

Evidence for a Role of Prolactin in Calcium Homeostasis: Regulation of Intestinal Transient Receptor Potential Vanilloid Type 6, Intestinal Calcium Absorption, and the 25-Hydroxyvitamin D₃ 1 α Hydroxylase Gene by Prolactin

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Increased calcium transport has been observed in vitamin D-deficient pregnant and lactating rats, indicating that another factor besides 1,25-Dihydroxyvitamin D₃ (1,25(OH)₂D₃) is involved in intestinal calcium transport. To investigate prolactin as a hormone involved in calcium homeostasis, vitamin D-deficient male mice were injected with 1,25(OH)₂D₃, prolactin, or prolactin + 1,25(OH)₂D₃. Prolactin alone (1 μ g/g body weight 48, 24, and 4 h before termination) significantly induced duodenal transient receptor potential vanilloid type 6 (TRPV6) mRNA (4-fold) but caused no change in calbindin-D_{9k}. Combined treatment with 1,25(OH)₂D₃ and prolactin resulted in an enhancement of the 1,25(OH)₂D₃ induction of duodenal TRPV6 mRNA, calbindin-D_{9k} mRNA, and an induction of duodenal calcium transport [$P < 0.05$ compared with 1,25(OH)₂D₃ alone]. Because lactation is associated with an increase in circulating 1,25(OH)₂D₃, experiments were done to determine whether prolactin also has a direct effect on induction of 25-hydroxyvitamin D₃ 1 α hydroxylase [1 α (OH)ase]. Using AOK B-50 cells cotransfected with the prolactin receptor and the mouse 1 α (OH)ase promoter -1651/+22 cooperative effects between prolactin and signal transducer and activator of transcription 5 were observed in the regulation of 1 α (OH)ase. In addition, in prolactin receptor transfected AOK B-50 cells, prolactin treatment (400 ng/ml) and signal transducer and activator of transcription 5 significantly induced 1 α (OH)ase protein as determined by Western blot analysis. Thus, prolactin, by multiple mechanisms, including regulation of vitamin D metabolism, induction of TRPV6 mRNA, and cooperation with 1,25(OH)₂D₃ in induction of intestinal calcium transport genes and intestinal calcium transport, can act as an important modulator of vitamin D-regulated calcium homeostasis. (*Endocrinology* 151: 2974–2984, 2010)

Calcium, an essential ion in all organisms, is an indispensable constituent of diverse physiological processes (1). To serve these multiple functions it is imperative that the body maintain a tight regulation of calcium homeostasis. Maintenance of calcium homeostasis involves

coordinated interplay between intestine, bone, and kidney (2). The intestine is the sole source of new calcium, supplying dietary calcium to the body. 1,25-Dihydroxyvitamin D₃ [1,25(OH)₂D₃] is the principal hormone regulating intestinal calcium absorption (2–4). 1,25(OH)₂D₃, the

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Abbreviations: bw, Body weight; C/EBP β , CCAAT enhancer-binding protein- β ; DN, dominant negative; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; h, human; JAK2, Janus kinase 2; 1 α (OH)ase, 25-hydroxyvitamin D₃ 1 α hydroxylase; 1,25(OH)₂D₃, 1,25-Dihydroxyvitamin D₃; PTHrP, PTH related peptide; STAT, signal transducer and activator of transcription; TRPV6, transient receptor potential vanilloid type 6; VDR, vitamin D receptor.

biologically active form of vitamin D, is produced by two sequential hydroxylations of vitamin D: first, in the liver by vitamin D 25-hydroxylase; and then, in the kidney by 25-hydroxyvitamin D₃ 1 α hydroxylase [1 α (OH)ase]. The actions of 1,25(OH)₂D₃ are mediated by the vitamin D receptor (VDR), which acts as a heterodimer with the retinoid X receptor, binds to vitamin D-response elements within the promoter of target genes, and together with coactivators, affects gene transcription (2–4). Active intestinal calcium transport is a transcellular process involving three 1,25(OH)₂D₃-regulated steps: apical entry of calcium via the apical calcium channel, transient receptor potential vanilloid type 6 (TRPV6), cytoplasmic translocation of calcium through the interior of the enterocyte (it has been suggested that the calcium-binding protein, calbindin-D_{9k}, acts to facilitate calcium diffusion), and basolateral extrusion of calcium by the intestinal plasma membrane calcium pump plasma membrane Ca²⁺-ATPase 1b (2–6). As the body's demand for calcium increases from pregnancy, lactation, and/or from a diet low in calcium, the synthesis of 1,25(OH)₂D₃ is increased, stimulating active intestinal calcium absorption (2, 6).

Although 1,25(OH)₂D₃ is the principal hormone regulating intestinal calcium absorption, increased intestinal calcium transport has been observed in vitamin D-deficient pregnant and lactating rats, suggesting that hormonal factors besides 1,25(OH)₂D₃ are involved in intestinal calcium absorption (7–9). Studies by Van Cromphaut *et al.* (10) showed that pregnancy and lactation enhance TRPV6 expression in the duodenum equally in wild-type and VDR null mutant mice, further suggesting vitamin D-independent effects during pregnancy and lactation on duodenal calcium absorption mechanisms. It has been suggested that prolactin, a lactogenic polypeptide hormone of the anterior pituitary that is elevated during pregnancy and lactation, acts as a calcium regulating hormone (11). Besides mammary gland, prolactin receptors or binding sites are widely distributed in vertebrates and include binding sites in duodenum, jejunum, ileum, osteoblasts, and kidney cortex (12). The prolactin receptor most commonly signals through the Janus kinase 2 (JAK2)/signal transducer and activator of transcription (STAT)5 pathway (13, 14). Prolactin has been shown to stimulate active intestinal calcium transport in vitamin D-deficient rats (15). In addition, a direct effect of prolactin on duodenal transcellular calcium transport was reported in studies using the Ussing chamber technique and prolactin applied to the incubation solution (16). Prolactin has also been reported to increase paracellular calcium permeability and passive calcium transport in the intestine (17, 18). In addition to direct effects on the in-

testine, an effect of prolactin on stimulating the production of 1,25(OH)₂D₃ has been suggested. Lactation is associated with an increase in circulating 1,25(OH)₂D₃ (7, 19, 20). Bromocriptine, which inhibits pituitary prolactin secretion, was reported to significantly reduce plasma 1,25(OH)₂D₃ levels in lactating animals and prolactin administered with bromocriptine resulted in a restoration of the 1,25(OH)₂D₃ plasma levels (21). Thus, it has been suggested that prolactin can stimulate 1,25(OH)₂D₃ production, which would lead to enhanced intestinal calcium absorption. Although it has been shown that prolactin can affect intestinal calcium transport, mechanisms whereby prolactin modulates vitamin D-mediated calcium homeostasis are largely unknown. In this study, we used vitamin D-deficient mice injected with prolactin alone or prolactin and 1,25(OH)₂D₃ to determine whether prolactin may up-regulate intestinal vitamin D target genes and whether this regulation is dependent or independent of 1,25(OH)₂D₃. In addition, because lactation is associated with an increase in circulating 1,25(OH)₂D₃, experiments were done to determine whether prolactin has a direct effect on induction of 1 α (OH)ase. Our findings indicate that prolactin can regulate TRPV6 and the 1 α (OH)ase gene and that prolactin has cooperative effects with 1,25(OH)₂D₃ in regulating intestinal calcium transport proteins, as well as intestinal calcium transport. Thus, prolactin, by different mechanisms, can act as a calcium regulating hormone and modulate the effect of 1,25(OH)₂D₃.

Materials and Methods

Materials

⁴⁵Ca (39.49 mCi/ml), γ [³²P] deoxy-ATP [3000 Ci (111 TBq)/mmol] was purchased from PerkinElmer Life Sciences (Waltham, MA). Random Primers DNA Labeling kit was purchased from Life Technologies, Inc. (Gaithersburg, MD). Prestained protein molecular weight markers and electrochemiluminescent detection system were obtained from Invitrogen (Carlsbad, CA) and Denville Scientific (Metuchen, NJ), respectively. T4 polynucleotide kinase for labeling double-stranded oligonucleotides for EMSA was purchased from Invitrogen. Ovine prolactin was obtained from Sigma (St. Louis, MO). Human prolactin was obtained from A. F. Parlow (The National Hormone and Peptide Program, Harbor-University of California, Los Angeles, Medical Center, Torrance, CA). Antiserum against rat calbindin-D_{9k} was obtained from Swant Swiss Antibodies (Bellinzona, Switzerland). STAT5 antibody (9363) was purchased from Cell Signaling Technologies (Danvers, MA). β -Actin antisera and secondary antibodies against mouse and rabbit antisera were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The 1 α (OH)ase antiserum was provided by Harvey J. Armbrecht (St. Louis Veterans Affairs Hospital, St. Louis, MO). Chemically synthesized 1,25(OH)₂D₃ was a gift from M. Uskokovic of Hoffmann-La Roche (Nutley, NJ). All other chemicals were pur-

chased from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified.

Animals

C57BL/6J mice (Taconic Farms, Germantown, NY) were exposed to a 12-h light, 12-h dark cycle. Food and water were given *ad libitum*. All of the animal experiments were approved by the University of Medicine and Dentistry of New Jersey Animal Care and Use Committee. For prolactin and vitamin D administration studies, 60-d-old male mice were fed a 0.8% strontium, 0.02% calcium, vitamin D-deficient diet (Teklad diet TD00562) for 7 d to inhibit endogenous renal synthesis of $1,25(\text{OH})_2\text{D}_3$ (22). Male mice were used to avoid alterations due to fluctuations of female hormones. The serum calcium levels of the strontium fed mice before injection with vehicle or hormone/s were less than 7.5 mg/dl. In one experiment the mice fed the strontium vitamin D-deficient diet were randomly separated into three groups and injected ip with either 0.1 ml vehicle (9:1 mix of propylene glycol and ethanol; $-D$ mice), $1,25(\text{OH})_2\text{D}_3$ [1 ng/g body weight (bw) in a 9:1 mix of propylene glycol and ethanol] or prolactin (1 $\mu\text{g/g}$ bw in saline) three times over the next 48 h (48, 24, and 4 h before being terminated). The three dose protocol was used to study both short-term and long-term effects of $1,25(\text{OH})_2\text{D}_3$ or prolactin. In another study, vitamin D-deficient mice were randomly separated into four groups and were injected with either vehicle, $1,25(\text{OH})_2\text{D}_3$ (ip 2 ng/g bw), or prolactin (1 $\mu\text{g/g}$ bw at 12 or 4 h before termination, respectively) or $1,25(\text{OH})_2\text{D}_3$ + prolactin (at 12 and 4 h before termination, respectively). For $1,25(\text{OH})_2\text{D}_3$, 12 h was chosen because suboptimal induction of intestinal TRPV6 and calbindin- D_{9k} mRNA results after one injection and termination after 12 h (23). For prolactin, 4 h was chosen because previous studies noted changes in gene expression within 4 h of *in vivo* prolactin treatment (24). Tissues were harvested, and RNA and protein were isolated as previously described (23, 25). Duodenum of mice under the same protocol but with 6-h treatment with prolactin before termination were also used for the determination of intestinal calcium transport using the everted gut sac assay. A longer time for prolactin treatment was used in these studies because previous studies related to the effect of prolactin on intestinal calcium absorption indicated that between 4 and 8 h after prolactin injection intestinal calcium transport is maximal (15). An additional group of mice injected with $1,25(\text{OH})_2\text{D}_3$ (1 ng/g bw at 48, 24, and 6 h before being terminated) was included in the intestinal calcium transport studies as a positive control.

Intestinal calcium transport

Intestinal calcium transport was determined by the everted gut sac assay using a 5-cm segment of duodenum removed from the mice proximally to the pyloric junction as previously described (25). The active accumulation of ^{45}Ca in the inside (serosal) fluid was expressed as a ratio of the final concentration of ^{45}Ca inside/outside (external incubation medium). The fold stimulation of intestinal calcium transport observed in these studies in $1,25(\text{OH})_2\text{D}_3$ -treated mice is consistent with previous studies (25). The everted gut sac assay selectively measures active intestinal calcium transport [the calcium transport is against a concentration gradient, is saturable, is sensitive to intestinal segment, dietary calcium, and $1,25(\text{OH})_2\text{D}_3$ treatment (25, 26–28)].

Northern blot analysis

Total RNA was prepared with RNazol RNA extraction solution according to the manufacturer's instructions (Tel-Test, Friendswood, TX), and 20 μg of total RNA were analyzed by Northern blot analysis for *calbindin-D_{9k}* gene expression as previously described (23). All membranes were stripped and rehybridized to [^{32}P] β -actin cDNA. The relative OD obtained using the test probe was divided by the relative OD obtained after probing with β -actin to normalize for sample variation.

RT-PCR analysis

Two micrograms of total RNA, prepared as described above, were reverse transcribed using Superscript II Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol. TRPV6 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were assessed by semiquantitative RT-PCR as previously described (25). For each primer set, PCR cycle numbers were chosen so that the amplification was in the linear range of amplification efficiency. Primers and annealing temperatures are the following: TRPV6, forward, 5'-ATCGATGGCCCTGC-GAACT-3' and reverse, 5'-CAGAGTAGAGGCCATCTTGT-TGCTG-3', $T_a = 60$ C; GAPDH, forward, 5'-TCACCATCT-TCCAGGAGCG-3' and reverse, 5'-CTGCTTCACCACCTT-CTTGA-3', $T_a = 60$ C.

Cell culture

Fetal bovine serum (FBS) and charcoal-stripped FBS were from Gemini Biological Products (Calabasas, CA). Cell culture media, 0.25% trypsin-EDTA and penicillin, streptomycin, and neomycin mixture were purchased from Invitrogen. Green monkey kidney cell lines (COS-1 and COS-7) and the human colon adenocarcinoma cell line (Caco-2) were obtained from the American Type Culture Collection (Manassas, VA). AOK-B50 cells, porcine renal proximal tubular cells [LLCPK1 cells that express PTH/PTHrP type I receptors] were obtained from F. R. Bringhurst (29). MPCT cells (mouse proximal tubule cells) were provided by Peter Friedman (30). Cells were cultured in DMEM supplemented with 10% heat-inactivated FBS and 1% penicillin, streptomycin, and neomycin antibiotic mixture. Caco-2 cells were further supplemented with 1% nonessential amino acids (Invitrogen). All cells were grown in a humidified incubator with an atmosphere of 95% air-5% CO_2 at 37 C. For treatments, cells were grown to desired confluency, and their medium was changed to DMEM supplemented with 2% charcoal-dextran-treated FBS. Treatments with vehicle or test compounds were done for the durations and with concentrations mentioned in *Results* and figure legends.

Plasmids, transfections, and assay of luciferase activity

For transfection studies, the human TRPV6 promoter luciferase reporter constructs were from the J. W. Pike lab (31). The mouse $1\alpha(\text{OH})\text{ase}$ promoter $-1651/+22$ placed upstream of a luciferase reporter gene in the pGL2b vector and the deletion constructs $-477/+22$, $-233/+22$, $-144/+22$, $-85/+22$, as well as constructs containing various lengths of the distal $1\alpha(\text{OH})\text{ase}$ promoter region [$-1631/-1064$ (SR2) and $-1631/-1386$ (BDC5')] were kindly provided by H. F. DeLuca (University of Wisconsin at Madison) (32). $-2300/+490$ β -casein promoter subcloned into pGL2 luciferase vector used as a pos-

itive control in some studies was from J. Rosen (Baylor College of Medicine, Houston, TX) (33). The glucocorticoid receptor expression vector, used in studies related to the casein promoter, was provided by Keith Yamamoto (University of California, San Francisco, San Francisco, CA). Prolactin receptor expression vector (pECE-PRL-R-L, which binds ovine and human lactogenic hormones) was from the lab of Paul Kelly (Institut National Santé Recherche Médicale, Paris, France). Expression vectors for STAT5a and STAT5b (pRcCMVSTAT5a and pcDNA3STAT5b) were from J. Rosen (Baylor College of Medicine). pMEX-CCAAT/enhancer-binding protein- β (C/EBP β) was a gift of Simon Williams (Texas Tech University, Lubbock, TX). STAT1 expression vector was provided by James Darnell (Rockefeller University, New York, NY). Dominant negative (DN) STAT5a (STAT5a truncated at amino acid 713 STAT5a DN) was from Jim Ihle. Cells were seeded in a 24-well culture dish 24 h before transfection at 70–80% confluence. Cells were transfected using Lipofectamine 2000 (Invitrogen) and treated as described in *Results*. Time-course studies with prolactin and STAT5 for activity of $1\alpha(\text{OH})\text{ase}$ transcription indicated a 5-fold induction in transcriptional activity using the $-1651/+22$ promoter construct at 4 h after prolactin treatment (400 ng/ml) and a peak at 12 h. Studies were done at 24 h (suboptimal conditions) consistent with previous studies examining transcriptional effects of prolactin (33, 34). After treatment, cells were harvested, and dual luciferase assay was performed as previously described (35).

Site-directed mutagenesis and EMSA

Putative STAT5-binding sites were mutated using the QuikChange site-directed mutagenesis kit (Stratagene, La

Jolla, CA). The mutated constructs were confirmed by DNA sequencing. For EMSAs, complementary oligonucleotides were synthesized based on the region of the mouse $1\alpha(\text{OH})\text{ase}$ promoter containing the putative wild-type or the mutated STAT5 sites (prepared by the University of Medicine and Dentistry of New Jersey Molecular Resource Facility). The complementary oligonucleotides were annealed, end-radiolabeled, and purified as described before and were used for the EMSA (35, 36).

Western blot analysis

Total protein were prepared and analyzed for protein concentration by the Bradford method (37), and 50 μg of protein from total cell or tissue extracts was loaded onto a 15% sodium dodecyl sulfate-polyacrylamide gel, separated by electrophoresis, and transferred onto a polyvinylidene fluoride membrane (Bio-Rad Laboratories, Inc., Hercules, CA). Western blot analysis of calbindin- $\text{D}_{9\text{k}}$ in intestine and $1\alpha(\text{OH})\text{ase}$ in AOK-B50 cells was performed as previously described (23, 38). All membranes were stripped and reprobed with β -actin antibody.

Serum calcium

Serum concentrations of calcium were determined using a standard calcium chloride solution (Fisher Scientific, Fair Lawn, NJ) by atomic absorption spectrophotometry (39).

Statistical analysis

Results are expressed as means \pm SE, and significance was determined by analysis by Student's *t* test for two-group comparison or by ANOVA for multiple-group comparison.

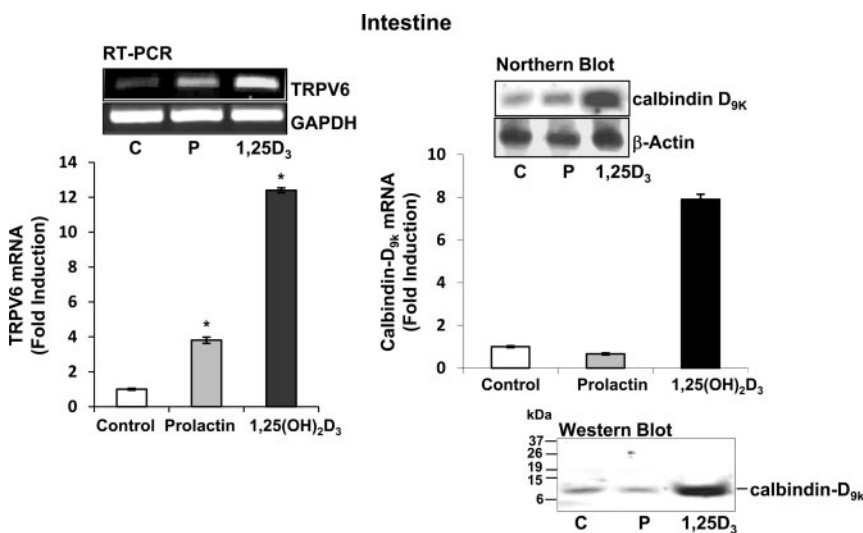


FIG. 1. The effect of repeated administration of prolactin or $1,25(\text{OH})_2\text{D}_3$ on duodenal TRPV6 and calbindin- $\text{D}_{9\text{k}}$ mRNA levels. Sixty-day-old mice were made $1,25(\text{OH})_2\text{D}_3$ deplete by feeding 0.8% strontium diet for 7 d. Mice were injected with prolactin [1 $\mu\text{g}/\text{g}$ bw, vehicle (control), or $1,25(\text{OH})_2\text{D}_3$ (1 ng/g bw as a positive control)] three times over 48 h (48, 24, and 4 h before termination). Serum calcium levels: control $-D$ mice, 6.3 ± 0.04 ; prolactin-treated mice, 7.1 ± 0.1 ; and $1,25(\text{OH})_2\text{D}_3$ -treated mice, 8.2 ± 1.5 mg/dl [$P < 0.05$ prolactin and $1,25(\text{OH})_2\text{D}_3$ -treated mice vs. control $-D$]. *Left panel*, RT-PCR analysis of TRPV6 mRNA in duodenum. *Right panel*, Summary of densitometric scans of Northern blot analyses of calbindin- $\text{D}_{9\text{k}}$ mRNA in duodenum. Data were corrected for GAPDH or β -actin mRNA expression and are presented relative to control (vitamin D-depleted mice). *Bottom right*, Western blot analysis of calbindin- $\text{D}_{9\text{k}}$ protein C, Control; P, prolactin; $1,25\text{D}_3$, $1,25(\text{OH})_2\text{D}_3$. Bars represent the mean \pm SEM, $n = 6$ –8/group. *, $P < 0.05$ compared with control.

Results

Prolactin induces duodenal TRPV6 mRNA

To investigate possible mechanisms involved in prolactin as a hormone involved in calcium homeostasis, vitamin D-deficient male mice were injected with vehicle, $1,25(\text{OH})_2\text{D}_3$ (as a positive control), or prolactin at 48, 24, and 4 h before termination to study both short-term and long-term effects of $1,25(\text{OH})_2\text{D}_3$ or prolactin administration. Three injections of $1,25(\text{OH})_2\text{D}_3$ significantly induced duodenal TRPV6 mRNA and protein as previously reported (23, 25). Three injections of prolactin significantly induced duodenal TRPV6 mRNA (4-fold; $P < 0.05$ compared with control) (Fig. 1, *left panel*). Using the same protocol, duodenal calbindin- $\text{D}_{9\text{k}}$ mRNA and protein levels were unaltered by prolactin (Fig. 1, *right panel*).

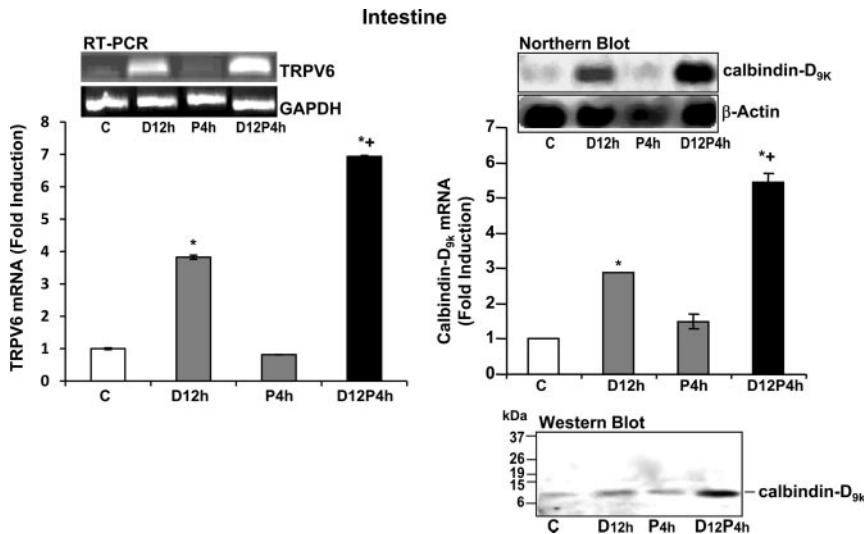


FIG. 2. Prolactin (P) has cooperative effects with $1,25(\text{OH})_2\text{D}_3$ (D) in regulating duodenal calcium transport genes. RT-PCR analysis of TRPV6 mRNA expression (*left panel*) or Northern blot analysis of calbindin- $\text{D}_{9\text{k}}$ mRNA (*right panel*) in the duodenum of vitamin D-deficient male mice injected with either vehicle (C, control), $1,25(\text{OH})_2\text{D}_3$ (2 ng/g bw D12 h), or prolactin (1 $\mu\text{g/g}$ bw P4 h) at 12 or 4 h before termination, respectively, or $1,25(\text{OH})_2\text{D}_3$ + prolactin (D12, P4 h) at 12 and 4 h before termination, respectively. *Bottom right*, Western blot analysis of calbindin- $\text{D}_{9\text{k}}$ protein. Bars represent the mean \pm SEM, $n = 4\text{--}6/\text{group}$. *, $P < 0.05$ compared with control; +, $P < 0.05$ compared with D12 h, P4 h vs. C. $P > 0.5$; a single injection of prolactin and termination at 2, 6, or 12 h after prolactin injection also did not result in a significant effect on TRPV6 mRNA or calbindin- $\text{D}_{9\text{k}}$ mRNA (data not shown).

Prolactin has cooperative effects with $1,25(\text{OH})_2\text{D}_3$ in regulating duodenal calcium transport genes and duodenal calcium transport

To determine whether there is a possible cooperative effect between $1,25(\text{OH})_2\text{D}_3$ and prolactin, vitamin D-deficient male mice were injected with $1,25(\text{OH})_2\text{D}_3$ or prolactin (12 or 4 h before termination, respectively) or $1,25(\text{OH})_2\text{D}_3$ + prolactin (at 12 and 4 h before termination). A single injection of prolactin alone was unable to induce TRPV6 mRNA or calbindin- $\text{D}_{9\text{k}}$ mRNA and protein (Fig. 2). $1,25(\text{OH})_2\text{D}_3$ given as a single injection significantly induced duodenal TRPV6 mRNA and duodenal calbindin- $\text{D}_{9\text{k}}$ mRNA and protein. Combined treatment with $1,25(\text{OH})_2\text{D}_3$ and prolactin resulted in a significant enhancement of the $1,25(\text{OH})_2\text{D}_3$ induction of duodenal TRPV6 mRNA and calbindin- $\text{D}_{9\text{k}}$ mRNA and protein [$P < 0.05$ compared with $1,25(\text{OH})_2\text{D}_3$ treatment alone] (Fig. 2). To evaluate the physiologic relevance of the enhanced gene and protein expression using combined treatment with $1,25(\text{OH})_2\text{D}_3$ and prolactin, everted gut sac assays were performed. As a positive control, three injections of $1,25(\text{OH})_2\text{D}_3$ administered to vitamin D-deficient mice resulted in a significant 2.3-fold induction in active duodenal calcium transport, as previously reported by our laboratory (25). Within the limits of sensitivity of our assay no change in active duodenal calcium transport was observed between vehicle injected vitamin D-deficient mice and vitamin D-deficient mice receiving a sin-

gle injection of either prolactin or $1,25(\text{OH})_2\text{D}_3$ (Fig. 3). Combined treatment with $1,25(\text{OH})_2\text{D}_3$ and prolactin (at 12 and 6 h before termination, respectively) resulted in a significant induction in active duodenal calcium transport [$P < 0.05$ compared with vehicle or treatment with a single injection of $1,25(\text{OH})_2\text{D}_3$ alone or prolactin alone Fig. 3].

Lack of transcriptional activation of the $-7\text{ kb}/+160$ TRPV6 promoter construct by prolactin

To further investigate the nature of prolactin regulation of TRPV6, we examined the effects of prolactin on the activity of the human (h)TRPV6 $-7000/+160$ promoter construct. The prolactin receptor and the hTRPV6 $-7\text{ kb}/+160$ promoter construct were transiently co-transfected in Caco-2 cells. Caco-2 cells, human colon adenocarcinoma cells, were used because they are the only intestinal cell line that differentiates in culture

and acquires the characteristics of intestinal enterocytes (40). They also have a functional VDR, and TRPV6 is regulated by $1,25(\text{OH})_2\text{D}_3$ in these cells (31, 41).

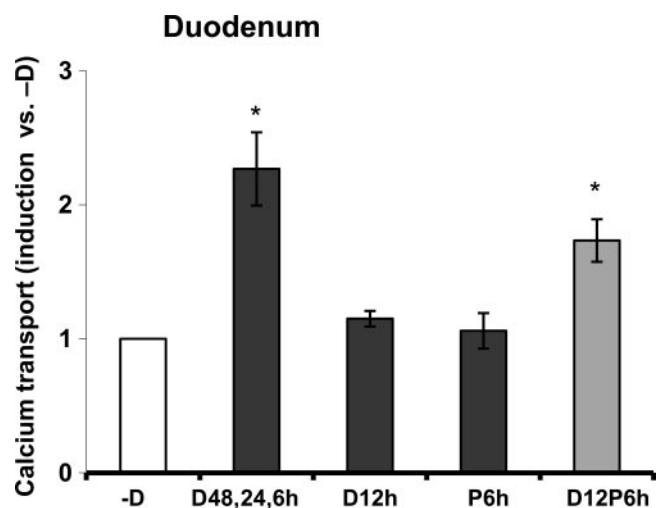


FIG. 3. Active intestinal calcium transport in the duodenum of vitamin D-deficient mice. Calcium transport was measured using everted intestinal sacs from the duodenum of vitamin D-deficient male mice injected with vehicle (-D) or $1,25(\text{OH})_2\text{D}_3$ (D; 1 ng/g bw) three times over 48 h (48, 24, and 6 h before termination) as a positive control. Mice were also injected with $1,25(\text{OH})_2\text{D}_3$ (2 ng/g bw D12 h) or prolactin (P; 1 $\mu\text{g/g}$ bw P6 h) at 12 or 6 h before termination, respectively, or with a combination of both $1,25(\text{OH})_2\text{D}_3$ and prolactin (D12P6 h) at 12 and 6 h before termination, respectively. Data are expressed relative to duodenal calcium transport in the deficient mice injected with vehicle (-D). Values represent the mean \pm SEM, $n = 12\text{--}16/\text{group}$. *, $P < 0.05$ compared with -D, vehicle-treated mice.

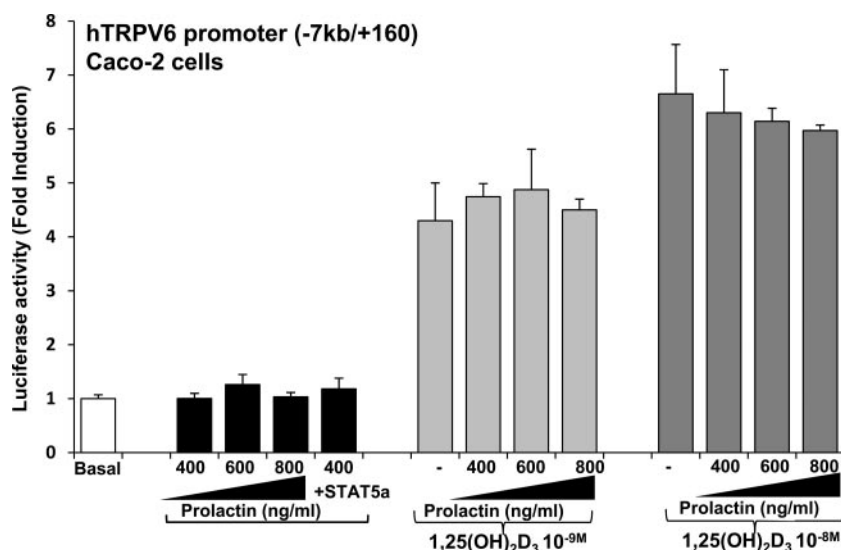


FIG. 4. Lack of transcriptional activation of the -7 kb/+160 TRPV6 promoter construct by prolactin. Caco-2 cells were transfected with 50 ng prolactin receptor and 0.3 μ g hTRPV6 promoter (-7 kb/+160) in the presence or absence of STAT5. Empty vectors were used to keep the total DNA concentration the same. After 24 h, cells were treated with vehicle, prolactin, $1,25(\text{OH})_2\text{D}_3$ (10^{-9} or 10^{-8} M), or prolactin + $1,25(\text{OH})_2\text{D}_3$ for 24 h. TRPV6 promoter activity was normalized to values for Renilla luciferase activity as an internal control and is expressed as fold induction by comparison with basal levels; mean \pm SEM, $n = 6$ –8 separate experiments. Prolactin alone (400–800 ng/ml) or in the presence of STAT5a (400 ng/ml prolactin + 50 ng STAT5a) did not affect TRPV6 transcriptional activation above basal levels ($P > 0.5$). In addition, prolactin did not enhance $1,25(\text{OH})_2\text{D}_3$ -induced TRPV6 transcriptional activation [$1,25(\text{OH})_2\text{D}_3$ (10^{-9} or 10^{-8} M) + prolactin (400, 600, or 800 ng/ml) vs. $1,25(\text{OH})_2\text{D}_3$ alone (10^{-9} or 10^{-8} M); $P > 0.5$]. Time-course studies (4–24 h) also did not indicate an effect of prolactin at earlier times in the presence or absence of $1,25(\text{OH})_2\text{D}_3$ on TRPV6 transcription (data not shown). When human prolactin, which is also an agonist for the long form of prolactin receptor, was substituted for ovine prolactin (used in these studies), similar results were observed.

Treatment with increasing concentrations of prolactin or transfection of STAT5a followed by prolactin treatment had no significant effect on luciferase activity (Fig. 4). Similar results were observed using STAT5b (data not shown). As previously reported (31), $1,25(\text{OH})_2\text{D}_3$ treatment alone resulted in a 4- to 7-fold increase in TRPV6 transcription. A combination of prolactin and $1,25(\text{OH})_2\text{D}_3$ did not result in a change in promoter activity as compared with $1,25(\text{OH})_2\text{D}_3$ alone. Unlike prolactin and hydrocortisone regulation of the casein gene, which requires cooperation between STAT5, glucocorticoid receptor, and C/EBP β (33), a combination of prolactin and $1,25(\text{OH})_2\text{D}_3$ in the presence of STAT5a, as well as C/EBP β , also did not result in a change in promoter activity compared with $1,25(\text{OH})_2\text{D}_3$ alone (data not shown). Similar results were observed using a COS-1 or COS-7 cell reconstitution system and transfection of VDR and the prolactin receptor (data not shown). In studies in COS-1 cells the β -casein promoter was used as a positive control (for β -casein promoter activation, transcription factor expression and treatment were done as previously described) (33). These findings suggest that prolactin may be regulating TRPV6 at a posttranscriptional level or

that TRPV6 is regulated by prolactin at other sites yet to be defined.

Prolactin is a regulator of the $1\alpha(\text{OH})ase$ gene

Because lactation is associated with an increase in plasma $1,25(\text{OH})_2\text{D}_3$ (7, 19, 20), in addition to effects on the intestine, the possibility that prolactin may have a direct effect on the transcription of the $1\alpha(\text{OH})ase$ gene was examined. AOK B-50 cells were cotransfected with the prolactin receptor and the mouse $1\alpha(\text{OH})ase$ promoter $-1651/+22$. Treatment with prolactin alone or transfection of STAT5a alone resulted in a 1.5- to 2-fold increase in luciferase activity. A combination of prolactin and STAT5a resulted in a maximum induction of $1\alpha(\text{OH})ase$ transcription of 13.2 ± 1.0 fold ($P < 0.05$ compared with prolactin alone or STAT5 alone), indicating a cooperative effects between STAT5 and prolactin in the regulation of $1\alpha(\text{OH})ase$ (Fig. 5A). Similar transactivation of $1\alpha(\text{OH})ase$ in the presence of STAT5b (50 ng) and prolactin was observed (data not shown). DN STAT5 inhibited prolactin/STAT5 induction of $1\alpha(\text{OH})ase$ transcription (Fig. 5A). Prolactin treatment in combination with STAT1 (PRL + STAT1) (Fig. 5A) did not

affect $1\alpha(\text{OH})ase$ transcription above levels observed with STAT1 alone. The prolactin/STAT5 induction of $1\alpha(\text{OH})ase$ transcription was also inhibited by the AG490, an inhibitor of JAK2 (Fig. 5B). Cooperative effects between STAT5 and prolactin were observed using the $-1651/+22$ construct but not the deletion constructs ($-477/+22$, $-233/+22$, $-144/+22$, and $-85/+22$) (Fig. 6A), suggesting that the prolactin/STAT5 responsive region is in the distal region between -1651 and -477 . To further determine which sequences in the distal region of the mouse $1\alpha(\text{OH})ase$ promoter are involved in the prolactin/STAT5 transactivation, constructs containing various lengths of the distal $1\alpha(\text{OH})ase$ promoter region fused to the minimal $1\alpha(\text{OH})ase$ promoter ($-85/+22$) upstream of the luciferase reporter gene were used (32). Cooperative effects between STAT5 and prolactin were observed using a $1\alpha(\text{OH})ase$ promoter construct containing $-1631/-1064$ (SR2) (32) but were not observed when a construct containing $-1631/-1386$ (BDC5') (32) was used, suggesting that the prolactin/STAT5 responsive region is between -1386 and -1064 . Consen-

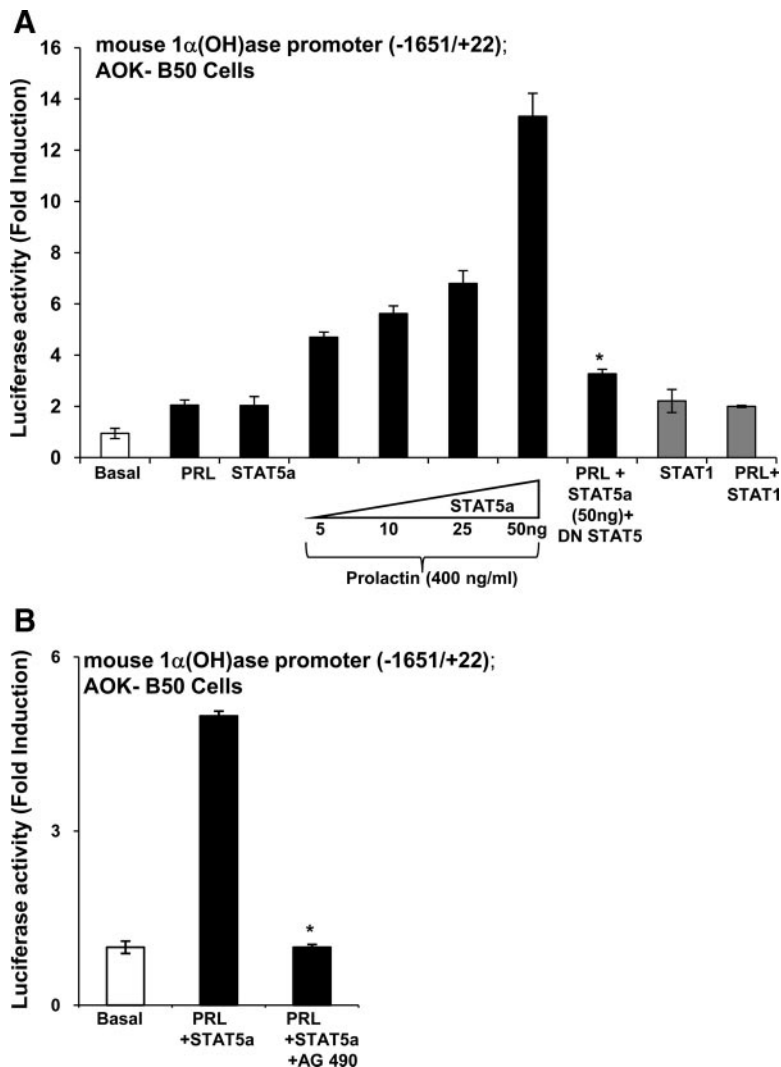


FIG. 5. Induction of $1\alpha(\text{OH})\text{ase}$ transcription by prolactin/STAT5. AOK B-50 cells were cotransfected with the prolactin receptor and the mouse $1\alpha(\text{OH})\text{ase}$ promoter construct (–1651/+22) in the presence or absence of STAT5a. After 24 h, cells were treated with vehicle or prolactin (400 ng/ml) for 24 h. For all transcription experiments, empty vectors were used to keep the total DNA concentration the same, Renilla luciferase was used as an internal control, and $1\alpha(\text{OH})\text{ase}$ promoter activity is represented as fold induction by comparison with basal levels. A, Prolactin alone (PRL) or STAT5 alone (50 ng) had a minimal effect on $1\alpha(\text{OH})\text{ase}$ transcription (1.5- to 2-fold increase in luciferase activity). However, a combination of prolactin and STAT5a resulted in a maximum induction of transcription of 13.2 ± 1.0 fold ($P < 0.05$ compared with prolactin alone or STAT5 alone at all concentrations of STAT5). Cotransfection with DN STAT5 (25 ng) resulted in a significant decrease in prolactin/STAT5 induction of $1\alpha(\text{OH})\text{ase}$ transcription (*, $P < 0.05$ PRL + STAT5a + DN STAT5 vs. PRL + STAT5a). There was no effect of DN STAT5 at the concentration used on basal levels of $1\alpha(\text{OH})\text{ase}$ transcription (data not shown). Prolactin treatment (400 ng/ml) in combination with STAT1 (PRL + STAT1) did not affect $1\alpha(\text{OH})\text{ase}$ transcription above levels observed with STAT1 alone [PRL + STAT1 (50 ng) vs. STAT1 (50 ng); $P > 0.5$]. Results represent the mean \pm SEM, $n = 4$ –10 observations/group. Similar results were observed using mouse proximal tubule (MPCT) cells (data not shown). B, The JAK2 inhibitor AG490 inhibits the prolactin/STAT5 induction of $1\alpha(\text{OH})\text{ase}$ transcription. AOK B-50 cells were cotransfected with the prolactin receptor and the mouse $1\alpha(\text{OH})\text{ase}$ promoter as described in A. Cells were also transfected with STAT5a expression vector (50 ng). AOK B-50 cells were pretreated for 1 h with JAK2 inhibitor AG490 (200 μM) followed by treatment with prolactin (PRL; 400 ng/ml) for 4 h. Results are expressed as mean \pm SEM of at least three observations/group. The JAK2 inhibitor AG490 significantly suppresses prolactin/STAT5 induction of $1\alpha(\text{OH})\text{ase}$ transcription ($P < 0.05$ PRL + STAT5a + AG490 vs. PRL + STAT5a).

STAT5-binding sites (TTCNNNGAA) were not identified in the mouse $1\alpha(\text{OH})\text{ase}$ promoter (1621/+22) (TRANSFAC database). However, within the prolactin/STAT5 responsive region (–1386/–1064), we identified two regions that are similar to the STAT5 consensus sequence (–1297/–1287 TTC-CCCAAGAA and –1236/–1229 TTCCA-GAA). Although EMSAs indicated binding of STAT5a to these sites but not to mutated sites, mutation of –1297/–1287 to TTtCCCAaAA, mutation of –1236/–1229 to TTtCAaAA (similar to reported mutations of other STAT sites) (42), or mutation of both putative STAT5 sites within the –1651/+22 $1\alpha(\text{OH})\text{ase}$ promoter construct did not affect the 13- to 15-fold induction in luciferase activity in response to prolactin/STAT5. Thus, these sites are not essential to activation by prolactin/STAT5 of $1\alpha(\text{OH})\text{ase}$ transcription. Although sites other than a STAT5 site or more divergent nonconsensus sites may be involved, these findings indicate for the first time that STAT5 and JAK2 mediated regulation by prolactin of $1\alpha(\text{OH})\text{ase}$ transcription. In addition, in AOK B-50 cells transfected with the prolactin receptor, prolactin treatment (400 ng/ml), and STAT5 transfection significantly induced $1\alpha(\text{OH})\text{ase}$ protein as determined by Western blot analysis (maximum induction 5.3-fold at 6 h after prolactin treatment) (Fig. 6B).

Discussion

In this study, we demonstrate novel mechanisms whereby prolactin can act as a calcium regulating hormone. Prolactin can induce TRPV6 mRNA in the intestine and has cooperative effects with $1,25(\text{OH})_2\text{D}_3$ in regulating intestinal calcium transport proteins, as well as intestinal calcium transport. In addition, we report for the first time that prolactin has a direct effect on the transcription of the $1\alpha(\text{OH})\text{ase}$ gene and that prolactin and STAT5 cooperate in regulating $1\alpha(\text{OH})\text{ase}$. Thus, prolactin, by different mechanisms, can modulate the effects of $1,25(\text{OH})_2\text{D}_3$.

Earlier reports have shown regulatory effects of prolactin on duodenal calcium transport (11, 15, 43). Studies in vitamin D-deficient male rats indicate that the effects of prolactin on duodenal active calcium transport can occur in a vitamin D-independent manner (15). This

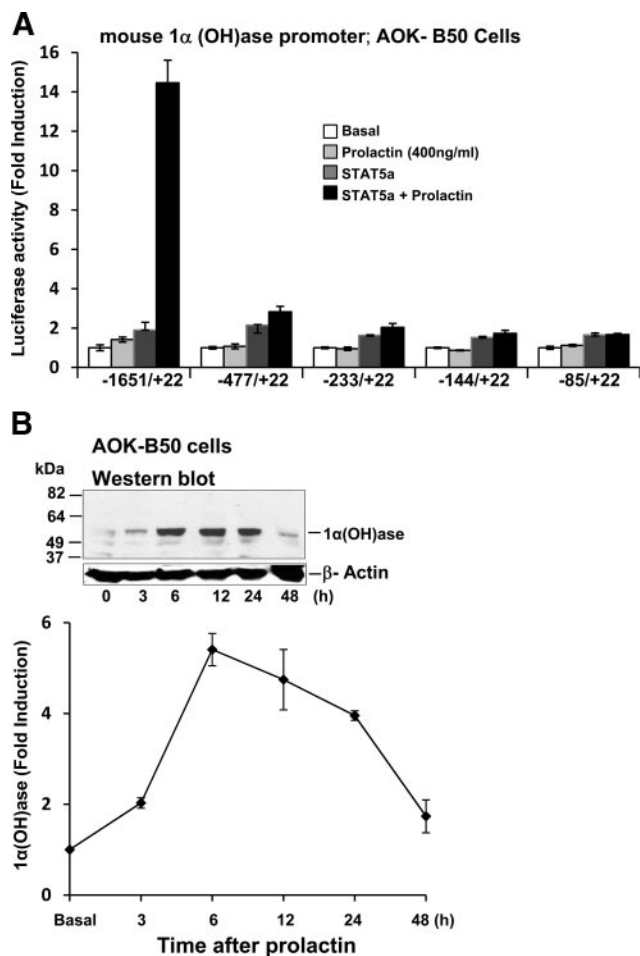


FIG. 6. Regulatory region for prolactin/STAT5 stimulation of 1 α (OH)ase transcription and induction of 1 α (OH)ase protein by prolactin. A, AOK B-50 cells were transfected with 0.3 μ g mouse 1 α (OH)ase promoter $-1651/+22$ or deletion constructs $-477/+22$, $-233/+22$, $-144/+22$, and $-85/+22$. In some wells, STAT5a expression vector (50 ng) was also transfected. After 24 h, cells were treated with vehicle or prolactin (400 ng/ml) for 24 h. Results are the mean \pm SEM of six to eight observations/group. B, Western blot analysis was performed using total extracts from AOK B-50 cells. Cells were transfected with STAT5a and prolactin receptor and treated with vehicle (0) or prolactin (400 ng/ml) for 3–48 h. Prolactin treatment resulted in a significant increase in 1 α (OH)ase protein levels at 3, 6, 12, and 24 h after prolactin treatment ($P < 0.05$ compared with vehicle).

is supported by studies demonstrating increased calcium absorption in pregnant and lactating vitamin D-deficient animals (7–9). However, the mechanisms involved in the effects of prolactin on calcium transport are not well defined and whether the expression of calcium transporter genes is affected by prolactin was not known. Studies using female vitamin D replete rats have suggested a non-genomic action of prolactin on duodenal active and passive transport (prolactin enhanced calcium uptake within 8 min, and effects on active duodenal calcium transport were observed within 1 h of ip injection of prolactin) (16, 44, 45). In our studies, we did not examine rapid actions of prolactin on the intestine but rather the effects of prolactin over a time period consistent with genomic actions.

Although TRPV6 mRNA expression in the duodenum is known to be induced by low dietary calcium and 1,25(OH) $_2$ D $_3$ (23, 25), in this study, we show that prolactin can also regulate duodenal TRPV6 mRNA. Three injections of prolactin over 48 h were required to observe induction of TRPV6 mRNA, suggesting that more long-term exposure, rather than acute administration, is needed for prolactin mediated regulation of TRPV6. Administration of prolactin alone either acutely or over 48 h was unable to affect duodenal calbindin-D $_{9k}$ expression however. This is consistent with previous findings in VDR null mutant mice, indicating that duodenal TRPV6 mRNA expression, but not calbindin-D $_{9k}$ expression, is markedly induced during lactation and late pregnancy (10, 46). Prolactin and PTHrP, which remain high during lactation, were suggested as candidate hormones that result in the major up-regulation of TRPV6 (10). Although it is possible that PTHrP may also affect TRPV6 expression, our findings indicate that at least part of the marked vitamin D-independent up-regulation of TRPV6 mRNA observed in the intestine during lactation is due to an effect of prolactin.

In addition to vitamin D-independent effects of prolactin on TRPV6 expression, cooperative effects were noted between prolactin and 1,25(OH) $_2$ D $_3$ in the regulation of both TRPV6 and calbindin-D $_{9k}$. Also, at concentrations that did not result in a measurable effect for each hormone alone, combined treatment with 1,25(OH) $_2$ D $_3$ and prolactin resulted in a significant induction in duodenal calcium transport as measured by the everted gut sac assay. Because both 1,25(OH) $_2$ D $_3$ and prolactin serum levels are increased during lactation (7, 19, 20, 47), prolactin may act together with 1,25(OH) $_2$ D $_3$ for some of its effects related to calcium homeostasis. Cooperative effects between prolactin and 1,25(OH) $_2$ D $_3$ have previously been reported for growth suppression of prostate cancer cells (48). Studies in prostate cancer cells showed an induction of VDR by prolactin (48). In our studies, however, we did not observe an increase in VDR in the intestine of vitamin D-deficient mice injected with prolactin alone, 1,25(OH) $_2$ D $_3$ alone (as previously reported) (49, 50), or 1,25(OH) $_2$ D $_3$ + prolactin, suggesting that the mechanism of the enhancement was not due to an induction of VDR in the intestine. Because the binding of prolactin to its receptor regulates transcriptional activation of milk protein genes (51), we examined the possibility that prolactin may transcriptionally activate the $-7000/+160$ TRPV6 promoter construct. The expression of TRPV6 is regulated by 1,25(OH) $_2$ D $_3$ /VDR at the transcriptional level through two active vitamin D-response elements at -2.1 and -4.3 kb (31). Although a cooperative effect was observed between prolactin and 1,25(OH) $_2$ D $_3$ in the regulation of TRPV6 mRNA, a combination of prolactin and

$1,25(\text{OH})_2\text{D}_3$ in the presence or absence of C/EBP β and STAT5 did not affect $1,25(\text{OH})_2\text{D}_3$ -induced TRPV6 promoter activity. It is possible that prolactin regulates TRPV6 at other sites in the TRPV6 promoter yet to be identified. It is also possible that prolactin may affect TRPV6 expression by posttranscriptional mechanisms. Prolactin induces casein gene transcription by both transcriptional and posttranscriptional mechanisms. Prolactin has been reported to induce a 2- to 3-fold increase in the rate of casein mRNA transcription and a 17- to 25-fold increase in the half life of casein mRNA (52). Thus, both mechanisms are involved in the marked accumulation of casein mRNA after prolactin treatment of mammary organ culture. More recent studies have shown that prolactin plus insulin can induce poly(A) elongation of β -casein (53). A previous study, which examined PTH regulation of 25-hydroxyvitamin D_3 24-hydroxylase, also noted posttranscriptional regulation of a $1,25(\text{OH})_2\text{D}_3$ target gene by a peptide hormone (54). With regard to calbindin, the large induction of calbindin- D_{9k} mRNA in intestine in response to $1,25(\text{OH})_2\text{D}_3$ has been shown to be due primarily to posttranscription mechanisms (55). Thus, it is possible that prolactin may enhance posttranscriptional mechanisms involved in an effect of $1,25(\text{OH})_2\text{D}_3$ on increasing calbindin- D_{9k} mRNA half life. It is also possible that the effect of prolactin may be secondary to a primary effect of prolactin on other factors that in turn affect TRPV6 expression or $1,25(\text{OH})_2\text{D}_3$ -induced calbindin- D_{9k} or TRPV6 expression.

Although in this study we focused on duodenum and calcium transport proteins involved in active calcium transport, prolactin may also have an effect on intestinal calcium absorption via the paracellular path. An effect of prolactin on the paracellular path has previously been suggested (17, 18, 45). Recent studies have shown that $1,25(\text{OH})_2\text{D}_3$ can regulate tight junction and transmembrane proteins in the intestine, including claudin-2, claudin-12, and cadherin-17, suggesting a role for $1,25(\text{OH})_2\text{D}_3$ in the transjunctional movement of calcium (56, 57). It will be of interest, in future studies, to determine whether prolactin can also affect the regulation of these proteins in the duodenum, as well as in other regions of the intestine.

Besides direct effects of prolactin on the intestine, an effect of prolactin on $1\alpha(\text{OH})\text{ase}$ expression and transcription in kidney cells was also observed. Early studies showed that circulating levels of $1,25(\text{OH})_2\text{D}_3$ are increased in pregnancy and during lactation (7, 19, 20). However, the mechanisms underlying the increase in serum $1,25(\text{OH})_2\text{D}_3$ and whether prolactin has a direct effect on $1\alpha(\text{OH})\text{ase}$ have been a matter of debate. During pregnancy and lactation, there is a significant flux of calcium to the fetus and the neonate, respectively. The drain on the plasma calcium pool results in a drop

in serum calcium (19, 20). Thus, it has been suggested that the drop in serum calcium in late pregnancy and lactation may stimulate the renal $1\alpha(\text{OH})\text{ase}$ via PTH (19, 20). Parathyroidectomy of lactating rats caused a 70% inhibition of the $1,25(\text{OH})_2\text{D}_3$ increase (20). However, it has been reported that hypoparathyroid women during lactation treated with the usual dose of calcitriol develop hypercalcemia, and in the absence of treatment, serum $1,25(\text{OH})_2\text{D}_3$ levels remain within the normal range, suggesting that another factor besides PTH stimulates $1\alpha(\text{OH})\text{ase}$ during lactation (58–60). Previous studies suggested that prolactin can influence $1\alpha(\text{OH})\text{ase}$. Early studies were done in chicks and showed that prolactin increased $1\alpha(\text{OH})\text{ase}$ activity after either long-term or short-term administration (61). In addition, prolactin was shown to act directly on chick renal cells to stimulate $1\alpha(\text{OH})\text{ase}$ activity (62, 63). Further, in lactating rats, bromocriptine treatment significantly reduced $1,25(\text{OH})_2\text{D}_3$ levels (21). We now report that prolactin has a direct effect on the transcription of the $1\alpha(\text{OH})\text{ase}$ gene. Using the mouse $1\alpha(\text{OH})\text{ase}$ promoter, we report for the first time a STAT5-mediated regulation by prolactin of $1\alpha(\text{OH})\text{ase}$ transcription. Our findings indicate that prolactin regulation of $1\alpha(\text{OH})\text{ase}$ transcription involves JAK2 and STAT5 (Fig. 5B), similar to other genes regulated by prolactin in mammary gland, breast cancer cells, and in adipocytes (34, 42, 64, 65). However, no consensus STAT5-binding sites were noted in the $-1651/+22$ region of the mouse $1\alpha(\text{OH})\text{ase}$ promoter, and mutations of regions that were similar to the consensus sequence did not affect activation of the $1\alpha(\text{OH})\text{ase}$ promoter by STAT5/prolactin. It is possible that STAT5 affects $1\alpha(\text{OH})\text{ase}$ transcription by binding to sites other than the STAT5 site [STAT5 has been reported to bind to sites that resemble the γ interferon-activated sequence (GAS TTNCNNNA) (66, 67)]. However, γ interferon-activated sequence sites were not identified within the region of the $1\alpha(\text{OH})\text{ase}$ promoter that responded to stimulation of transcription by prolactin/STAT5. Recent studies indicated that the majority of STAT5-binding sites are paired sites (68). In addition, it has been noted that the repertoire of potential binding sites for STAT5 is broader than expected (67). Thus, although more divergent, paired, nonconsensus sites may be involved in prolactin/STAT5 regulation, our findings nevertheless demonstrate a direct effect of prolactin via STAT5 and JAK2 on $1\alpha(\text{OH})\text{ase}$ transcription. Prolactin was also shown to enhance $1\alpha(\text{OH})\text{ase}$ protein expression. Therefore, it is likely that both PTH and prolactin have a physiological function to increase $1,25(\text{OH})_2\text{D}_3$ levels in lactation.

In summary, our findings indicate for the first time mechanisms whereby prolactin modulates vitamin D-mediated calcium homeostasis. Prolactin can regulate the

intestinal epithelial calcium channel TRPV6 and can cooperate with $1,25(\text{OH})_2\text{D}_3$ in regulating TRPV6 and calbindin- D_{9k} . In addition, prolactin has a direct effect on the *1\alpha(\text{OH})ase* gene, which is mediated by STAT5 and JAK2. These mechanisms may have physiological importance during lactation when plasma levels of prolactin are markedly elevated and there is increased calcium requirement for the neonate.

Acknowledgments

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