Prostaglandin E-Prostanoid-3 Receptor Activation of Cyclic AMP Response Element-Mediated Gene Transcription

LAURENT P. AUDOLY, LIJUN MA, IGOR FEOKTISTOV, STEPHANIE K. De FOE, MATTHEW D. BREYER, and RICHARD M. BREYER

Departments of Medicine (Divisions of Nephrology and Cardiology), Pharmacology (L.A., R.M.B.), Molecular Physiology and Biophysics (M.D.B.), Veterans Administration Medical Center (M.D.B.), and Vanderbilt University School of Medicine Nashville, Tennessee

Accepted for publication October 30, 1998

ABSTRACT

The prostaglandin E-prostanoid (EP)3 receptor signals primarily through the inhibitory G protein Gi, thereby decreasing intracellular cAMP levels. To study the signal transduction properties of the rabbit EP3 receptor, five splice variants were expressed in HEK293tsA201 cells: 72A, 74A, 77A, 80A and the novel splice variant NT, which lacks the C-terminal sequence. The ability of the EP3 receptor splice variants to modulate the expression of a β-galactosidase reporter gene under the control of a promoter containing cAMP response elements (CRE) was assessed. Each splice variant induced sulprostone-mediated increase in β-galactosidase enzymatic activity with EC50 ranging from 0.8 nM for the NT splice variant to 3.1 nM for the 77A splice variant. Substitution of either Asp338 with Ala, or Arg329 with Ala or Glu in the 77A splice variant resulted in a loss of receptor-evoked increases in β-galactosidase activity, whereas substitution of Lys300 with alanine had no effect on signal transduction. These phenotypes correlate with the inhibition of cAMP generation by direct cAMP measurement. Signal transduction was insensitive to pretreatment of cells with pertussis toxin, suggesting that a nonGi/Go pathway is activated by the EP3 receptor. Direct measurement of second messenger levels confirmed that there was no increase in cAMP levels mediated by the 77A splice variant, however, there was a modest increase in intracellular Ca2+. Partial blockade of the reporter activity with kinase inhibitors demonstrates that CRE activation is mediated in part by a Ca2+-dependent kinase pathway. These data suggest that the EP3 receptor signals through a novel CAMP response element binding protein/CRE pathway.

Received for publication July 29, 1998.

ABBREVIATIONS: fura-2 AM, acetoxymethyl ester of fura-2; CRE, cAMP response element; CREB, cAMP response element binding protein; HA, hemagglutinin; PGE2, prostaglandin E2; PK, protein kinase; RIA, radioimmunoassay; Ptx, pertussis toxin; ELISA, enzyme-linked immunosorbent assay; UAS, upstream activating sequence; EP, E-prostanoid; PCR, polymerase chain reaction; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; IBMX, 3-isobutyl-1-methylxanthine.
the cloned mouse, bovine, and human receptors were shown to signal through this pathway when expressed in cell culture systems (Sugimoto et al., 1992; An et al., 1993; Namba et al., 1993). Surprisingly, a subset of EP3 receptor splice variants also signaled via stimulation of either cAMP and/or phosphatidylinositol hydrolysis when expressed in CHO cells. These unexpected signaling effects were only observed at agonist concentrations five orders of magnitude higher than the concentration required for Gi-mediated effects. Moreover, the observed EP3 receptor signal transduction properties are dependent not only upon the C terminus but also the cellular background in which they are expressed (Namba et al., 1993; Katoh et al., 1996).

Although the rabbit 77A EP3 splice variant has previously been demonstrated to decrease intracellular cAMP concentrations ([cAMP]) when expressed in HEK293tsA201 cells, signal transduction of the other rabbit splice variants had not been reported. To test the signal transduction properties of each of these splice variants, a transient receptor expression assay in mammalian cells was developed using a β-galactosidase reporter gene. We tested the cAMP response element (CRE)/β-galactosidase-mediated activity of each of the EP3 receptor splice variants 77A, 72A, 74A, 80A, as well as a novel splice variant designated NT. Additionally, we correlated the ability of the 77A splice variant to modulate β-galactosidase activity with direct measurement of second messenger levels. Data presented here suggest that each EP3 receptor splice variant can stimulate the CRE-reporter system, and that this stimulation appears to be independent of cAMP generation. The CRE/β-galactosidase system can be applied to rapidly analyze EP3 receptor signal transduction and will be useful in facilitating further functional and structural studies.

**Experimental Procedures**

**Materials.** Sulprostone was purchased from Cayman Chemicals (Ann Arbor, MI). Indomethacin and forskolin were purchased from Sigma (St. Louis, MO). Chlorphenol red-β-D-galactopyranoside and the kinase inhibitors staurosporine, PD 98059, and bisindolymale-
imide I were purchased from Calbiochem (La Jolla, CA). Triton X-100 was purchased from Pierce (Rockford, IL). Acetoxyethyl ester of fura-2 (fura-2 AM) was purchased from Molecular Probes, Inc. (Eugene, OR). Lipofectamine and OPTI-MEM were purchased from Life Technologies (Grand Island, NY). pCRE/lacZ plasmid was a kind gift from Dr. R. Cone (Vollum Institute, Portland, OR). cAMP enzyme-linked immunosorbent assay (ELISA) reagents and the PathDetect trans reporting system for cAMP response element binding protein (CREB) activation were purchased from Stratagenne, Inc. (La Jolla, CA).

**Construction of EP3 Receptor Expression Vectors.** The hemagglutinin (HA)-tagged 77A vector pRc/CMV77AHA expressing the wt, RA329, RE329, DA338, and KA300 mutants were constructed previously (Audoly and Breyer, 1997). HA-tagged 72A splice variant expression vector was constructed in the following manner: Plasmid pRc/CMV77AHA encoding the HA-tagged 77A splice variant and the 72A cDNA subcloned in pBluescript (Breyer et al., 1994) were each digested with BsmI and ClaI restriction endonucleases. The small BsmI-ClaI fragment, which contains the unique C-terminal protein sequence of the 72A splice variant, was isolated and ligated to the 77A backbone, resulting in a chimeric construct that encodes the HA-tagged receptor with the 72A-specific C terminus. HA-tagged 74A and 80A were constructed as follows: the full length 74A splice variant subcloned into pCRII (Breyer et al., 1994) was digested with NcoI and SpeI and the 1.5-kb fragment cloned into the NotI/XhoI sites in the polylinker of pRc/CMV. The resultant plasmid was digested with MluI and the small 1.3-kb MluI fragment was replaced with the MluI fragment of pRc/CMV77AHA, thus fusing the HA tag to the N terminus of the 74A splice variant. The vector expressing the HA-tagged 80A receptor was created by a three part ligation of the 0.9-kb NcoI and SpeI fragment of the 80A splice variant (subcloned into pCRII) (Breyer et al., 1994), which contains the unique C terminus of the 80A variant, the 0.5-kb NotI/NotI fragment containing the HA tag and N terminus of the EP3 common region, and the pRc/CMV expression vector.

**Isolation of the NT Receptor Splice Variant.** Female New Zealand White rabbits weighing between 1.5 and 2.0 kg were treated with indomethacin (2 mg/ml, 2.5 mg/kg) twice a day for 3 consecutive days. Rabbits were then anesthetized using i.m. ketamine and xylazine (44 and 10 mg/kg, respectively). After surgical anesthesia was achieved, rabbits were sacrificed by decapitation and kidneys were harvested. Cortical collecting duct cells were isolated by immunodissection as described previously (Noland et al., 1992). Total RNA was isolated from cortical collecting duct cells using a modification of the TRIzol method (Chirgwin et al., 1979).

Reverse transcription-polymerase chain reaction (PCR) was carried out using a 3′ rapid amplification of cDNA ends protocol essentially as described (Frohman et al., 1988). The reverse transcription step was performed with a dT17-adapter oligonucleotide with the sequence 5′-GAC TGC CAG TCG ACA TCG ATT TTG TTG TTT TTG 3′. cDNAs were amplified using 5′ GCC ATC ACC TCG TGC 3′, as an upstream primer at nt 715 of the coding region, and the rapid amplification of cDNA ends adapter oligonucleotide, 5′ GAC TGC CAG TCG ACA TCG ATT 3′, as a downstream primer. The PCR conditions were: 70°C 5′; 95°C 1; 35 cycles: 95°C 15′, 52°C 15′, 72°C 2′; 72°C 10′. A second round of hemi-nested PCR was performed using 5′ ACA TCA TTG GAC CAC TGC 3′ at nt 916 of the coding region as the upstream primer and the adapter oligonucleotide as the downstream primer using the same PCR conditions as described above. The resulting products were subcloned into the pCRII vector. Colonies were isolated and screened by hybridization with the internal normal region oligonucleotide sequence 5′-GGA AAG CAG AAA GAA TGC 3′ at nt 946 of the coding region. Candidate clones were sequenced by the dideoxy termination reaction. A novel clone CDDI-5 was isolated, which encoded a naturally occurring “truncated” EP3 receptor in which a stop codon encoded by a previously undescribed exon was identified immediately after the splice junction at Q355. This unique 3′ sequence was cloned into the pRc/CMV77AHA expression vector, creating a chimeric receptor that ends at Q355. A 520-bp BamHI/ClaI fragment was isolated from the CDDI-5 clone in PCR. pRc/CMV77AHA was digested with NotI/BamHI and a 1064-bp fragment encoding the common region of EP3 receptor splice variants was isolated. The 520- and 1064-bp fragments were ligated with the NotI/ClaI fragment of vector pRcCMV (5.5 kb); this resultant-expressed protein was designated NT (No-Tail).

**Transient Coexpression of the EP3 Receptor and pCRE/lacZ.** HEK293tsA201 cells plated at approximately 50% confluence were cotransfected with 6 μg of the cDNA of interest and 6 μg of pCRE/lacZ plasmid using lipofectamine. Five hours after the addition of DNA-lipofectamine complex, the media was aspirated and replaced with Dulbecco’s modified Eagle’s medium (DMEM/10% fetal bovine serum (FBS)/20 μM indomethacin). Forty-eight hours after transfection, cells were plated in 96-well plates at a density of 5 × 104 cells/well in 100 μl DMEM/10% FBS/20 μM indomethacin containing 5 mM sodium butyrate, and cells were incubated an additional 12 to 16 h, at which point the cells had reached confluence. In some cases, the media contained 500 ng/ml pertussis toxin to inactivate Gα proteins before assay.

**CREB/β-Galactosidase Assay.** On the day of the assay, prosta- noid agonists dissolved at varying concentrations in OPTI-MEM containing 0.5 mM 3-isobutyl-1-methylxanthine (IBMX)/20 μM indomethacin were added to cells and incubated for an additional 6 h at 37°C, 5% CO2. In some cases, kinase inhibitors were added 1 h before prostanoid addition and incubated throughout the agonist incubation. Medium was aspirated and the cells incubated for 10 min in 25 μl of lysis buffer (10 mM sodium phosphate, 2 mM MgSO4, 0.1 mM MgCl2, pH 8.0). One hundred microliters of assay buffer (10× lysis buffer containing 0.5% Triton X-100 and 40 mM β-mercaptoethanol) was added to the lysis buffer and incubated for an additional 10 min. The β-galactosidase substrate chlorophenol red-β-D-galactopyranoside was dissolved in assay buffer at 4 mg/ml, and 25 μl of substrate solution was added to each well to determine enzymatic activity (König et al., 1991). Plates were incubated for 5 to 6 h and the absorbance read was measured at 570 nm on a Dynex MRX multiwell plate reader (Dynex Technologies, Inc., Chantilly, VA).

**CREB-trans Reporting System.** Activation of CREB was assayed independently using the PathDetect trans-activation reporter system (Stratagene). HEK293tsA201 cells were plated in 6-well plates at a density of 5 × 105 cells/well. After incubation for 24 h, each well of cells was transfected with 50 ng of plasmid pFA-CREB encoding the Gal4 DNA binding domain (aa 1–147) fused to CREB (aa 1–280) under the control of the CMV promoter; 1 μg of reporter plasmid pFR-Luc, which encodes the luciferase gene under transcriptional control of five repeated upstream activating sequence (UAS)Gal4 binding elements, and 50 ng of pRc/CMV77AHA encoding the 77A EP3 splice variant. Five hours after transfection, the medium was replaced with 2 ml of fresh DMEM/1% FBS and changed with 2 ml of fresh DMEM/0.5% FBS 18 to 24 h later. After incubating an additional 18 to 24 h, the medium was replaced with 2 ml of DMEM/1 mM IBMX/20 μM indomethacin medium containing varying concentrations of sulprostone or forskolin and incubated for an additional 6 h. Luciferase was extracted from the cells and activity was measured using the luciferase Assay Kit (Stratagene) according to the manufacturer’s directions.

**cAMP ELISA Measurements.** HEK293tsA201 cells plated in 100-mm dishes at approximately 50% confluence were transfected with 3 μg of plasmid pRc/CMV77AHA using the lipofectamine method. Five hours later the medium was replaced with 10 ml DMEM/10% FBS. Cells were cultured for 48 h, changing the medium every 24 h, and then the cells were distributed into a 24-well plate at 5 × 104 cells/well in DMEM/10% FBS/5 mM sodium butyrate. After 16 h, when the cells had reached confluence, the medium was replaced with 450 μl of DMEM/0.25 mM IBMX/20 μM indomethacin and incubated for 1 h. Fifty microliters of the same medium containing various concentrations of PGE2 or forskolin was added to each well and incubated for times up to 6 h. The reactions were stopped by
addition of 500 µl of 10% trichloroacetic acid. cAMP measurements of the cell lysates were performed by an ELISA according to the manufacturer’s instructions (Stratagene).

Measurement of Intracellular Ca\(^{2+}\). Cytosolic free Ca\(^{2+}\) concentrations were determined by the fluorescent dye technique. Cells were plated on glass coverslips 24 h post-transfection, and assayed 24 to 72 h later. Cells were loaded with 5 µM of fura-2 AM for 60 to 120 min at 37°C. After cells were loaded, they were superfused with a HCO\(_3\)/CO\(_2\) buffered solution (pH 7.4) at a flow rate of 2.5 ml/min after an equilibration period of 20 to 30 min. In some studies, 1 µM sulprostone or PGE\(_2\) was added to the superfusing medium. Fluorescence excitation was accomplished using continuous rapidly alternating illumination (20 ms per reading) from dual monochromators set at 340 and 380 nm, respectively (Deltascan; Photon Technology International, Melville, NY). The emission intensity (435 long pass filter), using 340/380 nm excitation, was continuously monitored. In situ calibration of \([\text{Ca}^{2+}]_i\) was accomplished by the addition of 0.4% digitoxin. Minimal fluorescence was determined by the addition of 10 µM 4Br-A23187 to the 1.8 mM Ca\(^{2+}\) buffer. The minimum 340/380 ratio was determined by changing to Ca\(^{2+}\)- and Mg\(^{2+}\)-free isotonic bath medium containing 2 mM EGTA and 10 µM 4Br-A23187. Free cytosolic Ca\(^{2+}\) concentration was estimated as previously reported (Gryniewicz et al., 1985; Hebert et al., 1991).

For cuvette measurement of free intracellular Ca\(^{2+}\), cells were loaded with fura-2 AM for 60 min at 23°C, washed to remove excess fura-2 AM, and resuspended at a density of 2 × 10\(^6\) cell/ml. Just before each experiment, 0.1 ml of cells were diluted into 2 ml and maintained at 37°C in the cuvette. Cells were monitored at an emission of 510 nm, using excitation wavelengths of 340 and 380 nm. Maximal fluorescence was determined by the addition of 0.4% digitoxin. Minimal fluorescence was determined by the addition of 40 µl of 1 M EGTA. Fluorescence was measured with a Fluorolog-2 spectrophotometer (SPEX Industries, Inc., Edison, NJ).

Results

Characterization of 77Awt-Evoked CRE/β-Galactosidase Activity. The EP\(_3\), 77A receptor splice variant mediates a well characterized agonist-dependent inhibition of [cAMP], when expressed in HEK293tsA201 cells (Audoly and Breyer, 1997). To assess whether a parallel decrease in forskolin-stimulated β-galactosidase activity could be measured with the CRE/β-galactosidase reporter system, the 77Awt splice variant was transfected into HEK293tsA201 cells. Initial studies examined a single high concentration of the EP\(_3\) agonist sulprostone (100 nM) on forskolin-stimulated β-galactosidase activity (Fig. 2A). Unexpectedly, the presence of 100-nM sulprostone resulted in a further increase in the forskolin-mediated up-regulation of β-galactosidase activity. Next, we examined the dose-dependent effects of sulprostone in the presence of 0.32 µM forskolin (approximately the EC\(_{50}\) concentration). No detectable sulprostone-mediated decrease in CRE/β-galactosidase activity of cells was observed. Surprisingly, agonist stimulation of the 77A receptor splice variant with sulprostone lead to a dose-dependent increase in CRE-mediated β-galactosidase activity (Fig. 2B). Similarly, sulprostone alone caused a dose-dependent increase in β-galactosidase activity with an EC\(_{50}\) of 3.1 nM (Fig. 2C). To determine whether the increase in β-galactosidase activity was mediated via a G\(_i\)-coupled pathway with β\(\gamma\) subunit stimulation of adenyl cyclase (AC; Tang and Gilman, 1991) the effect of pertussis toxin pretreatment on signal transduction was examined. As shown in Fig. 2 and Table 1, pertussis toxin (Ptx) had no detectable effect on sulprostone-stimulated β-galactosidase activity, with an EC\(_{50}\) for stimulation of

![Fig. 2](https://example.com/fig2.png)

Data are from a single experiment where each point was measured in triplicate and are representative of three independent experiments. B, cells transiently transfected with either pRc/CMV vector only (○) or pRc/CMV 77AHA (●) were incubated with or without 100 nM sulprostone and the indicated dose of forskolin for 6 h as described. Filled symbols were incubated in the absence of sulprostone, and open symbols had sulprostone included. Absorbance was monitored at 570 nm. Data are from a single experiment where each point was measured in triplicate and are representative of three independent experiments. C, experiments were performed as in (B) except the forskolin was omitted. pRc/CMV vector only (○), pRc/CMV 77AHA (●) were incubated with 0.32 µM forskolin and the indicated dose of sulprostone for 6 h as described. Filled symbols were incubated in the absence of sulprostone, and open symbols had sulprostone included. Absorbance was monitored at 570 nm. Data are from a single experiment where each point was measured in triplicate and are representative of three independent experiments.
3.1 ± 0.6 nM without Ptx pretreatment and EC\textsubscript{50} of 2.6 ± 0.5 nM with Ptx pretreatment, suggesting that neither G\textsubscript{i} nor G\textsubscript{o} heterotrimeric G proteins are involved in this signal transduction pathway.

**Signal Transduction of RA329, RE329, KA300, and DA338 EP\textsubscript{3} Receptors.** We had previously generated a series of point mutations RA329 (i.e., Arg→Ala at residue 329), RE329, KA300, and DA338, and reported their ability to inhibit [cAMP]\textsubscript{i} generation when expressed in HEK293tsA201 cells (Audoly and Breyer, 1997). The positively charged, conserved transmembrane residue R\textsubscript{329} had been identified as a residue that potentially interacts with the C1 carboxylate moiety of PGE\textsubscript{2}. Additionally, K\textsubscript{300}, although not conserved among all prostanoid receptors, is conserved among EP\textsubscript{3} receptors in each of the five species from which it has been cloned and so was also mutated. D\textsubscript{338} lies within the DPXXY motif, which is conserved among each of the prostanoid residues and has been implicated in signal transduction. Residues R\textsubscript{329} (TMVII), D\textsubscript{338} (TMVII), and K\textsubscript{300} (TMVI) had been mutated, in turn, to alanine to assess the role of these conserved charged amino acid residues in EP\textsubscript{3} receptor function. In addition, R\textsubscript{329} had been mutated to a glutamate to test whether introduction of a negative charge into the putative ligand-binding pocket caused constitutive receptor activation, as has been observed for a similar substitution in bovine rhodopsin (Robinson et al., 1992). Using a radioimmunoassay (RIA), we had determined that, although KA300 inhibited cAMP generation indistinguishably from wild-type receptor, the RA329, RE329, and DA338 receptors each lost the ability to inhibit cAMP at sulprostone concentrations up to 1 μM. The ability of each of these proteins to signal through the CRE/β-galactosidase pathway when expressed in HEK293tsA201 cells was assessed. Using the CRE/β-galactosidase system, KA300 receptors displayed a sulprostone-dependent increase in enzimatic activity with an EC\textsubscript{50} of 3.7 nM, which was essentially similar to the wild-type receptor (Table 2, Fig. 3). In contrast, neither the RA329, RE329, nor DA338 displayed any detectable increase in β-galactosidase activity. Thus, the results obtained using the CRE reporter system showed increased enzymatic activity in an EP\textsubscript{3} agonist-dependent manner, paralleling the decrease in [cAMP] measured determined by RIA (Table 2).

**Measurement of cAMP Levels.** Although the increase in CRE-mediated transcription directly contradicts the previously observed decreases in cAMP levels, differences in the time of agonist exposure for the CRE reporter experiments (6 h) versus the direct cAMP measurement (15 min) might account for the discrepancy in these two systems. Receptor-evoked cAMP generation was therefore measured directly over the 6-h time course used for the reporter assay. As shown in Fig. 4, there was no significant increase in cAMP generation in response to 100 nM sulprostone at incubation times up to 6 h, although forskolin potently increased [cAMP]. Moreover, in the absence of forskolin there was no increase in cAMP generation at the 6-h time point using sulprostone concentrations up to 1 μM, extending our earlier finding that this EP\textsubscript{3} splice variant does not cause agonist-dependent increases in forskolin or receptor-stimulated [cAMP] (Fig 4B).

**Trans Activation of CREB-GAL4 Fusion Reporter System.** The lack of cAMP generation, although not unexpected, raised the possibility that the pCRE/β-galactosidase response was being elicited by a nonCREB-mediated pathway. To test this possibility, an independent reporter construct, which does not rely on endogenous cellular CREB for signal transduction, was used. The reporter plasmid pFR-Luc encodes the luciferase gene under transcriptional control of five repeated UAS\textsubscript{GAL} binding elements. UAS\textsubscript{GAL} is a yeast-specific enhancer element that is activated by the Gal4 protein. The UAS\textsubscript{GAL} element is not activated in HEK293tsA201 cells in the absence of transfected Gal4 protein (data not shown). However, when a chimera comprised of the Gal4 DNA binding domain (aa 1–147) fused to CREB (aa 1–280) is coexpressed, trans activation of UAS\textsubscript{GAL}·luciferase via CREB, resulting in an increase in luciferase activity, may be observed. Receptor-evoked increases in luciferase activity by the 77A EP\textsubscript{3} splice variant therefore allows unambiguous assessment of the ability of the EP\textsubscript{3} receptor to activate the CREB pathway. When coexpressed with the Gal4·luciferase reporter system, the 77A receptor demonstrated an agonist-dependent increase in luciferase reporter activity with an EC\textsubscript{50} of 2.9 ± 1.4 nM (Fig. 5), similar to the EC\textsubscript{50} value of 3.1 nM observed for the CRE/βgal plasmid. Taken together with the absence of increase in [cAMP], by direct measure-

### Table 1

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Not treated</th>
<th>Ptx-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>72A</td>
<td>1.5 ± 0.4 (n = 5)</td>
<td>1.7 ± 0.9 (n = 4)</td>
</tr>
<tr>
<td>74A</td>
<td>1.3 ± 0.2 (n = 5)</td>
<td>3.7 ± 1.1 (n = 4)</td>
</tr>
<tr>
<td>77A</td>
<td>3.1 ± 0.6 (n = 11)</td>
<td>2.6 ± 0.5 (n = 6)</td>
</tr>
<tr>
<td>80A</td>
<td>2.2 ± 0.8 (n = 5)</td>
<td>3.6 ± 1.4 (n = 4)</td>
</tr>
<tr>
<td>NT</td>
<td>0.8 ± 0.2 (n = 7)</td>
<td>1.2 ± 0.4 (n = 4)</td>
</tr>
</tbody>
</table>

### Table 2

<table>
<thead>
<tr>
<th>Receptor</th>
<th>RIA\textsuperscript{a}</th>
<th>CRE/lacZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>0.2 ± 0.1</td>
<td>3.1 ± 0.6 (n = 11)</td>
</tr>
<tr>
<td>KA300</td>
<td>0.2 ± 0.1</td>
<td>3.7 ± 1.1 (n = 4)</td>
</tr>
<tr>
<td>RA329</td>
<td>not detected</td>
<td>not detected</td>
</tr>
<tr>
<td>RE329</td>
<td>not detected</td>
<td>not detected</td>
</tr>
<tr>
<td>DA338</td>
<td>not detected</td>
<td>not detected</td>
</tr>
</tbody>
</table>

\textsuperscript{a} RIA values were obtained from (Audoly and Breyer, 1997).
ment, this observation indicates that the CREB activation is mediated through a noncAMP-mediated pathway.

**Signal Transduction Characteristics of EP<sub>3</sub> Receptor Splice Variants.** Earlier work with EP<sub>3</sub> splice variants isolated from other mouse, human, and cow (Namba et al., 1993; Sugimoto et al., 1993; An et al., 1994) demonstrated differential signal transduction from individual splice variants. The ability of each of the isolated rabbit splice variants was therefore tested for its ability to activate the CREB-promoted β-galactosidase. cDNAs encoding five distinct rabbit EP<sub>3</sub> receptor splice variants, differing only in their intracellular carboxyl domains (Fig. 1), were assayed for their ability to activate the CREB/β-galactosidase. In addition to the previously cloned 77A, 74A, 72A, and 80A splice variants, a novel splice variant designated NT was isolated by reverse transcription-PCR from indomethacin-treated rabbit cortical collecting duct cells. Each of the five splice variants tested were capable of eliciting receptor-mediated increases in CREB-promoted β-galactosidase activity (Fig. 6). The EC<sub>50</sub> values for each of the receptors ranged 4-fold, from 0.8 nM for the NT variant to 3.1 nM for the 77A splice variant. Although the difference between the 77A and NT variants was statistically significant (p < .05), the functional significance of this difference is uncertain. Each of the other splice variants displayed intermediate EC<sub>50</sub> values (Fig. 6 and Table 1). We further examined the pertussis toxin sensitivity of the pathway activated by each individual receptor splice variant. None of these splice variants displayed any statistically significant differences in EC<sub>50</sub> values when exposed to Ptx (Table 1), suggesting that this signal transduction pathway is independent of G<sub>i</sub>/G<sub>o</sub> proteins.

**Receptor-Evoked Ca<sup>2+</sup> Increases Mediated Signal Transduction CREB.** To identify alternate signaling pathways that might be mediating the receptor-evoked increases in CREB, the ability of the 77A receptor to increase intracellular Ca<sup>2+</sup> was determined. Fura-2-loaded HEK293 cells transfected with the 77A splice variant of the EP<sub>3</sub> receptor were superfused with 1 μM sulprostone. A modest rise in Ca<sup>2+</sup> was observed, which was absent in cells transfected...
with the vector alone (Fig. 7) or the inactive RA329 mutant receptor (data not shown). Similar results were obtained with cells superfused with PGE$_2$ or assayed in suspension using the cuvette method (data not shown).

The ability of protein kinase (PK) inhibitors to block the CRE-mediated transcription was assessed. The CREB/βgal reporter signal transduction was partially inhibited by the PKC inhibitor bisindolylmaleimide (Toullec et al., 1991; Fig. 8). In contrast, PD98059, an inhibitor of the mitogen-activated protein kinase cascade (Pang et al., 1995), yielded only a very slight inhibition of sulprostone-evoked reporter activity. Treatment of cells with staurosporine, a very potent inhibitor of PKC, PKA, as well as other serine/threonine kinases (Schächtele et al., 1988; Rüegg and Burgess, 1989), completely abolished signal transduction (Fig. 8).

**Discussion**

These studies characterize a novel system for the analysis of signal transduction of EP$_3$ receptor splice variants and EP$_3$ receptor mutations. Moreover, these studies provide evidence for an alternative pathway by which these receptors might mediate their diverse physiological effects in vivo. Interestingly, although the EP$_3$ receptor is classically thought to be

---

**Fig. 7.** Effect of sulprostone on [Ca$^{2+}$]$_i$ in cells expressing the EP$_3$ receptor 77A splice variant. [Ca$^{2+}$]$_i$ was measured in fura-2 loaded, transfected HEK293tsA201 cells treated with 1 μM sulprostone. Top, cells transfected with wild-type 77A receptor. Bottom, cells transfected with vector alone.

**Fig. 8.** Signal transduction blockade of the 77A EP$_3$ receptor splice variant expressed in HEK293tsA201 cells using selective kinase inhibitors. Effects of staurosporine, PD98059, and bisindolylmaleimide I on the sulprostone-dependent response evoked in cells expressing the 77A receptor splice variant. Cells were transiently transfected with either pRc/CMV 77AHA were incubated with the indicated dose of sulprostone and appropriate kinase inhibitor for 6 h as described. Sulprostone alone, no inhibitor, sulprostone plus 2 μM staurosporine, 100 μM PD98059, or 2 μM bisindolylmaleimide I. Absorbance was monitored at 570 nm, and normalized to the A$_{570}$ maximum for sulprostone alone. Data are from a single experiment where each point was measured in triplicate and are representative of two or three similar experiments.
coupled to the G family of heterotrimeric GTP binding proteins, activation of the CRE pathway by each of the receptor splice variants was pertussis toxin-insensitive, suggesting that this pathway is not mediated by the G family.

Based on our previous findings that the 77A receptor splice variant was capable of receptor-mediated inhibition of [cAMP] in a dose-dependent manner in HEK293tsA201 cells, the ability of the 77A receptor splice variant to inhibit cAMP response element-driven/β-galactosidase activity was assessed. The unexpected finding that forskolin and sulprostone additively increased CRE/β-galactosidase activity precluded the detection of EP-mediated inhibition of CRE/β-galactosidase activity. Nonetheless, receptor-evoked stimulation of CRE/β-galactosidase activity appears to be a reliable indicator of EP receptor activation. The activity of each of the 77A receptor point mutations completely corresponded to their capacity to inhibit cAMP generation as described previously. This result suggests that similar upstream receptor-mediated mechanisms direct both the inhibition of adenylyl cyclase and CRE/β-galactosidase activation. Furthermore, it suggests that the CRE/β-galactosidase system may be applicable to the study of signaling properties of EP receptor mutations.

Activation of CREB was observed in two independent reporter plasmid systems including activation via the Gal4-CREB/UASβ-gal-luciferase system, which responds exclusively to the activation of CREB. Although the precise mechanism of CREB activation remains unclear, the observed increase in intracellular Ca2+, taken together with bisindolylmaleimide I blockade of reporter activity, suggests that a Ca2+/PKC-mediated pathway is involved in signal transduction. These findings are similar to previously reported signal transduction pathways observed in B-cells. In that case, cross-linking of surface Ig lead to activation of CREB by PKC in the absence of increases in [cAMP] (Xie and Rothstein, 1995). The observed partial inhibition of CRE transcription of CRE reporter transcription by the PKC inhibitor bisindolylmaleimide I raises the possibility that other kinases may also play a role in this signal transduction pathway; however, the lack of effect of PD98059 suggests that the MAP kinase cascade is not involved.

Analysis of EP-mediated signal transduction in heterologous expression systems is complicated by the finding that EP receptor splice variants cloned from several species have different signaling properties, depending on the intracellular carboxyl tail and cellular environment in which the cDNAs are expressed. To determine whether the CRE-reporter system is activated by each of the EP splice variants, signal transduction by several previously uncharacterized rabbit EP receptor splice variants was assessed. The tail-less (NT) splice variant lacks any additional amino acid residues past the point of divergence. Nevertheless, it and each of the receptors tested was capable of receptor-evoked increases in β-galactosidase activity. Inasmuch as the NT receptor is capable of receptor-evoked signaling in an agonist dependent fashion, the carboxyl tail of EP receptors is not essential for signal transduction through this pathway. This finding is at variance with the signal transduction properties of the murine EP receptor, which was truncated at the analogous position by site-directed mutagenesis. In that case, the murine receptor was alternately reported to be inactive (Irie et al., 1994) or constitutively active (Hasegawa et al., 1996), but in neither case did the receptor demonstrate agonist-dependent signal transduction. In both of these mutagenesis studies, the authors examined G-mediated mechanisms, as opposed to the pertussis toxin-insensitive mechanism involved in the current study. Taken together, these data support the notion that the C-terminal sequence plays a role in differential signaling by the EP receptors (Namba et al., 1993).

The EC50 value for CRE/β-galactosidase activation was approximately 15-fold higher (less sensitive) than the EC50 values of receptor-mediated cAMP inhibition measured by RIA. This may reflect the difference in intrinsic affinity of the receptor-G protein interaction for the two signaling pathways activated, or it may be the result of differences in direct measurement of cAMP concentration versus indirect measurement of cAMP via transcription-translation. In contrast to the higher EC50 values found for the EP3 receptor when measured by reporter system versus RIA, Liaw et al. (1994) observed the reverse phenomenon where the EC50 values of the corticotropin releasing factor receptor as measured by β-galactosidase activity was more than 10-fold lower than that measuring cAMP directly. Similarly, using the mouse melanocortin receptor mMC5-R, Chen et al. (1995) found the CRE/β-galactosidase assay to be, in general, 3 to 5 times more sensitive than those obtained with the adenylyl cyclase assay. Similar findings were observed for the bombesin receptor where the reporter gene was more sensitive than direct calcium measurements. The reversal of sensitivity observed in the current studies relative to these previous reporter gene studies may reflect fundamental differences in the signal transduction pathway activated by these different receptors.

In conclusion, using a CRE/β-galactosidase system, the signaling properties of several mutated EP receptor splice variants were described. The activation of CRE/β-galactosidase by EP receptor splice variants expands the repertoire of signal transduction cascades mediated by these proteins. Increases in CRE phosphorylation have been associated with drug addiction and regulation of neuronal gene expression (Nestler, 1993) and circadian rhythms (Ginty et al., 1993). In addition to these phenomena, parallel work on memory implicates CREB as a key molecule in converting short-term environmental stimuli into long-term changes in cell physiology (Hyman, 1996). These results suggest that prostaglandin EP receptor splice variants may participate in both short- and long-term regulation of cellular events, via independent signaling pathways.

References


1999

Prostaglandin EP3 Receptor Signaling

147

Downloaded from jpet.aspetjournals.org on June 26, 2017