Expression cloning and characterization of a renal organic anion transporter from winter flounder

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Abstract The cDNA coding for a renal *p*-aminohippurate (PAH) transporter from winter flounder (*Pseudopleuronectes americanus*), designated fROAT, was cloned by functional expression in *Xenopus laevis* oocytes. fROAT is approximately 2.8 kbp in length and encodes a protein of 562 amino acids, related to the rat renal organic anion transporter ROAT1/OAT1 and the organic cation transporters OCT1 and OCT2. In oocytes, fROAT mediated probenecid-sensitive PAH uptake, with a K_m for PAH of about 20 μM, and inhibited by external glutarate (GA) (1 mM). The functional characteristics suggest that fROAT is the basolateral PAH/dicarboxylate exchanger of the flounder kidney.

Key words: Organic anion transport; *p*-Aminohippurate; Kidney; Expression cloning; *Pseudopleuronectes americanus*

1. Introduction

The secretory renal organic anion transport pathway, which handles *p*-aminohippurate (PAH) and a wide variety of other substrates, plays a vital role in the elimination of potentially toxic compounds, including xenobiotics and drugs, from the body (reviewed in [1]). PAH secretion, which is also noteworthy for its effectiveness, has been demonstrated in a number of both non-mammalian and mammalian species, including humans. While the mode of the luminal exit of PAH varies among species [1], the uphill basolateral uptake into the epithelial cell appears to always occur via the same mechanism, i.e. a coupled system involving Na^+-driven dicarboxylate uptake and PAH/dicarboxylate exchange [2,3]. Thus, evidence for the operation of this tertiary active uptake system has been obtained for rat [2,3], pig [4], bovine [5] and human [6] renal proximal tubules with isolated basolateral membrane vesicles, as well as crab urinary bladder [7] and proximal tubules of marine teleosts [8,9], snake [10], rat [11] and rabbit [12] in intact tissue preparations. This suggests that the basolateral PAH/dicarboxylate exchanger is a transport mechanism that has been highly conserved through evolution.

Flounder kidney tubules provide a particularly suitable starting material for a cloning strategy based on functional expression, since they consist almost exclusively of proximal tubules [13], the region which actively secretes PAH [14]. This study reports the expression cloning of a cDNA coding for a renal PAH transporter from the winter flounder (*Pseudopleuronectes americanus*), fROAT. The functional characteristics of the transporter as expressed in *Xenopus laevis* oocytes suggest that it corresponds to the basolateral PAH/dicarboxylate exchanger of the flounder kidney.

2. Materials and methods

2.1. mRNA preparation, size fractionation, cDNA library construction and screening

mRNA from winter flounder (*Pseudopleuronectes americanus*) kidney was isolated and size-fractionated as previously described [15]. A 2–4 kbp size fraction of flounder renal tubule mRNA was used to construct a unidirectional cDNA library [15]. About 50 000 recombinants were screened. The bacteria were plated on nitrocellulose filters (Protran BA 85, 0.45 μm, Schleicher and Schuell) [16] and replicas of each master filter were incubated overnight for plasmid DNA purification. The colonies from each filter were then detached by shaking in liquid medium (1 h, 60 rpm, 37°C), and the plasmid DNA was purified using the plasmid mini kit (Qiagen). The plasmid DNA was linearized with *Not*I, and capped cRNA was synthesized using the mMessage mMachine-T7 in vitro transcription kit (Ambion).

2.2. Xenopus laevis oocytes and transport experiments

Stage V–VI oocytes in follicles from *Xenopus laevis* were manually dissected and incubated overnight at 18°C in modified Barth's solution (in mM: 88 NaCl, 1 KCl, 0.3 $Ca(NO_3)_2$, 0.41 $CaCl_2$, 0.82 $MgSO_4$, 15 HEPES/NaOH, pH 7.6) containing 10 μg/ml streptomycin sulfate. They were then injected with cRNA (40 ng in a volume of up to 50 nl), or an equivalent volume of H_2O (controls). After 3 days of incubation at 18°C in modified Barth's solution with streptomycin sulfate, transport of [^{3}H]PAH (5 μCi/ml) (aminohippuric acid, P-[glycyl-2-^{3}H], 1–5 Ci/mmol, NEN) or [^{14}C]urate (2.5 μCi/ml) (uric acid, [8-^{14}C], 50–60 mCi/mmol, American Radiolabeled Chemicals, Inc.) was assayed at room temperature in uptake buffer (in mM): 90 NaCl, 3 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 5 HEPES/Tris, pH 7.6. After the indicated time period, the uptake was terminated by aspiration of the incubation medium and 3×3 ml washes with ice-cold uptake buffer. Each oocyte was then dissolved in 0.1 ml of 1 N NaOH and, after neutralization with 0.1 ml of 1 N HCl, the ^{3}H or ^{14}C content assayed by liquid scintillation counting.

2.3. cDNA sequencing and analysis

For sequencing, deletion clones were generated using the Erase-a-Base system (Promega). Both strands of the fROAT cDNA were sequenced by dye terminator cycle sequencing (Applied Biosystems) with M13/pUC sequencing primers (Fermentas) (automatic sequencer: ABI Prism, Applied Biosystems). The sequence was assembled and analyzed with the Genetics Computer Group (GCG) software package, unless otherwise indicated. Sequence homology searches were performed at the National Center for Biotechnology Information using the BLAST network service.

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Abbreviations: PAH, *p*-aminohippurate; GA, glutarate; PKC, protein kinase C; PKA, protein kinase A; CK-2, casein kinase II; SITS, 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonate; TEA, tetraethylammonium

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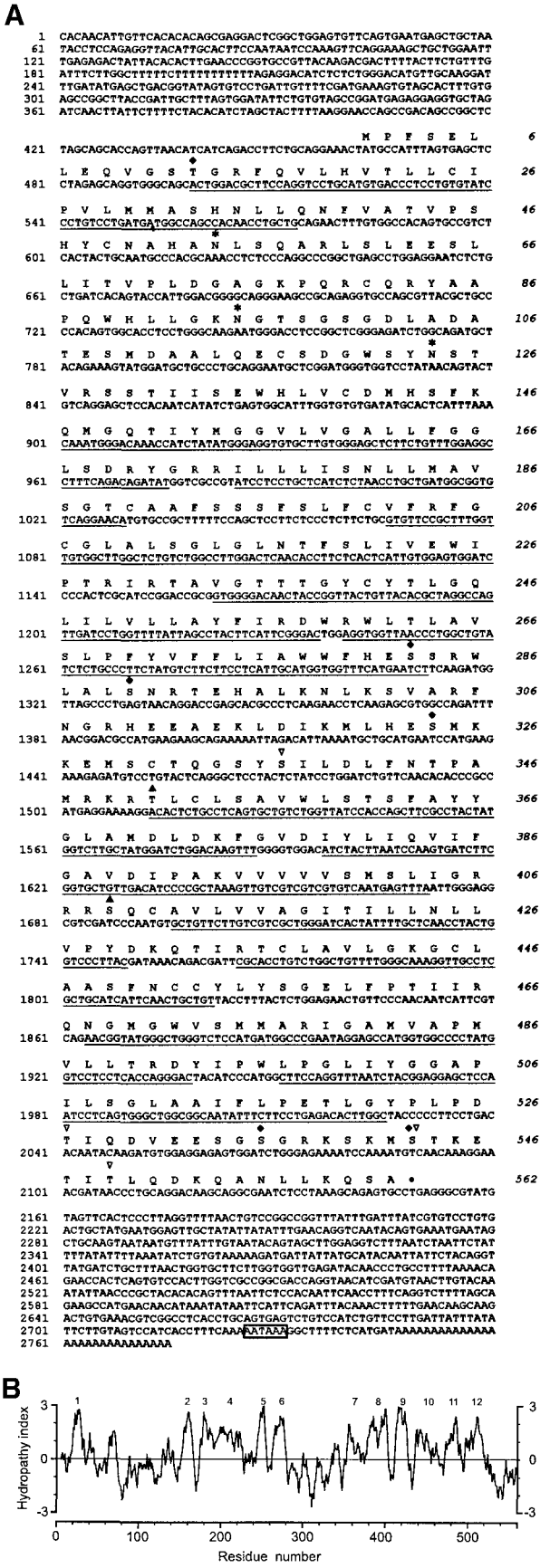

Fig. 1. Nucleotide (below) and deduced amino acid sequence (above) (A) and Kyte-Doolittle hydropathy analysis (B) of fROAT. A: The nucleotides are numbered on the left and the amino acids in italics on the right. Predicted transmembrane domains, as obtained using the SOSUI system (Tokyo University of Agriculture and Technology), are underlined, and potential *N*-linked glycosylation sites are indicated by asterisks. Putative protein kinase A (▲), protein kinase C (♦) and casein kinase II (▽) phosphorylation sites are indicated. The polyadenylation signal is boxed, and ● denotes the first in-frame termination codon. The sequence has been submitted to the EMBL database and was assigned the accession no. Z97028. B: Kyte-Doolittle [18] hydropathy analysis using a window setting of 9 amino acids. The putative membrane spanning domains are numbered.

3. Results

3.1. Expression cloning of fROAT cDNA

A winter flounder kidney cDNA library [15] was screened for expression of probenecid-sensitive PAH transport. A single cDNA clone was isolated which increased PAH transport of up to 30-fold over controls (cRNA: 81.7 ± 10.2 pmol/h per oocyte; controls: 5.4 ± 0.7 pmol/h per oocyte; means ± S.E.M., $n = 6$ independent experiments). This cDNA was called fROAT or flounder renal organic anion transporter.

3.2. Nucleotide and deduced amino acid sequence of fROAT

The fROAT cDNA is 2775 nucleotides in length, with a major open reading frame encoding a protein of 562 amino acids (Fig. 1A) with a calculated molecular mass of 62 kDa. This reading frame contains several potential AUG initiation codons, which lie in favorable context for translation initiation according to Kozak's rules [17]. Due to its 5′-proximal location, the first of these was tentatively assigned as the first codon. Kyte-Doolittle [18] hydropathy analysis of the fROAT protein predicts 12 transmembrane spanning domains (Fig. 1B). Secondary structure predictions based on this analysis suggest an intracellular location of both the N and C termini. A large extracellular loop between putative transmembrane domains 1 and 2 contains three potential *N*-glycosylation sites (NXS/T) and four cysteine residues, Cys^{49}, Cys^{81}, Cys^{117}, and Cys^{140}, possibly involved in the formation of disulfide bridges. Potential target sites for protein kinase C (PKC) and casein kinase II (CK-2) phosphorylation [19] are localized predominantly in the large intracellular loop between transmembrane domains 6 and 7 and in the C-terminal domain (Fig. 1A).

3.3. Related database sequences

Apart from similarities to hypothetical proteins from *Drosophila melanogaster* and *Caenorhabditis elegans*, database searches revealed the greatest homology of the fROAT protein to five recently cloned proteins of suggested or known organic ion transport function: ROAT1/OAT1, a rat renal organic anion transporter [20–22], the kidney-specific protein NKT [23], the liver-specific protein NLT [24], and the renal cation transporters OCT1 [25] and OCT2 [26] (Fig. 2). All six sequences also have common structural features: the predicted number of transmembrane domains, the large extracellular loop between transmembrane domains 1 and 2 with four conserved cysteines and several potential glycosylation sites, and the large intracellular loop between transmembrane domains 6 and 7. Additionally, they share some CK-2 (fROAT: Ser^{337}, Thr^{527}) and PKC consensus sites (fROAT: Ser^{283}). However,

Fig. 2. Comparison of the fROAT amino acid sequence with other proteins of known or suggested organic ion transport function. The alignment was performed using the Clustal method and the PAM250 residue weight table (DNAstar, Lasergene, Madison, WI). Amino acids identical to fROAT are shaded in black.

the overall homology to ROAT1 and NKT (46.9% and 47.9% identity, respectively) was significantly greater than to the other three proteins (38.2%, 33.4%, and 31.6% identity, respectively), with highly conserved regions in putative transmembrane domains 1, 7, 8 and 10. Interestingly, they also share one PKC consensus site (fROAT: Ser^{290}) not found in NLT, OCT1 and OCT2. On the other hand, of the target sites for protein kinase A (PKA) phosphorylation – not present in ROAT1 and NKT – one appears to be conserved between fROAT (Arg^{348}) and the organic cation transporters, the other between fROAT (Arg^{406}) and NLT. Whether any of these sites is of physiological importance remains to be determined.

3.4. *Functional characterization of the fROAT protein expressed in Xenopus laevis oocytes*

As a first step toward associating fROAT with one of the known renal organic ion transport systems, a variety of agents were tested for their ability to inhibit fROAT-mediated PAH uptake (Fig. 3). PAH transport by fROAT was greatly reduced by probenecid, 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonate (SITS), a 20-fold excess of unlabeled PAH, the loop diuretic bumetanide, and the dicarboxylate glutarate. The organic anion urate produced only a moderate inhibition (45%), and was itself not transported by fROAT at 50 μM under the same conditions (cRNA: 1.44 ± 0.10 pmol/h per oocyte, $n = 9$ oocytes; controls: 1.70 ± 0.33 pmol/h per oocyte, $n = 10$ oocytes; means ± S.E.M.). In contrast, the organic cation TEA (5 mM), a model substrate for the organic cation transporters OCT1 [25] and OCT2 [26], did not inhibit PAH uptake expressed from fROAT.

In order to more accurately compare the inhibitory potency of various dicarboxylates, initial rates of PAH uptake (5 min) were measured. Uptake of 50 μM PAH had previously been determined to be linear through this period (not shown). Of the dicarboxylates tested, only α-ketoglutarate (C5) and suberate (C8) significantly reduced PAH uptake by fROAT (> 70%), while malonate (C3), succinate (C4) and fumarate (C4) did not. Citrate (C6) produced a moderate but not statistically significant inhibition of about 45% (data not shown).

Trans-stimulation by dicarboxylate (GA) could only be demonstrated when oocytes were preloaded for 90 min with 1 mM GA and initial rate PAH uptake was then determined in the absence of GA (not preloaded: 19.26 ± 2.00 pmol/5 min per oocyte, $n = 11$ oocytes; GA-preloaded: 30.26 ± 2.87 pmol/

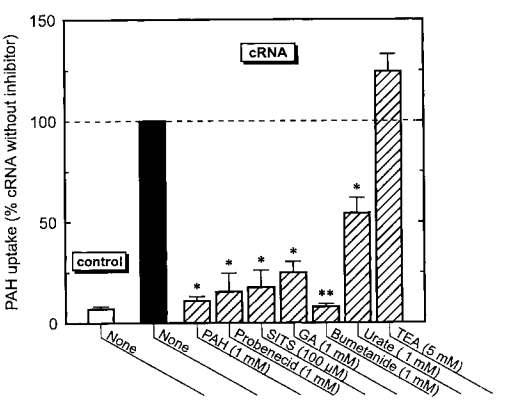

Fig. 3. Sensitivity of PAH uptake to various potential inhibitors in oocytes injected with fROAT cRNA. 1 h uptakes of 50 μM [^{3}H]PAH were assayed in the absence or presence of organic test ions. Data are means ± S.E.M. of $n = 3$ (H_2O controls $n = 7$) independent determinations, each carried out on 7–14 oocytes per treatment. Significance of differences in Student's *t*-test relative to the corresponding uptake of cRNA-injected oocytes in the absence of test ion is indicated (*$P < 0.05$, **$P < 0.01$).

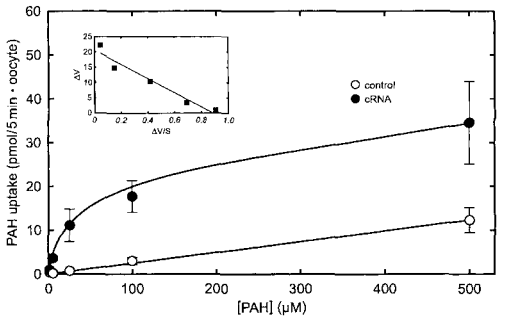

Fig. 4. Concentration dependence of PAH uptake in oocytes injected with fROAT cRNA. Oocytes were injected with fROAT cRNA or H_2O (control), and the initial rate (5 min) of uptake was assayed at 1–500 μM PAH. The inset shows an Eadie-Hofstee plot of the corrected uptake rates expressed from fROAT (uptake of control oocytes subtracted) (ΔV). Using linear regression, an apparent K_m for PAH of about 20 μM was calculated from this analysis. Data are means ± S.E.M. of $n = 3$ independent experiments, each carried out on 8–14 oocytes per treatment.

5 min per oocyte, $n = 15$ oocytes; means ± S.E.M.; Student's t-test: $P < 0.01$). Uptake by control oocytes was unaffected by GA preloading (not shown). Micromolar concentrations of external glutarate, however, did not stimulate PAH uptake (not shown).

The initial rate of PAH transport by fROAT was saturable and could be described by Michaelis-Menten type kinetics (Fig. 4). In contrast, uptake by control oocytes was linearly related to PAH concentration. In three independent experiments an apparent K_m of fROAT for PAH of 21 ± 4 μM was obtained by Eadie-Hofstee analysis (Fig. 4, inset).

4. Discussion

This study reports the expression cloning and partial functional characterization of a PAH transporter from flounder kidney, fROAT. The sequence and secondary structure of the 562 amino acid protein indicates that it belongs to a family of organic ion transporters comprising the rat renal organic anion transporter ROAT1/OAT1, as well as the organic cation transporters OCT1 and OCT2, and two proteins of as yet unknown function, NKT and NLT. The functional characteristics of fROAT suggest that it represents the basolateral PAH/dicarboxylate exchanger.

With ROAT1, which appears to be the basolateral organic anion carrier of the rat kidney and was cloned independently using the same approach, the fROAT protein shares not only a high sequence homology but also strikingly similar functional properties: saturable probenecid-sensitive PAH transport inhibited by external α-KG and *trans*-stimulated by internal GA, but not significantly affected by 1 mM TEA. Notably different between the two transporters is only the affinity of fROAT for the organic anion urate, which does not significantly affect PAH transport by ROAT1. Most recently, Sekine et al. [22] published an amino acid sequence 100% identical ROAT1, and also demonstrated that this protein functions as a PAH/dicarboxylate exchanger. The high homology of NKT to ROAT1 (94.9% identity), as well as – considering the evolutionary distance between the two species – to fROAT (47.9% identity), strongly suggests that NKT represents the mouse analogue of the flounder renal organic anion transporter and the rat ROAT1. Similarly, Lopez-Nieto et al. [23] speculated – based on sequence comparison with OCT1 – that NKT corresponds to an organic ion carrier with different substrate specificity, even though they could not demonstrate any transport activity by NKT expressed in *Xenopus laevis* oocytes. On the other hand, the homology and conserved PKA consensus sites between fROAT and NLT, or OCT1 and OCT2, suggests a common ancestor of all these organic ion transporters. In contrast, the fROAT protein shows little homology with the recently cloned multispecific organic anion carrier from rat liver, oatp [27] or the closely related rat kidney methotrexate transporter, OAT-K1 [28]: 14.9% and 15.7% identity, respectively.

Basolateral uptake of PAH appears to occur via exchange for dicarboxylates in both winter and Southern flounder proximal tubule cells [8,29]. Consistent with a transporter accepting both PAH and dicarboxylates, PAH uptake by fROAT was strongly *cis*-inhibited by GA, α-ketoglutarate and suberate. Moreover, the pattern of inhibition by dicarboxylates, requiring a chain length of at least 5 carbons, is compatible with the results obtained in isolated rat renal basolateral membrane vesicles [2,3,30] and rat perfused proximal tubules in situ [31]. Unlike the results obtained with rat renal basolateral membrane vesicles [2,3] and cortical slices [11], no significant stimulation of PAH uptake by low external GA was observed at any GA concentration in fROAT-expressing oocytes. Similarly, Dawson and Renfro [29], using winter flounder renal primary cultures, could not demonstrate stimulation of secretory PAH flux by low external GA, but only significant inhibition at higher GA concentrations. With regard to the oocytes, this discrepancy is most likely due to the low rate of endogenous GA uptake [32]. However, the *trans*-stimulation of fROAT-mediated PAH uptake in oocytes preloaded with GA again supports that this system operates as PAH/dicarboxylate exchanger.

The apparent K_m of fROAT for PAH (about 20 μM) is lower than previously reported for isolated winter flounder renal tubules in vitro [33] and primary monolayer cultures [29], 157 μM and 400 μM, respectively. These differences in affinity might be due to different composition of the surrounding membrane or to different posttranslational processing of the protein in oocytes. Similar discrepancies have been observed with flounder kidney Na^+-phosphate transporter, NaP_i-II [15] and rabbit intestinal Na^+-glucose cotransporter, SGLT1 [34].

In summary, a PAH transporter has been cloned from flounder kidney by functional expression in *Xenopus laevis* oocytes. The characteristics of fROAT-mediated PAH uptake in the oocytes – high sensitivity to probenecid, *cis*-inhibition by dicarboxylates with ≥ 5 carbon atoms, in particular glutarate and α-ketoglutarate, and *trans*-stimulation by GA – resemble those of the basolateral PAH/dicarboxylate exchanger of the renal proximal tubule of flounder, snake and a number of mammalian species, including humans. Together with ROAT1/OAT1 and NKT, fROAT provides important information about conserved regions, useful not only in the determination of functional domains but also in the search for a human analogue of this protein.

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References

[1] Pritchard, J.B. and Miller, D.S. (1993) Physiol. Rev. 73, 765–796.
[2] Pritchard, J.B. (1988) Am. J. Physiol. 255, F597–F604.
[3] Shimada, H., Moewes, B. and Burckhardt, G. (1987) Am. J. Physiol. 253, F795–F801.
[4] Werner, D. and Roch-Ramel, F. (1991) Am. J. Physiol. 261, F265–F272.
[5] Schmitt, C. and Burckhardt, G. (1993) Pflügers Arch. 423, 280–290.
[6] Guisan, B. and Roch-Ramel, F. (1995) Experientia 51, A93 (Abstract).
[7] Smith, P.M., Miller, D.S. and Pritchard, J.B. (1990) Am. J. Physiol. 259, R147–R156.
[8] Miller, D.S. and Pritchard, J.B. (1991) Am. J. Physiol. 261, R1470–R1477.
[9] Miller, D.S., Letcher, S. and Barnes, D.M. (1996) Am. J. Physiol. 271, F508–F520.
[10] Chatsudthipong, V. and Dantzler, W.H. (1991) Am. J. Physiol. 261, F858–F867.
[11] Pritchard, J.B. (1990) J. Pharmacol. Exp. Ther. 255, 969–975.
[12] Chatsudthipong, V. and Dantzler, W.H. (1992) Am. J. Physiol. 263, F384–F391.
[13] Kinter, W.B. (1975) Fortschr. Zool. 23, 223–231.
[14] Woodhall, P.B., Tisher, C.C., Simonton, C.A. and Robinson, R.R. (1978) J. Clin. Invest. 61, 1320–1329.
[15] Werner, A., Murer, H. and Kinne, R.K.H. (1994) Am. J. Physiol. 267, F311–F317.
[16] Hanahan, D. and Meselson, M. (1980) Gene 10, 63–67.
[17] Kozak, M. (1987) Nucleic Acids Res. 15, 8125–8148.
[18] Kyte, J. and Doolittle, R.F. (1982) J. Mol. Biol. 157, 105–132.
[19] Kennelly, P.J. and Krebs, E.G. (1991) J. Biol. Chem. 266, 15555–15558.
[20] Sweet, D.H., Wolff, N.A. and Pritchard, J.B. (1997) FASEB J. 11, A278 (Abstract).
[21] Sweet, D.H., Wolff, N.A. and Pritchard, J.B. (1997) J. Biol. Chem. (in press).
[22] Sekine, T., Watanabe, N., Hosoyamada, M., Kanai, Y. and Endou, H. (1997) J. Biol. Chem. 272, 18526–18529.
[23] Lopez-Nieto, C.E., You, G., Bush, K.T., Barros, E.J.G., Beier, D.R. and Nigam, S.K. (1997) J. Biol. Chem. 272, 6471–6478.
[24] Simonson, G.D., Vincent, A.C., Roberg, K.J., Huang, Y. and Iwanij, V. (1994) J. Cell Sci. 107, 1065–1072.
[25] Gründemann, D., Gorboulev, V., Gambaryan, S., Veyhl, M. and Koepsell, H. (1994) Nature 372, 549–552.
[26] Okuda, M., Saito, H., Urakami, Y., Takano, M. and Inui, K. (1996) Biochem. Biophys. Res. Commun. 224, 500–507.
[27] Jacquemin, E., Hagenbuch, B., Stieger, B., Wolkoff, A.W. and Meier, P.J. (1994) Proc. Natl. Acad. Sci. USA 91, 133–137.
[28] Saito, H., Masuda, S. and Inui, K. (1996) J. Biol. Chem. 271, 20719–20725.
[29] Dawson, M.A. and Renfro, J.L. (1990) J. Pharmacol. Exp. Ther. 254, 39–44.
[30] Burckhardt, G., Schmitt, C. and Ullrich, K.J. (1991) in: Nephrology (Hatano, M., Ed.), Vol. II, pp. 1380–1390, Springer Verlag, Tokyo.
[31] Fritzsch, G., Rumrich, G. and Ullrich, K.J. (1989) Biochim. Biophys. Acta 978, 249–256.
[32] Pajor, A.M. and Sun, N. (1996) Am. J. Physiol. 271, F1093–F1099.
[33] Pritchard, J.B. and James, M.O. (1979) J. Pharmacol. Exp. Ther. 208, 280–286.
[34] Ikeda, T.S., Hwang, E., Coady, M.J., Hirayama, B.A., Hediger, M.A. and Wright, E.M. (1989) J. Membrane Biol. 110, 87–95.