Regulation of human organic cation transporter hOCT2 by PKA, PI3K, and calmodulin-dependent kinases

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Çetinkaya, Ibrahim, Giuliano Ciarimboli, Gülay Yalcınkaya, Thomas Mehrens, Ana Velic, Jochen R. Hirsch, Valentin Gorboulev, Hermann Koepsell, and Eberhard Schlatter. Regulation of human organic cation transporter hOCT2 by PKA, PI3K, and calmodulin-dependent kinases. Am J Physiol Renal Physiol 284: F293–F302, 2003. First published October 15, 2002; 10.1152/ajprenal.00251.2002.—Properties and regulation of the human organic cation (OC) transporter type 2 (hOCT2) expressed in HEK-293 cells were extensively characterized using the fluorescent OC 4-[4-(dimethylamino)styryl]-N-methylpyridinium (ASP⁺). ASP⁺ uptake was electrogenic and inhibited by TPA⁺ (EC50 = 2.7 μM), tetraethylammonium (EC50 = 35 μM), cimetidine (EC50 = 36 μM), or quinine (EC50 = 6.7 μM). Stimulation with carbachol or ATP decreased initial uptake by 44 ± 3 (n = 14) and 34 ± 4% (n = 21), respectively, independently of PKC but dependent on phosphatidylinositol 3-kinase (PI3K). PKA stimulation decreased uptake by 18 ± 4% (n = 40). Inhibition of calmodulin (CaM), Ca²⁺/CaM-dependent kinase II, or myosin light chain kinase decreased uptake by 63 ± 2% (n = 15), 40 ± 4% (n = 30), and 31 ± 4% (n = 16), respectively. Inhibition of CaM resulted in a significant change in the EC50 value for the inhibition of ASP⁺ uptake by tetraethylammonium. In conclusion, we demonstrate that the hOCT2 is inhibited by PKA and PI3K and activated by a CaM-dependent signaling pathway, probably via a change in substrate affinity.

organic cation transport; human organic cation transporter type 2; human; calcium/calmodulin; calcium/calmodulin-dependent kinase II; myosin light chain kinase; phospholipase C; protein kinase C; protein kinase A; phosphatidylinositol 3-kinase; electrophysiology; fluorescence microscopy; 4-[4-(dimethylamino)styryl]-N-methylpyridinium

The term organic cations refers to a broad class of compounds of endogenous (e.g., choline, N³-methylnicotinamide, monoamine neurotransmitters such as dopamine and noradrenaline) and exogenous (e.g., cytokotatics, antibiotics, opiates, antihypertensives, antiarrhythmics, antihistamines, and sedatives) origin. The kidney is one of the most important organs in maintaining the homeostasis of organic cations (16, 17, 46). Besides substrate-specific, Na⁺-dependent organic cation transporters, Na⁺-independent polyspecific organic cation transporters (OCTs) have been postulated for the epithelia in the intestine, liver, kidney, and also for the brain (15–17, 46).

In 1994, the first organic cation transporter from the rat (rOCT1) was cloned by expression cloning (6). rOCT1 was shown to be the electrogenic organic cation transporter in the basolateral plasma membrane of kidney and liver (2, 6, 14, 21, 34). The successive identification of several OCTs of different species by homology cloning techniques (5) allowed (15–17, 19, 24, 36, 46–48) the investigation of molecular and physiological properties of single OCTs. A common feature of the OCTs is the presence of several potential protein kinase phosphorylation sites in the intracellular loops, suggesting that their activity can be subjected to regulation. We could indeed show that activation of PKC with subsequent phosphorylation of a serine residue of rOCT1 resulted in a stimulation of organic cation transport with an increase in substrate affinity and that rOCT1-mediated organic cation transport was also activated by PKA and endogenously stimulated by tyrosine kinases (21). Whereas rOCT1 is the principal OCT in the rat kidney, hOCT2 has been suggested to be the most important one in the human kidney (5). The more sensitive RT-PCR analysis showed additional transcription of hOCT2 mRNA in intestine, brain, spleen, and placenta (5). We also localized hOCT2 by RT-PCR in human isolated proximal tubules, and our functional studies with this preparation showed that hOCT2 is localized in the basolateral membrane (25). These results were recently confirmed by real-time PCR and immunohistochemical analysis of the human proximal tubule (22).

Functional characterization of hOCT2 expressed in HEK-293 cells or Xenopus laevis oocytes with tracer flux measurements and patch-clamp studies showed...
that hOCT2, like other transporters of the OCT type (OCT1, OCT2 and OCT3), mediates electrogenic transport of small organic cations such as choline, tetaethylammonium (TEA\(^{+}\)), \(N\)-methyl-nicotinamide (NMN\(^{+}\)), and 1-methyl-4-phenylpyridinium (MPP\(^{+}\)). hOCT2 and other OCT-type transporters differ in their substrate specificities and affinities (1, 5, 15, 17, 46).

Regulation of organic cation transport is important for the secretion of multiple cationic endogenous substances, drugs, and other xenobiotics and might explain the differences reported so far in substrate affinities and differences in pharmacokinetics of organic cations. To date, only a few studies have addressed the regulation of organic cation transport and none that of hOCT2. Regulation of the human extraneuronal monamine transporter hEMT (also named hOCT3) by phosphorylation/dephosphorylation mechanisms was recently shown (20). hEMT was inactivated by phosphorylation, especially by inhibition of MAP kinases, recently shown (20). hEMT was inactivated by phosphorylation, especially by inhibition of MAP kinases, recently shown (20).

The purpose of the present study was to investigate the properties of hOCT2 expressed in HEK-293 cells, with special emphasis on its regulation by protein kinases.

**METHODS**

**HEK-293 cell culture.** hOCT2 was stably expressed in HEK-293 cells (human embryonic kidney cortex cells, CRL-1573; American Type Culture Collection, Rockville, MD). Cells were grown at 37°C in 50-ml culture flasks (Greiner, Frickenhausen, Germany) in Dulbecco's modified Eagle's medium (Biochrom, Berlin, Germany) containing 3.7 g/l NaHCO\(_3\), 1.0 g/l D-glucose, and 2 mM L-glutamine (GIBCO Life Technologies, Eggenstein, Germany) gassed with 8% CO\(_2\). To this medium, 100,000 U/l penicillin, 100 mg/l streptomycin (Biochrom), 10% fetal calf serum, and 0.8 mg/ml gentamicin (GIBCO BRL/Life Technologies) were added. Experiments were performed after 4–8 days with cells grown on glass coverslips from passages 11–42. Culture and functional analysis of these cells were approved by the Landesumweltamt Nordrhein-Westfalen, Essen, Germany (521.-M-1.14/00).

**Experimental solutions.** As the superfusion solution, a HCO\(_3\)-free, Ringer-like solution was used containing (in mM) 145 NaCl, 1.6 K\(_2\)HPO\(_4\), 0.4 KH\(_2\)PO\(_4\), 5 D-glucose, 1 MgCl\(_2\), and 1.3 calcium gluconate, pH adjusted to 7.4. In some experiments, the superfusion solution was modified to (in mM) 95 NaCl, 50 KCl, 1.6 Na\(_2\)HPO\(_4\), and 0.4 NaH\(_2\)PO\(_4\) or 0 NaCl, 145 KCl, 1.6 Na\(_2\)HPO\(_4\), and 0.4 NaH\(_2\)PO\(_4\) with all other components kept constant.

**Fluorescence measurements with 4-[4-(dimethylamino)styr-**

yl]-N-methylpyridinium. As a substrate for hOCT2, the fluorescent organic cation 4-[4-(dimethylamino)styril]-N-methylpyridinium (ASP\(^{+}\)) was used (26, 29, 39). The fluorescence measurement device and experimental procedure were as customary in our laboratory (13, 21, 32, 33). In short, measurements were performed in the dark with an inverted microscope (Axiovert 135, Zeiss, Oberkochen, Germany) equipped with a ×100 oil-immersion objective. Cells were excited pulserly at 450–490 nm, and emission was measured at 575–640 nm with a photon-counting tube (Hamamatsu H 3460–04, Herrsching, Germany).

![Fig. 1](http://ajprenal.physiology.org/)

**Fig. 1.** Concentration dependence of the uptake of 4-[4-(dimethylamino)styril]-N-methylpyridinium (ASP\(^{+}\)) by HEK-293 cells expressing human organic cation transporter type 2 (hOCT2). Values are means ± SE of initial fluorescence increase (10–30 s) and maximal fluorescence, with the no. of observations in parentheses. Inset: 2 original recordings of fluorescence increase by nontransfected and hOCT2-expressing HEK-293 cells after addition of ASP\(^{+}\) (1 \(\mu\)M) to the bath solution.

After addition of ASP\(^{+}\), fluorescence increased rapidly due to cellular accumulation of ASP\(^{+}\) via hOCT2 (Fig. 1). As transport parameters, we evaluated the initial linear slope of the first 10–30 s (Fig. 1). In the initial experiments examining the concentration dependence of an ASP\(^{+}\)-induced fluorescence increase, we analyzed in addition the maximal fluorescence reached. While the initial slope directly represents the ASP\(^{+}\) uptake across the plasma membrane via hOCT2, the maximal fluorescence is the sum of ASP\(^{+}\) uptake into the cells, exit of ASP\(^{+}\) from the cells, intracellular compartmentalization with changes in the emission spectrum, and bleaching of the dye (21, 25, 32, 33). In all experiments with other substrates and regulators, only the initial slope of the curves was determined as a transport parameter. To study regulation of ASP\(^{+}\) uptake, monolayers were incubated for 10 min with the respective agonists or inhibitors before ASP\(^{+}\) was added in the continued presence of these substances.

**Fluorescence measurements with fura 2.** Intracellular Ca\(^{2+}\) concentrations ([Ca\(^{2+}\)]) of HEK-293 cells were measured with the Ca\(^{2+}\)-sensitive dye fura 2, as described previously (31). Cells were incubated with the acetomethyl ester (fura 2-AM; 5 \(\mu\)M) dissolved with 0.1 g/l pluronic F-127 in standard solution for 40 min at 37°C in the dark. Measurements were done with the same fluorescence system as described above (excitation: 340, 360, and 380 nm; emission: 500–530 nm). The ratio of the emissions after excitations at 340 and 380 nm was calculated. Calibration of [Ca\(^{2+}\)], was done by incubating the cells with the Ca\(^{2+}\) ionophore ionomycin (1 \(\mu\)M) in the presence (1.3 mmol/l) and nominal absence of Ca\(^{2+}\) with 5 mM EGTA present, according to standard methods (8).

**Patch-clamp experiments.** Membrane voltages (\(V_m\)) were measured with the slow whole cell patch-clamp technique as performed before for rOCT1 (21). Pipettes were filled with a solution containing (in mM) 95 K\(^+\) gluconate, 30 KCl, 4.8 Na\(_2\)HPO\(_4\), 1.2 Na\(_2\)HPO\(_4\), 5 D-glucose, 0.73 Ca\(^{2+}\) gluconate, 1 EGTA, 1.03 MgCl\(_2\), and 1 ATP, pH adjusted to 7.2. To this solution, 162 \(\mu\)M nystatin was added before use to permeabilize the membrane patch under the pipette. Patch pipettes
had an input resistance of ~10 MΩ. V_m was measured in the current-clamp mode of a patch-clamp amplifier (U. Fröbe, Physiologisches Institut, Universität Freiburg, Freiburg, Germany).

Chemicals. BAPTA, forskolin, sn-1,2-dioctanoyl glycerol (DOG), calphostin C, 1-[N-(6-Aminoquinolin-3-yl)-N-(4-methylbenzenesulfonyl)-N-methyl-1-tyrosyl]-4-phenylpiperazine (KN-62), 2-[N-((4-methoxybenzenesulfonyl)amino-N-(4-chlorocinnamyl)-N-methylbenzylamine phosphate (KN-92), calmidazolium, 1-(4-iodonapthalene-1-sulfonyle)homopiperazine (ML-7), 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene (U0126), 1-[6-(17β-3-methoxyestra-1,3,5(10)-tri-en-17-yl)-amino]hexyl]-2,5-pyrrolidinedione (U-73122), wortmannin, and pluronic were purchased from Calbiochem (Bad Soden, Germany). Human atrial natriuretic peptide was kindly provided by Dr. Knut Adermann (Niedersächsisches Institut für Peptid-Forschung, Hannover, Germany). ASP⁺ was purchased from Molecular Probes (Leiden, The Netherlands). All other substances and standard chemicals were obtained from Sigma (Taufkirchen, Germany) or Merck (Darmstadt, Germany).

Statistical analysis. Data are presented as originals or means ± SE, with n referring to the number of monolayers (fluorescence measurements) or cells (patch-clamp experiments). EC₅₀ values were obtained by sigmoidal curve fittings (GraphPad InStat, San Diego, CA). Unpaired (fluorescence measurements) or paired (patch-clamp experiments) two-tailed Student’s t-test was used to prove statistical significance of the effects. Entire concentration-response curves were compared using an F-test. A P value <0.05 was considered statistically significant.

RESULTS

Concentration dependence of ASP⁺ uptake. First, we determined the concentration dependence of ASP⁺ uptake by HEK-293 cells via hOCT2. At ASP⁺ concentrations between 0.1 μM and 1 mM, the cellular fluorescence increased concentration dependently (Fig. 1). Saturation of the fluorescence signal was only reached for the maximal fluorescence but not for the initial rate. Concentrations of ASP⁺ higher than 1 mM could not be dissolved in aqueous solutions. Because 1 μM ASP⁺ already resulted in a significant cellular fluorescence increase (Fig. 1), all further fluorescence experiments were performed with this ASP⁺ concentration. Figure 1 also demonstrates that ASP⁺ uptake was mediated by hOCT2 because the nontransfected HEK-293 cells showed only a very small and slow fluorescence increase with time.

Substrate specificity. Next, we investigated the substrate specificity of hOCT2 by studying inhibition of ASP⁺ uptake by known substrates of organic cation transport, like TEA⁺, TPA⁺, and cimetidine, and by the inhibitor of organic cation transporters, quinine (23). TPA⁺, TEA⁺, and cimetidine inhibited ASP⁺ uptake concentration dependently, with EC₅₀ values of 2.7, 35, and 36 μM, respectively (Fig. 2). ASP⁺ uptake was also concentration dependence inhibited by quinine, with an EC₅₀ value of 6.7 μM (Fig. 2).

Electrogeneity of ASP⁺ uptake. Previous investigations showed the electrogeneity of hOCT2 (5), a known feature of the OCT-type transporters (2, 6, 15, 21, 46). We confirmed this property of hOCT2 in two ways.

First, we measured the cellular fluorescence increase via hOCT2 by raising the K⁺ concentration in the superfusion solution from 3.6 to 50 and 145 mM. Under these conditions, the membrane voltage of transfected HEK-293 cells depolarized from -44 ± 2 to -17 ± 1 and to +6 ± 1 mV (n = 6) (21). Initial ASP⁺ uptake was inhibited by 22 ± 8 (n = 8) and 55 ± 4% (n = 9), respectively (Fig. 3, left). Second, we proved the electrogeneity of hOCT2 by determining the dependence of membrane depolarization on the ASP⁺ concentration in the bath with the slow whole cell patch-clamp technique. Increasing ASP⁺ concentrations from 1 μM to 1 mM resulted in increasing membrane depolarizations (Fig. 3, right).

Regulation by PLC and PKC. To investigate the involvement of the PLC and the subsequent PKC signaling pathway in the regulation of hOCT2, we tested different agonists. Incubation of cells with the muscarinic agonist carbachol resulted in a concentration-dependent inhibition of ASP⁺ uptake at concentrations between 0.1 and 100 μM (Fig. 4). Maximal inhibition at 100 μM carbachol was 44 ± 3% (n = 14). Without incubation, this carbachol concentration had no significant effect on ASP⁺ uptake (n = 10), indicating that the positively charged carbachol did not interfere significantly with hOCT2 as substrate.

As another activator of the PLC signaling pathway, we used ATP, which activates PLC through purinergic receptors. ATP led to a concentration-dependent inhibition of ASP⁺ transport at concentrations between 0.1 and 100 μM. The maximal ATP concentration of 100 μM led to an inhibition of ASP⁺ transport by 34 ± 4% (n = 21) (Fig. 4).

To examine whether the effect of carbachol on ASP⁺ uptake was mediated via PKC, we incubated cells with the PKC activator DOG, which is a membrane-permeable analog of diacylglycerol. DOG (1 μM) had no significant effect on ASP⁺ transport (-3 ± 5%, n = 26) (Fig. 5). Thus involvement of PKC in organic cation transport via hOCT2 is unlikely. We verified this con-
conclusion by simultaneous incubation of the cells with carbachol and either one of the PKC inhibitors calphostin C (0.1 µM, n = 12) or tamoxifen (20 µM, n = 5). There were no significant differences between the effects of carbachol in the absence and presence of these PKC inhibitors (Fig. 5). We also excluded endogenous activation of PKC by incubating the cells with calphostin C alone (n = 6), which again had no significant effect on ASP^+ transport (Fig. 5).

Regulation by PKA. To investigate the involvement of the cAMP-dependent protein kinase (PKA) in the regulation of organic cation transport via hOCT2, we used the adenylate cyclase activator forskolin. Compared with carbachol or ATP, forskolin had a weaker concentration-dependent effect on ASP^+ uptake (Fig. 4). At 1 µM forskolin, ASP^+ uptake was significantly reduced by 18 ± 4% (n = 40).

Regulation by Ca^{2+} and the Ca^{2+}/CaM complex. Because activation of G protein-coupled receptors by carbachol or ATP reduced ASP^+ uptake most likely independently of PKC, we further investigated whether this effect involves an increase in cytosolic Ca^{2+} and the Ca^{2+}/CaM complex. To verify an increase in [Ca^{2+}], after carbachol or ATP in HEK-293 cells and to estimate its magnitude, Ca^{2+} measurements with fura 2 were conducted. Carbachol (100 µM) and ATP (100 µM) increased [Ca^{2+}], by 300 ± 40 (n = 5) and 420 ± 62 nM (n = 5), respectively; therefore, the role of [Ca^{2+}] in the regulation of hOCT2 was examined. Reduction of the extracellular Ca^{2+} concentration from 1.3 mM to 1 µM Ca^{2+} resulted in an inhibition of ASP^+ transport by 50 ± 5% (n = 16) (Fig. 6). Reduction of [Ca^{2+}] by incubation of cells with the Ca^{2+} chelator BAPTA (5 µM) led to a reduction in ASP^+ uptake by 41 ± 5% (n = 15) (Fig. 6). The incubation of cells with carbachol in the presence of BAPTA resulted in a...

Fig. 3. Electrogeneity of ASP^+ uptake by hOCT2. Left: decrease in initial ASP^+ (1 µM) fluorescence increase when cells were depolarized by increasing the extracellular K^+ concentration from 3.6 to 50 or 145 mM. Right: change in membrane voltages (V_m) of HEK-293 cells expressing hOCT2 induced by addition of ASP^+ to the bath solution at concentrations between 1 and 1,000 µM. Values are means ± SE, with the no. of observations in parentheses. All effects are significant (P < 0.05).

Fig. 4. Concentration-response curves for the effects of carbachol, ATP, and forskolin on initial ASP^+ uptake (1 µM). Values are means ± SE, with the no. of observations in parentheses.

Fig. 5. Effects of PKC [sn-1,2-diocatoyl glycerol (DOG); 1 µM] and PLC (carbachol; 100 µM) activation on initial ASP^+ uptake (1 µM). Calphostin C (0.1 µM) or tamoxifen (20 µM) was used as a PKC inhibitor. Values are means ± SE, with the no. of observations in parentheses. *Statistically significant effect (P < 0.05).

Fig. 6. Effects of inhibition of calmodulin (CaM; calmidazolium; 5 µM), PLC activation (carbachol; 100 µM), lowering of intracellular Ca^{2+} concentration (low Ca^{2+}; extracellular Ca^{2+} = 1 µM), or chelation of intracellular Ca^{2+} (BAPTA; 5 µM) on initial ASP^+ uptake (1 µM). The independence of the effects of carbachol (100 µM) and calmidazolium (5 µM) or BAPTA (5 µM) is also demonstrated. Values are means ± SE, with the no. of observations in parentheses. *Statistically significant effect (P < 0.05).
significantly, even larger, reduction in ASP⁺ uptake compared with either carbachol or BAPTA alone, indicating an independence of the carbachol effect of [Ca²⁺]i (Fig. 6).

Inhibition of CaM with the CaM inhibitor calmidazolium (5 μM) reduced ASP⁺ transport by 63 ± 2% (n = 15) (Fig. 6), indicating a significant endogenous activation of hOCT2 via this complex. To exclude a possible interference of the cationic calmidazolium with hOCT2, the effects of 1, 5, and 50 μM calmidazolium were tested acutely without incubation. Inhibition of ASP⁺ uptake was 65 ± 4 (n = 10), 64 ± 3 (n = 18), and 65 ± 5% (n = 6), respectively. When CaM was inhibited by calmidazolium (5 μM) and the cells were treated simultaneously with carbachol (100 μM), ASP⁺ uptake was more significantly reduced than with either carbachol or calmidazolium alone (28 ± 2%, n = 16) (Fig. 6). This suggests that at least part of the carbachol effect is mediated via a Ca²⁺/CaM-independent pathway. To test whether activation of PLC is involved in the carbachol-mediated inhibition of ASP⁺ uptake, carbachol (100 μM) was added in the presence of the PLC inhibitor U-73122 (5 μM). Under these conditions, carbachol still reduced ASP⁺ uptake by 55 ± 11% (n = 18) (Fig. 7). U-73122 per se did not significantly inhibit basal transport activity (−10 ± 7%, n = 17) (Fig. 7). To identify the kinases that are involved in the Ca²⁺/Calmodulin-mediated regulation of hOCT2, we examined ASP⁺ uptake after incubation of cells with KN-62, an inhibitor of the multifunctional Ca²⁺/CaM-dependent protein kinase II (CaMKII). KN-62 (1 μM) inhibited ASP⁺ transport significantly by 40 ± 4% (n = 30) (Fig. 8). The inactive analog KN-92 (1 μM) had no statistically significant effect on ASP⁺ uptake (−4 ± 6%, n = 21) (Fig. 8), indicating the specificity of the effect of KN-62. Because the Ca²⁺/CaM complex also activates the myosin light chain kinase (MLCK), we tested the effect of the MLCK inhibitor ML-7. Incubation with ML-7 (3 μM) also led to an inhibition of ASP⁺ transport by 31 ± 4% (n = 16) (Fig. 8). These findings suggest that CaMKII and MLCK are endogenously activated in HEK-293 cells and stimulate ASP⁺ uptake via hOCT2.

To examine whether the effect of carbachol on hOCT2 is independent not only of Ca²⁺/CaM but also of CaMKII, cells were simultaneously incubated with carbachol (100 μM) and KN-62 (1 μM). This resulted in an inhibition of ASP⁺ transport by 64 ± 3% (n = 12) (Fig. 8), which was significantly greater than the effect of carbachol or KN-62 alone, indicating the additivity of these two signaling pathways.

Regulation by the phosphatidylinositol 3-kinase. Because activation of G protein-coupled receptors can also stimulate phosphatidylinositol 3-kinase (PI3K), we tested whether the effect of carbachol is influenced by inhibition of PI3K. Incubation with the PI3K inhibitor wortmannin (0.1 μM) alone significantly stimulated basal transport activity of HEK-293 cells (55 ± 17%, n = 16) (Fig. 7), indicating endogenous activation of this enzyme. Pretreatment of the cells with wortmannin could almost completely prevent the effect of simultaneous incubation with carbachol (100 μM) on initial ASP⁺ uptake (−7 ± 5%, n = 11) (Fig. 7). Further downstream in the signaling cascade are also the mitogen-activated kinase kinases MEK1 and MEK2. Inhibition of these kinases by UO126 (1 μM) had no effect on basal ASP⁺ transport (−4 ± 12%, n = 6) or on carbachol-induced inhibition of ASP⁺ uptake (−42 ± 4%, n = 6).

Influence of protein kinase activation on substrate affinities. For the OCT1 of rat (rOCT1), we reported that PKC activation results in a modulation of the

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![Fig. 7](http://ajprenal.physiology.org/DownloadedFrom)  
**Fig. 7.** Effect of inhibition of phosphatidylinositol 3-kinase by wortmannin (0.1 μM) on carbachol-mediated (100 μM) transport reduction and independence of carbachol-mediated (100 μM) transport reduction of PLC inhibition by U-73122 (5 μM). Values are means ± SE, with the no. of observations in parentheses. The effects of carbachol in the absence or presence of U-73122 are not significantly different. *Statistically significant effect (P < 0.05).

![Fig. 8](http://ajprenal.physiology.org/DownloadedFrom)  
**Fig. 8.** Effects of inhibition of Ca²⁺/CaM-dependent kinase II (CaMKII) by 1-[N,O-bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62; 1 μM) and its inactive analog 2-[N-(4-methoxybenzenesulfonyl)amino-N-(4-chlorocinnamyl))-N-methylbenzylamine phosphate (KN-92; 1 μM) or of myosin light chain kinase by 1-[5-idonaphthalene-1-sulfonlyl]homopiperazine (ML-7; 3 μM) on initial ASP⁺ uptake (1 μM). The independence of the effects of carbachol (100 μM) and of KN-62 (1 μM) is also demonstrated. Values are means ± SE, with the no. of observations in parentheses. *Statistically significant effect (P < 0.05).
transporters were cloned (5, 7, 35, 43, 47). Organic cation transporters after stable transfection in different cell models (15, 16, 21, 46). Functional and morphological studies suggested that the transporters of the OCT type are polycspecific organic cation transporters localized in the basolateral membrane (2, 6, 14, 21, 23, 34). In 1997, the first human organic cation transporters were cloned (5, 7, 35, 43, 47).

In this study, we investigated the properties and regulation of hOCT2 stably transfected in a human embryonic kidney cell line (HEK-293). Dynamic microfluorimetry with the fluorescent organic cation ASP+, as adapted in our laboratory (13, 21, 25, 32, 33), and the slow whole cell patch-clamp technique were used. We confirm the electrogeneity of this transporter and describe its substrate specificity for some classic organic cations. Most importantly, we demonstrate for the first time that hOCT2 is regulated by various intracellular signaling pathways. It is inhibited by PKA, PLC, and PI3K activation but not by PKC activation. It is endogenously activated by the Ca2+/CaM complex or by carbachol also results in altered substrate affinities, EC50 values for TEA+ were determined under these conditions and compared with those obtained under control conditions. Activation of the G protein-coupled receptor signaling pathways by carbachol (100 μM) or inhibition of the CaMKII by KN-62 or of PI3K by wortmannin induced no significant changes in the concentration-response curves for the inhibition of ASP+ uptake by TEA+ (Fig. 9). Conversely, inhibition of CaM by the CaM antagonist calmidazolium (5 μM) significantly reduced the apparent affinity of hOCT2 for TEA+ (EC50 = 249 μM) compared with 35 μM under basal conditions.

DISCUSSION

Organic cation transport in epithelia is mediated by several polycspecific transporters, which initially have been examined functionally using different experimental models and techniques (15–17, 46). In the renal proximal tubule, three main transport systems for organic cations were functionally differentiated: a basolaterally localized electrogenic uniporter, a luminaly localized electroneutral H+ /cation exchanger, and a luminaly localized electrogenic uniporter with high affinity to choline (17). The cloning of the first organic cation transporter from the rat (rOCT1) in 1994 (6) made it possible to specifically characterize single organic cation transporters after stable transfection in different cell models (15, 16, 21, 46). Functional and morphological studies suggested that the transporters of the OCT type are polycspecific organic cation transporters localized in the basolateral membrane (2, 6, 14, 21, 23, 34). In 1997, the first human organic cation transporters were cloned (5, 7, 35, 43, 47).

Previous radioactive tracer or electrophysiological studies on hOCT2 expressed in HEK-293 cells or X. laevis oocytes showed that hOCT2 transports small organic cations, such as TEA+, TPA+, NMN+, and choline, and is noncompetitively inhibited by larger organic cations, such as quinine, d-tubocurarine, and vecuronium (15–17, 42, 46). The EC50 values obtained for the inhibition of ASP+ uptake in the present study by TEA+ (EC50 = 35 μM), TPA+ (EC50 = 2.7 μM), cimetidine (EC50 = 36 μM), and quinine (EC50 = 6.7 μM) are similar to Km or Ki values determined with radiolabeled substrates for hOCT2 expressed in X. laevis oocytes: Km for radiolabeled TEA+ was 76 μM, and Ki for the inhibition of radiolabeled TEA+ uptake by TPA+ or quinine were 1.5 and 3.4 μM, respectively (5).

Electrogeneity of hOCT2. A common feature of all OCT-type transporters is their electrogeneity (15–17, 46). In the present study, we confirmed this electrogeneity in two ways: 1) in microfluorimetric studies, ASP+ uptake was dependent on membrane voltage and decreased with membrane depolarization induced by increases in extracellular K+ concentration (Fig. 3); and 2) in electrophysiological studies, increasing ASP+ concentrations led to increasing depolarization of the membrane voltage of transfected HEK-293 cells due to the influx of positive charges. While the first finding

![Fig. 9. Concentration-response curves for the inhibition of initial ASP+ uptake (1 μM) by TEA+ in the absence or presence of carbachol (CCH; 100 μM), KN-62 (1 μM), wortmannin (0.1 μM), or calmidazolium (5 μM). Only the curve for calmidazolium differs significantly from the control curve. The EC50 value increased from 35 (control) to 249 μM (calmidazolium). Values are means ± SE, with the no. of observations in parentheses.](http://ajprenal.physiology.org/DownloadedFrom/1022.20.11.25.3/10.1152/ajprenal.00350.2003)
could also be due to changes in substrate binding, the second clearly indicates electrogenic transport.

**Regulation of hOCT2.** The regulation of organic cation transport has been under investigation only recently (13, 21, 25). Organic cation transport across the basolateral membrane of proximal tubules was activated in rabbits (12) and inhibited in humans (25) by PKC stimulation. An increase in transport mediated by the rOCT1 was shown by us for PKC and PKA and the tyrosine kinase p56 \(^{\text{fck}}\) (21). Similar differences in regulation were also demonstrated by us for organic cation transport across the apical membrane of human and porcine proximal tubule cell lines (13). These findings suggest species- and also subtype-specific differences in transporter regulation. For OCT2 as well as for OCT1 transporters from rats and humans, several potential phosphorylation sites for PKA, PKC, and tyrosine kinases were described; some of them are conserved through all four isoforms (5, 6).

**cAMP-dependent PKA.** Addition of the adenylate cyclase activator forskolin leading to activation of PKA resulted in a small concentration-dependent inhibition of ASP\(^+\) uptake via hOCT2. Recently, we demonstrated that in freshly isolated human proximal tubules organic cation transport across the basolateral membrane, probably mainly mediated via hOCT2, is also downregulated by PKA to a similar extent (25). hEMT (hOCT3) again expressed in HEK-293 cells showed no cAMP-dependent regulation, and only nonspecific high concentrations of forskolin (250 \(\mu\)M) led to a small reduction in radiolabeled MPP\(^+\) transport in that study (20).

**G protein-coupled receptor and PKC.** To investigate the effect of activation of G protein-coupled receptor on ASP\(^+\) uptake in hOCT2-transfected HEK-293 cells, we used the purinergic agonist ATP and the muscarinic agonist carbachol. Receptors for both agonists have been demonstrated in HEK-293 cells (27, 37). ATP and carbachol resulted in inhibition of ASP\(^+\) uptake by \(-34\) and 44\%, respectively. We excluded the involvement of PKC in this effect by using the direct PKC activator DOG, a membrane-permeable analog of diacylglycerol. DOG had no significant effect on hOCT2-mediated ASP\(^+\) uptake. The fact that PKC was not involved in the effect of carbachol was further verified by simultaneous incubation of the cells with carbachol and the PKC inhibitors calphostin C or tamoxifen. Neither PKC inhibitor significantly influenced the carbachol-induced inhibition of ASP\(^+\) transport. This absence of a PKC-mediated regulation of hOCT2, therefore, parallels similar observations for hOCT3 (20) but is in contrast to the PKC-mediated activation of rOCT1 (21) or organic cation transport across the basolateral membrane of isolated rabbit proximal tubules (PKC-mediated activation) (12) or isolated human proximal tubules (PKC-mediated inhibition) (25). These findings also underline the marked differences in transport properties of these organic cation transporters between the different species and, possibly, the expression systems. Furthermore, it should be kept in mind that for hOCT1 and hOCT2 various splice variants were identified and that, especially in the human proximal tubule, expression of a splice variant to the originally cloned hOCT2 as the relevant transporter was described recently (11, 40).

**CaM and Ca\(^{2+}\)/CaM-dependent kinases.** After exclusion of PKC involvement in the regulation of hOCT2, we further investigated whether CaM and CaMKII or MLCK is involved in the regulation of hOCT2-mediated ASP\(^+\) uptake. Inhibition of CaM with calmidazolium resulted in a marked reduction in hOCT2 transport by 63\%, indicating an endogenous activation of the Ca\(^{2+}\)/CaM pathway. In line with this effect of CaM inhibition is the reduced activity of hOCT2 when cellular Ca\(^{2+}\) was decreased by using the Ca\(^{2+}\) chelator BAPTA or by lowering extracellular Ca\(^{2+}\) to 1 \(\mu\)M, which also leads to reduction in cellular Ca\(^{2+}\) levels. Activation of CaM by Ca\(^{2+}\) stimulates the multifunctional CaMKII, which is known to phosphorylate many target proteins, leading to changes in their activity. Consequently, also an inhibition of CaMKII by KN-62 reduced hOCT2-mediated ASP\(^+\) transport by 40\%. We verified the specificity of this inhibition by KN-62 with the inactive analog KN-92, which was without a significant effect. Because KN-62 inhibits not only CaMKII but also CaMKIV and CaMKV with similar affinities, these kinases may be involved in the observed effects as well. In addition to CaMKII, MLCK is also activated by CaM. Inhibition of MLCK by ML-7 reduced ASP\(^+\) uptake via hOCT2 again by 31\%. Together these data indicate that hOCT2 is endogenously activated via CaM-dependent CaMKII and MLCK stimulation. A similar regulation was shown for the renal basolateral PAH transporter by CaMKII in isolated proximal tubules of the rabbit (3). The involvement of CaMKII was also demonstrated for the regulation of the Na\(^+\)/H\(^+\) exchanger (41), the Na\(^+\)-HCO\(_3\) co-transporter, or proline uptake in the proximal tubule (30, 44, 45). A CaM-dependent regulation of organic cation transport was only described for hOCT3 (20) but has not been examined for any other cloned OCT so far.

Simultaneous activation of the G protein-coupled receptor signaling pathway by carbachol and inhibition of either CaM by calmidazolium or CaMKII by KN-62 had additive effects on ASP\(^+\) uptake, indicating the independence of the carbachol-mediated inhibition and the CaM-mediated stimulation of hOCT2 transport. Carbachol or ATP increased \([\text{Ca}^{2+}]\) in this and previous reports from HEK-293 cells (27, 37). This increase in \([\text{Ca}^{2+}]\) should have increased CaM activity and, therefore, stimulated hOCT2 transport; however, the opposite was true. This would indicate an effect of carbachol or ATP independently of Ca\(^{2+}\) and overriding the stimulation of CaM. This was further supported by the increased inhibition of hOCT2-mediated ASP\(^+\) uptake when the G protein-coupled receptor signaling pathway was activated by carbachol in the presence of BAPTA compared with either one alone. Additional evidence for the independence of the carbachol inhibition of ASP\(^+\) uptake from the PLC-activated signaling pathway derives from experiments performed under inhibition of the agonist-induced PLC activation.
activation with U-73122: inhibition of ASP\(^+\) uptake was still present even after simultaneous incubation of cells with carbachol and U-73122.

The observed effects of carbachol and calmidazolium on ASP\(^+\) uptake by hOCT2 could be also explained by a direct interaction at the transport binding site because these drugs are organic cations. However, for carbachol this can be excluded for the following reasons: acute administration of carbachol had no significant effect on ASP\(^+\) uptake; published affinity of carbachol for the basolateral organic cation transport system is 12.2 mM (39); carbachol had no effect on ASP\(^+\) uptake by hOCT1 or rOCT1 (Ciarimboli and Schlatter, unpublished observations); and carbachol incubation caused no change in the concentration-response curve for the inhibition of ASP\(^+\) uptake by TEA\(^+\). Acute addition of calmidazolium resulted in an inhibition of ASP\(^+\) uptake similar to that after incubation. This fast effect within seconds is compatible with data for this inhibitor in other systems (18). A significant direct interaction of calmidazolium with hOCT2, however, can again be excluded for the following reasons: inhibition of uptake did not differ between 1 and 50 µM calmidazolium; maximal inhibition of transport reached only \(\sim 60\%\), whereas other substrates inhibited hOCT2 by up to 100%; and calmidazolium incubation caused a rightward shift in the concentration-response curve for the inhibition of ASP\(^+\) uptake by TEA\(^+\), whereas a leftward shift should have been expected if calmidazolium were to be a substrate for the transporter. Therefore, the observed inhibition of ASP\(^+\) uptake by calmidazolium is due to regulation via CaM.

**PI3K.** Stimulation of G protein-coupled receptors mediates intracellular signaling via GTP-bound G protein \(\alpha\)-subunits or \(\beta\gamma\)-complexes. The GTP-bound G protein \(\alpha\)-subunits initiate the classic cascade involving diacylglycerol and inositol 3,4,5-trisphosphate, whereas the \(\beta\gamma\)-complexes activate a cascade that connects G receptor activation to PI3K and the subsequent MAPK signaling pathway (9, 10). We have therefore tested whether the signal pathway mediated by \(\beta\gamma\)-complexes is involved in the regulation of hOCT2 by carbachol. The incubation of the cells with wortmannin, a potent inhibitor of PI3K activity, significantly stimulated the ASP\(^+\) uptake via hOCT2, showing that the enzyme is endogenously activated. Stimulation of the G protein-coupled receptor by carbachol and the contemporaneous inhibition of the PI3K activity by wortmannin almost completely prevented the reduction of initial ASP\(^+\) uptake observed with carbachol alone, suggesting that the PI3K signaling pathway can be directly involved in the regulation of hOCT2 after stimulation of the G protein-coupled receptor.

In conclusion, we have shown that several signaling pathways are involved in the regulation of hOCT2-mediated organic cation transport. Whereas PKA stimulation resulted in inhibition, endogenously active CaM or the CaM-dependent kinases CaMKII and MLCK led to activation, and inhibition of this pathway led to a reduction, of hOCT2-mediated transport. Activation of PKC had no effect on this transporter. Activation of the G receptor-coupled signaling pathway by carbachol inhibited hOCT2-mediated transport, probably acting through PI3K. Regulation of organic cation transporters may alter the secretion and absorption of multiple endogenous substances, drugs, and other xenobiotics, leading to changes in pharmacokinetics of these organic cations.

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