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Primary Cultures of Renal Epithelial Cells from X-linked Hypophosphatemic (*Hyp*) Mice Express Defects in Phosphate Transport and Vitamin D Metabolism

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Summary

Mutation in a gene (symbol *Hyp*) on the X chromosome causes hypophosphatemia in the mouse. The murine phenotype is a counterpart of X-linked hypophosphatemia in man. Both exhibit impaired renal reabsorption of phosphate in vivo. In vitro studies in the *Hyp* mouse have shown decreased Na^+-dependent phosphate transport at the brush border membrane and abnormal mitochondrial vitamin D metabolism. To determine whether the mutant renal phenotype is intrinsic to the kidney or dependent upon putative extrinsic humoral factor(s) for its expression, we established primary cultures of renal epithelial cells from normal and *Hyp* male mouse kidneys. The cells are derived from proximal tubule. Initial uptake rates of phosphate and α-methyl-D-glucopyranoside (α-MG), a metabolically inert analogue of D-glucose, were measured simultaneously in confluent monolayers exhibiting epithelial polarity and tight junctions. The mean phosphate/α-MG uptake ratio in *Hyp* cultures was 82% of that in normal cells ($P < 0.01$, $n = 96$). Moreover, the production of 24,25-dihydroxyvitamin D_3 was significantly elevated in confluent cultures of *Hyp* cells relative to normal cells. These results imply that the *Hyp* gene is expressed in situ in renal epithelium and suggest that humoral factors are not necessary for the mutant renal phenotype in X-linked hypophosphatemia of mouse and man.

Introduction

X-linked hypophosphatemia is the most prevalent form of inherited rickets in man (Rasmussen and Anast 1983). Although first described in 1937 (Albright et al. 1937), the exact nature of the primary defect is unknown. Much of our knowledge regarding the pathophysiology of X-linked hypophosphatemia has been derived from studies of an inbred strain of laboratory mouse with hypophosphatemia caused by an X-linked mutant gene (symbol Hyp) (Eicher et al. 1976). The murine phenotype is a counterpart of the human disorder and is characterized by hypophosphatemia, rickets, and a specific defect in Na^+-dependent phosphate transport at the renal brush border membrane (Tenenhouse and Scriver 1978; Tenenhouse et al. 1978). Recent studies have demonstrated that the regulation of renal mitochondrial 25-hydroxyvitamin D_3 (25-OH-D_3) metabolism is also impaired in the X-linked *Hyp* mouse. Mutant mice exhibit abnormal renal synthesis of 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2$ D_3), the vitamin D hormone, in response to phosphate deprivation (Meyer et al. 1980; Lobaugh and Drezner 1983; Yamaoka et al. 1986), PTH infusion (Nesbitt et al. 1986), calcium restriction (Nesbitt et al 1986; Tenenhouse 1984*b*), and vitamin D deficiency (Tenenhouse 1983, 1984*a*). Moreover, the renal synthesis of 24,25-dihydroxyvitamin D_3 (24,25$(OH)_2D_3$) is significantly elevated in *Hyp* mice when compared with normal littermates (Cunningham et al. 1983; Tenenhouse 1983; Tenenhouse and Jones 1987).

Micropuncture studies have localized the phosphate transport defect to the proximal tubule of *Hyp* kidney (Giasson et al. 1977; Cowgill et al. 1979). Because production of 1,25$(OH)_2D_3$ and 24,25$(OH)_2D_3$ has

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also been localized to the proximal tubule of rat kidney (Kawashima et al. 1981), it is likely that the abnormality in 25-OH-D_3 metabolism in the *Hyp* mouse resides in the same region of the nephron shown to express the phosphate transport defect.

The precise relationship between the renal abnormalities in brush border membrane phosphate transport and mitochondrial vitamin D metabolism is still unclear. It has not been established whether either of the above defects is the primary consequence of the mutant gene, or whether the renal phenotype is secondary to a mutation involving an intrinsic or extrinsic regulatory factor, capable of modulating both vitamin D metabolism and phosphate transport. The purpose of the present study was to determine whether the mutant renal phenotype is expressed in primary cultures of renal epithelial cells derived from *Hyp* mice. We have developed methods to culture cells, derived from proximal tubule fragments from mouse kidney cortex, in a hormonally defined serum-free medium (Bell et al. 1985, 1986, and in press; Bell 1986). The epithelial monolayers exhibit polarity, domes, tight junctions, and several differentiated functions of the proximal tubule. We report here that cultures derived from *Hyp* mouse kidney express abnormal phosphate transport and vitamin D metabolism when compared with normal cultures. The results support the hypothesis that the *Hyp* gene is expressed in kidney.

Material and Methods

Mice

Normal (+/Y) and mutant (*Hyp*/Y) C57Bl/6 male mice, 21 to 30 days old, were obtained from Jackson Laboratory (Bar Harbor, ME) and R. A. Meyer, Marquette University (Milwaukee) or were bred in our laboratory. The initial breeding pairs, *Hyp*/+ heterozygous females and +/Y normal males, were obtained from the Jackson Laboratory and R. A. Meyer. The breeders were maintained on Wayne Breeder-Blox diet (Allied Mills, Inc., Chicago) containing 1.2% (wt/wt) calcium, 1.1% phosphate, and 4.5 IU vitamin D_3/g. After weaning, animals were sexed, separated, and placed on Wayne Lab-Blox diet containing 1.2% (wt/wt) calcium, 0.93% phosphate, and 4.4 IU vitamin D_3/g. Additional normal weanling C57Bl/6 males were obtained from Charles River Canada, Inc., St. Constant, Quebec. Serum phosphorus was measured routinely with kits from Pierce (Rockford, IL). Normal female (+/+) and mutant heterozygous female (*Hyp*/+) mice were used in some experiments. *Hyp*/+ females, like *Hyp*/Y males, exhibit a 50% decrease in renal brush border membrane phosphate transport (Tenenhouse and Scriver 1979*a*).

Material

Tissue culture media and fetal bovine serum (FBS) were purchased from Flow Laboratories, Mississauga, Ontario. Hormones and transferrin, for culture medium, and rotenone were obtained from Sigma Chemical Co., St. Louis. Collagenase (from *Clostridium histolyticum* grade II; 0.3 Wunsch U/mg) was purchased from Boehringer Mannheim, Dorval, Quebec, and arginine vasopressin from Parke-Davis, Scarborough, Ontario. Falcon 35-mm tissue culture dishes were purchased from Becton Dickinson, Mississauga, Ontario. Carrier-free $H_2[^{32}P]O_4$, α-[^{14}C]-methyl-D-glucopyranoside (275 mCi/mmol), [^{3}H]-D-glucose, [^{3}H]-25-OH-D_3, [^{3}H]-1,25$(OH)_2D_3$, and [^{3}H]-24,25$(OH)_2D_3$ were purchased from New England Nuclear, Boston. 25-OH-D_3 was a gift of Upjohn Co., Kalamazoo, and 1,25$(OH)_2D_3$ and 24,25$(OH)_2D_3$ were gifts of Hoffmann-LaRoche, Inc., Nutley, NJ. All other materials were obtained from Fisher Scientific, Montreal, or Sigma Chemical Co., St. Louis.

Tubule Preparation and Initiation of Primary Cultures

Tubule fragments were prepared from kidney cortex slices of normal and *Hyp* mice as described previously (Bell et al., in press). Renal cortical slices were digested at 22 C or 37 C in buffer containing collagenase (1%) and bovine albumin (0.5%) for 45 min. Tubule fragments were obtained by serial density sedimentation (Goldman et al. 1982). For initiation of primary cultures, tubules were washed in medium RK-1 (basal medium supplemented with insulin, transferrin, and hydrocortisone [Taub and Livingston 1981; Chung et al. 1982]) containing 10% FBS and were inoculated into 35-mm Falcon tissue culture dishes. The primary cultures were maintained in a humidified 5% CO_2/95% air incubator at 37 C. Twenty-four hours after plating, the medium was removed and replaced with serum-free medium RK-1. Culture medium was changed routinely every 2 days. The presence of proximal tubule cells in the primary cultures was confirmed by (1) alkaline phosphatase activity, (2) PTH-stimulated cAMP synthesis, (3) 24,25$(OH)_2D_3$ production, (4) phlorizin-inhibitable Na^+-dependent transport of α-methyl-D-glucopyranoside (α-MG), an inert glucose analogue, and (5) Na^+-dependent phosphate transport with an appar-

ent K_m similar to that obtained in renal brush border membrane vesicles (Bell et al. 1985, 1986, and in press).

Transport Studies

Brush border membrane vesicles.—Brush border membrane vesicles (BBMVs) were prepared by the method of Booth and Kenny (1974), as modified by Tenenhouse and Scriver (1978), from homogenates of kidney cortex (control BBMVs) or from tubule fragments prepared by incubation of kidney slices with collagenase for 45 min at 37 C (tubule-derived BBMVs). Fifteen-second initial rate uptakes of 0.01 mM [^{3}H]-D-glucose and 0.1 mM ^{32}P were measured simultaneously at 22 C in the presence of NaCl or KCl by a Millipore filtration method as described previously (Tenenhouse and Scriver 1978).

Tubule fragments.—Tubule fragments prepared as described above were resuspended in buffer containing (in mM) 5.4 KCl, 2.8 $CaCl_2$, 1.2 $MgSO_4$, 14 Tris HCl, 2.5 glutamine, 0.5 β-hydroxybutyrate, and 137 NaCl (pH 7.5), to yield a concentration of approximately 90 mg tissue/ml. For Na^+-free assays, NaCl was replaced by choline chloride. The initial rates of phosphate and α-MG uptake were measured using a centrifugal technique (Kimmich 1975) to separate cells from radioactive medium after 5 min incubation at 37 C. Uptake was initiated by the addition of 500 µl of tissue suspension (≈5 to 10 mg cell protein) to 1 ml of buffer containing ^{32}P or [^{14}C]α-MG, to yield a final concentration of 0.1 mM phosphate and 0.5 mM α-MG, each at a specific activity of 0.5 µCi/ml. To terminate uptake, 150-µl aliquots of the cell suspension were removed and diluted with 1.35 ml of ice-cold buffer. The samples were centrifuged for 30 s in a rapidly accelerating Eppendorf centrifuge at 4 C. The supernatants were discarded and pellets (≈0.5 to 1.0 mg protein) were washed with an additional 1.35 ml ice-cold buffer and dissolved in 1 ml of 0.2 N NaOH. A 500-µl aliquot was neutralized and counted. The remainder was used for protein determination (Lowry et al. 1951). All results were corrected for zero time uptake, defined as the radioactivity measured with immediate termination of incubation after addition of tissue at 4 C.

Uptakes of phosphate and α-MG were also measured in the presence of the metabolic inhibitor rotenone. Tubules were preincubated in a Na^+-free incubation buffer containing 20 µM rotenone for 20 min at 37 C. Rotenone was added to the buffer as an ethanolic solution. Identical amounts of ethanol were added to control buffers. Uptake at 37 C was initiated by the addition of rotenone-treated or control tubules to uptake buffer containing final concentrations of 0.1 mM ^{32}P or 0.5 mM [^{14}C]α-MG and 20 µM rotenone. The centrifugation technique, as described above, was used to separate cells from buffer. The final pellet was extracted with 1 ml 10% trichloroacetic acid (TCA) (wt/vol). The extracts were centrifuged at 4 C in an Eppendorf centrifuge. An aliquot of the supernatant was counted to determine total TCA-soluble phosphate (Pt) and α-MG uptake. An aliquot of the supernatant was extracted by the method of Vestergaard-Bogind (1964) to determine inorganic phosphate (Pi) by measuring the ^{32}P content in the isobutanol/benzene phase. Organic phosphate (Po) was then calculated by subtracting Pi from Pt. The TCA-insoluble fraction was washed two times with 10% TCA and dissolved in 1 ml 0.2 N NaOH for protein determination. The efficiency of the extraction procedure was monitored by measuring the recovery of [γ-^{32}P]-ATP and ^{32}P.

Cultured cells.—The initial rates of phosphate and α-MG uptake were measured in confluent kidney epithelial cell cultures exhibiting domes, usually at day 5 or 6 of culture. Unless stated otherwise, the uptakes of phosphate (0.1 mM) and α-MG (0.5 mM) were measured simultaneously in the same culture dish. Interaction between phosphate and glucose during net uptake was shown not to be significant (data not shown). To measure uptake, the medium was removed from the monolayer and the cells were washed with transport buffer containing (in mM) 137 NaCl, 5.4 KCl, 2.8 $CaCl_2$, 1.2 $MgSO_4$, and 14 Tris HCl (pH 7.5). Transport was initiated by adding 1 ml of the medium containing the appropriate concentration of ^{32}P and [^{14}C] α-MG, each at a specific activity of 0.5 µCi/ml. After 5 min incubation at 37 C or 22 C, uptake was terminated by aspirating the radioactive medium and washing the dish three times with ice-cold stop solution (100 mM NaCl, 1 mM Tris/HCl, 1 mM Na^+-arsenate, pH 7.5). The cells were then solubilized overnight in 1 ml of 0.2 N NaOH. An aliquot was removed for radioactive counting. For dual label experiments a ^{32}P/^{14}C double-label quench curve was used. The remainder of each sample was used for protein determination. All results were corrected for the values observed at zero time, defined as the radioactivity measured with immediate termination of incubation after addition of incubation medium. To measure transport in the absence of Na^+, NaCl in the transport buffer was replaced by choline chloride. The Na^+-dependent component of uptake was determined by subtraction of the diffusional

component, measured in the presence of choline chloride, from uptake in the presence of NaCl.

Uptakes of phosphate and α-MG were also measured in the cultured cells in the presence of the metabolic inhibitor rotenone. Cultures were preincubated in buffer containing choline chloride, 20 μM rotenone, 2.5 mM glutamine, and 0.5 mM β-hydroxybutyrate for 30 min at 37 C. Rotenone was added to the buffer as an ethanolic solution. Identical amounts of ethanol were added to control buffers. The final ethanol content was less than 0.1%. Uptakes of 0.1 mM phosphate and 0.1 mM [^{14}C]α-MG were measured simultaneously in the presence of rotenone, in a NaCl or choline chloride buffer for 1 or 5 min at 37 C or 22 C. The TCA extracts of washed cells were used to determine the distribution of phosphate as described above.

Metabolism of 25-Hydroxyvitamin D_3 (25-OH-D_3)

The metabolism of 25-OH-D_3 by primary cultures was examined as described by Trechsel et al. (1979). One milliliter Medium RK-1 was added to confluent monolayers in 35-mm culture dishes. The reaction was initiated by adding 50 pmoles ^{3}H-25-OH-D_3 (specific activity ≈2,500 cpm/pmol) in 10 μl 99% ethanol. After appropriate times of incubation at 37 C, the reaction was stopped by the addition of 1.25 ml methanol. The cells were scraped, and the cell suspension and medium were transferred to a glass vial. The dish was rinsed with 1.25 ml methanol; 1.25 ml chloroform was then added to the methanol washes. Samples were stored at −20 C under nitrogen until extraction. Boiled monolayers, incubated with substrate as described above, were used as background in the quantitation of product formation. The vitamin D_3 metabolites were extracted with methanol-chloroform (2:1) as described previously (Bligh and Dyer 1959). The organic phases were pooled and evaporated to dryness under a stream of nitrogen; 85% to 90% of the radioactivity was recovered in the lipid extract. The residue was reconstituted in hexane:isopropanol:methanol (92:7:1), and an aliquot was injected into a Zorbax Sil column (25 cm × 6.2 mm; Dupont Co., Wilmington, DE) equilibrated with hexane:isopropanol:methanol (92:7:1) as described by Jones (1980). Fractions were collected and counted. Elution positions of 25-OH-D_3, 24,25 $(OH)_2D_3$, and 1,25$(OH)_2D_3$ were determined with radioactive standards.

Statistical Analyses

Data are expressed as the mean ± SEM or SD. In figures where error bars are used, the absence of a bar indicates that the measured error lies within the size of the symbol.

The Wilcoxon signed rank test was used to determine whether or not a significant difference existed between the *Hyp*/Y and +/Y cultures in table 1. The Student *t*-test was used to determine statistical significance in all other experiments.

Results

Transport of Phosphate and α-Methyl-D-Glucopyranoside BBMVs

At the outset it was necessary to establish whether proximal tubule fragments prepared from *Hyp* mice, used to initiate the primary cultures of renal epithelial cells, expressed the phosphate transport defect at the brush border membrane as previously reported (Tenenhouse and Scriver 1978; Tenenhouse et al. 1978). Accordingly, the effect of genotype on the initial rates of phosphate and glucose uptake in tubule-derived BBMVs

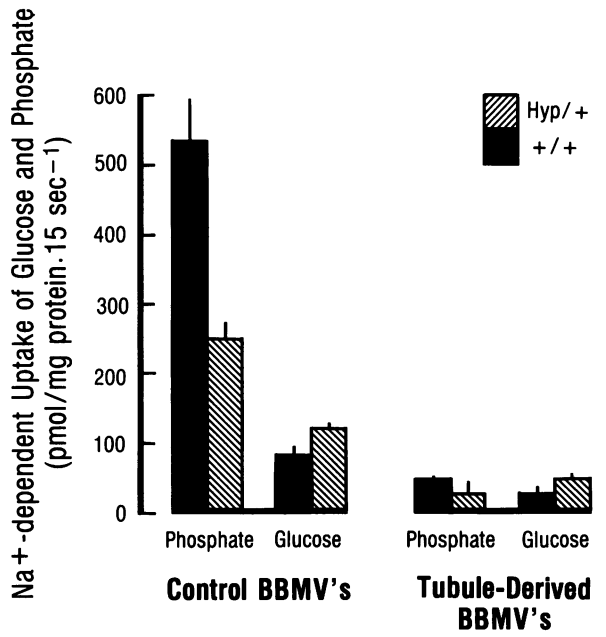

Figure 1 Na^+-dependent phosphate and D-glucose transport in BBMVs from normal +/+ and *Hyp*/+ (female) mice. Uptakes of 0.1 mM ^{32}P and 0.01 mM [^{3}H]-D-glucose were measured simultaneously at 15 s at 22 C in BBMVs prepared from homogenates of kidney cortex (control) and in BBMVs derived from tubule fragments prepared by incubation of kidney slices with collagenase for 45 min at 37 C (tubule-derived BBMVs). The Na^+-dependent uptake component was obtained by subtracting uptake in KCl from total uptake in NaCl. Results are expressed as mean ± SD of six determinations.

and in BBMVs prepared from homogenates of kidney cortex (control BBMVs) were compared. As shown in figure 1, tubule-derived BBMVs from both genotypes exhibit significantly less Na^+-dependent phosphate and D-glucose transport when compared with control BBMVs. Phosphate transport was decreased by 90%, while glucose transport was reduced by 40%, suggesting that the phosphate transporter is more sensitive to the procedure used for tubule preparaton, which includes a 45-min collagenase digestion at 37 C.

Figure 1 shows also that in spite of compromised transport in tubule-derived BBMVs, the effect of the *Hyp* mutation is still apparent, i.e., Na^+-dependent phosphate transport is one-half of normal in both tubule-derived and control BBMVs prepared from *Hyp* mice. Na^+-independent uptake is not impaired in *Hyp* BBMVs (data not shown). However, the magnitude of the *Hyp* genotype difference is much less apparent in the tubule-derived BBMVs. In contrast to phosphate transport, renal brush border membrane Na^+-dependent glucose transport is not significantly reduced in *Hyp* mice, in either mode of vesicle preparation.

Tubule Fragments

In view of compromised brush border membrane phosphate and glucose transport in brush border membranes derived from proximal tubule fragments prepared by collagenase treatment at 37 C (fig. 1), the temperature of collagenase digestion was lowered to 22 C. Tubule fragments prepared at 22 C exhibited a 225% increase in Na^+-dependent phosphate transport and a 35% decrease in the transport of α-MG, (a nonmetabolizable analogue of D-glucose) when compared with fragments prepared at 37 C (n = 8; data not shown). In all subsequent experiments, proximal tubule fragments were prepared at 22 C to maximize phosphate transport activity.

To examine the effect of the *Hyp* mutation on phosphate uptake in tubule fragments, experiments were performed in rotenone-treated tubules to minimize the influence of phosphate metabolism on net uptake. Figure 2 shows the effect of rotenone on the distribution of ^{32}P-labeled intracellular phosphate in tubule fragments from normal mice after a 5-min uptake. Preincubation with rotenone decreases the relative proportion of uptake into the organic phosphate (Po) pool; virtually all the phosphate taken up by the tubules is present as inorganic phsophate (Pi). It should be noted, however, that total TCA-soluble phosphate uptake is reduced by rotenone treatment (legend to fig. 2).

Figure 3 depicts the effect of the *Hyp* mutation on

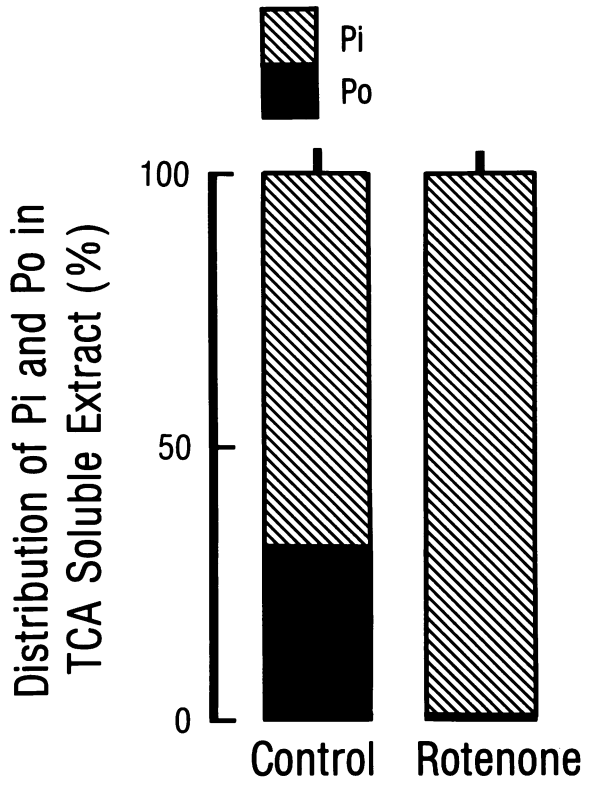

Figure 2 Effect of rotenone on ^{32}P metabolism by renal tubules from normal mice. Tubules prepared by collagenase digestion for 45 min at 22 C were preincubated in Na^+-free buffer containing 20 μM rotenone for 20 min at 37 C. Five-minute uptake at 37 C was performed in buffer containing NaCl, 0.1 mM ^{32}P, and 20 μM rotenone. Total phosphate (Pt) was determined by measuring the radioactivity in an aliquot of the TCA–soluble fraction. Inorganic phosphate (Pi) was measured in the isobutanol/benzene phase obtained following extraction of an aliquot of Pt in the presence of ammonium molybdate. Organic phosphate (Po) was calculated by subtraction of Pi from Pt. Na^+-dependent uptake values of phosphate (Pt) in control and rotenone-treated tubules are 0.61 ± 0.002 and 0.21 ± 0.02 nmol · mg protein^{-1} · 5 min^{-1}, respectively, and represent 100%. Results are expressed as mean ± SEM of two separate experiments, each performed in triplicate ($P < .001$).

5-min uptakes of phosphate and α-MG in rotenone-treated tubules. Tubules isolated from *Hyp*/Y mice exhibit a significant reduction in Na^+-dependent phosphate transport when compared with +/Y tubules ($P < .001$). Na^+-dependent uptake of α-MG is not different in tubules prepared from the two genotypes, confirming that the observed transport defect is specific for phosphate and is not related to any putative difference in cellular metabolism of phosphate in the two genotypes.

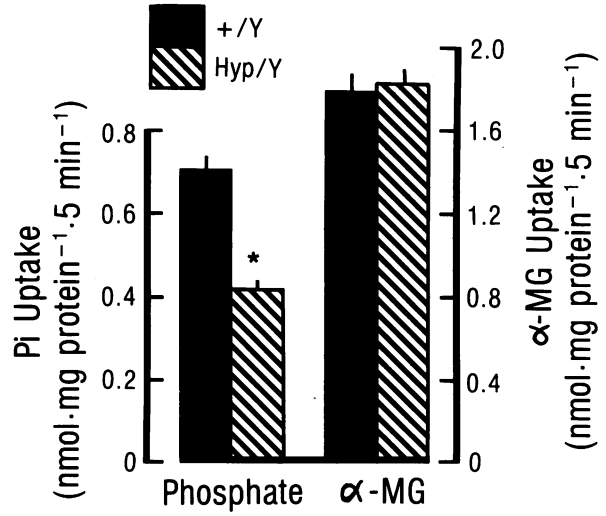

Figure 3 Na^+-dependent phosphate and α-MG uptake in tubules isolated from +/Y and *Hyp*/Y (male) mice. Tubule fragments were prepared by collagenase (1 mg/ml) digestion for 45 min at 22 C. Tubules were then incubated with 20 μM rotenone for 20 min at 37 C, and 5-min uptakes of 0.1 mM ^{32}P and 0.5 mM [^{14}C] α-MG were measured separately at 37 C. Results are expressed as mean ± SEM of 12 determinations measured in three individual experiments (*$P < .001$).

Cultured Cells

We then measured phosphate uptake by rotenone-treated primary cultures of renal epithelial cells. Figure 4 demonstrates that rotenone not only inhibits total phosphate uptake but also markedly reduces the proportion of ^{32}P entering the organic phosphate pool (18% vs. 82%). Phosphate transport was also examined at 22 C, in the presence and absence of rotenone. Figure 4 shows that although lowering the temperature from 37 C to 22 C decreased phosphate uptake in cultured cells, the relative distribution of ^{32}P in the organic and inorganic phosphate pools was not altered by temperature, either in the presence or absence of rotenone.

Phosphate and α-MG uptake was then examined in paired primary cultures derived from tubule fragments of normal and *Hyp* mice. Figure 5 depicts the results of four separate preparations of cultured renal epithelial cells from each genotype. Variation in phosphate and α-MG uptake is apparent between preparations. To correct for this variation we normalized phosphate uptake to α-MG uptake (phosphate/α-MG uptake ratio). In all four preparations the uptake ratio was less in cultured renal cells derived from *Hyp* mice, compared with normal cells.

Table 1 summarizes 23 experiments (4–5 replicates/experiment), comparing phosphate/α-MG uptake ratios for paired normal and *Hyp* cultures. The uptake measurements were performed under various incubation conditions, for example, in the presence and absence of rotenone and at different temperatures and times of incubation; variation in conditions explains the fivefold difference in uptake ratio values. In the aggregate, there was a significant difference in the uptake ratio value between the two genotypes ($P < .01$, $n = 96$; Wilcoxon signed rank test). The *Hyp* cultures had a lower phosphate/α-MG uptake ratio, about 82% of normal cultures. The deficiency of phosphate transport activity in *Hyp* cultures is smaller in magnitude than in isolated BBMVs (fig. 1) and in isolated tubule fragments (fig. 3) derived from *Hyp* mice.

Metabolism of 25-OH-D_3

We have previously demonstrated that confluent monolayers of mouse kidney epithelial cultures con-

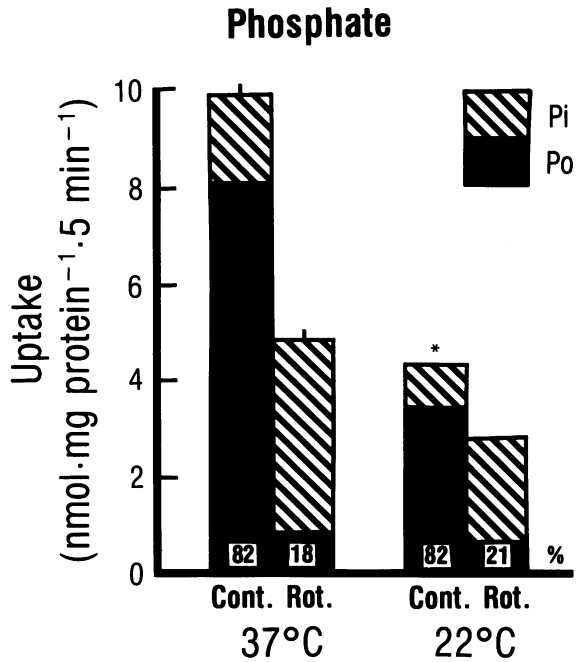

Figure 4 Effect of rotenone on ^{32}P metabolism by cultured cells. Cultures were preincubated in Na^+-free buffer containing 20 μM rotenone for 30 min. The uptake of 0.1 mM ^{32}P was performed for 5 min at 22 C or 37 C in a NaCl uptake buffer containing 20 μM rotenone. Total phosphate (Pt) was determined by measuring the radioactivity in an aliquot of the TCA-soluble fraction; inorganic phosphate (Pi) was measured in the isobutanol/benzene phase obtained following extraction of Pt in the presence of ammonium molybdate. Organic phosphate (Po) was calculated by subtraction of Pi from Pt. Results are expressed in nmol · mg protein^{-1} · 5 min^{-1} and represent the mean ± SEM of quadruplicate measurements (*$P < .001$).

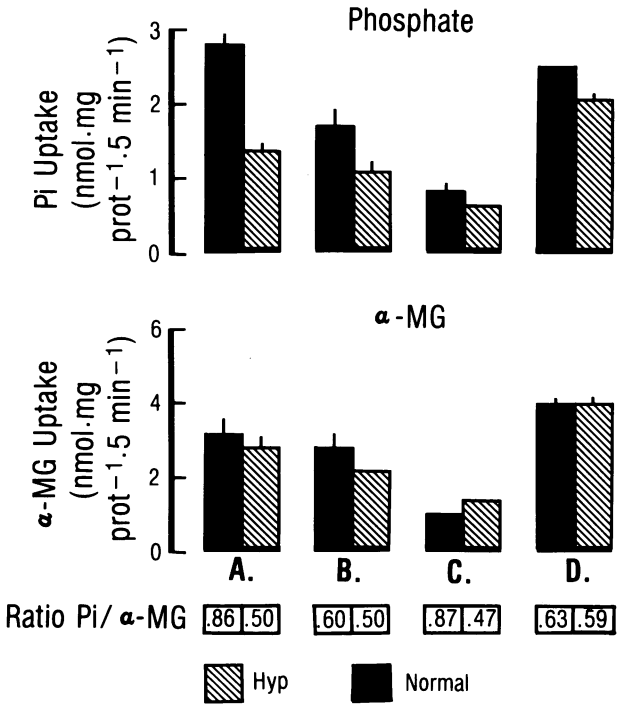

Figure 5 Phosphate and α-MG uptake by rotenone-treated primary cultures from normal and *Hyp* male mouse kidneys. Cultures were preincubated in Na^+-, phosphate-, and α-MG-free buffer containing glutamine and β-hydroxybutyrate for 30 min, followed by 30-min incubation in the same buffer containing 20 μM rotenone. Five-minute uptakes of 0.1 mM ^{32}P and 0.5 mM [^{14}C] α-MG were measured separately in a NaCl or choline chloride uptake buffer containing 20 μM rotenone at 37 C. Results are expressed in nmol · mg protein^{-1} · 5 min^{-1} and represent the mean ± SEM of quadruplicate measurements. Each data set (A–D) represents a different preparation of paired normal and *Hyp* primary cultures.

vert 25-OH-D_3 to 24,25$(OH)_2D_3$ and 1,25$(OH)_2D_3$ (Bell et al. 1985, 1986, and in press). Here we compare the production of 24,25$(OH)_2D_3$ in primary renal epithelial cultures established from *Hyp* and normal mice. Cultured cells from *Hyp* mice produce more 24,25-$(OH)_2D_3$ (5.97 ± 0.17 pmol · mg protein^{-1} · min^{-1}) than normal cultures (4.05 ± 0.27 pmol · mg protein^{-1} · min^{-1}) (n = 5; $P < .01$).

Discussion

The *Hyp* mouse is a counterpart of human X-linked hypophosphatemia (Eicher et al. 1976). Biochemical studies have demonstrated a specific phosphate transport defect at the brush border membrane (Tenenhouse and Scriver 1978; Tenenhouse et al. 1978) and an abnormality in the regulation of mitochondrial 25-OH-D_3 metabolism in *Hyp* kidney (Meyer et al. 1980; Cunningham et al. 1983; Lobaugh and Drezner 1983; Tenenhouse 1983, 1984*a*, 1984*b*; Nesbitt et al. 1986; Yamaoka et al. 1986; Tenenhouse and Jones 1987). What remains unclear is whether these renal defects are intrinsic to the kidney or whether their expression is dependent upon circulating or humoral factors. To address this question, primary epithelial cell cultures initiated from renal proximal tubular fragments prepared from normal and *Hyp* mice were used to examine phosphate transport and 25-OH-D_3 metabolism after 5–6 days in culture in a hormonally defined, serum-free medium. The present demonstration of decreased phosphate/α-MG uptake ratio and increased production of 24,25$(OH)_2D_3$ in *Hyp* cultures provides evidence for intrinsic renal defects in phosphate transport and vitamin D metabolism in X-linked hypophosphatemia.

The proximal tubule fragments used to initiate primary cultures were prepared by collagenase digestion, a procedure previously reported to damage rat renal brush border membranes (Vinay et al. 1981) and blunt PTH-stimulated cAMP production in mouse tubules (Brunette et al. 1979). By lowering the temperature of the collagenase digestion from 37 C to 22 C, we were able to increase phosphate transport activity in isolated proximal tubules and observe a larger genotype difference in phosphate uptake. It is of further interest that the glucose transporter, in the brush border membrane of proximal tubules, is less sensitive to collagenase digestion.

The measurement of solute transport in preparations of metabolically active cells is complicated by metabolic "runout" of solute, a process which may act as a driving force for solute uptake. To inhibit the incorporation of intracellular phosphate into organic phosphates, transport studies were performed in tubules and cultured cells which had been treated with the metabolic inhibitor rotenone as described previously (Carter-Su and Kimmich 1979). Rotenone-treated cells behave like giant vesicles and are amenable to transport studies.

To correct for the variability between preparations of primary cultures, we made measurements in parallel cultures of normal and *Hyp* cells, prepared at the same time. Furthermore, we normalized the phosphate uptake values to those for uptake of α-MG, an inert analogue of D-glucose, the transport of which is not modified by the *Hyp* mutation in either BBMVs (Tenenhouse and Scriver 1978; Tenenhouse et al. 1978; and fig. 1) or in proximal tubule fragments (fig. 3). We found

Table 1

Phosphate/α-MG Uptake Ratio in Paired Primary Cultures from +/Y and *Hyp*/Y Mouse Kidney

Experimental Conditions[a]	Normal	*Hyp*	*Hyp*/Normal
Rotenone (20 min), 37 C, 5-min uptake	.86	.50	.58
	.60	.50	.83
	.99	.47	.47
	1.70	.89	.52
	.63	.59	.94
	1.40	1.30	.93
	1.70	1.36	.80
SITS[b] (20 min), 37 C, 5-min uptake	2.40	2.10	.88
SITS + Rotenone (20 min), 37 C, 5-min uptake	1.50	1.60	1.07
37 C, 5-min uptake	1.80	1.90	1.06
37 C, 1-min uptake	2.40	2.00	.83
	1.40	1.40	1.00
22 C, 5-min uptake	4.30	3.30	.77
	3.50	2.90	.83
	1.85	1.65	.89
	1.42	1.50	1.06
1 mM phosphate,[c] 22 C, 5-min uptake	4.00	3.20	.80
	4.30	3.90	.91
	4.00	3.00	.75
2 mM phosphate, 22 C, 5-min uptake	3.20	2.30	.72
	6.50	4.10	.63
	3.20	2.60	.81
LiCl,[d] preincubation 22 C, 5-min uptake	.66	.49	.74
Mean ± SEM			.82 ± .04 ($P < .01$)[e]

[a] Uptakes of phosphate and α-MG were measured in paired primary cultures from normal and *Hyp* kidney. The values are means of four to five measurements (intraexperimental).

[b] SITS (4-acetomido-4′-isothiocyanostilbene-2,2′-disulfonate), an inhibitor of anion transport at the basolateral membrane, was used as described (Cole et al. 1984).

[c] Phosphate concentration refers to growth medium and not to uptake medium.

[d] Cultures were preincubated at 37 C for 30 min in transport buffer in which 120 mM NaCl was replaced by 120 mM LiCl. The values are means of four to five measurements (intraexperimental).

[e] *Hyp* values, related to their paired normal values, are significantly lower ($P < .01$; $n = 96$; Wilcoxon signed rank test).

that renal epithelial cultures from *Hyp* mice have 82% normal phosphate/α-MG uptake ratio. Because the genotype difference observed in primary cultures is less than the 50% decrement seen in purified BBMVs (Tenenhouse and Scriver 1978; Tenenhouse et al. 1978; and fig. 1) and proximal tubule fragments (fig. 3) from *Hyp* mice, the discrepancy must be explained. There are several possible explanations.

1. Measurement of brush border membrane phosphate transport in whole cells is complicated not only by cellular metabolism (see above) but also by transport across a variety of other membrane systems, such as the basolateral membrane and intracellular membranes. It is known that the *Hyp* mutation does not impair basolateral membrane phosphate transport (Tenenhouse et al. 1978) and probably has no direct effect on mitochondrial and microsomal phosphate transport. Accordingly, normal phosphate transport across these membranes may dilute the genotype effect. However, because the transport defect was fully expressed in intact proximal tubule cells, it is unlikely that this argument can account for the smaller genotype effect in cultured cells.

2. Cultured cells may not express the phenotype of the tubules from which they were derived owing to their inability to differentiate fully in their new environment. Dedifferentiation in culture may also occur, resulting in attenuation of the mutant transport phenotype.

3. Nephron heterogeneity in the preparation may be another explanation for our observations in the cul-

tured cells. The phosphate transport defect in the *Hyp* mouse has been localized to the proximal tubule, a heterogeneous structure composed of three functionally and morphologically distinct regions: the early and late proximal convoluted tubule, and the pars recta. Although the primary cultures appear to be enriched in proximal tubule cells, one cannot exclude that the presence of proximal tubule cells or of distal tubule cells, which do not express the mutation, may obscure the phosphate transport defect in the cultured cells derived from the *Hyp* mouse.

It is noteworthy that the defect in 25-OH-D_3 metabolism was more readily observed in *Hyp* primary cultures. This finding can be ascribed to the fact that, in contrast to phosphate transport, renal 25-OH-D_3 metabolism has been localized exclusively to the proximal tubule (Kawashima et al. 1981). Therefore, contamination by cells from other segments of the nephron would not "dilute" the expression of the mutant phenotype.

4. Our studies do not rule out the possibility that, in contrast to cultured cells, freshly prepared BBMVs and proximal tubule fragments are still under the influence of humoral or circulating factors which may modulate phosphate transport in vivo and therefore may contribute to the expression of the phosphate transport defect in vitro. Although expression of the defect in renal phosphate reabsorption in *Hyp* mice is apparently independent of PTH (Cowgill et al. 1979; Kiebzak et al. 1981), the somewhat elevated plasma PTH levels in *Hyp* mice (Kiebzak et al. 1982; Posillico et al. 1985) may further impair renal phosphate transport in situ and in the freshly prepared vesicles and tubules. Other extrinsic phosphaturic agents, not yet identified, may also play a role in the expression of the renal phenotype. It is well known that in patients with oncogenic hypophosphatemic osteomalacia, phosphaturic agents elaborated by tumor tissue are responsible for renal phosphate wasting and that renal handling of phosphate can be restored to normal after tumor excision (Agus 1983).

The present demonstration of abnormal phosphate transport and 24,25$(OH)_2D_3$ production in cultured cells derived from proximal tubules of *Hyp* mice supports the notion that the mutant renal phenotype is intrinsic to the kidney and independent of circulating or humoral factors for expression. Two previous reports are consistent with our findings, although, in those studies, the cultures contained cells derived from both proximal and distal tubule segments. Fukase et al. (1984) reported that the regulation of 1,25$(OH)_2D_3$ synthesis by calcium and calcitonin was impaired in renal epithelial cultures derived from *Hyp* mice. In the present study, we did not examine the effect of mutation on 1,25$(OH)_2D_3$ production in cultured cells because vitamin D hormone synthesis by renal cells from vitamin D–replete animals is very low and at the level of assay sensitivity (Tenenhouse 1983). However, we did demonstrate increased 24,25$(OH)_2D_3$ production in cultured *Hyp* cells, as had been previously reported for isolated renal mitochondria of *Hyp* mice (Cunningham et al. 1983; Tenenhouse 1983; Tenenhouse and Jones 1987). 24,25$(OH)_2D_3$ is the predominant metabolite synthesized by kidneys of vitamin D–replete mice (Tenenhouse 1983). More recently, Kinoshita et al. (1987) showed decreased phosphate accumulation in primary *Hyp* cultures deprived of phosphate, again supporting the notion of an intrinsic renal defect. It is of interest that, in contrast to cultured cells, BBMVs derived from *Hyp* mice exhibit a twofold increase in Na^+-dependent phosphate transport in response to phosphate deprivation (Tenenhouse and Scriver 1979*a*, 1979*b*). The present study appears to be the first to have measured phosphate transport and vitamin D metabolism together in the same renal culture system.

In summary, we have demonstrated that monolayers of renal proximal tubule epithelial cells from *Hyp* mice exhibit abnormal phosphate uptake and 24,25$(OH)_2D_3$ production after 5–6 days in culture. These results provide evidence for an intrinsic renal defect in *Hyp* mice and suggest that humoral factors are not required for the expression of the mutant renal phenotype in X-linked hypophosphatemia of mouse and man.

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