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Digital Fluorescence Imaging of Organic Cation Transport in Freshly Isolated Rat Proximal Tubules

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Running title:

Digital imaging of OC transport

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Abstract

The secretion of cationic drugs and endogenous metabolites is a major function of the kidney. This is accomplished by organic cation transport systems, mainly located in the proximal tubules. Here, we describe a model for continuous measurement of organic cation transport. In this model, organic cation transport in individual freshly isolated rat proximal tubules is investigated by use of digital fluorescence imaging. To directly measure organic cation transport across the basolateral membrane the fluorescent organic cation 4-(4-dimethylaminostyryl)-*N*-methylpyridinium (ASP^+) is used with a customized perfusion chamber. ASP^+ uptake in this model displayed the characteristics of organic cation transport. Over the tested range of 1 to 50 µM it showed a concentration dependent uptake across the basolateral membrane. In the presence of competitive inhibitors of OC transport like N^1-methylnicotinamid$^+$, tetraethylammonium$^+$ and choline$^+$ a concentration dependent and reversible inhibition of ASP^+ uptake could be documented. In conclusion, continuous measurement of organic cation transport in freshly isolated rat proximal tubules by digital fluorescence imaging using ASP^+ is a useful tool for investigation of drug transport and interactions, and furthermore, may be helpful for investigation of organic cation transport under pathophysiological conditions.

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Introduction

Elimination of organic compounds like drugs or metabolic waste products is a major function of the kidney limiting the body's exposure to toxic substances of exogenous and endogenous origin. The elimination is mainly accomplished by net secretion of the various compounds via the proximal tubules. According to their physicochemical properties the secreted substances can be classified either as organic anions or as organic cations. Many clinically used drugs like beta-blockers, calcium antagonists, antibiotics, hypoglycemic agents, diuretics, morphine analogues and endogenous substances like catecholamines and neurotransmitters are organic cations (OC).

Secretion of OC by the kidney occurs from the blood into the tubular lumen. Therefore, OC have to be taken up from the blood across the basolateral membrane into the proximal tubular cells. For most of these OC this entry involves an electrogenic transport driven by the electrochemical gradient generated by the inside negative membrane potential (Koepsell et al., 2003; Wright and Dantzler, 2004). This transport across the basolateral membrane can also function as an electroneutral antiport in exchange for other OC (Dantzler et al., 1991; Budiman et al., 2000). This transport is enabled by several polyspecific organic cation transporters, which all belong to the solute carrier drug transporter family 22 (SLC 22) (Koepsell and Endou, 2004).

Investigations of OC transport previously have been performed in cellular substructures, like cell culture. However, these models possess particular limitations. Cultured cells during their adaptations necessary to survive in vitro, undergo a variety of phenotypic changes. These changes are particularly problematic for proximal tubular cells, which change from their physiological dependence on oxidative metabolism to glycolysis under culture conditions. In contrast, freshly isolated proximal tubules retain the biochemical properties of the in vivo state. In addition, they also retain a high degree of structural integrity as well as

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the highly polarized and fully differentiated functions of the normal proximal tubular epithelium (Lieberthal and Nigam, 2000). In fact, the model of freshly isolated proximal tubules ideally combines properties of in vitro as well as in vivo preparations in that external manipulations comparable to in vitro studies can be applied, while many of the in vivo characteristics of proximal tubules are retained.

In isolated proximal tubules OC transport has been estimated by the radiolabeled substrates N^1-methylnicotinamide$^+$ ($NMeN^+$) or tetraethylammonium (TEA^+) (Schali et al., 1983; Tarloff and Brand, 1986; Besseghir et al., 1990; Dantzler et al., 1991; Groves et al., 1994; Goralski and Sitar, 1999). By using radiolabeled substrates, investigations are limited to distinct time points, and cannot be followed continuously. Fluorescent substrates may serve as an advantageous alternative, which allow for a continuous measurement of OC transport.

We identified 4-(4-dimethylaminostyryl)-*N*-methylpyridinium (ASP^+) as a fluorescent substrate for the organic cation transport systems in the kidney with an apparent inhibition constant (K_i) of 0.10 ± 0.02 mmmol/l for the luminal *N*-methyl-4-phenylpyridinium (MPP^+) transport, and of 0.28 ± 0.12 mmol/l for the contraluminal $NMeN^+$ transport (Pietruck and Ullrich, 1995). Using the fluorescent substrate ASP^+, together with the use of digital fluorescence microscopy, enables a continuous real time monitoring of OC transport.

To selectively investigate the transport of organic cations in freshly isolated rat proximal tubules we used a customized microperfusion chamber, where peritubular conditions can be varied. The aim of the study was to validate this model by investigation of organic cation transport under different conditions, including reversible transport inhibition by different inhibitors.

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Methods

Freshly isolated proximal tubules

Rat proximal tubules were freshly isolated as recently described (Kribben et al., 2003; Pietruck et al., 2003). In brief, after perfusion of the renal arteries with an oxygenated buffer containing collagenase and hyaluronidase, cortices of male Sprague-Dawley rat kidneys (220-300 g of body weight) were minced on ice. Tissue separation with hyaluronidase and collagenase was continued at 37°C. Proximal tubules were obtained by percoll gradient centrifugation, using 45% and 90% Percoll (Pharmacia, Uppsala, Schweden). After washing, tubules were suspended in an oxygenated solution, containing (in mM): 106 NaCl, 20 $NaHCO_3$, 5 KCl, 1 $CaCl_2$, 2 NaH_2PO_4, 1 $MgSO_4$, 5 glucose, 10 HEPES, 2 glutamine, 10 sodium butyrate, 4 sodium lactate, adjusted to pH 7.05 at 4°C, and gassed on ice with 95% O_2/5% CO_2.

Microperfusion chamber and transport measurements of the organic cation transport with ASP^+ in freshly isolated individual tubules

To investigate individual tubules, a microperfusion chamber was used, customized on the basis of a Leiden chamber, with a complete exchange of the fluid in the chamber in less than 30 seconds, as previously described (Pietruck et al., 2003). An aliquot of the tubule suspension was pipetted onto a Cell-Tak (Collaborative Biomedical Products, Bedford, MA, USA) coated cover slip, mounted to the base of the microperfusion chamber. The chamber was then fixed to the stage of an epifluorescence microscope (Zeiss Axiovert, Jena, Germany). The tubule suspension inside the chamber was gassed for 10 min with 95% O_2/5% CO_2 at room temperature. Meanwhile, the tubules settled down and attached to the cover slip. Afterwards the chamber was perfused continuously with 2 ml/min of 95% O_2/5% CO_2 oxygenated buffer containing (in mmol/l): 106 NaCl, 25 $NaHCO_3$, 5 KCl, 1 $CaCl_2$, 2 NaH_2PO_4, 1 $MgSO_4$, 5 glucose, 2.5 HEPES, 1 glutamine, 1 sodiumbutyrate, and 1 sodium

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lactate, pH 7.35-7.40. During this period the temperature was gradually increased with an air stream incubator to 37°C.

After an accommodation period of 10 minutes, transport experiments were started, by switching to a buffer additionally containing different concentrations of the fluorescent organic cation 4-(4-dimethylaminostyryl)-*N*-methylpyridinium (ASP^+) (Molecular Probes, Leiden, The Netherlands). Initial uptake of ASP^+ was quantified as the increase of fluorescence between 30 and 60 seconds after switching to perfusion with ASP^+ (Stachon et al., 1996).

Digital video imaging

Images were acquired by a 12-bit cooled slow-scan-frame-transfer CCD camera (Imago, T.I.L.L. Photonics, Planneg, Germany) and processed by using image processing software (VisIon, T.I.L.L. Photonics, Planneg, Germany). Light with an excitation wavelength of 470 nm (bandwidth of 10 nm) was generated by a mesh monochromator (T.I.L.L. Photonics, Planneg, Germany) and was inserted in the light path of an inverted microscope (Axiovert 100, Zeiss, Jena, Germany) via a fluorescence objective with 20-fold magnification (Fluar, Zeiss, Jena, Germany). Emitted light, collected through a dichroic mirror was transmitted through an emission filter (> 605 nm) to the camera. To prevent photo bleaching the exposure time was limited to 30 ms every 10 seconds. 2 – 5 Tubules were individually analyzed in each experiment. Fluorescence of the tubules is expressed as relative fluorescence intensity.

Reversible inhibition of ASP^+ transport by N^1-methylnicotinamide$^+$ ($NMeN^+$), tetraethylammonium (TEA^+) and choline$^+$

$NMeN^+$, TEA^+, and choline$^+$ were used to investigate competitive inhibition of ASP^+ transport. In the first experiments, inhibitors and ASP^+ were superfused together. This resulted in a constant tubular fluorescence, reflecting a complete inhibition of ASP^+ transport.

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Therefore, we further on superfused the tubules first with 5 µM ASP^+ only, and after 1 min switched to a perfusion buffer containing inhibitors in addition to ASP^+.

For testing the reversibility of inhibition, after 8 minutes of superfusion with ASP^+ together with inhibitor, the buffer was again switched to a buffer containing ASP^+ without inhibitor.

Two-Photon Microscopy for imaging tubules

To acquire images of freshly isolated proximal tubules, envisioning the uptake of ASP^+ into the tubular cells, two-photon microscopy was applied. Freshly isolated tubules were investigated in a glass bottom dish coated with Cell-Tak. After settling of the tubules and attaching to the glass bottom of the dish, ASP^+ was added to the oxygenated buffer at different concentrations. Images were generated over a time period of at least 10 min.

Statistical analysis

Data are expressed as mean. SEM (in all experiments ranging between 10 – 35% of the mean) is not shown in the figures for better visibility. Whenever possible, experiments were performed as paired experiments, i. e. the experiments were performed with tubules isolated from the same kidney. To quantify transport inhibition, the increase of tubular ASP^+ fluorescence was compared over two minutes. Unless indicated otherwise, the time interval between two and four minutes of the respective experiment was used. The increase of fluorescence over two minutes was compared between different conditions with either paired, or unpaired t-test as applicable. A p-value of < 0.05 was considered significant.

Results

Concentration dependency of ASP^+ transport

After starting perfusion of the chamber with buffer containing ASP^+, fluorescence of the tubules increased. The increase of ASP^+ fluorescence was time and concentration

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dependent over the tested range between 1 and 50 µM ASP^+ (Fig. 1). In this concentration range a linear increase of tubular fluorescence was observed within the first 2 – 3 minutes, whereas the uptake rates decreased in the later phase of the experiment approaching equilibration (Fig. 1). We additionally evaluated the initial uptake rates of ASP^+ as these better reflect the transport across the basolateral membrane, whereas the maximal fluorescence in the later phase could also be influenced by other factors, like extrusion of ASP^+ from the cells, intracellular compartmentalization, or bleaching of ASP^+. As can be seen from the concentration response curve in Fig. 2, the initial rate of ASP^+ uptake was not saturated at 50 µM, the maximal ASP^+ concentration used. Due to the experimental setup, no higher concentrations were used.

Intracellular localization of the fluorescent substrate ASP^+

Fig. 3 depicts the translocation of ASP^+ across the basolateral membrane into the proximal tubule. No unspecific binding to the outer membrane occurred even when ASP^+ was used in concentrations up to 50 µM. By imaging freshly isolated proximal tubules with two-photon microscopy, a time dependent uptake into the cytoplasm of proximal tubular cells could be documented.

Effect of N^1-methylnicotinamide on ASP^+ transport

The addition of $NMeN^+$, 1 min after starting superfusion with 5 µM ASP^+, resulted in a dose dependent inhibition of ASP^+ uptake, as shown in Fig. 4. 10 mM $NMeN^+$ led to a complete inhibition of ASP^+ transport. 1 mM $NMeN^+$ did not decrease ASP^+ fluorescence, whereas the addition of 5 mM $NMeN^+$ induced partial inhibition of ASP^+ fluorescence. In the presence of 5 mM $NMeN^+$ in addition to inhibition of the inwardly directed ASP^+ transport in the further course of the experiment, a negative slope of the fluorescence intensity occurred.

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Effect of choline$^+$ and TEA$^+$ on ASP$^+$ transport

The addition of 10 mM choline$^+$, as well as of 10 mM TEA$^+$, 1 min after starting ASP$^+$ perfusion showed an inhibition curve similar to that observed with 10 mM NMeN$^+$. All inhibitors led to a rapid loss of incremental ASP$^+$ fluorescence. In the presence of inhibitors, tubular fluorescence, after reaching its maximum, even decreased, possibly reflecting an outwardly directed transport or leakage (Fig. 5).

Reversibility of ASP transport inhibition

After perfusion with the inhibitors for 7 min the perfusion buffer was switched to a buffer containing ASP$^+$ without inhibitor, a steeply rising fluorescence intensity could be observed, demonstrating the reversibility of uptake inhibition by NMeN$^+$, choline$^+$ and TEA$^+$ (Fig. 5).

Discussion

The aim of the present study was to establish a model for continuous measurement of organic cation transport in individual freshly isolated rat proximal tubules. This was accomplished by using the fluorescent organic cation 4-(4-dimethylaminostyryl)-*N*-methylpyridinium (ASP$^+$) together with digital fluorescence microscopy and video image analysis.

We have proven different variables of ASP$^+$ transport reflecting the physiological function of OC transport in this model. We first investigated the concentration dependency of ASP$^+$ uptake. In the tested range between 1 and 50 µM, intracellular fluorescence of ASP$^+$ reflected a concentration dependent transport across the basolateral membrane, without reaching saturation at 50 µM, the highest feasible concentration. These findings indicate that the K_m for ASP$^+$ in this model must be much higher than 50 µM. This is consistent with data determined for ASP$^+$ uptake across the basolateral membrane of rat proximal tubules in situ (280 µM) (Pietruck and Ullrich, 1995). The time pattern in general was similar to earlier

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experiments with radiolabeled TEA^+ in rabbit proximal tubules (Groves et al., 1994). For further experiments we chose an ASP^+ concentration of 5 µM, which showed no saturation in the first five minutes, when ASP^+ transport was quantified.

Secondly, we investigated, whether organic cation transport in freshly isolated proximal tubules could be inhibited by N^1-metyhlnicotinamide, a well-known inhibitor of organic cation transport (Besseghir et al., 1990; Ullrich et al., 1991). When $NMeN^+$ was administered before or simultaneously with ASP^+, no relevant increase in fluorescence could be registered, reflecting a complete inhibition of ASP^+ uptake (data not shown). Therefore, in the following experiments we started ASP^+ perfusion without inhibitor for a short period of time (1 min), so that an initial tubular uptake of ASP^+ occurred, and then immediately switched to a perfusion buffer additionally containing the inhibitor $NMeN^+$. Shortly after switching to the inhibitor, a strong inhibition of ASP^+ transport could be observed with the higher concentrations of 5 and 10 mM $NMeN^+$, whereas 1 mM $NMeN^+$ did not change intracellular fluorescence, reflecting the dose dependency of the inhibition of OC transport.

Thirdly, to prove the specificity of this inhibition by $NMeN^+$ we tested the inhibitory effect of two structurally different inhibitors of OC transport TEA^+ and $choline^+$ (Ullrich et al., 1991). Both inhibitors showed a similar pattern of ASP^+ transport inhibition with an immediate effect after switching to the inhibitor perfusion. Especially in the presence of TEA^+ and $choline^+$, and for $NMeN^+$ in lower concentrations, tubular fluorescence after reaching its maximum, demonstrating complete transport inhibition, even decreased in the further course, reflecting the loss of some of the intracellular ASP^+. These data are consistent with an outwardly directed transport of ASP^+ at the basolateral membrane, while at the outside of the cell membrane a complete inhibition still persisted. A quantitavely relevant secretion into the luminal space is rather unlikely, because in this model the lumen of the proximal tubules is collapsed.

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Fourthly, we demonstrated the reversibility of ASP^+ transport inhibition by switching from the perfusion buffer with inhibitor back to a perfusion buffer containing ASP^+ alone, which resulted in another strong increase in ASP^+ fluorescence, reflecting OC transport across the basolateral membrane (Fig. 5).

In conclusion, continuous measurement of organic cation transport in freshly isolated rat proximal tubules by digital fluorescence imaging using ASP^+ is a useful tool for investigation of drug transport and interactions, it may furthermore allow continuous measurement of organic cation transport under pathophysiological conditions in the future.

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Footnotes

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Legends to figures

Figure 1. Time dependent increase of tubular fluorescence in freshly isolated rat proximal tubules under different concentrations of ASP^+. Tubules were superfused with perfusion buffer containing ASP^+ between 1 and 50 µM ($n = 3 – 6$) as indicated.

Figure 2. Concentration dependence of ASP^+ uptake in freshly isolated proximal tubules. Values are means ± SEM of initial fluorescence increase with $n = 3 – 6$ experiments.

Figure 3. Freshly isolated proximal tubule, imaged by two-photon microscopy, 1 min (left) and 5 min (right) after addition of 50 µM ASP^+. Length of the bar corresponds to 20 µm.

Figure 4. Effect of different concentrations of $NMeN^+$ on ASP^+ uptake in freshly isolated proximal tubules. Perfusion was started with perfusion buffer containing 5 µM ASP^+ without $NMeN^+$ and after 1 min (arrow) switched to a perfusion buffer additionally containing different concentrations of $NMeN^+$ as indicated. 10 mM $NMeN^+$ ($n = 4$, $p < 0.005$) and 5 mM $NMeN^+$ ($n = 4$, $p < 0.005$) resulted in a significant inhibition of ASP^+ uptake, whereas 1 mM $NMeN^+$ did not significantly inhibit ASP^+ transport ($n = 4$, $p =$ n. s.).

Figure 5. Effect of $NMeN^+$, TEA^+, and $choline^+$ on ASP^+ uptake, and its reversibility. 1 min (arrow) after starting perfusion with 5 µM ASP^+, perfusion buffer was switched to a buffer containing 10 mM of either $NMeN^+$, TEA^+, or $choline^+$, and after 8 min (arrow) again switched to buffer without inhibitors ($n = 3 – 5$). Perfusion with 5 µM ASP^+ served as control ($n = 5$).

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Fig. 1

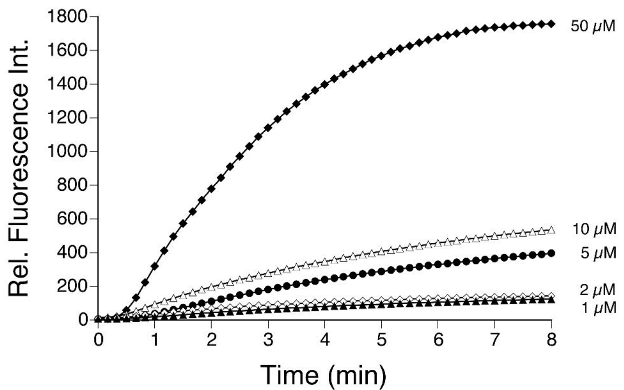
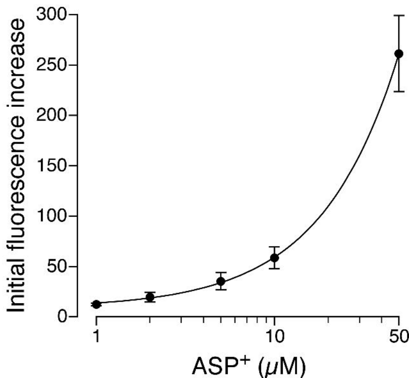


Fig. 2



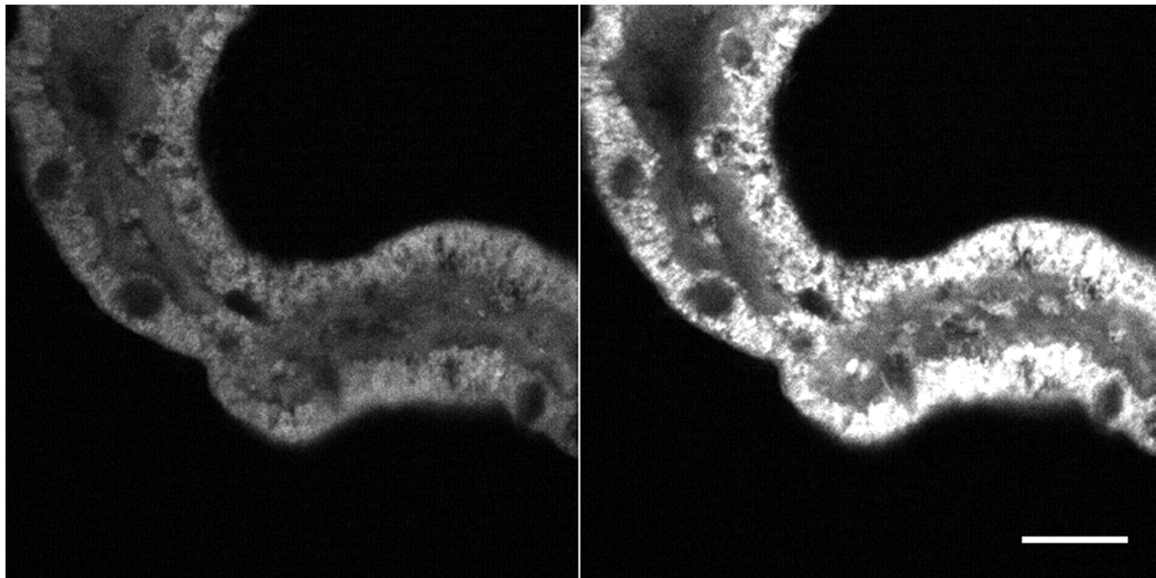


Fig. 3

Fig. 4

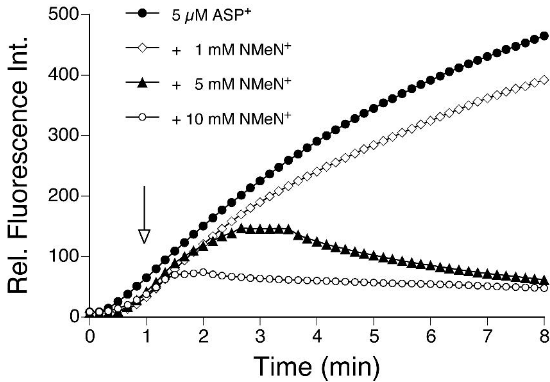


Fig. 5