Original Paper

Regulation of Organic Cation Transport in Isolated Mouse Proximal Tubules Involves Complex Changes in Protein Trafficking and Substrate Affinity

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Key Words

Organic cation transport • Protein kinases • Trafficking • Substrate affinities • Proximal tubule • Microfluorimetry

Abstract

This study characterizes the complex mechanisms of acute regulation of organic cation (OC) transport across the basolateral membrane of isolated mouse proximal tubules. The fluorescent substrate ASP⁺, 4-(-4-(dimethylamino) styryl-N-methylpyridinium), was used to quantify OC transport using a microtiter plate based fluorescence reader method. Inhibition of phosphatidylinositol-3-kinase, of p56 tyrosine kinase, stimulation of PKC and inhibition of PKA reduced ASP⁺-uptake. ASP⁺-kinetic and Dixon plot analyses revealed effects on transporter trafficking as explanation for the inhibition of ASP⁺-uptake by these pathways. Angiotensin II (AII) via stimulation of Ca²⁺/calmodulin increased ASP⁺-uptake. This effect aroused from an altered substrate affinity. Bafilomycin, an inhibitor of the vacuolar H⁺-ATPase and thus endosomal and lysosomal function, reduced ASP⁺-uptake, but did not prevent the AII effect on ASP⁺-uptake. Bafilomycin seemed to diminish the recycling rate of OCTs and hence to reduce the amount of transporters in the membrane. AII via Ca²⁺/calmodulin increased the substrate affinity of the remaining OCTs. The involvement of the cytoskeleton in acute regulation of OCTs became obvious as colchicine induced inhibition of microtubule polymerisation reduced ASP⁺-uptake. Acute regulation of mouse OCTs mostly involves changes in trafficking from and to the plasma membrane and only in the case of AII/CaM changes in substrate affinity.

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Introduction

Organic cation transporters (OCTs) are membrane proteins, which translocate organic cations (OCs) across the plasma membrane. In mammals the proximal tubule (PT) serves as the main renal elimination site of endogenous (e.g. choline, dopamine) and exogenous (e.g. cimetidine, procainamide, quinine and vecuronium) OCs [1, 2]. For this reason, OCTs have important physiological, pharmacological, toxicological and clinical implications. Specifically, their great importance becomes obvious by e.g. taking into account their pivotal role in the elimination of environmental toxins [3] and in reducing the exposure of the body to specific drug toxicities [4] such as antibiotics, antihistamines [5], cytostatics [6-9] or antiarrhythmics [10]. However, OCTs are also involved in toxic side effects of such compounds as they can lead to specific cellular accumulation [6-9, 11]. In the long run the targeted modulation of their activity by diverse inhibitors or protein kinases could e.g. reduce toxic side effects and improve treatment efficacy instead. But first of all further detailed insight into the intracellular signalling cascades, which acutely affect OC transport, has to be provided. OC transport has been characterized as bidirectional [12, 13], polyspecific [14] and electrogenic [12, 13, 15-17], but pH- and Na⁺ independent [12, 13, 15]. Three different isoforms have been identified each in rat, mouse and man (OCT1, OCT2 and OCT3). All of them belong to the solute carrier 22A superfamily (SLC22A) [14]. They physiologically occur predominantly in intestine, liver, brain and kidney [4, 17-19]. However, the expression and distribution of these isoforms differ in dependence of species and organs [14]. In rat PTs OCT1, which was the first OCT to be cloned, and OCT2 are highly expressed in parallel [16] whereas in human kidneys OCT2 is the predominant isoform [15]. In mouse kidney OCT1 and OCT2 are the dominant isoforms on mRNA level, while OCT3 shows only very minor expression [20]. The molecular structure of OCTs consists of twelve alpha helical transmembrane domains (TMDs) with a large hydrophilic extracellular loop between the first and second TMD and a large intracellular loop between the sixth and seventh TMD. Especially the intracellular domain and the c-terminal end contain several potential phosphorylation sites [2, 21], which are targets for protein kinases. So far several pathways have been identified which acutely regulate OCTs, mostly without identifying the involved mechanisms of molecular interaction. Although transgenic mouse models are commonly used for e.g. translational research, the functional properties, particularly detailed mechanisms of short-term regulation of OCTs in the mouse, are not yet sufficiently examined and described. This is especially true for the *in/ex vivo* situation. Therefore this study aims to characterize the complex pattern underlying mechanisms of acute regulation of OC transport across the basolateral membrane of freshly isolated mouse PTs focussing on protein kinase dependent signalling pathways and kinetic analyses. Moreover, we put emphasis on the role of cellular protein degradation and the involvement of essential components of the cytoskeleton, e.g. microtubules, in these regulatory mechanisms. Due to the fact that PTs express OCT1, OCT2 and to a minor extent also OCT3 all data presented in this study reflect the sum of effects on all three isoforms as present in the *in vivo* situation.

Materials and Methods

Animals

Nine week-old male C57BL/6 mice (n= 65, Charles River, Sulzfeld, Germany) were kept under temperature-, light- and humidity-controlled conditions and had free access to water and standard mice chow (Sniff, Soest, Germany). Experiments were approved by a governmental committee on animal welfare and were performed in accordance with national animal protection laws (06705.2011.256, LANUV NRW Recklinghausen, Germany).

Isolation of tubular segments and experimental solutions

In most cases, up to 800 PTs (S2 and S3 segments) were mechanically isolated by two experimenters from one or both kidneys of a mouse within 2 hours after nephrectomy. Experiments were performed within the next 2-4 hours as previous experiments validated that the specificity of ASP⁺-uptake remains stable at least up to 4 hours after tubule isolation [20]. During the dissection under the microscope tubules were kept in MEM-EARLE medium (Biochrom, Berlin, Germany) at 4°C and later transferred to HCO₃⁻-free Ringer-like control solution containing (in mM): NaCl 145, K₂HPO₄ 1.6, KH₂PO₄ 0.4, D-glucose 5, MgCl₂ 1, calcium gluconate 1.3, and pH adjusted to 7.4. Afterwards three tubule segments (total length approximately 500 µm) were transferred into one well of a 384 well microtiter plate (Deep Well Small Volume, Greiner, Frickenhausen, Germany) with 10 µl of control solution. To remove air bubbles in the wells which could disturb the measurements and to make the tubules gently settle down at the bottom of the wells the microtiter plate was mildly centrifuged for 10 min before starting the experiments. For regulatory experiments PTs were incubated with another 10 µl of control solution containing the respective agonists or antagonists at 37°C. These agonists and antagonists were present throughout the whole experiment. For all other measurements 10 µl of control solution were added to each well. In all experiments 5 µl ASP⁺ dissolved in control solution were injected by the fluorescence reader to achieve a final concentration of 1 µM (except indicated differently). In addition competitive organic cations were added to this ASP⁺-solution.

Fluorescence measurements

As customary in our laboratory ASP⁺, 4-(4-(dimethylamino)styryl)-N-methyl-pyridinium, a positively charged fluorescent dye [17, 21, 22], served as substrate in order to quantify OC transport across the basolateral membrane of isolated PTs. Microfluometric measurements were exerted with a fluorescence plate reader (Infinity M200, Tecan, Crailsheim, Germany) (excitation at 465 ± 35 nm, emission at 590 ± 20 nm) at 37°C to semi-automatically analyse OC transport [20]. Fluorescence was measured dynamically in each well before and after ASP⁺-injection and plotted versus time. ASP⁺ and other organic cations were added after the third sampling interval. Emission from the complete area of the well bottom was analysed four times and averaged for each well. After being taken up by the cell ASP⁺ shows a shift in its emission spectrum [23]. Therefore the initial linear fluorescence increase, measured by linear regression within the first 100 seconds, visualizes the specific cellular uptake of ASP⁺ across plasma membranes and is not significantly influenced by transport of ASP⁺ out of the cells, intracellular compartmentalization and bleaching of the dye [17, 22, 24]. Background fluorescence was measured for each plate in wells with identical solutions but no tubules and subtracted from each well containing tubules. Experiments were performed with tubules isolated from one animal per day with controls and test substances in the same plate.

Chemicals

Tetrapentylammonium (TPA⁺), cimetidine, human angiotensin II, bafilomycin, sn-1,2-dioctanoyl glycerol and colchicine were purchased from Sigma. Calmidazolium, aminoguanidine, wortmannin, KT5720, calphostin C and EGF were obtained from Calbiochem (Bad Soden, Germany), insulin from Novo Nordisk (Mainz, Germany), IGF-1 from Roche Molecular Biochemicals (Basel, Switzerland) and IL-1α from R&D (Minneapolis, Minnesota, USA). ASP⁺ was purchased from Molecular Probes/Invitrogen, Karlsruhe, Germany. All other substances and standard chemicals were obtained from Sigma or Merck (Darmstadt, Germany). Compounds were dissolved in HCO₃⁻-free Ringer-like solution (see above) and if necessary with ethanol or dimethylsulphoxide (DMSO) as solvent. The final concentration of these solvents did not affect the results of the experiments (data not shown).

Statistical analyses

Data are presented as mean values ± SEM, with (n) referring to the number of wells. K_m - and V_{max} - values were obtained by sigmoidal concentration-response curve fitting or by Dixon plot analyses using GraphPad Prism, Version 5.3 (GraphPad Software, San Diego, USA). Unpaired two-sided Student's t-test was employed to prove statistical significance of the effects. For multiple comparisons ANOVA with Bonferroni post-test was used. A P-value < 0.05 was considered statistically significant.

Results

ASP⁺-transport

Cellular ASP⁺-accumulation across the basolateral membrane of PTs is concentration dependent and shows an OCT- and transporter-independent component. The mOCT specific

Fig. 1. Kinetics of ASP^+ -uptake. mOCT-dependent initial ASP^+ -uptake rates (continuous line, difference) were obtained by subtraction of the mOCT-independent ASP^+ -uptake in the presence of TPA^+ (3 mM) (dashed line, $\text{ASP}^+ + \text{TPA}^+$) from the total uptake (dashed line, ASP^+). Data are given as mean values $\pm$ SEM, with 64 to 103 observations at each data point. From this mOCT-dependent curve (difference) a k_m of $4.6 \pm 0.5 \mu\text{M}$ and a $V_{\max}$ of 28.3 ± 1.0 a.u. were calculated.

Fig. 2. Protein kinase dependent regulation of mOCT mediated ASP^+ -transport. Significant decrease of ASP^+ -uptake was seen with inhibition of PI3K (wortmannin, $0.1 \mu\text{M}$), of p56^{lc} tyrosine kinase (aminogienistein, $10 \mu\text{M}$), of $\text{Ca}^{2+}/\text{CaM}$ (calmidazolium, $5 \mu\text{M}$), PKA inhibition (KT5720, $1 \mu\text{M}$) and PKC-stimulation (DOG, $1 \mu\text{M}$) after an incubation time of 10 min at 37°C . Angiotensin II (1 nM) increased ASP^+ -uptake significantly. Data are given as mean values $\pm$ SEM of initial cellular fluorescence increase in percent of controls with numbers of observations in parentheses; * indicates statistically significant differences compared to individual controls.

component of ASP^+ -uptake can be determined by subtracting the TPA^+ -dependent uptake from the total uptake. At 3 mM TPA^+ , a known substrate of mOCTs, the transport of ASP^+ is completely inhibited. Therefore the difference of both curves resulted in a saturation kinetic for the mOCT specific ASP^+ -uptake with a k_m -value of $4.6 \pm 0.5 \mu\text{M}$ ($n = 64\text{--}103$). As cellular ASP^+ fluorescence was measured in arbitrary fluorescence units (a.u.) the $V_{\max}$ -value of 28.3 ± 1.0 can only be compared relatively within this study with the technical settings kept constant (Fig. 1).

Protein kinase dependent mechanisms of regulation

In this work we further examined the cellular mechanisms of acute regulation of OC transport in freshly isolated mouse PTs. Phosphatidylinositol-3-kinase (PI3K), the $\text{Ca}^{2+}/\text{calmodulin}$ complex ($\text{Ca}^{2+}/\text{CaM}$), p56^{lc} tyrosine kinase (p56^{lc}), protein kinase A (PKA) and protein kinase C (PKC) served as targets in this study. Confirming our first report [20] we again demonstrate an affection of OC transport in isolated mouse PTs by these pathways (Fig. 2) allowing us to study the mechanisms responsible for this regulation in tubules from the same animals under the same experimental conditions. These data suggest that OC transport in mouse PTs is activated by PI3K, p56^{lc} , PKA and $\text{Ca}^{2+}/\text{CaM}$ and inhibited by PKC. In the present consecutive study we focussed on the identification of the cellular mechanisms underlying this acute regulation of OC transport. Furthermore we put emphasis on the investigation of aspects of lysosomal degradation and of the cytoskeleton as vital cellular components and demonstrate that both are involved in the regulation of OC transport.

Regulation by phosphatidylinositol-3-kinase (PI3K)

Wortmannin ($0.1 \mu\text{M}$), a known inhibitor of PI3K, reduced ASP^+ -uptake significantly by $48 \pm 3 \%$ ($n=76$) after 10 min incubation (Fig. 2). As in these isolated PTs PI3K could

	k_m (μ M)	V_{max} (a.u.)	n
Control	4.6 ± 0.5	28.3 ± 1.0	67-103
Wortmannin (0.1 μ M)	9.4 ± 8.4	$19.3 \pm 7.7^*$	16-23
Aminogestien (10 μ M)	3.6 ± 1.8	$18.5 \pm 2.9^*$	16-26
KT 5720 (1 μ M)	4.4 ± 3.9	$16.8 \pm 4.7^*$	27-29
DOG (1 μ M)	4.3 ± 3.3	$15.8 \pm 4.7^*$	21-27
Angiotensin II (1 nM)	$2.9 \pm 2.3^*$	33.8 ± 7.7	9-15

Table 1. Effects of protein kinase inhibition/activation on ASP⁺ transport kinetics. Mean values $\pm$ SEM with n indicating number of observations; * indicates significantly different to control.

Fig. 3. Effects of protein kinase dependent regulation of ASP⁺-uptake on transport kinetics. Kinetics of mOCT-dependent initial ASP⁺-uptake without (Control) and with incubation with agonists and antagonists for 10 min at 37°C. Specific uptake rates were obtained as depicted in figure 1. Wortmannin (0.1 μ M), aminogestien (10 μ M), DOG (1 μ M) and KT5720 (1 μ M) influenced the trafficking (lowered V_{max}) of mOCTs whereas angiotensin II (1 nM) modulated the substrate affinity (increased k_m) of ASP⁺ transport. Data are given as mean values $\pm$ SEM. Calculated k_m and V_{max} -values and numbers of observations are summarized in Table 1.

not be further activated by insulin, a known activator in the insulin receptor pathway, as demonstrated by us before [20], we used EGF (50 ng/ml), IGF-1 (50 ng/ml) and IL-1 α (10 ng/ml) as possible stimulators of PI3K. Also these agonists did not significantly stimulate ASP⁺-uptake (data not shown) again indicating that PI3K is already maximally endogenously active in these isolated tubules and stimulates OC transport in PTs of mice.

To find out whether the observed inhibitory effect of wortmannin on OC transport arises from an altered substrate affinity of mOCTs or is rather based on changes in transport capacity via mOCT trafficking from and to the plasma membrane, kinetic measurements with ASP⁺ as substrate were performed. Using the same experimental approach as depicted in figure 1, incubating PTs with wortmannin (0.1 μ M) for 10 min before starting the experiments did not lead to a significant change in the k_m -value for ASP⁺, but to a significantly reduced V_{max} -value (Table 1) as calculated from the mOCT-dependent ASP⁺ dependent uptake curve shown in Figure 3. This suggests that PI3K influences OC transport by trafficking or turnover effects without modifying substrate affinity.

Regulation by angiotensin II and the Ca²⁺/CaM complex

Furthermore we studied the calmodulin associated regulation of mOCTs. Angiotensin II (1 nM) raises intracellular Ca²⁺, activates the Ca²⁺/CaM complex and thereby stimulated ASP⁺-uptake as shown by us before [20]. In the present study angiotensin II (1 nM) increased ASP⁺-uptake by $40 \pm 7\%$ (n = 139) after 10 min incubation (Fig. 2). The observed angiotensin II effect could again be abolished by coincubation with calmidazolium (5 μ M), a known inhibitor of the Ca²⁺/CaM complex, which reduced ASP⁺-uptake in the presence of angiotensin II by $33 \pm 6\%$ (n = 40), suggesting a partial endogenous activation of the Ca²⁺/CaM complex in these PTs.

Fig. 4. Protein kinase-dependent effects on k_i of cimetidine. A. Dixon plot analyses of the inhibition of ASP⁺-uptake (1 or 5 μ M) by cimetidine were performed to determine the inhibition constant (k_i). k_i was determined by the negative concentration at the point of intersection of the regression lines evoked by the two ASP⁺ concentrations. B. Dixon plot analyses of the inhibition of ASP⁺-uptake by cimetidine under incubation with wortmannin (0.1 μ M, 10 min) resulted in no significantly altered k_i -value compared to the control. C. Dixon plot analyses of the inhibition of ASP⁺-uptake by cimetidine under incubation with angiotensin II (1 nM, 10 min) resulted in a significantly reduced k_i -value compared to the control. D. Dixon plot analyses of the inhibition of ASP⁺-uptake by cimetidine under incubation with aminoginistein (10 μ M, 10 min) did not significantly alter the k_i -value compared to the control. Data are given as mean values $\pm$ SEM of cellular fluorescence increase with numbers of observations in parentheses.

ASP⁺-uptake kinetics were determined after incubation with angiotensin II and compared to those under unstimulated control conditions (Fig. 3). Under angiotensin II stimulation a significantly reduced k_m -value but no changes in the V_{max} -value were calculated (Table 1). Therefore angiotensin II might induce conformational changes at the substrate-binding site of mOCTs, which lead to an increased substrate affinity.

Regulation by p56 tyrosine kinase (p56^{lck})

Aminoginistein inhibited p56^{lck} and thus reduced ASP⁺-uptake in our previous report [20]. In the present study aminoginistein (10 μ M) decreased ASP⁺-uptake by $35 \pm 4\%$ (n=56) (Fig. 2). Analysing the kinetics of ASP⁺-uptake after incubation with aminoginistein (Fig. 3) resulted in no change in k_m - and a significantly reduced V_{max} -value (Table 1). As only the V_{max} -value differed significantly from the unstimulated control this emphasizes that this inhibitory effect is probably also evoked by modifying transporter trafficking.

Regulation by protein kinase A (PKA) and protein kinase C (PKC)

Sn-1,2-diocanoyl glycerol (DOG), a membrane permeable analogue of diacyl glycerol, which is known to stimulate PKC and KT5720, which inhibits PKA, both reduced ASP⁺-uptake

Fig. 5. Influence of bafilomycin on ASP⁺ transport. Concentration dependent decrease of ASP⁺-uptake by bafilomycin after 10 min of incubation. Data are given as mean values $\pm$ SEM of cellular fluorescence increase in percent of controls with numbers of observations in parentheses; * indicates statistically significant effects.

significantly in our previous report [20] and again in this study by $30 \pm 9\%$ ($n = 28$) and $20 \pm 8\%$ ($n = 27$), respectively (Fig. 2). ASP⁺-kinetic measurements (Fig. 3) unveiled that these protein kinase signalling pathways also regulate OCT mediated transport via trafficking effects because DOG and KT5720 significantly reduced the $V_{\max}$ -values for ASP⁺, without altering substrate affinity (Table 1).

ASP⁺ transport inhibition by cimetidine

To further check and confirm the results on mechanisms of regulation gained from the ASP⁺-kinetic measurements described above we implemented Dixon plot analyses to evaluate the inhibition constant (k_i) for the inhibition of ASP⁺-uptake by the known competitor substrate cimetidine. We plotted the reciprocal ASP⁺-uptake values obtained at increasing concentrations of cimetidine at two substrate concentrations (ASP⁺ 1 and 5 μM). This indirect way of kinetic analysis is required, as cimetidine cannot be measured directly using a fluorescence system. For cimetidine we obtained a k_i -value of $142 \pm 9\ \mu\text{M}$ ($n = 9$ -30) (Fig. 4a).

Effect of the PI3K: To confirm the mode of regulation of ASP⁺-transport by protein kinases we determined whether these kinases affect the k_i -value for cimetidine. The k_i -value for cimetidine of $141 \pm 3\ \mu\text{M}$ ($n = 8$ -15) obtained after incubation with wortmannin (0.1 μM) did not differ significantly from that under control conditions (Fig. 4b).

Effect of angiotensin II: The k_i -value for cimetidine of $72 \pm 26\ \mu\text{M}$ ($n = 21$ -35) achieved from Dixon plot analyses after incubation with angiotensin II (1 nM) differed significantly from that obtained under control conditions, emphasizing the assumption obtained from ASP⁺ kinetics, that OC transport via the Ca^{2+} /CaM pathway is regulated by an altered substrate affinity (Fig. 4c).

Effect of the p56 tyrosine kinase: Dixon plot analyses of the k_i -value for cimetidine after incubation with aminogynostein (10 μM) resulted in no significant change in this value ($138 \pm 3\ \mu\text{M}$; $n = 22$ -33) compared to controls. Therefore these findings underscore the conclusion that the regulation of OC transport via the PI3K and the p56^{lck} pathways is mediated by trafficking or turnover effects, but not by modification of substrate affinities (Fig. 4d).

Involvement of proteolytic pathways

We further examined the impact of proteolytic pathways on the regulation of OC transport by using bafilomycin. Bafilomycin inhibits the vacuolar type of H⁺-ATPase reversibly and thus stops early endosomal recycling and lysosomal protein degradation. The incubation of PTs with bafilomycin led to a concentration dependent reduction of ASP⁺-uptake (Fig. 5).

Bafilomycin and angiotensin II. Under simultaneous incubation of PTs with bafilomycin (0.2 μM) and angiotensin II (1 nM) as stimulator of the Ca^{2+} /CaM complex we could still demonstrate the stimulating effect of angiotensin II even on the bafilomycin induced reduced level of OC transport (Fig. 6a). These results confirm the above findings that the stimulating effect of angiotensin II on OC transport is probably based on an increased substrate affinity of the transporters in the plasma membrane whereas the bafilomycin dependent proteolysis reduces the number of active transporters in the plasma membrane.

Fig. 6. Influence of bafilomycin on the regulation of ASP⁺ transport. Effects of incubation with bafilomycin on the effects of angiotensin II or wortmannin on ASP⁺-transport. A. The effect of angiotensin II (1 nM, 10 min) on ASP⁺-uptake was not prevented by bafilomycin incubation (0.2 μM, 10 min). B. The inhibitory effect of wortmannin (0.1 μM, 10 min) on ASP⁺-uptake was inhibited at two concentrations of bafilomycin indicating the concerted interaction of both substances with trafficking. Data are given as mean values ± SEM of cellular fluorescence increase in percent of controls with numbers of observations in parentheses; * indicates statistically significant effects compared to control; # denotes significant difference between indicated bars.

Bafilomycin and wortmannin. We examined the interaction of bafilomycin with the PI3K dependent regulation of ASP⁺-uptake in the same way. As wortmannin reduced ASP⁺-uptake (Fig. 2) we used two concentrations of bafilomycin (0.1 or 0.2 μM) to test whether the effect of wortmannin (0.1 μM) can still be observed under simultaneous incubation with bafilomycin. Under both bafilomycin concentration no further change of ASP⁺-uptake was induced by wortmannin (Fig. 6b), which again suggests that the PI3K mediated regulation of OC transport involves a proteolytic pathway dependent trafficking.

Involvement of the cytoskeleton

We next focussed on the involvement of components of the cytoskeleton in the regulation of OC transport. For this purpose we chose colchicine due to its destructive impact on microtubules. A 30 min incubation with colchicine (100 μM) reduced ASP⁺-uptake significantly by $38 \pm 8\%$ (n=33) (Fig. 7), suggesting a significant role of the cytoskeleton in the regulation of mOCTs. To further demonstrate that this component does not influence the properties of mOCTs residing in the plasma membrane we examined whether the angiotensin II (1 nM) mediated effect on ASP⁺-affinity can still be observed. As summarized in Figure 7 even after colchicine incubation the stimulatory effect of angiotensin II was still present.

Discussion

The kidneys are involved in the excretion of many endogenous and exogenous organic cations. The levels of these substances in blood vary depending on metabolism and food or drug intake. Probably to adjust excretion of especially the endogenous substrates to such variations in plasma concentrations mechanism of short- as well as long-term regulation of the respective transport proteins are developed. Consequently such mechanisms will also influence the uptake or excretion of exogenous drugs or toxins. Good knowledge about the structure and function of different OCT isoforms in various species could already be gained. Within the last years special focus was put on the investigation of regulatory mechanisms of OCTs [2, 4, 14]. However, data on acute regulation of OC transport in the intact organ or

Fig. 7. Involvement of the cytoskeleton in acute regulation of ASP⁺ transport by angiotensin II. The incubation of PTs with colchicine (100 μ M) for 30 min led to a significantly decreased ASP⁺-uptake, suggesting the role of an intact microtubular network for OCT-mediated transport. Under simultaneous incubation with colchicine and angiotensin II (1 nM) the stimulating angiotensin II effect could still be observed, in line with the observed absence of trafficking effects on OC transport by this pathway. Data are given as mean values $\pm$ SEM of cellular fluorescence increase in percent of controls with numbers of observations in parentheses; * indicates statistically significant effects compared to control; # denotes significant difference between indicated bars.

in isolated proximal tubules are scarce and are limited to single studies from rabbit [25], rat [23] and human PTs [22]. Although transgenic mice play a preeminent role in several physiological, pharmacological, or translational studies, data on functional properties of OC transport in PTs of mice, especially concerning mechanisms of short-term regulation, are not yet sufficiently described. Only recently we could report the first basic data on OCT regulation in isolated mouse proximal tubules [20]. Concerning the lack of respective studies as well as the well-known fact of species, organ and genotype dependent differences of OCTs [14] we attempted to further examine the mechanisms involved in the complex pattern underlying acute regulation of OC transport across the basolateral membrane of freshly isolated mouse PTs.

Determination of kinetics of substrate transport

Since the positively charged fluorescent dye ASP⁺, a model substrate of OCTs, is not transported by any other OC transporter expressed at the basolateral membrane of PTs our data exclusively depict ASP⁺-transport mediated by OCTs. In order to calculate transport kinetics saturable transport of ASP⁺ solely mediated by mOCTs had to be distinguished from OCT-independent ASP⁺-uptake. mOCT dependent ASP⁺-uptake was obtained by applying TPA⁺ (3 mM) to the injection solution, which completely blocks mOCT-mediated ASP⁺-uptake even at a concentration of 1 mM [20]. The k_m -value of approximately 5 μ M for ASP⁺ under control conditions obtained in the present study is in the same range as that reported by us before for isolated mouse PTs [20], human proximal tubules [22] or other OCT isoforms expressed in HEK-293 cells [15, 26]. As OCTs accept a wide spectrum of structurally highly different substrates and different binding sites for these substrates have been suggested [27], we tested whether the observed regulatory effects on ASP⁺ kinetics can also be seen using a structurally different classical OCT substrate. Therefore we performed Dixon plot analyses. While k_m - and k_i -values can be determined as absolute concentrations and therefore compared within this study but also to respective data in the literature, the V_{max} -values obtained in this study are arbitrary fluorescence values, which depend on the experimental and technical conditions and thus, can only be compared within this study.

Effects of protein kinases on transport kinetics

PI3K. The involvement of PI3K in the regulation of OC transport has been reported for hOCT2 [21, 28] or rOCT2 [23] stably expressed in HEK-293 cells as well as for human (Schlatter E. and Ciarimboli G., unpublished results) and mouse PTs [20]. Due to the results of our kinetic measurements performed in this study, i.e. effects only on V_{max} and not on k_m or k_i we suggest that the inhibitory effect of wortmannin is based on trafficking or turnover

Fig. 8. Suggested mechanisms of acute regulation of mOCTs. Schematic representation of affinity and trafficking effects induced by the depicted signalling pathways. Regulation of mOCTs by the p56lck, PI3K, PKA and PKC pathways seems to involve an altered number of transporters in the plasma membrane due to a decreased OCT trafficking to the membrane and/or a high turnover rate of OCT-retrieval from the membrane, whereas the angiotensin II dependent regulation of mOCTs via the Ca^{2+} /CaM pathway rather influences the substrate affinity of mOCTs.

effects of PI3K. This may be e.g. due to a deceleration of intracellular trafficking of mOCTs to the membrane or an accelerated retrieval and degradation of mOCTs to or from the membrane and thus, a decreased amount of active transporters expressed in the cell membrane.

In line with our previous findings that insulin, a known activator in the insulin receptor pathway, does not further stimulate PI3K in these freshly isolated PTs, EGF, IGF-1 and IL-1 α did also not lead to an altered ASP^+ -uptake. Thus, we suggest that PI3K is already endogenously active and stimulates OC transport in these freshly isolated mouse PTs via an altered trafficking to or from the plasma membrane. Similar findings have been observed by us for rOCT2 [23] expressed in HEK-293 cells whereas opposite findings have been revealed for hOCT2, which seems to be inhibited by PI3K [21, 28].

Angiotensin II. Angiotensin II activates multiple signalling pathways including the Ca^{2+} /CaM complex as well as the PKC signalling pathway. Our present findings on a Ca^{2+} /CaM-dependent regulation are in line with observations published for rOCT2 [23], hOCT1 [29], hOCT2 [21] and hOCT3 [30]. Due to the fact that calmidazolium antagonized the angiotensin II effect we conclude that the PKC signalling pathway seems to play a subordinated role in transporter regulation by angiotensin II in mouse PTs as already demonstrated for HEK-293 cells expressing hOCT1 and hOCT2 [28, 29]. Due to the observed lower respective k_m - and decreased k_i -values with no change in the $V_{\max}$ -value we suggest that angiotensin II has an impact on the substrate affinity of mOCTs by probably inducing conformational changes at the substrate binding-sites and thus, increasing the transport rate of the transporters. A similar effect on substrate affinity could be demonstrated by us before for the effect of PKC on rOCT1 [17, 24]. Opposite effects have been reported by us for hOCT2 expressed in HEK-293 cells [28], where an inhibition of the Ca^{2+} /CaM complex led to a significantly decreased $V_{\max}$ -value, which indicates a reduction of membrane associated hOCT2 [28]. Taken together, these findings once more underscore species dependent differences between OCTs.

Aminogenistein. In this study aminogenistein, an inhibitor of p56^{lck}, reduced ASP⁺-uptake significantly, emphasizing our findings reported before [20] and indicating endogenous activation of mOCTs by this kinase. Moreover these observations gained from mouse PTs are in line with the effects of aminogenistein observed for rOCT1 [17], rOCT2 [23] and hOCT1 [29]. Due to the fact that the k_m -value for ASP⁺ and the k_i -value for cimetidine were not altered by aminogenistein, whereas the V_{max} -value for ASP⁺ was significantly decreased, these results indicate that also the regulation of mOCTs by p56^{lck} is probably mediated by trafficking effects.

Involvement of proteolytic pathways

To obtain more inside into the mechanisms responsible for the observed apparent trafficking effects induced by PI3K-, Ca²⁺/CaM-, and p56^{lck}-activation, effects of endosomal and lysosomal function and microtubule organisation were studied. Bafilomycin, which is known as a macrolide antibiotic isolated from *Streptomyces* sp., inhibits both endogenous early endosomal recycling and lysosomal protein degradation by highly specifically interfering with the H⁺-ATPase and thus, averts the acidification of organelles belonging to the central vacuolar system. The ability of vacuolar organelles to generate and maintain an acidic pH plays a crucial role in a variety of critical cellular functions, e.g. for the proper function of vesicular transport of proteins, for the fusion between autophagosomes and lysosomes and thus, for membrane protein recycling or its subsequent degradation. The involvement of the vacuolar H⁺-ATPase in the process of proximal renal tubule endocytosis and protein degradation has also been demonstrated in previous studies [31, 32]. Inhibition of vacuolar H⁺-ATPase by bafilomycin reduced ASP⁺-uptake in the present study. This effect seems to be due to a reduced number of mOCTs in the plasma membrane as a result of decreased OCT trafficking to the membrane. It also suggests a high turnover rate of OCT-retrieval from the membrane under normal conditions. Therefore it could be speculated that regulation of mOCTs by PI3K could also involve an altered number of transporters in the plasma membrane due to a change in the turnover rate rather than an influence on the substrate affinity of mOCTs (Fig.8). This assumption was confirmed by the fact that PTs incubated with bafilomycin did no longer respond to inhibition of PI3K by wortmannin. In contrast, as stimulation of Ca²⁺/CaM by angiotensin II only decreased k_m - and k_i -values but not V_{max} for ASP⁺ and cimetidine, respectively, the effect of angiotensin II was still observed in the presence of bafilomycin. Thus, despite the strong reduction in the amount of active OC transporters in the plasma membrane by bafilomycin angiotensin II was still able to increase transport activity probably via a change in the affinity of the remaining mOCTs in the plasma membrane.

Sodium phosphate type two cotransporters (NpT2a, NpT2c) similarly underlie complex regulatory mechanisms by diverse stimuli, which primarily lead to an altered expression of these transporters in the apical membrane of renal proximal tubule cells [33-35]. The vacuolar H⁺-ATPase seems to be involved in transporter trafficking of NpT2a in and out of the apical membrane [36]. Therefore the vacuolar H⁺-ATPase probably plays a crucial role for plasma membrane trafficking of various transporters including mOCTs.

Involvement of the cytoskeleton

Finally we addressed the question whether the cytoskeleton is involved in acute regulation of OCTs as well. Because of the observed inhibitory effect of colchicine, which destroys microtubules by inhibiting their polymerisation at the plus end, it has to be postulated that also an intact cytoskeleton is important for normal activity of OC transport. As expected, the stimulatory effect of angiotensin II, which altered substrate affinity but not trafficking of mOCTs, was still preserved under colchicine.

In conclusion, we examined the molecular mechanisms of protein kinase dependent short-term regulation of mOCTs in isolated proximal tubules. We demonstrate a modulation

of mOCT trafficking to/from the plasma membrane by PI3K, p56^{lck}, PKA or PKC and an involvement of the cytoskeletal network in the regulation of mOCT-dependent transport in freshly isolated mouse proximal tubules. In addition we could demonstrate that activation of Ca²⁺/CaM via angiotensin II increases the substrate affinity of these transporters without modifying its trafficking. Certainly additional studies are necessary to further unravel the complex mechanisms of mOCT regulation by direct effects on transporters or indirect trafficking effects in proximal tubules of the kidney.

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