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Localization of a Calcium-Stimulated ATPase in the Basal-Lateral Plasma Membranes of the Proximal Tubule of Rat Kidney Cortex*

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Summary. In the proximal tubule of the kidney, calcium is reabsorbed by an active transport mechanism. Recently, a Ca^{2+}-activated ATP-phosphohydrolase has been described in plasma membranes from rat kidney cortex, which is different from the Ca^{2+}-ATPase present in mitochondria. To elucidate the role of this enzyme in transepithelial calcium transport we studied its localization within the cell of the proximal tubule. For this purpose, a plasma membrane fraction was subdivided by preparative free flow electrophoresis and the distribution of alkaline phosphatase (brush border microvillus membranes) and $Na^{+}-K^{+}$-ATPase (basal-lateral plasma membranes) was compared with that of Ca^{2+}-ATPase. The distribution pattern obtained and the corresponding enrichment factors show that a nonmitochondrial Ca^{2+}-ATPase is – in analogy to the $Na^{+}-K^{+}$-ATPase – located only in the basal-lateral plasma membranes of the proximal tubule. Regarding the different substrate specificity and the insensitivity of the enzyme towards sodium, potassium and ouabain it seems to be possible to differentiate between the two enzymes at a molecular level. It is proposed that the Ca^{2+}-stimulated ATPase is involved in the active transtubular transport of calcium.

In the proximal tubule of the kidney, besides monovalent cations like sodium and potassium, the bivalent cation calcium is also reabsorbed from the primary urine [28]. The transport of calcium is performed against a concentration gradient and has many similarities with the sodium transport [8]. Thus, the kidney plays an important role in the regulation of the homeostasis of the divalent cation [26] which is essentially involved in processes like neuronal transmission, permeability of cell membranes, cell adhesion and bone formation [5].

The biochemical events taking place when calcium is transported through the cell have been studied extensively in the gut, mainly by the group of

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Wasserman [21] and by Melancon and DeLuca [18]. Wasserman proposes that the formation of a complex between calcium and a calcium-binding protein is the rate-limiting and Vitamin D-controlled step of the transepithelial calcium transport, whereas Melancon and DeLuca favor the view that a calcium-stimulated ATPase of the cell membrane acts as a transport enzyme for calcium.

In the kidney, the presence of a calcium-binding protein [20] as well as the presence of a calcium-stimulated ATPase in plasma membranes has been described [2, 19, 21, 29]. To answer the question whether these membrane components are indeed involved in the transport, information on the localization within the cell seems to be useful.

The kidney cell shows a marked morphological and functional polarity. For studies on cellular distribution it is essential to enrich and purify the plasma membranes of the two surfaces of the cell separately; that means the brush border microvillus membranes and the basal-lateral plasma membranes which are in contact with the interstitium. One possibility to achieve this is to use the different electrophoretic mobility of the membranes during free flow electrophoresis. Thereby it could be shown that the biochemical equivalent for the sodium transport, the $Na^+ - K^+$-ATPase is only present in the basal-lateral plasma membranes and not in the brush border microvillus membranes [14].

In this paper the distribution of the Ca^{2+}-ATPase is compared to that of alkaline phosphatase (= brush border microvillus membrane) and to that of $Na^+ - K^+$-ATPase after the separation of rat kidney plasma membranes by free flow electrophoresis. The results show that a nonmitochondrial Ca^{2+}-ATPase is located in the same membranes as the $Na^+ - K^+$-ATPase, the membranes derived from the basal and lateral parts of the cell. In analogy to the $Na^+ - K^+$-ATPase this location would support the view that the Ca^{2+}-ATPase is involved in the transepithelial calcium transport of the proximal tubule.

Materials and Methods

Preparation of Membrane Fraction from Rat Kidney Cortex

Plasma membranes of rat kidney cortex were obtained by differential centrifugation from the kidneys of 20 male Wistar rats (180 to 200 g body weight) in isotonic sucrose medium (250 mM sucrose, 10 mM triethanolamine-HCl, pH 7.6 at 20 °C) after homogenization with Teflon-glass homogenizers as described previously [14]. This plasma membrane fraction was then further subdivided in brush border microvilli and in basal-lateral plasma membranes by preparative free flow electrophoresis. The electrophoresis was carried out in the FF4 electrophoresis apparatus developed by Hannig [10] as manufactured by DESAGA, Heidelberg. The electrophoresis buffer (the buffer in the separa-

tion chamber) was prepared from 8.5 mM triethanolamine, 8.5 mM acetic acid and 280 mM sucrose, pH 7.4 at 20 °C, adjusted with NaOH. The conductivity was 5.2×10^2 µmho. The buffer for the electrode vessels consisted of 100 mM triethanolamine and 100 mM acetic acid, pH 7.4 at 20 °C, adjusted with NaOH. The conditions of the run were the following: $90 \pm 10\%$ V/cm, 85 mamp, temperature 6 °C, electrophoresis buffer flow-rate 2 ml per hr and a fraction. All samples were spun down three times at $1{,}000 \times g$ before separation, to remove aggregates.

Protein and Enzyme Assay

Protein was determined after precipitation of the membranes with 10% TCA and dissolution in 1 N NaOH according to the method of Lowry and co-workers [17]. Bovine albumin was used as standard solution. Alkaline phosphatase activity was measured in the presence of 0.5% albumin with the aid of the Merckotest ○ system. The activity of Mg^{2+} and Na^+-K^+-ATPase was determined in 75 mM Tris-(hydroxymethyl)amino-methan-buffer at pH 7.6 using two different assays: (1) in the presence of 6 mM $MgSO_4$, 100 mM NaCl and 20 mM KCl, and (2) with $MgSO_4$, NaCl, KCl and 2 mM ouabain in the incubation medium. A sample of 3 mM Tris-ATP was used as substrate. After 30 min of incubation at 37 °C the samples were heated for 1.5 min in boiling water, chilled and centrifuged. The amount of P_i liberated was determined in aliquots of the supernatant using the modified methods of Bartlett [1] and Fiske and SubbaRow [7]. After correction for the substrate blank and the blank obtained for the membranes without substrate, the second assay was taken as Mg^{2+}-ATPase activity and the difference between the first and the second assay was considered to represent the Na^+- and K^+-stimulated, ouabain-sensitive Na^+-K^+-ATPase. The membranes were all pretreated with desoxycholate and ethylendiamintetraacetate according to Joergensen [15].

The determination of the Ca^{2+}-ATPase was performed – unless otherwise stated – in 75 mM Tris-buffer, pH 7.6 at 20 °C, in the presence of 5 mM $CaCl_2$, 2 mM ouabain and 5 mM Tris-ATP. The samples were incubated for 30 min at 37 °C, then the reaction was terminated by the addition of 500 µliters of 10% TCA which resulted in a final concentration of 5% TCA. After removal of the precipitate by centrifugation the amount of P_i liberated was determined in aliquots of the supernatant as described for the Mg^{2+}-ATPase. The values were corrected for the substrate blank and for the activity found in the absence of any divalent cation. The succinic dehydrogenase was measured according to Gibbs and Reimer [9] after pretreatment of the samples with 0.1% desoxycholate.

Results

In the plasma membrane fraction isolated by differential centrifugation the specific activities of alkaline phosphatase (marker enzyme for microvilli) and $Na^+ - K^+$-ATPase (marker enzyme for basal-lateral plasma membranes) are higher than in the kidney cortex homogenate. Alkaline phosphatase is enriched by a factor of 4.5, $Na^+ - K^+$-ATPase by a factor of 3.8. The specific activity of Ca^{2+}-ATPase increases 2.2 times compared to the homogenate (Table 1). Since the specific activity of a mitochondrial marker, succinic dehydrogenase, decreases simultaneously from 24.9 to 4.0 µU/mg protein, the plasma membrane fraction shows an enrichment of a nonmitochondrial

Table 1. Specific activity and enrichment factor (ratio) of Ca^{2+}-ATPase and marker enzymes at various stages of membrane purification

Membrane fraction	Alkaline phosphatase (mU/mg pr.-	ratio)	Na^+-K^+-ATPase (mU/mg pr.-	ratio)	Ca^{2+}-ATPase (mU/mg pr.-	ratio)	Succinic dehydrogenase (μU/mg pr.-	ratio)
Homogenate of kidney cortex	0.24 ± 0.01	1.00	0.04 ± 0.01	1.00	0.17 ± 0.02	1.00	24.96 ± 2.91	1.00
Plasma membrane fraction	1.07 ± 0.20	4.45	0.16 ± 0.02	4.00	0.36 ± 0.05	2.11	4.00 ± 0.24	0.16
Basal infoldings	0.32 ± 0.07	1.33	0.42 ± 0.02	10.50	0.68 ± 0.07	4.00	3.56 ± 0.19	0.14
Brush border microvilli	1.79 ± 0.21	7.45	0.05 ± 0.02	1.25	0.29 ± 0.10	1.70	2.08 ± 0.12	0.08

Mean values ±SE of 4 experiments. For the calculation of the ratio the enzyme activities found in the different membrane fractions are compared to the enzyme activity measured in the kidney cortex homogenate.

Ca^{2+}-ATPase. However, part of the activity in the fraction still represents mitochondrial contamination since inhibitors of mitochondrial Ca^{2+}-ATPase such as oligomycin (0.5 μM) and azide (5 mM) reduce the enzyme activity by 30%.

Another contribution to the Ca^{2+}-ATPase activity in the membrane fraction could be the action of the highly active alkaline phosphatase on ATP as substrate at neutral pH. Some authors even claimed an identity of these two enzyme activities [13]. This seems not to be the case because 10 mM cysteine, which inhibits alkaline phosphatase completely, does not inhibit Ca^{2+}-ATPase activity found in the membrane fraction.

If this plasma membrane fraction is further subdivided into microvilli and basal-lateral membranes by free flow electrophoresis the distribution of alkaline phosphatase, Na^+-K^+-ATPase and Ca^{2+}-ATPase given in Fig. 1 is obtained. The corresponding enrichment factors gained after electrophoresis are given in Table 1. From the distribution pattern as well as from the enrichment of the Ca^{2+}-ATPase it seems to be clear that most of the enzyme activity present in the plasma membrane fraction is connected with the Na^+-K^+-ATPase localized in the basal-lateral plasma membranes. The differences observed in the fractions 30 to 37 in Fig. 1 between Na^+-K^+-ATPase and Ca^{2+}-ATPase are probably due to the contribution of mitochondria to the enzyme activity since in these fractions mitochondria accumulate [14].

If the fractions containing mainly the basal-lateral plasma membranes (nos. 25 to 29, Fig. 1) are combined and re-electrophorized, the distribution

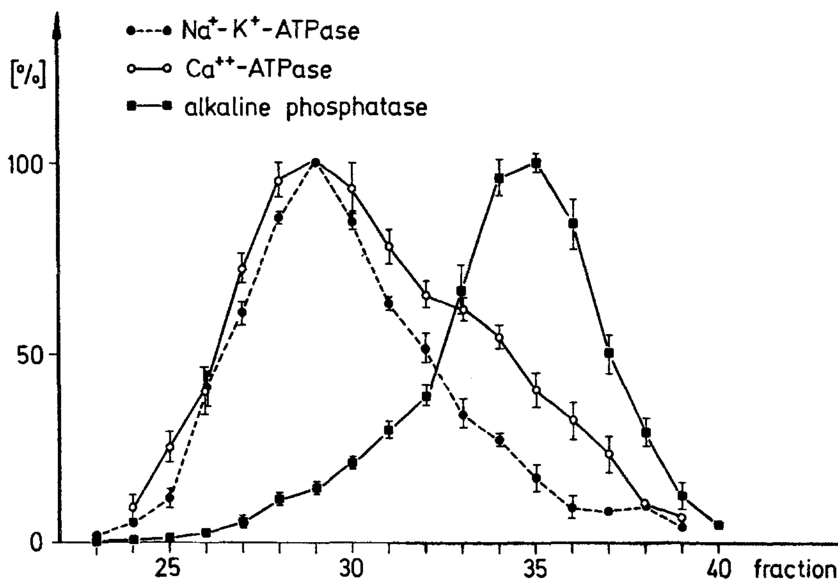

Fig. 1. Separation of a plasma membrane fraction from rat kidney cortex in a free flow electrophoresis. The activity (mU/ml) of Na^+-K^+-ATPase, Ca^{2+}-ATPase and alkaline phosphatase in the fractions collected are expressed as percent of the maximal activity. The mean values ±SE of 5 experiments are given

of Na^+-K^+-ATPase and Ca^{2+}-ATPase is completely identical (Fig. 2). A similar behavior is obtained when the same membrane fraction is subjected to another separation procedure such as centrifugation on a linear sucrose gradient. As shown in Fig. 3 again a close parallelism of the two enzymes is observed indicating the identity of the localization of both enzymes.

Knowing that Na^+-K^+-ATPase and Ca^{2+}-ATPase are present in the same membrane the question arises whether the activities measured are due to the existence of two different enzyme molecules or whether both activities are catalyzed by the same enzyme. This is especially important because it has been shown by Shamoo and Brodsky that a partial reaction of the Na^+-K^+-ATPase is stimulated as well by sodium as by calcium [25]. Therefore, the action of ouabain and potassium on the Ca^{2+}-ATPase in the membrane fraction was studied. In agreement with the results of Parkinson and Radde [19] the enzyme was found to be insensitive towards 2 mM ouabain and 10 to 50 mM potassium. In addition, the substrate specificity of Ca^{2+}-ATPase and Na^+-K^+-ATPase was tested. As shown in Table 2 the activity of both enzymes on trinucleotides are extremely different, so that it is very unlikely that both enzyme activities are catalyzed by the same molecule.

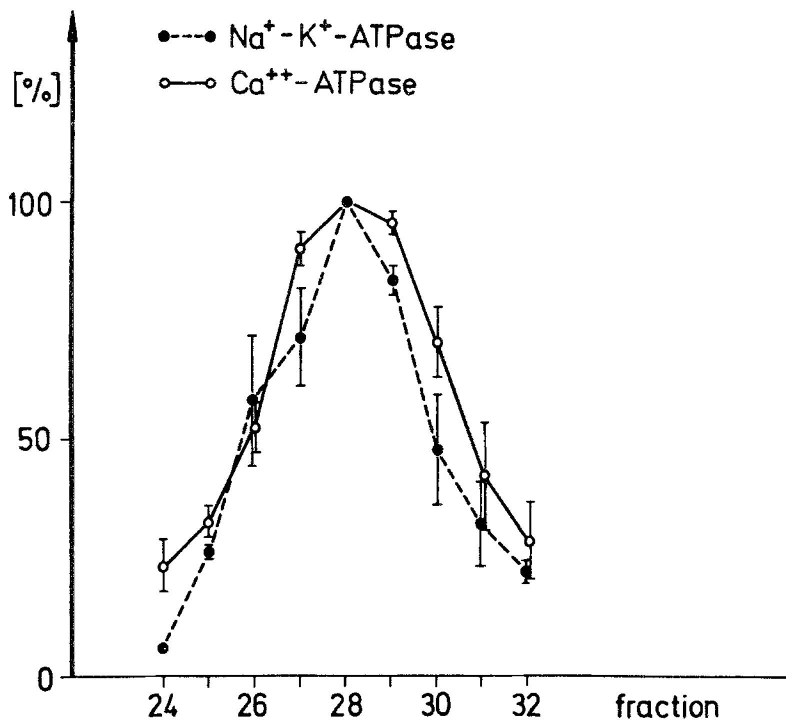

Fig. 2

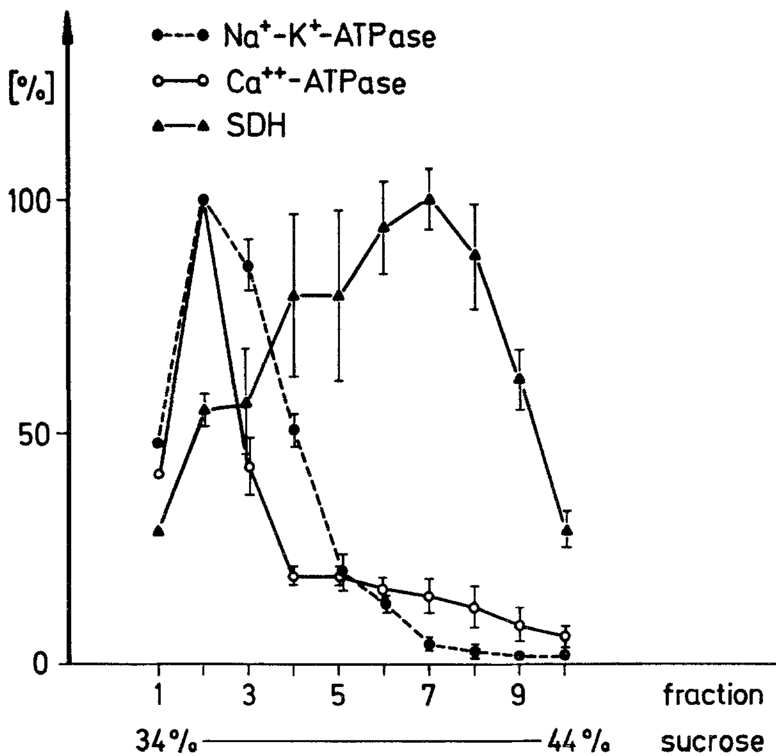

Fig. 3

Table 2. Activity of Na^+-K^+-ATPase and Ca^{2+}-ATPase of basal-lateral plasma membranes in the presence of different trinucleotides (3×10^{-3} M) in relation to the activity measured in the presence of ATP

Substrate	Na^+-K^+-ATPase (%)	Ca^{2+}-ATPase (%)
ATP	100	100
GTP	7.0	96.1
ITP	12.2	96.2
UTP	7.6	82.7

The mean values of 3 experiments are given.

Recently, Rostgaard [22] described the presence of actin-myosin filaments in the region of the basal plasma membranes of the proximal tubule of rat kidney cortex. Therefore, the possibility had to be tested whether the Ca^{2+}-ATPase activity of the basal-lateral plasma membranes could be due to attached filaments. Electron-microscopic controls performed together with Dr. H. Pockrandt-Hemstedt *(unpublished)* showed that no filaments are present in these fractions. In addition, the extraction procedure described by Hasselbach and Schneider [12] for actin and myosin was applied to the membranes. After treatment with 0.6 mM KCl at pH 6.4 at 0 °C for 20 min to remove myosin and further extraction for 18 hr at 3 °C with 0.6 M KCl, 0.04 M $NaHCO_3$ and 0.01 M Na_2CO_3 at pH 7.5 only 6% of the Ca^{2+}-ATPase activity was present in the supernatant whereas the sediment contained 94% of the enzyme activity. Nineteen per cent of the protein was removed by this procedure thereby increasing the specific activity of the Ca^{2+}-ATPase in the membranes which contradicts the assumption that Ca^{2+}-ATPase was removed specifically by the extraction of actin and myosin from the membranes.

Fig. 2. Re-electrophoresis of the fractions 25 to 29 pooled from the separation shown in Fig. 1. The activity (mU/ml) of Na^+-K^+-ATPase and Ca^{2+}-ATPase in the fractions collected is expressed as percent of the maximal activity. The mean values ±SE of 5 experiments are given

Fig. 3. Density gradient centrifugation of basal-lateral plasma membranes. The figure shows the distribution of Na^+-K^+-ATPase, Ca^{2+}-ATPase and succinic dehydrogenase in a continuous sucrose gradient (34 to 44%). Fraction 1 corresponds to the top of the gradient. The enzyme activities (mU/ml) are expressed in percent of the maximal activity. The mean values ±SE of 4 experiments are given

Table 3. Activity of Ca^{2+}-ATPase of basal-lateral plasma membranes in the presence of different concentrations of cyclic AMP in relation to the activity measured in the absence of cyclic AMP

cAMP (M)	Ca^{2+}-ATPase
10^{-3}	96.6% ±1.0
10^{-4}	95.9% ±4.6
10^{-5}	101.0% ±3.8
10^{-6}	103.8% ±3.3
10^{-7}	103.8% ±2.2
10^{-8}	104.3% ±8.8
10^{-9}	105.9% ±8.8

The mean values ±SE of 4 experiments are given.

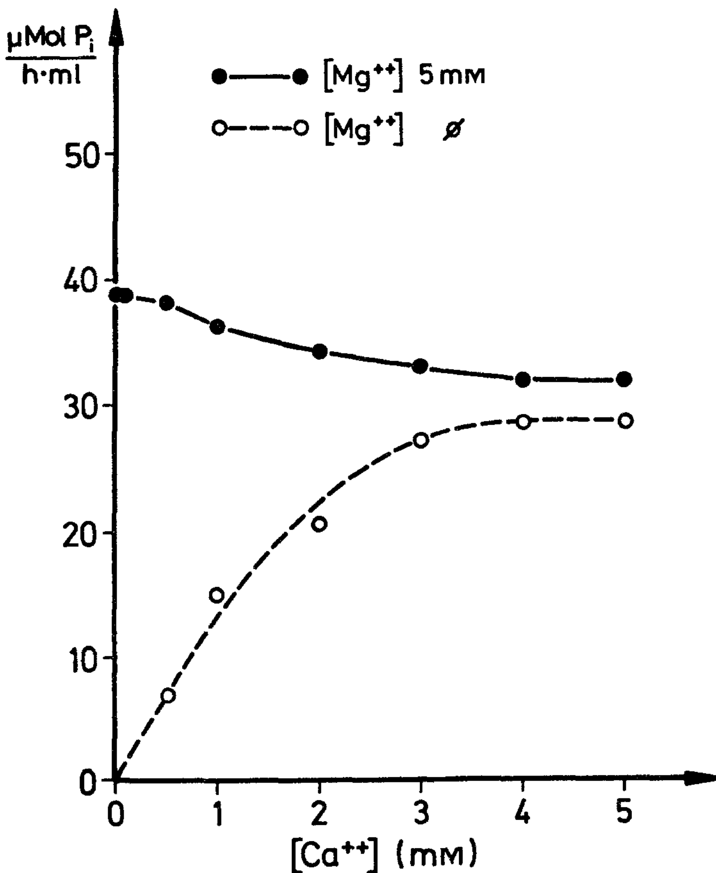

Fig. 4. Influence of calcium on the ATPase activity of isolated basal-lateral plasma membranes in the absence (o — o) and in the presence (• — •) of magnesium. Addition of calcium alone leads to an increase in activity, whereas in the presence of magnesium, calcium ions inhibit the ATPase. In this paper Ca^{2+}-ATPase stands for the activity found in the presence of 5 mM calcium alone (*see* Materials and Methods)

A further comparison was made with the enzyme described by Dietze and Hepp [6] to be present in the sarcolemma of heart. This enzyme is inhibited by cAMP up to 30%. The renal enzyme is not sensitive to cAMP as shown in Table 3. Also the activation pattern in respect to calcium differs for both enzymes; the sarcolemma enzyme is stimulated maximally by 2 mM calcium whereas the renal enzyme needs 5 mM calcium for maximal stimulation (Fig. 4).

The Ca^{2+}-ATPase of basal-lateral plasma membranes from rat kidney cortex is also not identical with the Ca^{2+}-ATPase of the erythrocytes [23, 30]. This enzyme is already activated by calcium concentrations as low as 2×10^{-6} moles/liter and it requires magnesium [23], whereas the renal enzyme is inhibited by calcium in the presence of magnesium (Fig. 4).

Discussion

Properties and Localization of Ca^{2+}-ATPase

A calcium-stimulated ATPase has been described in several plasma membrane fractions of kidneys from rat or rabbit [2, 19, 21, 29], most recently by Parkinson and Radde [19] and by Rorive and Kleinzeller [21]. The conclusion of the existence of a membrane-bound, nonmitochondrial Ca^{2+}-ATPase is confirmed by our results. Even after twice repeated free flow electrophoresis and density gradient centrifugation Ca^{2+}-ATPase and $Na^+ - K^+$-ATPase show an identical distribution which differs markedly from the distribution of succinic dehydrogenase.

Under the conditions of free flow electrophoresis both enzymes remain bound to the membranes. These are derived from the basal part of the cells of the nephron because, as shown by us using isolated brush border membranes and by Schmidt and Dubach [24] utilizing quantitative histochemistry, the apical portion of the cells does not show any $Na^+ - K^+$-ATPase activity. Furthermore we assume that these basal-lateral plasma membranes originate to a great extent from the proximal tubule since we could demonstrate the existence of a PTH-stimulated adenylate cyclase in the membranes [16].

The enzyme present in the renal plasma membranes differs in several properties from that found in the erythrocytes. Firstly, the amount of calcium needed for half maximal stimulation is much higher for the kidney enzyme than for the erythrocyte enzyme; secondly, ruthenium red which inhibits Ca^{2+}-ATPase of the erythrocytes [28] does not influence the Ca^{2+}-ATPase in renal membranes. Since there is also only little correspondence with the Ca^{2+}-ATPase of heart muscle plasmolemma [6], the transport

system for calcium through plasma membranes seems to be adapted to the specific requirement of the individual cell type and appears not to be as universal as the $Na^+ - K^+$-ATPase.

Function of Ca^{2+}-ATPase in Calcium Transport

Micropuncture studies in the proximal tubule have shown that the intratubular calcium concentration is lower than the concentration of free calcium in the plasma and that calcium transport and sodium transport show many identical characteristics [8]. Sodium is transported actively in the proximal tubule to about 40% using the transcellular pathway and to about 60% by other driving forces like solvent drag and electrical transference. The mechanism of the transcellular calcium transport has not yet been studied in detail for the tubule. Until now only experiments with non-polarized cultured kidney cells have been performed [3, 4]. All these investigations emphasize the important role of the mitochondrial accumulation and release of calcium for the regulation of the cytoplasmic calcium concentration [3, 4, 5]. In polarized cells, however, like the proximal tubule epithelial cell, in addition to cellular calcium homeostasis a vectorial calcium transport is maintained. From the similarities found between sodium and calcium transport one might conclude that the same mechanisms as for sodium are involved in the calcium absorption.

At the brush border membrane an electrochemical potential exists for calcium which is directed into the cell because the interior of the cell is negatively charged compared to the tubular lumen and because the intracellular concentration of calcium is thought to be much lower than that of the tubular fluid [3]. Thus, calcium, by following its electrochemical potential, can enter the cell by passive diffusion through the brush border membranes. Whether simple passive diffusion is the main process or a diffusion facilitated by a carrier molecule has not yet been elucidated. It is also not evident which role the calcium-binding protein isolated from the kidney [27] plays in the intracellular transfer of calcium.

At the basal cell side calcium has to overcome the electrochemical potential difference between the cell and the interstitial fluid by utilizing an active transport mechanism. This mechanism can be primarily active by direct transformation of chemical energy into osmotic work by means of an ATPase-system [11] or it can be coupled to the active transport of another solute, e.g. sodium. Until now, in the kidney, evidence for the latter case is lacking. A primary active transport system is also much more likely in the view of our findings that a calcium-activated ATPase is present in

the basal-lateral plasma membranes of the cell but not in the brush border membranes.

Another interesting feature is that the activities of $Na^{+} - K^{+}$-ATPase and Ca^{2+}-ATPase are in the same order of magnitude whereas the transport rates of the two cations are very different; i.e., the sodium net outward transport is about 8.9 μEquiv/mm^2 per sec, but the calcium absorption amounts only to 0.25 μEquiv/mm^2 per sec [8]. The high capacity of the Ca^{2+}-ATPase seems to be necessary to compensate the low affinity of the enzyme to calcium, since it has to be assumed that in the presence of the intracellular calcium concentrations of 10^{-5} to 10^{-6} moles/liter the Ca^{2+}-ATPase is saturated with the cation only to a very small extent.

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