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Involvement of EP1 and EP2 receptors in the regulation of the Na,K-ATPase by prostaglandins in MDCK cells

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Abstract

Prostaglandins are key regulators of ion transport in the kidney. In MDCK cells, which model distal tubule cells, the transcription of the Na,K-ATPase β 1 subunit is regulated by PGE₁ and PGE₂. To identify the EP receptors that mediate transcriptional regulation, transient transfection studies are conducted using the human β 1 promoter/luciferase construct, pH β 1-1141 Luc. The involvement of EP1 and EP2 receptors is indicated by studies with the EP1 selective agonist 17-phenyl trinor PGE₂, and the EP2 selective agonist butaprost (which stimulate), as well as by studies with the antagonists SC-51089 (EP1 specific) and AH 6809 (EP1 and EP2 specific). Consistent with the involvement of Gs coupled EP2 receptors, is that the PGE₁ stimulation is inhibited by the PKAI expression vector (encoding the protein kinase A (PKA) inhibitory protein), as well as by the myristoylated PKA inhibitory peptide PKI. In addition to this evidence (for the involvement of EP2 receptors), evidence for the involvement of EP1 receptors in the PGE₁ mediated stimulation of Na,K-ATPase β subunit gene transcription includes the stimulatory effect of 17-phenyl trinor PGE₂, as well as the inhibitory effects of SC-51089. Also consistent with the involvement of Gq coupled EP1 receptors, the PGE₁ stimulation is inhibited by the PKCI vector (encoding the PKC inhibitory domain), the PKC inhibitor Go 6976, thapsigargin, as well as the calmodulin antagonists W7 and W13.

Keywords

Prostaglandins; Kidney; MDCK; Na,K-ATPase; Transcription; EP receptors

1. Introduction

Prostaglandins (PGs) are biologically active lipid mediators, which are products of the metabolism of arachidonic acid by cyclooxygenase. Prostaglandins play important roles systemic blood pressure regulation and volume control [1]. In the kidney, endogenously produced prostaglandins regulate renal blood flow as well as ion transport, so as to maintain homeostasis under conditions of physiologic stress [2]. Prostaglandin E₂ (PGE₂) [2] the major renal prostaglandin, is produced by all of the nephron segments, the macula densa, and the renal interstitium [3]. The renal prostaglandins produced in these various locales have a variety of affects on different types of renal cells, acting in both an autocrine and paracrine manner.

PGE₂ in particular has profound effects on a number of the transport systems of the renal tubular epithelium [2,4], including the Na⁺/H⁺ antiport system [5], the Na,K-ATPase [4] and the epithelial Na⁺ channel [6,7]. The mechanisms by which prostaglandins affect transport systems

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Uncited references
[37,57,58,59,60].

are poorly understood, and the signaling pathways are to a large extent undefined. These issues have been further complicated by the results of microperfusion and micropuncture studies, that indicate that prostaglandin effects vary considerably between nephron segments. Some of the differences can be explained by the presence of different types of prostanoid receptors, and transport systems in the cell types that are in the different nephron segments.

One manner through which prostaglandin-mediated affects on transport can be more precisely defined is by means of studies with cultured renal cells. The Madin Darby Canine Kidney (MDCK) cell line is a well-defined cell culture system, which is amenable for studies concerning prostaglandin action [8]. MDCK cells retain the morphological polarity distinctive of renal tubule epithelial cells, including an apical surface which faces the culture medium, and a basolateral surface which faces the plastic dish [9]. Adjacent cells are interconnected by tight junctions. At confluence, MDCK monolayers possess the capacity for transepithelial ion transport [10–13]. The Ussing chamber studies of Cereijido et al. [14] have shown that (1) the entry of sodium into MDCK cells is dependent upon an amiloride-sensitive Na^+/H^+ antiport system, and (2) the active extrusion of Na^+ in the apical to the basolateral direction is dependent upon a ouabain-sensitive Na,K-ATPase, which is localized on the basolateral surface [13].

Typical of renal distal tubule cells, MDCK cells have been shown to retain appropriate hormone responses, as well as transport systems [9]. Indeed, arginine vasopressin, norepinephrine, glucagon, PGE_1 and PGE_2 all cause the activation of adenylate cyclase in MDCK cells [10, 15,16]. In vitro studies concerning the actions of such effector molecules are facilitated by a hormonally defined culture conditions. When MDCK cells are cultured in serum free Medium K-1, that contains insulin, transferrin, triiodothyronine, hydrocortisone and PGE_1 [17], the cells grow over the long term, at the same rate obtained in serum-supplemented medium [18].

Previously, we reported that PGE_1 , and 8-Bromocyclic AMP (8Br-cAMP) cause an increase in the Na,K-ATPase activity of MDCK cells [19], which was associated with an increase in the level of the Na,K-ATPase in this cell type [4]. The Na,K-ATPase is a protein which is composed of an α subunit with catalytic activity, as well as a β subunit, which facilitates the integration of the protein into the plasma membrane [20]. We observed that the level of both the α and the β subunit increased in PGE_1 treated MDCK cells [4,19]. In addition, the level of the mRNA for the α and β subunit of the Na,K-ATPase was increased [4,19]. Notably, the level of β subunit mRNA increased to a greater extent than α subunit mRNA [4,19]. As the β subunit level is known to limit the assembly of α/β heterodimers [21], the regulation of the $\beta 1$ subunit was examined in greater detail.

Transient transfection studies were conducted with MDCK cells, using a human Na^+,K^+ -ATPase $\beta 1$ subunit promoter/luciferase construct (pH $\beta 1$ -1141Luc) [22]. The results of these studies indicated that both PGE_1 and PGE_2 stimulated transcription [4,22]. Subsequently, we identified a Prostaglandin Response Element (PGRE) on the human $\beta 1$ subunit promoter that was responsible for the observed regulation by PGE_1 and PGE_2 [23,24]. A number of different classes of prostanoid receptors are involved in mediating signaling events initiated by prostaglandins. For this reason, in this report we further define the prostanoid receptors that are present in MDCK cells which are responsible for mediating signaling events initiated by PGE_1 and PGE_2 , which result in increased the transcription of the Na,K-ATPase $\beta 1$ subunit gene.

2. Experimental methods

2.1. Materials

Hormones, transferrin, prostaglandins, and other chemicals were from Sigma-Aldrich (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM), Ham's nutrient mixture F-12,

soybean trypsin inhibitor, molecular weight markers, and lipofectamine reagent were from Invitrogen (Carlsbad, CA). Nitrocellulose membranes, acrylamide, molecular weight markers, and other reagents for electrophoresis were from Bio-Rad (Hercules CA). The Galacto-Star™ system was obtained from Applied Biosystems (Bedford, MA). The pSVβ gal plasmid, and reporter lysis buffer were from Promega. The Prism 4 program was obtained from GraphPad Software, Inc. (San Diego, CA). The myristolated PKA inhibitory peptide (14–22) was from EMD Biosciences (La Jolla, CA). Prostaglandin agonists and antagonists were from Cayman Chemicals (Ann Arbor, MI).

2.2. Antibodies

Rabbit polyclonal antibodies against a synthetic peptide homologous to the C-terminal of the human EP1 receptor (amino acids 380–402; GLTPSAWESSLRSSRHSLSHF), the C-terminal of the human EP2 receptor (amino acids 335–358; SLRTQDATQTSCSTQSDASKQADL), amino acids 308–327 of the human EP3 receptor (NQTSVE-HCVKTHTEKQKECNF), and the C-terminal of the human EP4 receptor (amino acids 459–488; GSGRAGPAPKGSS-LVTFPSETLNLSEKCI) were from Cayman Chemicals (Ann Arbor, MI). Previously, the anti EP1 antibody does not cross-react with human EP2, EP3 and EP4, but is cross-reactive with the rat and murine EP1 receptor (which has 70% homology with amino acids 380–402 in human EP1). Canine EP1 (gi 50978908) has 90% homology with amino acids 380–402 in human EP1 (gi 38505193). The EP2, EP3 and EP4 antibodies have been shown to be cross-reactive with human EP2, EP3 and EP4 receptors, respectively, without cross-reacting with each of the remaining three classes of human EP receptors. The EP2, EP3 and EP4 antibodies have also been shown to be cross-reactive with mouse and rat EP2 (homology 83% (mouse), 72% (rat)), mouse and rat EP3 (homology of 65% for both mouse and rat) as well as mouse and rat EP4 (homology of 79% (mouse) and 76% (rat)), respectively. Canine EP2 (gi 50978908), EP3 (gi 50950179) and EP4 (gi 50978708) receptors are 80% homologous to amino acids 335–358 in human EP2 (gi 31881630), 95% homologous to amino acids 308–327 in human EP3 (gi 38505174), and 78% homologous to amino acids 459–488 in human EP4 (gi 4506259), respectively.

2.3. Expression vectors

The human Na,K-ATPase beta 1 promoter/Luciferase construct pHβ1-1141 Luc [22] was a gift from Dr. Jerry Lingrel (U. Cincinnati), and the constitutive β-galactosidase expression vector pSVβ gal was purchased from Promega (Madison, WI). The pKAI and the pKCI expression vectors were gifts from Dr. Te-Chung Lee, State University of New York at Buffalo. The pKAI vector was generated from the pTC21 expression vector [25] by inserting a cDNA to the PKA inhibitor [26], TCGACCACCATGACTTATGCCGATTTTCATTGCCTCTGGCCGCACTGGCCGC-CGCAACGCCATTTCATTAG, into the Sal I and Bam HI sites of PTC21 [27], while pKCI was generated by similarly inserting cDNA to the auto-inhibitory domain of rat PKC (AATTCCACCATGCGCTTCGCCCCGAAAGGCGCC-CTCCGGCAGAAGAACGTGTG, encoding for amino acid residues 19–31 of PKC as well as EwRI sequences) into the Eco RI site of PTC21.

2.4. Cell culture

The basal medium is a 50:50 mixture of DME and Ham's nutrient mixture F-12 supplemented with 15 mM HEPES (pH 7.4), 20 mM sodium bicarbonate, 92 units/ml penicillin, and 200 µg/ml streptomycin (DME/F12) [17]. The growth medium for stock cultures of MDCK cells (Medium K-1) consists of basal medium further supplemented with 5 µg/ml bovine insulin, 5 µg/ml human transferrin, 5×10^{-12} M triiodothyronine (T_3), 5×10^{-8} M hydrocortisone, 25 ng/ml PGE₁, and 5×10^{-8} M selenium. MDCK stock cultures were routinely subcultured by

detachment of the cells using 0.53 mM EDTA and 0.05% trypsin in phosphate-buffered saline (PBS), followed by inhibition of trypsin action using 0.1% soybean trypsin inhibitor in PBS [17].

2.5. Transient transfection studies

MDCK cells were plated at 10^5 cells/35-mm dish into basal medium supplemented with 5 μ g/ml bovine insulin and 5 μ g/ml human transferrin. The day after plating, the cultures were cotransfected utilizing lipofectamine, with a reporter plasmid (1 μ g), as well as pSV β gal (0.2 μ g), to adjust for transfection efficiency. During the transfection the cultures were maintained in antibiotic free basal medium supplemented with 5 μ g/ml insulin and 5 μ g/ml transferrin. The following day, the medium was changed to basal medium supplemented with 5 μ g/ml insulin and 5 μ g/ml transferrin. After a 2-h incubation, appropriate effector molecules were added, followed by a 4-h incubation (or, as otherwise indicated). At the end of the incubation, the cells were solubilized in reporter lysis buffer, and centrifuged for 1 min. Cell lysates were then utilized for luciferase and β -galactosidase assays.

To determine the luciferase activity, aliquots of the cell lysates were placed in 100 μ l of luciferase assay buffer (20 mM Tricine, 1.07 mM $\text{MgCO}_3 \cdot 4 \text{Mg(OH)}_2$, 2.67 mM MgSO_4 , 0.1 mM EDTA, 33.3 mM dithiothreitol, 270 μ M Coenzyme A, 470 μ M luciferin, and 530 μ M adenosine triphosphate), and the emitted light measured in a Packard Tri-Carb 4530 scintillation counter (coincidence off). The luciferase activity of the cell lysates was normalized with respect to β -galactosidase activity. The β -galactosidase activity was measured by incubating aliquots of the cell lysates in a reaction buffer containing Galacton-Star[®] substrate (provided in the Galacto-Star[™] system) for 90 min at 23 °C, and followed by measurements of the emitted light, as described above.

The values obtained from the luciferase assays were the mean ($\pm$ S.E.) of quadruplicate determinations. The level of stimulation (or inhibition) of luciferase activity, the values were divided by the indicated control value from the same experimental culture set. The significance of observed stimulation (or inhibition) was determined by a one-way analysis of variance (ANOVA), and the Newman–Keuls multiple comparison test, using Prism 4 software. Differences were significant when $p < 0.05$.

2.6. Western analysis

MDCK monolayers were washed with PBS, and lysed in 50 mM Tris, pH 7.4, 150 mM NaCl, Na^+ deoxycholate, 1% NP-40, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μ g/ml aprotinin, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin, 1 mM sodium orthovanadate and 1 mM NaF. The cell lysates were removed from the culture dishes with rubber policemen, and transferred to microfuge tubes. Samples were equalized for protein content by the Bradford method [28], separated on 10% SDS-polyacrylamide gels, and transferred to nitrocellulose membranes, using a Trans-Blot apparatus (Bio-Rad). The blots were blocked for 1 h in Tris-buffered saline (TBS) containing 0.1% (v/v) Tween 20 (TTBS), followed by a 2-h incubation with primary antibody, also in TTBS. After five washes with TTBS (5 min/wash), the blots were incubated 1 h in TTBS containing a goat anti-rabbit IgG-biotin conjugate, followed by five washes with TTBS. Finally, the blots were then incubated 1 h in TTBS containing a mouse anti-biotin-alkaline phosphatase conjugate. Signals were visualized using 0.33 mg/ml nitroblue tetrazolium (NBT) and 0.17 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in 0.1 M NaCl and 0.05 M MgCl_2 . Blots were scanned with a Bio-Rad scanning densitometer, and band intensities quantified using the Quantity One program.

3. Results

3.1. PGE₁ stimulation of the β 1 promoter: signaling through EP1 and EP2 receptors

To determine the effects of prostaglandins on Na,K-ATPase β 1-subunit gene expression in MDCK cells, transient transfection studies were conducted with pH β 1-1141Luc, a human β 1 promoter/luciferase construct [22]. Initially, the effects of PGE₁, carbaprostacyclin and fluprostanol were examined. Fig. 1 shows a 6.4 ± 0.6 -fold stimulation by 70 nM PGE₁, an EP receptor agonist, and a 2.8 ± 0.4 -fold stimulation by carbaprostacyclin (0.5 μ M), an IP receptor agonist, whereas fluprostanol (7.5 nM), an FP receptor agonist, had no significant effect.

The PGE₁ stimulation of transcription may be the consequence of the activation of a number of different subtypes of EP receptors, including EP1 and EP2. In order to evaluate the possibility of EP2 involvement, the effect of butaprost was examined. Butaprost is a structural analogue of PGE₁, which is a selective EP2 receptor agonist [29]. The effect of butaprost on transcription was examined as a function of the butaprost concentration from 0 to 4×10^{-6} M. Fig. 2A shows a stimulatory effect of butaprost on transcription, which increased as a function of concentration. The maximal stimulation (22 ± 5 -fold) was observed at a butaprost concentration of 4×10^{-7} M, which is close to the previously reported K_i value obtained for butaprost (1.1×10^{-7} M) for displacement of radiolabeled PGE₂ binding to the murine EP2 receptor [29].

In order to examine the possibility of EP2 receptor involvement further, the effect of AH6809 on the PGE₁ stimulation was examined. AH6809 is a competitive EP2 antagonist, which has been shown to be specific for the murine EP2 receptor subtype [29]. Because AH6809 is a competitive antagonist, the effect of increasing concentrations of AH6809 on the PGE₁ stimulation of transcription were examined when PGE₁ at a concentration of 0.028 μ M, which, as we previously showed, causes 67% of the maximal PGE₁ stimulation [4]. Fig. 2B shows that AH6809 did indeed inhibit the PGE₁ stimulation, and caused 50% inhibition at an AH6809 concentration of 6.2 μ M. When the AH6809 concentration was increased to 10 μ M, a $73 \pm 3\%$ inhibition of the PGE₁ stimulation was obtained. Similarly, 10 μ M AH6809 caused a 70% inhibition of the binding of 0.005 μ M ³H-PGE₂ to the recombinant human EP2 receptor [30].

In order to determine whether EP1 receptors are also involved, the EP1 receptor antagonist SC-51089 [32–34] was employed. Initially, the effect of increasing concentrations of SC-51089 on the stimulatory effect of 0.028 μ M PGE₁ was examined. Fig. 2C indicates that the PGE₁ stimulation was inhibited by SC-51089 at concentrations ≥ 10 μ M, unlike the case with the control level (data not included). An IC₅₀ value of 15 μ M was derived from the curve. Fig. 2C also shows that when the SC-51089 concentration was raised to 50 μ M, the PGE₁ stimulation was inhibited $91 \pm 4\%$. When utilizing a 30 μ M SC-19220 [29,35,36], an SC-51089 analogue, similarly a $40 \pm 5\%$ inhibition of the stimulatory effect of 0.028 μ M PGE₁ was obtained (unpublished observation).

In order to assess the involvement of EP1 receptors further, the effect of the EP1 agonist 17-phenyl trinor PGE₂ [29] was examined, as illustrated in Fig. 2D. A 1.6 ± 0.2 -fold stimulation was observed at 0.01 μ M, and increased to as high as 5.4 ± 0.7 when the 17-phenyl trinor PGE₂ concentration was raised to 50 μ M. In order to evaluate whether the stimulatory effect of 17-phenyl trinor PGE₂ is indeed mediated by EP1 receptors, the effect of SC-51089 on the 17-phenyl trinor PGE₂ stimulation was examined. Fig. 2E shows that the stimulatory effect of 10 μ M 17-phenyl trinor PGE₂ was inhibited by SC-51089 by up to $94 \pm 4\%$ at 50 μ M, and that the IC₅₀ value was 4 μ M.

Four major classes of EP receptors have been identified, including EP1, EP2, EP3 as well as EP4. In order to determine whether MDCK cells do indeed possess EP1, EP2, EP3 and/or EP4 receptors, a Western analysis was conducted. In addition, the effect of a 4-h incubation with

PGE₁ (70.5 nM) on the levels of these receptors was examined. Fig. 3A shows that all four classes of EP receptors were present in MDCK cells, and that the molecular weights of the EP receptors were between 52 and 53 kDa. The results (shown in Fig. 3A) also indicate that the level of EP1, EP2, EP3 and EP4 receptors increased by 6.5-, 3.1-, 7.9- and 3.0-fold, respectively, following a 4-h incubation with PGE₁. In contrast (shown in Fig. 3B) the level of GAPDH was unchanged in PGE₁ treated MDCK cells.

3.2. PGE₁ and 8Br-cAMP stimulates the Na⁺,K⁺-ATPase β1 promoter activity

All 4 classes of EP receptors are G-protein coupled, including EP2 and EP4 receptors (coupled to G_s, which activates adenylate cyclase (AC)), EP3 receptors (coupled to G_i, which inhibits AC), and EP1 receptors (coupled to G_q which activates Phospholipase C (PLC)) [29,31]. Consistent with the involvement of G_s coupled receptors was the observation (Fig. 4) that both 1.4 μM PGE₁ (Fig. 4A) and 1 mM 8Br-cAMP (Fig. 4B) were stimulatory. Also consistent with the involvement of G_s was the partial inhibition of the PGE₁ and 8 Br-cAMP stimulation by PKI, a cell-permeable myristo-lated PKA inhibitory peptide (36 nM) [4,38]. The PGE₁ stimulation was reduced from 6.4 ± 0.4-fold to 4.6 ± 0.2-fold by PKI (Fig. 4A), while the 8Br-cAMP stimulation was reduced from 8.4 ± 0.4-fold to 5.1 ± 0.4-fold (Fig. 4B).

In order to examine the role of PKA further, the effect of the PKAI vector (encoding for the Protein Kinase A inhibitory peptide) on the PGE₁ and the 8Br-cAMP stimulation was examined. Fig. 5A shows that in the presence of PKAI both the PGE₁ stimulation, and the 8Br-cAMP stimulation were reduced (45 ± 10%, and 52% ± 10%, respectively), when compared with control cultures cotransfected with the empty vector. However, the PGE₁ stimulation was also reduced by 65 ± 5% by the vector PKCI (which expresses the inhibitory domain of PKC) [27] (as shown in Fig. 5B). In the same experiment (Fig. 5B), PKCI reduced the TPA stimulation by 82 ± 2%, as compared with cultures cotransfected with the empty vector. Thus, the results in Fig. 5B suggest that a component of the PGE₁ stimulation is dependent upon PKC as well as PKA.

3.3. Other signaling pathways involved in mediating PGE₁ effects

Because PGE₁ was previously causes an increase in intracellular Ca²⁺, as well as cAMP in MDCK cells [2,16], the involvement of Ca²⁺ and Ca²⁺/calmodulin-dependent protein kinase (CaM kinase) was also examined. CaM Kinase II activity is affected by phosphorylation by CaM Kinase Kinase, and reduced following dephosphorylation by Protein Phosphatase I (PP-1) and Protein Phosphatase 2A (PP-2A). Okadaic acid, a PP-1 and PP-2A inhibitor, caused a 3.2-fold stimulation of β1 subunit gene transcription (Fig. 6A), consistent with the involvement of CaM Kinase in the PGE₁ stimulation.

The effects of the calmodulin antagonist *N*-(6 aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7) and W13 were also examined (Fig. 6B). The observation that W7 (56 μM) reduced the PGE₁ stimulation by 6.2 ± 0.7-fold (Fig 6B) was also consistent with the involvement of CaMK in mediating the PGE₁ stimulation. Even W13 (added at only 1 μM) caused a 1.5 ± 0.7-fold reduction in the PGE₁ stimulation. Fig. 6C shows that W7 and W13 also inhibited the 8Br-cAMP stimulation of transcription. These results may be explained if the PKA and CaMK pathways converge, and CaMK acts downstream of PKA.

Ultimately, CaMK activation is Ca²⁺ dependent. Consistent with the involvement of intracellular Ca²⁺, Fig. 6D shows that the PGE₁ stimulation (5.4 ± 0.3-fold) was reduced to 3.3 ± 0.3-fold in the presence of 0.1 μM thapsigargin. Thapsigargin releases sequestered Ca²⁺ by inhibiting endoplasmic reticulum Ca²⁺ ATPases, without affecting PKC [39–41].

4. Discussion

Previously, we reported that PGE₁ and PGE₂ stimulate transcription of the Na,K-ATPase β subunit gene in MDCK cells [24]. In this report we present evidence consistent with the involvement of at least two different classes of G protein coupled prostaglandin receptors in mediating the PGE₁ stimulation of β 1 subunit gene transcription, including the EP1 and the EP2 receptor subtypes. The hypothesis that EP2 receptors are involved in mediating the prostaglandin stimulation of Na,K-ATPase β 1 subunit gene transcription is supported by (1) the observed stimulatory effect of the EP2 agonist butaprost, and (2) the observed inhibitory effect of AH6809 on the PGE₁ stimulation. However, the EP1 selective agonist 17-phenyl trinor PGE₂ stimulated transcription by pH β 1-1141 Luc. In addition, the EP1 selective antagonist SC-51089 was also found to be capable of inhibiting the PGE₁ stimulation by up to $91 \pm 4\%$. Thus, EP1 receptors (coupled to Gq) as well as EP2 receptors (coupled to Gs) are very likely involved in mediating the stimulatory effect of PGE₁ on Na,K-ATPase β 1 subunit gene transcription. Consistent with this hypothesis are the results of our Western analysis which indicate that all four subtypes of PGE receptors (EP receptors) are expressed in MDCK cells, including EP1, and EP2, as well as EP3 and EP4.

While the binding of PGE₁ and PGE₂ to Gs coupled EP1 receptors results in the activation of adenylate cyclase, the binding of PGE₁ and PGE₂ to Gq coupled EP1 receptors results in the activation of PLC, an increase in the levels of diacylglycerol and inositol 1,4,5-trisphosphate (IP₃), and the activation of PKC [38]. In this report the involvement of EP1 as well as EP2 receptors in mediating the effects of PGE₁ and PGE₂ was also supported by our observations concerning the cellular signaling pathways involved in mediating the PGE₁ stimulation. Evidence for the involvement of both cAMP and PKC as mediators of the PGE₁ effect on β 1 subunit transcription includes the observed inhibitory effects the PKAI and the PKCI expression vectors on the PGE₁ stimulation. The PKAI and PKCI expression vectors encode for the PKA inhibitory peptide, and the inhibitory domain of PKC, respectively. Also consistent with the involvement of PKA, was the observation that a myristolated PKA inhibitory peptide (PKI) (like the PKAI vector) caused a partial inhibition of the PGE₁ stimulation. In contrast, the PGE₁ stimulation was completely inhibited by the PKCI vector. Similarly, we previously observed that the PKC inhibitor Go 6976 partially inhibited the PGE₁ stimulation of β 1 transcription in MDCK cells [4]. Because the PGE₁ stimulation was completely inhibited by PKCI, PKA may possibly act by augmenting the stimulation caused by PKC, rather than being responsible for a separate component of the PGE₁ stimulation. However, 8 Br-cAMP was individually observed to stimulate transcription by pH β 1-1141Luc, as was observed in the case of TPA.

Previous observations that PGE₁ and PGE₂ cause an increase in intracellular Ca²⁺ and cAMP in MDCK cells are consistent with the involvement of EP1 and EP2 receptors in mediating the effects of these two prostaglandins in MDCK cells [10,16,19,30,39–42]. The increase in intracellular Ca²⁺ that occurs in PGE₁ and PGE₂ treated MDCK cells may cause a number of Ca²⁺ mediated pathways to be activated (in addition to PKC), which may all contribute to the PGE₁ stimulation. Consistent with this hypothesis, Thapsigargin, which increases cytoplasmic Ca²⁺ (without affecting PKC), and okadaic acid caused a stimulation of β 1 subunit gene transcription. The calmodulin antagonists W7 and W13 inhibited the stimulatory effects PGE₁ on β 1 subunit gene transcription. Such calmodulin mediated effects on transcription can be explained by the activation of CaMK, and the phosphorylation of the cAMP regulatory element binding protein (CREB) by CAMK. However CaMK may also regulate β 1 subunit gene transcription indirectly, by modulating the effects of PKA and/or PKC. Consistent with this latter hypothesis is the observation that W7 and W13 inhibited the stimulatory effects of 8Br-cAMP on β 1 subunit gene transcription.

The results of our transient transfection studies have indicated the involvement of EP1 as well as EP2 receptors in regulating Na,K-ATPase β 1 subunit gene expression in our MDCK cell line, which is a distal tubule model. Previously, the results of in situ hybridization have indicated that EP1 receptor mRNA is found primarily in the collecting duct [43,44], while EP2 receptors are present predominantly in distal tubule cells as well as glomeruli [7,30]. Of particular interest in these regards, targeted disruption of EP2 was observed to cause salt-sensitive hypertension, supporting a role for EP2 in renal salt handling [44,45]. Our Western analysis also indicates that EP3 and EP4 receptors are present in MDCK cells. Previously studies indicate that in the kidney, EP3 is localized primarily in the collecting duct and thick ascending limb, while EP4 is primarily in the glomerulus [46].

There is an extensive literature regarding the effects of prostaglandins on renal transport, which consists primarily of in vivo microperfusion and micropuncture studies [47]. The results of these studies have indicated that the effects of prostaglandins on renal ion transport differ considerably as a function of nephron segment [47]. There are many conflicting reports, which may be explained by differences in responses between species, as well as other methodological details [47].

For this reason, in our studies utilizing EP receptor agonists and antagonists with MDCK cells, we cannot exclude the possibility of that the observed effects of prostaglandin agonists and antagonists on MDCK cells may differ from previously reported effects on human EP receptors. Indeed species differences in antagonist binding have been reported [29]. In addition is the possibility of different binding affinities of antagonists for the different isoforms of each receptor subtype. Two isoforms of the EP1 receptor, and eight isoforms of the EP3 receptor have been identified [3,30,48]. Altered responses to agonists and antagonists may also result from the cell culture situation. However, all of the EP receptors have been expressed in cultured cells as recombinant receptors [30], and the results obtained from these studies are generally in agreement with previous pharmacological and biochemical data [29].

Our investigations regarding PGE1 and PGE2 have been concerned with the effects of chronic treatments on MDCK cells [4]. PGE1 and PGE2 also have acute effects, exemplified by the reported inhibitory effects of PGE1 and PGE2 on the activity of the Na,K-ATPase in a clonal isolate of MDCK cells with collecting duct properties [49]. In this report the reduction in Na,K-ATPase activity following a 10 min incubation, was presumably due to a decrease in the level of the Na,K-ATPase in the plasma membrane, rather than to changes in overall gene expression. Such acute effects of PGE1 and PGE2 may possibly be mediated by EP3 receptors (which are localized in the collecting duct) [43,44,50,51]. However, the inhibitory effect of PGE2 on Na⁺ absorption in the collecting duct has been shown to occur via a Ca²⁺ dependent mechanism, and was not prevented by a potent EP3 agonist [44,51]. Nonsteroidal anti-inflammatory drugs (NSAIDs) enhance the urinary concentrating ability of the kidney by preventing such inhibitory effects of PGE2 in the collecting duct. However, even in this case EP3 receptors are not necessarily involved, because the targeted disruption of EP3 did not prevent such effects of NSAIDs [44,52,53].

Although PGE2 inhibits Na⁺ absorption in the collecting duct [52], PGE2 infusion studies with conscious dogs suggest that prostaglandins can also enhance renal Na⁺ absorption [54]. Consistent with this hypothesis are the results of studies with another subclone of MDCK cells (MDCK C7), which models renal collecting duct principal cells [7]. In these studies, PGE2 was observed to stimulate sodium reabsorption via an amiloride sensitive epithelial Na⁺ channel (ENaC) [7]. The investigators proposed that the stimulatory effect of PGE2 on ENaC was mediated by the EP2 receptors that they detected by RT-PCR.

Another proposed site for stimulatory effects of renal prostalandins on Na⁺ absorption is the distal tubule [46]. Indeed, enhanced Na,K-ATPase activity has been reported in isolated microdissected rat distal tubules following PGE₂ treatment [55]. In our own studies we observed that PGE₁ and PGE₂ increase the Na,K-ATPase activity of MDCK cells which model cells in the distal tubule. In addition our results indicate that the PGE₁ and PGE₂ stimulation are mediated by increases in cAMP and Ca²⁺. Ultimately, the transactivation CREB occurs as a consequence of PGE₁ treatment, presumably affecting the CREB which binds to the prostaglandin response element on the human β 1 subunit promoter [56]. The precise role played by specific EP receptors in modulating the effects of transcription factors on the β 1 subunit promoter will be the subject of future studies.

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Fig 1.

Prostaglandin effects on Na,K-ATPase $\beta 1$ subunit gene expression. MDCK cells were transiently transfected with pH $\beta 1$ -1141Luc. Twenty-four hours later, the effect of either 70 nM PGE₁, 7.5 nM fluprostanol or 0.5 μ M carbaprostacylin was examined. Luciferase activity was compared to untreated controls. Values are mean $\pm$ S.E.M.

Fig 2.

Effects of EP agonists and antagonists on Na^+, K^+ -ATPase $\beta 1$ subunit gene expression. MDCK cells were transiently transfected with pH $\beta 1$ -1141Luc. Twenty-four hours later, cultures were treated for 4 h with either: (A) butaprost at concentrations ranging from 0– 4×10^{-6} M; (B) AH6809 at concentrations ranging from 0– 10^{-5} M in either the presence or in the absence of 28 nM PGE_1 ; (C) SC-51089 at concentrations ranging from 0–50 μM in the presence of 28 nM PGE_1 or in the absence of PGE_1 ; (D) 17-phenyl trinor PGE_2 at concentrations ranging from 0–50 μM ; and (E) 10 μM 17-phenyl trinor PGE_2 in the presence of SC-51089 at concentrations ranging from 0 to 50 μM . In the experiments with AH6809 and with SC-51089 cultures were preincubated with these antagonists for 30 min prior to the initiation of the 4-h incubation with PGE_1 . Control experiments indicated that SC-51089 (0–50 μM) had no significant effect in the absence of PGE_1 . The luciferase activity of cell lysates was determined

in quadruplicate after the 4-h incubation period, and compared with untreated controls. At least three experiments were performed. Values are mean $\pm$ S.E.M.

Fig 3. EP receptors in MDCK Cells. Confluent MDCK monolayers were either treated with 70 nM PGE₁ for 4 h, or untreated. Cell lysates were analyzed by Western analysis using an antibody against either (A) EP1,EP2, EP3 or EP4, or (B) GAPDH.

Fig 4.

Role of PKA in the PGE₁ stimulation. MDCK cells were transfected with the pHβ1-1141Luc. Twenty-four hours later, the cells were treated for 4 h with either (A) 1.4 μM PGE₁ or 1 mM 8Br-cAMP. (B) 1.4 μM PGE₁ in the presence or the absence of 36 nM PKI. (C) 1 mM 8Br-cAMP in the presence or absence of 36 nM PKI. After the 4-h incubation the luciferase activity was determined in quadruplicate, and compared with untreated controls. At least three experiments were performed. Values are mean ± S.E.M. *, ●, $p < 0.001$ compared to control without PGE₁ and PGE₁ + PKI, or PGE₁ + 8Br-cAMP.

Fig 5.
Role of PKA and PKC in the PGE₁ stimulation. MDCK cells were cotransfected with p β 1-1141 Luc and either PKAI, PKCI or the empty vector PTC 21. (A) A portion of the PKAI and PTC 21 transfected cultures were then treated 3 h with either 1.4 μ M PGE₁ or 1 mM 8 BrcAMP. (B) A portion of the PKCI and PTC 21 transfected cultures were treated 3 h with either 1.4 μ M PGE₁ or 1 μ M TPA. Luciferase determinations (in quadruplicate) were compared with untreated controls. Values are mean $\pm$ S.E.M.

Fig 6.

Role of calcium. MDCK cells were transfected with the pHβ1-1141Luc. Cultures were treated for 4 h with either (A) 1.4 μM PGE₁ or 1 μM okadaic acid (B) 1.4 μM PGE₁ or (C) 1 mM 8Br-cAMP. (D) 1.4 μM PGE₁. In B and C a portion of the cultures were pretreated with either 56 μM W7 or, 1 μM W13 for 30 min prior to the 4-h incubation, as well as during the incubation period. In (D) a portion of the cultures were treated with 0.1 μM thapsigargin 30 min prior to, and during the 4-h incubation. Untreated controls were controls. Determinations were in quadruplicate. At least three experiments were performed. Values are mean ± S.E.M. * or •, $p < 0.001$ relative to control, PGE₁ + W7/W13, and for PGE₁ + Thapsigargin.