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The Organic Cation Transporters rOCT1 and hOCT2 Are Inhibited by cGMP

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Abstract. The electrogenic cation transporters OCT1 and OCT2 in the basolateral membrane of renal proximal tubules mediate the first step during secretion of organic cations. Previously we demonstrated stimulation and change of selectivity for rat OCT1 (rOCT1) by protein kinase C. Here we investigated the effect of cGMP on cation transport by rOCT1 or human OCT2 (hOCT2) after expression in human embryonic kidney cells (HEK293) or oocytes of *Xenopus laevis*. In HEK293 cells, uptake was measured by microfluorimetry using the fluorescent cation 4-(4-(dimethyl-amino)styryl)-N-methylpyridinium iodide (ASP^+) as substrate, whereas uptake into *Xenopus laevis* oocytes was measured with radioactively labelled cations. In addition, ASP^+-induced depolarizations of membrane voltages (V_m) were measured in HEK293 cells using the slow whole-cell patch-clamp method. Incubation of rOCT1-expressing HEK293 cells for 10 min with 100 μM 8-Br-cGMP reduced initial ASP^+ uptake by maximally 78% with an IC_{50} value of 24 ± 16 μM. This effect was not abolished by the specific PKG inhibitor KT5823, indicating that a cGMP-dependent kinase is not involved. An inhibition of ASP^+ uptake by rOCT1 in HEK293 cells was also obtained when the cells were incubated for 10 min with 100 μM cGMP, whereas no effect was obtained when cGMP was given together with ASP^+. ASP^+ (100 μM)-induced depolarizations of V_m were reduced in the presence of 8-Br-cGMP (100 μM) by 44 ± 11% ($n = 6$). Since it could be demonstrated that [^{3}H]cGMP is taken up by an endogeneous cyanine863-inhibitable transporter, the effect of cGMP is probably mediated from inside the cell. Uptake measurements with [^{14}C]tetraethylammonium and [^{3}H]2-methyl-4-phenylpyridinium in *Xenopus laevis* oocytes expressing rOCT1 performed in the absence and presence of 8-Br-cGMP showed that cGMP does not interact directly with the transporter. The data suggest that the inhibition mediated by cGMP observed in HEK293 cells occurs most likely via a mammalian cGMP-binding protein that interacts with OCT1-2 transporters.

Key words: Organic cation transport — HEK293 cells — Microfluorimetry — Regulation — ASP^+ — Nucleotides — Membrane voltage

Introduction

Several organic cation transporters that belong to a new transporter family (OCT1-family) have been cloned since the first member of this family was identified in 1994 (Gorboulev et al., 1997; Gründemann et al., 1997; Lopez-Nieto et al., 1997; Zhang et al., 1997b, 1997a; Koepsell, 1998; Terashita et al., 1998; Zhang, Brett & Giacomini, 1998; Koepsell et al., 1999; Dresser, Leabman & Giacomini, 2001). The organic cation transporters of this family contain three subtypes of electrogenic organic cation transporters (OCT1, OCT2, OCT3), an electroneutral cation transporter that may be a cation-proton antiporter (OCTN1) and a high-affinity Na^+-carnitine cotransporter that also translocates organic cations (Ohashi et al., 2001). The organic cation transporters translocate a variety of organic cations such as endogenous cationic metabolites, monoamine neurotransmitters, cationic drugs and xenobiotics. They are expressed in renal proximal tubules, small intestinal enterocytes, hepatocytes and neurons (Koepsell, 1998; Koepsell et al., 1999). The electrogenic transporters OCT1 and OCT2 have been localized to the sinusoidal

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membrane of hepatocytes (OCT1) and to the basolateral membrane of renal proximal tubules (Meyer-Wentrup et al., 1998; Sugawara-Yokoo et al., 2000; Karbach et al., 2001). They translocate cations in both directions and may mediate cellular uptake or release of organic cations dependent on the electrochemical driving force and substrate selectivity that may depend on the regulatory state of the transporter (Budiman et al., 2000; Mehrens et al., 2000; Arndt et al., 2001). Whereas OCTN2 has been localized to the brush-border membrane of renal proximal tubules (Meyer-Wentrup et al., 1998; Tamai et al., 1998; Wu et al., 1998), the membrane localization of OCTN1 and OCT3 has not been determined. While there is growing information on functional characteristics and localization of the organic cation transporters (Koepsell et al., 1999; Budiman et al., 2000; Arndt et al., 2001; Dresser et al., 2001), the examination of their regulation has just begun. We demonstrated that the OCT1 from rat (rOCT1) is regulated by the protein kinases PKA and PKC and by the $p56^{lck}$ tyrosine kinase (Mehrens et al., 2000). The activation of PKC leads to phosphorylation of the transporter and an increase in the affinity of this transporter for various substrates. Recently we reported that the uptake of organic cations across the basolateral membrane of isolated human proximal tubules is regulated by PKA, PKC and also by atrial natriuretic peptide (ANP) (Pietig et al., 2001). This transport is most likely mediated by the hOCT2. In a human proximal tubular cell line (IHKE-1) we observed stimulation of organic cation transport across the apical membrane by PKA, PKC and cGMP-dependent protein kinase PKG (Hohage et al., 1998). So far the role of cGMP or cGMP-activating peptides for the regulation of transport has not been studied with cloned transporters of the OCT1-family.

Here we examine the regulation of the rat organic cation transporter rOCT1 and the human form of the OCT2 (hOCT2) by cGMP. Transporters were stably expressed in transfected human embryonic kidney cells (HEK293). Employing dynamic fluorescence microscopy with the fluorescent organic cation 4-(4-(dimethylamino)styryl)-N-methylpyridinium (ASP^+) as substrate or electrophysiological analysis of membrane voltages (V_m), inhibition of cation transport by cGMP or 8-Br-cGMP was observed.

Materials and Methods

Cell Culture

HEK293-cells (CRL-1573, ATCC, Manassas, VA, USA) were stably transfected with rOCT1 (rOCT1-HEK293 cells) or hOCT2 (hOCT2-HEK293 cells), as described before (Busch et al., 1996; Busch et al., 1998). Cells (passages 17–38) were grown in 50-ml tissue culture flasks (Greiner, Frickenhausen, Germany) in Dulbeccco's modified Eagle's medium containing 10% (v/v) fetal calf serum and 0.6 mg/ml geneticin (GibcoBRL, Eggenstein, Germany). Cells were incubated at 37°C in an atmosphere of 95% air plus 5% CO_2. After 7 days the confluent monolayers were trypsinized with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline and 0.05% trypsin-EDTA (Biochrom, Berlin, Germany). For the fluorescence and patch-clamp experiments, cells were seeded on glass cover slips with areas of 2.25 cm^2. Cell confluency was reached after 3 to 5 days and measurements were carried out after 4 to 8 days.

ASP^+-Fluorescence Measurements in rOCT1-HEK293 and hOCT2-HEK293 Cells

Microfluorimetry was performed as described before, using an inverted microscope (Axiovert 135, Zeiss, Oberkochen, Germany) equipped with a 100× oil immersion objective (Stachon et al., 1996; Stachon, Schlatter & Hohage, 1997; Mehrens et al., 2000). The excitation light generated by a xenon-quartz lamp was passed through a 450–490 nm bandpass filter mounted in a filter wheel (U. Fröbe, Universität Freiburg, Germany) rotating at 10 Hz to generate a pulsating excitation light. The excitation light was reflected by a dichroic mirror (560 nm) to a perfusion chamber, with the cell monolayers on cover slips forming the bottom of the chamber. The cells were superfused at a rate of 10 ml/min with an HCO_3^--free Ringer solution containing in mM: NaCl 145; KH_2PO_4 0.4; K_2HPO_4 1.6; D-glucose 5; $MgCl_2$ 1; Ca-gluconate 1.3 and pH adjusted to 7.4 at 37°C. Fluorescence emission was measured after passing through a 575–640-nm bandpass filter by a photon counting tube (Hamamatsu H3460-04, Herrsching, Germany). Experiments were controlled and data analyzed with a computer-aided system and specific software (U. Fröbe). As a measurement of ASP^+ (0.5 μM) uptake, the increase of cellular fluorescence was plotted versus time. The signals of ten pulses every second were averaged giving a time resolution of 1 Hz. Since ASP^+ fluorescence is bleached by light, the whole device was protected from light. The initial slope of the fluorescence increase (linearly fitted during the first 30 seconds) was analyzed to quantify ASP^+ transport rates. Background fluorescence of 20 to 50 photon counts/sec, measured for each monolayer in the absence of ASP^+, was subtracted from every experiment. The fluorescence signal was obtained from approximately 5 cells using an adjustable iris diaphragm. A comparable number of control experiments and experiments with the test substances was always performed on the same day with cells of the same passage and age to reduce variability between the unpaired observations.

Patch-Clamp Studies in rOCT1-HEK293 Cells

Membrane voltages (V_m) were measured with the slow whole-cell patch-clamp technique (Kleta, Mohrmann & Schlatter, 1995). For this method, pipettes were filled with a solution containing (in mM): K^+-gluconate 95, KC1 30, Na_2HPO_4 4.8, NaH_2PO_4 1.2, D-glucose 5, Ca^{2+}-gluconate 0.73, ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) 1, $MgCl_2$ 1.03, ATP 1, pH 7.2. To this solution, 162 μM nystatin was added before use. V_m was measured in the current-clamp mode of a patch-clamp amplifier (U. Fröbe, Physiologisches Institut, Universität Freiburg, Germany) and recorded continuously on a pen recorder (WeKaGraph WK-250R, WKK, Kaltbrunn, Switzerland).

[^{3}H]cGMP-Uptake Studies in rOCT1-HEK293 Cells

The tracer uptake studies in rOCT1-HEK293 cells were performed with suspended cells as described earlier (Busch et al., 1996, 1998).

Confluent cells were washed and suspended at 37°C in PBS or K^+ buffer (139.7 mM KCl, 8 mM K_2HPO_4, 1.6 mM KH_2PO_4, pH 7.4). For uptake measurements, the cells were incubated for different time periods at 37°C in the absence or presence of 40 μM cyanine 863 with PBS or K^+ buffer containing 20 nM [^{3}H]cGMP. Some measurements were also performed in the presence of 100 μM probenecid. Uptake reactions were stopped with icecold PBS containing 100 μM quinine.

Tracer Flux Measurements in *Xenopus laevis* Oocytes Expressing rOCT1

rOCT1 was expressed in oocytes of *Xenopus laevis* and uptake measurements with radioactively labeled tetraethylammonium (TEA) or 1-methyl-4-phenylpyridinium (MPP) were performed as described earlier (Arndt et al., 2001). 10 ng of rOCT1 cRNA per oocyte were injected and the oocytes were incubated 2 days at 16°C in Ori buffer (5 (mM) 3-(*N*-morpholino)propanesulfonic acid-NaOH, pH 7.4, 100 NaCl, 3 KCl, 2 $CaCl_2$ and 1 $MgCl_2$). For uptake measurements, oocytes were incubated at room temperature for different times with Ori buffer containing either 10 μM [^{14}C]TEA or 0.5 μM [^{3}H]MPP. Parallel measurements were performed in the presence of 100 μM or 500 μM 8-Br-cGMP or in the presence of 100 μM cyanine 863.

Biochemicals

All standard chemicals, cGMP, 8-Br-cGMP, GMP, GDP, GTP, quinine, cyanine 863 and nystatin were obtained in highest available purity from Sigma (Taufkirchen, Germany); KT5823 was purchased from Calbiochem (Bad Soden, Germany), ASP^+ from Molecular Probes (Leiden, The Netherlands) and [^{3}H]cGMP (1.26 TBq/mmol) from Amersham Buchler (Braunschweig, Germany). [^{14}C]TEA (1.9 TBq/mmol) and [^{3}H]MPP (3.1 TBq/mmol) were obtained from Biotrend (Köln, Germany).

Statistics

Results are presented as mean values ± SEM. In the ASP^+ fluorescence measurements, *n* refers to the number of monolayers examined, in the patch-clamp experiments, *n* refers to the number of cells and is indicated in parenthesis. Uptake of [^{3}H]cGMP in HEK293 cells was calculated from four determinations in the absence and four determinations in the presence of 100 μM cyanine 863. For measurements of [^{14}C]TEA or [^{3}H]MPP uptake in *X. laevis* oocytes, 8–10 oocytes were analyzed per data point. IC_{50} values for inhibition of ASP^+ uptake by cGMP or 8-Br-cGMP and values for maximal inhibition were calculated by fitting the Hill equation for multisite inhibition to the data. Paired (patch-clamp experiments) and unpaired Student's *t*-test was used to test for statistical significance of effects and $P < 0.05$ was set as the significance level.

Results

Inhibition of ASP^+-Uptake in rOCT1-HEK293 Cells by 8-Br-cGMP and cGMP that is Independent of Protein Kinase G

The time-dependent accumulation of ASP^+ (0.5 μM) by rOCT1-HEK293 cells is shown in Fig. 1 for cells of 17 monolayers under control conditions. After a preincubation (10 min, 37°C) of 18 different monolayers from the same passages and days as the controls with 100 μM of the membrane-permeable second messenger analog of cGMP, 8-Br-cGMP, the cellular ASP^+-accumulation was significantly slowed down and reached a fluorescence intensity after 5 min of only around 20% compared to control conditions (Fig. 1). 8-Br-cGMP was continuously present throughout these experiments. Included in this figure is also the summary of 15 experiments, in which the monolayers were coincubated with 8-Br-cGMP (100 μM) and the specific protein kinase G (PKG)-inhibitor KT5823 (1 μM), demonstrating that blockade of PKG does not influence the 8-Br-cGMP-mediated inhibition of ASP^+-accumulation. The activity of KT5823 as PKG inhibitor was verified in several other preparations, including HEK293-cells in our laboratory. The data indicate that the effect of 8-Br-cGMP is not mediated by phosphorylation via PKG. Figure 2 shows the concentration response curves for the inhibition of initial ASP^+-uptake in HEK293 cells expressing rOCT1 by 8-Br-cGMP or cGMP. The cells were preincubated for 10 min with 8-Br-cGMP or cGMP before the uptake measurements with ASP^+ were performed. For inhibition, IC_{50} values of 24 ± 16 μM (8-Br-cGMP) and 16 ± 3 μM (cGMP) were calculated. The inhibition obtained with 100 μM 8-Br-cGMP or cGMP was 78 ± 4% and 40 ± 4%, respectively. To examine whether preincubation with cGMP is required for inhibition, cGMP (100 μM) was only added together with ASP^+ without prior incubation in another experimental series. The left column in Fig. 3 summarizes measurements from 25 monolayers and shows that without preincubation, ASP^+-uptake by rOCT1-HEK293 cells was not inhibited by cGMP.

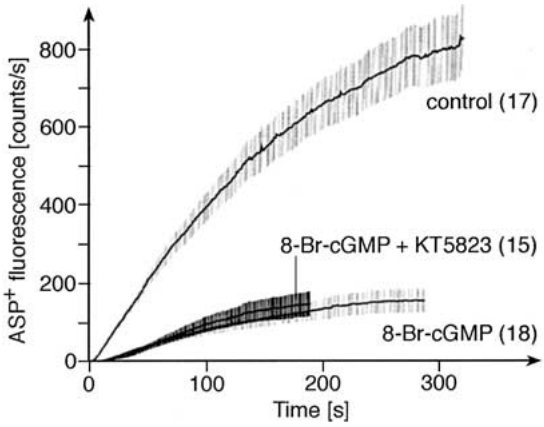

Fig. 1. Effect of 8-Br-cGMP on the cellular fluorescence increase in rOCT1-HEK293 cells after addition of ASP^+ (0.5 μM). rOCT1-HEK293 cells were incubated for 10 min with 100 μM 8-Br-cGMP or with 100 μM 8-Br-cGMP plus 1 μM KT5823. At time zero, 0.5 μM of ASP^+ was added and fluorescence change was analyzed. The curves represent the mean values ± SEM of several separate experiments. The numbers of monolayers used under different experimental conditions are indicated in parenthesis.

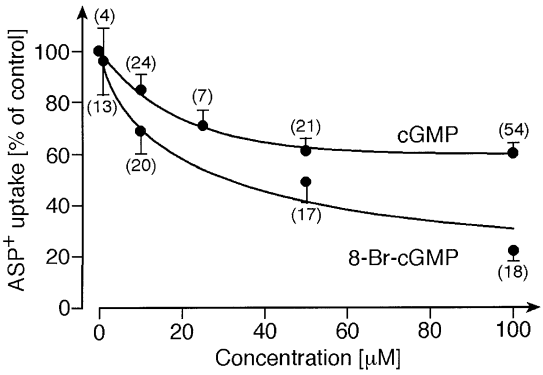

Fig. 2. Concentration-response curves of the inhibition of initial ASP^+ uptake in rOCT1-HEK293 cells by cGMP or 8-Br-cGMP. The experiments were performed as in Fig. 1. Here, the cells were incubated for 10 min with different concentrations of cGMP or 8-Br-cGMP and the initial slope of fluorescence change (uptake) was measured after the addition of 0.5 μM ASP^+. Data are presented as percentages of initial slope of uptake that was measured in the control experiments. The control experiments were performed on the same day with cells of the same passage and age. Mean values ± SEM are presented, and the number of monolayers used for each concentration is given in parenthesis. The curves were obtained by fitting the Hill equation to the data. The difference between ASP^+ uptake in the presence of cGMP or 8-Br-cGMP reached significance at nucleotide concentrations of 50 μM.

This excludes a direct extracellular interaction of cGMP with the transporter.

Lack of Effects of Other Nucleotides on ASP^+ Uptake by rOCT1-HEK293 Cells

To examine whether the observed effect of cGMP on ASP^+ uptake is specific for the cyclic form of this nucleotide, monolayers were preincubated for 10 min with 100 μM of GMP (n = 11), GDP (n = 13) or GTP (n = 11) and initial ASP^+ uptake was measured in the presence of the nucleotides (Fig. 3). At variance to cGMP, these nucleotides did not significantly reduce initial ASP^+ uptake.

cGMP also Inhibits ASP^+ Uptake by HOCT2-HEK293 Cells

Next we performed comparable experiments with hOCT2-HEK293 cells (Fig. 3), to evaluate whether the observed inhibition of rOCT1-mediated transport by cGMP is restricted to this member of the OCT1-family. Preincubation of monolayers for 10 min with 100 μM cGMP significantly reduced initial ASP^+ uptake by hOCT2, measured in the continued presence of cGMP by 17 ± 6% (n = 20) compared to control condition. This inhibition was significantly lower than the inhibition of ASP^+ transport in rOCT1-HEK293 cells, indicating some subtype selectivity. If the preincubation time was extended to 30 min, the inhibition of ASP^+-uptake increased to 33 ± 6% (n = 10). As in

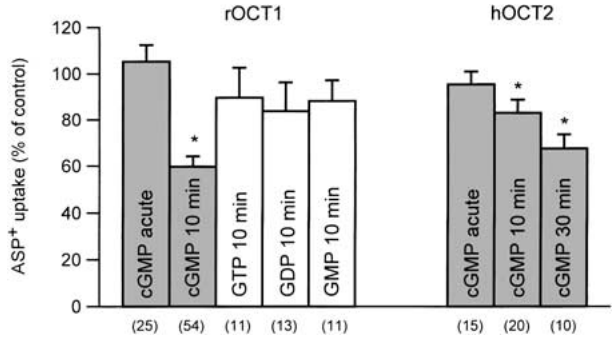

Fig. 3. Effects of cGMP without and with preincubation on initial ASP^+ uptake in HEK293 cells expressing rOCT1 or hOCT2, and nucleotide specificity of the cGMP effect on rOCT1. rOCT1-HEK293 cells or hOCT2-HEK293 cells were incubated for 10 min with 100 μM cGMP and the initial uptake of 0.5 μM ASP^+ was determined in comparison to control cells, as described in Fig. 2. In contrast to cGMP inhibition after 10 min preincubation (cGMP 10 min), no effect on ASP^+ uptake by rOCT1 or hOCT2 was observed when 100 μM cGMP was added simultaneously with ASP^+ (cGMP acute). In hOCT2-HEK293 cells, the cGMP effect was increased when the preincubation period was prolonged to 30 min (cGMP 30 min). To test the cGMP effect on ASP^+ uptake by rOCT1-HEK293 cells for nucleotide specificity, the cells were preincubated 10 min with GTP, GDP or GMP and the initial ASP^+ uptake was measured. The data are presented as mean values ± SEM (number of monolayers). Mean values that differed significantly from the controls are indicated by asterisks.

rOCT1-HEK293 cells, no significant effect of cGMP on initial ASP^+ uptake was observed when preincubation was omitted and cGMP was added simultaneously with ASP^+ (n = 15).

cGMP also Inhibits ASP^+-Induced Depolarizations of rOCT1-HEK293 Cells

To support the finding of a cGMP-mediated inhibition of ASP^+-uptake in the microfluorescence experiments, the effect of 8-Br-cGMP on ASP^+-induced depolarizations of V_m of rOCT1-HEK293 cells was examined. As shown before (Mehrens et al., 2000), ASP^+ (100 μM) depolarized V_m, indicating the electrogenic transport of ASP^+. As shown in Fig. 4, this depolarization was significantly reduced after incubation with 8-Br-cGMP by 44 ± 11% in 6 paired experiments. Basal V_m in these cells was −44 ± 3 mV.

Cyanine-Inhibitable Uptake of [^{3}H]cGMP in HEK293 Cells

To examine the hypothesis that preincubation with cGMP is required to observe inhibition of rOCT1 or hOCT2 because cGMP must be taken up before it can interact from the intracellular side, we measured the uptake of [^{3}H]cGMP in nontransfected and rOCT1-transfected HEK293 cells. In nontransfected and transfected cells, similar time-dependent uptake of [^{14}H]cGMP was observed between 1–30 min

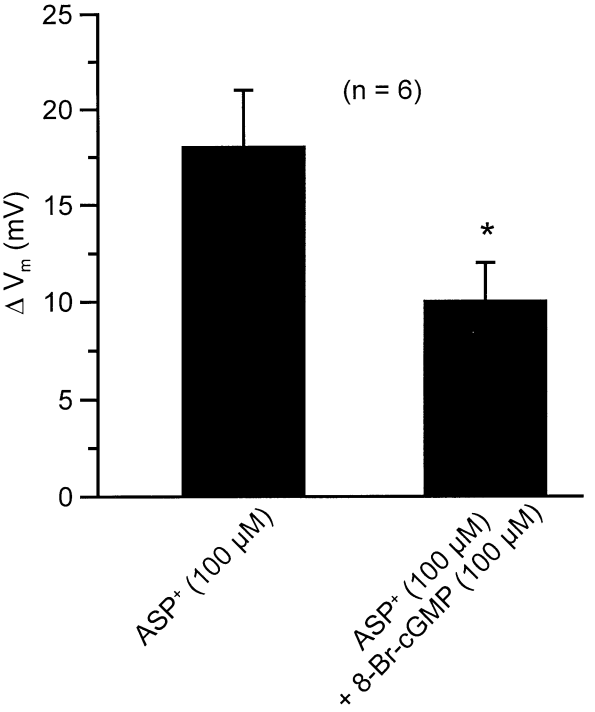

Fig. 4. Effect of 8-Br-cGMP (100 μM) on membrane depolarizations (ΔV_m) induced by ASP^+ (100 μM). The data are mean values ± SEM of 6 paired measurements where ASP^+ was added before and 10 minutes after 8-Br-cGMP, with the nucleotide still present. The statistically significant difference between the two bars is indicated by the asterisks.

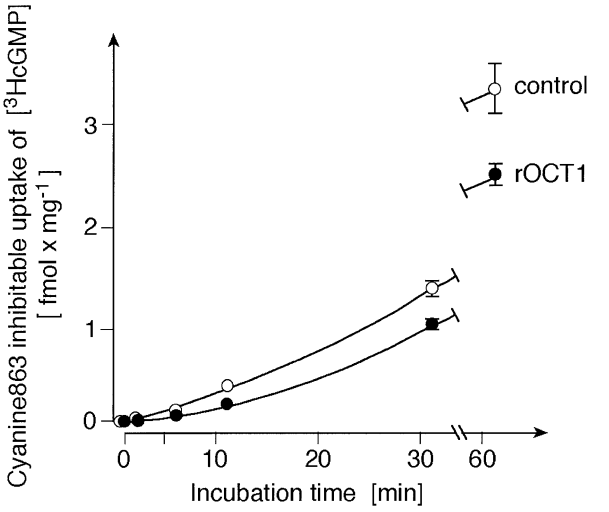

Fig. 5. Cyanine863-inhibitable uptake of [^{3}H]cGMP by HEK293 cells. Nontransfected HEK293 cells (control) and rOCT1-HEK293 cells (rOCT1) were incubated at 37°C with 20 nM of [^{3}H]cGMP in the absence and presence of 40 μM cyanine863. After different time intervals, the uptake was stopped and [^{3}H]cGMP in the cells was analyzed. Four parallel uptake measurements without cyanine683 and with cyanine863 were performed for each time point. The measurements without and with cyanine were subtracted and mean values ± SEM are presented.

incubation. This uptake showed no saturation up to 100 μM cGMP and was inhibited by 50–75% when the measurements were performed in the presence of 40 μM cyanine863. In Fig. 5 we present the time courses of the cyanine863-inhibited fraction of [^{3}H]cGMP uptake that were observed in nontransfected HEK293 cells compared to rOCT1-transfected HEK293 cells. After transfection with rOCT1, the cyanine-inhibited cGMP accumulation was, if anything, reduced compared to nontransfected cells, indicating that rOCT1 itself does not transport cGMP (*see* also below). In nontransfected HEK293 cells cyanine-inhibited uptake of [^{3}H]cGMP was not significantly different whether Na^+ or K^+ was the main cation in the incubation buffer (*data not shown*). Furthermore, changing the pH from 7.4 to 8.0 did not alter the [^{3}H]cGMP accumulation, and [^{3}H]cGMP uptake was not inhibited by 100 μM probenecid (*data not shown*). The data suggest that HEK293 cells contain an endogeneous Na^+-independent and pH-insensitive low-affinity uptake system for cGMP.

No Inhibition of rOCT1 Expressed in *Xenopus laevis* Oocytes by 8-Br-cGMP

To further characterize the PKG-independent inhibition of rOCT1 by cGMP and 8-Br-cGMP that was observed in HEK293 cells, we investigated whether this effect is also observed when rOCT1 is expressed in non-mammalian cells. We expressed rOCT1 in oocytes of *X. laevis* and investigated the effect of 8-Br-cGMP on organic cation transport employing tracer-flux measurements. Since previous data indicated that point mutations in rOCT1 differentially changed the interaction of different transported cations (e.g., of TEA and MPP) (Gorboulev et al., 1999) and that the activation of rOCT1 by protein kinase C changed the selectivity of rOCT1 for cations (Mehrens et al., 2000), we performed these measurements with [^{14}C] TEA and [^{3}H] MPP. Figure 6 shows that cyanine-inhibited uptake of TEA or MPP could not be inhibited by 100 μM or 500 μM 8-Br-cGMP. This indicates 8-Br-cGMP does not directly interact with rOCT1, neither from the extracellular nor from the intracellular side. This was important since we previously observed a much higher affinity of quinine to rOCT2 from the intracellular than from the extracellular side (Arndt et al., 2001). The result suggests the effect of cGMP and 8-Br-cGMP on rOCT1 and hOCT2 in HEK293 cells may be mediated by a cGMP-binding protein that is not present or effective in oocytes.

Discussion

In this study we analyzed transport activity of rOCT1 or hOCT2 expressed in HEK293 cells by measuring

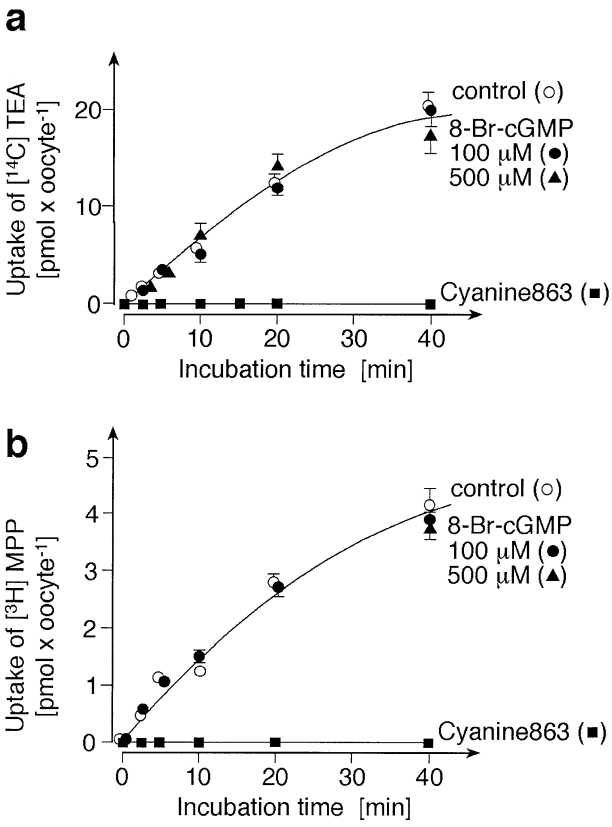

Fig. 6. Insensitivity against 8-Br-cGMP of organic cation uptake by rOCT1 expressed in *Xenopus laevis* oocytes. rOCT1 was expressed in oocytes of *X. laevis* and the uptake of 10 μM [^{14}C]TEA or 0.5 μM [^{3}H]MPP after different times of incubation was measured in the absence of nucleotides and cyanine863 (control), in the presence of 100 μM (●) or 500 μM 8-Br-cGMP (▲) or in the presence of 100 μM cyanine863 (■). The uptake of TEA or MPP in rOCT1-expressing oocytes measured in the presence of cyanine863 was not significantly different from the uptake in non-injected control oocytes. Mean values ± SEM of 8–10 oocytes are presented.

the change in cellular fluorescence after addition of the fluorescent substrate ASP^+ to the superfusion solution. As described before, the initial slope of the cellular ASP^+ fluorescence increase directly reflects transport of ASP^+ by rOCT1 or hOCT2 (Mehrens et al., 2000). With this method we recently observed that rOCT1 is stimulated by PKC, PKA, and $p56^{lck}$ tyrosine kinase (Mehrens et al., 2000). We now demonstrated that the transport activity of the organic cation transporters rOCT1, and to a lesser degree also of hOCT2, was decreased after elevation of cellular cGMP levels. In our experiments, intracellular cGMP was increased by adding the cGMP analog 8-Br-cGMP to the medium. However, a similar effect was obtained when the HEK293 cells were preincubated for at least 10 min with 100 μM cGMP. The inhibition of rOCT1 activity by 8-Br-cGMP was further demonstrated by the decrease of the ASP^+-induced depolarization of V_m of rOCT1-HEK293 cells after addition of the cyclic nucleotide. These parallel findings obtained with two completely different methods demonstrate that ASP^+ is electrogenically taken up by rOCT1.

Uptake measurements with [^{3}H]cGMP showed that cGMP was taken up by a cyanine863-inhibitable cGMP uptake system in the HEK293 cells. Since immunohistochemical and functional data showed that rOCT1 and hOCT2 are localized in the basolateral membrane of renal proximal tubules (Sugawara-Yokoo et al., 2000; Karbach et al., 2001; Pletig et al., 2001) the cGMP effects on transfected HEK293 cells grown on cover slips may have parallels to our previous observation that organic cation uptake across the basolateral membrane of isolated human proximal tubules was inhibited by atrial natriuretic peptide (Pietig et al., 2001). Interestingly, the inhibitory effect of 8-Br-cGMP was independent of cGMP-dependent protein kinase and was not observed when rOCT1 was expressed in *Xenopus* oocytes. From these data we conclude that cGMP does not interact directly with rOCT1 and that the inhibition of rOCT1 by cGMP observed in HEK293 cells is mediated via a cGMP-binding protein. This cGMP-binding protein may not be present or functional in *X. laevis* oocytes. The inhibition of rOCT1-mediated cation transport in HEK293 cells by 8-Br-cGMP or cGMP is supposed to be specific for this transporter family and perhaps other groups of transporters, but it is not generally observed, as, e.g., a cGMP-regulated K^+ channel did not show this regulation after expression in HEK293 cells (Schrøder et al., 2000). The inhibition of organic cation transport by cGMP via a change of the driving force can also be excluded, as an increase in cellular cGMP by ANP (100 nM) or 8-Br-cGMP (100 μM) led to a depolarization of the cells by only 2.5 ± 0.5 mV (n = 10) or 4.0 ± 3.0 mV (n = 6), respectively. This voltage change is far too small to account for the observed inhibition of ASP^+ uptake. In the proximal tubule, cGMP-dependent regulation of transport has been described in several studies, where either ANP or nitric oxide donors had been used to increase cytosolic cGMP concentrations. Several groups reported an inhibition of Na^+- and fluid-reabsorption in the proximal tubule via cGMP-dependent signalling pathways (Cantiello & Ausiello, 1986; Harris et al., 1996; Wang, 1997; Eitle et al., 1998; Linas & Repine, 1999). These effects may be mediated by an inhibition by ANP of the Na^+/H^+ exchanger or the Na^+-K^+-ATPase (Reddy et al., 1994; Guzman et al., 1995; Roczniak & Burns, 1996); also inhibition of albumin reabsorption (Jacobs et al., 1999) or phosphate reabsorption (Hammond et al., 1985) has been reported. At variance, an activation of Cl^- channels by ANP has been observed in rat proximal tubular cells (Darvish, Winaver & Dagan, 1995). In human cultured proximal tubule cells (IHKE-1), we previously demonstrated an activation of organic cation transport across the apical membrane by ANP via stimulation of a cGMP-de-

pendent protein kinase (Hohage et al., 1998). Thus, increases in cellular cGMP concentrations, either by activation of the membrane-bound particulate or the cytosolic soluble guanylate cyclases present in proximal tubular cells, lead to both a reduction or an activation of various transport processes in this nephron segment. In most of these studies, the involvement of a cGMP-dependent protein kinase in the transduction pathway, as is classical for ANP or NO, has not been addressed specifically so that the exact mechanism for the action of cytosolic cGMP remained unclear. We could recently demonstrate that the effect of ANP on K^+ channels in a human proximal tubular cell line is independent of such a kinase (Hirsch et al., 1999) and provided evidence that this K^+ channel can be directly inhibited by extracellular cGMP (Hirsch et al., 2001).

Taken together, based on the presented findings, an increase in cellular cGMP in the proximal tubule mediated by either natriuretic peptides or by NO could lead to a reduction in the uptake of organic cations across the basolateral membrane by inhibition of OCTs. Additional experiments are necessary to elucidate in what way the functional properties of the organic cation transporters are changed during this inhibition, and to define the specificity of the transporter for cyclic nucleotides in HEK293 cells.

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References

Arndt, P., Volk, C., Gorboulev, V., Budiman, T., Popp, C., Ulzheimer-Teuber, I., Akhoundova, A., Koppatz, S., Bamberg, E., Nagel, G., Koepsell, H. 2001. Interaction of cations, anions, and weak base quinine with rat renal cation transporter rOCT2 compared with rOCT1. *Am. J. Physiol.* **281:**F454–F468

Budiman, T., Bamberg, E., Koepsell, H., Nagel, G. 2000. Mechanism of electrogenic cation transport by the cloned organic cation transporter 2 from rat. *J. Biol. Chem.* **275:**29413–29420

Busch, A.E., Karbach, U., Miska, D., Gorboulev, V., Akhoundova, A., Volk, C., Arndt, P., Ulzheimer, J.C., Sonders, M.S., Baumann, C., Waldegger, S., Lang, F., Koepsell, H. 1998. Human neurons express the polyspecific cation transporter hOCT2, which translocates monoamine neurotransmitters, amantadine, and memantine. *Mol. Physiol.* **54:**342–352

Busch, A.E., Quester, S., Ulzheimer, J.C., Gorboulev, V., Akhoundova, A., Waldegger, S., Lang, F., Koepsell, H. 1996. Monoamine neurotransmitter transport mediated by the polyspecific cation transporter rOCT1. *FEBS* **395:**153–156

Cantiello, H.F., Ausiello, D.A. 1986. Atrial natriuretic factor and cGMP inhibit amiloride-sensitive Na^+ transport in the cultured renal epithelial cell line, LLC-PK1. *Biochem. Biophys. Res. Commun.* **134:**852–860

Darvish, N., Winaver, J., Dagan, D. 1995. A novel cGMP-activated Cl^- channel in renal proximal tubules. *Am. J. Physiol.* **268:**F323–F329

Dresser, M.J., Leabman, M.K., Giacomini, K.M. 2001. Transporters involved in the elimination of drugs in the kidney: Organic anion transporters and organic cation transporters. *J. Pharm. Sci.* **90:**397–421

Eitle, E., Hiranyachattada, S., Wang, H., Harris, P.J. 1998. Inhibition of proximal tubular fluid absorption by nitric oxide and atrial natriuretic peptide in rat kidney. *Am. J. Physiol.* **274:** C1075–C1080

Gorboulev, V., Ulzheimer, J.C., Akhoundova, A., Ulzheimer-Teuber, I., Karbach, U. 1997. Cloning and characterization of two human polyspecific organic cation transporters. *DNA Cell Biol.* **16:**871–881

Gorboulev, V., Volk, C., Arndt, P., Akhoundova, A., Koepsell, H. 1999. Selectivity of the polyspecific cation transporter rOCT1 is changed by mutation of aspartate 475 to glutamate. *Mol. Pharmacol.* **56:**1254–1261

Gründemann, D., Babin-Ebell, J., Martel, F., Örding, N., Schmidt, A., Schömig, E. 1997. Primary structure and functional expression of the apical organic cation transporter from kidney epithelial LLC-PK_1 cells. *J. Biol. Chem.* **272:**10408–10413

Guzman, N.J., Fang, M.Z., Tang, S.S., Ingelfinger, J.R., Garg, L.C. 1995. Autocrine inhibition of Na^+/K^+-ATPase by nitric oxide in mouse proximal tubule epithelial cells. *J. Clin. Invest.* **95:**2083–2088

Hammond, T.G., Yusufi, A.N.K., Knox, F.G., Dousa, T.P. 1985. Administration of atrial natriuretic factor inhibits sodium-coupled transport in proximal tubules. *J. Clin. Invest.* **75:**1983– 1989

Harris, P.J., Hiranyachattada, S., Antoine, A.M., Walker, L., Reilly, A.M., Eitle, E. 1996. Regulation of renal tubular sodium transport by angiotensin II and atrial natriuretic factor. *Clin. Exp. Pharmacol. Physiol.* **23:**S112–S118

Hirsch, J.R., Weber, G., Kleta, I., Schlatter, E. 1999. A novel cGMP-regulated K^+ channel in immortalized human kidney epithelial cells (IHKE-1). *J. Physiol.* **519:**645–655

Hirsch, J.R., Weber, G., Kleta, I., Schlatter, E. 2001. cGMP serves as an extracellular regulator of a Ca^{2+}-dependent K^+ channel in immortalized human proximal tubule cells. *Cell. Physiol. Biochem.* **11:**77–82

Hohage, H., Stachon, A., Feidt, C., Hirsch, J.R., Schlatter, E. 1998. Regulation of organic cation transport in IHKE-1 and LLC-PK_1 cells. Fluorimetric studies with 4-(4-dimethylaminostyryl)-N-methylpyridinium. *J. Pharmacol. Exp. Ther.* **286:**305–310

Jacobs, E.M.G., Vervoort, G., Branten, A.J.W., Klasen, I., Smits, P., Wetzels, J.F.M. 1999. Atrial natriuretic peptide increases albuminuria in type I diabetic patients: evidence for blockade of tubular protein reabsorption. *Eur. J. Clin. Invest.* **29:**109–115

Karbach, U., Kricke, J., Meyer-Wentrup, F., Gorboulev, V., Volk, C., Loffing-Cueni, D., Kaissling, B., Bachmann, S., Koepsell, H. 2001. Localization of organic cation transporters OCT1 and OCT2 in rat kidney. *Am. J. Physiol.* **279:**F679–F687

Kleta, R., Mohrmann, M., Schlatter, E. 1995. Effects of cell differentiation on ion conductances and membrane voltage in LLC-PK1 cells. *Pfluegers Arch.* **429:**370–377

Koepsell, H. 1998. Organic cation transporters in intestine, kidney, liver, and brain. *Annu. Rev. Physiol.* **60:**243–266

Koepsell, H., Gorboulev, V., Arndt, P. 1999. Molecular pharmacology of organic cation transporters in kidney. *J. Membrane Biol.* **167:**103–117

Linas, S.L., Repine, J.E. 1999. Endothelial cells regulate proximal tubule epithelial cell sodium transport. *Kidney Int.* **55:**1251–1258

Lopez-Nieto, C.E., You, G., Bush, K.T., Baros, E.J., Beier, D.R., Nigam, S.K. 1997. Molecular cloning and characterization of NKT, a gene product related to the organic cation transporter family that is almost exclusively expressed in the kidney. *J. Biol. Chem.* **272:**6471–6478

Mehrens, T., Lelleck, S., Çetinkaya, L., Knollmann, M., Hohage, H., Gorboulev, V., Bokník, P., Koepsell, H., Schlatter, E. 2000. The affinity of the organic cation transporter rOCT1 is increased by protein kinase C dependent phosphorylation. *J. Am. Soc. Nephrol.* **11:**1216–1224

Meyer-Wentrup, F., Karbach, U., Gorboulev, V., Arndt, P., Koepsell, H. 1998. Membrane localization of the electrogenic cation transporter rOCT1 in rat liver. *Biochem. Biophys. Res. Commun.* **248:**673–678

Ohashi, R., Tamai, I., Nezu, J.J., Nikaido, H., Hashimoto, N., Oku, A., Sai, Y., Shimane, M., Tsuji, A. 2001. Molecular and physiological evidence for multifunctionality of carnitine/organic cation transporter OCTN2. *Mol. Pharmacol.* **59:**358–366

Pietig, G., Mehrens, T., Hirsch, J.R., Cetinkaya, L, Piechota, H., Schlatter, E. 2001. Properties and regulation of organic cation transport in freshly isolated human proximal tubules. *J. Biol. Chem.* **276:**33741–33746

Reddy, S., György, A.Z., Dyne, M., Salipan-Moore, N., Pollock, C., Field, M.J., Cockayne, D.J.H. 1994. Effect of atrial natriuretic peptide on cellular element concentrations in rat proximal tubules: Evidence for inhibition of the sodium pump. *Clin. Exp. Pharmacol. Physiol.* **21:**775–780

Roczniak, A., Burns, K.D. 1996. Nitric oxide stimulates guanylate cyclase and regulates sodium transport in rabbit proximal tubule. *Am. J. Physiol.* **270:**F106–F115

Schrøder, R.L., Jensen, B.S., Strøbæk, D., Olesen, S.P., Christophersen, P. 2000. Activation of the human, intermediate-conductance, Ca^{2+}-activated K^+ channel by methylxanthines. *Pfluegers Arch.* **440:**809–818

Stachon, A., Hohage, H., Feidt, C., Schlatter, E. 1997. Characterization of organic cation transport across the apical membrane of proximal tubular cells with the fluorescent dye 4-Di-1-ASP. *Cell. Physiol. Biochem.* **7:**264–274

Stachon, A., Schlatter, E., Hohage, H. 1996. Dynamic monitoring of organic cation transport processes by fluorescence measurements in LLC-PK_1 cells. *Cell. Physiol. Biochem.* **6:**72–81

Sugawara-Yokoo, M., Urakami, Y., Koyama, H., Fujikura, K., Masuda, S., Saito, H., Naruse, T., Inui, K., Takata, K. 2000. Differential localization of organic cation transporters rOCT1 and rOCT2 in the basolateral membrane of rat kidney proximal tubules. *Histochem. Cell. Biol.* **114:**175–180

Tamai, I., Ohashi, R., Nezu, J., Yabuuchi, H., Oku, A., Shimane, M., Sai, Y., Tsuji, A. 1998. Molecular and functional identification of sodium ion-dependent, high affinity human carnitine transporter OCTN2. *J. Biol. Chem.* **273:**20378–20382

Terashita, S., Dresser, A., Zhang, L., Gray, A.T., Yost, S.C., Giacomelli, K. 1998. Molecular cloning and functional expression of a rabbit renal organic cation transporter. *Biochim. Biophys. Acta.* **1369:**1–6

Wang, T. 1997. Nitric oxide regulates HCO_3^- and Na^+ transport by a cGMP-mediated mechanism in the kidney proximal tubule. *Am. J. Physiol.* **272:**F242–F248

Wu, X., Prasad, P.D., Leibach, F.H., Ganapathy, V. 1998. cDNA sequence, transport function, and genomic organization of human OCTN2, a new member of the organic cation transporter family. *Biochem. Biophys. Res. Commun.* **246:**589–595

Zhang, L., Brett, C.M., Giacomini, K.M. 1998. Role of organic cation transporters in drug absorption and elimination. *Annu. Rev. Pharmacol. Toxicol.* **38:**431–460

Zhang, L., Dresser, M.J., Chun, J.K., Babbitt, P.C., Giacomini, K.M. 1997a. Cloning and functional characterization of a rat renal organic cation transport isoform (rOCT1A). *J. Biol. Chem.* **272:**16548–16554

Zhang, L., Dresser, M.J., Gray, A.T., Yost, S.C., Terashita, S., Giacomini, K.M. 1997b. Cloning and functional expression of a human liver organic cation transporter. *Mol. Pharmacol.* **51:**913–921