Abstract. Altered divalent cation homeostasis with bone mineral loss, hypercalciuria, and hypomagnesemia have been associated consistently with human diabetes mellitus. This study investigated functional, molecular, and biochemical determinants that accompany this condition in chronically (2 wk) streptozotocin (STZ)-diabetic rats. Catheterized, conscious, diabetic rats on servo-controlled fluid replacement exhibited an increased GFR (+70%) and a substantially raised urinary calcium output (+568%) when compared with control rats. In addition, fractional calcium reabsorption was reduced, indicating that the hypercalciuria was not due solely to an osmotic effect but may involve an actual tubular defect. The expression of proteins involved in renal distal calcium reabsorption and water transport in STZ-diabetic rats were then studied by Western analysis and immunofluorescence microscopy to investigate the molecular basis of the hypercalciuria. Extracellular Ca$^{2+}$-sensing receptor abundance was reduced to 52% of control in STZ-diabetes, whereas thiazide-sensitive NaCl cotransporter expression was increased by 192%. Subcutaneous insulin implant rectified both functional and molecular parameters. The levels of calbindin D$_{28k}$, plasma membrane Ca$^{2+}$-ATPase, and aquaporin 1 in whole kidney and of aquaporin 2 in inner medulla were unchanged in diabetic and/or insulin replacement. Blood levels of 1,25(OH)$_{2}$D$_{3}$ were reduced in diabetes as were levels of osteocalcin, a marker of bone formation. It is concluded that diabetic hypercalciuria in rats involves elevated GFR with raised urinary output, reduced Ca$^{2+}$ reabsorption, and impaired bone deposition. Changes in Ca$^{2+}$-sensing receptor and NaCl cotransporter protein expression could account for the altered divalent cation homeostasis seen during diabetes mellitus.

There is a large amount of evidence showing that early human diabetes mellitus can result in hypercalciuria (1–8) and reduced bone mass (osteopenia) (5,9,10). Delayed insulin treatment only partly rectifies the hypercalciuria, indicating that in early stages of diabetes mellitus some irreversible changes must take place (11). Previous studies from this laboratory have indicated that in the streptozotocin (STZ) model of type 1 diabetes mellitus, renal tubular Ca$^{2+}$ excretion is raised despite the maintenance of normal plasma calcium concentration (12). Urinary Ca$^{2+}$ output was much greater than that of any other electrolyte, suggesting that osmotic diuresis alone could not account for the substantial hypercalciuria (12,13). Thus, because diabetes mellitus can alter both renal calcium handling and bone calcification, it is important to characterize early phases of these changes and establish the renal contribution to the phenomenon.

Classically, rat renal function is studied by collection of 24-h urine samples in metabolic cages. However, an improvement on this method is to cannulate the rats to allow real-time collection of urine from the bladder and to permit infusion of [$^{3}$H]inulin for the accurate measurement of GFR. In this model, fluid balance is maintained during the time period in which urine and blood samples are collected for analysis by replacing fluid lost in the urine with a servo-controlled infusion pump (13,14). In addition, using this model, data can be obtained from conscious rats, with the advantage of preventing the depressive effects of both acute surgery and anesthesia on renal hemodynamics. In the first series of experiments, we therefore studied the effect of chronic (2 wk) diabetes on the renal function in conscious catheterized rats.

To determine the molecular mechanisms that could be affected by the diabetic condition and that could therefore explain the altered mineral ion metabolism during diabetes, we looked at changes in the renal expression of proteins involved in renal calcium and water transport in control versus STZ-diabetic rats and/or in diabetic rats on insulin replacement in the second series of experiments. A central protein in divalent cation homeostasis is the cell surface, calcium/polyvalent cation-sensing receptor (CaR) (15,16). In the distal nephron, the CaR maintains normocalcemia by integrating signals that arise from divalent cation excretion and water preservation (17). Calbindin-D$_{28k}$ is an intracellular Ca$^{2+}$-binding protein that regulates cellular calcium transport in the late distal convoluted tubule (DCT) and connecting tubule (CNT) cells (18). Plasma membrane Ca$^{2+}$-ATPase (PMCA) is found on the basolateral surface of DCT and CNT cells and can actively extrude Ca$^{2+}$.
against a concentration gradient (18). The thiazide-sensitive 
NaCl cotransporter (NCCT) is expressed on the apical mem-
brane of DCT and early connecting segment cells (19,20), and
in the DCT, Na⁺ reabsorption through NCCT is inversely
related to urinary Ca²⁺ excretion (21). Aquaporin 1 (AQP1) is
a constitutively active water channel located in renal proximal
tubules and part of the descending thin limbs. AQP2, the
vasopressin-regulated water channel, is localized to the apical
side of collecting ducts and is specifically inserted into the
apical membrane in response to vasopressin (reviewed in ref-
eration 22). Accordingly, the expression levels of CaR, NCCT,
calbindin-D₂₅₈₉, PMCA, and AQP1 and 2 proteins were inves-
tigated in the development of diabetic hypercalciuria. Finally,
in the same experimental rats, we measured bone formation
(osteocalcin) and resorption (urinary deoxypyridinoline
crosslinks) markers, as well as circulating levels of parathyroid
hormone (PTH) and 1,25-dihydroxy vitamin D₃ (1,25(OH)₂D₃).

Materials and Methods

Materials

[¹H]inulin and ECLplus were purchased from Amersham Interna-
tional Plc. (Little Chalfont, Bucks, UK). All other chemicals were
purchased from Sigma-Aldrich (Poole, Dorset, UK).

Animals

Experiments were performed in accordance with the UK Animals
(Charles River Laboratories, Wilmington, Kent, UK) were individu-
ally housed in wire-bottom cages and were maintained under a 12-h
light photoperiod (lights on at 0800 h) and an environmental tem-
perature of 21 to 23°C (55 ± 10% humidity). Rats had free access to food
(Beekay Rat and Mouse Diet, Bantin & Kingman, Hull, UK) and tap
water throughout the study. Experiments were performed on two
separate series of rats, the first of which was used for the renal
function measurements and the second for the protein expression
and for biochemical determination of 25(OH)D₃, 1,25(OH)₂D₃, PTH, and
bone formation and resorption markers.

Induction of Diabetes Mellitus with STZ

Eight-wk-old Sprague-Dawley rats were rendered diabetic with
STZ (60 mg/kg intraperitoneally in citrate buffer) (13). Control rats
received citrate buffer alone. Diabetes was confirmed by the develop-
ment of glycosuria within 36 h (Uristix; Ames DVN, Miles Ltd.,
Slough, UK) and hyperglycemia (blood glucose concentration > 15
mM). Blood for the latter was obtained from a tail vein (or from the
venous cannula in cannulated rats) and assayed using a blood glucose
analyzer (HemoCue, Sheffield, UK) and tap water throughout the study. Experiments were performed on two
separate series of rats, the first of which was used for the renal
function measurements and the second for the protein expression
and for biochemical determination of 25(OH)D₃, 1,25(OH)₂D₃, PTH, and
bone formation and resorption markers.

In Vivo Measurements of Renal Function

The methodology used for the servo-controlled fluid replacement
system is described extensively elsewhere (13,14). Briefly, sterilized
cannulae were implanted into the femoral artery (for blood sampling
and measuring BP) and vein (for infusion) of rats under anesthesia.
Custom-made titanium bladder catheters (AstraZeneca Pharmaeuti-
cals, Alderley Park, UK) were implanted exteriorized through the
ventral abdominal wall. The rats were then left to regain presurgical
body weight, which took approximately 5 d.

Each rat was placed in a restraining cage and allowed to settle for
1 h before administration of a bolus infusion of 6 μCi of [¹H]inulin.
The mass of voided urine was measured at 5-min intervals, and an
adjustable infusion pump then delivered an infusion of 2.5% dextrose
solution at a rate matching the spontaneous urine output. Preliminary
experiments measuring urinary ion excretion over a 5-h period indi-
cated that the period of the experiment that gave the most stable rates
of ion excretion occurred between 3 and 4.5 h after beginning the
renal clearance measurements (tₚ; data not shown). Thus, results
shown represent the data obtained during the period 3.5 and 4 h after
injection of [¹H]inulin. Blood samples were taken at the midpoint
of the urine collection periods (t = 3, 3.75, and 4.5 h). At the end of the
experiment, the rats were returned to their cages. On the following
day, rats were injected intravenously with or without STZ as described
above, and after 1 wk and then again after 2 wk, the above renal
function protocol was repeated.

Parameters of renal function were quantified as described previ-
ously (12,13). Briefly, GFR was determined by [¹H]inulin clearance,
urinary calcium and magnesium contents were analyzed using an
atomic absorption spectrophotometer, and urinary glucose was deter-
mined using a commercially available kit (Ames Sera-Pak, Bayer
Diagnostics, Basingstoke, UK). Calculations of renal function were
performed as described previously (13,14).

Preparation of Kidney Crude Membranes

After 14 d, the noncatheterized rats were killed and single kidneys
from each were excised and homogenized in buffer containing 12 mM
HEPES (pH 7.6), 300 mM mannitol, pepstatin, leupeptin, and phe-
nylmethylsulfonyl fluoride. To reduce between-group variability, we
processed kidneys in groups of three, one from each experimental
condition (i.e., control, diabetic, and insulin-diabetic rats). The ho-
mogenate was centrifuged at 2500 × g for 15 min, and an aliquot of
this postnuclear supernatant was centrifuged at 100,000 × g for 30
min to give a particulate protein pellet. Samples were normalized for
protein content by assaying according to the method of Bradford (23).
The protein equivalency of the subsequent sample loading volumes
was demonstrated by staining of a sodium dodecyl sulfate-polyacryl-
amide gel electrophoresis (SDS-PAGE) gel with Coomassie blue (not
shown).

Immunoblotting

Immunoblotting was performed as described previously (24) with
specific conditions for each protein as follows. All samples, except for
CaRa, were denatured at 100°C for 5 min in the presence of 143 mM
β-mercaptoethanol before SDS-PAGE. For CaRa, samples were dena-
tured in the absence of reducing agent at room temperature. We
previously showed that CaRa resolved under nonreducing conditions
migrates as a 240- to 310-kD dimeric species (24). Thus, to improve
on the method of quantification of CaRa, we resolved the protein under
nonreducing conditions here so as to have only one band to quantify
as opposed to three. The antisera used included affinity-purified
anti-CaRa (1:800 dilution, raised in rabbit to amino acids 214 to 236 of
the extracellular domain of the rat kidney CaRa [Loftstrand, Inc.,
Bethesda, MD] and affinity purified as described elsewhere (25)),
monoclonal anti-calbindin-D₂₅₈₉ (1:2500 dilution; Sigma-Aldrich),
monoclonal anti-PMCA (1:1500 dilution; Cambridge Bioscience,
Cambridge, UK), affinity-purified polyclonal anti-thiazide-sensitive
NCCT (1:5000 dilution; a gift of Dr. Steven Hebert, Vanderbilt University, Nashville, TN), and affinity-purified polyclonal anti-AQP1 and AQP2 (1:5000 dilution; Chemicon Int., Harrow, UK).

**Immunofluorescence Microscopy**

Assessment of immunoreactivity for both CaR and NCCT proteins in kidneys from normal versus diabetic rats was performed as described previously (25). Briefly, rat kidneys were perfusion fixed with 4% paraformaldehyde and cryoprotected in sucrose solution. Four-μm cryosections were antigen-retrieved using citrate buffer, permeabilized with 1% SDS (for CaR immunostaining only), and stained with affinity-purified anti-CaR or anti-NCCT polyclonal antibodies. As a secondary fluorescence antibody, an anti-rabbit IgG conjugated with Texas Red was used according to the manufacturer’s instructions. Slides were viewed using a Zeiss Axioplan 2 microscope with 10 to 40× objectives. Images were acquired using a Hamamatsu digital camera and processed using the software package KS300 version 3.0 (Carl Zeiss Ltd., Hertfordshire, UK).

**Measurement of Serum 25(OH)D₃, 1,25(OH)₂D₃, PTH, Deoxypyridinoline Cross Links, and Osteocalcin Levels**

Serum levels of 25(OH)D₃, and 1,25(OH)₂D₃ were assayed by in-house methods as previously published (26). Rat serum PTH was assayed using a kit supplied by Nicholls Institute Diagnostics (San Juan Capistrano, CA). Urinary deoxypyridinoline cross links and rat osteocalcin were measured using kits provided by Metra Biosystems Inc. (Mountain View, CA). Serum calcium levels in the same experimental rats were used according to the manufacturer’s instructions. Slides were viewed using a Zeiss Axioplan 2 microscope with 10 to 40× objectives. Images were acquired using a Hamamatsu digital camera and processed using the software package KS300 version 3.0 (Carl Zeiss Ltd., Hertfordshire, UK).

**Statistical Analyses**

Data are presented as means ± SEM, and statistical significance was determined either by multivariate ANOVA test for the urine and plasma in vivo data or by unpaired t test for the semiquantitative immunoblots.

**Results**

Two series of experimental rats were used in the study. In the first series, the rats were catheterized and their renal function was studied in conscious rats under conditions of servo-controlled fluid replacement. In the second series, control, diabetic, and insulin-treated diabetic rats were caged normally for the 14 d before collection of their kidneys for study of their protein distribution and expression. Finally, the same experimental rats were used for the biochemical determination of calcitropic hormones and of bone markers. In both series of experiments, non–insulin-replaced STZ-injected rats exhibited marked hyperglycemia, glycosuria, and attenuated body weight gain (series 2 data shown in Table 1, series 1 data similar but not shown). Upon tissue collection, it was found that the diabetic kidneys weighed significantly more than those from the control or insulin-treated diabetic groups. The data demonstrate that diabetes mellitus was successfully induced in these rats by STZ and was fully corrected by the slow-release insulin implants.

**Measurements of Renal Function**

In the first part of the study, the values for GFR, urine flow rate, fractional fluid, and Ca²⁺ reabsorption and urinary calcium excretion for control, STZ-diabetic, and insulin-treated STZ-diabetic rats were measured (Figures 1 and 2). As expected, at day 0, i.e., before any treatment, all of these parameters were statistically not different from each other for the three experimental groups.

As shown in Figure 1A, GFR was increased by STZ diabetes from 2510 ± 231 μl/min in control rats to 4189 ± 454 μl/min, and this effect was greatly ameliorated by insulin replacement. The urine output of the STZ-diabetic rats was substantially higher than in control rats (Figure 1B). By day 14, the polyuria was still evident but was lower than at day 7 and was again normalized by insulin replacement. Expressed as percentage of fractional fluid reabsorption, water reuptake was reduced in the STZ-diabetic rats after 14 d from 98.3 ± 0.4% in control to 95.1 ± 1.1%.

There was a sixfold increase in urinary calcium output in the STZ-diabetic rats by day 7 that remained stable until day 14 (0.167 ± 0.022 versus 0.025 ± 0.003 μmol/min control; Figure 2). Again, insulin replacement fully normalized the hypercalcuria. The hypercalcuria was associated with significantly reduced fractional calcium reabsorption in STZ-diabetic rats (day 14, 96.9 ± 0.6% versus 99.1 ± 0.2% control). Levels of total plasma Ca²⁺ were unchanged by STZ diabetes (day 14, control 2.06 ± 0.05 mM versus STZ-diabetic 2.0 ± 0.02 mM; N = 5, NS). Similarly, the levels of ultrafiltrable

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**Table 1. In vivo parameters**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Body Wt Day 1 (g)</th>
<th>Body Wt Day 14 (g)</th>
<th>Blood Glucose Day 3 (mM)</th>
<th>Blood Glucose Day 5 (mM)</th>
<th>Blood Glucose Day 14 (mM)</th>
<th>Kidney Wt Day 14 (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>251 ± 6</td>
<td>377 ± 9</td>
<td>–</td>
<td>–</td>
<td>7.7 ± 0.4</td>
<td>1.34 ± 0.05</td>
</tr>
<tr>
<td>Diabetic</td>
<td>247 ± 2</td>
<td>311 ± 5b</td>
<td>34 ± 1.2</td>
<td>–</td>
<td>37.7 ± 1.3b</td>
<td>1.84 ± 0.05b</td>
</tr>
<tr>
<td>Insulin-diabetic</td>
<td>253 ± 4</td>
<td>374 ± 5</td>
<td>34 ± 2.0</td>
<td>6.6 ± 0.9</td>
<td>7.0 ± 1.2</td>
<td>1.46 ± 0.06</td>
</tr>
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</table>

*In vivo* parameters of the animals used in the second series of experiments (i.e., molecular and biochemical observations, data reported in Figures 3 through 7). After injection of streptozotocin on day 1, the onset of diabetes in rats was confirmed on day 3 by the observation of glycosuria (not shown) and hyperglycemia. In half of the diabetic rats, insulin replacement was then begun on day 3; by day 5, these rats exhibited normoglycemia. Similar *in vivo* parameters (not shown) were obtained for the animals used for the functional studies (data reported in Figures 1 and 2). Values are means ± SEM.

* P < 0.001 *versus* control; N = 7 for each experimental condition.
Ca\textsuperscript{2+} in the blood, i.e., free ionized Ca\textsuperscript{2+}, were not significantly altered by STZ diabetes (day 14, control 1.31 ± 0.08 mM versus STZ-diabetic 1.11 ± 0.12 mM; N ≥ 5, NS).

In the servo-controlled, fluid-replaced STZ-diabetic rats, there was a more modest increase in urinary magnesium excretion that failed to reach significance versus day 14 control rats (0.36 ± 0.033 µmol/min STZ-diabetic versus 0.209 ± 0.047 µmol/min control; N ≥ 6, NS). However, the magnesium excretion rate in day 14 STZ-diabetic rats was significantly higher than in day 14 insulin-treated diabetic rats (0.174 ± 0.056 µmol/min; n = 7; P < 0.05). Despite this apparent increase in urinary magnesium excretion, there was no change in fractional magnesium reabsorption (STZ-diabetes, 80.7 ± 1.4% versus 79.3 ± 5.2% control; N ≥ 5, NS). Also, there were no significant changes in STZ diabetes in either total plasma Mg\textsuperscript{2+} levels (0.58 ± 0.02 mM in day 14 control versus 0.63 ± 0.02 in day 14 STZ-diabetic; N ≥ 5, NS) or ultrafilterable Mg\textsuperscript{2+} levels (0.44 ± 0.02 mM in day 14 control versus 0.52 ± 0.05 in day 14 STZ-diabetic; N ≥ 5, NS).

Urinary sodium excretion did not change significantly in the servo-controlled rats during diabetes either in the presence or in the absence of insulin (1.26 ± 0.30 µmol/min control versus 0.58 ± 0.19 µmol/min STZ-diabetic, 1.57 ± 0.50 insulin-treated STZ-diabetic; N ≥ 6, NS). Similarly, percentage of fractional sodium reabsorption did not differ between experimental groups (99.65 ± 0.06% control versus 99.91 ± 0.03% STZ-diabetic, 99.54 ± 0.19% insulin-treated STZ-diabetic; N ≥ 6, NS).

**Figure 1.** Effect of streptozotocin (STZ)-diabetes on GFR, urinary flow rate, and percentage of fractional fluid reabsorption. Histograms show the GFR (A), urinary flow rate (B), and percentage of fractional fluid reabsorption (C) in three experimental groups of rats: untreated control rats (lanes 1 to 3), non–insulin-treated STZ-diabetic rats (lanes 4 to 6), and STZ-diabetic rats treated with insulin (from day 3 onward, lanes 7 to 8). Measurements were made before the STZ treatment (or citrate injection for controls) on day 0 (lanes 1, 4, and 7) and then again 1 wk (lanes 2 and 5) and 2 wk after treatment (lanes 3, 6, and 8). For all parameters measured, no statistical difference was observed at day 0 between each experimental condition. Results are from a minimum of six animals per group. Statistical significance is indicated as follows: *, P < 0.05; **, P < 0.01; ***, P < 0.001.

**Figure 2.** Increased urinary calcium excretion and reduced percentage of fractional calcium reabsorption in STZ-diabetic rats. Histograms show the urinary calcium excretion (A) and percentage of fractional calcium reabsorption (B) in control, diabetic, and insulin-treated diabetic conscious catheterized rats on servo-controlled fluid replacement (as described in Figure 1). Results are from a minimum of six animals per group with statistical significance indicated as for Figure 1.
In contrast, there was a significant reduction in plasma sodium concentration in STZ-diabetic rats but not in insulin-treated diabetic rats (148 ± 4.9 mM control, 131.8 ± 3.7 mM STZ-diabetic, 141 ± 3 mM insulin-treated STZ-diabetic; N ≥ 5; P < 0.05 control versus STZ-diabetic).

Molecular and Biochemical Characterization of Diabetic Hypercalciuria

Expression of the Extracellular CaR. To assess the effect of diabetes on rat renal CaR expression, we used semi-quantitative immunoblotting to measure the whole kidney CaR content. By this method, we determined that the whole kidney content of CaR is reduced by STZ diabetes to 52% of control levels (Figure 3, B and C). In contrast, the mean renal CaR content of the insulin-treated diabetic rats was normal. No signal was detected in rat kidney membranes when the anti-CaR antibody was preabsorbed by the antigenic peptide (Figure 3A).

Previously, we showed that the strongest CaR-specific immunoreactivity is expressed in the proximal tubules, thick ascending limb (TAL), DCT, and the collecting ducts (25). Immunofluorescence microscopy on rat kidney cryosections showed a uniform reduction in CaR staining throughout the kidney section in the control versus diabetic rats (Figure 4). At higher magnification, the reduction in CaR immunostaining in cortical TAL (Figure 4, C and D) seemed to be due to a decrease in both the intensity of the immunoreactivity and the number of cells expressing the receptor. In addition, Figure 4, E and F, show that the reduction did not seem to be region specific, as it could also be observed in proximal tubule, collecting duct, and DCT cells and also in medullary regions (not shown). There was no apparent change in CaR immunoreactivity in control versus insulin-treated diabetic rats, and no signal was detected in slides in which the primary antibody was preabsorbed with the antigenic peptide (not shown).

Expression of Calbindin-D_{28k} and PMCA. The monoclonal anti–calbindin-D_{28k} antibody detected a single protein band (Mr approximately 26 kD) in the postnuclear supernatants of whole kidneys (Figure 5A, upper). The relative abundance of this calbindin-D{sub 28k}–reactive species was the same as control in both non–insulin-treated and insulin-treated STZ-diabetic rats (Figure 5B, left). The anti-PMCA monoclonal antisera detected a broad band of 128 to 155 kD as well as a smaller band of 89 kD (Figure 5A, lower). There was no change in PMCA protein abundance in the particulate fractions of total kidneys from control versus STZ-diabetic rats and STZ-diabetic rats on insulin replacement (Figure 5B, right).

Expression of the Thiazide-Sensitive NCCT. The anti-NCCT antibody detected a broad immunoreactive band of approximate molecular mass 138 to 181 kD (Figure 6) similar to NCCT immunoreactivity previously reported (19). In the STZ-diabetic rats, NCCT levels were significantly raised in all of the rats tested (+192%), and in particular, it was the lower portion of the NCCT band that was most markedly upregulated (Figure 6A and not shown). Figure 6B shows that membrane particulate from kidneys of insulin-treated diabetic rats exhibited levels of NCCT immunoreactivity similar to control.

In agreement with the Western blot data, NCCT immunoreactivity in rat kidney cryosections was enhanced in the DCT of diabetic versus control rats (Figure 6, C and D). No staining was detected in sections in which the primary antibody was omitted, and no difference in NCCT immunoreactivity was observed in kidney cryosections from control versus insulin-treated diabetic rats (not shown).

Expression of AQP1 and AQP2. In preliminary experiments (not shown), there was a great deal of kidney-to-kidney variability between total kidney membrane samples when im-
Figure 4. CaR immunoreactivity in normal and diabetic rats. Indirect immunofluorescence micrographs of 4-μm rat kidney cryosections from cortical regions of control (A, C, and E) and diabetic (B, D, and F) rats stained with an affinity-purified CaR polyclonal antibody (representative of three rats). (A through D) CaR-specific (i.e., peptide-protectable) indirect immunofluorescence: cortical thick ascending limbs (CTAL) exhibit the brightest signal. Low-magnification images exhibit a uniformly reduced CaR immunoreactivity throughout the sections of diabetic (B) compared with control rats (A). In the TAL, the decrease seems to be due to a reduction in both the intensity of immunostaining and the number of cells expressing CaR protein. C, control; D, diabetic. (E and F) The reduction in CaR immunoreactivity in diabetic rats is distributed uniformly throughout the kidney as it can also be seen in proximal tubules (p), collecting ducts (cd), and distal convoluted tubules (dct). Arrow, TAL-DCT junction. The pictures in A and B, C and D, and E and F were taken with the same exposure times. Magnifications: ×85 in A and B; ×170 in C and D; ×340 in E and F.
Figure 5. Calbindin-D$_{28k}$ and plasma membrane Ca$^{2+}$-ATPase (PMCA) expression is unchanged in STZ-diabetic rat kidneys. (A) Immunoblot containing either 50 µg of whole kidney postnuclear supernatant protein per lane (upper) or 100 µg of whole kidney particulate (lower) probed with anti-calbindin and anti-Ca$^{2+}$-ATPase antisera, respectively. (B) Calbindin-D$_{28k}$ (left, n = 7 rats from each experimental condition) and 128 to 155 kD anti-Ca$^{2+}$-ATPase immunoreactivity (right, n = 4), quantified as described in Figure 3 and displayed graphically.

m bunoblotted against anti-AQP2 antisera. Accordingly, when we quantified AQP2 abundance in STZ diabetes, only the inner medulla was studied. Anti-AQP1 and anti-AQP2 antisera both detected protein species of approximate molecular masses 29 (nonglycosylated) and 35 to 45 kD (glycosylated) in total kidney membrane proteins and in kidney inner medulla, respectively (Figure 7A). STZ diabetes did not affect renal AQP1 protein expression in total kidney or AQP2 abundance in inner medulla (Figure 7B).

Measurements of 1,25(OH)$_2$D$_3$ and Osteocalcin Levels in STZ Diabetes. 1,25(OH)$_2$D$_3$ levels were significantly lower in sera from STZ-diabetic rats, whereas 25(OH)D$_3$ levels did not change significantly (Table 2). PTH values were extremely variable and did not reach significant differences between the two experimental groups (221.83 ± 200.98 pg/ml STZ-diabetic, n = 6, versus 216 ± 135.24 pg/ml control, n = 4, NS). Given this variability, we cannot be sure that PTH levels were completely unaffected by the diabetes, because a modest change in PTH levels could be masked by the statistical error. Nevertheless, we saw no evidence of even a qualitative trend suggesting altered PTH secretion in STZ diabetes, although further work would be required to confirm this. As for the in vivo measurements, serum Ca$^{2+}$ levels were not significantly different in diabetic rats (Table 2). The bone formation marker osteocalcin was reduced in STZ diabetes by 53%, whereas levels of the biochemical marker for bone resorption, urinary deoxypyridinoline cross-links, was not significantly altered (Table 2).

Discussion

In humans, hypercalciuria, hypomagnesemia, and osteopenia often accompany type I diabetes mellitus (1–8). To investigate the source of urinary calcium, we used the STZ diabetes model to correlate functional, molecular, and biochemical profiles that accompany the onset of experimental diabetes mellitus. STZ diabetes resulted in a significant hypercalciuria quantified as a sixfold increase in urinary calcium excretion. This hypercalciuria occurred in the presence of normocalcemia as there was no concomitant change in total Ca$^{2+}$ or ultrafilterable ionized Ca$^{2+}$ levels in the blood. This is consistent with previous data from this laboratory (12,13), with the findings of a number of clinical studies (1,27), and in micropuncture studies using anesthetized rats (28–31). Indeed, using micropuncture techniques, Garland et al. (32) showed that urinary calcium output was increased almost fivefold after 2 wk of diabetes. The glomerular hyperfiltration associated with diabetes delivers an increased load of calcium to the tubules, and this may in itself cause an increased rate of excretion of this ion.

However, in the present study, fractional reabsorption of calcium was significantly lower in diabetic than in control rats, suggestive of an altered tubular reabsorption of calcium in diabetes.

When rats were examined under conditions of servo-controlled fluid replacement, it was found that the induction of STZ diabetes increased the glomerular flow rate and decreased fractional fluid reabsorption, producing a polyuria consistent with that seen in early human diabetes. Urinary sodium excretion was not significantly affected during diabetes in either the presence or the absence of insulin. In contrast, there was a significant reduction in plasma sodium concentration in diabetic versus control rats, which was normalized by insulin treatment. These and previous studies (11,12) clearly indicate that urinary sodium output cannot account for the strong hypercalciuria and that the latter is not simply due to a glomerular hyperfiltration, because there was no change in percentage of fractional sodium reabsorption. The most obvious explanation for the hypercalciuria seen in STZ-diabetic rats is that in diabetes, GFR is raised by the glycosuria-mediated osmotic diuresis, increasing the delivery of Ca$^{2+}$ to the tubules. If renal calcium reabsorption is occurring at a close-to-maximum rate, then the diuresis alone could explain the hypercalciuria. However, previous micropuncture experiments from both this and other laboratories suggest that there is a real Ca$^{2+}$ handling defect in diabetes that is not localized to the proximal tubule, where Ca$^{2+}$ reuptake occurs passively, and that rather it seems to be a distal tubular lesion (31,32).

In a previous study, we showed that in 2-wk diabetic rats, urinary calcium output is qualitatively similar to that obtained in the conscious rats on servo-controlled fluid replacement (12). Therefore, in the second series of experiments, we inves-
tigated the renal expression of proteins involved in mineral ion handling by the distal nephron in noninfused rats. Under these conditions, we found that after 2 wk of diabetes, rats exhibited an increase in NCCT protein. This could either represent a compensation mechanism for the additional Na\(^+\) delivery to the distal nephron caused by the elevated GFR or be a direct consequence on the DCT cells of the hyperglycemia/hypoinsulinemia. This upregulation per se could also account for the apparent decrease in Ca\(^{2+}\) reabsorption by the distal tubule (21). It is known that thiazide diuretics promote calcium reabsorption in the DCT in addition to inhibiting NaCl reabsorption through NCCT. In the NCCT gene knockout mouse, one observes very similar urinary sodium levels between the wild-type and homozygote animals, yet the homozygotes excrete 75\% less Ca\(^{2+}\) (33). This is also true in patients with Gitelman’s syndrome, who express mutant NCCT protein resulting in limited salt wasting but marked hypocalciuria (34). This suggests that it is not unusual for a change in NCCT activity to produce a relatively small change in urinary Na\(^+\) excretion yet a substantial alteration in renal Ca\(^{2+}\) excretion. The upregulation in NCCT would also explain the much greater effect on urinary Ca\(^{2+}\) excretion compared with Mg\(^{2+}\) excretion observed in the in vivo experiments. Whereas urinary excretion of magnesium was raised in the STZ-diabetic rats, there was no significant change in its fractional reabsorption, suggesting that the increased Mg\(^{2+}\) excretion is predominantly an osmotic effect. 
Hypomagnesemia has long been known to be associated with diabetes mellitus and has been confirmed in nearly one third of diabetic outpatients (35). Despite the hypermagnesiuria in our study, we did not detect a significant decrease in serum Mg\(^{2+}\) in diabetic rats. This could be due to the duration of diabetes studies here, because hypomagnesemia has been previously reported in STZ rats after a longer period of diabetes (36).

In our study, we identified reduced CaR protein expression in diabetes mellitus (approximately 50%), which can be seen throughout the whole kidney section and does not seem to be region specific. The important question is whether the renal CaR downregulation is a cause or a consequence of the increase in renal divalent cation excretion. Ho et al. (37) previously reported that adult CaR gene knockout mice exhibit increased renal reabsorption of Ca\(^{2+}\), leading to reduced Ca\(^{2+}\) clearance, i.e., hypocalciuria. Because the reduced CaR activity in the knockout model is due to a gene dosage effect (37), the uniform CaR downregulation seen throughout the diabetic kidneys should produce hypocalciuria instead of the observed hypercalciuria. Conversely, a downregulation secondary to hypercalciuria could explain reduced CaR expression in regions where the receptor is present at the luminal aspect, i.e., proximal tubules and collecting ducts (25), but it would not explain the clear reduction in CaR immunoreactivity in the TAL, where the receptor is basolateral. This means either that in the STZ-diabetic rat, other changes such as raised GFR or NCCT upregulation have a much greater effect on renal Ca\(^{2+}\) clearance than renal CaR or that the changes in renal CaR reported here are either secondary to the hypercalciuria or even unrelated to it. The diabetic condition could affect CaR expression in other organs involved in extracellular calcium homeostasis that express the CaR (reviewed in reference 38), i.e., the parathyroid glands, the gastrointestinal tract, and the bone. Schwartz et al. (39) reported a mild reduction in the set point for PTH secretion in patients with insulin-dependent diabetes mellitus, although their PTH levels were comparable to control patients’. Because the parathyroid CaR controls PTH secretion, this study suggests that in diabetics, there might be an altered sensitivity to serum calcium levels by the parathyroid CaR. It would be interesting to test CaR expression levels in parathyroid glands from poorly controlled diabetic versus control patients.

Whatever the mechanism for diabetic hypercalciuria, it does not seem to result from altered calbindin-D\(_{28k}\) expression. A fall in the levels of the intracellular Ca\(^{2+}\)-binding protein theoretically could contribute to reduced Ca\(^{2+}\) reabsorption by impairing transcellular Ca\(^{2+}\) transport, but such a change in expression was not observed. Similarly, there was no change observed in the renal expression of PMCA, indicating that the reduced Ca\(^{2+}\) reabsorption cannot be explained by decreased basolateral active Ca\(^{2+}\) exit. It is perhaps surprising that renal calbindin-D\(_{28k}\) expression is unchanged given the fall in 1,25(OH)\(_2\)D\(_3\) levels. However, in a previous study that re-

**Table 2.** Altered levels of vitamin D and bone formation in STZ diabetes

<table>
<thead>
<tr>
<th></th>
<th>1,25(OH)(_2)D(_3) (pg/ml)</th>
<th>25(OH)D(_3) (ng/ml)</th>
<th>Total Ca(^{2+}) (mM)</th>
<th>Osteocalcin (ng/ml Sera)</th>
<th>Urinary Deoxypyridinoline Cross Links (nM/mM Creatinine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>121.0 ± 65.3</td>
<td>14.2 ± 2.6</td>
<td>2.55 ± 0.09</td>
<td>45.4 ± 13.7</td>
<td>121.0 ± 90.1</td>
</tr>
<tr>
<td>STZ diabetic</td>
<td>27.2 ± 6.6(^c)</td>
<td>10.5 ± 2.0</td>
<td>2.62 ± 0.09</td>
<td>21.4 ± 10.6(^b)</td>
<td>148.5 ± 43.3</td>
</tr>
</tbody>
</table>

\(^a\) Levels of the calcitropic hormones 1,25(OH)\(_2\)D\(_3\), 25(OH)D\(_3\), and calcium and of the biochemical markers for bone resorption (urinary deoxypyridinoline cross links) and bone formation (osteocalcin) were measured as described in the Materials and Methods section. Values are means ± SEM; N = 4 to 6 for each experimental condition.

\(^b\) P < 0.05.

\(^c\) P < 0.0001.
ported a similar observation (40), it was suggested that either the 1,25(OH)₂D₃ levels, although reduced, may still have been above threshold levels for inducing gene expression or that local 1,25(OH)₂D₃ levels were actually higher than the circulating levels measured. It is also possible that renal calbindin-D₂₈k gene expression may be induced by another calcitropic agent, because vitamin D receptor–ablated mice exhibit only modestly reduced renal calbindin-D₂₈k expression (41).

In several experimental conditions, including chronic lithium treatment (42), hypokalemia (43), low-protein diet (44), and chronic dihydrotachysterol treatment (45), a polyuria that can be explained by downregulation of AQP2 in inner medullary collecting ducts is observed. Therefore, we examined whether the polyuria seen with STZ diabetes also involves a reduction in the expression of either AQP1 or AQP2. As we observed no change in the protein levels of AQP1 in the total kidney or AQP2 in the inner medulla, we conclude that diabetes mellitus–induced diuresis does not involve downregulation of these water channels. The AQP2 result is consistent with data previously reported by Klein et al. (46), who showed that in male Sprague-Dawley rats, STZ treatment failed to alter AQP2 levels in the inner medullary tip. In contrast, a recent study that examined STZ-induced diabetes in female Wistar rats reported an increase in AQP2 levels, presumably representing a compensation mechanism to combat their severe diuresis (47). In addition to strain and gender differences between this study and the two former studies, Nejsum et al. (47) measured AQP2 expression in whole kidney rather than in inner medulla alone and thus may have detected AQP2 upregulation in cortical collecting ducts not measured here. Important, though, is that all three studies consistently observed no downregulation of AQP2.

It is still unclear what precisely is the source of the additional calcium excreted in the diabetic urine. Is osteopenia secondary to a diabetes-induced impairment of renal calcium reabsorption, or does the hypercalcemia follow impaired bone metabolism? A recent study showed that chronic furosemide treatment in rats effected a renal hypercalcuria sufficient to reduce bone mineral content and bone mineral density measured after 7 wk (48). Therefore, hypercalcemia of primarily renal origin does seem to be capable of inducing osteopenia in rats. In the last part of the study, we therefore related the observed functional and molecular changes with circulating levels of calcitropic hormones and bone markers. The bone formation marker osteocalcin was significantly reduced during diabetes, whereas the bone resorption marker deoxyypyridinoline was unchanged. Serum levels of 1,25(OH)₂D₃ were significantly reduced during STZ diabetes, whereas levels of 25(OH)D₃ were not, indicating that the fall in 1,25(OH)₂D₃ was not due to a lack of substrate. Together, these data indicate that the reduced bone formation and mineralization could account for the hypercalcemia with normocalcemia and explain, at least in part, the progressive osteopenia seen in diabetic patients. In addition or alternatively, osmotic diuresis and increased dietary calcium and carbohydrate intake secondary to hyperphagia (12) can account for the hypercalcemia. This would also explain the reduction of circulating levels of 1,25(OH)₂D₃ during diabetes observed in the current and previous studies (49). However, control osmotic diuresis with insulin therapy and hyperphagia with paired feeding only partly corrects the hypercalcuria (31), which indicates that changes in 1,25(OH)₂D₃ are specific to the diabetic condition. Further studies using a Ca²⁺-deprived diet will help understand the contribution of the gastrointestinal tract toward diabetic hypercalcemia.

In summary, we demonstrated that hypercalcemia and impaired bone deposition together with renal NCCT upregulation and CaR downregulation are features of experimental diabetes mellitus. It should therefore be possible to test in vivo whether pharmacologic modulators of NCCT and CaR function exert any corrective effect on diabetic hypercalcemia in either an animal model of diabetes or human patients.

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We dedicate this work to the memory of Dr. Hugh Garland (1948 to 1999).

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