Chronic metabolic acidosis stimulated transcellular and solvent drag-induced calcium transport in the duodenum of female rats

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Chronic metabolic acidosis stimulated transcellular and solvent drag-induced calcium transport in the duodenum of female rats. Am J Physiol Gastrointest Liver Physiol 291: G446–G455, 2006. First published May 11, 2006; doi:10.1152/ajpgi.00108.2006.—Chronic metabolic acidosis results in a negative calcium balance as a result of bone resorption and renal calcium loss. However, reports on the changes in intestinal calcium transport have been controversial. The present investigation therefore aimed to study the effects of chronic metabolic acidosis induced by 1.5% NH4Cl administration on the three components of duodenal calcium transport, namely, solvent drag-induced, transcellular active, and passive paracellular components, in rats using an in vitro Ussing chamber technique. The relative mRNA expression of genes related to duodenal calcium transport was also determined. We found that 21-day chronic metabolic acidosis stimulated solvent drag-induced and transcellular active duodenal calcium transport but not passive paracellular calcium transport. Our results further demonstrated that an acute direct exposure to serosal acidic pH, in contrast, decreased solvent drag-induced calcium transport in a pH-dependent fashion but had no effect on transcellular active calcium transport. Neither the transepithelial resistance nor duodenal permeability to Na+ and Ca2+ via the passive paracellular pathway were altered by chronic metabolic acidosis, suggesting that widening of the tight junction and changes in the charge-selective property of the tight junction did not occur. Thus the enhanced duodenal calcium transport observed in chronic metabolic acidosis could have resulted from a long-term adaptation, possibly at the molecular level. RT-PCR study revealed that chronic metabolic acidosis significantly increased the relative mRNA expression of duodenal genes associated with solvent drag-induced transport, i.e., the β1-subunit of Na+–K+–ATPase, zonula occludens-1, occludin, and claudin-3, and with transepithelial active transport, i.e., transient receptor potential vanilloid family Ca2+ channels 5 and 6 and plasma membrane Ca2+-ATPase isoform 1b. Total plasma calcium and free ionized calcium and magnesium concentrations were also increased, whereas serum parathyroid hormone and 1α,25-dihydroxyvitamin D₃ levels were not changed. The results indicated that 21-day chronic metabolic acidosis affected the calcium metabolism in rats partly through enhancing the mRNA expression of crucial duodenal genes involved in calcium absorption, thereby stimulating solvent drag-induced and transcellular active calcium transport in the duodenum.

Besides affecting vital organs, metabolic acidosis has been shown to profoundly impair calcium and phosphate metabolism, leading to defective mineralization (5), increased calcium loss from bone (9), and hypercalciuria (8). The final outcome includes growth retardation, abnormal bone growth, osteomalacia, nephrocalcinosis, and negative calcium balance (3, 58). Nevertheless, little is known regarding the effect of metabolic acidosis on the gastrointestinal system, which is the major organ supplying calcium to the body.

Although several investigators have provided evidence that metabolic acidosis also influences intestinal calcium absorption (16, 20, 21, 34), the results are still controversial and inconclusive. Goulding and Campbell (21) reported an in vivo stimulatory effect of 16-day chronic metabolic acidosis induced by 2.0% NH4Cl on intestinal calcium absorption in rats; however, the intestinal transepithelial calcium flux was not measured directly. In contrast, Gafter and colleagues (20), using metabolic balance study, reported that 9-day chronic metabolic acidosis did not have a significant effect on calcium absorption. Interestingly, an in vitro acidic pH experiment that emulated acute metabolic acidosis showed a decrease in the net calcium absorption in both the duodenum and ileum (16). It is possible that acute and chronic metabolic acidosis differentially alter intestinal calcium absorption, but the mechanism has not been established.

The duodenum is the most effective site for calcium transport (19, 27) because of its high capability to extract and absorb luminal calcium by passive and active mechanisms to mitigate a negative calcium balance, for example, during pregnancy, lactation, and low calcium intake (4, 7, 39). The duodenal passive mechanism transports calcium paracellularly in a transepithelial calcium gradient-dependent manner (6). Passive paracellular calcium transport predominates during high calcium intake and is absent after the transepithelial calcium gradient is eliminated (6, 10). Duodenal active calcium transport utilizes two major metabolically energized components, i.e., solvent drag-induced paracellular and transepellular active calcium transport, which are more important during negative calcium balance (49). The transepithelial component is a three-step process that consists of apical calcium uptake via transient receptor potential vanilloid family Ca2+ channel 6 (TRPV6) (22, 47), cytoplasmic calcium translocation in a calbindin-D9K-bound form (17), and basolateral calcium extrusion via plasma membrane Ca2+-ATPase isoform 1b (PMCA1b) (19, 22). Administration of calmodulin-dependent PMCA inhibitors, e.g., trifluoperazine, can abolish transepithelial active calcium transport in the duodenum (10).

On the other hand, solvent drag-induced calcium transport depends on a paracellular sodium gradient generated by Na+-K+-ATPase lining the lateral membrane (12, 13, 33). There-
fore, it can be abolished by reducing the transepithelial sodium uptake, which maintains the paracellular hyperosmotic environment (10, 49). Widening of the tight junction as well as changes in the charge-selective property of the tight junction could also affect the solvent drag-induced paracellular transport of small ions and nutrients (18, 36, 37, 54). Several duodenal tight junction proteins, including zonula occludens (ZO)-1, occludin, and claudins, are involved in the regulation of its size and charge selectivity (1, 23, 24). We (10, 49) have previously demonstrated that the solvent drag component accounts for ~70% of the total active duodenal calcium transport, suggesting a physiological significance of solvent drag-induced calcium transport in the duodenum. Until now, the effects of metabolic acidosis on each component of duodenal calcium transport and expression of the major calcium-transporting genes have not been elucidated.

The objectives of the present study were therefore to 1) investigate the effect of 21-day chronic metabolic acidosis and acute direct exposure to serosal acidic pH on each component of duodenal calcium transport, 2) determine the electrical properties and charge-selective characteristics of the duodenal epithelium after exposure to chronic metabolic acidosis for 21 days, and 3) demonstrate chronic metabolic acidosis-modulated mRNA expression of important genes pertaining to duodenal calcium transport.

MATERIALS AND METHODS

Animals

Female Sprague-Dawley rats, weighing 180–200 g (10 wk old), were obtained from the National Laboratory Animal Center (Bangkok, Thailand). They were housed in the laboratory animal husbandry unit for at least 5 days before the experiments under a 12:12-h light-dark cycle. The room had a temperature of 20–25°C and humidity of 50–60%. Animals were fed regular pellets containing 1.0% calcium and 0.9% phosphorus (CP Limited, Bangkok, Thailand) and water ad libitum. In the 21-day chronic acidosis experiment (protocols 1, 3, and 4), rats were provided with 1.5% (wt/vol) NH₄Cl (Sigma, St. Louis, MO) in their drinking water ad libitum for 21 days, thus introducing acid-loading chronic metabolic acidosis (20, 40, 42). In the direct acid exposure experiment (protocol 2), rats were killed after a 21-day rest period. All animals were cared for in accordance with the principles and guidelines of the Laboratory Animal Ethics Committee of Mahidol University (Bangkok, Thailand) and the American Physiological Society “Guiding Principles in the Care and Use of Animals.”

Duodenal Preparation

Under 50 mg/kg ip pentobarbital sodium (Abbott Laboratory, North Chicago, IL) anesthesia, a median laparotomy was performed. A 10-cm duodenal segment was obtained distal to the pyloric sphincter. After being rinsed in an ice-cold bathing solution pregassed with 5% CO₂-95% O₂, a longitudinal incision was made along the radix mesenterii to expose the mucosa as described previously (50). The tissue was then mounted in a modified Ussing chamber with an exposed surface area of 0.69 cm² to measure calcium fluxes. The tissue was incubated for 20 min before the 60-min experiment was carried out. For mRNA isolation (protocol 4), duodenal enterocytes were collected by scraping the mucosal surface of a 10-cm duodenal segment with an ice-cold glass slide (41).

Bathing Solution

The bathing solution contained (in mM) 118 NaCl, 4.7 KCl, 1.1 MgCl₂, 1.25 CaCl₂, 23 NaHCO₃, 12 d-glucose, and 2 mannitol (all purchased from Sigma). The solution, which was continuously gassed with humidified 5% CO₂-95% O₂, was maintained at 37°C, pH 7.4, and had an osmolality of 290–295 mmol/kg water. The osmolality of the solution and plasma was measured by a freezing point-based osmometer (model 3320: Advanced Instruments, Norwood, MA). Water used in the present work had a resistance of >18.3 MΩ·cm, organic carbon of <10 μg/l, and free Ca²⁺ of <2.5 nM.

Electrical Measurement

As previously described (10, 50), a pair of Ag/AgCl electrodes was mounted in a modified Ussing chamber with an exposed area of 0.69 cm² to measure the tissue for measurement of the transepithelial potential difference (PD). The other ends of the electrodes were connected to a preamplifier (model EVC-4000; World Precision Instruments, Sarasota, FL) and finally to a PowerLab/4SP (ADInstruments, Colorado Springs, CO). Another pair of Ag/AgCl electrodes was placed at the end of each hemichamber to supply a short-circuit current (Iₛₑ), which was also measured by a PowerLab/4SP connected in series to the EVC-4000 current-generating unit. The PowerLab/4SP operated with Chart 5.2.2 for Mac OS X (ADInstruments). Transepithelial resistance (TER) and conductance were calculated by the Ohm’s equation.

Calcium Flux Measurement

Calcium fluxes were determined by the modified methods of Karbach (27) and Charoenphandhu et al. (10). After a 20-min incubation in the Ussing chamber, the bathing solution was changed to a fresh one. One side was ⁴⁵Ca-containing solution (initial amount of 5 mCi/ml, final specific activity of ~450–500 mCi/mmol; Amersham, Buckinghamshire, UK). Seven samples were collected per each setup to calculate the unidirectional flux (Jᵤ→𝑐) in mmol·h⁻¹·cm⁻² from the hot side (H) to the cold side (C), as follows (46):

\[ J_{H→C} = R_{H→C}/(S_H \times A) \]

where \( R_{H→C} \) is the rate of tracer appearance in the cold side (in counts·min⁻¹·h⁻¹); \( S_H \) is the specific activity in the hot side (in counts·min⁻¹·mmol⁻¹); \( A \) is the surface area of the tissue (in cm²); \( C_{H,C} \) is the mean of radioactivity in the hot side (in counts·min); and \( C_T \) is the total calcium in the hot side (in mmol).

At the end of each experiment, d-glucose was added to the mucosal solution to give a final concentration of 30 mM to check the tissue viability (10). The radioactivity of ⁴⁵Ca was analyzed by liquid scintillation spectrophotometry (model 1219: LKB Wallac, Turku, Finland). The total calcium concentration of the bathing solution was analyzed by atomic absorption spectrophotometry (model SpectraAA-300; Varian Techtron, Springvale, Australia).

To measure solvent drag-induced calcium flux, 0.1 mM trifluoperazine (Sigma) was added to the serosal solution to inhibit calmodulin-independent PMCA activity, thereby diminishing the transcellular active calcium flux (15, 50). To measure transcellular active calcium flux, 12 mM mucosal glucose was replaced with the same concentration of mannitol to minimize sodium entry into the cells, thus disrupting the paracellular sodium gradient and abolishing solvent drag-induced calcium flux (10, 49). The passive paracellular fluxes were measured by determining the calcium fluxes in the presence of various mucosal calcium concentrations (29), i.e., 1.25, 2.5, 5, 10, and 20 mM, in the absence of solvent drag-induced and transcellular active calcium transport.

Permeability Measurement

The permeability of sodium and chloride ions was measured by the dilution potential technique, which was modified from the methods of Kahle et al. (26) and Hou et al. (23). In brief, a duodenal segment was equilibrated in bathing solution containing 145 mM NaCl for 20 min before the mucosal solution was substituted with 72.5 mM NaCl-
containing solution. Osmolality was maintained by an equivalent amount of mannitol. Changes in the electrical parameters during fluid substitution were recorded. The ion permeability ratio (p) was calculated from the dilution potential (Vb) by using the following Goldman-Hodgkin-Katz equation (44):

\[
V_b = \frac{RT}{F} \ln \frac{P_{Na}C_m + P_{Cl}C_s}{P_{Na}C_s + P_{Cl}C_m}
\]

where \(P_{Na}\) is the absolute permeability of sodium, \(P_{Cl}\) is the absolute permeability of chloride, \(C_m\) is the mucosal NaCl concentration, \(C_s\) is the serosal NaCl concentration, \(R\) is the gas constant, \(T\) is temperature, and \(F\) is Faraday’s constant.

When given \(p = P_{Na}/P_{Cl}\), \(\phi = C_j/C_m\), and \(v = FV_b/RT\), then Eq. 3 can be rewritten as follows:

\[
p = (\phi - e')/(\phi e' - 1)
\]

\(P_{Na}\) and \(P_{Cl}\) were calculated from the conductance (G) and permeability ratio (\(P_{Na}/P_{Cl}\)) using a simplified Kimizuka-Koketsu equation (26), as follows:

\[
P_{Na} = \frac{GRT}{C_mF^2} \times \frac{p}{1 + p}
\]

\[
P_{Cl} = P_{Na}/p
\]

The duodenal permeability to calcium (\(P_{Ca}\)) via the passive paracellular pathway was calculated from the following equation (48):

\[
P_{Ca} = J_C/\Delta C
\]

where \(J_C\) is the passive paracellular calcium flux and \(\Delta C\) is the difference in concentration between mucosal and serosal calcium concentrations.

mRNA Isolation and RT-PCR

By using TRizol RNA extract reagent (Invitrogen, Carlsbad, CA), total RNA was prepared from duodenal cells according to the manufacturer’s instructions. One microgram of total RNA extract was reverse transcribed by RT-PCR (MyCycler; Bio-Rad, Hercules, CA) to be cDNA with the ImProm-II kit (Promega, Madison, WI). The primers of TRPV5, TRPV6, calbindin-D9K, PMCA1b, Na+/Ca\(^{2+}\) exchanger 1 (NCX1), the \(\beta_1\)-subunit of Na\(^{+}/K\(^{+}\)-ATPase, ZO-1, occludin, and claudin-3 are shown in Table 1. GAPDH was used as an internal control during the detection of mRNA expression. Sense and antisense primers for NCX1, ZO-1, claudin-3, and GAPDH were designed by OLGIO 6 (Molecular Biology Insights, Cascade, CO) and Primer Validator 1.3 (Naratt Software, Bangkok, Thailand). PCR products were visualized on a 2% agarose gel stained with ethidium bromide under an ultraviolet transilluminator (Alpha Innotech, San Leandro, CA) and were quantified by AlphaEase 4.1 for Microsoft Windows (Alpha Innotech).

Blood Chemistry

Total plasma calcium and inorganic phosphate were determined by modified o-cresolphthalein complexone and phosphomolybdate-based kits, respectively, using a Dimension RxL analyzer (Dade Behring, Marburg, Germany). Free ionized calcium and magnesium were measured by ion-selective electrodes (model Stat Profile CCX; Nova Biomedical, Waltham, MA) under an anaerobic environment. Serum parathyroid hormone and 1α,25-dihydroxyvitamin D3 [1α,25-(OH)\(_2\)D3] concentrations were measured by ELISA (Amersham, Buckinghamshire, UK) and radioimmunassay (Immunodiagnostic Systems, Tyne & Wear) kits, respectively.

Experimental Protocols

Protocol 1. The objective of this protocol was to investigate the effects of chronic metabolic acidosis on duodenal calcium transport. NH\(_4\)Cl (1.5%) was given to experimental rats for 21 days to induce chronic metabolic acidosis. Blood gas analysis (model Ultra C; Nova Biomedical, Waltham, MA) and plasma osmolality confirmed the success of acidic induction. Solvent drag-induced, transepithelial active, and passive paracellular calcium transport in the duodenum were determined after the 21-day period. Changes in the levels of total plasma calcium, free ionized calcium, free ionized magnesium, and inorganic phosphate were determined. Serum parathyroid hormone and 1α,25-(OH)\(_2\)D\(_3\) were also measured.

Protocol 2. Because chronic metabolic acidosis had adverse effects on various physiological systems that led to long-term adaptations in the affected cells, it was not known whether changes in the duodenal functions in chronic metabolic acidosis resulted from a direct action of acid. To clarify this point, the serosal side of the duodenum in this

Table 1. Rattus norvegicus oligonucleotide sequences used in the RT-PCR experiment (protocol 4)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession No.</th>
<th>Primer</th>
<th>Product length, bp</th>
<th>Reference</th>
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<tr>
<td>TRPV5</td>
<td>NM_053787</td>
<td>Forward: 5’-TTACAGGGTGTAGACAGCAGCA-3’</td>
<td>163</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: 5’-TTGGCAAGACGGAGCTCTTA-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRPV6</td>
<td>NM_053686</td>
<td>Forward: 5’-ATCGCCTGCTATGCAC-3’</td>
<td>80</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: 5’-AGTTTTTCTGTGACTGTTTTTG-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CaBP</td>
<td>X16635</td>
<td>Forward: 5’-CCCGAGAAATGAAGAGGATTT-3’</td>
<td>174</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: 5’-TTCCTGCACTACGCTTGTTATCG-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PMCA1b</td>
<td>NM_053311</td>
<td>Forward: 5’-GCCCATTTTCCTGGCAATATT-3’</td>
<td>109</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: 5’-CGCGATTTGGATTGAAGTC-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCX1</td>
<td>NM_019268</td>
<td>Forward: 5’-TTTGCTTGTGCTGGTGCTTG-3’</td>
<td>163</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: 5’-GGTGGAGTGTGCTACCTTTT-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NKA</td>
<td>X63375</td>
<td>Forward: 5’-CCACTGTCCTGAGAAGCACCT-3’</td>
<td>79</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: 5’-CCGACTGTCAGAATCTTTC-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZO-1</td>
<td>XM_218747</td>
<td>Forward: 5’-CATCCTGATGTTGTTGCGTC-3’</td>
<td>270</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: 5’-CCTGGCCTGCTGCTGTTGTC-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Occludin</td>
<td>NM_031329</td>
<td>Forward: 5’-CGACCTGAAAGTGTTGCTGA-3’</td>
<td>188</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: 5’-CCTTTGCTCATATAGCCT-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Claudin-3</td>
<td>NM_031700</td>
<td>Forward: 5’-GGTGGCTGCGGGTGTGACTG-3’</td>
<td>246</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: 5’-GGTGGCTGCGGGTGTGACTG-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>NM_017008</td>
<td>Forward: 5’-GGTGGCTGCGGGTGTGACTG-3’</td>
<td>301</td>
<td>52</td>
</tr>
</tbody>
</table>
Protocol was directly exposed to bathing solution with pHs of 7.1, 7.2, 7.3, or 7.4 according to the modified methods of Favus et al. (16), whereas the mucosal pH was kept at 7.4. The pH of the bathing solution was continuously monitored throughout the 80-min experiment. Solvent drag-induced and transcellular active calcium transport were determined.

Protocol 3. To demonstrate that the enhanced solvent drag-induced calcium flux after 21-day chronic metabolic acidosis was not associated with the widening of the tight junction or changes in the charge-selective property of the duodenal epithelium, epithelial electrical parameters and dilution potential were determined. The permeability ratio, \( P_{Na} \), and \( P_{Cl} \) were calculated from Eqs. 4, 5, and 6, respectively.

Protocol 4. Because the enhanced calcium transport observed in response to chronic metabolic acidosis might have arisen from adaptive changes at the molecular level, the objective of this protocol was therefore to demonstrate changes in the mRNA expression of genes known to be involved in duodenal calcium transport (22, 32). Relative expression levels of important genes for transcellular active calcium transport, i.e., TRPV5, TRPV6, calbindin-D9K, PMCA1b, and NCX1, were determined by the RT-PCR technique. The expression of genes that encoded major paracellular proteins involved in either paracellular calcium transport or overall calcium metabolism, i.e., the \( \beta_1 \)-subunit of Na\(^+\)-K\(^+\)-ATPase, ZO-1, occludin, and claudin-3, was also studied.

Statistical Analyses

Results are expressed as means \( \pm \) SE. Two sets of data were compared using the unpaired Student’s t-test. Multiple comparisons were performed by one-way ANOVA with the Newman-Keuls post-test. The level of significance for all statistical tests was \( P < 0.05 \). Data were analyzed by GraphPad Prism 4.0 for Mac OS X (GraphPad Software, San Diego, CA).

RESULTS

Administration of 1.5% NH\(_4\)Cl in Drinking Water for 21 Days Induced Sustained Metabolic Acidosis

During the 21-day acid loading period, rats were given 1.5% NH\(_4\)Cl ad libitum to induce chronic metabolic acidosis. Arterial blood gas analysis revealed an acidic plasma pH of 7.34 \( \pm \) 0.02 in the 1.5% NH\(_4\)Cl-treated group compared with 7.41 \( \pm \) 0.01 in the control group (Table 2). The water consumption of all animals exceeded 60 ml/day. Plasma osmolality in the 1.5% NH\(_4\)Cl-treated group was not increased, indicating that all treated animals consumed sufficient amounts of NH\(_4\)Cl-containing water to produce sustained acid-loading metabolic acidosis.

Chronic Metabolic Acidosis Stimulated Calcium Transport in the Duodenum

After the administration of 1.5% NH\(_4\)Cl for 21 days to induce chronic metabolic acidosis, three components of duodenal calcium transport, i.e., solvent drag-induced, transcellular active, and passive paracellular transport, were studied separately. In the present investigation, the solvent drag-induced calcium transport in the normal duodenum accounted for \( \sim 80\% \) of the total active duodenal calcium transport. Under the acidic condition, the results showed that 21-day chronic metabolic acidosis markedly stimulated solvent drag-induced calcium transport by 2.5-fold from 35.84 \( \pm \) 4.09 to 91.81 \( \pm \) 8.42 mmol·h\(^{-1}\)·cm\(^{-2}\) (Fig. 1). Transcellular active duodenal calcium transport was also enhanced from the control value of 8.03 \( \pm \) 0.59 to 10.40 \( \pm \) 0.66 mmol·h\(^{-1}\)·cm\(^{-2}\) (Fig. 2). Passive paracellular calcium transport, on the other hand, was not changed by chronic metabolic acidosis. However, Fig. 3A demonstrates that passive paracellular calcium transport linearly increased with the mucosal calcium concentration, which produced a significantly higher flux when its level was \( \leq 5 \) mM.

Enhanced Duodenal Calcium Transport Was Not Due to a Direct Action of Acidic pH

Because chronic metabolic acidosis is a systemic condition, and several organs would have had acted in concert to reduce the detrimental consequences, enhanced duodenal calcium transport could be a part of the body response that may involve adaptive changes at both the molecular and organ levels. To verify this hypothesis, a direct effect of acid on calcium transport was studied by directly exposing the serosal side of the duodenum to various acidic pHs in vitro. We found that a

Table 2. Arterial blood gas analysis of rats after 21-day chronic metabolic acidosis

<table>
<thead>
<tr>
<th>Condition</th>
<th>( n )</th>
<th>Plasma pH</th>
<th>( P_{CO_2} ), kPa</th>
<th>Plasma [HCO(_3)(^-)], mM</th>
<th>Plasma osmolality, mmol/kg water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>7.41±0.01</td>
<td>7.05±0.17</td>
<td>33.89±1.44</td>
<td>289.50±1.56</td>
</tr>
<tr>
<td>Acidosis</td>
<td>10</td>
<td>7.34±0.02*</td>
<td>7.52±0.52</td>
<td>30.93±1.04</td>
<td>287.50±1.25</td>
</tr>
</tbody>
</table>

Values are means \( \pm \) SE; \( n \), no. of animals/group. \( P_{CO_2} \), arterial \( P_{CO_2} \). *\( P < 0.01 \) compared with the control group.

Fig. 1. Effects of 21-day chronic metabolic acidosis on solvent drag-induced calcium transport in the duodenum of rats. Mucosal and serosal solutions had equal calcium concentrations of 1.25 mM to diminish passive paracellular calcium transport. The experiment was performed in the presence of 0.1 mM trifluoperazine, which abolished transcellular active calcium transport. ***\( P < 0.001 \) compared with the control group; \( n \) denotes the number of experimental animals.
reduction of serosal pH from 7.4 to 7.1, in contrast to chronic metabolic acidosis, significantly decreased solvent drag-induced duodenal calcium transport in a pH-dependent manner (Fig. 4) while having no effect on transcellular active calcium transport (Fig. 5). The present results thus indicated that the enhanced solvent drag-induced and transcellular active duodenal calcium transport seen in chronic metabolic acidosis were likely to result from long-term cellular or molecular adaptive responses and not acidic pH per se.

Chronic Metabolic Acidosis Did Not Induce Widening of Tight Junctions for Paracellular Calcium Transport

Generally, increases in paracellular ion transport involve changes in the size- and charge-selective properties of the tight junction (37, 54). In this study, electrical parameters and permeabilities to various ions were determined to demonstrate whether widening of the tight junction occurred concurrently with enhanced solvent drag-induced calcium transport. Table 3 shows that chronic metabolic acidosis had no effect on PD, $I_{sc}$, or TER. However, the lower PD and $I_{sc}$ of both the control and acidotic groups in the transcellular experiment compared with those of the solvent drag experiment were due to the absence of transepithelial sodium transport, as seen when mucosal sodium was replaced by mannitol (49, 50). In the study of passive paracellular calcium transport, $P_{Ca}$ was also not altered by chronic metabolic acidosis (Fig. 3B). In the dilution potential experiment (protocol 3), the dilution potential, $P_{Na}/P_{Ca}$, $P_{Na}$, and $P_{Cl}$ in the 21-day chronic metabolic acidic group were comparable with those in the control group (Table 4), indicating an intact charge-selective property of the tight junction. We also demonstrated that the duodenal epithelium slightly favored the movement of positively charged ions, because $P_{Na}/P_{Cl}$ was >1.0 (Table 4).

Chronic Metabolic Acidosis Enhanced the Expression of Genes Involved in Duodenal Calcium Transport

The effects of chronic metabolic acidosis on the expression of essential genes involved in solvent drag-induced calcium transport, i.e., the $\beta_{1}$-subunit of Na$^+$-K$^+$-ATPase, ZO-1, occludin, and claudin-3, and transcellular active calcium transport, i.e., TRPV5, TRPV6, calbindin-D$_{9K}$, PMCA$_{1b}$, and NCX1, were investigated (Fig. 6). We found that the relative
mRNA expression of the β₁-subunit of Na⁺-K⁺-ATPase, an important transporter generating paracellular hyperosmotic milieu for the solvent drag, was increased to 117.04 ± 3.65% of control after 21-day chronic metabolic acidosis. Regarding the tight junction-associating proteins, chronic metabolic acidosis significantly enhanced the relative mRNA expression of ZO-1, occludin, and claudin-3 to 146.32 ± 9.29%, 125.93 ± 7.23%, and 136.52 ± 8.77%, respectively. The relative mRNA expressions of TRPV6 and PMCA₁B, the crucial transporters contributing to transcellular active duodenal calcium transport, were increased to 154.32 ± 8.64% and 148.42 ± 8.58%, respectively. The relative mRNA expression of TRPV5 was also increased to 118.92 ± 7.43%. However, the relative expression levels of calbindin-D₂₀ and NCX₁ were not altered.

**Chronic Metabolic Acidosis Affected Some Plasma Components Related to Calcium Metabolism**

As shown in Table 5, after 21-day chronic metabolic acidosis, the total plasma calcium concentration was significantly increased from 2.46 ± 0.06 to 2.59 ± 0.03 mM. Plasma free ionized calcium and magnesium concentrations increased from 1.23 ± 0.02 to 1.34 ± 0.01 mM and from 0.71 ± 0.03 to 0.84 ± 0.03 mM, respectively. However, plasma inorganic phosphate and serum parathyroid hormone remained unchanged. In addition, chronic metabolic acidosis tended to elevate serum 1α,25-(OH)₂D₃, but the difference was statistically insignificant.

**DISCUSSION**

The present work showed, for the first time, that 21-day chronic metabolic acidosis induced by 1.5% NH₄Cl enhanced solvent drag-induced and transcellular active calcium transport but had no effect on passive paracellular calcium transport. It was important to study each component of calcium transport separately because each component contributed significantly to the calcium balance in different situations. For example, transcellular active calcium transport becomes essential during high calcium demand or negative calcium balance, such as during pregnancy, lactation, and low calcium intake (2, 4), whereas passive paracellular transport is responsible for the major portion of calcium absorption during high calcium intake (6). Although the presence and significance of solvent drag have been controversial in some leaky epithelia (30), a number of investigations have confirmed its existence (13, 28, 33, 38). In the duodenum, solvent drag-induced calcium transport contributed ~70% under normal conditions (49). The present results also confirmed our previous reports (49, 50) showing that solvent drag-induced calcium transport was 4.5-fold higher than transcellular active calcium transport.

The effects of chronic metabolic acidosis on intestinal calcium transport have not been extensively studied in the past three decades, and the findings remain inconclusive. By measuring calcium intake and fecal calcium excretion after 16-day NH₄Cl-induced chronic metabolic acidosis, Goulding and Campbell (21) reported an increase in fractional intestinal calcium absorption. However, Gafter and colleagues (20), also using the calcium balance study, reported no changes in intestinal calcium transport after 9 days of NH₄Cl-induced chronic metabolic acidosis. In contrast, other in vivo studies (34, 56) in humans have reported a decrease in intestinal calcium absorption during similar conditions. The discrepancy could be partially explained by the different techniques used in the measurement of calcium absorption. The in vivo calcium balance study used by previous investigators was not a sensitive method for determining intestinal calcium transport, because it could be affected by blood flow as well as by intestinal motility (14, 45). Importantly, in vivo passive paracellular calcium transport...
transport, which was linearly dependent on the transepithelial calcium gradient (28), could overwhelm any effects of chronic metabolic acidosis on active calcium transport, especially at a luminal calcium concentration of >5 mM (Fig. 3A).

It was not known whether the increase in calcium transport observed in the present investigation resulted from a direct action of acid. Enhanced active duodenal calcium transport might represent a complex adaptation of epithelial cells after a long-term exposure to acidic extracellular fluid, as seen in several tissues (40, 58). As shown in Figs. 4 and 5, an acute exposure to acidic pH decreased solvent drag-induced calcium transport, whereas transcellular active calcium transport remained unchanged. Our findings confirmed that acute and chronic metabolic acidosis exerted different effects by an unidentified mechanism. We speculated that decreases in calcium fluxes after acute acid exposure might relate to the protonation of the negatively charged extracellular amino acids that determine the cationic selectivity of tight junction proteins (53). Favored and coworkers (16) also reported a decrease in the total duodenal calcium transport when the medium pH was reduced. On the basis of this evidence, we further postulated that a long-term adaptation could occur at the molecular or cellular levels in response to chronic metabolic acidosis, e.g., an increase in the expression of important genes related to calcium transport (40, 42), or changes in tight junction-related properties.

The size- and charge-selective properties of the tight junction have been documented to modulate solvent drag-induced ion transport (37, 38). Nevertheless, our previous finding, that solvent drag-induced duodenal calcium transport was not increased in the presence of cytochalasin E, which is known to induce widening of the tight junction (49), suggested an absence of a direct association between solvent drag-induced paracellular calcium transport and size selectivity of the tight junction. The intact TER reported in the present work confirmed that widening of the tight junction did not occur during acidosis.

Herein, the charge selectivity was also determined by using the dilution potential method. Most epithelia, including the duodenal epithelium, were slightly cation selective, as indicated by the relative permeability for Na\(^+\) to Cl\(^-\) being >1.0 (54), i.e., \(P_{Na}/P_{Cl}\) of 1.23 in the normal duodenum (Table 4).

Charge selectivity was determined by the presence of charge-selective integral membrane proteins of the claudin family on the tight junction (53). Changes in the expression of claudins and the charge-selective property of the tight junction could alter the rate of paracellular ion transport in both intact and cultured epithelia (53). However, in the present study, \(P_{Na}/P_{Cl}\) as well as the absolute duodenal \(P_{Na}\) and \(P_{Cl}\) were not affected by chronic metabolic acidosis, indicating that the charge selectivity of the tight junction had remained unchanged. The similar duodenal \(P_{Cl}\) of the passive paracellular pathway in the control and acidotic groups (Fig. 3B) also suggested that chronic metabolic acidosis did not upregulate any component involved in the passive paracellular calcium movement. However, facilitated paracellular calcium transport induced by other unknown proteins in the presence of solvent drag could not be excluded.

Furthermore, because changes in the size- and charge-selective properties of the tight junction after 21-day chronic metabolic acidosis were not detected, we proceeded to examine the mRNA expression of genes associated with solvent drag-induced calcium transport. \(Na^{+}-K^{+}\)-ATPases, the crucial transporters lining the paracellular space (12, 33), were responsible for generating a hyposmotnic paracellular environment, which could be 15 mM higher than the surrounding medium (11), thereby inducing solvent drag. The increase in the expression of the \(\beta_1\)-subunit of \(Na^{+}-K^{+}\)-ATPase in this study, therefore, could partly explain the enhanced solvent drag-induced calcium transport and confirmed the concept of a long-term adaptation to chronic acidosis at the molecular level. Our results also showed increases in the mRNA expression of three tight junction-associated proteins, namely, ZO-1, occludin, and claudin-3. Virtually nothing was presently known regarding the exact roles of these proteins in duodenal calcium transport. A report (43) on occludin knockout mice with a thinner femoral compact bone and abnormal calcification suggested the possible involvement of occludin in normal calcium metabolism. However, that study did not address changes in intestinal calcium absorption. ZO-1, a cytoplasmic protein with the ability to bind with both occludin and claudins (25), was another protein associated with the structure dynamic and

<table>
<thead>
<tr>
<th>Condition</th>
<th>(PD, \text{mV})</th>
<th>(I_{sc}, \mu\text{A/cm}^2)</th>
<th>(TER, \Omega\text{cm}^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.22±0.47</td>
<td>36.75±8.52</td>
<td>129.64±16.31</td>
</tr>
<tr>
<td>Acidosis</td>
<td>4.24±0.48</td>
<td>39.70±8.57</td>
<td>131.96±26.49</td>
</tr>
</tbody>
</table>

Values are means ± SE; \(n\), no. of animals/group. \(P_{Na}/P_{Cl}\), permeability ratio; \(P_{Na}\), absolute sodium permeability; \(P_{Cl}\), absolute chloride permeability.
experimental animals. All samples were performed in triplicate. The expression of claudin-3 mRNA was found to be related to calcium transport. Claudins, on the other hand, were more closely related to calcium transport than to the regulation of intestinal calcium absorption (24). A physiological role of ZO-1 in the regulation of intestinal calcium absorption (24) has not been established, whereas binding of ZO-1 to claudin-16 augments renal tubular calcium and magnesium reabsorption (24). The increase in ZO-1 expression observed in the present study might implicate its role in the regulation of intestinal calcium transport. Claudins, on the other hand, were more closely related to calcium transport. By using the microarray technique, the expression of claudin-3 mRNA was found to be $\alpha_{3,25}(OH)_2D_3$ dependent (32), but its physiological role pertaining calcium transport has not been investigated. It was interesting that claudin-16, found only in the kidneys, was essential for paracellular calcium and magnesium transport in the thick ascending limb (57). However, a recent mutagenesis study (23) has suggested that claudin-16 does not actually form a functional calcium/magnesium-selective paracellular channel but prevents dissipation of the positive potential, a driving force for calcium/magnesium reabsorption. Although calcium-selective characteristics of claudin-3 or other claudins have not been identified, it is possible that, among the growing members of the claudin family, there is a calcium-selective claudin existing in the duodenum. In addition, activities of $\text{Na}^+\text{K}^+\text{ATPase}$ were required for the functions of tight junction proteins (55); therefore, the enhanced expressions of $\text{Na}^+\text{K}^+\text{ATPase}$, ZO-1, occludin, and claudin-3 seen in acidosis might act in concert to modulate the tight junction microstructure and paracellular driving force, thereby facilitating solvent drag-induced duodenal calcium transport.

Regarding transcellular active calcium transport, the mRNA expressions of TRPV6 and PMCA$_{1b}$, which are transporters for apical calcium entry and basolateral calcium extrusion, respectively, were stimulated by chronic metabolic acidosis. Although parathyroid hormone and $\alpha_{3,25}(OH)_2D_3$ have been reported to stimulate intestinal calcium transport and upregulate several calcium-transporting genes (22), we, like other previous reports (8, 31), did not find any changes in the serum levels of the two hormones during chronic metabolic acidosis. Hence, the enhanced duodenal calcium transport as well as the upregulation of TRPV6 and PMCA$_{1b}$ in the present study could not be explained by the actions of parathyroid hormone and $\alpha_{3,25}(OH)_2D_3$. However, there have been reports (20, 31) of elevated serum $\alpha_{3,25}(OH)_2D_3$ in severe acidosis and during the administration of $\text{NH}_4\text{Cl}$ of concentrations >1.5%.

Concomitantly, the expression of TRPV5, which is important in renal tubular calcium reabsorption but was thought to have little role in the duodenum (22), was also increased in the present study. There have been reports of TRPV5 mRNA expression in the rat duodenum being increased by $\alpha_{3,25}(OH)_2D_3$ (32) and a low-calcium diet (7), suggesting a possible role of TRPV5 in transcellular duodenal calcium transport. Recently, $\text{NH}_4\text{Cl}$-induced chronic metabolic acidosis was also found to modulate the mRNA expression of TRPV5 in the kidneys (40). On the other hand, the absence of change in the mRNA expression of NCX was not unexpected, because this

![Fig. 6.](image)

**Fig. 6.** Relative mRNA expression of genes related to solvent drag-induced paracellular calcium transport (A), i.e., the $\beta_1$-subunit of $\text{Na}^+\text{K}^+\text{ATPase}$ (NKA), zonula occludens (ZO)-1, occludin, and claudin-3, and to transcellular active calcium transport (B), i.e., transient receptor potential vanilloid family $\text{Ca}^{2+}$ channels 5 (TRPV5) and 6 (TRPV6), calbindin-D$_{9K}$ (CaBP), plasma membrane $\text{Ca}^{2+}$-ATPase isof orm 1b (PMCA$_{1b}$), and Na$^{+}$/Ca$^{2+}$ exchanger 1 (NCX1), in duodenal enterocytes of rats. *$P<0.05$, **$P<0.01$, and ***$P<0.001$ compared with their respective controls; $n$ denotes the number of experimental animals. All samples were performed in triplicate.

### Table 5. Changes in total plasma calcium, free ionized calcium, free ionized magnesium, inorganic phosphate, serum parathyroid hormone, and serum $\alpha_{3,25}(OH)_2D_3$ in rats after 21-day chronic metabolic acidosis

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Acidosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>$n$</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Total plasma calcium, mM</td>
<td>2.46±0.06</td>
<td>2.59±0.03*</td>
</tr>
<tr>
<td>Free ionized calcium, mM</td>
<td>1.23±0.02</td>
<td>1.34±0.01†</td>
</tr>
<tr>
<td>Free ionized magnesium, mM</td>
<td>0.71±0.03</td>
<td>0.84±0.03*</td>
</tr>
<tr>
<td>Inorganic phosphate, mM</td>
<td>0.47±0.02</td>
<td>0.42±0.01</td>
</tr>
<tr>
<td>Serum parathyroid hormone, pg/ml</td>
<td>26.96±2.73</td>
<td>25.06±2.93</td>
</tr>
<tr>
<td>Serum $\alpha_{3,25}(OH)_2D_3$, pM</td>
<td>49.64±8.25</td>
<td>57.92±5.42</td>
</tr>
</tbody>
</table>

Values are means ± SE; $n$, no. of animals/group. $\alpha_{3,25}(OH)_2D_3$, $\alpha_{3,25}$-dihydroxy vitamin D$_3$. *$P<0.05$ and †$P<0.001$ compared with the control group.
transporter is important for transepithelial calcium transport in the kidneys but not in the small intestine (22). As for vitamin D-dependent calbindin-D9K, the unaltered mRNA expression agreed with the unchanged serum level of 1α,25-(OH)2D3 in chronic metabolic acidosis. The present finding in the duodenum was in contrast to that in the rat distal tubule, where NH4Cl-induced chronic metabolic acidosis was found to increase calbindin-D28K abundance (42). It is possible that the normal expression of duodenal calbindin-D9K was already sufficient for the enhanced transcellular active calcium transport. The present findings of increases in the total plasma calcium, free ionized calcium, and free ionized magnesium after 21-day chronic metabolic acidosis were consistent with previous reports in rats and dogs (8, 35). López and colleagues (35) have demonstrated that metabolic acidosis increased free ionized calcium within 10 min after in vivo acidotic induction. In addition to high bone turnover and impaired mineralization (8, 9), an increase in the intestinal calcium absorption could also explain the resultant hypercalcemia. The enhanced duodenal calcium transport in chronic metabolic acidosis may have a physiological significance, because it would help in replenishing the body store of calcium, which was severely depleted by bone resorption and renal calcium loss. In other word, our findings showed that compensatory adaptations in intestinal calcium absorption occurred to counteract calcium loss after acidosis-induced bone and renal dysfunction.

In conclusion, the present study elucidated the stimulatory effects of 21-day chronic metabolic acidosis on solvent dragged induced duodenal calcium transport in rats. Its effects were explained, in part, by an increase in the mRNA expression of Na+-K+-ATPase and tight junction proteins, namely, ZO-1, occludin, and claudin-3. Widening of the tight junction and alterations in the charge-selective paracellular structure were not required for the enhanced solvent drag. Furthermore, chronic acidosis also stimulated transcellular active duodenal calcium transport, which could have resulted from increases in the mRNA expression of important transporters such as TRPV5, TRPV6, and PMCA1b. Because an acute direct exposure to acidic pH did not stimulate calcium transport, the increased transport must have resulted from a long-term adaptive response of the duodenal epithelium to chronic metabolic acidosis.

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