Acute regulation of OAT3-mediated estrone sulfate transport in isolated rabbit renal proximal tubules

S. Soodvilai, V. Chatsudthipong, K. K. Evans, S. H. Wright, and W. H. Dantzler. Acute regulation of OAT3-mediated estrone sulfate transport in isolated rabbit renal proximal tubules. Am J Physiol Renal Physiol 287: F1021–F1029, 2004. First published July 6, 2004; doi:10.1152/ajprenal.00080.2004.—We investigated the regulation of organic anion transport driven by the organic anion transporter 3 (OAT3), a multispecific OAT localized at the basolateral membrane of the renal proximal tubule. PMA, a PKC activator, inhibited uptake of estrone sulfate (ES), a prototypic substrate for OAT3, in a dose- and time-dependent manner. This inhibition was reduced by 100 nM bisindoylmaleimide I (BIM), a specific PKC inhibitor. The α1-adrenergic receptor agonist phenylephrine also inhibited ES uptake, and this effect was reduced by BIM. These results suggest that PKC activation downregulates OAT3-mediated organic anion transport. In contrast, epidermal growth factor (EGF) increased ES uptake following activation of MAPK. Exposure to PGE2 or dibutyryl (db)-cAMP also enhanced ES uptake. Stimulation produced by PGE2 and db-cAMP was prevented by the PKA inhibitor H-89, indicating that this stimulation required PKA activation. In addition, inhibition of cyclooxygenase 1 (COX1) (but not COX2) inhibited ES uptake. Furthermore, the stimulatory effect of EGF was eliminated by inhibition of either COX1 or PKA. These data suggest that EGF stimulates ES uptake by a process in which MAPK activation results in increased PGE2 production that, in turn, activates PKA and subsequently stimulates ES uptake. Interestingly, EGF did not induce upregulation immediately following phenylephrine-induced downregulation; and phenylephrine did not induce downregulation immediately after EGF-induced upregulation. These data are the first to show the regulatory response of organic anion transport driven by OAT3 in intact renal proximal tubules.

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THE KIDNEY PLAYS AN IMPORTANT role in the excretion of a structurally diverse array of compounds from the body. A great number of the potentially toxic compounds in the human body are organic anions, including both endogenous compounds and xenobiotics that entail a variety of environmental toxins and pharmaceutical agents (2, 7, 24, 39, 40, 43). The active secretory process of anionic compounds to the tubular lumen appears to be restricted to the proximal tubule. This process involves the following steps: transport of compounds from the blood into the cells across the basolateral membrane against an electrochemical gradient and movement from the cells into the lumen down an electrochemical gradient (23, 43). Transport into the cell at the basolateral membrane, which is the rate-limiting step in secretion, is a tertiary active process. This step involves countertransport of an organic anion into the cell against its electrochemical gradient in exchange for mediated efflux of α-ketoglutarate (α-KG). The outwardly directed gradient for α-KG is not only maintained by intracellular metabolic generation of α-KG but is also fueled by active α-KG uptake across the basolateral membrane via a Na-dicarboxylate cotransporter (NaDC) that is, in turn, driven by the transmembrane sodium gradient established by the Na-K-ATPase (21, 22, 41).

Many cDNAs encoding renal organic anion transporters (OATs) have been cloned, including OAT1, OAT2, OAT3, OAT4, OAT-P1, OAT-K1, and OAT-K2 (43). However, of these, only OAT1 and OAT3 have been identified as playing a major role in the cellular uptake of organic anions across the basolateral membrane of renal proximal tubules (33, 34). OAT3 shows high amino acid sequence identity with OAT1 (~99%) (4, 14). Moreover, OAT1 (31, 35) and OAT3 (1, 33) share a common energetic mechanism (i.e., OA/α-KG exchange) (35) and have overlapping substrate specificities (1, 2, 34, 37). However, activity of the two transporters in rabbit renal proximal tubules can be distinguished using the homolog-selective substrates estrone sulfate (ES) and p-aminohippurate (PAH); whereas basolateral ES transport is effectively restricted to OAT3, PAH transport is effectively restricted to OAT1 (17). Basolateral organic anion transport in renal cells has been shown to be regulated by several hormones that activate protein kinases, including bradykinin, phenylephrine, ANG II, and parathyroid hormone (10, 20, 32). The phosphorylation cascades induced by protein kinases result in either the activation or inhibition of specific enzymes or in the activation of the transcription of specific genes (39). Activation of protein kinase C (PKC) by PMA and 1,2-dioctanoyl-sn-glycerol (DOG) downregulates organic anion transport mediated by several orthologs of OAT1 (16, 19, 36, 42, 44) and rat OAT3 (38). In addition, activation of PKC, either directly with PMA or DOG or indirectly with ligands of physiological receptors coupled to the PKC pathway (e.g., the α1-receptor agonist phenylephrine; or the peptide hormone bradykinin), inhibits basolateral uptake and transepithelial transport of fluorescein (FL) in the S2 segment of rabbit renal proximal tubules (10, 32).

Recent data indicate that epidermal growth factor (EGF), which appears to be important in normal tubulogenesis and tubular regeneration after injury, stimulates basolateral uptake of PAH in both cell cultures (28, 29) and intact renal proximal tubules (27). This effect of EGF on the basolateral uptake of organic anions occurs via the MAPK pathway. Administration...
of EGF leads to phosphorylation of mitogen-activated/extracellular signal-regulated kinase kinase (MEK), ERK1/2, and phospholipase A2 (PLA2), resulting in an increased release of arachidonic acid. Arachidonic acid is subsequently metabolized to prostaglandin via cyclooxygenase 1 (COX1), which then mediates EGF-induced stimulation of basolateral PAH uptake (29). In S2 segments of rabbit renal proximal tubule, prostaglandin enhances basolateral PAH uptake via adenylyl cyclase activation and causes protein kinase A (PKA) activation (27). Previous data demonstrated the effect of protein kinases, including tyrosine kinase, phosphatidylinositol-3-kinase (PI3-K), MAPK, and calcium/calmodulin-dependent multifunctional protein kinase II, on organic anion transport driven by OAT1 in the intact rabbit proximal tubule (9). These results suggest that a number of physiological factors regulate basolateral organic anion transporters through the activation of an array of phosphorylation pathways.

At present, although substantial information concerning the regulation of organic anion transport in heterologous systems is available, little is known about the regulation of organic anion transport in the intact renal proximal tubule, and nothing is known about regulation of OAT3-mediated transport in renal tubules. It is imperative to study regulation of organic anion transport with the transporter expressed in its native, intact system to be certain that regulatory responses observed in other systems reflect, in at least a qualitatively significant way, those occurring under comparatively normal physiological circumstances. Although previous data showed that PKC activation inhibits FL uptake in intact rabbit tubules (10), we now know that FL is a substrate for both OAT1 and OAT3 (Zhang X, Droker B, and Wright S, unpublished observations). The regulation of each transporter needs to be investigated by using specific substrates for that transporter. For this purpose, we studied the regulatory mechanism of organic anion transport driven by OAT3 in nonperfused S2 segments of rabbit renal proximal tubules, using [3H]estrone sulfate ([3H]ES) as a comparatively specific substrate for this pathway in rabbit tubules (17). We present data indicating that activation of PKC downregulates OAT3 activity. In contrast, EGF activates MAPK leading to PGE2 production, subsequent PKA activation, and finally OAT3 upregulation.

METHODS

Chemicals. [3H]ES (43.5 Ci/mmol) was purchased from Perkin-Elmer Life Science Products (Boston, MA). Bisindolylmaleimide I (BIM), EGF, and phenylephrine were purchased from Sigma (St. Louis, MO). PGE2, dibutyryl (db)-cAMP, and H-89 were purchased from Calbiochem. U-0126 was obtained from Promega (Madison, WI), whereas PMA was purchased from Alexis Biochemical. All other chemicals were purchased from Sigma and were generally of the highest purity available.

Preparation of isolated tubules. New Zealand White rabbits (1.5 kg; Harlan, Indianapolis, IN) were killed by intravenous injection of pentobarbital sodium, and all protocols employing rabbits were conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health. The kidneys were flushed via the renal artery with an ice-chilled HEPES-sucrose buffer containing 250 mM sucrose and 10 mM HEPES, adjusted to pH 7.4 with Tris-HCl, and bubbled with 100% O2 before use. They were then gently removed and sliced transversely using a single-edge razor. A kidney slice was transferred to the lid of a plastic petri dish on ice, which contained the standard solution (nutritionally enriched bicarbonate buffer) used for dissecting and bathing tubules (in mM: 110 NaCl, 25 NaHCO3, 5 KCl, 2 NaHPO4, 1 MgSO4, 1.8 CaCl2, 10 Na-acetate, 8.3 D-glucose, 5 L-alanine, 0.9 glycine, 1.5 lactate, 1 malate, and 1 sodium citrate). This standard solution was aerated continuously with 95% O2-5% CO2 to maintain pH at 7.4. The osmolality of the solutions averaged ~290 mosmol/kgH2O.

S2 segments of proximal tubules were individually dissected from the cortical zone without the aid of enzymatic agents as described by others (3). All dissections were performed at 4°C, and all experiments were performed at 37°C. Transport rates were normalized to tubule surface area based on tubule lengths and average diameters as determined from photographs taken through a dissecting microscope equipped with a digital image capture system (Snappy, Play).

Measurement of transport of [3H]ES in S2 segments of nonperfused, isolated renal proximal tubule. These experiments were performed in a manner similar to that used previously (5, 6, 11). Briefly, an appropriate number of tubule segments (3–5 for each condition to be studied) were teased from fresh renal tissue and maintained in oxygenated (95% O2-5% CO2) nutritionally enriched bicarbonate buffer solution at 4°C that was covered with a layer of mineral oil to prevent evaporation until the start of each experiment. The standard solution was warmed to 37°C before the tubules were transferred to baths containing the different activators or inhibitors of protein kinases. At the end of the preincubation period, each tubule was transferred to a new bathing medium containing [3H]ES (~0.12 μM) for 30 s. This time period was chosen because in preliminary experiments it was confirmed that it permitted an adequate approximation of the initial rate of ES uptake (see Ref. 17). No putative stimulators or inhibitors were present in the medium during these 30-s uptakes. The uptake was stopped by transferring each tubule into individual wells containing 7 μl 1 N NaOH. Accumulated labeled substrate was determined by liquid scintillation counting. Control and experimental uptakes were determined alternately and sequentially in tubules from the same kidney.

Data analysis. Data are summarized as means ± SE. The n value indicates the number of experiments (each using tubules from a different rabbit). The differences between rates of uptake for the different regimens were analyzed with the one-way ANOVA. In all analyses, differences were considered statistically significant when P < 0.05.

RESULTS

Effect of PKC activation by PMA on basolateral uptake of ES. We first examined the time course of the effect on transport of preincubation in 100 nM PMA, a concentration shown previously to decrease ES uptake in cultured cells that stably express rat OAT3 (38). The tubules were preincubated for different lengths of time in control medium (nutritionally enriched bicarbonate buffer) containing 100 nM PMA, followed by 30-s uptake in control medium containing [3H]ES (~0.12 μM). There was a modest decrease in ES uptake following a 15-min exposure to 100 nM, and significant decreases were produced by both 25- and 45-min exposures. The initial rate of uptake was reduced by 34 ± 7% of control after 25 min, and there was no significant difference between the uptakes measured after 25- and 45-min exposures. The initial rate of uptake was reduced by 34 ± 7% of control after 25 min, and there was no significant difference between the uptakes measured after 25- and 45-min exposures to PMA (Fig. 1A). Figure 1B shows the dose-response of the PMA effect on basolateral uptake of ES in S2 segments of renal proximal tubules. The tubules were preincubated with control medium containing increasing concentrations of PMA for 25 min and then incubated with control medium containing [3H]ES (~0.12 μM) for 30 s. The inhibitory effect of PMA was dose dependent, with 100 nM proving to be the smallest
effective concentration (decrease of 35 ± 7%, compared with control). Increasing the concentration to 1 μM led to further inhibition (decrease of 46 ± 6%). To determine whether the inhibitory effect produced by PMA on basolateral uptake of ES reflected activation of PKC, we examined the effect of BIM, a specific PKC inhibitor, on the PMA-induced inhibition of basolateral ES uptake. As shown in Fig. 2A, exposure to 100 nM BIM for 25 min had no effect on basolateral uptake of ES. For the PMA plus BIM treatment, the tubules were first preincubated with BIM alone for 5 min and then transferred into medium containing 100 nM PMA plus 100 nM BIM for 25 min. The effect of PMA in the presence of BIM (23% decrease of ES uptake) was significantly less than that of PMA alone (37% decrease; P < 0.05), suggesting that inhibition of PKC reduced the inhibitory effect of PMA. These results support the contention that the downregulation of OAT3-mediated organic anion transport induced by exposure to PMA involves activation of PKC.

Effect of PKC activation by phenylephrine on basolateral uptake of ES. To investigate whether PKC-mediated regulation of OAT3 also takes place after receptor-coupled activation of PKC, we examined the effect of phenylephrine, which is known to activate PKC via an α1-adrenergic receptor (15). As shown in Fig. 2B, a 25-min exposure to phenylephrine (1 μM) inhibited the subsequent 30-s uptake of ES by 38 ± 9%. Furthermore, BIM significantly reduced the effect of phenylephrine on ES uptake (19% decrease following exposure to phenylephrine plus BIM), indicating that (at least) a fraction of the inhibitory effect of phenylephrine was prevented by BIM. These data demonstrate that ES uptake is also inhibited by receptor-mediated activation of PKC.

Effect of EGF on basolateral uptake of ES. S2 segments of the renal proximal tubule were preincubated for 10 min with control medium or control medium containing EGF (10 to 50 ng/ml). As shown in Fig. 3A, EGF (10 ng/ml) significantly increased the initial rate of ES uptake (by 32 ± 7% of control).
Increased concentrations of EGF did not further increase ES uptake, indicating that EGF at 10 ng/ml was sufficient to produce a maximum effect on ES uptake. The effect of EGF on ES uptake observed here was similar to its effect on PAH uptake in renal proximal tubules, a response shown to involve activation of the MAPK pathway (27). Therefore, we examined the influence of U-0126, a specific inhibitor of MEK, on the response of tubules to EGF exposure. The tubules were preincubated in four conditions: 1) control medium, 2) control medium containing 10 ng/ml EGF for 10 min, 3) control medium containing 10 μM U-0126 for 20 min, and 4) control medium containing 10 μM U-0126 for 10 min followed by control medium containing 10 ng/ml EGF and 10 μM U-0126 for 10 min. As shown in Fig. 3B, EGF significantly stimulated ES uptake (by 30 ± 8% of control), whereas U-0126 alone significantly inhibited ES uptake by (30 ± 3% of control). The stimulatory effect of EGF on ES uptake was eliminated in the presence 10 μM U-0126 and ES uptake was reduced to approximately the same level (62 ± 8% of control) as with U-0126 alone (Fig. 3B). These results are consistent with the stimulatory effect of EGF on ES uptake reflecting activation of the MAPK pathway.

Effect of PGE₂ and PKA activation on basolateral uptake of ES. Previous studies showed that EGF stimulates OAT1 activity via MAPK activation followed by increased production of PGE₂ via COX1 and the subsequent activation of PKA (27, 29, 30). Therefore, because the effect of EGF on OAT3 activity apparently involved activation of the MAPK pathway, we investigated whether the additional steps in the stimulation pathway for OAT3 were the same as those for the stimulation of OAT1. First, we examined the effect of PGE₂ on the initial rate of ES uptake and, then, if PGE₂ had an effect, whether that effect could be blocked by H-89, an inhibitor of PKA. For these studies, the tubules were preincubated in four conditions: 1) control medium, 2) control medium containing 2 μM PGE₂ for 10 min, 3) control medium containing 2 μM H-89 for 10 min, and 4) control medium containing 2 μM H-89 for 5 min followed by control medium containing 2 μM PGE₂ and H-89 for 10 min. As shown in Fig. 4A, PGE₂ stimulated ES uptake by 22 ± 7% of control and this stimulation was abolished by H-89, whereas exposure to H-89 alone had no effect on ES uptake. These data support the idea that PGE₂ stimulates the basolateral uptake of ES and that this stimulation involves activation of PKA.

To further examine the involvement of PKA as a stimulus of ES uptake, we examined the effect of db-cAMP, a direct PKA activator, on ES uptake in the presence and absence of the PKA inhibitor H-89. The preincubation protocol was the same as that used with PGE₂ and H-89. The tubules were preincubated in four conditions: 1) control medium, 2) control medium containing 10 μM db-cAMP for 10 min, 3) control medium containing 2 μM H-89 for 15 min, and 4) control medium containing 2 μM H-89 for 5 min followed by control medium containing 10 μM db-cAMP and H-89 for 10 min. As shown in Fig. 4B, db-cAMP significantly stimulated ES uptake by 23 ± 9% of control and this stimulation was eliminated by the
presence of H-89. These results indicate that direct activation of PKA also leads to stimulation of ES uptake and further supports its role in the pathway for OAT3 upregulation.

PGE$_2$ is the major metabolite of arachidonic acid produced in the kidney by the activity of COX, which consists of two isoforms (COX1 and COX2) (27). To determine whether production of PGE$_2$ by one or both of these COX isoforms normally plays a role in OAT3 activity, we determined the effect of SC-560, a COX 1 inhibitor, and indomethacin n-heptyl ester (IHE), a COX2 inhibitor, on the initial rate of ES uptake. The tubules were preincubated with various concentrations of COX inhibitors for 15 min before ES uptake was measured. As shown in Fig. 5, exposure to 0.1 mM SC-560 led to the inhibition of ES uptake by $\sim 28 \pm 5\%$ of control. Increasing the concentration of SC-560 uptake up to 10 mM did not produce any greater inhibition of ES uptake, suggesting that 0.1 mM SC-560 was sufficient to produce the maximum effect on ES uptake. In contrast to exposure to SC-560, exposure to IHE had no effect on ES uptake. These data indicate that PGE$_2$ produced by the activity of COX 1, but not COX 2, is partly responsible for the basal OAT3-mediated organic anion transport in renal proximal tubules.

To determine whether the stimulation of ES uptake by EGF was indeed mediated via PGE$_2$ (produced by COX1) and finally via PKA activation, we examined the effects of SC-560 and H-89 on ES uptake during EGF stimulation. The tubules were preincubated under four conditions: 1) control medium, 2) control medium containing 10 ng/ml EGF for 10 min, 3) control medium containing 0.1 mM SC-560 for 5 min followed by control medium containing 10 ng/ml EGF and 0.1 mM SC-560 for 10 min, and 4) control medium containing 2 mM H-89 for 5 min followed by control medium containing 10 ng/ml EGF and H-89 for 10 min. The results are shown in Fig. 6. As expected, EGF stimulated ES uptake (by 26 $\pm$ 9% of the control value). This stimulation was eliminated and the control rate of uptake was reduced by either SC-560 or H-89. These results support the idea that the stimulatory effect produced by EGF involves PGE$_2$ production and PKA activation.

Effect of EGF and phenylephrine on basolateral uptake of ES after PKC and MAPK activation. As mentioned above, activation of different regulatory pathways was capable of either increasing or decreasing the rate of OAT3-mediated ES transport. The range of transport activity from the lower limit of “downregulation” to the upper limit of “upregulation” was greater than twofold. The following experiments were performed to determine whether OAT3 activity could be rapidly shifted over the obtainable dynamic range by exposure to different physiological stimuli. First, we examined the effect of EGF on the initial rate of uptake of ES after OAT3 activity was downregulated by phenylephrine. Before measurement of ES uptake, the tubules were preincubated in: 1) control medium, 2) control medium containing 1 mM phenylephrine for 25 min, 3) control medium containing 1 mM phenylephrine for 25 min followed by control medium for 10 min, and 4) control medium containing 1 mM phenylephrine for 25 min followed by 10 ng/ml EGF in control medium for 10 min. As shown in Fig. 7A, exposure to 1 mM phenylephrine caused the expected inhibition of ES uptake and, after the tubules were transferred from medium containing 1 mM phenylephrine to control medium, ES uptake returned to the control value. However, exposure to EGF during the recovery period failed to increase transport to a level above the control value. We also examined the effect of phenylephrine on the initial rate of ES uptake after OAT3 activity was upregulated by EGF. The tubules were preincubated in four different conditions before ES uptake was measured: 1) control medium, 2) control medium containing 10 ng/ml EGF for 10 min, 3) control medium containing 10 ng/ml EGF for 10 min followed by control medium for 25 min, and 4) control medium containing 10 ng/ml EGF for 10 min followed by control medium containing 1 mM phenylephrine for 25 min. As shown in Fig. 7B, exposure to 10 ng/ml EGF stimulated ES uptake, which returned to the control value after the tubules were transferred to control medium. Interestingly, similar to the situation noted above, exposing the “recovering” tubules to phenylephrine had no effect; the rate of ES uptake decreased to the control value, but no lower. These data suggest that the mechanism(s) that result in the stimulation or inhibition of OAT3 activity following exposure to EGF or...
phenylephrine are, at least temporarily, not accessible to cells that have recently had PKC or MAPK activated, respectively.

**DISCUSSION**

Although regulation of organic anion transport can be examined with cloned transporters expressed in heterologous cell systems, it is important to study the regulatory responses of these processes when expressed in their native, intact system. For example, whereas activation of PKC can reduce activity of flounder NaDC-3 by 50 to 90% when expressed in *Xenopus laevis* oocytes (12), there is no evidence of a similar change in basolateral activity of NaDC-3 in intact rabbit proximal tubules following activation of PKC (26). As mentioned previously, although substantial information is available concerning the regulation of organic anion transport by several orthologs of OAT1 and OAT3 expressed in heterologous cell systems, little is known about the regulation of organic anion transport in intact renal proximal tubules, especially that mediated by OAT3. Previous studies reported that activation of PKC led to downregulation of OAT1 and OAT3 activity in cultured cells systems (10, 16, 20, 32, 36, 42, 44). Significantly, FL uptake in intact S2 segments of renal proximal tubules is downregulated by activation of PKC (10, 32). However, FL is a substrate for both OAT1 and OAT3 (Zhang, Droker, and Wright, unpublished observations); consequently, it is not clear if the decrease in FL uptake in tubules reflects regulation of activity of OAT1, OAT3, or both. Exposure to EGF, on the other hand, has been shown to stimulate OAT1 activity in opossum kidney cells (28, 29) and intact renal proximal tubules (27), an effect mediated by activation of MEK and ERK1/2. In the present study, we investigated the regulatory mechanism of OAT3-mediated organic anion transport in S2 segments of rabbit renal proximal tubule. We took advantage of the previous observation (17) that basolateral transport of ES in S2 segments of rabbit renal proximal tubule is effectively restricted to an interaction with OAT3, an observation further supported by results obtained with OAT3-null mice (34).

As shown in the results section, exposure to PMA inhibited \[^{3}H\]ES uptake in a dose- and time-dependent manner in isolated, nonperfused S2 segments of renal proximal tubule (Fig. 1). These findings are consistent with a previous study with rat OAT3 expressed in mouse S2 cells (38). The inhibitory effect was significantly reduced by inhibition of PKC by BIM. Exposure to BIM alone, however, had no effect on ES uptake, implying that under basal physiological conditions OAT3 activity is not under the tonic influence of PKC. We also investigated whether indirect activation of PKC through a receptor-mediated mechanism results in a downregulation of OAT3 activity. The \(\alpha_1\)-receptor agonist phenylephrine stimulates PKC in renal proximal tubular cells via a ligand-receptor coupling reaction (15). Exposure to 1 \(\mu\)M phenylephrine inhibited ES uptake and this inhibitory effect was reduced by BIM. The inhibitory effect of phenylephrine was reversible as evidenced by the fact that after the removal of phenylephrine, ES uptake returned to control levels (Fig. 7A).

Although BIM did not eliminate the inhibitory effect of PMA or phenylephrine on activity of OAT3, the significant reduction of those effects following exposure to BIM supports the conclusion that activation of PKC does exert a regulatory influence on OAT3 activity in proximal tubule cells. The incomplete block of the effect of PMA or phenylephrine by BIM may have been due to the concentration of BIM (i.e., 100 nM) used in our experiments. In at least some cells, the 50% inhibitory concentration of BIM for the inhibition of PKC-induced effects is 200–250 nM (10). However, we chose not to use concentrations as large as this because BIM loses its specificity for PKC at these levels (10). In addition, PMA typically activates all isoforms of PKC, whereas BIM is most effective at inhibiting PKC group A (18). Nevertheless, we cannot rule out the possibility that some part of the PKC or phenylephrine effect involved something other than activation of PKC. Our results showed that activation of PKC, both through direct activation by PMA and physiological activation by an \(\alpha_1\)-receptor agonist, lead to downregulation of OAT3 activity in S2 segments of rabbit renal proximal tubules.

Basolateral uptake of organic anions mediated by OAT3 is a tertiary active process, i.e., it involves the parallel activity of three transport processes: 1) OA/\(\alpha_\)-KG exchange (OAT3), 2) Na-\(\alpha_\)-KG cotransport (NaDC-3), and 3) the Na-K-ATPase. Modulation of any of these processes may be expected to result in a change in basolateral uptake of an organic anion such as ES (33). Indeed, manipulation of the activity of basolateral Na-\(\alpha_\)-KG cotransport has been shown to exert a profound effect on organic anion transport (22, 41). It is, however, unlikely that the decrease in ES uptake that followed activation of PKC is solely the result of a decrease in Na-\(\alpha_\)-KG cotrans-
port. As noted earlier, previous studies found that activation of PKC appears to have little or no effect on basolateral dicarboxylate transport in rabbit tubules (10, 26), suggesting that acute effects of PKC on either NaDC-3 or the Na-K-ATPase are sufficiently minor to have no influence on the outwardly directed α-KG gradient (at least over the time course of the experiments in the current study). Thus it seems likely that the observed effect of activation of PKC on activity of OAT3 noted in the present study reflected a direct, rather than indirect, effect on activity of OAT3. One possible mechanism of the inhibitory effect of PKC might be a reduction in the number of transporters on the basolateral cell surface following membrane internalization. The PKC-mediated downregulation of OAT1 activity in LLC-PK1 cells that stably express the mouse ortholog of this process was found to reflect a decrease in the 

\[ J_{\text{max}} \]

for uptake, rather than the \( K_t \) (44). Interestingly, the decrease in mOAT1 activity was not correlated with a change in phosphorylation of the protein. These observations led to the suggestion that the decrease in OAT1 activity reflected a PKC-mediated internalization of cell surface transporters. Recent studies of the regulation by PKC of human OAT1 expressed in X. laevis oocytes confirmed these observations (42).

The decrease in PAH uptake into hOAT1-expressing oocytes that followed activation of PKC was correlated with the internalization of immunoreactive OAT1 protein. Furthermore, elimination of putative PKC phosphorylation sites by site-directed mutagenesis did not influence the regulatory response to PKC activation. The several parallels in the molecular characteristics of OAT1 and OAT3 suggest that mechanisms of PKC-based regulation of OAT1 may also influence OAT3 activity, but confirmation of this hypothesis will require further work.

Pertinent to this last suggestion is the observation that, as with OAT1 (27–29), EGF activation of MAPK stimulated basolateral OAT3 activity, as shown by the significant increase in ES uptake into S2 segments of renal proximal tubules (Fig. 3B). The stimulatory effect of EGF on PAH uptake (i.e., OAT1) requires activation of MAPK. Therefore, we investigated whether EGF stimulated ES uptake via MAPK activation. We examined the effect of U-0126, a specific MEK inhibitor, on basolateral ES uptake in S2 segments of renal proximal tubule. Inhibition of MAPK completely blocked the stimulatory effect of EGF (Fig. 3B). These results indicate that EGF increased ES uptake via MAPK activation, consistent with similar effects on PAH uptake. Unlike PKC, which had no effect on OAT3 activity under basal conditions, MAPK appears to play a role in OAT3 regulation under basal conditions, as evidenced by the fact that exposure to U-0126 alone inhibited ES uptake (Fig. 3B). EGF stimulates OAT1 and, subsequently, the basolateral uptake of PAH via MAPK activation and then through a series of steps involving activation of phospholipase A₂, increased release of arachidonic acid, and metabolism of arachidonic acid to PGE₂ via COX1. PGE₂ then stimulates adenylate cyclase resulting, finally, in activation of PKA (27, 29, 30).

Our study indicates that EGF stimulates OAT3 via the MAPK pathway; therefore, it seems likely that the additional steps in this pathway are the same as those for EGF stimulation of OAT1. We demonstrated the effect of PGE₂ and PKA on the initial basolateral uptake of ES. Exposure to PGE₂ stimulated ES uptake, and this stimulation was completely blocked by PKA inhibitor H-89 (Fig. 4A). These data indicate that PGE₂ stimulation of ES uptake requires PKA activation. As evidenced by the fact that PGE₂ stimulated ES uptake via PKA activation, direct activation of PKA should lead to the stimulation of ES uptake. Therefore, we examined the effect of db-cAMP, a PKA activator, on ES uptake. db-cAMP did, in fact, enhance ES uptake and this stimulation was abolished by PKA inhibitor H-89 (Fig. 4B). This effect mimicked the effect of PGE₂ on ES uptake. These data support the idea that stimulation of ES requires PKA activation. In addition, we also investigated the effect of COX on ES uptake. Exposure to SC-560 (COX1 inhibitor) inhibited ES uptake. In contrast, IHE (COX2 inhibitor) had no effect on ES uptake (Fig. 5). These results indicate that COX1 but not COX2 appears to play a role in OAT3-mediated organic anion transport under the basal condition.

PGE₂ is a substrate for the organic anion transport system involving OAT3 (13); therefore, PGE₂ could trans-stimulate basolateral uptake of ES when present inside the cells. However, it was found that PKA inhibition completely eliminated the PGE₂ action on ES uptake, whereas PKA inhibition alone had no effect on ES uptake (Fig. 4A). If the stimulation produced by PGE₂ was due to trans-stimulation, inhibition of PKA should not have had any effect on its action. These data exclude the possibility of trans-stimulation as a mechanism of PGE₂ action on the basolateral uptake of ES in the renal proximal tubules.

Our study also showed that the stimulatory effect of EGF on ES uptake was blocked by inhibition of COX1 and PKA (Fig. 6). Taken together, our data in this study indicate that the stimulation of EGF on ES uptake requires the activation of

![Fig. 8. Proposed model for the regulatory mechanism of organic anion transport mediated by organic anion transporter 3 (OAT3). PE stimulates PKC leading to downregulation of OAT3 by an unknown mechanism(s). As shown in previous studies and the present study, EGF stimulates OAT3 activity via MAPK, MEK, and ERK1/2, which activates PLA₂ leading to the release of arachidonic acid (AA). AA is then metabolized to prostaglandins (PGE₂), which activates PKA via adenylate cyclase and finally stimulates OAT3 by an unknown mechanism(s).](http://ajprenal.physiology.org/DownloadedFrom/10.220.33.1/June10,2017)
MAPK followed by PGE₂ production, leading subsequently to PKA activation.

One possible mechanism for PKA stimulation of OAT3 activity could involve transfer of additional OAT3 transporters from an intracellular compartment to the basolateral cell membrane. It is possible that PKA may enhance the translocating of these transporters to the cell membrane by phosphorylation of unknown regulatory proteins that are responsible for the enhanced trafficking of the transporter. Finally, it also seems unlikely that the stimulatory effect of EGF involves an increase in NaDC activity in light of studies showing that dicarboxylate uptake into isolated rabbit tubules did not change following inhibition of the MAPK pathway (9).

The stimulatory effect of EGF on ES uptake could not be elicited in tubules in which ES uptake had just been inhibited by phenylephrine (Fig. 7A). In addition, the inhibitory effect of phenylephrine could not be produced when EGF had just stimulated the OAT3 activity (Fig. 7B). We did not determine the time required for recovery before exposure to EGF or phenylephrine would have an effect. However, the data certainly show that changes in opposite directions cannot be produced very rapidly by these physiological stimuli. One possible theory to explain why the OAT3 activity could not be rapidly adjusted between up- and downregulation by physiological stimuli is that the activity of OAT3 is regulated by interaction between two opposing pathways (stimulatory and inhibitory pathways). When EGF activates MAPK and subsequently activates PKA, PKA may not only stimulate OAT3 activity but also inhibit the processes that are normally set into motion by activation of PKC, thereby accounting for the failure to see the effect of phenylephrine after the tubules were preincubated with EGF (Fig. 7B). A previous study using normal rat kidney cells (NRK cells) supports this idea, as evidenced by the finding that the phosphorylation activity of PKC was inhibited after NRK cells were preincubated with forskolin, a PKA activator (8). Moreover, in endocrine cells, PKC not only stimulates cell proliferation but also inhibits the PKA activity that is responsible for cell differentiation (25).

This evidence may explain why EGF could not produce its effect after PKC is activated. Another possibility is that phenylephrine or EGF may suppress the membrane expression or activity of EGF receptor and α₁-adrenergic receptor, respectively, but this possibility has yet to be examined.

From the information in the literature on the regulation of organic anion transport and the results of the current study, we propose a model of the regulatory mechanism of organic anion transport mediated by OAT3 (Fig. 8). In the model, physiological activation of PKC, e.g., via phenylephrine interaction to the α₁-adrenergic receptor, results in downregulation of OAT3 activity by an unknown mechanism. In contrast, EGF stimulates MEK, ERK1/2, and PLA₂, leading to increased production of arachidonic acid, which is then metabolized to PGE₂ via COX 1 (27, 29, 30). PGE₂ activates adenylyl cyclase, which produces cAMP leading to activation of PKA and a subsequent increase in OAT3 activity.

In conclusion, OAT3-mediated organic anion transport was downregulated by both direct and indirect (physiological) activation of PKC. In contrast, this transporter was upregulated by EGF through activation of the MAPK pathway and subsequently through PKA activation. Although the precise cellular processes of OAT3 regulation that follow activation of PKC and PKA remain uncertain, these data provide the first evidence that OAT3 is differentially regulated by protein kinases. Regulation of transporter activity, including OAT1 and OAT3, may influence the total profile of renal organic anion transport in intact renal proximal tubules and thereby influence renal clearance of both toxic and therapeutic xenobiotics.

**REFERENCES**


