Short-Term Regulation of Basolateral Organic Anion Uptake in Proximal Tubular Opossum Kidney Cells: Prostaglandin E₂ Acts via Receptor-Mediated Activation of Protein Kinase A

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Abstract. It was shown previously that EGF induces release of the important prostanoid prostaglandin E₂ (PGE₂) in proximal tubular opossum kidney (OK) cells and PGE₂ then stimulates initial basolateral uptake of organic anions (OA) dose dependently. PGE₂ is a receptor agonist and a known substrate for the basolateral exchanger mediating OA uptake (OAT1 and/or OAT3). This study investigated the mechanism of short-term PGE₂ action on initial basolateral OA uptake in OK cells. PGE₂ stimulation of OA uptake was abolished by selective inhibition of adenylate cyclase (by MDL-12, 330A) or protein kinase A (PKA; by H89). PGE₂ stimulation of OA uptake persisted after preloading the cells with glutarate and was still abolished by inhibition of PKA. Selective activation of adenylate cyclase by forskolin led to identical results. These data contradicted the hypothesis that PGE₂ action on OA uptake is due to its action as a counter ion. Therefore, we tested whether the PGE₂ receptors (EP1 to 4) are involved in stimulation of OA uptake in OK cells by PGE₂. Because of their intracellular signaling profile, EP1 and EP3 were not taken into account as possible receptors for mediation of PGE₂-induced OA uptake. With the use of selective agonists (11-deoxy PGE₁ and butaprost), EP4 was pharmacologically identified as the receptor responsible for PGE₂-mediated stimulation of OA uptake. By reverse transcription–PCR, cloning, and subsequent sequencing, a homologue fragment to EP4 was identified in OK cells. EGF-induced stimulation of basolateral organic anion uptake was abolished by inhibition of adenylate cyclase or PKA. This indicates that EGF action is mediated by generation of PGE₂. The following model is proposed: PGE₂ generated in the cells does not act as a counter ion but activates adenylate cyclase. This is mediated by a homologue of EP4 receptor. cAMP then activates PKA, which stimulates initial basolateral uptake of OA in OK cells by a not-yet-known mechanism. PGE₂ is an organic anion, a potential stimulator of organic anion excretion, and an important mediator of inflammation all at once. Thus, the mechanism presented here may contribute to a limitation of inflammatory events in the kidney cortex interstitium.

The organic anion transport system of the renal proximal tubule plays a crucial role in the excretion of a variety of potentially toxic compounds (1,2). This system consists of a basolaterally located, polyspecific organic anion (OA) exchanger and a less well-characterized transport step at the apical membrane (3,4).

The basolateral OA exchanger is a tertiary active transport system, dependent on an inward-directed Na⁺ gradient to drive the uptake of α-ketoglutarate, which is then exchanged for OA (2,3,5,6). The basolateral exchanger for OA and dicarboxylates was cloned by three independent groups (7–9) in 1997 and named OAT1. The homologous protein was cloned from human kidney and was called hOAT1 (10,11) or hPAHT (12). The genomic DNA from human hOAT1 is organized in 10 exons, and up to now, four isoforms have been described (13).

Furthermore, it was shown that OAT1 represents characteristics of the basolateral, polyspecific transporter for OA (14), which has been functionally described for a substantial amount of time (6). Meanwhile, there is evidence that the basolaterally located OAT3 from rat also acts as an exchanger for dicarboxylates and OA as OAT1 does (15).

Furthermore, three additional homologues were cloned and named hOAT2, hOAT4, and hOAT5 (16). hOAT4 is expressed in kidney and placenta (17). Up to now, it seemed that hOAT5 was not expressed in the kidney (16). hOAT2 shows 37% homology in amino acid sequence compared with hOAT1 (16) and differs from OAT1 in substrate specificity. There is also evidence that OAT2 is located in the luminal membrane of the proximal tubular epithelium (18). Furthermore, these proteins are no exchangers like OAT1 or OAT3 but seem to work as facilitators (19).

Little is yet known about the modulation of this transport system (2,3,20). In fact, most of the publications available up to now investigated the effect of protein kinase C (PKC) in different species, setups, or cell lines. Thereby, it was shown that stimulation of PKC (e.g., by parathyroid hormone, bradykinin, or phenylephrine) leads to an inhibition of basolateral OA transport (21–23). You et al. (24) showed that PKC inhibi-
its murine OAT without direct phosphorylation of the transport protein itself. As we could show only recently, EGF stimulates the basolateral OA transport via mitogen-activated protein kinases (MAPK) (25). MAPK then activate phospholipase A2, generating arachidonic acid, which is converted to prostaglandin E2 (PGE2) by cyclooxygenase 1 (COX1). PGE2 then dose-dependently stimulates basolateral OA uptake by a not-yet-known mechanism (26) (see Figure 1).

Prostanoids are important regulatory factors in the kidney. Renal tubular epithelia and interstitial cells synthesize and release predominantly PGE2 in a regulated manner. PGE2 affects urine concentration ability; promotes salt and water concentration ability; promotes salt and water excretion by the renal medulla; maintains renal blood flow and GFR, particularly under neurohumoral activation; and stimulates renin release (27). Furthermore, PGE2 is involved in inflammatory events in kidney tissue (28). PGE2 initiates and maintains its physiologic effects through interaction with specific receptors on target cell surface membranes. At least four different PGE2 receptor isoforms exist, designated EP1 to 4, which all are expressed in the kidney (29,30). The EP2 and EP4 subtypes act via activation of adenylate cyclase, whereas EP3 inhibits adenylate cyclase activity and stimulation of EP1 leads to activation of PKC. In addition, it is known that PGE2 is a substrate for the OA secretory transport system (31) and also may serve as substrate and/or counter ion for the basolateral OA exchanger (which may consist of OAT1 and/or OAT3, as already mentioned) (32).

Thus, we investigated the mechanisms of PGE2-induced stimulation of basolateral OA uptake in more detail. For this stimulation to occur, two mechanisms are possible. First, PGE2 could act as a counter substrate and thus stimulate the uptake of OA. Second, PGE2 could act via coupling to specific receptors present at the membrane of proximal tubular OK cells (see Figure 1). As a model system, we chose the proximal tubule-derived OK cell line cultured on permeable supports, a functionally well-characterized cell line to investigate basolateral OA transport via the dicarboxylate exchanger mechanism (25,33). Meanwhile, the functionally predicted OAT1 from OK cells is partly cloned (Dr. Y. Hagos, Goettingen; GenBank AJ308236), showing 88% sequence homology to human OAT1. In the following, we present data showing that PGE2 stimulates basolateral OA uptake by binding a specific receptor, which, via cAMP, stimulates protein kinase A (PKA). PKA then stimulates initial basolateral OA uptake.

Materials and Methods

Cell Culture

OK cells were obtained from Dr. Biber (Department of Physiology, University of Zurich, Zurich, Switzerland). Cells were maintained in culture at 37°C in a humidified 5% CO2, 95% air atmosphere. The growth medium was MEM (pH 7.4), supplemented with Earl’s salts, nonessential amino acids, 10% (vol/vol) FCS (Biochrom KG, Berlin, Germany), and 26 mmol/L NaHCO3. Cells were cultured on permeable supports (3-μm pore diameter; Falcon, Becton Dickinson Labware, Franklin Lakes) for transport measurements. The effective growth area on one permeable support was 4.3 cm2/filter. All studies were performed between passages 60 and 100. The seeding density was 0.4 · 106 cells/cm2. The medium was changed every third day, and the cells were washed twice with ice-cold PBS and OA uptake was determined afterward. Obtained trans-stimulation by glutarate preload was nearly threefold. Trans-stimulation means uptake of a substrate in exchange with a counter ion from the other (trans) side of the cell membrane. Thus, cis inhibition herein stands for acute inhibition of

Transport Measurements

General Setup. The volumes of the apical and basolateral compartment were 1.3 ml and 2.5 ml to avoid hydrostatic pressure differences. Before each experiment, the cells were washed three times with PBS (138 mmol/L NaCl, 1 mmol/L Na2HPO4, 4 mmol/L NaH2PO4, 4 mmol/L KCl, 1 mmol/L MgCl2, 1 mmol/L CaCl2, 5 mmol/L glucose [pH 7.4]). Transport measurements were performed in phosphate-buffered ringer at pH 7.4 and 37°C.

Glutarate Preincubation Experiments. The preincubation scheme used in glutarate preload experiments was the following: Cells were incubated in PBS containing 5 mM glutarate (pH 7.4) for 50 min. After that, the cells were incubated with the respective activators or inhibitors in the presence of glutarate for 10 min. After that, the cells were washed twice with ice-cold PBS and OA uptake was determined afterward. Obtained trans-stimulation by glutarate preload was nearly threefold. Trans-stimulation means uptake of a substrate in exchange with a counter ion from the other (trans) side of the cell membrane. Thus, cis inhibition herein stands for acute inhibition of

Figure 1. Summary indicating the main questions addressed in this article. Former studies indicated that EGF stimulates the PAH/dicarboxylate exchanger via activation of the mitogen-activated protein kinases (MAPK) MEK and ERK1/2 (23). ERK1/2 then activates phospholipase A2 (PLA2), increasing the release of arachidonic acid. Arachidonic acid is then metabolized into prostaglandin E2 (PGE2) which then stimulates initial organic anion (OA) uptake (24). This article investigates the mechanisms by which PGE2 stimulates basolateral OA uptake in proximal tubular opossum kidney (OK) cells. Does PGE2 act as a counter substrate (I) or via a receptor-mediated pathway (II)?

Figure 1
substrate uptake by a compound located at the same (cis) side of the cell membrane.

**Tracer Flux Method.** The concentrations of the radiolabeled substrates applied to the basolateral bath were as follows: $15 \cdot 10^{-6}$ mol/L $[^{14}]$C$[^{3}]$PAH and $55 \cdot 10^{-6}$ mol/L $[^{3}]$H$[^{3}]$mannitol. Mannitol was used to correct secretion for paracellular fluxes and to determine extracellular water space. After 1 min in the transport buffer, the cells were washed twice with ice-cold PBS. Subsequently, the filters containing the cells were washed twice with ice-cold PBS and cut from the supports. Radioactivity of the cells was measured using a liquid scintillation counter (Packard Instruments, Frankfurt, Germany). Counts of cells on filters were corrected for nonspecific binding on filters by subtraction.

**Fluorescein Uptake Method.** OA transport was also determined by measuring the uptake of $10^{-6}$ M fluorescein after 1 min from the basolateral bath, according to a modified protocol (34); $10^{-6}$ M was used because of the better signal-noise ratio compared with $10^{-6}$ M fluorescein. After that, cells were washed six times with ice-cold PBS until no fluorescein was detectable in the washing solution. After that, the cells were lysed in 1 ml of 0.1% Triton-X100 in 20 mM 3-(N-morpholino)propanesulfonic acid and fluorescence was counted in a multiwell plate reader (Victor2, Wallac Instruments, Finland). Counts were corrected for extracellular binding and unspecific adhesion to the cells by subtraction of fluorescein counts on cells at 4°C. Fluorescein counts were normalized to protein content in the lysate measured by BCA protein assay (Pierce, Rockford, IL).

**Compatibility of the Methods.** PAH uptake under control conditions was 29 pmol/mg per min (±5.37), and fluorescein uptake was 0.9 pmol/mg per min (±0.353). Data are presented as mean of all experiments (±SEM). The amount of $[^{14}]$C$[^{3}]$PAH in the cells is 15-fold the amount of fluorescein, which perfectly reflects the 15-fold higher concentration in the basolateral bath. Thus, as $[^{14}]$C$[^{3}]$PAH and fluorescein behaved identically, the transport data were pooled and are all presented as OA uptake in percentage of control. The method used for generation of the data is given in the respective figure legend. With respect to the action of PGE$_2$, uptake of $[^{14}]$C$[^{3}]$PAH or fluorescein also behaved identically. As for example in Figure 4A, PGE$_2$ stimulation of OA uptake consists of PGE$_2$ stimulation of $[^{14}]$C$[^{3}]$PAH uptake to 154% (±15.6; n = 5) of control and of PGE$_2$ stimulation of fluorescein uptake to 195% (±62.5; n = 6) of control. Both statistically different from control but not from each other.

**Reverse Transcription–PCR**

RNA from OK cells was extracted using Trizol reagent (Invitrogen, Carlsbad, CA). In brief, reverse transcription–PCR (RT-PCR) was performed according to Superscript One-Step RT-PCR system protocol (Invitrogen) with a final MgSO$_4$ concentration of 1.45 mM. cDNA was generated at 55°C for 15 min, then the samples were denatured at 94°C for 2 min. PCR amplification was performed in 35 cycles of 94°C for 15 s, then 55°C for 30 s and 72°C for 30 s. For EP4, primers were 5'-GGGAAGACCTTGCTCAGTA-3' (sense) and 5'-GGAAGCAAATCTGCAGCTC-3' (antisense), covering bases 1007 to 1246 of rat EP4 cDNA, 240 bp. For EP2, primers were 5'-TTCCGGAGCAAAGAAAGCC-3' (sense) and 5'-GAGGCGATTAGTGTCCAG-3' (antisense), covering bases 725 to 1025, 301 bp, both according to Jensen et al. (35).

**PCR.** RT-PCR products were re-amplified using Platinum Taq polymerase kit (Invitrogen). MgSO$_4$ concentration was 1.5 mM. PCR amplification was performed in 35 cycles of 94°C for 15 s, then 55°C for 30 s and 72°C for 30 s. EP4 primers were used as described above.

**Generation of Sequence Data**

PCR product of choice was eluted (Qiagen Gel Extraction Kit, Qiagen, Germany) and then cloned into the pCR3.1 vector using Invitrogen TA cloning Kit. Sequencing was done by MWG Biotech (Munich, Germany). Homology search was done using BLAST. Homology was quantified using ALIGNplus software.

**Statistical Analyses**

If not stated otherwise, data are presented as mean ± SEM. n is given in the text or in the figures. n is the number of culture plates or filters used to perform the measurements. Statistical significance was determined by unpaired t test or ANOVA as appropriate. Results were considered statistically different at P < 0.05. Significant difference is indicated by asterisks.

**Materials**

$[^{14}]$C$[^{3}]$PAH and $[^{3}]$H$[^{3}]$mannitol were purchased from American Radiolabeled Chemicals (St. Louis, MO). PD98059 and H89 were from Alexis Corp. (Läufelfingen, Switzerland). Butaprost and 11-deoxy-PGE$_2$ were from Cayman Chemical (Ann Arbor, MI). MDL-12,330A was from Calbiochem (Merck, Darmstadt, Germany). If not stated otherwise, all other chemicals were from Sigma (St. Louis, MO).

**Results**

**PGE$_2$ cis Inhibits Basolateral OA Uptake**

It is well known that prostaglandins are substrates for the proximal tubular OA transport system and in particular for the basolateral rate-limiting step mediated by OAT1 (31) or, as indicated only recently, OAT3 (15,32). To address the question of how PGE$_2$ stimulates OA uptake in OK cells, we investigated whether PGE$_2$ acutely interacts with OA uptake, because if it does, then PGE$_2$ may also act as a counter ion for OA uptake. As shown in Figure 2, PGE$_2$ acutely inhibits basolat-
eral OA uptake in OK cells. (For control uptake rates of PAH and fluorescein, see Transport Measurements and Compatibility of the Methods). According to its acutely inhibitory potency and the mentioned literature, PGE₂ may possibly serve as counter ion for OA uptake. Thus, a trans-stimulatory action of intracellularly generated PGE₂ increasing basolateral OA uptake rate cannot be ruled out up to this point.

**PGE₂ Action Is Totally Abolished by Inhibition of Adenylate Cyclase**

PGE₂ stimulation of OA uptake cannot be mediated via EP₁, as per the literature (22,23,36) and our own data (25), activation of PKC inhibits basolateral uptake of OA. Because in all possible receptor-signaling pathways remaining adenylate cyclase is involved, we investigated the effect of adenylate cyclase inhibition (by MDL-12,330A) on PGE₂-mediated stimulation of basolateral OA uptake in OK cells. MDL-12,330A completely abolished the stimulatory effect of PGE₂, whereas MDL-12,330A alone had no effect on basolateral OA uptake in OK cells (Figure 3). In addition, stimulation of adenylate cyclase activity by forskolin (5 μM) led to an increase of OA uptake rate in OK cells (Figure 4B), whereas forskolin did not have a competitive effect on OA uptake (Figure 2). This is the first evidence that PGE₂ does not act by a trans-stimulatory mechanism. In addition, as EP₃ acts by adenylate cyclase inhibition, EP₃ can be excluded as a possible receptor mediating PGE₂ action.

**Effect of PGE₂ or Forskolin Is Abolished by Inhibition of PKA**

Increased activity of adenylate cyclase leads to generation of cAMP, which then activates PKA. Thus, we investigated whether the increase of OA uptake induced by PGE₂ or forskolin is affected by inhibition of PKA activity (1 μM H₈⁹). Preincubation and uptake measurement was performed as mentioned in Materials and Methods. Cells were treated with PGE₂ (A)/forskolin (B), MDL-12,330A alone, or PGE₂ (A)/forskolin (B) in combination with MDL-12,330A for 10 min, respectively. After washing, uptake of OA was determined after 1 min. *P < 0.05 versus the respective control. (A) Effect of H₈⁹ (1 μM) on the PGE₂ (100 nM) mediated stimulation of basolateral OA uptake. Substrate used was [¹⁴C]PAH or fluorescein; n = 10 to 11. (B) Effect of H₈⁹ (1 μM) on the forskolin (5 μM) mediated stimulation of basolateral OA uptake. Substrate used was [¹⁴C]PAH or fluorescein; n = 11 to 12.

**Figure 4.** Effect of inhibition of protein kinase A (PKA) by H₈⁹ on basolateral OA uptake in OK cells in the absence or presence of PGE₂ or forskolin. Preincubation and uptake measurement was performed as mentioned in Materials and Methods. Cells were treated with PGE₂ (A)/forskolin (B), MDL-12,330A alone, or PGE₂ (A)/forskolin (B) in combination with MDL-12,330A for 10 min, respectively. After washing, uptake of OA was determined after 1 min. *P < 0.05 versus the respective control. (A) Effect of H₈⁹ (1 μM) on the PGE₂ (100 nM) mediated stimulation of basolateral OA uptake. Substrate used was [¹⁴C]PAH or fluorescein; n = 10 to 11. (B) Effect of H₈⁹ (1 μM) on the forskolin (5 μM) mediated stimulation of basolateral OA uptake. Substrate used was [¹⁴C]PAH or fluorescein; n = 11 to 12.

**PGE₂ or Forskolin also Stimulates OA Uptake in Glutarate-Preloaded Cells**

To gain more information on whether the effects of PGE₂ or forskolin are partly trans-stimulatory, we investigated their
effect in glutarate-preloaded cells. OK cells were preloaded with glutarate (5 mM; 50 min), a nonmetabolizable analogue of α-ketoglutarate that drives OA uptake under physiologic conditions. As shown in Figure 5, preloading the OK cells with glutarate leads to a nearly threefold increase of OA uptake rate. Under this condition, 100 nM PGE₂ (Figure 5A) or 5 μM forskolin (Figure 5B) still stimulated OA uptake. The latter stimulation was still inhibitable by inhibition of PKA in both cases (Figure 5). This is additional important evidence that PGE₂ stimulates OA uptake via a mechanism different from trans-stimulation.

**MAPK Are Not Involved in PGE₂ or Forskolin Action**

In OK cells, it is described that activation of the MAPK ERK is dependent on the cAMP level of the cells after α2 adrenergic stimulation (37). To investigate whether MAPK are involved in downstream signaling of PGE₂ or whether feed back loops are present, we investigated the effect of inhibition of MEK by 5 μM PD98059. As indicated in Figure 6A, neither was the stimulation of OA uptake by PGE₂ affected by PD98059 nor did 5 μM PD98059 alone have any effect. The

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**Figure 5.** Effect of glutarate preload on basolateral OA uptake in OK cells and its stimulation by PGE₂ or forskolin. Incubation was performed as mentioned in Materials and Methods. In brief, cells were incubated with 5 mM glutarate for 50 min. Then, cells were treated with PGE₂/forskolin, H89 alone, or PGE₂/forskolin in combination with H89 for 10 min in the presence of glutarate. After intense washing, uptake of OA was determined after 1 min. Data are presented as percentage of the unstimulated (not glutarate-preincubated) control. Thus, glutarate control indicates cells solely preincubated with glutarate. Substrates used were [14C]PAH or fluorescein. *P < 0.05 versus the respective control. (A) Effect of H89 (1 μM) on the PGE₂ (100 nM) mediated stimulation of basolateral OA uptake in OK cells preloaded with glutarate (5 mM; 50 min); n = 6 to 9. (B) Effect of H89 (1 μM) on the forskolin (5 μM) mediated stimulation of basolateral OA uptake in OK cells preloaded with glutarate (5 mM; 50 min); n = 6 to 9.

**Figure 6.** Effect of inhibition of ERK1/2 by PD98059 on basolateral OA uptake in OK cells in the absence or presence of PGE₂ or forskolin. Incubation was performed as mentioned in Materials and Methods. In brief, cells were treated with PGE₂ (A)/forskolin (B), PD98059 alone, or PGE₂ (A)/forskolin (B) in combination with PD98059 for 10 min. OA used was [14C]PAH. *P < 0.05 versus the respective control; n = 6. (A) Effect of PD98059 (5 μM) on the PGE₂ (100 nM) mediated stimulation of basolateral OA uptake. (B) Effect of PD98059 (5 μM) on the forskolin (5 μM) mediated stimulation of basolateral OA uptake.
same was true for forskolin (Figure 6B). Thus, ERK1/2 activation is not involved in PGE$_2$-mediated stimulation of basolateral OA uptake in OK cells. This is in good agreement with our previous data (26) on signaling hierarchy, showing ERK upstream of prostaglandin generation.

**Prostaglandins Act via EP2 or EP4**

Following what was mentioned before, PGE$_2$ should act by binding to prostanoid receptor subtypes EP2 or EP4. Thus, we tested whether the EP2,3,4 selective prostanoid 11-deoxy PGE$_2$ stimulates basolateral OA uptake in OK cells. As shown in Figure 7A, 11-deoxy PGE$_2$ (10 min) dose-dependently stimulates OA uptake. In addition, 11-deoxy PGE$_2$ did not show any acute, cis-inhibiting effect on OA uptake (data not shown).

![Graph showing OA uptake with different concentrations of 11-deoxy PGE$_2$](image)

**Figure 7.** Effect of selective EP receptor agonists on basolateral OA uptake in OK cells. OK cells were incubated with various concentrations of the respective agonist for 10 min. After washing, basolateral uptake of OA was determined after 1 min. OA used was fluorescein. *P < 0.05 versus the respective control. (A) Effect of increasing concentrations of 11-deoxy PGE$_2$ on basolateral OA uptake; n = 8 to 17. (B) Effect of increasing concentrations of butaprost on basolateral OA uptake; n = 6 to 8.

Taken together, this is additional pharmacologic evidence that PGE$_2$ acts via the EP2 or EP4 receptor.

**Prostaglandins Act via a Homologue of EP4**

To gain more detailed information about the nature of PGE$_2$ action, we investigated whether the EP2 selective prostanoid butaprost affects basolateral OA uptake in OK cells. Reasonable concentrations of butaprost (Km = 100 nM) have no effect on OA transport rate (Figure 7B). Butaprost also has no competitive effect on OA uptake (data not shown). As no data on the presence or absence of prostanoid receptors in OK cells are available, we determined whether an EP2 or EP4 analogue is detectable in OK cells. No EP2 signal was detectable, whereas RT-PCR against EP4 (as described in Materials and Methods) led to a fragment of approximately 240 bp (Figure 8A). Sequencing led to a fragment of 239 bp in length, which hit rat EP4 prostaglandin receptor in BLAST search (Figure 8B) as well as the human type (see Discussion). Local homology search using ALIGNplus software indicated a 64% homology.

![Graph showing butaprost effect on OA uptake](image)

**Figure 8.** Detection of a rat EP4 receptor homologue in OK cells. (A) Reverse transcription–PCR (RT-PCR) product from OK cell mRNA. Primers against EP4 and conditions were as described in Materials and Methods. M, marker lane; EP4, lane with RT-PCR product. (B) Graph showing the location and grade of homology of the OK cell RT-PCR product to the rat EP4 receptor. (C) Alignment of the OK cell 239 bp RT-PCR product to the local homology region of rat EP4 receptor DNA sequence. EP4-1B-1.TXT, RT-PCR product from OK cell mRNA; RATEP4-1.TXT, rat EP4 receptor DNA sequence.
of the cloned fragment to the sequence segment predicted by the primers used in PCR (Figure 8, B and C). Thus, most likely, an EP4 receptor homologue to which PGE<sub>2</sub> can bind is present in OK cells.

**EGF-Induced Stimulation of OA Uptake Is Mediated by Adenylate Cyclase and PKA**

Finally, we investigated whether the stimulatory action of EGF on proximal tubular OA uptake described before (25,26) includes the PGE<sub>2</sub> mechanism presented here. Therefore, we investigated the effect of inhibition of PKA (by H89; Figure 9A) or adenylate cyclase (by MDL-12,330A; Figure 9B) on EGF-induced stimulation of basolateral OA uptake in OK cells. Both inhibition of PKA and adenylate cyclase completely abolished EGF-induced stimulation of OA uptake. Thus, the stimulatory mechanism of EGF on OA uptake includes the PGE<sub>2</sub>-induced mechanism presented herein.

**Discussion**

As already mentioned in the introduction, only little is known about the regulation of basolateral uptake of OA. In previous studies, we reported that EGF stimulates basolateral OA uptake in OK cells via MEK and ERK1/2 (25), which then activates phospholipase A<sub>2</sub> (PLA<sub>2</sub>), leading to arachidonic acid generation. COX1 then transforms arachidonic acid into PGE<sub>2</sub>, which dose-dependently activates basolateral OA uptake (26). For this stimulation to occur, two mechanisms are possible. First, PGE<sub>2</sub> could act as a counter substrate and thus stimulate the uptake of OA. Second, PGE<sub>2</sub> could act via coupling to specific receptors present at the membrane of proximal tubular OK cells. In the following, both possibilities are discussed.

Literature (19,31,32) indicates that PGE<sub>2</sub> is a substrate for the basolateral OA uptake exchanger OAT1. PGE<sub>2</sub> is also shown to be a substrate for the human type OAT3 (4). Our own data (Figure 2) show that PGE<sub>2</sub> acutely interacts with OA uptake. Thus, the possibility emerges that PGE<sub>2</sub> may cis-inhibit basolateral uptake of OA competitively (Figure 2) and may trans-stimulate basolateral OA uptake when present inside the proximal tubular cells (for definition of the cis- and trans-nomenclature, see Materials and Methods). In addition, cAMP generated when PGE<sub>2</sub> binds to EP2 or EP4 receptors is a substrate for basolateral organic uptake mechanisms (8,19). Thus, it may cis-inhibit and trans-stimulate with respect to basolateral OA uptake. Therefore, we decided to investigate the action of PGE<sub>2</sub> under circumstances when trans-stimulation by glutarate occurs. Preincubation of OK cells with 5 mM glutarate for 50 min is shown to increase OA uptake approximately threefold (Figure 5). This trans-stimulatory mechanism is well described for OAT1 (7,8,19,38) and also for OAT3 (15). After preincubation with glutarate, PGE<sub>2</sub> still shows stimulatory action. In detail, after preincubation, relative stimulation of basolateral OA uptake by the same total amount of PGE<sub>2</sub> (100 nM) is approximately 1.7-fold compared with the respective control (Figure 5A; glutarate control). In cells not preloaded with glutarate (Figure 4A; control set as 100%), a 1.8-fold increase compared with the respective control occurred. Preincubation of OK cells with glutarate leads to an increase of counter ions for basolateral OA uptake. Thus, if acting in a trans-stimulatory manner, the same amount of PGE<sub>2</sub> should exert a decreased relative stimulatory action in cells preloaded with glutarate. As this is not the case, it is evident that PGE<sub>2</sub> action presented herein is not due to trans-stimulation. In addition, if stimulation by PGE<sub>2</sub> was due to trans-stimulation, then inhibition of adenylate cyclase should not have any effect on its action. Moreover, PGE<sub>2</sub> action was mimicked by adenylate cyclase activator forskolin in both control and glutarate-preloaded OK cells. Forskolin is not a potential counter ion for the basolateral OA exchanger as no acute inhibitory interaction with OA uptake was observed (Figure 2). The PKA inhibitor H89 completely abolished PGE<sub>2</sub> action in both control and glutarate-preloaded OK cells, which
is evidence that cAMP does not act as counter ion with respect to stimulation of basolateral OA uptake. In addition, forskolin-induced stimulation of OA uptake persists after glutarate pre-load as compared with the control cells (in parallel to what was mentioned before for PGE2). Moreover, forskolin action on OA uptake is completely abolished by inhibition of PKA (in control and glutarate-preloaded OK cells), which in addition contradicts a trans-stimulatory mechanism. Taken together, these data exclude the possibility of trans-stimulation as a putative mechanism of the dose-dependent PGE2 action on initial basolateral OA uptake in OK cells described by Sauvant et al. (26).

Thus, PGE2 action should be mediated by activation of one of its receptors. Four isoforms of the PGE2 receptors, named EP1 to EP4, are known, and all of them are present in the kidney and act via G-proteins (35,39). EP1 is known to act via Gq after activation of PKC. As already mentioned in the introduction, it is well known from literature and from our own experiments that activation of PKC inhibits basolateral OA uptake. Thus, in our case, EP1 is not a possible receptor for mediation of PGE2 action. The remaining possibilities include EP2 and EP4, both activating adenylate cyclase and PKA, or EP3, which inhibits adenylate cyclase and PKA. If PGE2 would stimulate OA uptake via EP3, then inhibition of adenylate cyclase or PKA should stimulate uptake as activation of EP3 leads to inhibition of adenylate cyclase and PKA. However, as indicated in Figures 3 and 4, both maneuvers mentioned have no effect on OA uptake in OK cells. In addition, if PGE2 would stimulate OA uptake in OK cells via the EP3 receptor, then inhibition of both adenylate cyclase and PKA in the presence of PGE2 should lead to increased stimulation as compared with PGE2 action alone. As this also is not the case, it seems highly unlikely that PGE2 is acting via the EP3 receptor subtype. This is confirmed by the fact that forskolin stimulates OA uptake and that this stimulation is again prevented by inhibition of PKA. Thus, stimulation of OA uptake by PGE2 cannot be mediated by a mechanism inhibiting adenylate cyclase or PKA, as EP3 receptor stimulation does.

Thus, the stimulatory action of PGE2 should be mediated by interaction with the EP2 or EP4 receptor subtype. This hypothesis was confirmed using 11-deoxy PGE1, an EP2,3,4 selective agonist (Km approximately 30 to 50 nM for EP2 or EP4 (40)). Figure 7 shows the stimulatory action of 11-deoxy PGE1 on basolateral OA uptake. In addition, no acute inhibitory interaction with OA uptake was observed for 11-deoxy PGE1 at a concentration being at least twice as high as affinity constant (40). Thus, 11-deoxy PGE1 is not a possible substrate or a counter ion with respect to basolateral uptake of OA. 11-Deoxy PGE1 has also been described to bind to EP3 with high affinity (Km approximately 1.5 nM (40)), but as a result of the observed stimulation of OA uptake by 11-deoxy PGE1 and the above-mentioned data, excluding EP3 as a possible receptor for mediating stimulation of OA uptake, this possibility can be ruled out. In addition, reasonable amounts of the EP2 selective agonist butaprost (Km approximately 100 nM; (40)) do not affect basolateral organic uptake rate, neither after 10 min nor acutely. The EP selective agonists used were shown to act not only in rodents but also in cells from pigs (41) and humans (15). Thus, their pharmacologic profile seems not to be strictly species dependent. Thus, all pharmacologic data presented give evidence that the receptor-mediating stimulation of basolateral OA uptake by PGE2 is of the EP4 type.

As no molecular evidence for the presence of EP receptors in OK cells was available, we decided to determine whether homologues to the known rat receptor subtypes EP2 or EP4 are expressed. As mentioned in Results, no mRNA for EP2 could be detected in OK cells with the methods used herein. Certainly, this is not proof for the absence of EP2 in OK cells. However, these data are in good agreement with the fact that butaprost has no effect on OA uptake. However, using EP4 selective primers and RT-PCR, we were able to clone a 239-bp fragment showing 64% homology to the respective part of the rat EP4 receptor DNA sequence. Thus, an mRNA homologue to EP4 is expressed in the proximal tubular OK cell line. Moreover, the cloned EP4 fragment from OK shows not only 64% homology to the respective part of the EP4 receptor DNA sequence from rat but also a 67% homologue to respective human sequence. Comparison of the EP4 receptor sequences from rat and human leads to a homology of 65%. Thus, the OK cell fragment cloned is as homologous to rat or human as the EP4 receptors from rat and human are compared with each other. Together with the pharmacologic data of butaprost and 11-deoxy PGE1 presented above, this is clear evidence that prostanoids stimulate basolateral OA uptake in OK cells by a mechanism activating adenylate cyclase and PKA. This is in good agreement with data showing an increase in cAMP in human proximal tubular cells (42) after incubation with PGE2. The receptor mediating this regulatory cascade is most likely a homologue of the EP4 receptors described in rats or humans.

As already mentioned, EGF-mediated stimulation of basolateral OA transport in OK cells is due to a signaling pathway leading to generation of PGE2 (26). Herein PGE2 was shown to stimulate OA uptake by activation of PKA and adenylate cyclase. In addition, EGF stimulation of OA uptake was abolished by inhibition of adenylate cyclase or PKA. Thus, PGE2 generation links EGF action and PKA activation. Moreover, this is again evidence contradicting a trans-stimulatory mechanism of PGE2, because in this case, EGF action should not be abolished by the above-mentioned inhibitory mechanisms.

The observed stimulatory effects of PGE2 on initial basolateral uptake of OA presented before (26) can be explained by using the following model (Figure 10): PGE2 is generated in response to EGF (via a signaling pathway shown by Sauvant et al. (26)) in the cells or externally, then most likely binds to a EP4 receptor leading to G-protein–mediated activation of adenylate cyclase. cAMP then activates PKA, which stimulates basolateral OA uptake by an as-yet-unknown mechanism. Meanwhile, PKA phosphorylation sites were shown to exist on human OAT1 but up to now are not indicated in human or rat OAT3 (32). However, this does not exclude the possibility that OAT3 is stimulated by PKA as this action may also be mediated via the phosphorylation of auxiliary proteins interacting with the respective transport protein. In addition, You et al. (24) showed that PKC inhibits murine OAT without direct
proximal tubular cells from pigs and humans, PGE₂ is shown to be upregulated in the kidney, PGE₂ and its receptors are known to be produced in the renal cortex and in particular in inflammatory disease of the kidney. Additionally, PGE₂ is considered to be the major renal cyclooxygenase metabolite of arachidonic acid, which is known to bind to the EP4 receptor homologue, leading to an increase in adenylate cyclase activity. Thus, the generation of cAMP is enhanced, and PKA activity is stimulated. PKA then stimulates the basolateral OA uptake by an as-yet-unknown mechanism. For reasons mentioned in Discussion, OAT1 and OAT3 are the most likely candidates for the observed stimulation. EGF stimulation of basolateral OA uptake in OK cells in the short time range. PGE₂ plays a crucial role in inflammation. EP4 receptors have been detected in the proximal tubule and are known to be upregulated in inflammatory disease of the kidney, PGE₂ and its receptors are shown to be upregulated in proximal tubular cells (35, 39).

PGE₂ plays a crucial role in inflammation (43). EP4 receptors have been detected in the proximal tubule (35, 39). In addition, PGE₂ is considered to be the major renal cyclooxygenase metabolite of arachidonic acid (27), which is known to be produced in the renal cortex (44) and in particular in proximal tubular cells (45). In inflammatory disease of the kidney, PGE₂ and its receptors are shown to be upregulated (28). In proximal tubular cells from pigs and humans, PGE₂ is generated by reactive oxygen species (42, 46) induced by stress and may directly contribute to renal lesions and loss of kidney function. As basolateral uptake is considered to be the rate-limiting step of OA secretion, we expect that PGE₂ induces stimulation of OA secretion into the proximal tubular lumen. As PGE₂ is an OA itself, this mechanism may reduce the concentration of PGE₂ in the kidney interstitium, which contradicts its inflammatory potency. We hypothesize that this may represent a mechanism to limit PGE₂-induced inflammatory events in the kidney cortex interstitium. Therefore, we further hypothesize that inhibition of OA secretion during kidney inflammation may contribute to an increase in severity of inflammation. This will have to be investigated in further experiments.

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References

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**Figure 10.** Summary showing the mechanism of prostaglandin action on basolateral OA uptake in OK cells in the short time range. PGE₂ binds to the EP4 receptor homologue, which leads to an increase in adenylate cyclase activity. Thus, the generation of cAMP is enhanced, leading to an increase in PKA activity. PKA then stimulates the basolateral OA uptake by an as-yet-unknown mechanism. For reasons mentioned in Discussion, OAT1 and OAT3 are the most likely candidates for the observed stimulation. EGF stimulation of basolateral OA uptake described before is shown to be mediated via the mentioned mechanism.


