Effects of vitamin D-induced chronic hypercalcemia on rat renal cortical plasma membranes and mitochondria

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Departments of Medicine, Dallas Veterans Administration Medical Center, and University of Texas Health Science Center at Dallas, Dallas, Texas 75235; and Denver Veterans Administration Medical Center, and University of Colorado Health Science Center, Denver, Colorado 80220

Levi, Moshe, Bruce A. Molitoris, Thomas J. Burke, Robert W. Schrier, and Francis R. Simon. Effects of vitamin D-induced chronic hypercalcemia on rat renal cortical plasma membranes and mitochondria. Am. J. Physiol. 252 (Renal Fluid Electrolyte Physiol. 21): F267-F275, 1987.—In vivo vitamin D-induced chronic hypercalcemia on rat renal cortical brush-border and basolateral membranes and mitochondria. In the brush-border membrane, hypercalcemia caused significant decreases in alkaline phosphatase-specific activity, total phospholipid molar content, and phosphatidylethanolamine percent molar composition and increases in the cholesterol-to-total phospholipid molar ratio and phosphatidylcholine percent molar composition. In the basolateral membrane, hypercalcemia caused significant decreases in Na⁺-K⁺-ATPase-specific activity and total phospholipid molar content and increases in the cholesterol-to-total phospholipid molar ratio and phosphatidylinositol percent molar composition. In the mitochondrial membranes, hypercalcemia caused mild increases in the mitochondrial calcium content, but no alterations in succinic dehydrogenase-specific activity, succinate-ADP-phosphatase, or uncoupler-induced respiration. Thus hypercalcemia caused alterations in brush-border and basolateral membrane enzyme activity and lipid composition, but no functional changes were detected in mitochondria. These hypercalcemia-induced plasma membrane biochemical alterations may be markers of early cell injury and suggest a role for calcium in causing or predisposing to renal tubular cell injury.

Methods

General. All studies were performed on male Fischer 344 rats (Charles River, Wilmington, MA) weighing between 225 and 275 g. Rats were fed a commercially obtained control diet (ICN Nutritional Biochemicals, Cleveland, OH). Hypercalcemia was induced by supplementation of the control diet with dihydrotachysterol (DHT, Roxane Laboratories, Columbus, OH) (4.25 mg DHT/kg of diet). In addition, to determine the effects of DHT in the absence of changes in serum calcium, a third group of rats was fed a diet supplemented with a non-hypercalcemic dose of DHT (0.425 mg DHT/kg of diet). In pilot studies we determined that this was the maximal dose of DHT that caused hypercalcemia, but did not cause an increase in serum total or ionized calcium concentration. All rats on the control and DHT-supplemented diets were weight and age matched at the onset of each experiment, and were pair fed throughout the experiments.
terial period. All studies were performed after 8 days on the control or DHT diets.

**Determination of serum calcium concentration, cortical homogenate, brush-border membrane, basolateral membrane, and mitochondrial calcium content.** Serum total calcium concentration was measured by an atomic absorption spectrophotometer (Perkin Elmer, Norwalk, CT), and serum ionized calcium concentration was measured by an ionized calcium analyzer (Nova 2, Nova Biochemical, Newton, MA). For measurement of tissue calcium content, aliquots of cortical homogenate, brush-border membrane, basolateral membrane, and mitochondria were digested with concentrated nitric acid, and the contents were diluted with distilled and deionized water in acid-washed plastic tubes prior to measurement by atomic absorption spectrophotometer. Calcium content is expressed as nanomoles Ca per milligram protein.

**Isolation of brush-border and basolateral membranes and mitochondria.** Brush-border and basolateral membranes from the renal cortical tissue of hypercalcemic and normocalcemic control rats were simultaneously isolated by a differential centrifugation, magnesium precipitation, and discontinuous sucrose gradient method as previously described by Molitoris and Simon (26). Rats were lightly anesthetized with ether, the kidneys were removed through a midabdominal incision and placed in an ice-chilled isolation buffer containing 300 mM mannitol, 5 mM ethyleneglycol-bis (P-aminoethylether)-N,N',N",N"'-tetaacetic acid (EGTA), 0.1 mM phenyl methylsulfonyl fluoride (PMSF), and 18 mM tris(hydroxymethyl)aminomethane (Tris), pH 7.4. EGTA, a chelator of calcium, and PMSF, a protease inhibitor, were added to the isolation buffer to minimize potential effects of calcium, phospholipases, and proteases during the isolation procedure on the resultant brush-border and basolateral membrane enzyme activity and lipid composition. Thin cortical sections were obtained and homogenized using a polytron (Brinkman, Westbury, NY). The brush-border and basolateral membranes were then isolated from the cortical homogenate as previously described (26).

Mitochondria from the renal cortical tissue of hypercalcemic and normocalcemic control rats were isolated by a differential centrifugation method as previously described by Wilson et al. (36). Rats were lightly anesthetized with ether, the kidneys were removed through a midabdominal incision and placed in an ice-chilled isolation buffer containing 210 mM mannitol, 70 mM sucrose, 200 μM ruthenium, 0.5% fat-free bovine serum albumin (BSA), and 2 mM K-N-2-hydroxyethylpiperazine ethanesulfonic acid (HEPES), pH 7.4. Ruthenium red, a specific inhibitor of mitochondrial calcium influx via the electrophoteric mitochondrial calcium uniporter, was added to the isolation buffer to exclude any role for mitochondrial calcium influx during the isolation procedure on the resultant mitochondrial calcium content and mitochondrial respiration. EGTA, a chelator of calcium, was not added to the isolation buffer, to avoid mitochondrial calcium efflux during the isolation procedure. Thin cortical sections were obtained and homogenized using a Potter Elvehjem tissue homogenizer. The mitochondria were then isolated from the cortical homogenate as previously described (36).

**Determination of enzyme activity.** Enzyme activity determinations were made on homogenates and membrane fractions frozen at -20°C for 12 h. Enzyme activity assays were performed under conditions of substrate concentration and reaction time so that the rate of each reaction was linearly related to the amount of sample protein added. Specific activities of Na+-K+-ATPase (basolateral membrane-bound), alkaline phosphatase and leucine aminopeptidase (brush-border membrane-bound), succinic dehydrogenase (mitochondrial membrane-bound), NADPH cytochrome c reductase (endo-plasmic reticular membrane-bound), and N-acetyl-β-D-glucosaminidase (lysosomal membrane-bound) were measured in samples of homogenates and basolateral, brush-border, and mitochondrial membrane fractions, as previously described (25-27), using a Beckman spectrophotometer (Fullerton, CA) equipped with a kinetic and temperature control unit.

Na+-K+-ATPase activity was measured using an assay system coupled to pyruvate kinase, phosphoenolpyruvate, and lactate dehydrogenase at 340 nm and 37°C. Alkaline phosphatase activity was measured by a kinetic assay, monitoring the production of p-nitrophenol and phosphate from p-nitrophenyl phosphate at 405 nm and 37°C. Leucine aminopeptidase activity was measured by a kinetic assay, monitoring the conversion of L-leucine-4-nitroanilid at 380 nm and 37°C. Succinic dehydrogenase activity was measured by a kinetic assay, monitoring the production of 2,6-dichlorophenolindophenol at 600 nm and 37°C. NADPH cytochrome c reductase activity was measured by a kinetic assay, monitoring the increase in absorbance produced by the reduction of cytochrome c at 550 nm and 37°C. N-acetyl-β-D-glucosaminidase activity was determined spectrophotometrically at 405 nm and 37°C by measuring the conversion of p-nitrophenyl-N-acetyl-β-D-glucosaminid to p-nitrophenol. Enzyme activities are expressed as micro moles per hour per milligram protein. Protein was determined by the method of Lowry et al. (21) using crystalline BSA as standard.

Enrichment (specific activity in membrane fraction/specific activity in homogenate) and recovery (total activity in membrane fraction/total activity in homogenate) of each membrane fraction was determined using the above enzymes as markers of specific subcellular organelles.

**Lipid extraction and determination of membrane lipid composition.** Total lipids were extracted by the method of Bligh and Dyer (4). One milligram of homogenate, brush-border and basolateral membrane protein was used for the extraction. Coprostanol (Supelco, Bellefonte, PA) was included as an internal standard for cholesterol determination. The original sample was extracted twice and the combined extract evaporated to residue under nitrogen. The residue was resuspended in chloroform and divided into aliquots for cholesterol, total, and individual phospholipid determinations.

The cholesterol sample was evaporated to a residue and resuspended in hexane and injected into a 30% SP
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2250 glass-packed column (Supelco) in a gas liquid chromatograph (Perkin-Elmer model 3920B, Norwalk, CT), run isothermally at 285°C, with coprostanol serving as internal standard. Peak height and area ratios were used for quantitation and cholesterol expressed in nanomoles per milligram protein.

The phospholipid sample was dried to residue and resuspended in chloroform. Phospholipid content in the total lipid extract was determined by measuring the phosphorus content by the method of Ames and Dubin (2). Individual phospholipid polar head group species were isolated by two-dimensional thin-layer chromatography (TLC) (10). The phospholipid sample was spotted onto Kieselgel Silica Gel 60 precoated TLC plates (Merck, Darmstadt, FRG). The plates were developed in two dimensions. The first dimension solvent system consisted of chloroform:methanol:acetic acid (65:25:10, vol/vol) and the second, chloroform:methanol:88% formic acid (65:25:10, vol/vol). This system resulted in an excellent separation of sphingomyelin, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol, as well as lysophosphatidylcholine and lysophosphatidylethanolamine. After development, chromatograms were allowed to dry and then were exposed to iodine crystals. Individual phospholipids were identified by comparison with cochromatography of authentic standards (Supelco). Areas of silica gel contained to be free of phospholipids. Phospholipid content is expressed as nanomoles per milligram protein. The percent recovery of phospholipids from the TLC plate and reextraction from the gel was determined by dividing the sum amount of individual phospholipids by the total phospholipid applied to the TLC plate.

For the analysis of anionic phospholipids including phosphotidylglycerol, phosphatidylglycerol 4-phosphate, phosphatidylinositol 4,5-bisphosphate, and phosphatidic acid, 2 mg of brush-border or basolateral membrane protein was extracted by the method of Grove et al. (12) in 6 ml chloroform:methanol (1:1, vol/vol) containing 10 mM tetrabutylammonium sulfate (TBAS). The extracts were evaporated under nitrogen, chloroform was added and washed once with 0.1 N HCl and three times with theoretical upper phase made with chloroform:0.1 N HCl (1:1, vol/vol). In preliminary studies we found that extracting the anionic phospholipids by the amphiphilic ion-pairing reagent TBAS was far more efficient than extracting with chloroform:methanol solvent systems that included either KCl or HCl. For the separation of phosphatidylglycerol, phosphatidylglycerol 4-phosphate, and phosphatidylinositol 4,5-bisphosphate, lipid samples were applied to silica gel HL prechanneled TLC plates with preabsorbent phase (Analtech, Newark, DE) and run in one dimension in a solvent system of chloroform:methanol:20% aqueous methylamine (60:36:10, vol/vol) (31). For the separation of phosphatidic acid, lipid samples were applied to the silica gel HL TLC plates and were run in one dimension in a solvent system of chloroform:pyridine:formic acid (50:30:5, vol/vol) (31). The phospholipid spots were visualized, identified, extracted from the gel, and quantified by the Ames and Dubin phosphate assay as described above.

Determination of mitochondrial respiration. Mitochondrial respiration was measured polarographically in a closed reaction vessel fitted with a Clark O2 electrode (Yellow Springs Instrument, Yellow Springs, OH) at 25°C in a volume of 1.7 ml (36). The incubation medium was composed of 120 mM KCl, 2 mM KH2PO4, 4 µM rotenone, 1 mM EGTA, and 5 mM Tris- HCl, pH 7.1. Mitochondria (2 mg protein) were energized with the addition of 5 mM K-succinate. After a short equilibration period, state 3 respiration (S3) was induced by the addition of 300 nmol of adenosine diphosphate (ADP). After all the added ADP was phosphorylated to ATP, state 4 respiration (S4) was measured. The ratio of O2 consumption in the presence of ADP to that in its absence (S3/S4) was termed the acceptor control ratio (ACR). In addition, maximal rate of O2 consumption was measured with the addition of 0.2 nmol of carboxyl cyanide p-trifluoromethoxyphenyl hydrasone (FCCP), an uncoupler of oxidative phosphorylation. Mitochondrial respiration is expressed as natoms O2 per minute per milligram protein.

Statistical analyses. All values reported represent the mean ± SE. The unpaired t test (two-tailed) was used to evaluate the significance of differences between the normocalcemic and hypercalcemic rats. Significance was accepted at P < 0.05.

RESULTS

Effects of vitamin D diets on serum calcium concentration, cortical homogenate, brush-border membrane, basolateral membrane, and mitochondrial calcium content. Dietary vitamin D supplementation (4.25 mg DHT/kg diet) significantly increased serum total calcium concentration to 12.8 ± 0.2 vs. 10.0 ± 0.1 mg/dl in normocalcemic control rats, P < 0.01, and serum ionized calcium concentration to 6.7 ± 0.2 vs. 5.4 ± 0.1 mg/dl in normocalcemic control rats, P < 0.01. As per design of the study, dietary vitamin D supplementation with lower DHT doses (0.425 mg DHT/kg diet) did not cause significant changes in serum total (9.7 ± 0.1 mg/dl) or ionized (5.3 ± 0.1 mg/dl) calcium concentration.

Dietary vitamin D supplementation also significantly increased cortical homogenate calcium content to 28.5 ± 4.1 vs. 15.0 ± 2.3 nmol Ca/mg cortical protein in normocalcemic control rats, P < 0.01. There was also a minor but significant increase in the mitochondrial calcium content of the hypercalcemic rats, 14.6 ± 0.5 vs. 11.4 ± 0.2 nmol Ca/mg mitochondrial protein in normocalcemic control rats, P < 0.01. There was no measurable calcium in the brush-border or basolateral membranes from the hypercalcemic or normocalcemic rats. This is most likely due to the isolation of the brush-border and basolateral membranes in a buffer containing...
excess amounts of EGTA.

Effects of hypercalcemia on brush-border and basolateral membrane, and mitochondrial isolation. Brush-border membranes isolated from normocalcemic and hypercalcemic rats had similar enrichment and recovery for alkaline phosphatase and leucine aminopeptidase activities (Table 1). Contamination of the brush-border membrane with the other subcellular organelles was minimal and similar, except for a slightly higher contamination with the basolateral membrane in the hypercalcemic rats (Table 1).

Basolateral membranes isolated from normocalcemic and hypercalcemic rats were similarly enriched for Na⁺-K⁺-ATPase activity (Table 1). Recovery of Na⁺-K⁺-ATPase activity and contamination of the basolateral membrane with the other subcellular organelles were similar in normocalcemic and hypercalcemic rats (Table 1).

Mitochondria isolated from normocalcemic and hypercalcemic rats had similar enrichment and recovery for succinic dehydrogenase activity (Table 2). Contamination of the mitochondria with the other subcellular organelles was similar, except for a slightly higher contamination with the basolateral membrane in the hypercalcemic rats (Table 2).

Effects of hypercalcemia on cortical homogenate, brush-border, basolateral, and mitochondrial membrane enzyme activity. In the cortical homogenate, hypercalcemia caused a 29% decrease in the specific activity of alkaline phosphatase (P < 0.01), a 22% decrease in the specific activity of Na⁺-K⁺-ATPase (P < 0.01), and a 210% decrease in the specific activity of N-acetyl-β-D-glucosaminidase (Table 3). Hypercalcemia did not cause significant decreases in the specific activities of leucine aminopeptidase, succinic dehydrogenase, or NADPH cytochrome c reductase (Table 3).

In the brush border membrane, hypercalcemia caused a 34% decrease in the specific activity of alkaline phosphatase (P < 0.01) (Fig. 1); there was no significant decrease in the specific activity of leucine aminopeptidase.

In the basolateral membrane, hypercalcemia caused a 26% decrease in the specific activity of Na⁺-K⁺-ATPase (P < 0.01) (Fig. 1).

In the mitochondria, hypercalcemia did not cause a significant decrease in the specific activity of succinic dehydrogenase (P = NS) (Table 7).

Nonhypercalcemic doses of vitamin D did not cause any significant alterations in the alkaline phosphatase specific activity in the cortical homogenate or brush-border membrane, and Na⁺-K⁺-ATPase specific activity in the cortical homogenate or basolateral membrane. Therefore, the effects of vitamin D-induced hypercalcemia on alkaline phosphatase and Na⁺-K⁺-ATPase specific activities are apparently caused by the hypercalcemia rather than nonhypercalcemic effects of vitamin D.

Effects of hypercalcemia on cortical homogenate, brush-border and basolateral membrane lipid composition. In the cortical homogenate, hypercalcemia caused no significant change in the cholesterol molar content, but a 16% decrease in the total phospholipid molar content (P < 0.01), resulting in a 17% increase in the cholesterol-to-total phospholipid molar ratio (P < 0.01) (Table 4).

In the brush-border membrane, hypercalcemia caused no significant change in the cholesterol molar content, but a 10% decrease in the total phospholipid molar content (P < 0.05) (Table 5), resulting in a 15% increase in the cholesterol-to-total phospholipid molar ratio (P < 0.05) (Fig. 2). Hypercalcemia also caused significant decreases in the molar content of phosphatidylethanolamine and phosphatidylethanolamine, but when the individual phospholipid content was expressed as the percent molar composition of the total phospholipid content, only the change in the percent composition of phosphatidylinerine achieved significance (Table 5). In addition, hypercalcemia caused a significant increase in the molar content and percent molar composition of phosphatidylinositol (P < 0.05), but no changes in the molar content of the other anionic phospholipids (Table 6). The TLC recovery for the phospholipids did not significantly differ between the hypercalcemic (78.7 ± 2.4%) and normocalcemic (77.6 ± 4.2%) rats.

In the basolateral membrane, hypercalcemia caused no significant change in the cholesterol molar content, but a 12% decrease in the total phospholipid molar content (P < 0.05) (Table 5), resulting in a 16% increase in the cholesterol-to-total phospholipid molar ratio (P < 0.05) (Fig. 2). Hypercalcemia also caused significant decreases in the molar content of sphingomyelin, phosphatidylcholine, and phosphatidylethanolamine, but when the individual phospholipid content was expressed as the percent molar composition of the total phospholipid content, these changes did not achieve significance (Table 5).

### Table 1. Enrichment and contamination of brush-border and basolateral membranes isolated from normocalcemic and hypercalcemic rats

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Membrane</th>
<th>Brush-Border Membrane</th>
<th>Basolateral Membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Normocalcemic</td>
<td>Hypercalcemic</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>Brush-border</td>
<td>9.0±0.9</td>
<td>8.7±1.5</td>
</tr>
<tr>
<td>Leucine aminopeptidase</td>
<td>Brush border</td>
<td>9.7±0.9</td>
<td>9.0±0.9</td>
</tr>
<tr>
<td>Na⁺-K⁺-ATPase</td>
<td>Basolateral</td>
<td>1.5±0.2</td>
<td>2.3±0.3*</td>
</tr>
<tr>
<td>NADPH cytochrome c reductase</td>
<td>Endoplasmic reticulum</td>
<td>0.24±0.09</td>
<td>0.30±0.05</td>
</tr>
<tr>
<td>Succinic dehydrogenase</td>
<td>Mitochondria</td>
<td>0.24±0.02</td>
<td>0.25±0.02</td>
</tr>
<tr>
<td>Glucosaminidase</td>
<td>Lysosome</td>
<td>0.34±0.02</td>
<td>0.43±0.03*</td>
</tr>
</tbody>
</table>

Results are expressed as means ± SE; n = 12 in each group. Enrichment or contamination of each subcellular membrane marker enzyme is defined as the ratio of specific activity of the enzyme in the membrane fraction/specific activity of the enzyme in the homogenate. * P < 0.05.
Enrichment or contamination of each subcellular membrane marker mitochondria isolated from normocalcemic and Na+-K+-ATPase Basolateral $0.51 \pm 0.06$ $0.70 \pm 0.08^*$ Leucine aminopeptidase Brush-border $0.24 \pm 0.03$ $0.29 \pm 0.10^*$ Glucosaminidase Lysosome $0.83 \pm 0.09$ $0.86 \pm 0.11$ Succinic dehydrogenase Mitochondria $4.20 \pm 0.20$ $4.80 \pm 0.34^*$ NADPH cytochrome c Endoplasmic $0.51 \pm 0.03$ $0.29 \pm 0.03^*$

Results are expressed as means ± SE; n = 12 in each group. Enrichment or contamination of each subcellular membrane marker enzyme is defined as ratio of specific activity of enzyme in mitochondrial fraction/specific activity of enzyme in homogenate. * P < 0.05.

### TABLE 3. Effect of hypercalcemia on renal cortical homogenate enzyme-specific activity

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Membrane</th>
<th>Normocalcemic</th>
<th>Hypercalcemic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaline phosphatase</td>
<td>Brush-border</td>
<td>$97.4 \pm 4.1$</td>
<td>$69.5 \pm 5.6^*$</td>
</tr>
<tr>
<td>Leucine aminopeptidase</td>
<td>Brush-border</td>
<td>$5.4 \pm 0.3$</td>
<td>$5.7 \pm 0.7$</td>
</tr>
<tr>
<td>Na$^+$-K$^+$-ATPase</td>
<td>Basolateral</td>
<td>$25.9 \pm 1.6$</td>
<td>$20.2 \pm 0.4^*$</td>
</tr>
<tr>
<td>Succinic dehydrogenase</td>
<td>Mitochondria</td>
<td>$15.9 \pm 0.8$</td>
<td>$14.9 \pm 0.6$</td>
</tr>
<tr>
<td>NADPH cytochrome c reductase, reticulum</td>
<td>Endoplasmic</td>
<td>$1.00 \pm 0.04$</td>
<td>$0.97 \pm 0.07$</td>
</tr>
<tr>
<td>Glucosaminidase, $\mu$mol $\cdot$ h$^{-1}$ $\cdot$ mg protein$^{-1}$</td>
<td>Lysosome</td>
<td>$9.9 \pm 0.5$</td>
<td>$7.9 \pm 0.6^*$</td>
</tr>
</tbody>
</table>

Results are expressed as means ± SE; n = 12 in each group. * P < 0.05, † P < 0.01.

### FIG. 1. Brush-border membrane alkaline phosphatase specific activity ($\mu$mol $\cdot$ h$^{-1}$ $\cdot$ mg protein$^{-1}$), A, and basolateral membrane Na$^+$-K$^+$-ATPase specific activity ($\mu$mol $\cdot$ h$^{-1}$ $\cdot$ mg protein$^{-1}$), B, in normocalcemic (open bar) and hypercalcemic (solid bar) rats. n = 12 in each group and values represent means ± SE.

### TABLE 4. Effect of hypercalcemia on renal cortical homogenate lipid composition

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Normocalcemic</th>
<th>Hypercalcemic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol, nmol/mg</td>
<td>$68 \pm 3$</td>
<td>$66 \pm 1$</td>
</tr>
<tr>
<td>Total phospholipid, nmol/mg</td>
<td>$192 \pm 3$</td>
<td>$102 \pm 1.5^*$</td>
</tr>
<tr>
<td>Cholesterol/total phospholipid</td>
<td>$0.35 \pm 0.02$</td>
<td>$0.41 \pm 0.02^*$</td>
</tr>
</tbody>
</table>

Results are expressed as means ± SE; n = 6 in each group. * P < 0.01.

In addition, hypercalcemia caused a significant increase in the molar content and percent molar composition of phosphatidylinositol 4,5-bisphosphate ($P < 0.05$), but there were no changes in the molar content of the other anionic phospholipids (Table 6). The TLC recovery for the phospholipids did not significantly differ between the hypercalcemic (65.6 ± 2.4%) and normocalcemic (63.3 ± 1.5%) rats.

Nonhyperglycemic doses of vitamin D did not cause any significant alterations in cortical homogenate, brush-border, or basolateral membrane cholesterol content, total phospholipid content, cholesterol-to-total phospholipid molar ratio, or individual phospholipid content. Therefore, the effects of vitamin D-induced hypercalcemia on lipid composition is apparently a consequence of the hypercalcemic rather than nonhypercalcemic effects of vitamin D.

### DISCUSSION

Intracellular and mitochondrial calcium overload that occurs in ischemic and toxic acute renal failure (3, 7, 24, 34, 36) has been proposed to play a role in renal tubular cell injury and dysfunction (7). The experimental models of ischemic and toxic acute renal failure so far studied usually result in severe cell damage and necrosis. The associated cellular calcium overload and the cellular functional impairments and biochemical alterations (13, 27, 36) could be a consequence, rather than the cause, of tubular cell injury. It has been, therefore, difficult on the basis of these studies to assign a primary role for calcium in causing cell injury. Recent preliminary studies, however, have shown that acute and chronic hypercalcemia in the rat enhances ischemic and toxic acute renal failure (8, 16, 28, 33), suggesting an important role for calcium in predisposing to renal cell injury. Hypercalcemia, therefore, seems to be a suitable model to examine the cellular effects of calcium-mediated cell injury.

The present study used a model of vitamin D-induced chronic hypercalcemia in the rat that has previously been shown to be associated with only mild decrements in renal blood flow, glomerular filtration rate (17), and tubular function (18), and therefore allows the study of the effects of calcium on the renal tubular plasma membrane and mitochondria in the absence of severe cell injury and prior to the manifestations of acute renal failure.

Hypercalcemia resulted in a mild but significant increase in the mitochondrial calcium content. Since, in the present study, the mitochondria were isolated in the presence of Ruthenium red, which would inhibit in vitro mitochondrial calcium influx, but in the absence of EGTA, the increase in mitochondrial calcium content...
TABLE 5. Effect of hypercalcemia on brush-border and basolateral membrane lipid composition

<table>
<thead>
<tr>
<th></th>
<th>Brush-Border Membrane</th>
<th>Basolateral Membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normocalcemic</td>
<td>Hypercalcemic</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>409±7</td>
<td>421±6</td>
</tr>
<tr>
<td>Total phospholipid</td>
<td>482±17</td>
<td>433±18*</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>184±4</td>
<td>186±10*</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>102±2</td>
<td>94±4*</td>
</tr>
<tr>
<td>Phosphatidylyethanolamine</td>
<td>116±5</td>
<td>113±5</td>
</tr>
<tr>
<td>Phosphatidyserine</td>
<td>71±6</td>
<td>43±9*</td>
</tr>
<tr>
<td>Total anionic phospholipid</td>
<td>346±26</td>
<td>377±27</td>
</tr>
</tbody>
</table>

Results are expressed as means ± SE; n = 12 in each group. Cholesterol, total, and individual phospholipid levels are expressed as nanomoles lipid phosphorus per milligram protein. Individual phospholipids are also expressed as molar percent of total phospholipid content (in parenthesis). * P < 0.05.

TABLE 6. Effects of hypercalcemia on brush-border and basolateral membrane anionic phospholipid composition

<table>
<thead>
<tr>
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<tbody>
<tr>
<td></td>
<td>Normocalcemic</td>
<td>Hypercalcemic</td>
</tr>
<tr>
<td>Total anionic phospholipid</td>
<td>346±26</td>
<td>377±27</td>
</tr>
<tr>
<td>Phosphatidylinositol</td>
<td>46.0±3.3</td>
<td>57.4±2.4*</td>
</tr>
<tr>
<td>Phosphatidylinositol 4-phosphate</td>
<td>9.7±0.7</td>
<td>11.2±0.7</td>
</tr>
<tr>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
<td>10.1±0.7</td>
<td>9.5±0.8</td>
</tr>
<tr>
<td>Phosphatidic acid</td>
<td>7.6±0.7</td>
<td>7.0±1.1</td>
</tr>
</tbody>
</table>

Results are expressed as means ± SE; n = 12 in each group. Total and individual phospholipid levels are expressed as nanomoles lipid phosphorus per milligram protein. Individual phospholipids are also expressed as molar percent of total phospholipid content (in parenthesis). * P < 0.05.
by its interaction with the brush-border membrane phosphatidylinositol. In addition, alkaline phosphatase is an intrinsic membrane enzyme, and the activity of the enzyme is modulated by changes in the composition or fluidity of the membrane bilayer (6, 9). In this regard, hypercalcemia caused a significant increase in the brush-border membrane cholesterol to total phospholipid molar ratio. An increase in this ratio is associated with a decrease in membrane fluidity (25). These findings are compatible with results obtained using fluorescence polarization techniques that have shown calcium ion to decrease the fluidity of rat hepatocyte and intestinal plasma membranes (9, 19, 34). Taken together, therefore, hypercalcemia may cause a decrease in alkaline phosphatase activity by a direct effect on the enzyme molecule, by interacting with the brush-border membrane phosphatidylinositol, or by potentially causing a decrease in brush-border membrane fluidity. Leucine aminopeptidase, on the other hand, is an extrinsic membrane enzyme, and as in the present study, calcium-phosphoinositide interactions may have adverse effects on membrane fluidity (19, 34). Therefore, membrane phospholipids, including lysophosphatidylcholine and lysophosphatidylethanolamine, may have adverse effects on membrane function. In the present study we did not detect lysophospholipids including lysophosphatidylcholine and lysophosphatidylethanolamine, but we cannot rule out the presence of increased free fatty acid accumulation in the cortical homogenate or membranes from hypercalcemic rats, since we did not use a TLC solvent system that would separate and identify these lipid breakdown products. The present model of vitamin D-induced hypercalcemia is associated with a fivefold increase in urinary prostaglandin E2 excretion rate (17, 18), which certainly suggests enhanced arachidonic acid release from membrane phospholipids. In fact, in vitro incubation of rat hepatocyte plasma membranes with calcium is associated with a selective decrease in plasma membrane arachidonic acid content, which also results in a decrease in membrane fluidity (19, 34). Therefore, membrane phospholipid hydrolysis and enhanced fatty acid metabolism may be an important cause of the decrease in the membrane phospholipid content and the impairment in membrane enzyme activity in chronic hypercalcemia.

In the present model of vitamin D induced hypercalcemia, the effects of calcium on enzyme activity and lipid composition cannot be attributed to calcium-independent effects of vitamin D, since nonhypercalcemic doses
of vitamin D resulted in no significant alterations in brush-border or basolateral membrane enzyme activity or lipid composition. In addition, in vivo administration of vitamin D has been shown to increase the phosphatidylcholine and the unsaturated fatty acid content of rat intestinal and renal brush-border membranes (11, 22), and in vitro incubation with vitamin D to lower the transition temperature of alkaline phosphatase and to increase the fluidity of rat intestinal brush-border membranes (9), results contrary to those observed and/or suggested in the present study. In addition, it is highly unlikely that the effects of hypercalcemia on enzyme activity and lipid composition are caused by in vitro effects of membrane-bound calcium, since the brush-border and basolateral membranes were isolated in a buffer containing excess amounts of EGTA (~500 nmol EGTA/mg cortical homogenate protein vs. 20 nmol Ca/mg cortical homogenate protein). Indeed, the final membrane fractions did not have any measurable calcium. Furthermore, the final membrane fractions were resuspended in the same buffer containing 5 mM EGTA prior to enzyme and lipid assays.

The renal cortical brush-border and basolateral membrane enzyme activity and lipid compositional alterations caused by hypercalcemia could result in impairment of critical cellular transport processes, altered intracellular ion homeostasis, and thus predispose to renal tubular cell injury. In fact, in a preliminary study, renal prostaglandin secretion inhibition with indomethacin resulted in acute renal failure in hypercalcemic, but not in normocalcemic rats (16). In other preliminary studies, hypercalcemia has been shown to significantly enhance ischemia (8), gentamicin (28), and Bence Jones protein-induced acute renal failure in the rat (33).

The present studies, therefore, demonstrate that hypercalcemia induces significant alterations in renal cortical brush-border and basolateral membrane enzyme activity and lipid composition. The plasma membrane functional and compositional changes occur prior to any demonstrable impairment in mitochondrial function. These plasma membrane biochemical changes could be cellular markers of early cell injury and suggest a role for calcium in causing renal tubular dysfunction and predisposing to renal tubular cell injury.

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