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Detection of EP_2, EP_4, and FP Receptors in Human Ciliary Epithelial and Ciliary Muscle Cells

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ABSTRACT. We have examined the expression of three prostaglandin (PG) receptors, EP_2, EP_4, and FP, in a nonpigmented ciliary epithelial cell line (ODMCl-2) and in human ciliary muscle (HCM) cells. Total RNA preparations from either ODMCl-2 or HCM cells were subjected to reverse transcription-polymerase chain reaction (RT-PCR) with sense and antisense primers for each of the three PG receptors. The RT-PCR generated DNA products of predicted sizes corresponding to the EP_2, EP_4, and FP receptors in both ODMCl-2 and HCM cells. PCR products corresponding to each receptor were hybridized with specific ^{32}P-labeled probes and, for further confirmation, digested with appropriate restriction enzymes. Pharmacological studies with the EP_2 receptor-selective agonist butaprost resulted in a significant increase in the cyclic AMP level in ODMCl-2 cells. The stimulation of cyclic AMP in ODMCl-2 cells by PGE_2 and 11-deoxy PGE_1, the respective $EP_1/EP_2/EP_3/EP_4$ and $EP_2/EP_3/EP_4$ receptor agonists, was concentration-dependently inhibited by the EP_4 receptor-selective antagonist AH23848. These results conclusively demonstrate the presence of both mRNA and protein for EP_2, EP_4, and FP receptors in ODMCl-2 and HCM cells. BIOCHEM PHARMACOL 53;9:1249–1255, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. human nonpigmented ciliary epithelial cells; ciliary muscle cells; RT-PCR; adenylyl cyclase

Specific PG[‖] receptors have been pharmacologically detected in a variety of tissues, including those of the eye of different animal species [1, 2]. By now, all of the pharmacologically described PG receptors have been cloned and their presence has been detected in human and animal tissues. Hirata *et al.* [3] cloned the thromboxane A_2 (TP) receptor. Subsequently, the EP_1, EP_2, EP_3, EP_4, FP, IP, and DP receptors were cloned [4–17]. A human EP receptor subtype cloned by Bastien *et al.* originally was referred to as the human EP_2 receptor, but later was determined to be the EP_4 subtype [17]. Regan *et al.* [17] cloned the pharmacologically defined EP_2 receptor. All these receptors have a deduced structure containing the seven-transmembrane domain that typifies G-protein coupled receptors.

PGs are important as inflammatory mediators, but they also have other actions. In the eye, for example, $PGF_{2\alpha}$ and its derivatives reduce intraocular pressure in animals and humans [18–20]. Selective analogues of $PGF_{2\alpha}$ are now undergoing clinical trial as ocular hypotensive agents for the treatment of glaucoma [21]. The EP_2 receptor detected in the iris-sphincter and ciliary muscles of the eye [22] has been reported to mediate the reduction of intraocular pressure by 11-deoxy PGE_1 [23]. The regional distribution and characteristics of PG-specific binding sites and prostanoid receptors in ocular tissues suggest that there are high-affinity binding sites for PGE_2 and $PGF_{2\alpha}$ in the iris-sphincter muscles and ciliary bodies of cows and rabbits [24–26]. Bhattacherjee *et al.* [27] have reported the presence of PGE_2-specific binding sites in rabbit nonpigmented ciliary epithelial cells. Liu *et al.* [28] and Jumblatt *et al.* [29] have reported that ODMCl-2 cells respond to PGE_2 with an increase in intracellular cyclic AMP. Autoradiographic and biochemical studies have demonstrated that FP and EP_2 receptors are expressed in human ciliary muscles and cells [30, 31]. EP_4, the most recently identified subtype of EP receptors, was first detected in porcine saphenous vein, where it was found to mediate PG-induced smooth muscle relaxation [32]. No selective agonist has been identified for this receptor subtype. PGE_2 is the only natural PG that is

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‖ *Abbreviations:* PG, prostaglandin; ODMCl-2, Simian-Virus-40-transformed human nonpigmented ciliary epithelial cell line; HCM, human ciliary muscle; RT-PCR, reverse transcription-polymerase chain reaction; PGE_2, prostaglandin E_2; 11-deoxy PGE_1, 11-deoxy prostaglandin E_1; AH23848, [1α(Z), 2β, 5α]-(±)-7-[5-[[(1,1′-biphenyl)-4-yl]methoxy]-2-(4-morpholinyl)-3-oxocyclopentyl]-4-heptenoic acid; AH22921, [1α(Z), 2β, 5α]-(±)-7-[5-[[(1,1′-biphenyl)-4-yl]methoxy]-2-(4-morpholinyl)-3-oxocyclopentyl]-5-heptenoic acid; D-MEM, Dulbecco's Modified Eagle's Medium; mRNA, messenger ribonucleic acid; cDNA, complementary deoxyribonucleic acid; SSPE, sodium chloride sodium phosphate ethylenediaminetetra acetic acid; IL-2, interleukin-2; and $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$.

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1000- to 6000-fold more potent than other PGs in causing smooth muscle relaxation in porcine saphenous vein. However, this EP_4-mediated response can be differentiated from other EP receptor-mediated effects through use of the selective inhibitors AH22921 and AH23848 [2, 32]. In the present study, we have examined the expression of EP_2, EP_4, and FP receptors in ODMCl-2 and HCM cells at the molecular and pharmacological levels.

MATERIALS AND METHODS

PGE_2 and 11-deoxy-PGE_1 were obtained from Cayman Chemicals (Ann Arbor, MI). Butaprost and AH23848 were gifts from Bayer (West Haven, CT) and Glaxo (Hartfordshire, U.K.), respectively. Trypsin, calf serum, antibiotic-antimycotic and D-MEM were supplied by Gibco-BRL (Grand Island, NY). The ODMCl-2 cell line was a gift from Dr. M. Coca-Prados of Yale University Medical School (New Haven, CT). The cyclic AMP assay kit was obtained from Amersham International (Arlington Heights, IL). All other chemicals were purchased from the Sigma Chemical Co. (St. Louis, MO). The plasmids containing the cDNA sequences of the human FP receptor and human EP_4 receptor were provided by Merck Frosst Canada, Inc., (Quebec, Canada). The pGEM DNA markers were purchased from the Promega Corp. (Madison, WI).

Culture of Human Ciliary Muscle and Nonpigmented Ciliary Epithelial Cells

Human eyes were obtained 6 hr postmortem from the University of Louisville Kentucky Lions Eye Bank. As described in detail elsewhere [30], ciliary muscles were separated from their adjacent tissues, cut into 5 × 5 mm explants, washed 2–3 times in culture medium, and plated on 6-well culture plates. The culture medium was D-MEM containing 10% fetal bovine serum and antibiotic-antimycotics. All explants were covered with coverslips and incubated in an atmosphere of 95% air and 5% CO_2 at 37° in the culture medium, which was changed twice a week. When primary cultures reached confluency, cells were collected from individual wells by trypsinization, then subcultured at a ratio of 1:3. Isolated cells were passaged, and their identity was established by immunocytochemistry using desmin antibody (Pierce, Rockford, IL). Human nonpigmented ciliary epithelial cells were cultured to confluency in 6-well plates using D-MEM containing 10% fetal bovine serum and antibiotic-antimycotics.

Determination of Cyclic AMP

After human nonpigmented ciliary epithelial cells had been cultured to confluency, the medium was aspirated from each well. The cells were then incubated in the presence of selected concentrations of PG agonists in 2 mL of Kreb's pyruvate buffer at 37° for 15 min in a shaking water bath. Cells not exposed to agonists or antagonists incubated as above were used as controls to ascertain the basal level of cyclic AMP. At the end of the incubation, cells were transferred to tubes, to which 0.5 mL of hot 0.1 N NaOH was then added. Five minutes later, 0.5 mL of 0.1 N HCl was added to neutralize the solution. Samples were then centrifuged for 5 min at 15,000 *g*. The supernatant was removed for the assay of cyclic AMP using the protein-binding kit (Amersham International). The net amount of cyclic AMP formed (stimulated-basal) was expressed as picomoles per milligram protein per 15 min. Protein concentration was determined according to the method of Lowry *et al.* [33].

Isolation of RNA

Confluent ODMCl-2 or HCM cells were collected by scraping in a guanidinium thiocyanate homogenization buffer (4 M guanidinium thiocyanate, 0.5% *N*-sodium lauryl sarcocinate, 25 mM sodium citrate, and 0.7% 2-mercaptoethanol) at pH 7.0. Total RNA was prepared according to the guanidinium thiocyanate method [34]. The final RNA pellet was stored as an alcohol precipitate at −70° until used.

Oligonucleotide PCR Primers

Sense and antisense specific primers were synthesized at Life Technologies (Grand Island, NY). For the human FP receptor, the sense primer [5′-GCGGCTCCGTCTTCT-GCTCCTCA-3′] corresponded to nucleotide sequences −126 to −104 (in the 5′ untranslated region of the mRNA); the antisense primer [5′-ACCGTCCAATGGCCATCA-CACTGC-3′] corresponded to nucleotide sequences 377–400 of the human FP receptor sequence [13]. For the EP_4 receptor, the sense primer [5′-GGTCATCTTACTCATT-GCCACC-3′] and the antisense primer [5′-AGATG-AAGGAGCGAGAGTGG-3′] corresponded, respectively, to nucleotide sequences 1197–1218 and 1512–1531 of the human EP_4 receptor sequence [16]. For the EP_2 receptor, the sense primer [5′-GCTGCTGCTTCTCATT-GTCTCG-3′] corresponded to nucleotide sequences 765–786; the antisense primer [5′-TCCGACAACAGAG-GACTGAACG-3′] corresponded to nucleotide sequences 1135–1156 of the human EP_2 receptor sequence [17].

RT-PCR

Using the Perkin-Elmer GeneAmp RNA PCR kit from Roche Molecular Systems, Inc. (Branchburg, NJ) total RNA (2 μg) from each cell type was reverse-transcribed to synthesize the first-strand cDNA using random hexanucleotide primers. The reverse transcription reaction (final volume 20 μL) contained 5 mM $MgCl_2$, 2 μL 10× PCR buffer, 2 μg total RNA, 1 mM each of dATP, dCTP, dGTP, and dTTP, 1 μL RNase inhibitor (20 U/μL), 1 μL MuLV

reverse transcriptase (50 U/μL) and 2.5 μM random hexamers. The reaction was carried out at 42° for 15 min and then, consecutively, for 5 min at 99° and 5°. The first-strand cDNA was used as a template for PCR amplification with sense and antisense primers. The PCR reaction mixture (final volume, 100 μL) contained 20 μL from the reverse transcription reaction, 8 μL 10× PCR buffer, 2 μL (2 μM) of each sense and antisense primer, 0.5 μL AmpliTaq DNA polymerase (Perkin-Elmer, 5 U/μL), and 2 mM $MgCl_2$. For the amplification of EP_2 and EP_4 receptors from ODMCl-2, and HCM, 40 PCR cycles were carried out, each for 1 min at 94°, 2 min at 55°, and 2 min at 72°. For the human FP receptor, 40 PCR cycles were carried out, each for 1 min at 94°, 2 min at 57°, and 2 min at 72°. In each case, the last cycle ended with 7 min at 72°. The PCR products were purified using the QIAquick PCR purification kit (QIAGEN, Chatsworth, CA) and were electrophoresed on 1.5% agarose gel for Southern transfer. A portion of each PCR product was digested with appropriate restriction enzymes and analyzed by agarose gel electrophoresis.

Southern Blot Hybridization

cDNA probes for Southern hybridization were excised from the plasmids by digestion with specific endonucleases. For example, in the case of the FP receptor, a 1.5 kb *Pst*I fragment (nucleotide 123 to 1675) and, for the EP_4 receptor, a 0.4 kb *Sma*I fragment (nucleotide 733 to 1165) were digested out. These fragments were then labeled with [α-^{32}P]dCTP (3000 Ci/mmol; DuPont-NEN, Boston, MA), using a random priming labeling system according to the manufacturer's instructions (Boehringer-Mannheim, Indianapolis, IN). The purified PCR product corresponding either to the FP or the EP_4 receptor was electrophoresed on 1.5% agarose gels, transferred to nylon membranes (GeneScreen, NEN Research Products, Boston, MA), and hybridized with a ^{32}P-labeled probe at 45° in a solution containing 5× SSPE (0.9 M NaCl, 50 mM $NaHPO_4$, pH 7.7, 5 mM EDTA), 5× Denhardt's solution, 100 μg/mL denatured salmon sperm DNA, and 50% formamide. After hybridization, the filters were washed with 2× SSPE/0.1% SDS followed by 0.2× SSPE/0.1% SDS at 65° and then were exposed to the phosphoimager screen. Images were scanned using a Molecular Dynamics (Sunnyvale, CA) phosphoimager.

A 39-mer oligonucleotide (5′-CTCAACCTCATCCGCATGCACCGCCGAAGCCGG-3′) was designed for the EP_2 receptor, based on the sequence from nucleotide 816 to 853 of the published cDNA sequence for that receptor [17]. The oligonucleotide was labeled with [γ-^{32}P]ATP (3000 Ci/mmol) and used as a hybridization probe. Hybridization was done at 50° in a solution containing 5× SSPE, 2× Denhardt's solution, and 100 μg/mL denatured salmon sperm DNA. The filter was washed with 2× SSPE/0.1% SDS followed by 0.2× SSPE/0.1% SDS at 37°, and then were exposed to the phosphoimager screen.

RESULTS

The FP Receptor mRNA

Human ciliary muscle and ODMCl-2 cells tested for the presence of FP receptor mRNA by RT-PCR demonstrated the formation of PCR products of the predicted size (526 bp) (Fig. 1, lanes 1 and 2). In the absence of reverse transcriptase, no PCR product was detected, indicating that the PCR product was derived from RNA rather than DNA (Fig. 1, lanes 6 and 7). Restriction digestion of the purified PCR product with MscI yielded fragments of the correct sizes: 331, 149, 31, and 15 bp (data not shown).

The EP_2 Receptor mRNA

RT-PCR with RNA from either HCM or ODMCl-2 cells and EP_2 primers led to formation of the PCR product of the expected size, 394 bp (Fig. 2, lanes 1 and 2). No product was detected in the absence of reverse transcriptase, confirming that the product was derived from RNA (Fig. 2, lanes 6 and 7). Fragments of the predicted sizes, 264 and 126 bp, were obtained on restriction digestion of the PCR products with *Sma*I (data not shown).

The EP_4 Receptor mRNA

ODMCl-2 and HCM cells were also examined by RT-PCR for the expression of EP_4 receptor mRNA. As can be seen in Fig. 3, a PCR product of the predicted size, 334 bp, was

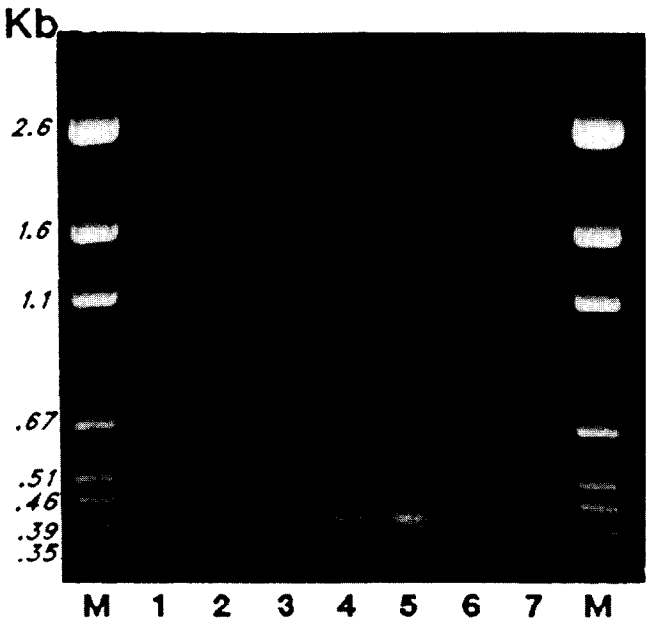

FIG. 1. Detection of FP receptor mRNA by RT-PCR, showing a 526-bp FP receptor product: (1) ODMCl-2 RNA; (2) HCM RNA; (3) human ovary RNA (positive control); (4 and 5) positive controls with human IL-1α primers; (6) ODMCl-2 RNA without reverse transcriptase; and (7) HCM RNA without reverse transcriptase. Lanes marked as "M" are pGEM DNA size markers from Promega.

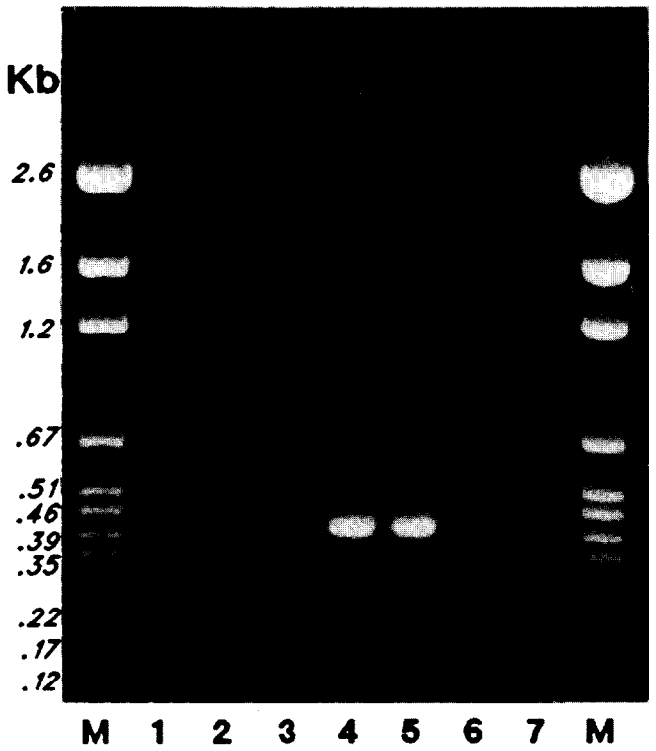

FIG. 2. Detection of EP_2 receptor mRNA by RT-PCR, showing a 394-bp EP_2 receptor product: (1) ODMCl-2 RNA; (2) HCM RNA; (3) human lung RNA (positive control); (4 and 5) positive controls with human IL-1α primers; (6) ODMCl-2 RNA without reverse transcriptase; and (7) HCM RNA without reverse transcriptase. Lanes marked as "M" are pGEM DNA size markers from Promega.

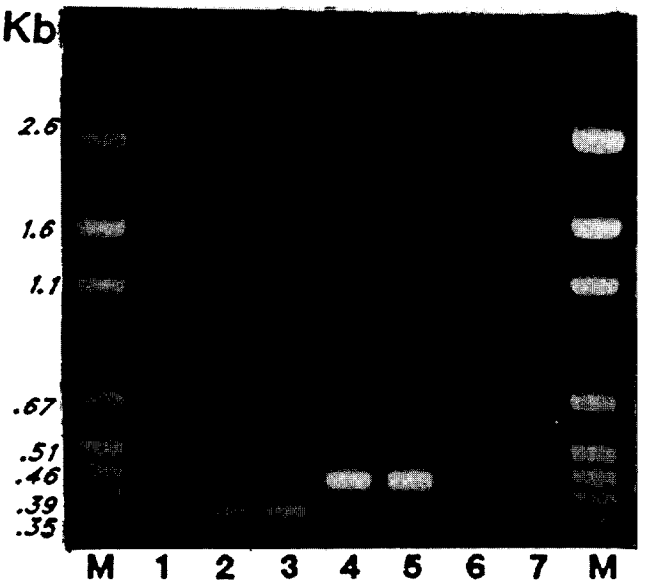

FIG. 3. Detection of EP_4 receptor mRNA by RT-PCR, showing a 334-bp EP_4 receptor product: (1) HCM RNA (8 μg total RNA); (2) ODMCl-2 RNA (2 μg total RNA); (3) human lung RNA (2 μg total RNA, positive control); (4 and 5) positive controls with human IL-1α primers; (6) HCM RNA without reverse transcriptase; and (7) ODMCl-2 RNA without reverse transcriptase. Lanes marked as "M" are pGEM DNA size markers from Promega.

obtained from both sources (Fig. 3, lanes 1 and 2). Restriction digestion of the purified PCR products with *Eco*RI generated fragments of the expected sizes, 138 and 196 bp (data not shown). However, it should be noted that the PCR products for EP_4 receptors in HCM cells required 8 μg of total RNA to produce a distinct band. The amount of total RNA used in HNPE or human lung cells was only 2 μg. This amount of RNA from HCM cells generated a barely detectable band.

Southern Hybridization

Using Southern blot analysis to determine whether the PCR products hybridized with probes for the appropriate PG receptors, we obtained positive signals for FP, EP_2, and EP_4 receptors in ODMCl-2, as well as in HCM cells. This confirmed the presence of these receptor mRNAs in these cells. The intensity of the signal for EP_4 receptor PCR product from HCM cells was much less than that from HNPE cells. This probably indicates a lower abundance of EP_4 receptor mRNA in HCM cells.

Stimulation of Adenylyl Cyclase

In ODMCl-2 cells, PGE_2, 11-deoxy PGE_1, and butaprost markedly increased cyclic AMP levels in a concentration-dependent manner (Fig. 4a). Of these agonists, 11-deoxy PGE_1 appeared to be the least potent in generating cyclic AMP in the concentration range of 0.1 to 1.25 μM. The production of cyclic AMP in response to PGE_2 and 11-deoxy PGE_1 was concentration-dependently reduced by the EP_4 antagonist AH23848 (Fig. 4b). At the highest concentration of the antagonist used, the reductions were 36% for PGE_2 and 77% for 11-deoxy PGE_1.

DISCUSSION

Previous pharmacological and biochemical studies have suggested the presence of EP_2 receptors in ODMCl-2 cells [28, 29]. Our present studies at the molecular level confirm this suggestion, providing direct evidence of the expression of EP_2 receptors in this cell line. The generation of cyclic AMP by butaprost, the EP_2 receptor agonist (Fig. 4a), also confirmed the RT-PCR evidence of the expression of the EP_2 receptor mRNA. We demonstrated that the generation of cyclic AMP by PGE_2 and 11-deoxy PGE_1 was inhibited by an EP_4 receptor antagonist, AH23848 (Fig. 4b). This antagonist, although not highly potent, is highly selective for EP_4 receptors [32]. Therefore, we have provided pharmacological evidence that EP_4 receptors are present in ODMCl-2 cells. This pharmacological evidence was confirmed by RT-PCR and Southern hybridization studies. The relevance of the presence of EP_2, EP_4, and FP receptors in ODMCl-2 cells is largely speculative, although it has been suggested that the disruption of the blood-aqueous barrier

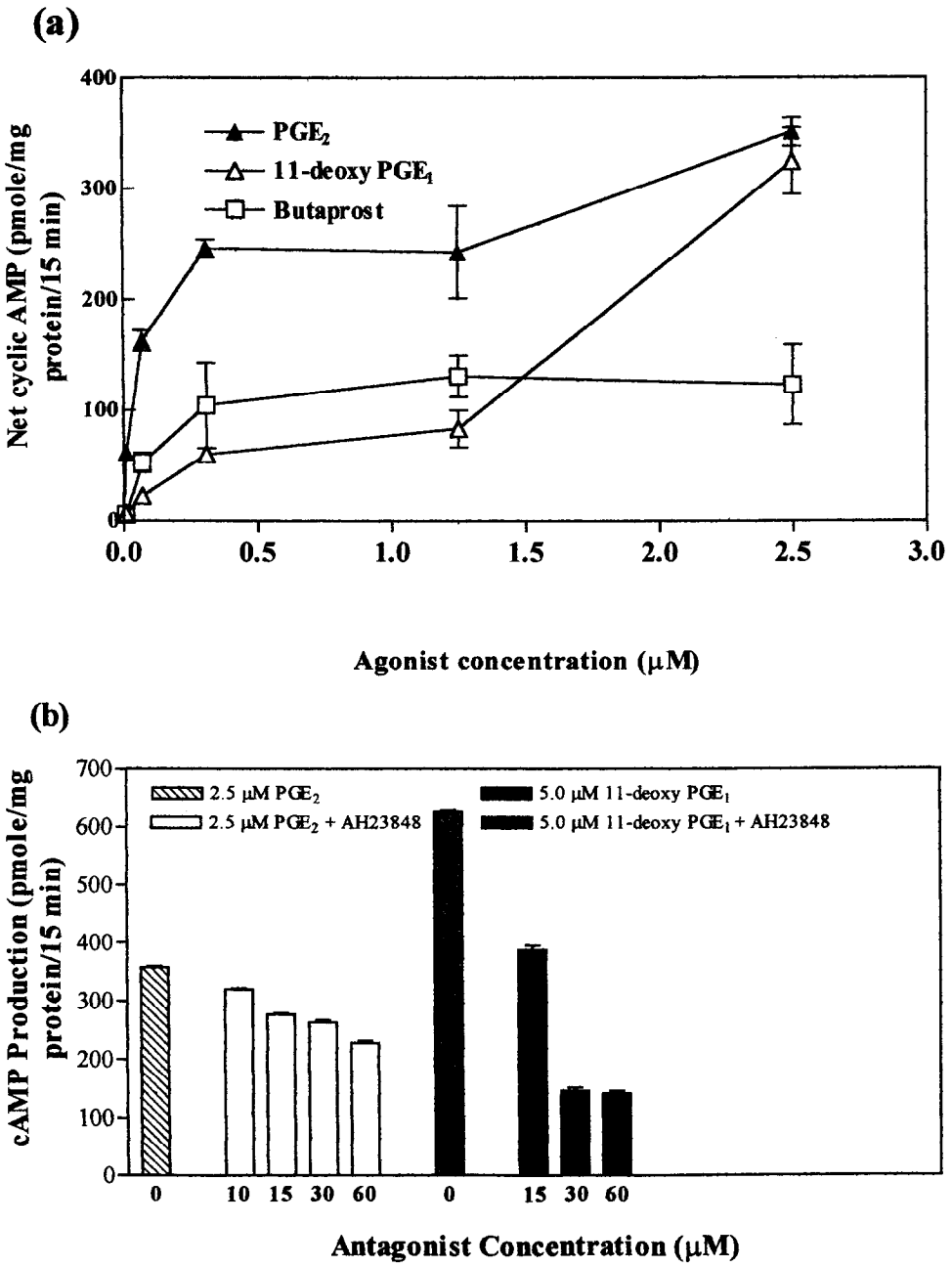

FIG. 4. (a) Formation of cyclic AMP in ODMCl-2 cells in response to butaprost, PGE_2, and 11-deoxy PGE_1, expressed as the net amount formed/mg protein/15 min. The columns represent means ± SEM of three experiments with triplicates at each concentration. (b) Inhibition of cyclic AMP formation in response to prostaglandin by the EP_4-selective antagonist AH23848 in ODMCl-2 cells in response to PGE_2 and 11-deoxy PGE_1. The columns represent means ± SEM of three experiments with triplicates at each concentration.

by 11-deoxy PGE_1 is mediated by EP_2 receptors [35]. Now that the agonist 11-deoxy-PGE_1 has been found to have affinity for EP_4, in addition to EP_2 and EP_3, it is conceivable that this receptor subtype is also involved in disruption of the blood-aqueous barrier.

PGs have other pharmacological actions, such as anti-inflammatory [36–40] and immunomodulatory effects [41–43]. PGs are generated and released during ocular inflammation. Lymphocytes and cytokines also play an active role in the initiation and sustenance of inflammatory reactions in experimental immune and autoimmune uveitis. PGs cause suppression of B and T cell proliferation and IL-2 generation probably via cyclic AMP. This second messenger is formed following stimulation of EP_2 and EP_4 receptors. Therefore, it is possible that elevated cyclic AMP level by stimulated EP_2 and EP_4 receptors in the ocular tissues underlines the anti-inflammatory and immunomodulatory actions of PGs in the eye and elsewhere. Physiological significance of FP receptor expression in ODMCl-2 cells is yet to be elucidated.

Our use of the RT-PCR and Southern hybridization techniques confirmed the presence of EP_2, FP, and EP_4 receptor mRNAs in HCM cells. Ciliary muscle is thought to be the site of the ocular hypotensive action of $PGF_{2\alpha}$ in humans [21, 44]. Therefore, the FP receptors detected in HCM cells may be directly involved in the ocular hypotensive action of $PGF_{2\alpha}$. The stimulation of EP_2 receptors not only mediates the relaxation of smooth muscle cells [2]

but also causes relaxation of the feline ciliary muscle, with concomitant elevation of intracellular cyclic AMP [45]. It is possible that EP_2 receptor stimulation may also cause relaxation of human ciliary muscles. EP_4 receptors also act via G_s protein elevating cyclic AMP and, hence, may also play a role in the relaxation of human ciliary muscles.

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