Individual PKC-Phosphorylation Sites in Organic Cation Transporter 1 Determine Substrate Selectivity and Transport Regulation

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To elucidate the molecular mechanisms underlying stimulation of rat organic cation transporter type 1 (rOCT1) by protein kinase C (PKC) activation, functional properties and regulation of rOCT1 stably expressed in HEK293 cells after site-directed mutagenesis of putative PKC phosphorylation-sites were compared with wild-type (WT) rOCT1 using microfluorometric measurements with the fluorescence organic cation 4-(4-(dimethylamino)styryl)-N-methylpyridinium (ASP*). Either substitutions of single (S286A, S292A, T296A, S328A, and T550A) or of all five PKC-sites (5x-PKC) with alanine suppressed PKC-induced stimulation of ASP* uptake, whereas regulation by p56lk tyrosine kinase was conserved in all mutants. Remarkably, the apparent affinities for TEA*, TPA*, and quinine were changed differently in each mutant (EC50 in WT, S286A, S292A, T296A, S328A, T550A, and 5x-PKC in μmol: TEA*: 105, 153, 56, 1135, 484, 498, 518; TPA*: 0.1, 2.1, 0.3, 1.0, 43, 0.3, 2.2; quinine: 1.5, 3.0, 2.5, 4.8, 81, 7.6, 8.9, respectively). After mutations, no effects of PKC activation on apparent affinity of rOCT1 for these substrates could be detected, in contrast to what was observed in WT. PKC activation had no significant effect on rOCT1 trafficking from intracellular pools to the cell membrane. Substitution of all PKC sites suppressed PKC-induced phosphorylation of rOCT1. In conclusion, it was found that the presence of all five potential PKC phosphorylation sites is necessary for the PKC-induced stimulation of rOCT1. The different effects on the EC50 values by the different mutations suggest that the large intracellular loop participates in building the substrate binding pocket of rOCT1 or specifically modulates its structure.

way. These findings suggest different regulation pathways of OCTs. Regulation of these transporters has great physiological and even clinical significance for the secretion of multiple endogenous metabolites, drugs, and xenobiots. Stimulation of this process can accelerate detoxification, whereas inhibition can prolong the exposition of the body to dangerous substances (13).

The rOCT1 is a 556–amino acid membrane protein (14) mapped to chromosome 1q11-12 (15), which has been identified by Northern blot analysis in rat kidney, small intestine, colon, and liver (14). In the kidney, it has been localized at the basolateral membrane of S1 and S2 segments of proximal tubules (16). The transport mediated by rOCT1 has been characterized as electrogenic, independent of Na⁺ and pH, and bidirectional by radioactive tracer flux measurements with oocytes from *Xenopus laevis* expressing rOCT1 (17). rOCT1 can translocate organic cations like TEA⁺ and choline (18), catecholamines (19), and nucleosides like 2’-deoxytubercidin (20), whereas cations like tetraptayedammonium (TPA⁺) and cyanine 863 are nontransported inhibitors of the transporter (21). For this transport protein 12 transmembrane domains, a large hydrophilic extracellular loop between the first and the second predicted transmembrane domain and an intracellular localization of the N- and C-termini have been described (22). rOCT1 possesses five putative PKC phosphorylation sites (S286, S292, T296, S328, and T550) (23) and, according to the PROSITE prediction program (24), three PKA phosphorylation sites (T297, T348 and T550) (23) and, according to the PROSITE prediction program—and T537 (Fig-}

Figure 1. Proposed rat organic cation transporter type 1 (rOCT1) topology. The positions of putative phosphorylation sites for protein kinase C (PKC) are indicated with a circle, for protein kinase A (PKA) with a square and for tyrosine kinase with a hexagon. Modified from (23).

Materials and Methods

Construction of rOCT1 Mutants

rOCT1 variants carrying mutations in the putative PKC-phosphorylation sites were constructed by the PCR using as a template the plasmid rOCT1/pRcCMV (25). All single mutants were prepared by the overlap extension method (26). The PCR amplificates were digested with NheI and EcoRII (for mutants S286A/S292A/T296A/S328A/T550A) and cloned directly into the plasmid rOCT1/pRcCMV treated with the same restriction endonucleases. To create the rOCT1 variant in which all five PKC-sites are mutated (5x-PKC), at first the triple mutant S286A/S292A/T296A was performed using megaprimer strategy (27). PCR amplificate was digested with restriction endonucleases NheI and EcoRIII and cloned into the rOCT1/pBluescript II SK plasmid cut with the same enzymes. The same strategy was used to combine these three mutations and S328A in one construct, quadruple mutant S286A/S292A/T296A/S328A. The quadruple mutant was then cloned into the eukaryotic expression vector pRcCMV (Invitrogen, The Netherlands). The 5x-PKC variant was finally obtained combining the quadruple mutant and T550A into one construct in the pRcCMV vector using EcoRIII and HindIII.

HEK293 Cell Culture

The HEK293 cells (human embryonic kidney cortex cells, CRL-1573; American Type Culture Collection, Rockville, MD) stably expressing WT, 5x-PKC, and single-mutant rOCT1 were transfected as described previously (25). Cells were grown at 37°C in 50-ml cell culture flasks (Greiner, Frickenhausen, Germany) in DME (Biochrom, Berlin, Germany) containing 3.7 g/L NaHCO₃, 1.0 g/L D-glucose, and 2 mmol L-glutamine (Life Technologies BRL/Life Technologies, Eggenstein, Germany) gassed with 8% CO₂. To this medium 100,000 U/L penicillin, 100 mg/L streptomycin (Biochrom), 10% fetal calf serum, and 0.8 mg/ml geneticin (Life Technologies BRL/Life Technologies) were added. Experiments were performed after 4 to 8 d with cells grown on glass cover slips of the passages 11 to 42. Culture and functional analysis of these cells was approved by the Landesumweltamt Nordrhein-Westfalen, Essen, Germany (521-M-1.14/00).

Fluorescence Measurements with 4-(4-(dimethylamino)styryl)-N-methylpyridinium

As substrate for OCTs, the fluorescence organic cation 4-(4-(dimethylamino)styryl)-N-methylpyridinium (ASP⁺) was used. Fluorescence measurement device and experimental procedures were as customary in our laboratory (7,8,11). Briefly, measurements were performed in the dark with an inverted microscope (Axiovert 135; Zeiss, Oberkochen, Germany)
equipped with a 100× oil immersion objective. Excitation light (450 to 490 nm) was reflected by a dichroic mirror (560 nm) to a perfusion chamber. Cell monolayers on cover slips formed the bottom of the chamber. The preparations were superfused at a rate of 10 ml/min with a HCO₃⁻/Na⁺-free Ringer-like solution containing (in mmol): NaCl 145, K₂HPO₄ 1.6, KH₂PO₄ 0.4, D-glucose 5, MgCl₂ 1, calcium gluconate 1.3, and pH adjusted to 7.4 at 37°C. Fluorescence emission (575 to 640 nm) was measured by a photon counting tube (Hamamatsu H 3460-04; Herrsching, Germany). The initial slope of fluorescence increase represents directly the ASP⁺ uptake across plasma membrane via OCTs and is not significantly influenced by exit of D-glucose 5, MgCl₂ 1, calcium gluconate 1.3, and pH adjusted.  

The rate of 10 ml/min with a HCO₃⁻/Na⁺-free Ringer-like solution containing (in mmol): NaCl 145, K₂HPO₄ 1.6, KH₂PO₄ 0.4, D-glucose 5, MgCl₂ 1, calcium gluconate 1.3, and pH adjusted to 7.4 at 37°C. Fluorescence emission (575 to 640 nm) was measured by a photon counting tube (Hamamatsu H 3460-04; Herrsching, Germany). The initial slope of fluorescence increase represents directly the ASP⁺ uptake across plasma membrane via OCTs and is not significantly influenced by exit of D-glucose 5, MgCl₂ 1, calcium gluconate 1.3, and pH adjusted.  

Effect of PKC Stimulation on Trafficking of rOCT1
The same number of cells (WT or 5x-PKC) of same passage and age were incubated 10 min with or without 1 μmol sn1,2-dioctanoyl glycerol (DOG) at 37°C. After incubation, cells were washed three times with ice-cold Ringer buffer and fixed for 1 h with 4% paraformaldehyde pH 7.4. After washing three times with ice-cold Ringer buffer, the unspecific binding sites were blocked by 1 h incubation at 22°C with Ringer buffer containing 0.5% gelatin (Amersham Biosciences Europe, Freiburg, Germany). Finally, cells were incubated overnight at 4°C with affinity-purified antibodies against the extracellular region of the transporter (22) diluted 1:250. After washing, cells were incubated 45 min in the dark with Alexa Fluor 488 F(ab')₂ fragment of goat anti-rabbit IgG diluted 1:500. Cell-associated fluorescence was measured with a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). To evaluate the intracellular transporter pool, after fixation the cells in some experiments were permeabilized with 0.1% saponin for 20 min at 4°C.

Immunoprecipitation of rOCT1 and Detection of Phosphorylated Transporters
Immunoprecipitation with anti rOCT1 antibodies was performed as described previously (28). Briefly, after 15 min incubation of the same number of WT or of 5x-PKC cells of same passage with or without 1 μmol DOG at 37°C, cells were washed three times with cold PBS and solubilized in lysis buffer (in mmol: 20 Tris-HCl pH 7.5, 25 NaCl, 50 NaF, 15 Na₃P₂O₇, 1 EDTA, 2 Na₃VO₄ containing 1% Triton X100, 44 μg/ml PMSE, and a cocktail of protease inhibitors). After centrifugation at 10,000 × g for 15 min, lysates were incubated with gentle shaking 2 h with 10 μl affinity-purified antibodies against the extracellular region of the transporter (22) and then 2 h with 40 μl protein G-Agarose. All procedures were performed at 4°C. The immunocomplexes bound to the beads were recovered by centrifugation, washed five times with lysis buffer, resuspended in Laemmli buffer, denatured at 95°C for 5 min, and loaded onto 8 to 16% SDS-polyacrylamide gels (PAGEr Gold Precast Gels; Cambrex, Rockland, ME) for Western blot analysis or phosphoprotein determination. For Western blot analysis, the proteins on the gel were transferred to a polyvinylidene difluoride (PVDF) membrane incubated with blocking reagent (Amersham, Freiburg, Germany). After incubation with the primary antibody (goat-polyclonal antibody to hOCT1; Santa Cruz Biotechnology, Santa Cruz, CA), membranes were incubated with rabbit anti-goat horseradish peroxidase-conjugate (Southern Biotechnology, Birmingham, AL) and covered with SuperSignal (Pierce, Bonn, Germany) before exposure (Lumimag, Roche Diagnostics, Mannheim, Germany). For in-gel detection of phosphate groups attached to tyrosine, serine, or threonine and of total proteins, the Pro-Q Diamond phosphoprotein and the Sypro Ruby gel stains (Molecular Probes, Leiden, the Netherlands), respectively, were sequentially used with the same gel according to the instructions of the manufacturer. Gels were imaged with a Typhoon 9400 scanner (Amersham, Freiburg, Germany).

Chemicals
Forskolin was obtained from Sigma (Taufkirchen, Germany); aminogenestein and DOG were purchased from Calbiochem (Bad Soden, Germany); gelatin was obtained from Amersham (Freiburg, Germany); ASP⁺ and Alexa Fluor 488 F(ab')₂ fragment of goat anti-rabbit IgG were obtained from Molecular Probes (Leiden, The Netherlands). All other substances and standard chemicals were obtained from Sigma or Merck (Darmstadt, Germany).

Statistical Analyses
Data are presented as mean values ± SEM with n referring to the number of monolayers used in fluorescence measurements. EC₅₀ values were obtained by sigmoidal curve fittings (GraphPad InStat, San Diego, CA) and were compared using one-way ANOVA. Effects of treatment on the EC₅₀ of concentration response curves were compared using GraphPad Prism, San Diego, CA. Unpaired or paired two-sided t test was used to prove statistical significance of the effects. P < 0.05 was considered statistically significant.

Results
Regulation of rOCT1 by Protein Kinases
Stimulation of PKC with 1 μmol DOG resulted in a significant increase of ASP⁺ uptake in cells expressing WT-rOCT1 (+92 ± 26%, n = 18), whereas no significant effect could be observed in cells expressing mutated transporters (S286A: −9 ± 4%, n = 22, S292A: −14 ± 6%, n = 19, T296A: −9 ± 13%, n = 20, S328A: −11 ± 6%, n = 15, T550A: −15 ± 20%, n = 21, 5x-PKC: −7 ± 24%, n = 11) (Figure 2a). Activation of PKA with 1 μmol forskolin significantly stimulated ASP⁺ uptake in WT (+60 ± 21%, n = 13), T296A (+59 ± 27%, n = 19), and T550A (+69 ± 22%, n = 13), but not in S286A (−8 ± 8%, n = 31), S292A (−7 ± 7%, n = 31), S328A (−8 ± 10%, n = 15), and 5x-PKC cells (+8 ± 12%, n = 12) (Figure 2b). Inhibition of the p56lck tyrosine kinase with 10 μmol aminogenestein significantly reduced ASP⁺ uptake in all cells tested (WT: −47 ± 8%,
Substrate Specificity

The substrate specificities of the different forms of rOCT1 have been determined by studying inhibition of ASP uptake by known substrates or inhibitors of organic cation transport (Figure 3), like TEA (Figure 4), TPA (Figure 5), and quinine (Figure 6). TEA inhibited ASP uptake in a concentration-dependent manner in WT and all rOCT1 mutants. The S292A mutant displayed the highest and T296A mutant the lowest apparent affinity for TEA (Table 1). When compared with ANOVA, the EC50 values for TEA of WT were not different from those obtained in S286A and S292A, but were significantly different from those obtained with the other mutants. The inhibition curves obtained with T296A, S328A, and T550A had a shallower slope than those obtained with WT (Figure 4). TPA and quinine also inhibited ASP uptake in a concentration-dependent manner in all forms of rOCT1 (Figures 5 and 6, respectively). The WT had the highest apparent affinity for TPA and quinine, and S328A the lowest (Table 1). When compared with ANOVA, all EC50 values obtained with mutated rOCT1 were significantly different from those obtained with WT (Table 1); moreover, the curves obtained with T296A were shallower than the others (Figures 5 and 6, respectively). We have previously demonstrated that activation of PKC shifted the concentration-response curves for the inhibition of ASP uptake by TEA, TPA, and quinine to the left, causing a decrease in the EC50 value by more than one order of magnitude (8), indicating that the increase in transport is caused by an increase in rOCT1 apparent affinity. In this study, we have therefore tested also whether mutations of the potential PKC-phosphorylation sites abolished this effect. The TEA and quinine inhibition curves of ASP uptake without or with DOG incubation in T296A (EC50 in μmol without DOG: TEA 1135, 5x-PKC: TEA -74 ± 4%, n = 12, 5x-PKC: -74 ± 4%, n = 11) (Figure 2c).

Figure 2. Effects of 10 min incubation with 1 μmol sn1,2-di-octanoyl glycerol (DOG) (panel a), 1 μmol forskolin (panel b) or 10 μmol aminogenestein (panel c) on uptake of 1 μmol ASP in wild-type (WT), S286A-, S292A-, T296A-, S328A-, T550A-, and 5x-PKC-mutants. Data are given as stimulation or inhibition in percent of control measurements, which were performed with the same batch of cells incubated for 10 min in control solution without DOG, forskolin or aminogenestein. Mean values ± SEM from the indicated number of monolayers (n) are presented. * indicates statistically (unpaired t test; P < 0.05) significant differences from control measurements.

Figure 3. Time course of the increase in cellular fluorescence of 5x-PKC cells with 10⁻⁴ mol, 10⁻⁵ mol and without TEA. At time 0, 1 μmol ASP⁺ was added to bath solution. The curves represent mean values ± SEM of several individual experiments. n indicates the number of monolayers used.
monolayers used. EC50 values are also indicated.

cells of the same passage and age.

percent of control experiments performed at the same day with cells of the same passage and age. n indicates the number of monolayers used. EC50 values are also indicated.

Figure 4. Concentration-response curves for TEA+ effects on uptake of 1 μmol ASP+ by WT (n = 9 to 34), S286A (n = 8 to 19), S292A (n = 4 to 13), T296A (n = 6 to 20), S328A (n = 9 to 34), T550A (n = 11 to 17) and 5x-PKC (n = 22 to 51) cells. Data are presented as initial slope (mean values ± SEM) of the uptake in percent of control experiments performed at the same day with cells of the same passage and age. n indicates the number of monolayers used. EC50 values are also indicated.

Figure 5. Concentration-response curves for TPA+ effects on uptake of 1 μmol ASP+ by WT-rOCT1 (n = 4 to 18), S286A (n = 4 to 14), S292A (n = 5 to 8), T296A (n = 4 to 19), S328A (n = 3 to 8), T550A (n = 8 to 28) and 5x-PKC (n = 6 to 13) cells. Data are presented as initial slope (mean values ± SEM) of the uptake in percent of control experiments performed at the same day with cells of the same passage and age. n indicates the number of monolayers used. EC50 values are also indicated.

Figure 6. Concentration-response curves for the quinine effects on uptake of 1 μmol ASP+ by WT (n = 5 to 17), S286A (n = 6 to 15), S292A (n = 7 to 13), T296A (n = 4 to 14), S328A (n = 7 to 11), T550A (n = 5 to 9) and 5x-PKC (n = 7 to 21) cells. Data are presented as initial slope (mean values ± SEM) of the uptake in percent of control experiments performed at the same day with cells of the same passage and age. n indicates the number of monolayers used. EC50 values are also indicated.

quarine 4.8; with DOG: TEA+ 1422, quinine 7.8; Figure 7a), T550A (EC50 in μM without DOG: TEA+ 498, quinine 7.6; with DOG: TEA+ 238, quinine 10.5; Figure 7b), and 5x-PKC (EC50 in μM without DOG: TEA+ 518, quinine 8.9; with DOG: TEA+ 368, quinine 8.1; Figure 7c) were not statistically different. The same was found with S286A, S292A, and S328A, in which PKC activation did not change the apparent affinity for TEA+ (EC50 in μM without DOG: 153, n = 4 to 13; 56, n = 4 to 13 and 484, n = 7 to 23; with DOG: 109, n = 9 to 14; 99, n = 5 to 9 and 291, n = 5 to 10, respectively; Table 1). Because the regulatory pathway linked to the p56/lck tyrosine kinase is active in WT and all mutants (as shown by the experiments with aminogensitein), we have tested if this regulation is also associated with changes in transporter affinity. The concentration-response curves for ASP+ uptake inhibition by TEA+ with or without aminogenestein incubation in WT were not significantly different (EC50 without aminogenestein: 68 μmol, n = 8 to 34; with aminogenestein: 69 μmol, n = 7 to 11).

Effect of PKC Stimulation on Trafficking of rOCT1

Figure 8a shows the effects of incubation with 1 μmol DOG on the membrane-associated fluorescence in WT and 5x-PKC cells. Activation of PKC with DOG caused no significant increase of cell-associated fluorescence in WT and also in 5x-PKC cells. Exposition of the intracellular transporter pool by cell permeabilization resulted in a small but significant increase of fluorescence (10 ± 2%, n = 4, Figure 8b).

Immunoprecipitation of rOCT1 and Detection of Phosphorylated Transporters

After stimulation of PKC with DOG, rOCT1 phosphorylation in WT increased substantially, whereas it remained unchanged in 5x-PKC (Figure 9a). Total protein was the same with or without DOG for WT- and also for 5x-PKC-cells (Figure 9b). Western blot analysis confirmed the presence of rOCT1 at an apparent molecular weight of 70 KDa (Figure 9c).

Discussion

OCT play an essential role in controlling concentration of endogenous and exogenous positively charged metabolites in the body fluids. In the kidney, OCT represent an important detoxification pathway for many potentially harmful sub-
For these reasons, regulation of their activity has a great physiological and pathophysiological significance. These transporters have a species-specific organ localization of the different isoforms: For example, in the rat the principal renal isoform is isoform 1, whereas in humans it is isoform 2 (29).

All OCT possess several potential PKC-phosphorylation sites in the intracellular loops of the molecule (5), suggesting a role for PKC in the regulation of OCT activity. However, different isoforms have a different pattern of PKC sites (29). This can explain why different isoforms are differently regulated. hOCT1 (11), hOCT2 (10), and hOCT3 (12), for example, are not activated by PKC stimulation, but by various other regulatory pathways such as PKA or Ca\(^{2+}\)-calmodulin (10,12). In this study, we attempted to determine which of the described five putative PKC sites (23) is responsible for PKC-mediated regulation of rOCT1 and whether transporter phosphorylation changes substrate affinities. Properties and regulation of cation transport of rOCT1 stably expressed in HEK293 cells after site-directed mutagenesis of the single PKC-sites S286, S292, T296, S328, and T550, or of all PKC-sites to alanine have been compared with wild-type rOCT1. The experiments on apparent substrate affinities show that each of the five mutations of the putative PKC-phosphorylation sites has different effects on the apparent affinity of the transporter for its substrates, even though the rank order of affinity was not changed being always TPA\(^+\) > quinine > TEA\(^+\). However, the ratios between the EC\(_{50}\) values of the mutants and of the WT-rOCT1 (Table 1) show that different mutations change apparent substrate affinities to different extents: T296A caused the highest change in TEA\(^+\) affinity, whereas for TPA\(^+\) and quinine the biggest

### Table 1. Effects of mutations of potential PKC-phosphorylation sites on the EC\(_{50}\) (μmol) for TEA\(^+\), TPA\(^+\), and quinine

<table>
<thead>
<tr>
<th>Transporter</th>
<th>TEA(^+) -DOG</th>
<th>TEA(^+) +DOG</th>
<th>TPA(^+)</th>
<th>Quinine</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>105</td>
<td>19*</td>
<td>0.1</td>
<td>1.5</td>
</tr>
<tr>
<td>S286A</td>
<td>153 (1.5)</td>
<td>109</td>
<td>2.1* (30.1)</td>
<td>3.0* (2.0)</td>
</tr>
<tr>
<td>S292A</td>
<td>56 (0.5)</td>
<td>99</td>
<td>0.3* (4.7)</td>
<td>2.5* (1.7)</td>
</tr>
<tr>
<td>T296A</td>
<td>1135* (10.8)</td>
<td>1422</td>
<td>1.0* (14)</td>
<td>4.8* (3.2)</td>
</tr>
<tr>
<td>S328A</td>
<td>484* (4.6)</td>
<td>291</td>
<td>43* (614)</td>
<td>81* (54)</td>
</tr>
<tr>
<td>T550A</td>
<td>498* (4.7)</td>
<td>238</td>
<td>0.3* (4.7)</td>
<td>7.6* (5.1)</td>
</tr>
<tr>
<td>5x-PKC</td>
<td>518* (4.9)</td>
<td>368</td>
<td>2.2* (31.9)</td>
<td>8.9* (5.9)</td>
</tr>
</tbody>
</table>

Ratios of EC\(_{50}\) values obtained with mutated transporters and with those of WT-rOCT1 are presented in italics. PKC indicates protein kinase C; WT, wild-type; DOG, sn1,2-dioctanoyl glycerol.

Effects of activation of PKC with 1 μmol DOG on the apparent affinities of the transporters for TEA\(^+\) are also reported.

*Significant differences from WT (one-way ANOVA, P < 0.05).

†Significant differences from nonstimulated cells (F test, P < 0.05; GraphPad Prism, San Diego, CA).

Figure 7. Concentration-response curves for the effects of TEA\(^+\) (squares) and quinine (triangles) on uptake of 1 μmol ASP\(^+\) by T296A- (a), T550A-mutants (b) and 5x-PKC (c) under control conditions (closed squares, n = 6 to 20; n = 11 to 17; n = 10 to 58 and closed triangles, n = 4 to 14; n = 5 to 8; n = 7 to 21 for T296A, T550A, and 5x-PKC respectively) and after incubation with 1 μmol DOG for 10 min (open squares, n = 8 to 14; n = 10 to 15; n = 6 to 17 and open triangles, n = 4 to 9; n = 6 to 11; n = 7 to 15 for T296A, T550A, and 5x-PKC, respectively). Data are presented as initial slope (mean values ± SEM) of the uptake in percent of control experiments performed at the same day with cells of the same passage and age. The curves in the presence of DOG are not significantly different from the respective curves without DOG.
change in affinity could be observed with S328A. The shift of apparent affinities was in T550A of the same order of magnitude for all tested substrates, suggesting that this mutation influences mainly the ASP*/H11001 recognition site in the binding pocket of the rOCT1. In S286A and S292A, there was no significant change of the apparent affinity for TEA*/H11001; however, those for quinine and, to a greater extent, for TPA*/H11001 were significantly lower than in WT. The results of this study show that mutations in the regulatory residues of the intracellular domains of the molecule influence to different extents the binding of various substrates and inhibitors of OCT types, as also proposed on the basis of other site directed mutagenesis experiments (30,31). Modeling of the substrate binding regions of rOCT1 using the tertiary structure of the lactose permease from Escherichia coli supports this view (31). More importantly, these findings indicate that the large intracellular loop containing the PKC sites is part of the binding pocket of the rOCT1 or closely interacts with it. Other evidence supporting and extending this view to other OCT types come from electrophysiological studies: In oocytes expressing rOCT2, tetrabutylammonium had a significantly higher affinity from the outside compared with the inside, whereas corticosterone had a higher affinity from the inside compared with the outside (32). These experiments with the mutated transporters confirm the importance of PKC in the regulation of rOCT1: After site-directed mutagenesis of all five putative PKC-phosphorylation sites, activation of PKC by DOG did not stimulate more ASP*/H11001 uptake. Detection of phosphorylated transporters after immunoprecipitation shows that PKC activation of the 5x-PKC mutant does not increase transporter phosphorylation, in contrast to what was observed with WT cells. Also, single substitutions of the S286, S292, T296, S328, or of T550 with alanine abolished PKC-mediated stimulation of ASP*/H11001 uptake. The PKC-mediated regulation of rOCT1 seems not to be associated with changes in transporter trafficking from an intracellular pool to the membrane, as demonstrated by FACS analysis using fluorescence-labeled antibodies directed versus the extracellular domain of the transporter. Membrane-associated fluorescence of WT and 5x-PKC cells was very similar, suggesting no or only minimal change in transporter incorporation into the membrane with PKC activation. Experiments after membrane permeabilization showed that the in-

Figure 8. (A) Effects of 10 min incubation with 1 μmol DOG on cellular fluorescence of WT cells and 5x-PKC-mutants. Transporters were labeled with an antibody against the extracellular region of rOCT1. Data are given as cell-associated fluorescence in arbitrary units as measured by FACS scanning (B) Effects of permeabilization with saponin obtained in four paired experiments, where WT cells were divided after fixation in two parts: One aliquot was further processed with and the other without permeabilization with saponin. Data are given as cell-associated fluorescence in arbitrary units as measured by FACS scanning. * indicates statistically significant differences from control measurements.

Figure 9. Effects of 10 min incubation with 1 μmol DOG on phosphorylation of WT cells and 5x-PKC mutants. Results of staining of phosphoproteins with Pro-Q-Diamond are illustrated in a. Staining for total protein is illustrated in b and Western blot analysis is shown in c. Before the assay, the same number of WT or 5x-PKC-mutant cells of same passage and age was incubated with or without DOG, immunoprecipitated, and separated with electrophoresis.
tracellular pool of rOCT1 accounts only for 10% of the total number of transporters. Because this intracellular pool is relative small, it is unlikely that recruitment of this pool can explain the large effect of PKC activation on rOCT1 activity. For many other transporters and ion channels, regulation via different mechanisms is long known. Direct phosphorylation of multiple canonical sites has been suggested for PKC-induced regulation of antidepressant-sensitive serotonin transporters (33). Stimulation of PKC decreased the activity of the human organic anion transporter 1 (hOAT1) transiently expressed in Xenopus laevis oocytes or HEK293 cells. This regulatory effect is achieved through carrier retrieval from the cell membrane and does not involve phosphorylation of the seven predicted hOAT1 PKC sites (34). Even if some of the consensus PKC sites are conserved in some OAT types and OCT types, there is a sharp divergence in their regulation. Therefore, rather than the presence of determined single residues, the overall complex of potential PKC sites (never the same in different isoforms of ion transporters) on the intracellular domain may be important to determine if and how a transporter is regulated. It also could be speculated that modifications in one or more putative PKC sites prevents phosphorylation of another site by steric alteration of the molecule. Such steric influences on functional properties of rOCT1 are also evident from changes in PKA sensitivity seen with some of these mutations in putative PKC sites. The PKA-mediated regulation of rOCT1 was still active in T296A and T550A mutants, but not in S286A, S292A, and S328A mutants. Inhibition of p56^Lck tyrosine kinase with aminogenestein significantly reduced ASP^+ uptake in all mutants tested. The regulation through this pathway is not influenced by PKC site mutations, probably because the regulatory effect of p56^Lck tyrosine kinase is not associated with affinity changes of the transporter, as demonstrated by experiments in which concentration-response curves for ASP^+ uptake inhibition by TEA^+ under aminogenestein incubation in WT were compared with those obtained without aminogenestein incubation. Further studies are necessary to demonstrate that regulation of rOCT1 activity via tyrosin kinases also involves direct phosphorylation of the transporter as in the case for PKC-mediated activation or whether this activation involves altered trafficking of the molecule to the plasma membrane as shown, e.g., for organic anion transporters (34).

Because different organic cations may have different interaction sites in rOCT1 binding pocket, such regulatory mechanisms may have more or less pronounced effects on different substrates. Activation of PKC with DOG did not change apparent affinities of mutated rOCT1 for TEA^+ and quinine more, in contrast to what was found with WT-rOCT1, confirming a direct phosphorylation of this transporter (8) and the importance of these residues for the regulation of the transporter by PKC activation. The data suggest that all putative PKC sites can be endogenously phosphorylated, as shown by the decrease of apparent affinity for the tested substances in every mutant (Table 1). However, endogenous phosphorylation of S286 and S292 influences the binding sites for TPA^+ and, to a lower extent, for quinine, but not that for TEA^+. These modulations of the substrate affinity of rOCT1 depending on phosphorylation of the molecule may explain the differences in apparent substrate affinities of OCTs determined in different organs or species or after expression of cloned transporters in different cellular systems (8,35).

In conclusion, we demonstrated that the presence of all five potential PKC-phosphorylation sites in the intracellular domain of rOCT1 is essential for its regulation by PKC. These data suggested that the large intracellular loop of rOCT1 is part of the binding pocket or closely interacts with it and that under basal conditions rOCT1 is partially endogenously phosphorylated. Phosphorylation of different residues influences the binding site for various substrates differently and occurs at the S286, S292, T296, S328, and T550 residues.

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