Regulation of Cyclooxygenases by Protein Kinase C

EVIDENCE AGAINST THE IMPORTANCE OF DIRECT ENZYME PHOSPHORYLATION*

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Cyclooxygenases (COXs) are key prostaglandin biosynthetic enzymes. While COX-1 expression is largely constitutive, COX-2 is highly regulated by cytokines, growth factors, and tumor promoters, such as the protein kinase C (PKC) activator, phorbole 12-myristate 13-acetate (PMA). While phosphorylation of transcription factors may regulate COX transcription, the existence of PKC consensus sequences suggests that direct enzyme phosphorylation might also regulate differential expression of the enzymes. Nevertheless, phosphorylation of both human recombinant COX-1 and COX-2 by rat brain PKC in vitro was minimal, as was phosphorylation of peptides based on PKC consensus sequences in COX-1 (less than 4% of the phosphorylation of the PKC-α pseudosubstrate peptide). Similarly, phosphorylation of the corresponding COX-2 peptides was not observed using either the phosphocellulose paper absorption method or electrospray mass spectrometry.

MEG-01 and NIH 3T3 cells were labeled with [32P]orthophosphate to investigate COX phosphorylation in vivo. COX-2 synthesis was induced by PMA (100 nM) or serum stimulation in NIH 3T3 cells. COX-1 was expressed constitutively in MEG-01 cells. Specific polyclonal antibodies raised against sequences of human COX-1 (Ala24–Cys35) and COX-2 (Asn 580–Lys598) were used for immunoprecipitation. Neither COX-1 nor COX-2 was phosphorylated in vivo, irrespective of the presence of a phosphatase inhibitor (1 μM okadaic acid).

Although COX-1 and COX-2 are differentially regulated, no differences were observed in terms of susceptibility to phosphorylation by PKC either in vitro or in vivo. Despite regulated expression of COX-2 by PMA and the existence of consensus sequences for PKC phosphorylation, it appears that it is an unfavorable substrate for this enzyme.

The unstable metabolites of arachidonic acid (prostaglandins, leukotrienes, and epoxyeicosatrienoic acids) are thought to be of importance in the modulation of complex biological responses, such as vascular homeostasis, ion transport, and inflammatory reactions in vivo (1). The major rate-limiting enzymes in prostaglandin formation are the cyclooxygenases (COXs).1 Two COX genes have been cloned. COX-1 is generally expressed constitutively; however, its expression may be regulated by sex hormones and certain cytokines (2). COX-2, by contrast, is rarely expressed constitutively but is highly regulated by tumor promoters, growth factors, and cytokines (1). Given this discrepancy between the enzymes, COX-2 is thought to be the form that predominates in inflammatory states (3–5) and, perhaps, in cancer (6, 7). Consequently, considerable effort has been invested in the development of selective inhibitors of this isoform (3, 5, 8).

The two human COX isoforms exhibit substantial (61%) amino acid identity. Much of the sequence disparity resides in the amino and carboxy-terminal ends of the enzymes (9, 10). Among the residues conserved are those critical to both peroxidase and cyclooxygenase activities and the serine target for aspirin acetylation (11). Resolution of the crystal structures of both enzymes (12, 13) confirms a more accommodating active site in COX-2, which may explain a differential substrate affinity and a different profile of products formed by the enzymes following acetylation by aspirin (14, 15).

The primary sequence of the COX cDNAs suggests several sites for posttranslational modification. Both enzymes contain multiple glycosylation sites (9, 16, 17). They may also be subjected to phosphorylation. Phosphorylation/dephosphorylation reactions are employed widely to regulate protein function (18). For example, the cytosolic phospholipase A2 is phosphorylated in a mitogen-activated protein kinase-dependent manner (19), although the functional consequences of this modification are unclear (20). The COX enzymes, particularly COX-2, are also potential targets for this form of regulation. The tumor promoter, phorbole 12-myristate 13-acetate (PMA), is a potent activator of protein kinase C (PKC) (21) and induces COX-2 expression in many cell types (1). Potential serine and threonine targets for PKC exist in both COX enzymes; however, the 18-amino acid carboxy-terminal extension of COX-2 contains several such target residues, including a PKC consensus sequence (22). Consistent with the possibility that PKC might regulate COX-2 expression, specific inhibition of the kinase or its depletion (23) prevents COX-2 induction by PMA or interleukin-1α in endothelial cells.

Given these observations, we wished to address the hypothesis that COX-2 was directly phosphorylated by PKC. Despite its regulation by PMA and the existence of potential target sequences for PKC, COX-2 is an unfavorable substrate for PKC, even during induction of the enzyme by PMA in vivo.

EXPERIMENTAL PROCEDURES

Materials

Aprotinin, leupeptin, pefabloc, soybean trypsin inhibitor, and the protein kinase A catalytic subunit were obtained from Boehringer endoperoxide synthase; BSA, bovine serum albumin; DTT, dithiothreitol; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PAGE, polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography; DMEM, Dulbecco's modified Eagle's medium; PGF2α, prostaglandin F2α.
In Vitro Phosphorylation Assays

In vitro phosphorylation of COX-1 and COX-2 was carried out as described previously (24) with some modifications. Two μg of human recombinant COX-1 or COX-2 (kindly donated by Dr. Jim Barnett of Syntex Laboratories; Ref: 25) or 2 μg of histone H1, as a positive control, was used as a substrate for purified rat brain PKC in 50 μl reactions containing 20 mM HEPES, pH 7.4, 10 mM MgCl₂, 0.5 mM CaCl₂, 20 μM ATP, 5 mM dithiothreitol (DTT), 8 μM/ml phosphatidyserine, 1 μM PMA, 2 μCi of [γ-32P]ATP (6000 Ci/mmol, 10 μCi/ml), and 1 μl of PKC.

Reactions were carried out at 37°C for the indicated times and terminated by trichloroacetic acid precipitation with 50 μl of ice-cold 10% trichloroacetic acid. After incubation for 30 min, the reactions were centrifuged at 12,000 × g for 10 min, and the pellets were dissolved in Laemmli sample buffer (26). Proteins were analyzed by SDS-PAGE (8% acrylamide). The gels were stained with Coomassie Blue, and the bands corresponding to COX were analyzed by densitometry. The gels were then dried and autoradiographed. The bands corresponding to COX were excised from the dried gel and counted for radioactivity, correcting for the amount of COX loaded in each lane. Accurate quantification of H1 phosphorylation is not possible in these experimental conditions, since the trichloroacetic acid precipitation of H1 is not complete; for this reason, the supernatants of H1 samples were also loaded in the gel. Quantitation of H1 phosphorylation was accomplished by summing the radioactivity of the precipitated sample with that of its supernatant.

Phosphorylation of COX-1 and COX-2 peptides containing the putative consensus sequences for PKC-mediated phosphorylation was also studied. The peptides had the following sequences: TWWLRNSLRPSP (COX-1; Thr75–Pro85); HQFKKTSGKMG (COX-1; His207–Gly217); VI- pseudosubstrate peptide (RFARKGSLRQKNV), as a control, were performed using a phosphocellulose paper absorption method (27, 28).

Reactions containing 12.5 mM HEPES, pH 7.3, 5 mM MgCl₂, 5 mM EGTA, 20 μM ATP, 2 μCi of [γ-32P]ATP, and 0.5 μl of protein kinase A catalytic subunit. The reactions were allowed to proceed at 37°C for different times (0, 15, 30, 45, and 60 min) and then terminated by trichloroacetic acid precipitation, as described above.

Reverse Phase HPLC/Electrospray Mass Spectrometry

Phosphorylation of the peptides was carried out as described above; at the end of the incubation time, samples were put on ice and then frozen and stored at −20°C until the analysis. A 20-μl aliquot of a 30 nM solution was separated using a 1.0 mm × 25-cm Phenomenex Primesphere C18 column (Phenomenex, Torrance, CA). The HPLC system (Applied Biosystems 1405B dual syringe pump; Applied Biosystems, Foster City, CA) was operated at a flow rate of 50 μl/min starting with a mobile phase composition of 90% solvent A (0.05% trifluoroacetic acid in water) and 10% solvent B (0.05% trifluoroacetic acid, 90% acetonitrile in water), which was increased linearly to 40% solvent B in 30 min. The separation was monitored by a UV detector (Applied Biosystems 756A UV detector; Applied Biosystems) set at 214 nm and a VG Quattro II mass spectrometer (Fison Instruments, Beverly, MA) equipped with a coxial electrospray probe and triple quadrupole analyzer further downstream. The sampling cone voltage was set to 30 V, the capillary voltage to 3.5 kV, and the source temperature to 75°C. The mass spectrometer was scanned continuously from m/z 300 to 1000 with a scan duration of 5 s. The relative amount of the peptide phosphorylation was calculated from the selected ion chromatography peak area obtained in the liquid chromatography/mass spectrometry experiments.

Measurement of COX-1 and COX-2 Activity in Vitro

PGF₂α production was measured after incubation of COX-2 with arachidonic acid to determine if PKC phosphorylation could affect enzymatic activity. Two μg of recombinant COX-2 were incubated with PKC as described above, with the difference that no [γ-32P]ATP was added. DTT was also omitted from the reaction mixture, because it impairs COX activity. The absence of DTT only slightly decreased phosphorylation, as detected by autoradiography, in a selected experiment in which [γ-32P]ATP was added to the reaction mixture. Phosphorylation reactions were stopped after a 1-h incubation at 37°C; half of the sample (25 μl) was mixed with 225 μl of a buffer containing 50 mM Tris-HCl, pH 8, 1 mM phenylmethylsulfonyl fluoride hydrochloride (pefabloc), 1 mM NaF, 1 mM sodium orthovanadate, and the bands corresponding to COX were analyzed by densitometry/mass spectrometry using a derivatized 18O₂-labeled Primesphere C18 column (Phenomenex, Torrance, CA). The HPLC system was monitored by a UV detector (Applied Biosystems 1405B dual syringe pump; Applied Biosystems, Foster City, CA) was operated at a flow rate of 50 μl/min starting with a mobile phase composition of 90% solvent A (0.05% trifluoroacetic acid in water) and 10% solvent B (0.05% trifluoroacetic acid, 90% acetonitrile in water), which was increased linearly to 40% solvent B in 30 min. The separation was monitored by a UV detector (Applied Biosystems 756A UV detector; Applied Biosystems) set at 214 nm and a VG Quattro II mass spectrometer (Fison Instruments, Beverly, MA) equipped with a coxial electrospray probe and triple quadrupole analyzer further downstream. The sampling cone voltage was set to 30 V, the capillary voltage to 3.5 kV, and the source temperature to 75°C. The mass spectrometer was scanned continuously from m/z 300 to 1000 with a scan duration of 5 s. The relative amount of the peptide phosphorylation was calculated from the selected ion chromatography peak area obtained in the liquid chromatography/mass spectrometry experiments.

In Vivo Phosphorylation Experiments

COX-2—NIH 3T3 fibroblasts were grown in 60-mm culture dishes in Dulbecco's modified Eagle's medium (DMEM) containing 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum at 70% confluence and then shifted to DMEM with 1% serum for 48 h. Cells were washed and incubated for 3 h at 37°C with 20 μg as orthophosphate (aqueous solution, carrier-free, specific activity 10 μCi/ml), 0.3 μCi/ml in DMEM without sodium phosphate. COX-2 synthesis was induced either by PMA (100 nM) stimulation of starved cells or by stimulation of the cells with 20% serum in the presence or absence of 100 μM PMA or of the phosphatase inhibitor, 0.5 μl of protein kinase A catalytic subunit (1 μM). Me2SO was added at the same concentration (0.1%), irrespective of whether the cells were treated with PMA. Cells were washed in phosphate-buffered saline and lysed with 150 μl of ice-cold lysis buffer (50 mM Tris-HCl, pH 8, 1 mM EDTA, 1% Nonidet P-40, 0.5% SDS, 0.5 mM DTT, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 0.5 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (pefabloc), 1 μg/ml NaF, 1 mM orthovanadate) at the end of the incubation period. Cells were scraped, and the DNA was sheared by passing the cell lysates through a 21-gauge needle. The lysates were then centrifuged for 15 min at 12,000 × g at 4°C, and the protein concentration in the supernatant was determined using a microbicinchonic acid assay (Pierce) with BSA as a standard. Immunoprecipitation was carried out as we have previously described (17), using a specific polyclonal antibody, kindly donated by Dr. Jacques Maclouf. Ten mg of protein A-Sepharose CL-4B was incubated with 25 μl of a specific rabbit polyclonal antibody raised against the C-terminal sequence of human COX-2 (Asn590–Lys594) in 500 μl of buffer (150 mM NaCl, 50 mM phosphate buffer, pH 7.4). After an overnight incubation at 4°C, protein A-Sepharose was washed five times with radioimmune precipitation buffer buffer (50 mM Tris-HCl, pH 8,
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150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS) and incubated for 3 h at 4°C with cell lysates (600 μg of the proteins) in 500 μl. After five washings in radioimmune precipitation buffer, protein A-Sepharose was mixed with Laemmli buffer (26) under reducing conditions, heated for 5 min at 95°C, and subjected to 10% acrylamide SDS-PAGE. Thirty μl of the supernatants of the immunoprecipitation reactions were also run in the gel. The gel was transferred onto a nitrocellulose membrane (Schleicher & Schuell, Göttingen, Germany) with a semidry transfer unit (Hoefer Scientific Instruments, San Francisco, CA). Transfer was performed in 25 mM Tris, 192 mM glycine buffer, pH 8.3, containing 0.01% SDS and 20% methanol for 1.5 h at 200 mA. Blots were stained with 0.4% Ponceau Red in 0.3% trichloroacetic acid and exposed to Kodak X-omat film at ~80°C. Immunoblotting was performed to assess the effectiveness of COX-2 immunoprecipitation. Western blots were saturated overnight in 5% nonfat dry milk in Tris-buffered saline (50 mM Tris-HCl, pH 7.4, 250 mM NaCl, 0.1% Tween 20) and incubated with a mouse monoclonal antibody (1:2000) in Tris-buffered saline containing 0.1% Tween 20 and 1% milk for 1 h at room temperature. Blots were washed in the same buffer without milk and incubated with a peroxidase-conjugated donkey anti-mouse IgG diluted 1:5000 in the buffer containing milk for 1 h. After washing to eliminate the excess of antibody, enhanced chemiluminescence substrates were used to reveal positive bands according to the manufacturer’s instructions, and bands were visualized after exposure to Kodak Bio-MAX films.

COX-1—COX-1 phosphorylation was assessed in MEG-01 cells rather than NIH 3T3 fibroblasts for two reasons. First, we found that MEG-01 cells express a higher amount of COX-1 compared with NIH 3T3 cells in preliminary immunoblotting experiments. Second, immunoprecipitation of NIH 3T3 cell lysates with a specific rabbit polyclonal antibody raised against the N-terminal sequence of human COX-1 (Ala24–Cys35), was suboptimal, possibly due to a low affinity of the antibody for the native murine protein.

MEG-01 cells were grown in RPMI 1640 containing 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum. Seven million cells/sample were washed and labeled in 3 ml of DMEM without sodium phosphate with 0.3 μCi/mM [32P]ATP as orthophosphate as described above, in the presence or in the absence of 100 μM PMA and 1 μM okadaic acid. 0.1% Me2SO was added to the control sample. Cells were incubated 3 h at 37°C, washed, and lysed in Nonidet P-40 buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 1% Nonidet P-40) containing protease and phosphatase inhibitors as described above. Nonidet P-40 buffer was chosen for its low detergent concentration to retain the antibody binding sites and to avoid solubilization of background proteins. For the lysis of the cells, double immunoprecipitation was carried out using a rabbit polyclonal antibody raised against the N-terminal sequence of human COX-1 (Ala24–Cys35), kindly donated by Dr. Jacques Maclouf. The immunoprecipitation steps were essentially those described above, with the exception that protein A-Sepharose was washed three times in Nonidet P-40 buffer instead of in radioimmune precipitation buffer; the first immunoprecipitation was performed for about 15 min, then precipitated proteins were eluted in Tris-buffered saline (50 mM Tris-HCl, pH 8.3, containing 0.01% SDS and 20% methanol for 1.5 h at 200°C). The eluates were precipitated with 0.1% sodium dodecyl sulfate and 10% trichloroacetic acid and then washed twice with 10% trichloroacetic acid. Each precipitate was dissolved in 50 μl of sodium dodecyl sulfate sample buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 1% Nonidet P-40) containing 5% β-mercaptoethanol, heated for 5 min at 95°C, and subjected to 10% acrylamide SDS-PAGE, and the gels were autoradiographed. Each panel is representative of four experiments. Molecular mass markers are in kilodaltons. Apparent time-dependent phosphorylation of bands at 70 kDa is observed.

Table I

|     | COX-1          | COX-2          | Histone          |
|-----|----------------|----------------|------------------|
|     | 186 ± 17       | 97 ± 15        | 664 ± 83         |

or COX-2 by PKC in concentrations of 0.3–10 μg/50 μl when the incubation period was prolonged for up to 30 min. Incubation of COX-2 with PKC did not modify enzyme activity as assessed by the formation of PGE2α, (data not shown).

To assess phosphorylation of the enzymes in more detail, we synthesized peptides based on the putative consensus sequences for PKC-dependent phosphorylation (22). Phosphorylation of the peptides was first evaluated by phosphocellulose paper absorption. The PKC-α pseudosubstrate peptide was highly phosphorylated by PKC, with a Km of 6.5 ± 1.5 μM (n = 3). The COX-2 peptides (3–100 μM), by contrast, were not phosphorylated by PKC. Similarly, phosphorylation of two of the four COX-1 peptides (HQFFKTSGKMG and VIRES-REMRL) was not observed. The other two COX-1 peptides that we studied (TWLRNSLRPSP and IVKTATLKKLV) were slightly phosphorylated by PKC. However, the radioactivity associated with these peptides was very low; it accounted for less than 4% of the radioactivity associated with the PKC-α pseudosubstrate peptide used at the same concentration (Table II). Given this observation and that we failed to reach a plateau of phosphorylation at concentrations of peptides up to 1.3 mM, we could not calculate an accurate K. The phosphorylation of peptide TWLRNSLRPSP (10 μM) remained low (0.4% of that observed in the positive control), even when the period for phosphorylation was extended to 30 min.

Peptides were also analyzed by electrospray mass spectrometry. The phosphorylation of the PKC-α pseudosubstrate peptide could be readily observed with this method. When the peptide was preincubated with PKC, a shift of the molecular mass (from 1559.6 to 1639.8 daltons) consistent with the addition of one phosphate group was observed in 85% of the molecules. By contrast, phosphorylation of the two COX-2 peptides or of two of the COX-1 peptides was not detected.

Consistent with the phosphocellulose paper absorption method, phosphorylation of two COX-1 peptides was also detected by electrospray mass spectrometry (Fig. 2). Thus, the molecular mass of TWLRNSLRPSP increased from 1325.2 to 1405.2 daltons in the presence of PKC, and the molecular mass of IVKTATLKKLV increased from 1212.4 to 1292.8 daltons.

FIG. 1. Time course of COX-1 and COX-2 phosphorylation by PKC in vitro. Two μg of recombinant COX-1 (upper panel) or COX-2 (lower panel) were used as substrates for PKC in the presence of [γ-32P]ATP. Reactions were carried out for 0, 15, 30, 45, and 60 min and terminated by trichloroacetic acid precipitation. Samples were analyzed by SDS-PAGE, and the gels were autoradiographed. Each panel is representative of four experiments. Molecular mass markers are in kilodaltons. Apparent time-dependent phosphorylation of bands at 70 kDa is observed.
However, only 12 and 1.9% of the molecules were phosphorylated, respectively.

In Vivo Phosphorylation Experiments—We also studied the possible phosphorylation of COX-1 and COX-2 in vivo. To determine whether COX-1 and COX-2 were phosphorylated, we immunoprecipitated these enzymes from 32P-labeled MEG-01 and NIH 3T3 cells, respectively. No phosphorylated bands were detected in the immunoprecipitated NIH 3T3 cell lysates, either when COX-1 synthesis was induced by PMA (100 nM) in serum-starved cells (Fig. 3A, upper part) or by serum stimulation, even in the presence of PMA (100 nM), okadaic acid (1 μM), or a combination of both (Fig. 3B, upper part). Okadaic acid, a serine/threonine phosphatase inhibitor, was added to exclude a rapid dephosphorylation reaction, which could theoretically account for a lack of detectable phosphorylation. To verify that COX-2 was actually immunoprecipitated, immunoblotting was performed, demonstrating the presence of COX-2 in the immunoprecipitated samples (Fig. 3, A and B, lower parts). The efficiency of cell labeling was demonstrated by the high protein phosphorylation observed in the supernatants of the immunoprecipitated samples (Fig. 3, A and B, upper parts).

Similar results were obtained when COX-1 was immunoprecipitated with a specific antibody from 32P-labeled MEG-01 cell lysates; no radioactivity associated with COX-1 was observed, even in cells treated with PMA (100 nM) with or without okadaic acid (1 μM) (Fig. 4, upper panel). The effectiveness of the immunoprecipitation was again verified by immunoblotting (Fig. 4, lower panel).

**DISCUSSION**

COX-2 regulation is thought to be of importance in the elaboration of prostanoids in inflammation and, perhaps, in cancer. It is likely that both the expression and function of such an enzyme is highly controlled. This may include phosphorylation of transcription factors, such as the nuclear factor-κB (NF-κB), c-Jun, c-Fos, and CREB (31). Although promoter analysis of the COX-2 gene has been relatively limited, recognition motifs for NF-κB, the nuclear factor for interleukin-6 expression (NF-IL6), PMA, and cyclic AMP have been described in the human COX-2 gene (32). In addition to a role for phosphorylation in transcriptional activation of the COX-2 gene, it is possible that the enzyme would be subject to posttranslational modification by direct phosphorylation. Thus, precedent has been established for the biological significance of such modifications, most notably modulation of the interaction of heptahelical receptors with G proteins during receptor desensitization (33, 34). Additionally, enzyme function or localization may be modified by

**TABLE II**

|                  | 3 μM    | 10 μM   | 30 μM   | 100 μM
|------------------|---------|---------|---------|--------
| TWLRNSLRPSP      | 0.24 ± 0.1 (n = 6) | 0.37 ± 0.1 (n = 7) | 0.98 ± 0.1 (n = 9) | 3.98 ± 1 (n = 5) |
| IVKTATLKKLV      | 0.3 ± 0.3 (n = 2)  | 0.34 ± 0.08 (n = 4) | 0.65 ± 0.06 (n = 6) | 3.62 ± 1 (n = 3) |

**FIG. 2.** Electrospray mass spectrometry analysis. Electrospray mass spectra of PKC-α pseudosubstrate peptide (A), TWLRNSLRPSP (B), and IVKTATLKKLV (C) analyzed alone (upper parts) or after preincubation with PKC (lower parts). The x axis represents the mass:charge ratio (Da/e) of the doubly charged ions, and the y axis represents the relative intensity for each species. A molecular mass shift of 80 daltons, i.e. 40 for the doubly charged ions is consistent with phosphorylation.

**TABLE II**

|                  | 3 μM    | 10 μM   | 30 μM   | 100 μM
|------------------|---------|---------|---------|--------
| TWLRNSLRPSP      | 0.24 ± 0.1 (n = 6) | 0.37 ± 0.1 (n = 7) | 0.98 ± 0.1 (n = 9) | 3.98 ± 1 (n = 5) |
| IVKTATLKKLV      | 0.3 ± 0.3 (n = 2)  | 0.34 ± 0.08 (n = 4) | 0.65 ± 0.06 (n = 6) | 3.62 ± 1 (n = 3) |
phosphorylation. Thus, putative inhibitors of PKC and of tyrosine kinases prevented COX-2 induction by PMA and interleukin-1α. Depletion of PKC by prolonged incubation with PMA had a similar effect. The potent induction of COX-2 expression by short term incubation with PMA, known to result in PKC activation (4, 9, 10, 17) also suggests a role for PKC-dependent phosphorylation in COX-2 regulation. The possibility of direct phosphorylation of COXs was suggested by the existence of some conventional PKC consensus sequences in the enzymes, together with additional potential target serines and threonines outside the boundaries of these motifs. Intriguingly, five serines and four threonines are contained within the 18-amino acid carboxyl-terminal tail extension that is peculiar to COX-2. It also includes a conventional PKC consensus sequence.

Several methodological approaches were used in our study to document COX phosphorylation. The first of these was conventional: incubation of the COX enzymes with PKC in the presence of radioactive ATP, electrophoresis, and autoradiography. Apparent phosphorylation of both enzymes was detected. However, this was modest when compared with a positive control (histone H1) substrate. Furthermore, when using the phosphocellulose paper absorption method, we failed to detect phosphorylation of either enzyme. These experiments indicated that quantitative phosphorylation of the whole enzymes by PKC was, at best, minimal and suggest that misleading results could be obtained for such in vitro assays. Experiments with protein kinase A also suggested that COXs were unfavorable substrates for this kinase.

To address the potential for phosphorylation in more detail, we synthesized peptides based on the putative consensus sequences for PKC in both enzymes. We chose peptides in which a serine or a threonine is surrounded by positively charged residues, such as arginine or leucine (37). Using the phosphocellulose paper method, we failed to phosphorylate in all but two of the COX-1-based peptides (TWLRLNSLRPSF and IVKTATLKLTV). These were slightly phosphorylated. Again, the degree of phosphorylation was trivial by comparison with that of a PKC-α pseudosubstrate peptide. Internally consistent results were obtained with electrospray mass spectrometry,

![Image](https://example.com/image1.png)

**Fig. 3. In vivo phosphorylation experiments to assess COX-2 phosphorylation.** A, NIH 3T3 fibroblasts were serum-starved for 48 h; cells were then labeled with 32P with or without simultaneous stimulation with PMA (100 nM) to induce COX-2 synthesis. Cells were lysed, and COX-2 was immunoprecipitated. Immunoprecipitated samples (ip) and their supernatants (s) were analyzed by SDS-PAGE. The gel was then blotted onto a nitrocellulose membrane. The upper part shows autoradiography of the membrane, while the lower part shows the immunoblot of COX-2 protein. The lane indicated as C represents the control for immunoprecipitation, obtained incubating an aliquot of protein A-Sepharose with the anti-COX-2 antibody but not with the cell lysate. Therefore, it shows the position of the antibody used for the immunoprecipitation. Molecular mass markers are in kilodaltons. The arrow represents the position of authentic COX-2 standard. This figure is representative of three experiments. B, NIH 3T3 fibroblasts were serum-starved for 48 h and then labeled with 32P in a medium containing serum 20%. A high concentration of serum was used to induce COX-2 synthesis in this set of experiments. Incubations were carried out in the presence (+) or in the absence (−) of PMA (100 nM) and okadaic acid (1 μM). Cells were lysed, and COX-2 was immunoprecipitated. Immunoprecipitated samples (ip) and their supernatants (s) were analyzed by SDS-PAGE. The gel was then blotted onto a nitrocellulose membrane. The upper part shows autoradiography of the membrane, while the lower part shows the immunoblot of COX-2 protein. The lane indicated as C represents the control for immunoprecipitation, obtained incubating an aliquot of protein A-Sepharose with the anti-COX-1 antibody but not with the cell lysate. Therefore, it shows the position of the antibody used for the immunoprecipitation. Molecular mass markers are in kilodaltons. The arrow represents the position of authentic COX-1 standard. This figure is representative of two to three experiments.

**Fig. 4. In vivo phosphorylation experiments to assess COX-1 phosphorylation.** MEG-01 cells were labeled with 32P in the presence (+) or in the absence (−) of PMA (100 nM) and okadaic acid (1 μM). Cells were lysed, and COX-1 was immunoprecipitated. Immunoprecipitated samples were analyzed by SDS-PAGE. The gel was then blotted on a nitrocellulose membrane. The upper panel shows autoradiography of the membrane, while the lower panel shows the immunoblot of COX-1 protein. The lane indicated as C represents the control for immunoprecipitation, obtained incubating an aliquot of protein A-Sepharose with the anti-COX-1 antibody but not with the cell lysate. Therefore, it shows the position of the antibody used for the immunoprecipitation. Molecular mass markers are in kilodaltons. The arrow represents the position of authentic COX-1 standard. This figure is representative of three experiments.
which confirmed trivial phosphorylation of only the two COX-1 peptides by PKC.

Although these experiments suggested that both COX enzymes were unfavorable substrates for PKC-dependent phosphorylation in vitro, we wished to address the issue in vivo. Particularly, we were interested in investigating this possibility during COX-2 induction by PMA, a known stimulator of PKC (21). We did this in NIH 3T3 cells in the presence and absence of okadaic acid, a phosphatase inhibitor. Rapid activation of phosphatases (38) could theoretically confound the interpretation of such experiments. COX-2 was induced, either by PMA in serum-starved cells or by incubation with serum. However, immunoprecipitated COX-2 was not phosphorylated in either case, despite presumptive activation of endogenous PKC by PMA (39, 40). Similar results were obtained in MEG-01 cells for COX-1. Induction is not necessary in these cells, since COX-1 is expressed constitutively. However, immunoprecipitated COX-2 was not phosphorylated by PKC inhibition and depletion, our results indicate that COX-2 is an unlikely substrate for direct phosphorylation by this enzyme in vitro or in vivo. Should phosphorylation play a role in COX expression, it is likely to involve other kinases and/or act at the level of transcription.

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