The molecules of proximal tubular transport: insights from electrophysiology

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Introduction

In proximal renal tubules a great variety of filtered substances are reabsorbed to partially or fully escape urinary excretion. The entry of the substances into the proximal tubule cells is accomplished by the respective transporters in the brush-border membrane. The transporters have previously been identified and characterized by micro-puncture techniques [1, 2], perfusion of isolated tubules [3] and by studying brush-border membrane vesicles [4-6]. Furthermore, electrogenic transport systems have been analyzed with electrophysiological techniques [7, 8]. These experiments allowed the identification of transport specificities, location, kinetic properties, coupling ratios, driving forces, regulation, etc. of the transport systems [9].

In the past few years another dimension of transport analysis has been achieved by the cloning and expression of distinct transporters [10]. Thus, the study of proximal renal tubule transport has now become amenable at the molecular level and experiments are being done aiming at understanding the structure-function correlation of the transporters.

The present paper reviews recent electrophysiological studies on several cloned renal transport proteins (fig. 1). The methods of molecular biology are not dealt with in detail here.

Fig. 1. Transport proteins with identified molecular structure in proximal renal tubules producing electrogenic transport. The abbreviation for the transporters are explained in the text for the respective transporters.
Advantageous Use of Voltage Clamp Studies in the
Xenopus Oocyte Expression System

In the past, the Xenopus oocyte expression system has proven useful for the expression of cloning and characterization of a number of proteins, including membrane receptors, ion channels and transport proteins [11]. Once the gene encoding a specific protein is cloned, the in vitro synthesis of cRNA of such proteins can be performed. Injection of in vitro synthesized cRNA results in the expression of the respective proteins in the injected oocytes. The main advantage of the oocyte expression system for the study of identified membrane proteins is obvious: The expression of endogenous membrane proteins in Xenopus oocytes is usually insignificantly low compared to the high expression of exogeneous proteins after cRNA injection. Advantage has already been taken of electrophysiological techniques for the study of transport processes in the proximal renal tubule [12–16]. However, the endogenous expression of transporters such as phosphate or sulfate transport is low and largely concealed by endogeneous ion channels and other electrogenic transport processes. As illustrated in this review, these transporters can be expressed in oocytes in concentrations yielding high transport-related currents thus facilitating the analysis of these tubular transport processes. The most common way of studying expressed transport proteins is the study of radioactive substrate transport. Although such tracer studies are highly sensitive and have been extremely useful for the expression cloning of a number of transporters, there are a number of dissatisfying disadvantages of this method: (1) Individual oocytes produce a variable expression of transporters, caused by the different size of the oocytes or by a somewhat variable cRNA injection. Because one oocyte can be used only for one transport measurement under a given condition, a variable expression consequently results in a scatter of transport data, which complicates a clean quantitative description of substrate transport and regulation thereof. (2) Transport studies are performed under non-voltage-clamp conditions, which means that the membrane potential of the oocyte is variable. However, individual oocytes have individual resting potentials and a number of transporters are known to transport their substrate in an electrogenic and voltage-dependent fashion. Consequently, a voltage-dependence of transport mediates distinct transport rates in distinct oocytes, and electrogenic transport itself alters its own rate. When performing voltage clamp in Xenopus oocytes, these disadvantages among others can be avoided for electrogenic transport systems. Using this technique (summarized in fig. 2), it is possible to perform a complete series of experiments, such as a substrate kinetic or transport regulation within one oocyte at given membrane potentials. Consequently, the results can be quantitatively analyzed in greater detail, because the level of expression or the membrane potential cannot perturb the results.

A caveat must be kept in mind during interpretation of electrophysiological transport data: the transport-induced current cannot be translated without reservations into transport of substrate. Thus, the elimination of current does not necessarily indicate cessation of transport and an increase of current may reflect an increase of charge carried along with the transported substrate. For instance, phosphate may apparently be transported in the divalent and the monovalent form, i.e. the unit charge carried by each transported phosphate may be either one or two (see below). A comparison of isotope transport studies with electrophysiological data indeed reveals any variability of charge transfer and allows deeper insight into the transport mechanism than either technique alone. Accordingly, with electrophysiological analysis additional critical information can be gained about the mechanisms of transport.

Procedures of Electrophysiological Analysis in Xenopus Oocytes

Voltage clamp recording can be performed for all electrogenic transport systems about 2–8 days after cRNA injection to warrant sufficient expression of the transport proteins. In voltage-clamp experiments the intracellular membrane voltage is clamped at a given command potential (usually around the resting potential). If an expressed transport system is electrogenic, the superfusion of the respective substrate results in the induction of a current. A general scheme describing the technique is given in figure 1. As the sample rate for slow substrate-induced currents can be very low, the signals can be filtered strongly to avoid electrical disturbances. This rather simple approach is efficient in determining the general properties of the transporters, such as the substrate kinetics or up- and down-regulation by specific second messengers.

Besides steady state voltage clamp studies there are more sophisticated voltage clamp approaches to gain information about the valence of transport proteins in the membrane and the role of membrane voltage in substrate binding and translocation [17–19]. For such experiments, an extremely fast control of the membrane voltage must be achieved to produce reliable measurements of the
membrane capacitance and currents induced by conformational changes of the transport protein within the membrane.

**Electrophysiology of Defined Transporters**

**Na⁺ Coupled Glucose Transport via SGLT-Type Transporters**

Proximal renal tubular transport of glucose is well established as an electrogenic transport system, driven by the electrochemical gradient for Na⁺. From studies on brush-border vesicles the existence of two types of renal glucose transporters have been postulated: a high-capacity, low-affinity system has been shown to couple the transport of one Na⁺ to one glucose, a low-capacity, high-affinity system couples the transport of 2 Na⁺ with one glucose.

The first glucose transporter (SGLT-I) was originally cloned from rabbit intestine [20]. An almost identical cDNA was later cloned from rabbit kidney [21, 22], indicating that almost the same protein is expressed in the kidney. In 2 patients with glucose/galactose malabsorption, a point mutation (exchange of aspartate to asparagine at position 28) has been identified, explaining the disorder at the molecular level [23]. These children, however, did not display grossly abnormal renal glucose transport, indicating that at least one additional glucose transporter must operate in the proximal renal tubules, which is capable to maintain proximal tubule glucose transport in the absence of intact SGLT-1. Nevertheless, SGLT-1 is likely to participate in proximal tubular glucose reabsorption. Recently, a membrane associated protein has been cloned regulating the activity of the glucose transporter [24].

In *Xenopus laevis* oocytes expressing SGLT-1, extracellular glucose leads to a depolarization of the cell membrane, as expected for a transport system carrying a positive charge. In voltage-clamped oocytes, addition of glucose indeed induces an inward current (Iᵢ). Steady state kinetic analysis [18, 19] and relaxation kinetics [17] have been performed and yielded the transport model, in which two Na⁺ ions bind to the carrier prior to glucose.

The carrier binding site for glucose and the two Na⁺ ions (SCNa₂) are translocated into the cell. Following the release of glucose and the two Na⁺ ions, the empty carrier binding site (C) is translocated towards the extracellular side of the cell membrane. Movement of both the empty carrier site (C) and the carrier site binding glucose and Na⁺ is in theory reversible, the steep electrochemical gradient for Na⁺ into the cell, however, drives the charged carrier site into the cell. Besides empty and fully charged carrier, the carrier binding Na⁺ only can traverse the membrane. Since transient charge transfers are eliminated by sugars, only the translocation of the empty carrier across the cell membrane and the binding of Na⁺ are considered to participate in the charge transfer.
Fig. 3. Phosphate-induced currents in oocytes expressing the NaPi-2. a Superfusion with Pi results in inward currents, which are dependent on both extracellular Pi (upper panel) and Na+ concentrations (lower panel). b Simplified model for Na+/Pi cotransport, which involves binding of 3 Na+ and one Pi on the luminal side and translocation of the substrates to the inside of the cell. Both monovalent and divalent Pi are substrate for NaPi, and H+ interferes with Na+ binding.

Na+ Coupled Transport of Inorganic Phosphate (Pi) via NaPi-Type Transporters

Renal proximal tubular phosphate transport has previously been described to involve a Na+-coupled process at the brush-border membrane. The coupling ratio was thought to be 2:1, which would suggest an electroneutral transport of divalent Pi. The transport rate is modified by phosphate, acid base and electrolyte balance and regulated by a great variety of hormones [25, 26].

Recently, rabbit (NaPi-1 [27]), rat (NaPi-2 [28]) and human (NaPi-3 [28]) renal Na+/Pi cotransporters have been identified by using the X. laevis expression cloning system. The proteins expressed by NaPi-1 are indeed incorporated into the brush-border membrane and indeed mediate transport across this membrane [29–31]. NaPi-2 and NaPi-3 are highly homologous, whereas the sequence of NaPi-1 is clearly distinct [27, 28].

Parallel to renal proximal tubular phosphate transport content of specific mRNA and protein of NaPi-2 (rat [32]; Biber and Murer [unpubl.]) is increased by dietary Pi deprivation. In contrast, NaPi-1 mRNA proved to be insensitive to a phosphate diet [33]. In a hypophosphatemic mouse model (Hyp mouse [34]) the content of NaPi-2-related mRNA and protein is reduced again paralleling renal tubular phosphate loss in those animals. Thus, NaPi-2-mediated phosphate transport probably accounts for altered renal phosphate handling in those conditions.

In X. laevis oocytes expressing NaPi-2, extracellular phosphate leads to a depolarization of the cell membrane, as expected for a transport system carrying a positive charge [35]. In voltage-clamped oocytes, addition of phosphate indeed induced an inward current (Ip; fig. 3a), depending on both extracellular concentration of Na+ and Pi. Thus Na+ must be carried in excess of negative charge. A kinetic analysis of the Na+ and/or Pi concentration dependence of Ip suggested indeed a stoichiometry of 3:1 (Na+:Pi). Accordingly, the carrier moves two charges during transport of monovalent phosphate and one charge during transport of divalent phosphate.

Na+/Pi transport is well known for its regulation by external pH, and there has been a controversy about the mechanism of how low pH inhibits transport and whether both mono- and divalent Pi can be substrate for the Na+/Pi cotransporter. From electrophysiological analysis [35], it became clear that the Na+ affinity for the transporter is dramatically decreased at low pH, probably accounting for most of the pH effect (fig. 3b). Further, a decrease of pH inhibits Pi-induced currents to a lesser extent than transport of labelled phosphate. In addition, at low Pi concentrations (0.01–0.03 mM) there were no differences observed for Ip at pH 6.5–7.5. Taken, together, these observations suggested that both mono- and divalent Pi are substrate for NaPi. The quantitative difference of transport and current inhibition by acidification can be accounted for by an increased transport of monovalent Pi at low pH, resulting in doubling the induced current for the transport of one Pi molecule.

Beside regulation of NaPi-2 expression by a low Pi diet, the first studies were completed on the functional role of
NaPj glycosylation [36] and protein phosphorylation via protein kinases A and C (unpublished results). Protein glycosylation in the extracellular region seems mainly responsible for a sufficient transport and insertion of the transporter into the membrane, while other properties of the transporter such as Pj or Na+ kinetic did not seem to be altered in transporter mutants lacking the glycosylation sites. However, while Pj-induced currents through the wild-type NaPj were strongly voltage-dependent, the voltage dependency of such currents through the mutants lacking glycosylation was much weaker [unpubl. results]. This could indicate a role of glycosylation for the translocation of the substrates.

**Na+ Coupled Transport of Inorganic Sulfate (Si) via NaSj-Type Transporters**

Transcellular transport of sulfate is accomplished by Na+-coupled uptake across the brush-border membrane and probably by anion exchange across the basolateral cell membrane [37]. The Na+/sulfate cotransporter at the brush-border membrane has been shown to interact with oxyanions such as thiosulfate and selenate, but not with phosphate [38-42]. Kinetic analysis revealed a Hill coefficient exceeding unity for Na+. Thus, it has been concluded that the transport of sulfate is coupled to two Na+ ions and is of electroneutral character.

Recently, a Na+-dependent sulfate transporter (NaSj-1) has been cloned from rat kidney [43], which — upon expression in oocytes of *X. laevis* — displays kinetics and substrate specificity characteristic for proximal tubule sulfate transport. The NaSj transporter has subsequently also been studied in voltage clamp experiments and a number of surprising observations were made.

Superfusion of *X. laevis* oocytes expressing NaSj is followed by a depolarization of the cell membrane, pointing to transport of a positive charge into the cell (fig. 4a) [44]. Accordingly, in voltage-clamped oocytes sulfate induced an inward current (I<sub>S</sub>). Thus, Na+ transport is in excess of the negative charge carried by sulfate, indicating transport of at least three Na+ ions with one sulfate (fig. 4b). Kinetic analysis revealed Hill coefficients of 1 and 3 for sulfate and Na+, respectively. These observations suggest electrogenic cotransport of sulfate and Na+ with a stoichiometry of 1:3. Experiments with thiosulfate and selenate indicate that the carrier accepts these anions with a similar affinity as sulfate, whereas phosphate is apparently not accepted by the carrier.

As expected for transport of a positive charge into the cell, I<sub>S</sub> was also shown to depend on the voltage across the membrane, being increased at more negative intracellular potentials. In summary, I<sub>S</sub> shows a number of similarities to I<sub>p</sub>, but also some differences, such as insensitivity to external pH within a range from pH 6.5 to 8.5.

**Na+ Independent Transport of Neutral and Dibasic Amino Acids Induced by the Protein rBAT**

Transport of amino acids across brush-border membranes of renal proximal tubules is accomplished by several distinct transport mechanisms [1]. One of these transport systems mediates the sodium-independent reabsorption of dibasic and some neutral amino acids. The transport system is expressed not only in kidney, but also in intestine, and its properties are similar to the system b<sup>0</sup> in mouse blastocytes [45].

Recently, a cDNA was cloned from rabbit, rat and human kidney cortex, which encodes for the protein rBAT. rBAT induces upon expression in oocytes the sodium-independent transport of dibasic and some neutral amino acids including L-cystine. The expressed transporter displays similar properties to system b<sup>0</sup> [46-50]. The expressed rBAT is expressed in brush-border membranes of both the renal proximal tubule and small intestine [51-54]. A mutation of rBAT has been demonstrated to occur in patients with classic cystinuria, an inherited disorder of epithelial dibasic amino acid and cystine reabsorption in the renal tubule and gastrointestinal tract [55].

If *Xenopus* oocytes expressing rBAT are exposed to extracellular neutral amino acids such as leucine, they hyperpolarize and display at voltage clamp conditions net
positive current from cell to extracellular space (outward current; fig. 5a) [56]. In contrast, if the same oocytes are exposed to dibasic amino acids in extracellular fluid, they depolarize and at voltage clamp conditions display net positive current into the cell (inward current). Unlike the current due to transport of neutral or dibasic amino acids, the current direction induced by L-histidine can be reversed by moderate alterations of ambient pH. Unlike L-arginine and L-leucine with pKa values around 2 and 10, respectively, the direction of L-histidine-induced currents can also be readily reversed by changes in the holding potential. The reversal potential for L-histidine-induced currents also displays an expected pH dependency.

A kinetic analysis reveals simple Michaelis-Menten kinetics for both neutral and dibasic amino acids. The affinity of the carrier is clearly higher for dibasic (half-maximal saturation at 0.01 mmol/l) than for neutral amino acids (half-maximal saturation at 0.1 mmol/l).

If the cells are exposed to dibasic amino acids for 5 min, the initially strong inward current decays slowly. However, outward currents induced by neutral amino acids are increased after such extended dibasic amino acid perfusion compared to the currents before such treatment. Conversely, preexposure to neutral substrates of the carrier leads to augmented inward currents upon addition of dibasic amino acids (fig. 5b). These observations pointed to the exchanger property of the carrier (fig. 5c), which was confirmed in subsequent tracer studies [unpubl. results]. The carrier binds dibasic amino acids or the neutral amino acids leucine, cysteine, etc., at both the intra- and extracellular sides. The intracellularly bound amino acids is then transported in exchange for the extracellular amino acid. If the intracellular and extracellular amino acids bound are neutral, their transport does not create any current. Similarly, the transport of dibasic amino acids in exchange for dibasic amino acids is electrically silent. Only if one of the two amino acids exchanged is dibasic is a current observed. Usually both neutral and dibasic amino acids are available and bound to the carrier at the intracellular side. If only dibasic amino acids are added to the extracellular space, an inward current is observed, since part of the amino acids exchanged for the extracellular dibasic amino acids are neutral. Conversely, if only neutral amino acids are added to the extracellular side, a net outward current is registered due to a proportion of carriers binding dibasic amino acids at the intracellular side.

In view of the exchange property of the carrier the question arises as to the functional significance of the carrier. The answer may be quite simple, since in parallel to the rBAT, Na⁺ coupled transport of neutral amino acids guarantees the efficient reabsorption of these amino acids and the maintenance of the driving force for the cellular accumulation of dibasic amino acids. The transport of dibasic amino acids by this transport system may be considered tertiary active, since the driving force for the transport comes partially from the chemical gradient of neutral amino acids, which themselves are accumulated into the cell by secondary active transport driven by the electrochemical gradient for Na⁺.
Conclusions and Future Perspectives

Functional analysis of cloned transporters by electrophysiological means opens a new dimension of insight into the mechanisms of transport. The electrophysiological approach allows the elucidation of all events involving the membrane during conformational changes. Since in a single oocyte multiple experimental conditions can be tested, it is most powerful to precisely define kinetic parameters or to identify factors inhibiting or stimulating transport. The transporters analyzed in this way thus far include Na+ coupled transport of glucose [17], phosphate [35], sulfate [44], glutamate [57], neutral amino acids [58] and GABA [59], but also Na+-independent amino acid transport via the systems y+ [60] and b0,+ [56]. Attractive future programs include the analysis of factors regulating transport and naturally occurring mutant transporters for changes in their electrophysiological properties.

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