Cyclooxygenase-2 Induction by Bradykinin in Human Pulmonary Artery Smooth Muscle Cells Is Mediated by the Cyclic AMP Response Element through a Novel Autocrine Loop Involving Endogenous Prostaglandin E$_2$, E-prostanoid 2 (EP2), and EP4 Receptors*

Received for publication, July 22, 2003, and in revised form, September 25, 2003
Published, JBC Papers in Press, September 29, 2003, DOI 10.1074/jbc.M307964200

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Bradykinin (BK) is an important mediator in several inflammatory and vascular diseases that acts in part via induction of cyclooxygenase-2 (COX-2). The mechanisms involved in BK-mediated COX-2 induction are unclear. Here we characterized the transcriptional mechanisms involved in human pulmonary artery smooth muscle cells. BK stimulated the activity of a transiently transfected 966-bp (−917 to +49) COX-2 promoter luciferase reporter construct. There was no reduction in BK-induced luciferase activity in cells transfected with COX-2 promoter constructs of 674, 407, 239, and 135 bp or constructs with mutated CCAAT/enhancer-binding protein- or NF-κB-binding sites. In contrast luciferase activity was reduced in cells transfected with a 407-bp COX-2 promoter fragment containing a mutated cAMP response element (CRE)-binding site, suggesting that the CRE binding site is critical. Electrophoretic mobility shift assays using oligonucleotides specific for the CRE-binding region of the COX-2 promoter and consensus oligonucleotides showed strong specific binding. Furthermore BK increased consensus cAMP-responsive luciferase reporter (p6CRE/luc)-mediated luciferase expression. CRE activation occurred by BK inducing cytosolic phospholipase A$_2$-mediated arachidonic acid release and rapid prostaglandin E$_2$ (PGE$_2$) production, thereby increasing cAMP. Indomethacin inhibited BK-induced PGE$_2$ production, cAMP accumulation, and CRE/luc reporter and COX-2 promoter luciferase activity. Exogenous PGE$_2$, and EP2 (ONO-AE1 259) and EP4 (ONO-AE1 239) PGE$_2$ receptor agonists mimicked the effect of BK. Collectively these studies indicate that COX-2 induction by BK in human pulmonary artery smooth muscle cells is mediated by the CRE through a novel autocrine loop involving endogenous PGE$_2$.

The kinin peptide bradykinin (BK)$^1$ is an important pro-inflammatory mediator associated with allergic and degenerative chronic inflammatory conditions such as asthma and rheumatoid arthritis (1, 2). BK together with Lys-BK or kallidin are also the principal kinins involved in the maintenance of cardiovascular homeostasis (3). BK binds to specific cell surface receptors, B$_1$ and B$_2$. The B$_1$ receptor is induced in response to inflammatory challenge, whereas B$_2$ receptor expression is constitutive. Both receptors are members of the seven-transmembrane G protein-coupled receptor family. Ligand binding to G protein-coupled receptors, which are expressed in many cell and tissue types, gives rise to diverse physiological responses including cytokine and growth factor production.

Cyclooxygenase (COX) catalyzes the production of prostaglandin, prostacyclin, and thromboxane from arachidonic acid. Arachidonic acid, which is released from membrane phospholipid by phospholipases, is converted to prostaglandin H$_2$ by COX and then to the specific prostaglandin by terminal synthases. There are different isoforms of COX: COX-1, whose expression in constitutive, and COX-2, which is induced in response to pro-inflammatory peptides and mediators (4). There is also the recently identified COX-3, whose function has been less well characterized (5). We have recently shown that BK induces COX-2 in pulmonary artery smooth muscle cells (PASMC), but the transcriptional mechanisms used by BK to induce COX-2 have not been defined (6). The COX-2 promoter has binding sites for a number of transcription factors including nuclear factor-κB (NF-κB), C/EBP, AP-2, and cAMP response element (CRE), which are involved in COX-2 induction in response to several other stimuli. For example NF-κB is involved in IL-1β- and hypoxia-induced COX-2 expression in lung epithelial cells and vascular endothelial cells (7, 8). In fibroblasts, COX-2 induction by both phorbol 12-myristate 13-acetate and IL-1β utilizes the C/EBP-binding domain (9). There are a number of candidate transcription factors for the effect of BK on COX-2 because BK has been shown to activate transcription factors that have binding motifs on the COX-2 promoter. BK induced IL-1β mRNA in a human lung fibroblast cell line by activation of NF-κB (10) and also activated NF-κB in HeLa cells transfected with BK B$_2$ receptors (11). Our own studies of regulation of the IL-8 gene have shown that BK can...
activate C/EBP (12). A CRE is also present on the human COX-2 promoter, but its role has been less well defined. Signaling via the CRE- and AP-1-binding motifs has been reported in phorbol 12-myristate 13-acetate-stimulated COX-2 induction in murine osteoblastic cells (15).

Here we have determined the mechanisms involved in the transcriptional regulation of the COX-2 promoter by BK in HPASMC. Mutational and deletional analysis of the COX-2 promoter showed that NF-κB and C/EBP sites were not required for the increase in COX-2 promoter activity produced by BK. In contrast mutations of the CRE site abolished the effect, suggesting that the CRE was essential. Consistent with this, BK increased activity of a CRE luciferase construct and CREB binding to the COX-2 promoter by EMSA. Furthermore studies with exogenous PGE₂, EP2 and EP4 PGE₂ receptor subtype agonists, and the COX inhibitor indomethacin showed that BK-induced activation of the CRE was due to an autocrine loop involving the early release of PGE₂ via cPLA₂ and action on EP2 and EP4 prostanoid receptors.

**MATERIALS AND METHODS**

**Cell Culture**—Proximal human HPASMC were purchased at passage 3 from Clonetics® (BioWhittaker UK Ltd., Wokingham, UK). The PASMC were cultured to passage 6 in smooth muscle cell growth medium-2 BulletKit® (Clonetics®, BioWhittaker UK Ltd.). All of the experiments were set up in triplicate or quadruplicate using passage 6 cells cultured in 6- or 24-well plates at 37 °C in a 5% CO₂, humidified incubator (Leece, Colwick, Nottingham, UK). The confluent cells were growth-arrested by serum withdrawal for 24 h. The medium was then replaced with fresh serum-free medium with or without bradykinin (Sigma). For the inhibition experiments the cells were pre-incubated for 30 min with either 1 μM indomethacin (Sigma) or 0.1% Me₃SO (Sigma) as the vehicle control. The highly selective EP2 and EP4 PGE₂ receptor subtype agonists ONO-AE1 259 and ONO-AE1 329, which were a gift from ONO Pharmaceuticals (Osaka, Japan), were used in the PGE₂ receptor studies (14, 15).

**Assessment of Cell Viability**—One well of the 24-well plate in each condition was detached using 0.025% trypsin and 0.01% EDTA. The cell number and percentage of viability were assessed using a hemocytometer and 0.4% Trypan blue (Sigma).

**PGE₂ Assay**—The PGE₂ levels in the culture supernatants were assessed by radioimmunoassay as described previously. The bound [³²P]PGE₂ (Amersham Biosciences) was measured using the Tri-Carb 2100TR liquid scintillation analyzer (Packard Bioscience Ltd., Pangbourne, UK). The PGE₂ levels were calculated with Riasmart software (Packard Bioscience Ltd.). PGE₂ was chosen because it is the main prostaglandin produced by pulmonary artery smooth muscle cells (16).

**cAMP Assay**—The cAMP was assayed after freon-amine (Sigma) extraction using [³H]adenosine 3',5'-cyclic phosphate, ammonium salt (Amersham Biosciences), and protein kinase A cAMP-dependent binding protein (Sigma) as described previously. This assay is based on the competitive binding between the unlabeled endogenous cAMP and a known, fixed quantity of [³H]-labeled cAMP for an added known amount of the 3',5'-cAMP-dependent protein kinase (17).

**[³H]Arachidonic Acid Release**—Confluent PASMC were growth-arrested by withdrawal of serum for 24 h. The medium was then replaced with serum-free medium containing 18.5 KBq/ml of [³H]arachidonic acid (Amersham Biosciences), and the cells were incubated for 16 h. The culture supernatant was removed, and the cells were washed three times in sterile serum-free medium. The cells were then treated with or without 10 μM BK and for 0.25, 0.5, 1, 2, 3, and 4 h. The antibiotic and calcium ionophore calcimycin A23187 was used as a positive control. After the incubation times the supernatants were removed, and the released [³H]arachidonic acid was counted using Tri-Carb 2100TR liquid scintillation analyzer and Emulsifier Safe liquid scintillation mixture (Packard Bioscience Ltd.). The remaining cells were lysed with 0.1% Triton X-100 (Sigma) and counted as above. The percentage [³H]arachidonic acid release was calculated as shown in the following equation.

\[
\%[³H]-\text{AA release} = \frac{\text{radioactivity in medium} \times 100}{\text{radioactivity in medium} + \text{radioactivity in cell lysate}} \quad \text{(Eq. 1)}
\]

**Western Blot Analysis**—Western blotting for COX-1 and COX-2 was performed as described previously (18). The goat anti-mouse horseradish peroxidase-conjugated secondary antibody was purchased from BD Biosciences (Cowley, Oxford, UK). The staining intensity of the bands was measured using a densitometer (Syngene, Braintrie, UK) together with Genesnap and Genetools software (Syngene). The figures depicting Western blotting are representative of four blots.

**RNA Isolation and Reverse Transcriptase-PCR**—HPASMC were cultured to confluence in 6-well plates and growth-arrested for 24 h by
FIG. 2. A, time course of BK mediated COX-2 mRNA induction. PASMC were incubated with 10 μM BK for 0, 15, 30, 60, 120, and 240 min. COX-2 and internal control, GAPDH, mRNA were measured by reverse transcriptase-PCR. The relative band density was calculated by dividing the density of COX-2 band by the density of GAPDH band at the same time points. COX-1 mRNA was constitutive. The figure is representative of three experiments. B, Preincubation with 5 and 10 μg/ml actinomycin D (ACT D), an inhibitor of transcription, reduces COX-2 mRNA induced by 60 min of incubation with 10 μM BK. The figure is representative of three experiments. C, Western blotting for COX-2 showing that 5 and 10 μg/ml actinomycin D inhibit BK mediated COX-2 protein induction. D, luciferase activity in PASMC transiently transfected for 2 h with either a 986-bp fragment of the COX-2 promoter (−917 to +49) (C2.1) or control vector (pGL3 basic) ligated to a luciferase reporter construct. The cells were cultured to confluence, growth-arrested, and transfected with 1 μl of LF2000 and 0.8 μg of DNA/well. There was a significant increase in luciferase in cells transfected with the COX-2 promoter and stimulated for 2 h with 10 μM BK compared with unstimulated and control cells. ***, p < 0.001. The cells were co-transfected with the internal control plasmid pRL-SV40 containing the Renilla luciferase gene and corrected for any variations in transfection efficiency. The figure represents the mean and S.E. of three experiments performed in triplicate.

| PE A3 | SP1 | GRE | NF IL-6 | GATA-1 | SP1 | K Bu | PE A3 | AP-2 | SP1 | K Bd | NF IL-6 | CRE | Lac |
|-------|-----|-----|---------|--------|-----|-----|-------|------|-----|------|---------|-----|-----|
| −810  | −767| −751| −728    |        | −490| −442|       | −312 | −265| −174 | −101     |     |     |

| SP1 | K Bu | PE A3 |
|-----|-----|-------|
| −490| −422|       |

| AP-2 | SP1 | K Bd |
|------|-----|------|
| −312| −265| −174 |

| NF IL-6 | CRE | Lac |
|---------|-----|-----|
| −174    |     |     |

FIG. 3. A schematic representation of the COX-2 promoter and constructs used in the transfection studies showing the positions of putative transcription factor-binding sites.
serum withdrawal. The cells were treated with BK at a final concentration of 10 μM and collected at 0, 0.25, 0.5, 1, 2, and 4 h, respectively. Total RNA was isolated using the RNeasy mini kit (Qiagen) following the manufacturer's protocol. 1 μg of total RNA was reverse transcribed in a total volume of 25 μl including 200 units of Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI), 25 units of RNase inhibitor (Promega), 0.5 μM of oligo(dT)15 primer, 0.5 mM of each dNTPs, and 1× first strand buffer. (Promega). The reaction was incubated at 42 °C for 90 min.

FIG. 4. A, increase in luciferase expression in PASM C transiently transfected with the deletion series of the COX-2 promoter luciferase constructs after 2 h of incubation with 10 μM BK. The cells were cultured in 24-well plates to confluence, growth-arrested, and transfected using 1 μl of LF2000 and 0.8 μg of DNA/well. The cells were co-transfected with the internal control plasmid pRL-SV40 containing the R enilla luciferase gene and corrected for any variations in transfection efficiency. The figure represents the mean and S.E. of three experiments performed in triplicate. There was no significant reduction in luciferase activity with the deletion series compared with the full-length construct, C2.1. B, the effect of mutations of the NF-κB-binding sites Kbu (−455/−428) and Kbd (−223/−214) on the COX-2 promoter activity in response to 10 μM BK. The graph shows the fold increase of luciferase activity in cells treated with BK compared with those with no BK treatment. The cells were cultured in 24-well plates to confluence, growth-arrested, and transfected using 1 μl of LF2000 and 0.8 μg of DNA/well. The figure represents the mean and S.E. of two experiments performed in quadruplicate. C, the effect of C/EBP and CRE-binding site mutations on the COX-2 promoter activity (Sty digest) in response to 10 μM BK. The cells were cultured in 24-well plates to confluence, growth-arrested, and transfected using 1 μl of LF2000 and 0.8 μg of DNA/well. The graph shows the fold increase of luciferase activity in cells treated with BK compared with those with no BK treatment. The figure represents the mean and S.E. of three experiments performed in triplicate and was analyzed by analysis of variance. **, p < 0.01.
AGG GGG CC-3'; cPLA
dtaxisense 5'-GCT ACC ACA GCA TCA CG-3'; COX-1 sense, 5'-TGC CCA GCT CCT GGC CCG CCG CT-3'; and COX-1 antisense 5'-TGT CAT CAA CAC AGG CGC CTC TTC-3'. Amplification was carried out with a PTC-100 programmable thermal controller (MJ Research Inc., Watertown, MA) after an initial denaturation at 94 °C for 3 min. This was followed by 30 cycles of PCR using the following temperatures and times: denaturation at 94 °C for 1 min, primer annealing at 58 °C for 2 min, primer extension at 72 °C for 1 min, and a final extension of 72 °C for 10 min. The PCR products were electrophoresed on 2% agarose gel in 0.5× TBE buffer Tris-boric acid-EDTA (89 mm Tris borate, 2 mm EDTA, pH 8.3) containing 0.5 μg/ml ethidium bromide and visualized using ultraviolet illumination and the GeneGenius gel documentation and analysis system (Syngene).

Transfection of COX-2 Promoter with Deletions and Mutations—The PASMC were transiently transfected for 2 h using a liposomal transfection system (LipofectAMINE LF2000; Invitrogen). Transfections of PASMC were transiently transfected for 2 h using a liposomal transfection system (LipofectAMINE LF2000; Invitrogen). Transfections of COX-2 promoter were performed using C2.2 (−2375/+49) 2424-bp or C2.1 (−917/+49) 966-bp fragments and C2.1 with a series of deletions or site-specific mutations ligated with a luciferase reporter plasmid pGL3 basic (Promega UK, Southampton, UK). Deletions consisted of Dra (−625/+49) 674-bp, Sty (−358/+49) 407-bp, Alu (−190/+49) 219-bp, and Rca (−86/+49) 135-bp fragments. Mutations consisted of C/EBP (−129/−124), CRE (−59/−53), and NF-κB motifs κB (−447/−438) and κBd (−224/−214) on the COX-2 promoter that bind p50/p65 NF-κB heterodimers.

The cells were cultured in 24-well plates to confluence, growth-arrested, and transfected using 1 μl of LF2000 and 0.8 μg of DNA/well according to the company instructions. The cells were co-transfected with 1 μg/well of the internal control plasmid pRL-SV40 (Promega UK) containing the Renilla luciferase gene (6). After 2 h of incubation with or without 10 μM bradykinin, the cells were harvested, and the firefly and Renilla luciferase activities were measured using a dual luciferase assay system kit (Promega UK) and a Microlumat Plus LB 96V luminometer (Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany). LipofectAMINE was shown to have a transfection efficiency of 10–20% under these conditions using HPASMC and green fluorescent protein (data not shown).

Transfection with p6CRE/luc Reporter—The p6CRE/luc construct contains the firefly luciferase gene under control of the minimal herpes simplex virus thymidine kinase promoter and six CREs and was obtained from S. Rees (Glaxo Wellcome, Stevenage, UK). This construct responds to agents that increase cAMP levels (19). Confluent, growth-arrested HPASMC in 24-well plates were transfected for 2 h using 2 μl of LF2000 and 1 μg of DNA/well. The medium was removed and incubated with serum-free medium containing either 1 μM indomethacin or 0.2% MeSO for 30 min. BK was added to give a final concentration of 10 μM. Medium was added to the control wells. The cells were incubated for 2 h, after which the supernatants were removed, the cells were washed twice in phosphate-buffered saline and lysed with lysine lysis buffer, and the luciferase levels were measured using a luciferase assay system kit (Promega UK) and the Microlumat Plus LB 96V luminometer (Berthold Technologies GmbH & Co. KG). In some of the wells 10 nm exogenous PGE2 was added.

Electrophoretic Mobility Shift Assay—The nuclear protein fractions for EMSA were prepared using Nu-Clear extraction kit (Sigma) following the manufacturer’s protocol. The protein concentrations were determined using the Bio-Rad protein assay. Oligonucleotides containing the CREB sequences 5'-AAC AGT CAT TGG TCA CGG TCT TTG-3' (sense) and 5'-CA AGC TGT GAC GAA ATG ACT GTT-3' (antisense) (Sigma) were labeled using [γ-32P]ATP (Amersham Biosciences) and T4 polynucleotide kinase (Promega UK). Fifty micrograms of nuclear protein, 50 P-labeled double-stranded probe (40,000 counts/min/ng), and 2 μl of 5× binding buffer (20% glycerol, 5 mM MgCl2, 2.5 mM EDTA, 2.5 mM dithiothreitol, 250 mM NaCl, 50 mM Tris-Cl (pH 7.5), 0.25 mg/ml poly(dI-dC)-poly(dI-dC)) were mixed in a total volume of 10 μl. In competition assays, 50× unlabeled competitors were added at the same time as probe addition. The mixture was incubated at room temperature for 30 min and then loaded on a 5% polyacrylamide gel in 0.5× TBE buffer and subjected to electrophoresis for 60 min. The gel was dried and exposed for autoradiography on Kodak XAR film at −70 °C for 19–48 h. Supershift was demonstrated using 400 ng of specific goat polyclonal and mouse monoclonal anti-human CREB-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Statistical Analysis—The results of the cAMP, PGE2, and luciferase levels were expressed as the means of the triplicate or quadruplicate wells for that experiment. The experiments were repeated at least three times, and the results shown represent the means ± S.E. Analysis of variance was used to determine significant differences. A p value of <0.05 (two-tailed) was regarded as statistically significant.

**Fig. 5.** A, BK increases consensus CREB binding and the addition of 4 μg of anti-CREB-1 antibody to the nuclear extracts from cells treated with 10 μM BK for 60 min resulted in a supershift. The figures shown are representative of three experiments. B, 10 μM BK for 60 min increased CREB binding to the COX-2 promoter by EMSA. The addition of anti-CREB-1 antibody to the nuclear extracts modified this CREB binding and induced a shift. The figures shown are representative of three experiments. C, for CRE binding competition, nuclear extracts from BK-treated cells were incubated with labeled CRE (Hot CRE) in the presence of a 50-fold excess of unlabeled CRE (Cold CRE) or unlabeled AP-1 (Cold AP-1). The figures shown are representative of three experiments.
and remained constant at all the time points (Fig. 1). COX-1 protein was constitutively expressed, protein detected at 2, 4, 8, 16, and 24 h, with the strongest control cells (Fig. 1). The figure shows the mean and S.E. of four experiments performed in triplicate. ***, p < 0.001. There was a significant difference in PGE2 levels in cells treated with 10 μM BK at 0, 15, 30, 60, 120, and 240 min time points. The figure is representative of three experiments. ***, p < 0.01; *, p < 0.05 by analysis of variance. C. BK stimulation for 30, 60, 90, and 120 min resulted in a significant increase in PGE2 levels in the BK-treated cells compared with control cells at all the time points. The figure shows the mean and S.E. of four experiments set up in quadruplicate. ***, p < 0.001.

RESULTS

BK Induces COX-2 Protein and PGE2 Release—We have previously reported that BK induces COX-2 protein and stimulates increased PGE2 release in cultured human PASMC (6). Here we also found that BK increased PGE2 release. There was a significant difference in PGE2 levels in cells treated with 10 μM BK at 2, 4, 8, 16, and 24 h compared with unstimulated control cells (Fig. 1A). COX-2 was induced by BK with COX-2 protein detected at 2, 4, 8, 16, and 24 h, with the strongest bands at 4 and 8 h. COX-1 protein was constitutively expressed and remained constant at all the time points (Fig. 1B).

BK Induction of COX-2 Is Transcriptional—Reverse transcriptase-PCR showed that BK increased COX-2 mRNA levels with time with a 2-fold increase at 60 min reducing to almost basal levels by 4 h (Fig. 2A). To confirm that this was due to COX-2 gene transcription rather than stabilization of mRNA, the cells were cultured for 1 h with 10 μM BK together with 5 and 10 μg/ml actinomycin D, an inhibitor of RNA polymerase II. The actinomycin D-treated cells showed a reduction in mRNA density (Fig. 2B). Western blotting demonstrated that incubation for 4 h with 10 μM BK together with 5 and 10 μg/ml actinomycin D inhibited COX-2 protein induction by BK (Fig. 2C). The cells were transfected with a 966-bp COX-2 promoter fragment (–917 to +49) ligated to a firefly luciferase reporter plasmid. A diagram representing the COX-2 promoter showing the key deletion sites and the positions where the restriction enzymes cleave the promoter to generate the series of deletions is shown in Fig. 3. BK increased the luciferase levels 5.4 ± 0.27 in the C2.1 construct. There was no reduction in luciferase activity with any of the deletions compared with the C2.1, suggesting that even the smallest Rsa fragment of the COX-2 promoter, which contains only the CRE transcription factor, was able to induce COX-2 (Fig. 4A).

Transfection studies using wild type and mutated C/EBP (−132/−124) and the NF-κB-binding sites from the COX-2 promoter xBu (−447/−438) and xBd (−224/−214) did not show reduction of the luciferase activity (Fig. 4, B and C). However, there was a significant inhibition (p < 0.01) with the mutated

![Figure 6](image6.png)

**Fig. 6.** A, confluent growth-arrested PASMC were incubated for 15, 30, 60, and 120 min with or without 10 μM BK. There was a significant increase in cAMP levels in the BK-treated cells compared with control cells. ***, p < 0.001. The cAMP levels in the unstimulated cells measured at the same times were below the level of detection of the assay. The figure shows the mean and S.E. of three experiments set up in triplicate. B, luciferase activity in PASMC cells transiently transfected for 2 h with pGL3.6CRE/luc containing six repeats of the CRE-binding site using 2 μl of LF2000 and 1 μg of DNA/well of a 24-well plate and incubated for 2 h with or without 10 μM BK. The figure represents the mean and S.E. of four experiments performed in triplicate. ***, p < 0.001.

These results suggest that induction of COX-2 by BK is transcriptional and not mediated by post-transcriptional stabilization of COX-2 mRNA.

Mutations in the CRE but Not the NF-κB- or C/EBP-binding Sites in the COX-2 Promoter Reduce BK-stimulated Luciferase Activity—To determine which transcription factors are involved, the cells were transfected with 966 bp of the wild type COX-2 promoter (C2.1) and a series of deletion constructs ligated to a firefly luciferase reporter plasmid. A diagram representing the COX-2 promoter showing the key deletion sites and the positions where the restriction enzymes cleave the promoter to generate the series of deletions is shown in Fig. 3. BK increased the luciferase levels 5.4 ± 0.27 in the C2.1 construct. There was no reduction in luciferase activity with any of the deletions compared with the C2.1, suggesting that even the smallest Rsa fragment of the COX-2 promoter, which contains only the CRE transcription factor, was able to induce COX-2 (Fig. 4A).

Transfection studies using wild type and mutated C/EBP (−132/−124) and the NF-κB-binding sites from the COX-2 promoter xBu (−447/−438) and xBd (−224/−214) did not show reduction of the luciferase activity (Fig. 4, B and C). However, there was a significant inhibition (p < 0.01) with the mutated
CRE construct (−59/−53), suggesting that this factor is necessary for COX-2 induction by BK (Fig. 4C).

**BK Increases CREB Binding to the COX-2 Promoter—**We used EMSA to determine whether BK treatment increased CREB binding to the COX-2 promoter. Incubation with 10 μM BK for 60 min induced CREB-1 binding activity with consensus and COX-2 promoter CRE oligonucleotides (Fig. 5, A and B). Supershift studies using a monoclonal antibody to CREB-1 produced gel retardation with the consensus sequence (Fig. 5A) and with the COX-2 promoter-specific primers (Fig. 5B). Competition with 50-fold excess unlabeled COX-2 promoter-specific CRE oligonucleotides blocked transcription factor binding, whereas excess AP-1 oligonucleotides did not block CRE transcription factor binding, demonstrating that the binding was specific (Fig. 5C).

**BK Increases cAMP Production and Activates a 6CRE/luc Reporter Construct—**To confirm that BK was increasing cAMP and thereby activating CRE-mediated transcription, we performed cAMP assays after BK stimulation and also measured activation of a 6CRE/luc reporter gene. BK rapidly increases intracellular cAMP levels (Fig. 6A) and increased the activity of the 6CRE/luc reporter construct (Fig. 6B), thereby demonstrating that BK was acting to increase cAMP and stimulate CRE-mediated transcription.

**BK-induced Release of PGE₂ via cPLA₂ Is Responsible for Generating the cAMP Signal—**BK is known to produce a rapid increase in PGE₂ by activating cPLA₂ (20). Because PGE₂ can increase cAMP via EP2 and EP4 receptors (21, 22), we postulated that cPLA₂ induced early PGE₂ production was the upstream signal to increase cAMP and stimulate cAMP-mediated transcription.

![Figure 8](image-url)
CRE activation and gene transcription.

We found that BK induced an increase in cPLA₂ mRNA that was maximal at 60 min. Furthermore we saw a significant increase in [³H]arachidonic acid release in the BK-treated cells at 30, 60, and 120 min (Fig. 7, A and B). This was accompanied by PGE₂ generation (Fig. 7C). To test the role of this early PGE₂ release, we determined the effect of its inhibition by indomethacin, a COX inhibitor. We found that PGE₂ release was inhibited by preincubation with 1 µM indomethacin (Fig. 8A). Moreover indomethacin also inhibited BK-mediated increases in cAMP levels (Fig. 8B) and BK increases in 6CRE/luc reporter luciferase activity (Fig. 8C). Furthermore indomethacin inhibited CRE transcription factor binding by EMSA (Fig. 8D). The inhibitory effects of indomethacin on PGE₂ release by cPLA₂, cAMP generation, 6CRE/luc reporter activity, and EMSA CRE binding strongly suggest that early PGE₂ release by cPLA₂ is critical in the signal transduction cascade.

Exogenous PGE₂ Mimics the Effect of BK—To confirm that the inhibitory effects of indomethacin are linked to the reduction in PGE₂ levels, we performed additional studies to determine whether exogenous PGE₂ would mimic the effect of BK. We found that exogenous PGE₂ stimulated cAMP production in
HPASMC in a concentration- and time-dependent manner (Fig. 9, A and B) and also increased 6CRE/luc reporter construct luciferase activity (Fig. 9C). Similarly exogenous PGE$_2$ activated CRE transcription factor binding to the COX-2 promoter by EMSA (Fig. 9D) and increased luciferase levels in cells transfected with the COX-2 promoter construct C2.1 (Fig. 9E).

**EP2 and EP4 Receptor Agonists Mimic the Effect of Exogenous PGE2**—To determine which PGE$_2$ receptors were important, we looked at the effect of PGE$_2$ receptor agonists on cAMP production together with luciferase activity in cells transfected with 6CRE/luc reporter construct. Both EP2 and EP4 receptors are known to couple to adenylyl cyclase. We found that both EP2 (ONO-AE1 259) and EP4 (ONO-AE1 329) receptor agonists increased cAMP production and luciferase activity in a concentration-dependent manner (Fig. 10) in the same way as exogenous PGE$_2$. This suggests that PGE$_2$ is acting via both EP2 and EP4 receptors. The specificity of these PGE$_2$ receptor agonists has been determined previously using Chinese hamster ovary cells (23). At the concentrations used here, the compounds are highly selective and do not cross-react with other prostanoid receptors.

Collectively these results suggest that BK induces COX-2 gene transcription by a cAMP-dependent mechanism involving an autocrine prostanoid loop. BK up-regulates cPLA$_2$, resulting in increased membrane-derived arachidonic acid release. Arachidonic acid is converted to PGE$_2$. PGE$_2$ binds to EP2 and EP4 receptors and increases intracellular cAMP production, which then phosphorylates CREB and induces transcription by binding to the CRE elements on the COX-2 promoter (Fig. 11).

**DISCUSSION**

This study shows that BK increases COX-2 expression through transcriptional mechanisms involving activation of the CRE region on the COX-2 promoter. Transfection experiments with ~1 kb of the COX-2 promoter construct and a series of deletions containing key transcription factor-binding sites showed that effective promoter activity was maintained with a construct having an isolated CRE region only. Mutational studies of the COX-2 promoter indicated that the NF-$\kappa$B- and C/EBP-binding sites were not involved in BK induction of COX-2 but that the CRE binding site was essential. Furthermore, EMSA showed that after BK stimulation, CREB bound to the COX-2 promoter. BK increased intracellular cAMP levels and cAMP-driven p6CRE/luc luciferase expression. Additional studies with the COX inhibitor indomethacin showed that it inhibited the elevations in cAMP, 6CRE/luc activity, and
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\[ \text{BK} \rightarrow \text{phosphorylation of cytosolic phospholipase A}\text{\textsubscript{2}} \rightarrow \text{Arachidonic acid release} \rightarrow \text{PGE}\text{\textsubscript{2}} \text{ production} \rightarrow \text{EP2/EP4 Receptor binding} \rightarrow \text{Increased intracellular cAMP} \rightarrow \text{CRE (cyclic AMP response element) transcription factor activation on the COX-2 promoter} \rightarrow \text{COX-2 induction} \]

Elevated cAMP levels were observed in cells treated with BK. This effect was consistent with studies in PASMC, where BK increased cAMP levels. We confirmed this effect using a COX-2 promoter construct and a firefly luciferase reporter, showing increased luciferase activity in BK-treated cells.

In our experiments when cells were pretreated with actinomycin D prior to BK treatment, COX-2 mRNA was reduced by actinomycin D, suggesting that COX-2 protein induction was transcriptional. This was confirmed in transfection experiments with a wild type COX-2 promoter ligated to a firefly luciferase reporter construct that had increased luciferase activity in BK-treated cells. We then proceeded to studies using a series of deletions of the 1-kb COX-2 promoter fragment. We found that deletions of the COX-2 promoter down to 0.135 kb all had maximal promoter activity, suggesting that a transcriptional binding site on the 0.135 kb was essential for promoter activity. Consistent with this, mutations of the NF-κB- or C/EBP-binding sites did not reduce activity. In contrast, when we used a COX-2 promoter construct with a mutated CRE site, BK-induced luciferase activity was markedly reduced compared with the wild type construct, suggesting involvement of the CRE transcription factor-binding site in BK-mediated COX-2 transcription. This observation was supported by the EMSA experiments showing that BK induced CREB binding to CRE consensus sequences and also the COX-2 promoter CRE.

We confirmed that binding was specific by studies with excess unlabeled CRE and AP-1 oligonucleotides and demonstrated supershift with a monoclonal antibody to CREB-1. Furthermore, we showed that BK increased cAMP levels and activated a CRE-luc construct. The lack of involvement of NF-κB and C/EBP in BK-induced COX-2 expression contrasts with studies with IL-1β and TNF-α. TNF-α enhanced COX-2 promoter activity in a mouse osteoblastic cell line, MC3T3-E1, by the activation of both NF-κB and C/EBP (26). COX-2 gene transcription by IL-1β in the bronchial epithelial cell line A549 involved NF-κB (7) and C/EBP and NF-κB co-regulation in rabbit chondrocytes (27).

The lack of involvement of NF-κB and C/EBP in BK-induced regulation also contrasts with our own studies showing that BK can activate IL-8 in airway smooth muscle cells via NF-κB and C/EBP (12). Other studies have shown that BK induces IL-1β gene expression by NF-κB activation in human epithelial cells and lung fibroblast cell lines (10, 28) and activates AP-1 in HEK 293 cells (29). Collectively, these studies suggest that BK can activate several transcription factors that then regulate several genes in a manner that is gene- and transcription factor-specific.

CRE has been implicated in COX-2 induction by phorbol ester in murine osteoblastic cells, (13) and in phorbol 12-myristate 13-acetate-mediated differentiation of the human monocyte U937 cells, (30), but none have looked at the involvement of the CRE in BK-mediated COX-2 induction. Our studies clearly indicate that the CRE is a critical part of BK signaling to the COX-2 promoter.

We then performed additional mechanistic studies to determine how BK was acting to increase cAMP and therefore CRE-mediated COX-2 transcription. BK is known to increase PGE\textsubscript{2} production in smooth muscle cells in two stages. The first stage involves up-regulation of cPLA\textsubscript{2}-derived arachidonic acid providing the substrate for constitutively expressed COX-1 (20) with the second more sustained phase being due to COX-2 induction (18). In the first cPLA\textsubscript{2}-mediated phase the released PGE\textsubscript{2} binds to cell surface receptors and stimulates intracellular cAMP synthesis. BK has also been shown to decrease the rate of cAMP degradation by inhibiting cellular phosphodiesterase activity, potentiating the effects of cAMP (31). Intracellular cAMP activates PKA resulting in phosphorylation of the CREB at Ser\textsuperscript{159}, which binds to the CRE resulting in gene transcription. Because PGE\textsubscript{2} can increase cAMP via EP2 and EP4 receptors, we hypothesized that early release of PGE\textsubscript{2} via cPLA\textsubscript{2} was responsible for the increase in cAMP, which then
activated CRE-mediated transcription. To test this hypothesis, we performed studies using the broad spectrum COX inhibitor indomethacin and measured cPLA2 mRNA expression, arachidonic acid release, and PGE2 production. We found that BK induced cPLA2 mRNA expression, arachidonic acid mobilization, and PGE2 release at early time points. Both the BK-induced PGE2 release and cAMP production was markedly reduced by indomethacin. Indomethacin also prevented CREB binding to the CRE binding site in BK-treated cells. These studies strongly suggested that endogenous prostanoid produced via cPLA2 were responsible for generating the cAMP signal leading to CRE activation. To further test the hypothesis we determined whether exogenous PGE2 would mimic the effect of BK on CRE-mediated COX-2 expression. We found that exogenous PGE2-stimulated cAMP production increased 6CRE/luc luciferase activity and induced CREB binding to the CRE-binding site on the COX-2 promoter. Similarly the PGE2 receptor subtype agonists EP2 and EP4 stimulated both cAMP production and increased 6CRE/luc luciferase. PGE2-activated EP2/EP4 receptors are known to stimulate adenylyl cyclase, resulting in increased cAMP and activation of PKA (22).

This study and our previous studies with BK in airway smooth muscle where BK stimulation of ASM resulted in increased IL-8 and vascular endothelial growth factor production that was dependent on the generation of endogenous prostanoid (12, 32) suggest that generation of prostanoids by BK strongly suggested that endogenous prostanoid produced via the COX-2 promoter CRE site in BK-treated cells. These studies furthered the idea that endogenous prostanoid production contributes to inflammatory and vascular diseases.

In conclusion our studies provide strong evidence that BK induces COX-2 via the CRE-binding site on the COX-2 promoter. Furthermore autocrine PGE2 generation by cPLA2 with subsequent binding to the EP2 and EP4 receptors is responsible for generating the cAMP signal leading to CRE binding and CRE activation of the COX-2 promoter. These studies provide new insight into the mechanisms whereby membrane G protein-coupled receptor ligands such as BK cause COX-2 induction.

Acknowledgment—We thank S. Rees (Glaxo Wellcome) for providing the 6CRE/luc reporter vectors.

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