligonucleotides to PKCζ, then we would be able to close the loop and demonstrate a functional consequence of PKCζ in the signal transduction mechanisms involved in PGE2 production. The experiment illustrated by Fig. 6, therefore, demonstrates that while the antisense oligonucleotides to PKCζ inhibit PGE2 formation in response to IL-1β by 37%, the sense oligonucleotides had no effect. Similarly, the antisense oligonucleotide inhibited CoxI protein expression by 40% while the sense oligonucleotide did not. Thus, we feel these experiments close the loop and draw a link between the activation of PKCζ and the induction of CoxI mRNA. Clearly, the production of PGE2 in renal mesangial cells is the result of activation and induction of phospholipase A2 (22) plus the induction and expression of CoxII. The antisense oligonucleotides that only inhibit PGE2 and CoxI expression by about 40% suggest that the effects of PKCζ are exerted entirely at the level of the cyclooxygenase.

These experiments, while intriguing, clearly illustrate the complexity of the signal transduction process and point to the fact that there are multiple factors and pathways that are involved in IL-1β signal transduction mechanisms. One key feature of these experiments is the fact that while it could be argued that if the PKCζ that is staurosporine-insensitive was the only isotype involved in this signal transduction process, then the use of staurosporine should not have potentiated the effect of IL-1β but rather should have had no effect on CoxI message production. In reality, it did potentiate CoxI mRNA and suggests that staurosporine was having an additional effect as well as that of failure to inhibit PKCζ.

We postulate that two additional mechanisms are possible to explain this phenomenon. The first is that staurosporine is having an additional effect that is unrecognized and that somehow leads to the potentiation of the effect of IL-1β on CoxI mRNA production. While we cannot rule out this possibility since the use of staurosporine, a pharmacological agent, carries with it the possibility of certain unknown functions, we feel other plausible explanations for this phenomenon should be considered. In experiments not shown but previously reported, IL-1β does not affect CoxI expression (16, 25). Second, there may be other staurosporine-sensitive PKC isotypes that are involved in the signaling process and that are inhibited by staurosporine. Such a phenomenon could be linked to the activation of a phosphatase that regulates the activity of another signal transduction process mediated by IL-1β that is also coupled to transcriptional activation leading to an increase in mRNA for coxI. There is evidence from other investigators suggesting that activation of PKC decreases phosphorylation of c-jun at sites that negatively regulate its DNA binding activity (23). In addition, there is evidence that activators of protein kinase C stimulate association of the Shc and the PEST tyrosine phosphatases (24). While we have no direct evidence that the Shc or PEST tyrosine phosphatases are involved in interleukin signaling mechanisms, we have provided evidence that vanadate alone, an inhibitor of protein tyrosine phosphatases, increases mRNA for coxI and that it potentiates the effect of IL-1β on coxI mRNA levels (25). The available data at least raise the issue of whether there may be a staurosporine-sensitive isoform that is coupled to the activation of a phosphatase that acts as a breaking phenomenon on the IL-1β stimulatory effect. The result of adding staurosporine to inhibit this protein kinase C would be to inhibit the effect of this phosphatase and, therefore, leave the positive effects mediated through PKCζ unchecked. This would clearly explain a potentiation of the effect of IL-1β and will also explain why the drug alone at 100 nM was able to induce the message for the coxI gene. We are currently addressing this issue in the laboratory by designing experiments directed at this possibility. We suggest, therefore, that a PKC isoform that is coupled to the activation of a phosphatase could influence the expression of the mRNA for coxI. Similarly, surprising observations have been described in PC12 cells where staurosporine markedly potentiated neurotransin and/or neuromedin mRNA accumulation in combination with other inducers (for example, NGF and PMA) and in some circumstances appeared to substitute for PMA (11). Furthermore, NGF-induced differentiation of PC12 cells utilizes the PMA-insensitive PKCζ isoform. This was demonstrated by activation of PKCζ by NGF and by attenuation of the effects of NGF on neurite outgrowth by antisense oligonucleotides to PKCζ (26).

In summary, we feel we have provided the evidence that PKCζ is involved in the signal transduction mechanism for IL-1β. We feel that this pathway is involved in the ability of IL-1β to increase mRNA expression for coxI. The recent data suggesting that PKCζ can phosphorylate IκB (27) and that inhibition of PKCζ blocks the activation of NF-κB-like activity in Xenopus laevis oocytes (28) suggest that this isoform of PKC may be very relevant to the effect of the transcriptional factor NF-κB on PGE2 production in response to IL-1β. This mechanism also has support in the observation that PKCζ mediates NF-κB activation in human immunodeficiency virus-infected monocytes (29).

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