Modulation of the basolateral and apical step of transepithelial organic anion secretion in proximal tubular OK cells: Acute Effects of EGF and MAPK.

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Running title: Acute effect of EGF and MAPK on PAH transport in OK cells.
Summary:
The organic anion transport system in the proximal tubule of the kidney is of major importance for the excretion of a variety of endogenous and potentially toxic exogenous substances. Furthermore, the clearance of model substrates (e.g. para-aminohippurate; PAH) of this system is used for the determination of renal blood flow. We investigated regulation of organic anion secretion in a way that allowed us to examine simultaneously regulation of overall transepithelial secretion and to estimate the separate contributions of regulation of the basolateral and apical transport steps to this overall regulation. The data were verified by measurement of initial basolateral uptake rate and initial apical efflux rate. OK cells were used as a suitable model system for proximal tubule cells and \([^{14}\text{C}]\text{PAH}\) was utilized as organic anion.

Stimulation of protein kinase C (PKC) inhibited transepithelial secretion due to inhibition of both, apical efflux and basolateral uptake. Inhibition of the mitogen activated protein kinase (MAPK) kinase MEK reduced transepithelial secretion via inhibition of basolateral uptake and apical efflux. Epidermal growth factor (EGF) enhanced transepithelial secretion via stimulation of basolateral uptake, but did not affect apical efflux. EGF induced stimulation of basolateral uptake was abolished by inhibition of MEK. EGF led to phosphorylation of ERK1/2, which was also abolished by inhibition of MEK. Thus, EGF stimulated basolateral uptake of organic anions via MAPKs.

Transepithelial organic anion secretion can be regulated at two sites, at least: basolateral uptake and apical efflux. Both steps are under control of PKC and MAPK. The pathophysiologically relevant growth factor EGF enhances transepithelial secretion via stimulation of basolateral uptake. EGF stimulates basolateral uptake via MEK and ERK1/2. Thus, renal organic anion extraction may be modulated, especially under pathophysiological conditions.
Introduction:

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). This system consists of a basolaterally located organic anion exchanger and a less well characterized transport step at the apical membrane (2). The basolateral organic anion exchanger is a tertiary active transport system, dependent on an inward directed Na⁺-gradient to drive the uptake of α-ketoglutarate (α-KG), which is then exchanged for organic anions (1-4). The basolateral exchanger for organic anions and dicarboxylates was cloned by three independent groups (5-7) in 1997, and called OAT1 (rat), ROAT1 (rat) or fROAT1 (winter flounder). Only recently, the homologous protein was cloned from human kidney and called hOAT1 or hPAHT (8;9). A number of mechanisms have been described for the apical efflux of organic anions (10), which differ with species and experimental setup used. For example, a PAH/dicarboxylate exchanger, a PAH/anion exchanger and a membrane potential dependent mechanism have been described (11). Furthermore there is evidence for the involvement of oatp, OAT-K1, and OAT-K2 (10;12-16). Thus, secretion of organic anions is mediated by a well described, tertiary active transport step at the basolateral membrane and a not yet settled, apical transport step.

Very little is yet known about the modulation of the secretory organic anion transport system. Nagai and coworkers showed an inhibition of basolateral uptake and transepithelial secretion of organic anions in OK cells by parathyroid hormone via a staurosporine sensitive mechanism (17). Inhibition of basolateral organic anion transport during stimulation of protein kinase C (PKC) was reported in isolated tubules of kilifish (18). The basolateral exchanger for organic anions and dicarboxylates in isolated proximal tubules of rabbit kidney was shown to be sensitive to inhibition of Ca²⁺/calmodulin dependent protein kinase II, tyrosine kinase, phosphatidylinositol-3-kinase and mitogen activated protein kinases (MAPKs) (19). Furthermore, inhibition of OAT1 by bradykinin and phenylephrine via PKC in isolated rabbit
proximal tubules has been described (20). In most of the studies, only regulatory events at the basolateral membrane were investigated. The study of Nagai and coworkers investigated transepithelial transport, but they used the rather unspecific kinase inhibitor staurosporine. Inhibition of net secretory transport (21) of organic anions by bradykinin and phenylephrine has been reported for isolated perfused rabbit proximal tubules. Recently, Henderson and Coworkers showed that PKC inhibits murine OAT without direct phosphorylation of the transport protein itself (22). Taken together, these studies give no detailed information concerning the contribution of the single transport steps to the regulation of transcellular secretion. In order to address this problem, we investigated transcellular secretion in combination with measurements of initial basolateral uptake rate and initial apical efflux rate.

In the present study, we determined the effect of epidermal growth factor (EGF) and mitogen activated protein kinases (MAPKs) on PAH transport in OK cells. EGF and its receptor are known to be expressed in proximal tubular cells (23). EGF has been suggested as a mediator of normal tubulogenesis and tubular regeneration after injury. A reduction of renal EGF expression and/or urinary excretion has been reported during acute and chronic tubular injury (24). Additionally, EGF led to an increase in PAH excretion in rats (25) indicating an influence of EGF on proximal tubular organic anion transport. MAPKs are known to be involved in renal stress response and represent an important downstream signal of the EGF pathway (26).

Our data show that activation of protein kinase C (PKC) inhibits both the basolateral and the apical step of PAH secretion. Moreover, we show that MAPK activity is required for a proper activity of basolateral uptake step and the apical exit of PAH. EGF stimulates transepithelial secretion via stimulation of the basolateral uptake, but does not affect the apical transport step. EGF leads to successive activation of the MAPKs, ERK kinase (MEK) and extracellular regulated kinase 1/2 (ERK1/2).
Experimental procedures:

Cell culture. OK cells were obtained from Dr. Biber, Dept. of Physiology, University of Zurich. Cells were maintained in culture at 37°C in a humidified 5% CO₂, 95% air atmosphere. The growth medium was Minimal Essential Medium (MEM), pH 7.4, supplemented with Earl’s salts, non-essential amino acids, 10% (v/v) fetal calf serum (Biochrom KG, 12213 Berlin, FRG) and 26 mmol/l NaHCO₃. Cells were cultured on permeable supports (3 µm pore diameter, Falcon, Becton Dickinson Labware, Franklin Lakes, USA) for transport measurements. The effective growth area on one permeable support was 4.3 cm²/filter. All studies were performed between passage 60 and 100. The seeding density was 0.4⋅10⁶ cm⁻². The medium was changed every third day and the monolayers were used for experiments at day 10 after seeding. The effect of 10⁻⁷ M PMA or 5⋅10⁻⁵ M DOG on PAH secretion was determined in non quiescent and quiescent cells, as shown in figure 1. OK cells were made quiescent by cultivating them in serum depleted cell culture medium for 24 hours before the experiments. In both cases, secretion (Fig. 1A) was reduced to a greater extent than cellular content (Fig. 1B), indicating that apical transport may be affected by PKC. Moreover, the effects were more pronounced in quiescent (means serum depleted for 24 hours) cells (Fig. 1; right panel) as compared to non quiescent cells (Fig. 1; left panel). In non quiescent cells EGF showed no effect on cellular PAH content or transepithelial PAH secretion (data not shown). As fetal calf serum contains a variety of chemokines, it is evident that investigation of the effect of an isolated chemokine is only possible in the absence of serum. As shown for PKC and mentioned for EGF, the effects on organic anion transport in OK cells were more pronounced or only apparent when cells were made quiescent. Thus, quiescent OK cells were used for all subsequent experiments.

Transport measurements. The volumes of the apical and basolateral compartment were
1.3 ml and 2.5 ml in order to avoid hydrostatic pressure differences. Before each experiment, the cells were washed three times with phosphate buffered Ringer (138 mmol/l NaCl, 1 mmol/l NaH₂PO₄, 4 mmol/l Na₂HPO₄, 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. The concentrations of the radiolabeled substrates applied to the basolateral bath were: 1.5·10⁻⁶ mol/l or 15·10⁻⁶ mol/l [¹⁴C]PAH and, 55·10⁻⁹ mol/l [³H]mannitol or 55·10⁻¹⁰ mol/l [³H]mannitol. [³H]mannitol was used to correct secretion for paracellular fluxes and to determine extracellular water space. At the end of the experiment, the apical and basolateral solutions were collected. Subsequently, the filters were washed twice with ice cold PBS and cut from the supports. Radioactivity of the solutions and the cells was measured using a liquid scintillation counter (Packard Instruments, Frankfurt, Germany). Counts of cells on filters were corrected for non specific binding on filters by subtraction.

In order to investigate PAH efflux, the cells were incubated with 15·10⁻⁶ mol/l [¹⁴C]PAH for 60 min. After washing, the efflux was determined during the first minute. Apical and basolateral solutions and the cellular compartment were collected separately. Radioactivity in the solutions and the cells was measured by liquid scintillation counting. The total amount of counted [¹⁴C]PAH was set as amount of [¹⁴C]PAH in the cells at time 0 of the efflux experiments.

**Western blot analysis.** OK cells were rinsed three times with PBS followed by a 10 min incubation with EGF and/or PD98059. Subsequently cells were washed with ice cold PBS three times and lysed in ice cold Triton X-100 lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 40 mM β-glycerophosphate, 200 µM sodium orthovanadate, 0.1 mM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, 1 µM pepstatin A, 1 % Triton X-100) for 25 min at 4°C. Insoluble material was removed by centrifugation at
12,000g for 15 min at 4°C. The protein content was determined using a microbicinchoninic acid assay (Pierce, Rockford, IL, USA) with BSA as a standard. Cell lysates were matched for protein, separated by 12% SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride microporous membrane. Subsequently, membranes were blotted with rabbit anti-pERK1/2 (p42/p44) antibody (New England Biolabs, USA). The primary antibody was detected using alkaline phosphatase conjugated goat anti rabbit IgG visualized by ECL Western blotting reagents and Hyperfilm ECL (Amersham Life Sciences International, Buckinghamshire, GB). According to the manufacturers handbook Hyperfilm ECL exhibits a linear response to the light produced from enhanced chemiluminescence. Additionally, linearity was verified for our experimental conditions by a dilution series with increasing amounts of total cell protein. Western blotting was performed with protein from five independent extractions from five independent cell culture passages. Blots were analyzed using SigmaGel 2000 Software (Jandel Scientific, USA).

**Processing of experimental data.**

10 min transport: (Fig. 2A) According to our measurements secretory transport of organic anions in the OK clone used, is detectable after 3-4 min and is then linear for at least one hour (data not shown). Thus, secretory transport after 10 min represents the linear phase of secretion. Therefore, the amount of radio labeled PAH in the apical compartment after 10 min was used to measure *PAH secretion*. The quantity of PAH in the cells is denominated *cellular content*. Summing up the values for PAH secretion and for cellular PAH content gives *basolateral uptake of PAH*, i.e. the total amount of PAH transported across the basolateral membrane during 10 min. Additionally we calculated the ratio of secretion to cellular content of PAH (*secretion-to-content ratio*). Introducing these parameters enabled us to gain information regarding the contribution of basolateral and/or the apical transport step(s) to the overall secretion of PAH.
**Basolateral uptake during the first minute:** Under our experimental conditions uptake of PAH is linear at least during the first 1.5 min as shown in figure 2B (right panel), and no net secretion of PAH occurs (data not shown). Thus, the cellular PAH content after one minute represents the initial basolateral uptake rate. Thus, the predictions concerning the basolateral part of PAH transport derived from the 10 min transport experiments were verified by the 1 min data.

**Apical efflux of PAH during the first minute:** After preloading the cells as described above, we determined the apical efflux of $[^{14}\text{C}]$PAH during the 1st min as percentage of cellular PAH content at time 0 min. As the volume of the apical compartment is about 1000 times that of the cells, there is a large outward-gradient during the entire experimental period. According to figure 2C (right panel) efflux into the apical compartment is linear at least during 1.5 min and thus, the gradient is not collapsed in the time frame investigated. Thus, the apical efflux during 1 min represents the initial apical efflux rate.

**Data analysis.** Data are presented as mean ± SEM. $n$ is given in the text or in the figures. $n$ represents the number of culture plates or filters used. Statistical significance was determined by unpaired Student’s $t$-test or ANOVA as appropriate. Results were considered statistically different at $p < 0.05$. Significant differences are indicated by asterisks.

**Materials.** $[^{14}\text{C}]$PAH (55 mCi/mmol) and $[^{3}\text{H}]$mannitol (15 mCi/mmol) were purchased from American Radiolabeled Chemicals Inc., St. Louis, USA. PD98059 was from Alexis Corp., Läufelfingen, Switzerland. U0126 from Promega Corp., Madison, Wisconsin, USA. Antibody against the phosphorylated form of ERK1/2 (pERK1/2) was from New England Biolabs Inc., USA. If not stated otherwise, all other chemicals were from Sigma. EGF from Sigma was used as human, recombinant substance.
Results:

Validation of the cell clone used.

We wanted to investigate regulation of organic anion secretion in a way that would allow us to examine regulation of overall transepithelial secretion and the separate contributions of the basolateral and apical transport steps to this overall regulation, simultaneously. For this purpose, we chose the proximal tubule derived OK cell line cultured on permeable supports, a well characterized model system to investigate organic anion secretion (27-29). Probenecid (10 mM), the classical inhibitor of organic anion transport, and a 1000-fold excess of unlabeled PAH, both inhibited uptake and secretion of $[^{14}C]$PAH by more than 95% (data not shown). Thus, our particular cell clone transports organic anions in accordance to the published data mentioned in the introduction.

PKC affects basolateral and apical transport.

Stimulation of PKC by $5 \times 10^{-5}$ M DOG reduced secretion of PAH (Fig. 3A), while the cellular content (Fig. 3B) was not significantly different from control. Adding up secretion and cellular content of PAH, gives the amount of PAH transported across the basolateral membrane (Fig. 3C). Transport across the basolateral membrane was reduced by DOG, in agreement with other studies (18-20). Furthermore, the decreased secretion-to-content ratio (Fig. 3D) indicates that the apical transport step is also inhibited by PKC. However, there is another possible explanation. The apical transport is carrier mediated and thus has a hyperbolic relationship to substrate concentration. A decrease in intracellular concentration could reduce this ratio, without inhibition of the apical step itself. Thus, experiments on initial transport rates will have to decide what kind of explanation applies to these data.

Simultaneous inhibition of apical and basolateral transport was confirmed by measurement of initial apical efflux rate and initial basolateral uptake rate. As shown in figure 4A, activation of PKC reduced the initial apical efflux rate, thereby leading to a decreased
relative amount of PAH in the apical bath as compared to control. In addition, figure 4B shows that stimulation of PKC reduced the initial basolateral uptake rate of PAH. By contrast, inhibition of PKC with $10^{-7}$ M BIM increased basolateral uptake of PAH (data not shown). Thus, the basolateral organic anion transport in OK cells is regulated by PKC in agreement with data published previously (19;20).

**EGF stimulates transepithelial secretion.**

EGF increased cellular content (Fig. 5B) and transepithelial secretion (Fig. 5A) of PAH. However, EGF did not affect secretion-to-content ratio for PAH (Fig. 5D) at all, indicating that EGF stimulates basolateral PAH transport in OK cells, but does not affect the apical transport step. Initial efflux experiments showed no change in PAH efflux across the apical membrane in EGF treated cells (Fig. 6A). These data confirm the predictions derived from the 10 minutes transport experiments with EGF (Fig. 5). As shown in figure 6B, EGF stimulates the initial basolateral uptake rate of PAH in agreement with the prediction derived from the 10 minute experiments.

**MAPKs affect basolateral and apical transport.**

It is known that EGF uses the MAPK pathway also in OK cells (30). Therefore, we investigated the effect of substances which inhibit MAPK activation. Transepithelial secretion (Fig. 7A) and basolateral uptake (Fig. 7C) of PAH were reduced by PD98059 (inhibitor of MEK), while cellular PAH content (Fig. 7B) was increased 3-fold as compared to control. The secretion-to-content ratio (Fig. 7D) was dramatically decreased in the presence of PD98059. These data indicate a strong inhibitory effect of PD98059 on the apical transport step of PAH secretion. Furthermore, it is possible that the increased cellular content of PAH results solely from the strong inhibition of the apical exit step of PAH, while the basolateral transport step remains unchanged. This is also true for the observed reduction of basolateral uptake in figure 7. In fact, even an increase in basolateral transport activity would be in
agreement with these data. Thus, in this particular configuration, it is impossible to draw a final conclusion concerning the basolateral transport step. As figure 8A clearly shows, the prediction concerning the apical step is confirmed by the data obtained from efflux experiments. PD98059 inhibited initial apical efflux rate significantly. Initial basolateral uptake rate of PAH (Fig. 8B) is also reduced by PD98059. These data show that basolateral uptake of PAH in OK cells is inhibited by inhibition of MEK. Increasing the concentration of PD98059 to 50 µM (Fig. 8B), which represents the maximal effective concentration, leads to an increased inhibition of initial basolateral uptake rate of PAH, indicating a dose dependent action of PD98059 on the basolateral transport.

In summary, transepithelial secretion of PAH in OK cells is under the stimulatory control of the mitogen activated protein kinase kinase (MAPKK) MEK. Increased secretion when MEK is active results from a stimulation of both, basolateral and apical transport (Fig. 9A). Similar results were obtained with another, structurally different, MEK inhibitor U0126 (data not shown).

**EGF acts on basolateral uptake via successive activation of MEK and ERK.**

As already mentioned, it is known that the MAPK pathway is stimulated by EGF in OK cells (30). Thus, we investigated whether the stimulatory effect of EGF on basolateral PAH transport is mediated by MAPK. As shown in figure 10, inhibition of the MAPK MEK by PD98059 or U0126 completely abolishes the stimulatory effect of EGF on initial basolateral PAH uptake. These data indicate that the stimulatory effect of EGF on basolateral uptake is mediated by the activation of MEK.

As shown in figure 11, EGF leads to increased phosphorylation of ERK1/2 in OK cells within 10 min. PD98059 alone, slightly but significantly, decreased the amount of pERK1/2 as compared to controls. These data are in good agreement with the effects on PAH transport. However, EGF increased phosphorylation of ERK1/2 is reduced by inhibition of MEK with
PD98059, whereas EGF stimulated uptake of PAH is totally abolished by PD98059. We explain this apparent discrepancy with the existence of intermediate signaling steps between ERK and basolateral organic anion transport, as discussed in detail later on.

Finally we tested whether preincubation for 10 min with EGF or PD98059 affects glutarate uptake in OK cells. Glutarate is a non-metabolizable analogue of the dicarboxylate α-ketoglutarate. EGF and PD98059 did not affect basolateral uptake of glutarate (control: 1.2 ± 0.1; n = 3; 10 ng/ml EGF: 1.4 ± 0.1; n = 3; 5 μM PD98059: 1.3 ± 0.3; n = 6; in pmol · cm⁻² · 10 min⁻¹). Furthermore, EGF did not affect apical glutarate uptake (control: 0.9 ± 0.1; n = 3; 10 ng/ml EGF: 1.1 ± 0.1; n = 3; in pmol · cm⁻² · 10 min⁻¹). Thus, availability of intracellular counterions for PAH uptake is not altered by EGF or PD98059. Previously published data showed no effect of PD98059 on basolateral glutarate uptake (19).

Taken together these data present strong evidence that EGF stimulates exchange of dicarboxylates and organic anions via stimulation of the ERK1/2 (see Fig. 9C).
Discussion:

The experimental setup

The purpose of the present study was (a) to gain more information concerning the overall regulation of the proximal tubule organic anion transport and (b) to investigate the contribution of the basolateral and apical transport steps to this regulation. As shown for EGF, we were able to determine their effects on the basolateral and apical transport steps simultaneously with their effects on transepithelial secretion. However, inhibition of MEK by PD98059 led to a configuration (reduced secretion, increased content, reduced uptake and reduced secretion-to-content ratio), where it was not possible to determine the contribution of the basolateral transport step simultaneously with secretion, indicating the limitations of the method. This is also true for the DOG effect presented. However, with the help of initial apical efflux experiments and determination of the initial basolateral transport rate it is possible to investigate apical and basolateral transport steps separately. Nevertheless, we show that it is possible to estimate the site of action of a chemokine on the secretory transport simultaneously with transepithelial transport, although transport occurs across two membranes (and the cytosol). Thus, the particular processing of the 10 min transport data presented here, is suitable to obtain a first estimate regarding the site of action of a given modulator of secretory transport.

Stimulation of PKC inhibits basolateral uptake rate and inhibition of MEK decreases basolateral uptake rate of PAH (Fig. 9A). These data are in agreement with the action of PKC (18-20) and MAPK (19) in isolated proximal tubules, and again show the suitability of the OK cell system used. Additionally, we show for the first time, that PKC inhibits and MEK stimulates the apical step of organic anion secretory transport in a proximal tubule derived cell line (Fig. 9A). Since the specific PKC inhibitors calphostin C and bisindolylmaleimide stimulate basolateral uptake of PAH, the observed inhibitory action of DOG is due to an interaction
with regulatory cascades and not due to non specific or even toxic action. Furthermore, the calphostin C induced stimulation of PAH uptake was prevented by both, PMA and DOG (data not shown). Systematic changes in mannitol flux were not observed with any of the substances used. Thus, no changes in epithelial tightness were induced and the measured changes in PAH transport are not due to altered paracellular flux. The unchanged epithelial tightness, together with the short exposure time (10 min) and the moderate concentrations used, make it highly unlikely, that any observed effect is due to unspecific toxic actions.

EGF stimulates organic anion secretion

As the above mentioned maneuvers act directly on intracellular signaling pathways, we investigated the effect of more physiologically or pathophysiologically relevant stimuli, namely EGF. We show for the first time that EGF increases the secretion of PAH in OK cells by a stimulation of the basolateral uptake step. EGF does not affect the apical transport step (see Fig. 9B).

The effect of EGF on basolateral uptake is mediated by MEK, because two specific, structurally distinct inhibitors of MEK (PD98059 and U0126) completely abolished EGF induced stimulation of initial basolateral PAH uptake. As shown in figure 8B 5 µM PD98059 led to a slight decrease of initial PAH uptake. This is in parallel with the decrease of ERK1/2 phosphorylation induced by 5 µM PD98059. However, the same concentration of PD98059 completely prevented the EGF induced stimulation of initial basolateral PAH uptake. This was also the case for the structurally distinct MEK inhibitor U0126. The fact that inhibition of MEK decreased uptake only slightly, but abolished EGF stimulated uptake completely, is strong evidence for the fact that EGF acts on basolateral transport via stimulation of MEK. Moreover, as already mentioned above, no secretion of organic anions in OK cells was detected during the 1st min. Thus, alterations of the apical transport step should not influence
initial basolateral uptake rate.

Inhibition of MEK prevented EGF induced stimulation of basolateral PAH uptake completely, however it reduced EGF induced ERK1/2 activation only partially. We consider this difference of action as evidence that ERK1/2 does not act directly on basolateral organic anion uptake, but via one or more intermediate signaling steps. In fact, preliminary data from our laboratory indicate that phospholipase A₂ (PLA₂) is involved in downstream signaling following ERK1/2 activation (31). These intermediate steps (as e.g. PLA₂) possibly modulate the signal downstream of ERK1/2 in a way, that a partial inhibition of ERK1/2 activation leads to a complete inhibition of PAH uptake stimulation. This could be explained by a certain ERK1/2 activation threshold which has to be exceeded in order to stimulate the downstream signals. Thus, we hypothesize that EGF stimulates basolateral organic anion uptake via the successive activation of MEK, ERK and additional downstream signaling steps (Fig. 9C). However, additional experiments will be necessary to clarify the downstream signal transduction and amplification network with respect to organic anion uptake in more detail.

As seen in the control lane of figure 11B, OK cells show a certain intrinsic ERK1/2 activation although they were serum depleted for 24 hours. Our observations indicate that OK cells with higher intrinsic ERK1/2 activation show higher control levels of basolateral PAH uptake as compared to those with a lower ERK1/2 activation (data not shown). These data again support the hypothesis that ERK1/2 activity regulates organic anion transport in OK cells.

Since the effects of EGF were studied after 10 min exposure, it can be excluded that the regulatory events observed resulted from changes in protein synthesis. However, up to now we can only speculate on the molecular events involved in the observed regulatory phenomena. Possible mechanisms include an increased insertion of preformed transport protein by fusion of vesicles with the basolateral membrane in response to EGF. Of course
EGF could also lead to stimulation of transport proteins in the cell membrane, by e.g. phosphorylation. Future experiments will investigate the effects of EGF on the affinity ($K_m$) and the maximum transport rate ($V_{max}$) of basolateral organic anion uptake in more detail.

By contrast to the events at the basolateral membrane, stimulation of MEK by EGF did not lead to a stimulation of the apical transport step, although PD98059 data indicate a regulatory role of MEK on the apical transport step, too. This apparent discrepancy could be explained by the fact that basal MEK activity already induces maximal stimulation of apical transport. Consequently, reduction of basal ERK1/2 activity by PD98059 (Fig. 11) leads to reduced apical and basolateral transport, while activation of MEK by EGF affects only the basolateral step. Another possible explanation is the involvement of additional signal pathways downstream of EGF, which might antagonize the MEK effect on apical transport, but not on basolateral transport. Finally there is the possibility of basolateral signaling microdomains, which confine the effect of EGF. Future experiments will have to explain this apparent discrepancy.

PAH clearance is routinely used for the determination of renal blood flow, based on the assumption of a high and constant renal PAH extraction. Since PAH extraction would vary according to the regulatory state of its transport system, it is conceivable that the determination of renal blood flow from PAH clearance may lead to under- or overestimation. Corrigan, for example, described a decreased renal PAH extraction after postischemic acute renal failure in humans, leading to severe underestimation of renal blood flow (32). After renal injury, a rapid fall of EGF mRNA in the kidney was measured (26). We could show that the basolateral step of PAH secretory transport is under stimulatory control of EGF, which is in agreement with excretion data from rat (25) and could explain, at least in part, the above mentioned data from Corrigan.

In summary, we were able to determine the site of regulation of the secretory transport
of organic anions. The results obtained were confirmed by measurements of initial basolateral uptake rate and initial apical efflux rate. Thus, we could show that activation of PKC not only inhibits the basolateral, but also the apical step of organic anion secretion. We also showed that MEK stimulates not only the basolateral, but also the apical transport step of organic anion secretion. Additionally, we presented data indicating a stimulation of the organic anion secretion by EGF. This stimulation resulted from an increase of the basolateral uptake rate only. Furthermore, we showed that EGF stimulates the basolateral uptake of organic anions via successive activation of MEK and ERK. In conclusion, the excretion of organic anions in proximal tubular cells seems to be a regulated and therefore variable process. This may be particularly important under pathophysiological conditions.
Reference list


Index words:
apical transport,
basolateral transport,
EGF,
ERK1/2,
MAPK,
MEK,
OK cells,
organic anion transport,
PKC,
proximal tubule,
regulation.

Abbreviations:
DOG - 1,2-dioctanoyl-sn-glycerol,
EGF - epidermal growth factor,
ERK1/2 - extracellular regulated kinase (isoforms) 1 and 2,
MAPK - mitogen activated protein kinase,
MAPKK - mitogen activated protein kinases kinase,
MEK - mitogen activated/extracellular-signal regulated kinase kinase,
OK cells - opossum kidney cells,
PAH - para-aminohippurate,
PKC - protein kinase C,
PLA2 - phospholipase A2
PMA - phorbol 12-myristate 13-acetate.
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**Figure legends:**

**Fig 1:** Effect of PKC stimulation in non quiescent (left panel) and quiescent (right panel) OK cells on secretion (A) and cellular content (B) after 10 minutes.  
PMA (10\(^{-7}\) M) and DOG (5\( \cdot \)10\(^{-5}\) M) were added to the transport buffer and were present throughout the ten minutes of the transport process. 1.5\( \cdot \)10\(^{-6}\) M [\(^{14}\)C]PAH was used as a substrate. Non quiescent cells were cultivated in medium containing 10 % (v/v) fetal calf serum until the cell epithelia were experimentally used. For quiescent cells, experimental medium was replaced with medium without fetal calf serum 24 hours prior to the experiments. \( n \) for every bar is shown in brackets. * = \( p < 0.05 \) versus control.

**Fig 2:** Model showing the measured and calculated parameters describing the secretory transport of [\(^{14}\)C]PAH in OK cells.  
(A) The cellular content of [\(^{14}\)C]PAH (1) and the transepithelial secretion of [\(^{14}\)C]PAH (2) are directly measured by liquid scintillation counting of the epithelium or the apical fluid respectively. The parameter "basolateral uptake" (3) is the sum of the cellular content and the secretion of [\(^{14}\)C]PAH after 10 min, and represents the total amount of [\(^{14}\)C]PAH that crossed the basolateral membrane during 10 min. The secretion-to-content ratio (4) is obtained by dividing transepithelial secretion by cellular content. (B) According to data showing linearity for at least 1.5 min (right panel), initial basolateral uptake rate of [\(^{14}\)C]PAH was measured after 1 min. (C) According to data showing linearity for at least 1.5 min (right panel), initial apical efflux rate was determined after 1 min.

**Fig 3:** Effect of PKC stimulation by DOG (5\( \cdot \)10\(^{-5}\) M) on 10 min transport of 1.5\( \cdot \)10\(^{-6}\) M
[14C]PAH in quiescent OK cells.

The following parameters describing transepithelial transport are shown: transepithelial secretion (A), cellular content (B), basolateral uptake (C) and the secretion-to-content ratio (D). The parameters were determined and generated as described in the materials and methods section. The cells were cultivated in medium without fetal calf serum starting 24 hours before the experiment. DOG was present in the transport buffer throughout the 10 min the transport was measured. n = 5 for every bar. * = p<0.05 versus control.

Fig 4: Effect of PKC on initial apical efflux rate of [14C]PAH (A) and initial basolateral uptake rate of [14C]PAH (B) in quiescent OK cells.

A: Apical efflux of [14C]PAH during 1 min was determined as described in the materials and methods section. DOG (5·10^-5 M) was present during the last 10 min before efflux. Apical efflux is presented in % of PAH content of the OK cells at efflux time 0. n = 3 for every bar.

B: Initial basolateral uptake of 15·10^-6 M [14C]PAH after 1 min was determined as described. The cells were incubated with DOG (5·10^-5 M) 10 minutes before uptake was determined. n for every bar is shown in brackets. * = p<0.05 versus control.

Fig 5: Effect of EGF (10 ng/ml) on 10 min transport of 1.5·10^-6 M [14C]PAH in quiescent OK cells.

The following parameters describing transepithelial transport are shown: transepithelial secretion (A), cellular content (B), basolateral uptake (C) and the secretion content ratio (D). The parameters were determined and generated as described in the materials and methods section. EGF was present in the transport buffer throughout the 10 min the transport was
measured. n = 9 for every bar. * = p<0.05 versus control.

**Fig 6:** Effect of EGF (10 ng/ml) on initial apical efflux rate of $[^{14}\text{C}]\text{PAH}$ (A) and initial basolateral uptake rate of $[^{14}\text{C}]\text{PAH}$ (B) in quiescent OK cells.

**A:** Apical efflux of $[^{14}\text{C}]\text{PAH}$ during 1 min was determined as described in the materials and methods section. EGF was present during the last 10 min before efflux. Apical efflux is presented in % of PAH content of the OK cells at efflux time 0. n = 3 for every bar.

**B:** Initial uptake of $15\cdot10^{-6}$ M during 1 min was determined as described. The cells were incubated with EGF 10 minutes before the uptake was determined. In between, the cell epithelia were washed once with PBS. n = 6 for every bar. * = p<0.05 versus control.

**Fig 7:** Effect of inhibition of MEK by PD98059 ($5\cdot10^{-6}$ M) on 10 min transport of $1.5\cdot10^{-6}$ M $[^{14}\text{C}]\text{PAH}$ in quiescent OK cells.

The following parameters describing transepithelial transport are shown: transepithelial secretion (A), cellular content (B), basolateral uptake (C) and the secretion content ratio (D). The parameters were determined and generated as described in the materials and methods section. PD98059 was present in the transport buffer throughout the 10 min the transport was measured. n = 5 for every bar. * = p<0.05 versus control.

**Fig 8:** Effect of PD98059 on initial apical efflux rate of $[^{14}\text{C}]\text{PAH}$ (A) and initial basolateral uptake rate of $[^{14}\text{C}]\text{PAH}$ (B) in quiescent OK cells.

**A:** Apical efflux of $[^{14}\text{C}]\text{PAH}$ during 1 min was determined as described in the materials and methods section. PD98059 ($5\cdot10^{-6}$ M) was present during the last 10 min before efflux.
Apical efflux is presented in % of PAH content of the OK cells at efflux time 0. n = 3 for every bar.

**B:** Initial uptake of 15\( \cdot 10^{-6} \) M during 1 min was determined as described. The cells were incubated with PD98059 (5\( \cdot 10^{-6} \) M or 5\( \cdot 10^{-5} \) M) for 10 minutes before the uptake was determined. Between this incubation and the uptake measurement, the cell epithelia were washed once with PBS. n for every bar is given in brackets. * = p<0.05 versus control.

**Fig 9:** Summary models: "+" indicating stimulatory, "-" indicating inhibitory effects.

**(A)** Effects of PKC and MEK and **(B)** EGF on the basolateral and the apical transport step of transepithelial secretion of PAH in the OK cell model of the proximal tubule.

**(C)** Hypothesized model of the stimulation of the basolateral exchange step of PAH and dicarboxylates by EGF. EGF stimulates the PAH/dicarboxylate exchanger via activation of the MAPKs MEK and ERK1/2. ERK1/2 action on the PAH/dicarboxylate exchanger is mediated via at least one intermediate signaling step.

**Fig 10:** Effect of inhibitors of MEK (PD98059 or U0126) on EGF stimulation of initial basolateral uptake rate of [14C]PAH.

The cells were incubated with EGF alone (10 ng/ml), and in presence of PD98059 (5\( \cdot 10^{-6} \)M) or U0126 (25\( \cdot 10^{-6} \) M) for 10 minutes before the uptake was determined. Reference values are untreated controls and uptake in presence of PD98059 or U0126 respectively. Thus the part of initial basolateral uptake rate of [14C]PAH that is stimulated by EGF is given by the difference in uptake after EGF and untreated controls, the difference in uptake after EGF in presence of PD98059 and PD98059 alone, and the difference in uptake after EGF in presence of U0126 and U0126 alone. Epithelia were washed once with PBS between incubation and
uptake measurement. n = 9 for every bar. * = p<0,05 versus 0.

**Fig 11:** Effect of EGF and/or PD98059 on phosphorylation of ERK1/2 in OK cells:

Western blot of OK cell protein with anti-pERK1/2 antibody, according to the method section. Cells were treated with 10 ng/ml EGF and/or 5 µM PD98059 for 10 min before protein was extracted as described. Amount of total OK cell protein per lane was 20 µg.

**(A)** Histogram of five Blots from five different protein extractions and cell culture passages of OK cells. EGF leads to a significant stimulation of pERK1/2 amount. Protein extracts from cells treated with PD98059 or with EGF are different from controls. Protein extracts from cells treated with PD98059 in combination with EGF do not differ from controls. * = p<0,05 versus control.

**(B)** Single most typical Western blot showing the effect of EGF and/or PD98059 on phosphorylation of ERK1/2 in OK cells. The antibody used leads to a specific staining of two protein bands at 42 kDa and 44 kDa representing pERK1 and pERK2. No other protein signals were detected.
**Figure 1**

**A**

- **non-quiescent**
  - [\(^{14}\text{C}\)]-PAH secretion [% of control]
  - (6) (3) (3)

- **quiescent**
  - (11) (6) (5)

**B**

- **control**
- **PMA 10^{-7} M**
- **DOG 5 \times 10^{-5} M**
**Figure 2**

**A Transepithelial transport (10 min)**

- **Apical**
  - PAH-content
  - PAH-secretion
  - \( \frac{1}{1} = 3 \text{ basolateral uptake: total amount of PAH transported across the basolateral membrane} \)
  - \( \frac{2}{1} = 4 \text{ secretion/content -ratio} \)

- **Basolateral**

**B Initial basolateral uptake (1 min)**

- **Apical**

- **Basolateral**

**C Initial apical efflux (1 min)**

- **Apical**

- **Basolateral**
Figure 3

(A) \[^{14}C\]-PAH secretion [pmol x cm\(^{-2}\) x 10min\(^{-1}\)]

(B) \[^{14}C\]-PAH content [pmol x cm\(^{-2}\) x 10min\(^{-1}\)]

(C) basolateral \[^{14}C\]-PAH uptake [pmol x cm\(^{-2}\) x 10min\(^{-1}\)]

(D) secretion/content ratio

n = 5; for every bar

- ■ control
- □ DOG 5 x 10\(^{-5}\) M
Figure 4

A

Initial apical efflux [% of PAH in the cell at t=0]

- Control: (3)
- DOG 50μM: * (3)

B

Initial basolateral uptake [pmol x cm⁻² x min⁻¹]

- Control: (6)
- DOG 50μM: * (3)
Figure 5

A

$[^{14}C] \text{PAH secretion}$

$[\% \text{ of control after 10 min}]$

B

$[^{14}C] \text{PAH content}$

$[\% \text{ of control after 10 min}]$

C

$[^{14}C] \text{PAH uptake}$

$[\% \text{ of control after 10 min}]$

D

secretion/content-ratio

$n = 9 \text{ for every bar}$

control

EGF 10 ng/ml
Figure 8

(A) Initial apical efflux [% of PAH in the cell at t=0] for control and PD98059 (5μM)

(B) Initial basolateral uptake [pmol x cm⁻² x min⁻¹] for control, 5μM, and 50μM PD98059
Figure 9

A

PKC

MEK

apical
cell
basolateral

B

EGF

apical
cell
basolateral

C

MEK

ERK1/2

OAT1

basolateral

EGF
Figure 11

A

![Graph showing pERK1/2 intensity (% of control) for different treatments: control, PD98059, EGF, EGF + PD98059. The graph indicates a significant increase in pERK after EGF treatment.]

B

![Western blot analysis showing pERK1 (42 kD) and pERK2 (44 kD) levels under various conditions. The blot shows the following: EGF 10 ng/ml (+ for control and EGF, - for PD98059). PD98059 5 μM (+ for control and PD98059, - for EGF). The blot also highlights the intensity and distribution of pERK bands.]
Modulation of the basolateral and apical step of transepithelial organic anion secretion in proximal tubular OK cells: Acute Effects of EGF and MAPK
Christoph Sauvant, Hildegard Holzinger and Michael Gekle

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