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A Role for Protein Kinase Cα in Stimulation of Prostaglandin G/H Synthase-2 Transcription by 14,15-Epoxyeicosatrienoic Acid

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Arachidonic acid, but not eicosapentaenoic acid, increased prostaglandin G/H endoperoxide synthase-2 transcription in cultured intestinal epithelial cells. This stimulatory effect on PGHS-2 synthesis was prevented by an AA utilization inhibitor, eicosatetraynoic acid. Specific inhibitors of the cycloxygenase or the lipoxygenase pathways of AA metabolism did not prevent AA-mediated induction of PGHS-2 synthesis; however, the involvement of cytochrome P450 monoxygenases (CYP450) was indicated as several CYP450 blockers, ketoconazole, miconazole, and metyrapone, inhibited the induction of PGHS-2 mRNA synthesis by AA. This blockade by CYP450 inhibitors could be overcome by the addition of the AA epoxygenase metabolite 14,15-epoxyeicosatrienoic acid (14,15-EET); other EET regioisomers were unable to elevate PGHS-2 mRNA level. Blockade of protein kinase C with a specific inhibitor, bisindolyl maleimide-1, or translational inhibition of protein kinase Cα by antisense oligonucleotides reduced PGHS-2 transcription, suggesting the involvement of protein kinase Cα in the signal transduction pathway.

Numerous factors such as mitogenic agents, cytokines, growth factors, hormones, oxidant stress and inflammatory mediators, known to release arachidonic acid (AA) from membrane phospholipids (1, 2), have also been shown to stimulate the transcription of prostaglandin G/H synthase-2 (PGHS-2), the inducible isozyme that catalyzes prostaglandin synthesis from arachidonic acid (3, 4). However, a role for AA in the signal transduction has not been established. AA can directly affect ion channels (5, 6) but most AA undergoes oxidation through the cyclooxygenase, lipoxygenase and epoxygenase pathways to yield prostaglandins (PG), prostacyclin, thromboxane, leukotrienes, lipoxins and hydoxy-, hydroperoxy- and epoxy-fatty acids. Products of these pathways have been shown to affect expression of immediate-early genes. For example, c-fos has been shown to be induced by PGE_2 (7), leukotriene B_4 (8) and epoxygenase metabolites (9). However, only PGs have been reported to induce the expression of PGHS-2 in osteoclasts (10); whereas the role of the other two AA pathways, lipoxygenases and monoxygenases, in eliciting PGHS-2 transcription is not known.

We therefore investigated if AA-induced PGHS-2 expression in rat intestinal crypt cell line is mediated by the products of the cyclooxygenase, lipoxygenase, or monoxygenase routes of AA metabolism. Our findings indicated that AA induced the synthesis of PGHS-2 mRNA through its utilization by monoxygenases to (±)14,15-epoxyeicosatrienoic acid (EET). Furthermore, PKCα appeared to be an integral part of signal transduction by (±) 14,15-EET leading to PGHS-2 transcription.

MATERIALS AND METHODS

Materials. The following reagents were purchased: peroxide-free AA, eicosatetraynoic acid (ETYA), metyrapone, methyl esters of (±)5,6-EET, (±)8,9-EET, (±)11,12-EET, (±) 14,15-EET (Cayman Chemical Ann Arbor, MI); [^{32}P]-CTP (3000 Ci/mmol); pGEM-4 plasmid vector, *in vitro* transcription kit (Promega, Madison, WI); protein assay and electrophoretic reagents (Bio-Rad, Mississuaga, Ontario); guanidine isothiocyanate, proteinase K, RNase A, RNase T_1, anti-PKCα serum (BRL Life Technologies, Burlington, Ontario); rat β-actin riboprobe (Ambion Inc., Austin, TX); ketoconazole, miconazole, nordihydroguaretic acid and caffeic acid (Sigma, St. Louis, MO). LY233569 was a gift from Eli Lilly & Co. (Indianapolis, IN). All other chemicals were of analytical reagent grade and were purchased from either Sigma (St. Louis, MO) or ICN Biochemicals (Montreal, Quebec).

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Abbreviations used: PGHS, prostaglandin G/H synthase; ETYA, eicosatetraynoic acid; EET, epoxyeicosatrienoic acid; PG, prostaglandin; AA, arachidonic acid; PKC, protein kinase C; NDGA, nordihydroguaretic acid.

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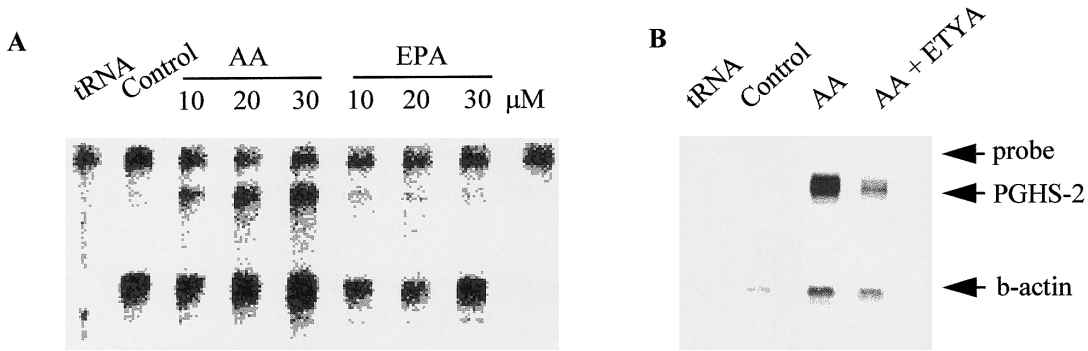

FIG. 1. (A) Effects of arachidonic acid (AA) and eicosapentaenoic acid (EPA) and (B) of AA in the presence of eicosatetraenoic acid (ETYA), an inhibitor of AA utilization, on PGHS-2 mRNA levels in quiescent IEC-18 cells. Total RNAs (10 μg) from serum-deprived IEC-18 cells treated with varying concentrations of AA or EPA or AA (10 μM) in the presence of ETYA (50 μM) for 1 h, were subjected to RNase protection assay as described in methods. The antisense probes for rat PGHS-2 and β-actin comigrate at the same position (200 nt). The protected fragments for rat PGHS-2 and β-actin were 176 nt and 127 nt respectively.

Cell culture and drug treatments. Rat intestinal epithelial primary cells, IEC-18 (American Type Culture Collection, Rockville, MD) were cultured in Dulbecco's Modified Eagle's medium with 10% fetal bovine serum and antibiotics, penicillin (10 U/ml) and streptomycin (10 μg/ml) in a humidified atmosphere containing 5% CO_2 at 37°C. Confluent primary cell cultures of IEC-18 (passages 16-20) were rendered quiescent by maintaining them in DMEM containing 0.5% fetal bovine serum for 24 h. Drugs and other chemicals were added to the cultures as described in the figure legends. Rat PKCα antisense oligonucleotide: 5′ GGC GTT GCC CAT CGC 3′ (flanking the ATG start site) and the control oligonucleotide (randomized sequence): 5′ ATC CTC GAC CCT TGG 3′ were added to the cells at 1 μM in DMEM containing 0.5% serum for 24 h.

RNase protection assays. The monolayers of cells were washed once with ice-cold phosphate-buffered saline (PBS) and the total RNA was extracted by using the acid phenol guanidine isothiocyanate method (11). The RNA was quantified by measuring the absorbance at 260 nm. [^{32}P]-Labelled cRNA probes for rat PGHS-2 and rat β-actin (used as control for input RNA) were prepared using an *in vitro* transcription kit.

Aliquots of the total RNAs were subjected to RNase protection assays according to a published protocol (12) with minor modifications. Briefly, 5–10 μg of total RNA was incubated overnight at 50°C with 5×10^4 cpm of cRNA probes in 20 μl of hybridization buffer (80% deionized formamide, 40 mM PIPES, pH 6.8, 1 mM EDTA and 0.4 M NaCl). The RNA hybrids were digested in 200 μl of digestion buffer (10 mM Tris- HCl, pH 7.5, 5 mM EDTA, and 0.3 M NaCl) containing ribonuclease A (10 μg/ml) and RNase T_1 (250 units/ml) for 30 min at 25°C. Proteinase K treatment followed by precipitation of protected fragments was conducted exactly as described (12). The protected RNA fragments were resolved on urea-8% polyacrylamide gels and the bands were visualized by phosphorimaging (Molecular Dynamics, Sunnyvale, CA).

Western blot analysis of PKCα. Monolayers of cells were scraped, collected and washed twice in ice-cold PBS. Total cell lysates were prepared by lysing cells directly in sample buffer (125 mM Tris-HCl, pH 6.8, 2% [w/v] SDS, 10% [v/v] glycerol). In order to prepare membranes, cells were resuspended in a hypotonic buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 10 μg/ml each of leupeptin and pepstatin, 0.2 mM benzamidine, 1 mM PMSF and 1 mM DTT) and lysed by brief sonication. Cell lysates were then centrifuged at 12,000 g for 10 min. Membranes were pelleted by centrifugation at 100,000 g for 1 h. The membrane pellets were solubilized in sample buffer as described above. Protein concentration was determined by using the Bio-Rad DC protein assay reagent. Aliquots of total protein (25-50 μg) were denatured in sample buffer containing 10% β-mercaptoethanol and 0.1 mg/ml bromophenol blue by boiling for 5 min before loading on SDS-polyacrylamide gels. Electrophoretic transfer of the proteins to PVDF membranes, successive incubations with PKCα specific antiserum and horse radish peroxidase-conjugated anti-rabbit IgG antibodies were conducted as described previously (13). The immunoreactive bands were visualized by using the enhanced chemiluminescence kit as instructed by the manufacturer.

RESULTS

Incubation of quiescent monolayers of IEC-18 cells with 10-30 μM arachidonic acid for 1 h increased PGHS-2 mRNA levels in a dose-dependent manner (Fig. 1). This effect was not produced by eicosapentanoic acid (precursor of leukotriene synthesis) at same concentrations (Fig. 1A), indicating an effect specific to AA. Furthermore, treatment of cells with 50 μM eicosatetraenoic acid (ETYA), an inhibitor of AA utilization, prevented the AA-induced increase in PGHS-2 mRNA levels (Fig. 1B). The increment in PGHS-2 mRNA abundance was due to an increase in transcription, as actinomycin D (10 μg/ml) inhibited PGHS-2 induction by AA (data not shown).

To investigate if the effects of AA are due to prostaglandins, 10 μM of each of PGE_2, 16,16-dimethyl PGE_2 (PGE_2 analogue), fenprostalene ($PGF_{2\alpha}$ analogue), PGD_2, carbaprostacyclin (PGI_2 analogue), and a thromboxane mimetic (U46619) were added to cells and PGHS-2 mRNA levels were compared. As shown in Fig. 2A, none of the compounds elicited the synthesis of PGHS-2 mRNA. However, PMA (0.1 μM), a PKC activator, induced PGHS-2 transcription in IEC-18 cells, whereas forskolin (0.1 μM), an adenylate cyclase activator, did not.

In agreement with the lack of induction of PGHS-2 mRNA by prostaglandins, treatment of IEC-18 cells with 10-100 μM each of cyclo-oxygenase inhibitors, diclofenac (Fig. 2B) or naproxen and ibuprofen (data not

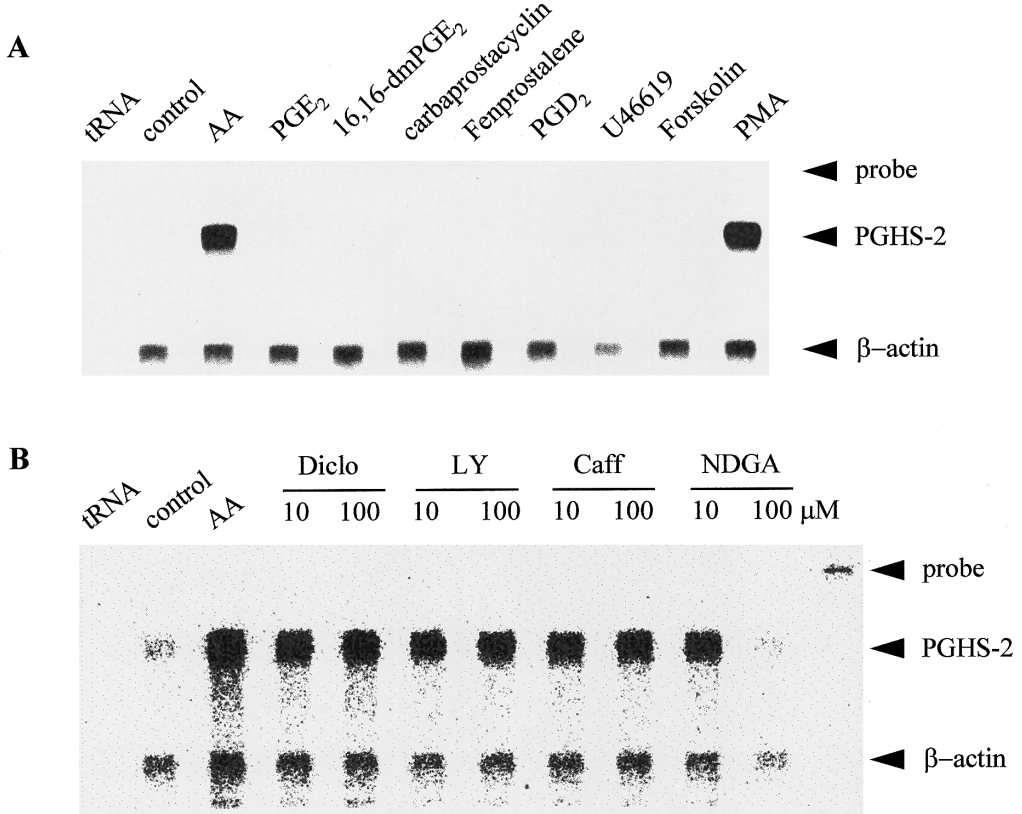

FIG. 2. (A) Effects of prostanoids (10 μM each) and protein kinase A and C stimulants (0.1 μM each) on PGHS-2 transcription in serum-deprived IEC-18 cells. (B) Effects of inhibitors of major pathways of AA metabolism on PGHS-2 mRNA levels measured by RNase protection assay: cyclooxygenase inhibitor, diclofenac (Diclo) and lipoxygenase inhibitors, LY233569 (LY) and caffeic acid (Caff), lipoxygenase/CYP450 monoxygenase inhibitor, nordihydroguaretic acid (NDGA). Aliquots of total RNA from IEC-18 (10 μg) were subjected to RNase protection assay using rat-specific cRNA probes as described in methods.

shown) or LY233569 (5-lipoxygenase inhibitor) and caffeic acid (non-selective 5-, 12- and 15-lipoxygenase inhibitors at the doses used), did not prevent the induction of PGHS-2 mRNA by AA. However, NDGA (a lipoxygenase/monoxygenase inhibitor) at 100 μM completely suppressed AA-induced PGHS-2 mRNA synthesis.

To test if CYP450 monoxygenase metabolism of AA is involved in PGHS-2 transcription in IEC-18 cells, the cells were treated with three distinct inhibitors of CYP450 enzymes before the addition of AA. As shown in Fig. 3A, All three inhibitors, ketoconazole, miconazole and metyrapone, prevented the induction of PGHS-2 transcription by AA. Of the CYP450 monoxygenase products of AA, epoxyeicosatrienoic acids (EETs) have been proposed to induce transcription of the immediate-early genes c-fos and Egr-1 (9). Hence, we examined if EETs can stimulate PGHS-2 transcription. Indeed, ±14,15-EET increased PGHS-2 mRNA levels (Fig. 3B); other regioisomers of EETs did not elicit PGHS-2 transcription. To test if ±14,15-EET could rescue AA-induced PGHS-2 mRNA synthesis in cells pretreated with CYP450 inhibitors, 10 μM of ±14,15-EET was added along with AA. As shown in Fig. 3A, addition of ±14,15-EET produced similar PGHS-2 mRNA levels as AA in cells pretreated with all three inhibitors, ketoconazole, miconazole and metyrapone.

PGHS-2 transcription in IEC-18 cells was elicited by PMA but not by forskolin (Fig. 2A), indicating that PKC activation is involved in ±14,15-EET-mediated signal transduction. To test the role of PKC, IEC-18 cells were pretreated with bisindolyl maleimide (BIM), a specific inhibitor of all PKC isozymes, before adding 10 μM AA. As shown in Fig. 4A, BIM reduced PGHS-2 transcription in a dose-dependent manner. Furthermore, addition of an antisense oligonucleotide (15-mer encompassing the ATG start site of rat PKCα) to cells at 1 μM for 24 h, resulted in 50-60% reduction in PKCα immunoreactive bands (75 kDa), but not in those that were treated with a control oligonucleotide (Fig. 4B), thus disclosing the efficacy of the anti-PKCα oligonucleotide in reducing PKCα protein expression. Moreover, anti-PKCα oligonucleztide also reduced ±14,15-EET-induced PGHS-2 mRNA, compared to the control oligonucleotide (Fig. 4D). Consistent with membrane translocation of PKC in the proximal stage of signal transduction, treatment of quiescent IEC-18 cells with

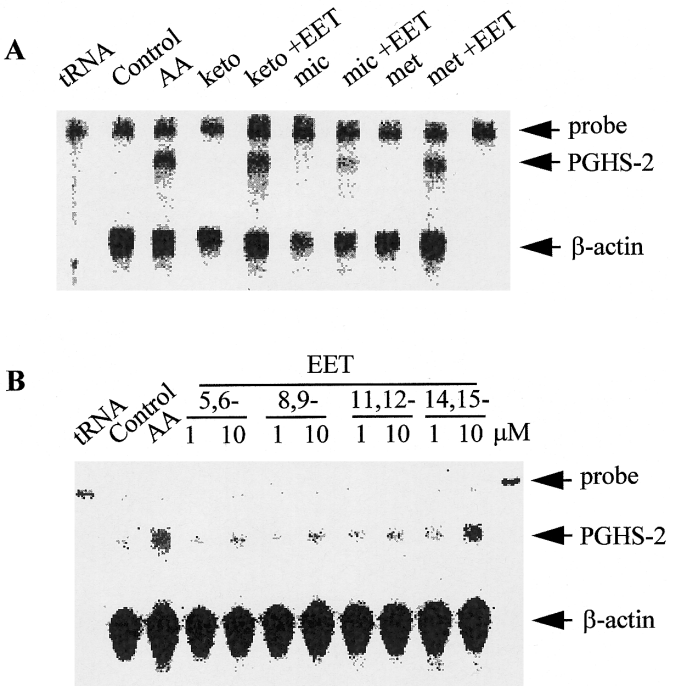

FIG. 3. (A) Inhibition of AA-induced PGHS-2 mRNA synthesis by 10 μM each of CYP450 monoxygenase blockers, ketoconazole (keto), miconazole (mic), and metyrapone (met). (±)14,15-EET was added at 10 μM where indicated. (B) Effects of (±)5,6- , (±)8,9- , (±)11,12-, and (±)14,15-EET methyl esters on PGHS-2 transcription. Total RNAs (5 μg) from treated cultures were subjected to RNase protection assay as described in methods.

10 μM of 14,15-EET for 10 min resulted in increased content of immunoreactive PKCα in the particulate fraction (Fig. 4C).

DISCUSSION

PGHS-2 has been suggested to play important roles in oxidant stress, inflammation, organogenesis and oncogenesis (3, 4, 14, 15). Numerous agents and inflammatory mediators which stimulate PGHS-2 expression also cause release of AA (1, 2); but the role and mode of action of AA in this process is not known. The data presented here indicate that AA induced the synthesis of PGHS-2 mRNA in rat intestinal epithelial cells requiring its metabolism by the monoxygenase pathway, but not by cyclooxygenases or by lipoxygenases. One of the AA-epoxygenase products, ±14,15-EET stimulated PGHS-2 transcription.

Specific CYP450 enzymes catalyze monoxygenation of AA leading to (a) epoxidation giving rise to four regioisomers, 5,6-, 8,9-, 11,12- and 14,15-EETs, which in turn are converted by epoxide hydrolases to corresponding dihydroxyeicosatrienoic acids (DiHETEs), (b) allylic oxidation to produce six regioisomers, 5-, 8-, 9-, 11-, 12-, and 15-HETEs, and (c) ω/ω1 hydroxylation to result in 19- and 20-HETEs (16, 17). Though CYP450 monoxygenases capable of AA oxidation are present in all tissues and cells, the spectrum of monoxygenase metabolites produced in various tissues and cells may depend upon the distribution of these enzymes and the type of stimulus. Since EETs are produced in response to various stimuli which release AA and have been proposed to activate immediate early genes, c-fos and Egr-1 (9), we investigated if these epoxygenase products could activate PGHS-2 transcription. Among the EETs, (±)14,15-EET elicited PGHS-2 mRNA expression; though these findings do not exclude a role for HETEs, but these seem to be unlikely mediators (9). Furthermore, the epoxide hydrolase inhibitor, 4-phenyl chalcone oxide (18) potentiated the ability of AA to induce PGHS-2 mRNA (data not shown), strongly implicating EETs, but not their vicinal diols, as the critical product/s involved in PGHS-2 induction.

PKC activation is an integral part of signal transduction initiated by many agents (19) including AA. However, this AA-induced PKC activation is not uniformly observed (20) nor is PKC systematically involved in stimulation of immediate early genes by AA or other agents (21, 22). The dependence of PGHS-2 transcription on a specific protein kinase is both agonist- and cell type-specific: for instance, on PKA in response to parathyroid hormone in osteoblastic cells (23), on PKC in response to PDGF in mesangial cells (24), and on both PKC and PKA in response to LH and GnRH in preovulatory follicles (25). Direct stimulation of adenylate cyclase by forskolin did not elicit PGHS-2 transcription in these cells, hence cAMP-dependent protein kinases may not participate in the signal transduction initiated by AA and 14,15-EET.

To ascertain whether PKC is involved in PGHS-2 induction by AA, we used a specific but a non-selective PKC blocker, BIM; this drug reduced PGHS-2 mRNA levels, suggesting a role for PKC in this process. Evidence for the role of a specific isozyme of PKC in this function was obtained by using PKCα-specific antisense oligonucleotide which reduced immunoreactive PKCα in treated cells and also the response of (±)14,15-EET in eliciting PGHS-2 mRNA synthesis. A role for PKCα in interleukin 1α-induced PGHS-2 transcription in endothelial cells was demonstrated based on the use of antisense oligonucleotides to PKCα (26). In addition, increased membrane localization of PKCα in response to (±)14,15-EET supporting a role for PKCα in the signal transduction. Taken together, our observations point to a pathway initiated by the epoxygenase product of AA, (±)14,15-EET, involving phosphorylation events by PKCα, resulting in PGHS-2 transcription.

In summary, PGHS-2 transcription in rat intestinal epithelial cells was elicited by its own substrate, AA, via its metabolism by the monoxygenase pathway. These data suggested a novel concept whereby induction of PGHS-2 expression by AA can occur not only

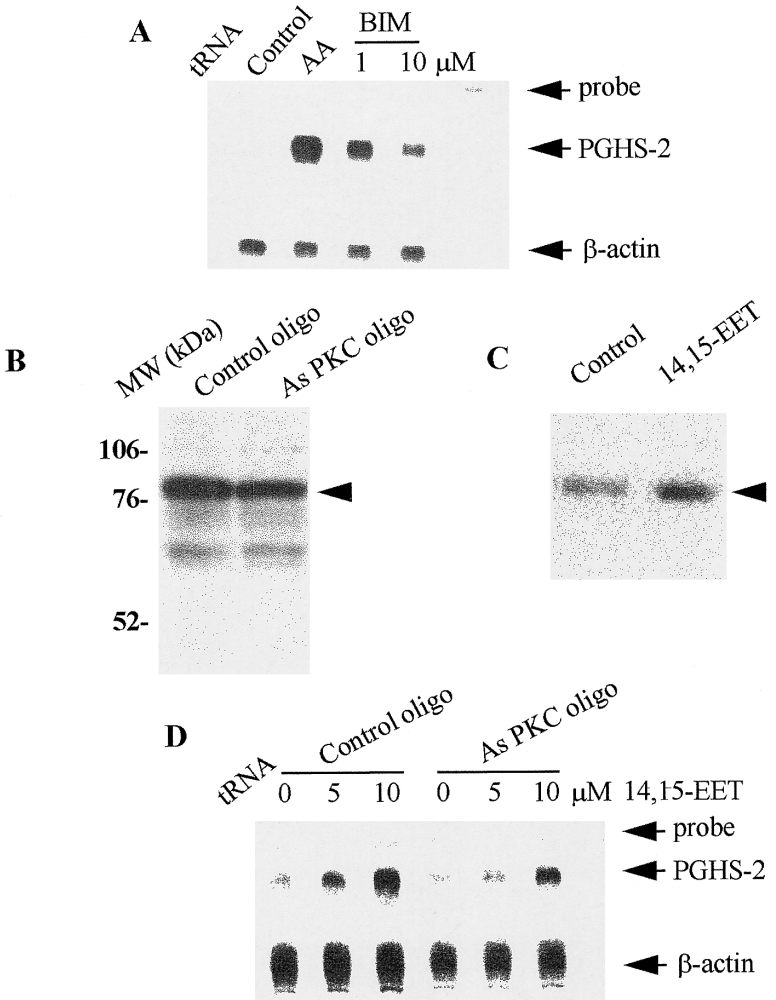

FIG. 4. Role of PKCα in AA-induced PGHS-2 transcription. (A) Effect of PKC-specific inhibitor, bisindolylmaleimide-I (BIM) on PGHS-2 mRNA expression by AA. (B) Immunoreactive PKCα (arrow indicates PKCα position) in control and anti-PKCα oligonucleotides (As PKC oligo) [1 μM each] treated IEC-18 cells. (C) Immunoreactive PKCα in particulate fraction of cells treated with 10 μM (±) 14,15-EET (arrowhead indicates PKCα position). (D) PGHS-2 mRNA levels elicited by 5-10 μM (±)14,15-EET in cells treated with control and anti-PKCα oligonucleotides (1 μM) as described in methods.

via products of the cyclooxygenase pathway, PGs [autologous regulation] (10) but also depend upon metabolites of the other AA pathways (heterologous regulation). It is interesting to note that various conditions which lead to PGHS-2 transcription, most notably oxidant stresses (27), also acutely stimulate EET production (28). This diverse regulation of PGHS-2 by different products of AA may be relevant in determining tissue response in oxidative stress, inflammation and other pathologies as well as during development (29).

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