Expression of a rat renal sodium-dependent dicarboxylate transporter in *Xenopus* oocytes

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Microinjection of mRNA isolated from rat kidney cortex into *Xenopus laevis* oocytes resulted in the expression of a Na⁺-dependent dicarboxylate transporter, as detected by uptake measurements with [¹⁴C]succinate as substrate. The expressed transporter showed an S-shaped Na⁺-dependence with half-maximal activation at 19–21 mM Na⁺ and a Hill coefficient between 2 and 3. Endogenous succinate uptake was not Na⁺-dependent. Na⁺-stimulated succinate uptake in mRNA-injected oocytes exhibited a maximum at pH 7.5, whereas endogenous Na⁺-independent transporter was fastest at pH 8.5. The expressed

dicarboxylate transporter also differed from the endogenous transporter in its sensitivity to citrate as well as dicarboxylates in *trans* and *cis* configurations. The expressed transporter resembled the renal basolateral transporter, especially with respect to affinity for succinate (K_m 28 μ M), activation by Na⁺, pH-dependence and substrate specificity. After injection of size-fractionated mRNA, succinate uptake was expressed by mRNA of 2–3 kb. Our results suggest expression of the basolateral Na⁺-dependent dicarboxylate transporter after injection of mRNA from rat kidney into *Xenopus* oocytes.

INTRODUCTION

In the mammalian kidney, Krebs-cycle intermediates serve as an important energy source for proximal tubule cells [1]. These intermediates are taken up from the primary urine as well as from the blood by Na⁺-coupled dicarboxylate transporters located in the brush-border and basolateral cell membranes [2–4]. The dicarboxylate transporter in the basolateral membrane deserves special attention because it may also be involved in cellular uptake of some drugs, e.g. the diuretic, furosemide [5]. In addition, α -oxoglutarate taken up from the blood into proximal tubular cells can leave the cells at the basolateral membrane in exchange for a great variety of organic anions including potentially toxic xenobiotics [6]. Hence, the renal Na⁺-coupled dicarboxylate transporters play a unique role in cell nutrition and in excretion of drugs and xenobiotics.

The dicarboxylate transporters in the brush-border and basolateral membranes differ markedly with respect to substrate affinity for succinate which is much higher in the basolateral-membrane transporter (10 compared with 610 μ M, [7]). The dicarboxylate-transport system of the basolateral membrane is inhibited by dimethylsuccinate [6] whereas the respective transporter in the brush-border membrane is only weakly affected. In the rat kidney, contraluminal dicarboxylate uptake is pH-dependent with maximum uptake at pH 7.0–7.5, whereas the luminal transporter seems to be independent of extracellular proton concentration [3,8]. Citrate is transported more effectively by the basolateral transporter at neutral pH than at lower pH, whereas citrate uptake by the luminal transporter is higher at lower pH than at neutral pH [4,9].

Although these transport systems have been investigated in great detail by physiological studies, information about the molecular structure of the dicarboxylate transporter is still lacking. *Xenopus laevis* oocytes are a widely used *in vitro* system for expression cloning (for review, see ref. [10]), and, for example, have been successfully used to identify a clone that encodes the Na⁺-dependent glucose transporter [11]. Therefore we used this system to express the Na⁺-dependent dicarboxylate transporter.

After injection of mRNA from rat kidney cortex a Na⁺-dependent transport system is expressed with characteristics of the renal basolateral dicarboxylate transporter. We provide evidence that, after size fractionation of mRNA, dicarboxylate transport is expressed by a 2–3 kb fraction, which can now be used to construct a library for this transporter. Preliminary results of this work have recently been published in abstract form [12].

EXPERIMENTAL

RNA preparation and fractionation

Total RNA was isolated as described by Chomczynski and Sacchi [13]. Cortex and outer medulla from kidneys of male Wistar rats were dissected and homogenized in guanidine isothiocyanate buffer followed by phenol extraction. The RNA was precipitated by adding propan-2-ol and incubation at –20 °C. The precipitated RNA was resuspended in 75% ethanol, centrifuged for 10 min, and the pellets dried under vacuum. The poly(A)⁺ mRNA was purified by chromatography on oligo(dT)-cellulose and precipitated in the presence of 0.3 M sodium acetate [14].

For size fractionation of mRNA, electrophoresis was performed with 175 μ g of mRNA on a 1.2% agarose gel in TAE buffer (40 mM Tris, 5 mM sodium acetate, 1 mM EDTA, pH 7.8). After 2 h electrophoresis at 80 V, the gel was cut in 21 horizontal strips. Each strip was placed in a dialysis bag (Serva, Heidelberg, Germany) filled with 400 μ l of TAE buffer and electroeluted at 100 V as described for DNA [14]. The mRNA-containing buffer was pipetted out of the dialysis bags and the RNA precipitated with 3 M sodium acetate and ethanol. The integrity and size of mRNA fractions were confirmed by electrophoresis on formaldehyde/agarose gel and ethidium bromide staining [14].

Oocytes, injection and uptake experiments

Xenopus laevis were purchased from Kähler (Hamburg, Germany). Oocytes were prepared from *Xenopus laevis* ovary by

treatment with collagenase and subsequent washing in Ca^{2+} -free oocyte Ringer's solution (ORi, see below) as described previously [15]. Total mRNA (50 ng/oocyte) was injected by a micropump (Bachofner, Reutlingen, Germany) into the oocyte (stages V and VI as defined by Dumont [16]). The injected oocytes were maintained for 2 days at 18 °C in Barth's medium [in mM: 90 NaCl, 2.4 NaHCO_3 , 1 K_2SO_4 , 0.8 MgSO_4 , 0.3 $\text{Ca}(\text{NO}_3)_2$, 0.4 CaCl_2 , 5 Hepes, pH 7.5] with 300 μl of gentamycin (Refobacin; Merck, Darmstadt, Germany) added per litre. The medium was changed daily and damaged oocytes were discarded.

For uptake experiments, five to ten oocytes were incubated for 30 min in ORi containing the substrate [^{14}C]succinate (NEN, Bad Homburg, Germany) at 74 kBq/400 μl of incubation solution. The standard solution (ORi) contained (in mM): 110 NaCl, 3 KCl, 2 CaCl_2 and 5 Hepes adjusted to pH 7.5. If a lower Na^+ concentration was used, Na^+ was replaced with tetramethylammonium (TMA). All experiments were performed at room temperature.

To describe the concentration-dependence of succinate uptake, the following equation was fitted to the data:

$$v = \frac{V_{\max} \times [\text{S}]^h}{[\text{S}]^h + (K_{0.5})^h}$$

where V represents rate of transport, h the Hill coefficient and $[\text{S}]$ the succinate or Na^+ concentration.

RESULTS

Xenopus oocytes exhibit an endogenous dicarboxylate transport system varying between different animals from 0.19 ± 0.04 to 2.58 ± 1.18 pmol of succinate/h per oocyte in 22 independent experiments with 5–15 oocytes each. A similar variation in endogenous uptake has been reported for a variety of substrates (for example biotin [17], glutamate [18] and phosphate [19]).

No difference in succinate uptake was found when non-injected oocytes were compared with oocytes injected with 50 nl of water (0.28 ± 0.06 compared with 0.26 ± 0.02 pmol/h per oocyte, $n = 20$). Therefore we used non-injected oocytes as controls in the following experiments.

As shown in Table 1, injection of 50 ng of mRNA per oocyte led to a stimulation of succinate uptake, which was already seen 1 day after the injection, with an increase at day 2 and no significant further change at day 3. The stimulated succinate transport was Na^+ -dependent, whereas there was no significant Na^+ -dependence of succinate uptake in control oocytes. In the following experiments uptake was tested 2 days after mRNA injection.

Expression of Na^+ /succinate co-transport was a saturable function of the amount of injected mRNA (Figure 1) with the highest expression in oocytes injected with 50 ng of mRNA. Injection of a higher amount of mRNA did not result in any further increase in expression of Na^+ -dependent succinate transport activity (results not shown). As a consequence we chose 50 ng of mRNA for injection in all further experiments.

Succinate uptake into mRNA-injected oocytes increased linearly with time for up to 60 min (Figure 2). As calculated from the slope, this amounted to 0.088 pmol/min per oocyte in the presence of Na^+ and 0.005 pmol/min per oocyte in the absence of Na^+ ($r = 0.988$). In non-injected oocytes, succinate uptake was also proportional to incubation time and was 0.005 pmol/min per oocyte in the absence or presence of Na^+ (results not shown).

Endogenous and expressed succinate uptake both showed a

saturable dependence on succinate concentration (Figure 3) with K_m values of 23 ± 4 μM (endogenous uptake) and 28 ± 6 μM succinate (expressed uptake), which agree reasonably well with the reported affinity constant of the basolateral transporter [2,3,7].

To characterize the Na^+ -dependence of the expressed succinate transporter, we varied the Na^+ concentration from 0 to 110 mM (Figure 4). Na^+ was iso-osmotically replaced by tetramethylammonium. In control oocytes, succinate uptake was low and independent of Na^+ . In contrast, [^{14}C]succinate uptake rose steeply with increasing Na^+ concentration in mRNA-injected oocytes, exhibiting sigmoidal kinetics. Using the Michaelis–Menten equation (see the Experimental section), data could be equally well fitted by assuming a co-transport of two or three Na^+ ions with one succinate molecule (solid and broken lines respectively in Figure 4). Half-maximal activation of succinate uptake occurred at 21 ± 5 mM Na^+ (assuming $n_H = 2$) or 19 ± 4 mM Na^+ ($n_H = 3$). When the data were replotted according to Hill (Figure 4b), a straight line was obtained ($r = 0.92$) with a slope of 1.40. This result also suggests the interaction of more than one Na^+ with one succinate. In this Hill plot, a $K_{0.5}$ of 19 mM Na^+ was calculated.

As rat renal basolateral and luminal dicarboxylate transporters differ in their pH-dependence [3,8], we compared succinate uptake

Table 1 Succinate uptake into *Xenopus* oocytes injected with mRNA from rat kidney cortex

Oocytes were injected with 50 ng of mRNA per oocyte and were assayed 1–3 days after injection in the presence or absence of 110 mM Na^+ for uptake of [^{14}C]succinate (42 μM in the incubation medium). Data represent means $\pm$ S.E.M. of ten oocytes of a representative experiment.

Day	Succinate uptake (pmol/h per oocyte)			
	mRNA injected		Control	
	110 mM Na^+	0 mM Na^+	110 mM Na^+	0 mM Na^+
1	1.08 ± 0.22	0.29 ± 0.09	0.06 ± 0.01	0.07 ± 0.01
2	1.67 ± 0.12	0.17 ± 0.04	0.17 ± 0.02	0.18 ± 0.03
3	1.72 ± 0.12	0.15 ± 0.06	0.16 ± 0.03	0.13 ± 0.04

Figure 1 Dependence of succinate uptake on the amount of injected mRNA

Oocytes were injected with 0, 16.7, 33.3 or 50 ng of mRNA per oocyte. After 2 days, uptake of [^{14}C]succinate (42 μM in the incubation medium) was measured in the presence (●) or absence (○) of 110 mM Na^+ . Data represent means $\pm$ S.E.M. of ten oocytes of a representative experiment.

Figure 2 Time course of succinate uptake

Oocytes were injected with 50 ng of mRNA each, and 2 days after injection uptake of [14 C]succinate (84 μ M in the incubation medium) was measured after 5, 10, 15, 30 and 60 min. Each symbol represents the mean $\pm$ S.E.M. of seven oocytes of a representative experiment. ●, Succinate uptake in the presence of 110 mM Na^+ ; ○, uptake in the absence of Na^+ .

Figure 3 Kinetics of succinate uptake into oocytes

The oocytes were injected with 50 ng of mRNA and 2 days later incubated in ORI containing various concentrations of succinate. ●, Succinate uptake into mRNA-injected oocytes; ○, succinate uptake into control oocytes. Michaelis-Menten kinetics were used to obtain K_m values for succinate of 28 ± 6 μ M (expressed transporter) and 23 ± 4 μ M (endogenous transporter). Results are means $\pm$ S.E.M. of ten oocytes of a representative experiment.

at pH 7.5 with uptake at pH 6.0 or pH 8.5 (Table 2). In the injected oocytes, maximum succinate uptake was found at pH 7.5, and uptake at pH 6.0 was higher than at pH 8.5 ($P < 0.03$). Thus the pH-dependence of the expressed succinate transporter resembles that of the renal basolateral dicarboxylate transporter. Endogenous succinate transport showed maximal uptake at pH 8.5 and decreased with increasing proton concentration, indicating a marked difference between endogenous and expressed succinate transport with respect to their pH-dependence.

In further experiments we characterized the specificity of the Na^+ -dependent succinate transporter in mRNA-injected oocytes (Table 3). The expressed transporter preferred substrates with a *trans* configuration (fumarate) over those with a *cis* configuration (maleate). As reported for the contraluminal transporter [4], the addition of dimethylsuccinate caused inhibition of succinate uptake. The tricarboxylate, citrate, inhibited [14 C]succinate uptake more effectively at neutral pH than at pH 6.0, indicating a prevalence for the trivalent form of citrate. The monocarboxylate

Figure 4 Dependence of succinate uptake on Na^+ concentration

(a) Succinate uptake into mRNA-injected oocytes (●) and control oocytes (○). mRNA (50 ng/oocyte) was injected and 2 days later uptake of [14 C]succinate (42 μ M) was measured in the presence of various Na^+ concentrations. To keep osmolality constant, Na^+ was replaced by tetramethylammonium. Results are means $\pm$ S.E.M. of ten oocytes. Michaelis-Menten kinetics was fitted to the data with a Hill coefficient of either 2 (solid line) or 3 (broken line). The calculated $K_{0.5}$ values were 21 ± 5 mM ($n_H = 2$) and 19 ± 4 mM ($n_H = 3$) Na^+ . (b) Hill plot of the data corrected for Na^+ -independent uptake.

Table 2 Influence of extracellular pH on succinate uptake

The oocytes were injected with 50 ng of mRNA per oocyte and 2 days later uptake of [14 C]succinate (84 μ M in the incubation medium) was measured in the presence and absence of 110 mM Na^+ at different pH values. Values are means $\pm$ S.E.M. of eight oocytes of a representative experiment. In the mRNA-injected oocytes, uptake at pH 6.0 and pH 7.5 differed significantly ($P < 0.03$) from uptake at pH 8.5.

pH	Succinate uptake (pmol/h per oocyte)			
	mRNA injected		Control	
	110 mM Na^+	0 mM Na^+	110 mM Na^+	0 mM Na^+
6.0	15.08 ± 2.23	2.67 ± 0.20	0.29 ± 0.03	0.37 ± 0.19
7.5	16.59 ± 1.87	0.66 ± 0.15	0.52 ± 0.08	0.45 ± 0.07
8.5	11.44 ± 1.02	0.80 ± 0.15	1.17 ± 0.28	1.56 ± 0.22

p-aminohippurate (PAH) did not cause significant inhibition of the expressed or the endogenous transporter.

Uptake of [14 C]succinate into control oocytes was also inhibited by fumarate and maleate. In contrast with mRNA-injected oocytes, fumarate was only slightly more effective than maleate,

Table 3 Influence of selected anions on succinate uptake into mRNA- and water-injected oocytes

Uptake of [14 C]succinate (42 μ M) was measured in the presence of Na $^{+}$ and the indicated anions in the incubation medium. Data represent means $\pm$ S.E.M. of seven oocytes. For citrate, the percentage inhibition was related to the respective succinate uptake at pH 6.0 or pH 7.5.

Anion (1 mM)	pH	Expressed succinate transporter		Endogenous succinate transporter	
		Succinate uptake (pmol/h per oocyte)	Inhibition (%)	Succinate uptake (pmol/h per oocyte)	Inhibition (%)
—	7.5	15.86 $\pm$ 1.88	—	0.52 $\pm$ 0.08	—
α -Oxoglutarate	7.5	2.16 $\pm$ 0.31	86.4	0.19 $\pm$ 0.02	63.5
Fumarate	7.5	0.61 $\pm$ 0.16	96.2	0.24 $\pm$ 0.01	53.9
Maleate	7.5	6.13 $\pm$ 0.16	61.4	0.30 $\pm$ 0.02	42.3
Dimethylsuccinate	7.5	8.15 $\pm$ 1.66	48.0	0.25 $\pm$ 0.08	51.9
Citrate	7.5	1.00 $\pm$ 0.22	93.7	0.48 $\pm$ 0.02	7.7
<i>p</i> -aminohippurate	7.5	14.12 $\pm$ 2.76	11.0	0.54 $\pm$ 0.12	0
—	6.0	14.61 $\pm$ 1.56	—	0.33 $\pm$ 0.07	—
Citrate	6.0	5.54 $\pm$ 0.37	62.0	0.29 $\pm$ 0.07	12.1

Figure 5 Expression of succinate uptake into oocytes injected with size-selected mRNA

Oocytes were injected with 5 ng of size-fractionated mRNA, and 2 days after injection the uptake of [14 C]succinate (42 μ M in the incubation medium) was measured in the presence ($\square$) and absence (hatched) of 110 mM Na $^{+}$. The range of injected mRNAs determined by denatured agarose-gel electrophoresis was as follows: 1.2–2.0 kb (F17–19), 2.0–3.0 kb (F16), 3–3.6 kb (F15), 3.6–4.1 kb (F14), > 4.1 kb (F9–13). Data represent means $\pm$ S.E.M. of ten oocytes.

indicating that the endogenous dicarboxylate transporter has a weaker preference for dicarboxylates in the *trans* configuration. In addition, inhibition of succinate uptake into control oocytes by 1 mM fumarate or 1 mM α -oxoglutarate was less than 54% or 64% respectively in comparison with 96% or 86% inhibition of the expressed dicarboxylate transporter. At both pH 6.0 and 7.5, citrate caused no or only a small inhibition of succinate uptake into control oocytes, suggesting a low affinity of the endogenous dicarboxylate transporter for citrate.

To determine the size of mRNA encoding the succinate transporter, total mRNA was fractionated on a 1.2% agarose gel and mRNA was electroeluted from 21 fractions as described in the Experimental section. After injection of 5 ng of fractionated mRNA per oocyte from different fractions (Figure 5), maximal stimulation of succinate uptake was obtained in oocytes injected with fraction 16. All of the expressed succinate uptake was Na $^{+}$ -dependent. There was no appreciable stimulation of succinate uptake in the other fractions. Thus the expressed Na $^{+}$ -dependent succinate transporter corresponds to an mRNA size of 2.0–3.0 kb as determined by gel electrophoresis on a 1% agarose gel.

This result was confirmed in a second independent mRNA fractionation (results not shown).

DISCUSSION

In this paper, we have demonstrated that injection of rat renal mRNA into *Xenopus laevis* oocytes results in the expression of Na $^{+}$ -dependent succinate uptake. *Xenopus* oocytes contain a variety of endogenous Na $^{+}$ -dependent and -independent transport systems as has been shown for glucose [11,15], phosphate [19,20] and amino acids (for example [18,21]). Here we show that oocytes also possess an endogenous succinate transporter. Therefore the increased succinate transport activity after injection of mRNA does not mean *a priori* the expression of a foreign carrier, but may be the result of stimulation of endogenous transport activity. To distinguish clearly between expression of a renal dicarboxylate transporter and stimulation of an endogenous system, succinate uptake into mRNA-injected and control oocytes was characterized with respect to affinity, specificity and pH-dependence.

We demonstrated that, after injection of mRNA, succinate uptake was markedly stimulated only in the presence of Na $^{+}$, in contrast with the endogenous dicarboxylate transport system which was Na $^{+}$ -independent. Stimulation of succinate uptake into mRNA-injected oocytes by Na $^{+}$ depended on Na $^{+}$ concentration, with sigmoidal kinetics indicating that two or three Na $^{+}$ ions are co-transported with one molecule of [14 C]succinate. Half-maximal activation of succinate uptake occurred at 19–21 mM Na $^{+}$. These values are close to those reported for the rat renal basolateral transporter (24 mM [3]). The calculated stoichiometry of ≥ 2 Na $^{+}$ /succinate agrees with the observations of Wright et al. [22] and Burckhardt [3].

In mRNA-injected oocytes, succinate uptake at pH 7.5 was higher than at pH 6.0 or 8.5, whereas the endogenous transporter exhibited highest rates of uptake at pH 8.5. The normal pH inside the oocytes is about 7.5 [23]. Therefore, if the pH is 6.0 outside, an inwardly directed proton gradient exists. As succinate uptake at pH 6.0 was significantly higher than at pH 8.5, a symport of one proton, one or two Na $^{+}$ ions and the succinate ion could take place as suggested earlier [3]. These results favour the hypothesis that the electrogenic dicarboxylate transporter is expressed from the basolateral membrane of renal proximal tubule cells. This co-transport system may carry three Na $^{+}$ ions

with one bivalent succinate or, perhaps, two sodium ions and one proton.

Measuring the dependence of succinate uptake on succinate concentration, an apparent K_m of $28 \pm 6 \mu\text{M}$ succinate was found for the expressed Na^+ -dependent transporter. This is close to the reported K_m value of the basolateral dicarboxylate transporter in rat and rabbit kidney [2,3,7] and much lower than the reported K_m value of $610 \mu\text{M}$ for the luminal transporter [7]. Interestingly, the endogenous uptake showed a similar affinity constant towards succinate. However, as already stressed, this dicarboxylate transporter is not Na^+ -dependent.

Studies with the stopped-flow capillary perfusion method [4] have suggested that the contraluminal, but not the luminal, dicarboxylate transporter can be inhibited by dimethylsuccinate. We demonstrated inhibition by dimethylsuccinate of the expressed transporter as well as the endogenous transporter. A preference for dicarboxylates with a *trans* configuration over substrates with a *cis* configuration has been reported for the luminal and contraluminal transporter [3,4]. In accordance with these results, we found that the expressed transporter had a preference for fumarate over maleate. The endogenous Na^+ -independent dicarboxylate transporter showed a less marked preference for fumarate over maleate, indicating differences in substrate specificity. In addition, inhibition by 1 mM fumarate or 1 mM α -oxoglutarate was small for the endogenous transporter compared with the expressed transporter, suggesting different affinities for these substrates.

In accordance with results reported for dicarboxylate transport in rat renal basolateral membrane vesicles [3], the monocarboxylate PAH had no significant influence on expressed or endogenous succinate transport. As reported recently, the PAH transporter is expressed after injection of total mRNA from rat kidney [24]. Therefore the small inhibition of the expressed dicarboxylate transporter by 1 mM PAH may be explained by a *trans*-stimulated export of succinate via the expressed PAH transporter in the injected oocytes.

Several studies [4,9,25] revealed that the contraluminal dicarboxylate transporter accepted citrate at neutral pH better than at lower pH, whereas citrate uptake by the luminal transporter was higher at lower pH than at neutral pH. In our experiments, the inhibition of endogenous transport by 1 mM citrate was only minimal at pH 7.5 and pH 6.0. In the mRNA-injected oocytes, citrate inhibition of succinate uptake was 93.7% at pH 7.5 and 62% at pH 6.0, suggesting again expression of the dicarboxylate transporter present in the basolateral membrane of renal proximal tubule cells.

In summary, the succinate transporter in the mRNA-injected oocytes differs from the endogenous transporter with respect to a number of features (Na^+ - and pH-dependence and affinities for di- and tri-carboxylates). The expressed transporter also shows remarkably similar properties to the renal dicarboxylate transporter of the basolateral membrane. This includes maximal

uptake at pH 7.5, inhibition by dimethylsuccinate and by citrate, preference for the trivalent form of citrate and high affinity for succinate (K_m 28 μM). Therefore we conclude that we have expressed the renal Na^+ -coupled dicarboxylate transporter of the basolateral membrane. We found maximal Na^+ -dependent succinate uptake after injection of rat renal mRNA of size 2–3 kb. Coady et al. [26] reported the expression of rabbit renal Na^+ -dependent amino acid and dicarboxylate transport by injection of mRNA of size 1.5–3.0 kb. However, these authors did not further characterize the expressed dicarboxylate transporter. Nevertheless it can be assumed that the rabbit and the rat renal transporter might be encoded by mRNA of similar size. Now the size-fractionated mRNA can be used to prepare a cDNA library to screen for the renal basolateral dicarboxylate transporter.

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