Citrate and succinate transport in proximal tubule cells

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Hering-Smith, Kathleen S., Cecilia T. Gambala, and L. Lee Hamm. Citrate and succinate transport in proximal tubule cells. Am. J. Physiol. Renal Physiol. 278: F492–F498, 2000.—Urinary citrate, which inhibits calcium nephrolithiasis, is determined by proximal reabsorption via an apical dicarboxylate transporter. Citrate is predominantly trivalent at physiological pH, but citrate\(^2\) is transported at the apical membrane. We now demonstrate that low-Ca solutions induce transport of citrate\(^2\) and succinate in opossum kidney cells. With 1.2 mM extracellular Ca, citrate uptake was pH insensitive and not competed by succinate\(^2\). In contrast, with low extracellular Ca, citrate uptake increased twofold, was inhibited by succinate (and other dicarboxylates), was stimulated by lowering extracellular pH (consistent with citrate\(^2\) transport), and increased further by lowering extracellular Mg. The effect of Ca was incrementally concentration dependent, between 0 and 1.2 mM. The effect of Ca was not simply complexation with citrate because succinate (which is complexed significantly less) was affected by Ca similarly. Incubation of cells for 48 h in a low-pH media increased citrate transport (studied at control pH) more than twofold, suggesting induction of transporters.

DICARBOXYLATE; CALCIUM; MAGNESIUM; ACID-BASE; NEPHROLITHIASIS

Citrate is usually considered as a component of the tricarboxylic acid cycle. However, renal handling and excretion of plasma citrate are additionally important from several perspectives. First, urinary citrate is one of the most important endogenous inhibitors of calcium nephrolithiasis (22). Citrate inhibits calcium stone formation by forming soluble complexes with calcium and also by inhibiting other steps in stone formation such as crystal growth and agglomeration (22, 23). Also, citrate is a significant renal metabolite substrate (16, 28). Finally, urinary citrate excretion influences acid-base balance (4, 13). Excretion of organic anions in the urine represents the loss of “potential” bicarbonate; and citrate is the predominant urinary organic anion.

Citric acid is a tricarboxylic acid with pKa values of 2.9, 4.3, and 5.6; therefore, in plasma (and in urine to a lesser extent) citrate exists predominantly as a trivalent anion citrate\(^3\) (9). Plasma citrate is filtered at the glomerulus and reabsorbed predominantly in the proximal tubule. Our previous studies using isolated perfused rabbit tubules demonstrated that citrate is primarily reabsorbed in the proximal tubule (proximal convoluted > proximal straight >> none in thick ascending limb and cortical collecting duct) (3). This citrate reabsorption is sodium dependent and unaccompanied by any measurable citrate secretion (3).

The mechanism of proximal tubule reabsorption of citrate across the apical membrane is a sodium-dependent dicarboxylate transporter that reabsorbs a variety of Krebs cycle intermediates such as alphaketoglutarate and succinate (31). In fact, succinate is the usual test substrate for this transporter (15, 31, 33). Citrate appears to be transported as the dicarboxylate species despite the presence of the more abundant tricarboxylic acid species (9, 31, 34).

Whole-animal and human studies have shown that a variety of factors influence citrate excretion. Principal among these factors is systemic acid-base status. As previously reviewed (9), a variety of mechanisms have been shown to contribute to the exquisite pH sensitivity of urinary citrate excretion. Among these mechanisms are mitochondrial uptake and metabolism (28), pH dependence of the concentration of the transported species citrate\(^2\) (2, 9, 34), increased maximal velocity (V\(_{\text{max}}\)) of the brush-border membrane for citrate transport with chronic acid loads (11), and recently the cytoplasmic enzyme ATP citrate lyase (18). In the proximal tubule, luminal pH is a key acute determinant of citrate reabsorption due to the higher concentration of citrate\(^2\) at a lower pH (2). A variety of other mechanisms also alter urinary citrate: potassium depletion, urinary calcium and magnesium, starvation, and lithium (9, 28). Changes in urinary citrate resulting from these factors are important in both determining the propensity for calcium nephrolithiasis at the clinical level and influencing systemic acid-base status.

To further understand the mechanisms and regulation of citrate transport, we investigated cell culture models. Opossum kidney (OK) cells (OK proximal tubule cell line) were found to have a sodium-dependent,
saturable citrate transport process (17). However, this transport process was found to involve a tricarboxylate transporter (probably located on the basolateral membrane in vivo) instead of the regulated apical dicarboxylate citrate transport process (17). The tricarboxylate citrate transport process is distinguished from the apical dicarboxylate transport process by relative pH insensitivity and lack of competition with succinate, a dicarboxylate with high affinity on the dicarboxylate transporter. We and others have previously been unable to clearly identify a cell culture system with dicarboxylate transport consistent with that in the apical membrane (17). The only exception to this is a recent report of increased short-circuit current on addition of succinate in immortalized proximal tubule cell culture lines (30). However, the present studies demonstrate that dicarboxylate citrate and succinate transport can be demonstrated in OK cells by lowering extracellular calcium. The present studies characterize this system and demonstrate that citrate transport in proximal tubule cells is not only regulated acutely and chronically by pH, but also by extracellular calcium and magnesium.

METHODS

OK cells between passages 90 and 100 were maintained in MEM containing 26 mM HCO3- and supplemented with 10% fetal bovine serum (GIBOC-BRL), 25 mM HEPES, 11 mM l-glutamine, 100 IU penicillin, and 100 mg/ml streptomycin in a humidified atmosphere of 5% CO2-95% air at 37°C. Cells monolayers were grown on 24-well plates (Corning Costar), wells = 2.0 cm2, with media changes every 2 days. After reaching confluence, cell monolayers were changed to serum-free media for a minimum of 24 h before study. In specified experiments, the cell culture media pH was made acidic by lowering the bicarbonate concentration as specified.

Citrate transport was measured by the uptake of radiolabeled citrate into cell monolayers. Just before uptake measurement, the cells were rinsed free of media and equilibrated for 2 min at 37°C in a buffer containing (in mM) 109 NaCl, 3 KCl, 2 KH2PO4, 1 MgSO4, 5 alanine, 8.3 glucose, l Na acetate, 25 HEPES, 1.2 CaCl2; osmolality was 290 mosmol/kgH2O and pH 7.4, unless otherwise specified. In experiments specified below, calcium was nominally absent (“zero”-calcium solutions); the above solution was prepared without the addition of calcium. Ionized calcium was measured to be 1.14 mM in the original calcium-containing solutions and <200 µM in the zero-calcium solutions.

Uptake was performed at 37°C and started by adding 0.4 ml of uptake buffer, the above-mentioned solution with 0.5 mM CI/ml [1,14C]citrate added to the individual wells. The final concentration of citrate was ~0.014 mM. The uptake solution also contained [3H]mannitol (NEN-DuPont) to determine the residual extracellular volume. After 3 min (unless specified otherwise) the uptake solution was removed and the wells were rinsed three times with ice-cold 0.1 M MgCl2; the monolayers were then lysed with 1 ml of 0.1 N NaOH. The lysate was transferred to vials for liquid scintillation counting. In some experiments the transport of [14C]succinate was determined; in these experiments the succinate concentration was 0.0106 mM unless specified.

Uptake was calculated from the measured 14C radioactivity per well; appropriate windows and cross-over calculations were used to distinguish [3H]mannitol and 14C. Uptake was further factored for the residual extracellular volume that was not removed by the triplicate rinsing; the residual extracellular volume was calculated from the residual [3H]mannitol. Experiments that had a residual extracellular volume of >1% of the initial uptake media were eliminated. Because of variability in absolute transport rates, each experiment had a simultaneous control from the same batch of cells.

Data are expressed as means ± SE. Each mean is derived from six or more individual wells studied on at least 3 separate days (and from separate batches of cells). Michaelis-Menten and Hill coefficients were calculated by using a nonlinear least square fit to the Hill equation in the SlideWrite program. Statistical comparisons were made by using either Student's t-test or ANOVA for multiple groups. Statistical significance was defined as P < 0.05. Unless noted otherwise, all chemicals, radioisotopes, and cell culture reagents were obtained from Sigma-Aldrich (St. Louis, MO).

RESULTS

Effects of calcium on citrate uptake (Fig. 1). As seen in our previous studies (17), in the presence of 1.2 mM calcium, citrate transport was not altered by 1 mM succinate, implying that citrate uptake is not occurring via an apical type dicarboxylate transporter (2 left bars of Fig. 1). However, shown in the third bar of Fig. 1, we now find that in zero-calcium solutions, citrate uptake is increased by ~100%, compared with that in the presence of 1.2 mM calcium (from 0.51 ± 0.12 to 1.01 ± 0.25 pmol/well). And the citrate uptake in the nominal absence of calcium was nearly completely inhibited by 1 mM succinate. This is consistent with competitive inhibition of a dicarboxylate transporter. To determine more directly the effect of calcium on dicarboxylate transport, additional studies were performed with

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1 The relative concentration of citrate changes little with pH in the range of pH 6.0-7.5; however, citrate concentrations change dramatically in this pH range. These concentration changes alone render citrate transport pH sensitive.
[14C]succinate. Figure 2 shows that the removal of calcium (0 Ca) also stimulates succinate transport, which can be competed with 1 mM unlabeled succinate (0 Ca + Suc).

To determine whether this dicarboxylate transport was sodium dependent, further experiments examined uptake rates with varying sodium concentration in the zero-calcium solutions (Fig. 3). Sodium concentration was varied from 0 to 150 mM by substituting choline chloride for NaCl. [14C]citrate uptake increased with increasing concentration of sodium, with an apparent Michaelis-Menten constant of $K_M = 52$ mM, intermediate between values for the rabbit and human cotransporters (20); the apparent Hill coefficient was 1.9.

An additional characteristic of dicarboxylate transport of citrate should be acute pH sensitivity of citrate transport. As shown in Fig. 4, lowering pH of the uptake solution to pH 7.1 acutely increases citrate uptake by about twofold, consistent with dicarboxylate transport. As shown also in Fig. 4, lowering pH in the uptake solution did not significantly alter succinate uptake as expected because succinate is predominantly in the divalent form at both pH values.

To compare the substrate specificity of the OK cell dicarboxylate transporter to previous studies in brush-border membrane vesicles, two other potential competitive substrates besides succinate were studied, $\alpha$-keto-glutarate and malate (both at 1 mM concentration). In zero calcium solutions, both $\alpha$-ketoglutarate and malate completely inhibited citrate uptake (uptake not significantly different from 0 with competitor); however, in 1.2 mM calcium solutions $\alpha$-ketoglutarate again inhibited citrate uptake completely, but malate inhibited citrate uptake insignificantly (23.8 ± 16.4%). The results in 1.2 mM calcium solutions are similar to our previous data (17) in which $\alpha$-ketoglutarate had a significantly higher affinity than malate for the tricarboxylate type transporter. The results in zero-calcium solutions are consistent with the previously characterized dicarboxylate transporter.

To further examine the influence of divalent cations on citrate transport, additional studies were performed with both calcium and magnesium. First, calcium was varied between the two extremes of zero (no added calcium) and 1.2 mM. As shown in Fig. 5, citrate uptake increased progressively as calcium was lowered. The effect of magnesium in the zero calcium solutions was also examined as shown in Fig. 6. In zero calcium solutions, progressive lowering of extracellular magnesium caused progressive increases in citrate uptake. Because both calcium and magnesium complex with citrate, one component of the effect of the divalent cations may be by complexation, reducing the concentration of the transported citrate. To address this issue, succinate transport was also studied. Succinate com-

![Fig. 2. Effect of calcium on succinate uptake. [14C]succinate uptake in OK cells in Ca or 0 Ca conditions (see Fig. 1). Methods were as in Fig. 1, except that [14C]succinate was used. Cells were exposed for 3 min to [14C]succinate at 37°C. Uptake solutions were pH 7.4 and 290 mosmol/kgH$_2$O. Total extracellular calcium was nominally 1.2 mM (left 2 bars) and $<200$ µM (right 2 bars). Experiments in bars 2 and 4 had 1 mM unlabeled succinate added as a competitor. Data were factored for unremoved extracellular fluid volume by using [3H]mannitol and are expressed as pmol/well. In absence of unlabeled succinate, isotope uptake was significantly greater in 0 Ca than with 1.2 mM Ca. *Significantly greater than with 1.2 mM Ca, P < 0.05.](http://ajprenal.physiology.org/)

![Fig. 3. Dependence of citrate uptake on sodium. [14C]citrate uptake in OK cells with 0 added calcium in presence of varying levels of extracellular sodium. Sodium concentration was varied from 0 to 150 mM by substituting choline chloride for NaCl. Cells were exposed for 3 min to $-0.014$ mM [1,5-14C]citrate at 37°C. Uptake solutions were pH 7.4 and 290 mosmol/kgH$_2$O. Total extracellular calcium was $<200$ µM. Data were factored for unremoved extracellular fluid volume using [3H]mannitol and are expressed as pmol/well.](http://ajprenal.physiology.org/)

![Fig. 4. Effect of pH on citrate uptake. [14C]citrate or [14C]succinate uptake in OK cells with 0 added calcium in experimental uptake solution at pH 7.4 or 7.1. Cells were exposed for 3 min to either [14C]citrate or [14C]succinate at 37°C. Uptake solutions were pH 7.4 or 7.1 and 290 mosmol/kgH$_2$O. Total extracellular calcium was $<200$ µM. Data were factored for unremoved extracellular fluid volume using [3H]mannitol and are expressed as pmol/well. Citrate uptake but not succinate uptake was significantly higher at pH 7.1 than at pH 7.4.](http://ajprenal.physiology.org/)
plexes with calcium ~100-fold less than citrate does (29). As shown in Fig. 7, succinate transport also progressively increased as calcium concentration was lowered.

In addition to the effects of acute acidosis, in vivo chronic acidosis also induces changes in brush-border membranes (and cellular metabolism of citrate) to augment citrate reabsorption (11). The mediator(s) of these effects (acidosis per se vs. secondary hormonal changes, and so on) has not been delineated. To study the effects of chronic acidosis in vitro on citrate uptake, OK cells were grown to confluence, kept in serum-free media for 24 h, and then were changed to MEM at pH 7.4 or 7.1. The pH 7.1 MEM was prepared by lowering the bicarbonate concentration to 12 mM and then titrating the culture media to a pH of 7.1. Cells were kept in this low-pH media or normal-pH media for 48 h before study. Cells were then studied under identical conditions by using the pH 7.4 uptake solution. In these experiments (Fig. 8), citrate uptake was increased by chronic exposure to the acidic culture media by 100%. This demonstrates that chronic acidosis in vitro, without the secondary in vivo effects of acidosis, induces citrate uptake even when studied at normal extracellular pH.

**DISCUSSION**

The present studies 1) describe and characterize a cell culture model for dicarboxylate citrate transport, 2) demonstrate regulation of citrate transport by extracellular calcium and magnesium, and 3) demonstrate that chronic acidosis in vitro induces citrate transport without any other in vivo factors. Both citrate and succinate transport in OK cells were found to be stimulated by low-calcium solutions; the resulting transport (or cell uptake) was characteristic of a dicarboxylate transport process. Proximal tubule apical membrane dicarboxylate transport has been studied best in brush-border membrane vesicles (15, 31, 33) and is characterized by competitive inhibition by succinate and other dicarboxyls, and by pH sensitivity of citrate transport (34).
These same characteristics were found in OK cells studied in low-calcium solutions. A cell culture model for proximal tubule transport of citrate or dicarboxylates has not been previously described in detail. In fact, only two prior reports have examined either citrate or dicarboxylate transport in cultured cells (17, 30). We previously reported that OK cells (in usual calcium solutions) transport citrate predominantly by a sodium-coupled tricarboxylate process that is relatively insensitive to pH (17). We hypothesized that the process in OK cells was that present in the basolateral membrane of the proximal tubule in vivo (17; similar to Ref. 35). In the previous studies, succinate transport was also reported but was not characterized in depth (17). The only other report of dicarboxylate transport in cultured cells was a report of succinate-stimulated current in an immortalized rat proximal tubule cell line (30); however, this apparent transport was not further characterized. The reason that dicarboxylate transport has been difficult to define in cultured cells is not clear. The present studies demonstrate that OK cells (studied in low-calcium solutions) represent a useful cell line to study the regulation of citrate and dicarboxylate transport.

Several lines of evidence indicate that citrate is being transported as citrate$^{-2}$ in OK cells in the low-calcium solutions. First, in contrast to findings in 1.2 mM calcium solutions, succinate and other dicarboxylates inhibited citrate transport. Also, lowering extracellular pH acutely stimulated citrate transport in low-calcium solutions as predicted for citrate$^{-2}$ transport. Therefore, in low-calcium solutions, OK cells exhibit a sodium-dependent dicarboxylate transporter with characteristics of that reported for the apical membrane of the proximal tubule studied by using membrane vesicles or perfused tubules (3, 31). This contrasts with our prior findings (consistent with citrate$^{-3}$ transport) in normal-calcium solutions: no inhibition of citrate transport by succinate and no increase in uptake in low-pH solutions (17). Possible reasons for this difference, on the basis of extracellular calcium, are discussed below.

The apical or basolateral location of the observed citrate transport in OK cells has not yet been identified. Several findings suggest an apical location. First, the cells are grown on a solid support, rather than a permeable support, limiting access of the apically applied citrate to the basolateral aspect of the cells. More importantly, the transport process in low-calcium solutions has features typical of the in vivo apical membrane transporter: transport of both citrate and succinate, and sensitivity to both acute and chronic lowering of pH. Regarding the transport of both succinate and citrate, the apical transporter in vivo transports dicarboxylates including citrate$^{-2}$, despite the low concentrations of this ionic species at physiological pH. The basolateral membrane also has a dicarboxylate transporter, but it appears to transport citrate with relatively lower affinity than the apical transporter (5, 8). Evidence from basolateral membranes suggests that citrate is transported predominantly as the trivalent ionic species, citrate$^{-3}$, probably via a distinct transporter that is pH insensitive (12, 35). Our prior findings in OK cells in normal-calcium solutions exhibited characteristics of a tricarboxylate transport process for citrate distinct from any dicarboxylate transporter (17). Therefore, our present data in OK cells in low-calcium solutions is most consistent with an apical dicarboxylate transport process. Proof of this is limited by the lack of opossum-specific probes (i.e., antibodies) and lack of information on opossum citrate transport. Although the data are consistent with two separate transporters in OK cells, best studied in two distinct conditions, the data could result from a single transporter that has drastically different features in the two conditions.

In vivo, the principal determinant of urinary citrate is acid-base balance; acidosis or acid loads dramatically increase citrate reabsorption and lower urinary citrate levels (9, 28). Similar effects of acute acidosis have been reported in several in vitro models including brush-border membrane vesicles and isolated perfused tubules (2, 34). A major mechanism of this acute effect is the influence of luminal pH on the divalent citrate ion, which is the ionic species transported at the apical membrane. The last pKa of citrate is 5.6 so that at pH 7.4, ~98.4% of the total citrate is the trivalent ionic species, and only 1.6% is the divalent ionic species. Lowering the pH only modestly to pH 7.1 nearly doubles the divalent ionic species to 3.1%. This increase in the transported substrate can increase citrate transport dramatically. Therefore, not surprisingly, acutely lowering extracellular pH increased citrate uptake (Fig. 4).

Chronically, acidosis increases citrate reabsorption by a variety of additional mechanisms. First, mitochondrial metabolism is altered by acidosis such that cellular citrate concentrations fall, increasing the lumen-to-cell citrate concentration gradient, favoring citrate reabsorption (28). Also, Melnick et al. (18) have recently shown convincingly that cytosolic ATP citrate lyase is increased by chronic acidosis and that this response is an important aspect of the chronic adaptation of citrate reabsorption to acidosis. Importantly, the apical dicarboxylate transporter also undergoes an adaptive increase with acid loads. Jenkins et al. (11) demonstrated that brush-border membrane vesicles obtained from acid-loaded rats have an increased $V_{\text{max}}$ for citrate transport. This tentatively suggests an increased number of dicarboxylate transporters present in the apical membrane of the proximal tubule in chronic acidosis. The present studies demonstrate that acidosis in vitro increases citrate transport in OK cells studied in low-calcium solutions. Therefore, our studies demonstrate that chronic acidosis can directly increase citrate transport rates independent of systemic factors such as glucocorticoids, which are important in some of the adaptive consequences of chronic acidosis (14). Because these studies examined initial rates, transport rates should be independent of intracellular metabolism (mitochondrial or citrate lyase). Therefore, our results are most consistent with an increased number of dicarboxylate transporters present in the apical membrane in response to acidosis.
The present studies also clearly demonstrate that extracellular calcium and magnesium can regulate citrate and succinate transport. Several interrelated issues in this regard merit attention. First, are the effects of calcium an in vitro artifact of extremely low extracellular calcium or are the effects physiologically relevant? The effect seems to be graded with varying levels of calcium (Fig. 6) and magnesium (Fig. 7). Also, some precedence for regulation by calcium has been previously reported; older in vivo studies had noted that conditions associated with increasing urinary calcium had increased urinary citrate (10, 27). Also, studies by Bushinsky et al. (6) demonstrate that urinary citrate increases in hypercalciuric rats as urinary calcium increases. One previous study directly addressed the effect of calcium on citrate transport in brush-border membrane vesicles and demonstrated that high concentrations of extracellular calcium (10 mM) were associated with decreased citrate transport in brush-border membrane vesicles, apparently via complexation of calcium with citrate (1). The present studies extend these observations to lower levels of calcium and also to magnesium. In addition, the present studies suggest that an additional mechanism, besides complexation, is also likely operative. Succinate transport, not just citrate transport, was affected by calcium concentration. Succinate is complexed much less than citrate (some 100-fold less), (29) and hence, the effects on succinate are unlikely the result of complexation. Therefore, besides complexation with divalent cations, another cellular mechanism must underlie the regulation of citrate transport by extracellular calcium and magnesium. Such regulation, if found in vivo, could be physiologically important in preventing nephrolithiasis. Decreased proximal tubule citrate reabsorption and increased urinary citrate with conditions of high urinary calcium would be beneficial in vivo in reducing the propensity for calcium nephrolithiasis.

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2 Two heavy metals, mercury and cadmium, have also been found to inhibit citrate transport in brush-border membrane vesicles (24, 25). Whether the mechanisms involved are related to the present findings with calcium and magnesium is unknown.