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1,25(OH)₂D₃ stimulates Mg²⁺ uptake into MDCT cells: modulation by extracellular Ca²⁺ and Mg²⁺

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Ritchie, Gordon, Dirk Kerstan, Long-Jun Dai, Hyung Sub Kang, Lucie Canaff, Geoffrey N. Hendy, and Gary A. Quamme. 1,25(OH)₂D₃ stimulates Mg²⁺ uptake into mouse MDCT cells: modulation by extracellular Ca²⁺ and Mg²⁺. *Am J Physiol Renal Physiol* 280: F868–F878, 2001.—The distal convoluted tubule plays a significant role in renal magnesium conservation. Although the cells of the distal convoluted tubule possess the vitamin D receptor, little is known about the effects of 1α,25-dihydroxyvitamin D [1,25(OH)₂D₃] on magnesium transport. In this study, we examined the effect of 1,25(OH)₂D₃ on distal cellular magnesium uptake and the modulation of this response by extracellular Ca²⁺ and Mg²⁺ in an immortalized mouse distal convoluted tubule (MDCT) cell line. MDCT cells possess the divalent cation-sensing receptor (CaSR) that responds to elevation of extracellular Ca²⁺ and Mg²⁺ concentrations to diminish peptide hormone-stimulated Mg²⁺ uptake. Mg²⁺ uptake rates were determined by microfluorescence in Mg²⁺-depleted MDCT cells. Treatment of MDCT cells with 1,25(OH)₂D₃ for 16–24 h stimulated basal Mg²⁺ uptake in a concentration-dependent manner from basal levels of 164 ± 5 to 210 ± 11 nM/s, representing a 28 ± 3% change. Pretreatment with actinomycin D or cycloheximide abolished 1,25(OH)₂D₃-stimulated Mg²⁺ uptake (154 ± 18 nM/s), suggesting that 1,25(OH)₂D₃ stimulates Mg²⁺ uptake through gene activation and protein synthesis. Elevation of extracellular Ca²⁺ inhibited 1,25(OH)₂D₃-stimulated Mg²⁺ uptake (143 ± 5 nM/s). Preincubation of the cells with an antibody to the CaSR prevented the inhibition by elevated extracellular Ca²⁺ of 1,25(OH)₂D₃-stimulated Mg²⁺ uptake (202 ± 8 nM/s). Treatment with an antisense CaSR mRNA oligodeoxynucleotide also abolished the effects of extracellular Ca²⁺ on 1,25(OH)₂D₃-responsive Mg²⁺ entry. This showed that elevated extracellular calcium modulates 1,25(OH)₂D-mediated responses through the CaSR. In summary, 1,25(OH)₂D₃ stimulated Mg²⁺ uptake in MDCT cells, and this is dependent on de novo protein synthesis. Elevation of extracellular Ca²⁺, acting via the CaSR, inhibited 1,25(OH)₂D₃-stimulated Mg²⁺ entry. These data indicate that 1,25(OH)₂D₃ has important effects on the control of magnesium entry in MDCT cells and these responses can be modulated by extracellular divalent cations.

1α,25-dihydroxyvitamin D; calcium/magnesium-sensing receptor; adenosine 3',5'-cyclic monophosphate measurements; intracellular magnesium determinations; magnesium uptake; fluorescence

THE HORMONALLY ACTIVE METABOLITE of vitamin D, 1,25-dihydroxyvitamin D [1,25(OH)₂D₃], has an important role in regulating mineral metabolism at the level of the intestine, bone, and kidney (1, 32, 51). It is clear that 1,25(OH)₂D₃ increases intestinal calcium and magnesium absorption and regulates bone formation, but the actions of 1,25(OH)₂D₃ on the kidney are not as well characterized (1, 31, 51). On balance, clinical and experimental observations support the notion that 1,25(OH)₂D₃ increases calcium and magnesium reabsorption within the kidney (1, 19, 25, 26, 33, 36, 38, 45). Nevertheless, some studies reported little, if any, affect on renal divalent cation conservation (9, 13, 30, 39, 40). This discrepancy may be due to associated vitamin D-induced changes in plasma calcium and magnesium concentrations that affect renal divalent cation handling. An extracellular Ca²⁺/Mg²⁺-sensing receptor (CaSR) that modulates calcium and magnesium reabsorption in the loop of Henle and distal convoluted tubule has been identified along the length of the kidney tubule (27, 43). The distal convoluted tubule plays an important role in renal magnesium conservation as it has final control of urinary magnesium excretion (43). We have shown that elevated extracellular Ca²⁺ or Mg²⁺ inhibits hormone-mediated Mg²⁺ uptake in a distal convoluted tubule cell line (3). Our evidence is that hypercalcemia and hypermagnesemia diminish distal reabsorption, in part, through CaSR inhibition of peptide hormone receptor-mediated responses (3). In the present study, we investigated whether 1,25(OH)₂D₃ stimulates Mg²⁺ uptake into distal convoluted tubule cells and whether elevated extracellular Ca²⁺ or Mg²⁺ may inhibit these responses. Our notion is that 1,25(OH)₂D₃ plays a role in magnesium conservation at both the intestinal and renal level and that the latter is modulated by the prevailing extracellular Ca²⁺ and Mg²⁺ concentrations through the CaSR. Accordingly, increases in 1,25(OH)₂D₃ may lead to enhanced magnesium conservation, no change, or increased urinary excretion, depending on the magnitude of changes in plasma calcium and magnesium concentration.

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To date, there are no experiments reporting the direct effects of vitamin D metabolites on cellular magnesium transport. In the present studies, we used mouse distal convoluted tubule (MDCT) cells to investigate the actions of 1,25-(OH)₂D₃ on Mg²⁺ uptake rates. MDCT cells have been extensively used to study cellular mechanisms of calcium and magnesium absorption within the distal tubule (23, 43). Parathyroid hormone (PTH), glucagon, and arginine vasopressin (AVP) stimulate Mg²⁺ uptake in MDCT cells (16, 17). In a recent study, we showed that MDCT cells also possess a polyvalent cation-sensing mechanism that is responsive to extracellular Mg²⁺ and Ca²⁺ (4). We documented the expression of the parathyroid/kidney Ca²⁺-sensing receptor (i.e., CaSR) in MDCT cells and suggested that it is responsible, either entirely or in part, for the extracellular polyvalent cation-sensing mechanism in these cells (4). We also found that activation of this mechanism with either extracellular Ca²⁺, Mg²⁺, or the polyvalent cation neomycin inhibited PTH-, glucagon-, and AVP-stimulated cAMP release (4) as well as hormone-stimulated Mg²⁺ uptake in MDCT cells (3). These studies indicated that this cation-sensing mechanism in immortalized MDCT cells plays an important role in modulating hormone-mediated intracellular signals in response to changes in extracellular Mg²⁺ as well as Ca²⁺. Yang et al. (54) have reported that MDCT cells also express 1,25(OH)₂D₃- and PTH-responsive 25(OH)D 24-hydroxylase (54), and Friedman and Gesek (22) have shown that 1,25(OH)₂D₃ accelerates PTH-dependent Ca²⁺ uptake, suggesting that vitamin D receptors are present in this cell line and play a role in mineral metabolism (22). Accordingly, these cells provide a useful model by which to determine the effects of 1,25(OH)₂D₃ on Mg²⁺ handling in the distal convoluted tubule, which is difficult to study *in situ* or dissect out for functional measurements. The present studies demonstrate that 1,25(OH)₂D₃ stimulates Mg²⁺ uptake in MDCT cells, providing evidence that the metabolite might affect magnesium absorption in the intact distal tubule. Moreover, elevation of divalent cations, acting through the CaSR, modulates 1,25(OH)₂D₃-mediated Mg²⁺ entry in this cell line.

MATERIALS AND METHODS

Basal DMEM and Ham's F-12 media were from GIBCO Laboratories, Grand Island, NY. FCS was from Flow Laboratories (McLean, VA). Mag-fura 2-acetoxymethyl ester (AM) was obtained from Molecular Probes (Eugene, OR). 1,25(OH)₂D₃ and other materials were from Sigma (St. Louis, MO).

Cell preparation. Immortalized MDCT cells were kindly provided by Dr. P. A. Friedman. They were cultured in DMEM-Ham's F-12 media, 1:1, supplemented with 10% FCS, 1 mM glucose, 5 mM L-glutamine, 50 U/ml penicillin, and 50 µg/ml streptomycin in a humidified environment of 5% CO₂-95% air at 37°C. For cAMP determinations, the MDCT cells were cultured to confluence in 24-well plastic dishes. Sixteen hours before the cAMP measurements were made, the culture medium was changed to one containing 0.2% BSA rather

than FCS. For the fluorescence studies, confluent cells were washed three times with PBS containing 5 mM EGTA, trypsinized, and seeded on glass coverslips. Aliquots of harvested cells were allowed to settle onto sterile glass coverslips in 100-mm Corning tissue culture dishes, and the cells were grown to confluence over 4–6 days in supplemented media as described above. The normal media contained 0.6 mM magnesium and 1.0 mM calcium. In the experiments indicated, the cells were cultured in nominally magnesium-free media (<0.01 mM) for 16 h before study (0.2% BSA replaced FCS during this period). Other constituents of the magnesium-free media were identical to those of the complete media.

cAMP measurements. cAMP was determined in confluent MDCT cell monolayers cultured in 24-well plates in DMEM-Ham's F-12 media without serum, as previously reported (4). After addition of various hormones, MDCT cells were incubated at 37°C for 5 min in the presence of 0.1 mM IBMX. cAMP was extracted with 5% trichloroacetic acid, which was removed with ether acidified with 0.1 N HCl. The aqueous phase was dried, dissolved in Tris-EDTA buffer, and then cAMP was measured with a radioimmunoassay kit (Diagnostic Products, Los Angeles, CA).

Cytoplasmic Mg²⁺ measurements. Coverslips were mounted within a perfusion chamber, and the attached subconfluent cells were incubated with 5 µM mag-fura 2-AM dissolved in pluronic acid F-127 (0.125%; Molecular Probes) for determination of intracellular Mg²⁺ concentration ([Mg²⁺]_i) in media for 20 min at 37°C. Cells were subsequently washed three times with a buffered salt solution containing (in mM) 145 NaCl, 4.0 KCl, 0.8 K₂HPO₄, 0.2 KH₂PO₄, 1.0 CaCl₂, 5.0 glucose, and 20 HEPES-Tris, at pH 7.4. The MDCT cells were incubated for an additional 20 min to allow for complete deesterification and washed once before measurement of fluorescence.

Epifluorescence microscopy was used to monitor changes in the mag-fura 2 fluorescence of the MDCT cell monolayer. The chamber was mounted on an inverted Nikon Diaphot-TMD microscope with a Fluor ×100 objective, and, under oil immersion, fluorescence within a single cell was monitored over the course of the study. Fluorescence was recorded at 1-s intervals by using a dual-excitation-wavelength spectrofluorometer (Delta-scan, Photon Technologies, Princeton, NJ) with excitation for mag-fura 2 at 335 and 385 nm (chopper speed set at 100 Hz) and emission at 505 nm. Media changes were made without interruption in recording.

The free [Mg²⁺]_i was calculated from the ratio of the fluorescence at the two excitation wavelengths as previously described (15) by using a dissociation constant (*K*_d) of 1.4 mM for the mag-fura 2-Mg²⁺ complex. The minimum (*R*_{min}) and maximum (*R*_{max}) ratios were determined for the cells at the end of each experiment by using 20 µM digitonin. *R*_{max} for mag-fura 2 was found by the addition of 50 mM MgCl₂ in the absence of Ca²⁺, and *R*_{min} was obtained by removal of Mg²⁺ and addition of 100 mM EDTA, pH 7.2. The excitation spectrum of the cellular mag-fura 2 under these conditions was similar to that of free mag-fura 2 in the same solutions.

Antibody block of the CaSR in MDCT cells. In those experiments where the CaSR was blocked with a specific antibody, MDCT cells were Mg²⁺ depleted for 16 h and then incubated with the antibody for 1.5–2 h at 36°C. A mouse monoclonal antibody (ADD) raised against a peptide comprising residues 214–236 of the CaSR was used (20). The antibody, which was provided by Drs. P. K. Goldsmith and A. M. Spiegel (National Institutes of Health) and K. V. Rogers (NPS Pharmaceuticals), has been extensively characterized with respect to specificity for the parathyroid/kidney CaSR (24). The antibody was diluted to a final concentration of 1.6 µg/ml in a buffer solution containing 0.02% BSA. The control cells were

incubated with mouse IgG. The cells were washed twice in an appropriate buffer solution, and intracellular cAMP content or microfluorescence experiments were performed.

Antisense oligodeoxynucleotides designed to the CaSR in MDCT cells. A second approach was used to block extracellular Ca²⁺/Mg²⁺ sensing in MDCT cells. Oligonucleotide (ODN) antisense methodology, a strategy designed to bind specifically and efficiently to the complementary sequence of a targeted mRNA, has been extensively used in *in vitro* systems. Twenty-base pair phosphorothioate-derivatized sense and antisense CaSR ODNs were synthesized on an automated solid-phase synthesizer by using standard phosphoramidite chemistry. The sequence of the sense ODN was 5'-GAGAAGGCAGAGCCATGGCATGG-3', and that of the antisense ODN was 5'-CCATGCCATGGCTCTGCCTTCTC-3'. The MDCT cells were incubated in buffer containing 0.1% BSA with the ODN, 1.0 nmol/ml, for 48–72 h before fluorescence measurements were performed. Cationic transfection agents, such as lipofectamine, were avoided because they interfere with Mg²⁺ transport, presumably because of membrane damage. Protein was extracted from the cells for Western blot analysis.

Western blots. Cells were lysed in triple detergent buffer [50 mM Tris·HCl (pH 8.0), 150 mM NaCl, 0.02% NaN₃, 0.1% SDS, 1 mM EDTA, 100 μg/ml polymethylsulfonyle fluoride, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 0.1% NP-40, and 0.5% sodium deoxycholate] for 5 min at 0°C. The cell lysates were spun at 1,200 g for 2 min at 4°C, and the supernatants were stored at –80°C. Aliquots were electrophoresed through 8% SDS-polyacrylamide gels and blotted onto polyvinylidene difluoride membranes (Bio-Rad). Membranes were rinsed in TBST (10 mM Tris·HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20), blocked with 5% dried milk powder in TBST for 1–2 h, and incubated with ADD, the antibody against the CaSR (24). As a control, immunoblotting was carried out as described above with the antiserum preabsorbed for 1 h with the peptide (10 μg/ml) against which it was raised. Blots were also incubated with a β-tubulin monoclonal antibody (Cedarlane Laboratories, Hornby, ON).

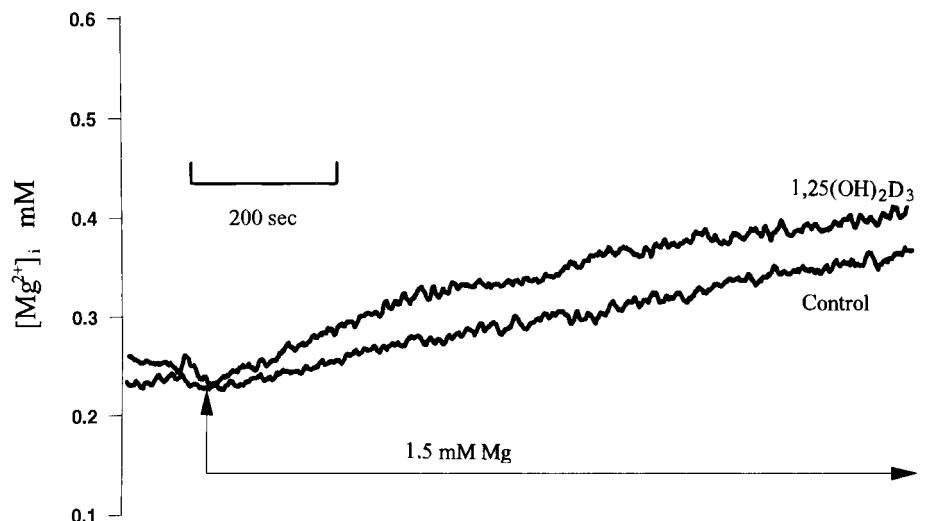
Statistical analysis. Representative tracings of fluorescent intensity ratios are given, and all results are means ± SE where indicated. Significance was determined by Tukey's analysis of variance where indicated, and comparisons among groups of data were made by using Student's *t*-test. A probability of *P* < 0.05 was taken to be statistically significant.

RESULTS

1,25(OH)₂D₃ stimulates Mg²⁺ entry in MDCT cells. MDCT cells were treated with 1,25(OH)₂D₃ for 16 h before experimentation. During this time they were incubated in media without magnesium to deplete them of intracellular Mg²⁺ (16). The cells were then placed in 1.5 mM MgCl₂, and [Mg²⁺]_i was monitored by fluorescence. Figure 1 shows a typical result of a fluorescence experiment of MDCT cells pretreated with 1,25(OH)₂D₃ compared with control cells. The rate of entry into control cells, measured over the initial 500 s, was 164 ± 5 nM/s, *n* = 6. Pretreatment with 1,25(OH)₂D₃ increased Mg²⁺ uptake by 59 ± 4% to 260 ± 15 nM/s, *n* = 5. Next, we determined that vitamin D-stimulated Mg²⁺ uptake required a minimum of 3 h for the largest response and was maintained for 24 h. We have previously shown that the channel blocker nitrendipine inhibits Mg²⁺ uptake into MDCT cells (36). Nitrendipine (50 μM) inhibited Mg²⁺ uptake to a similar extent in 1,25(OH)₂D₃-treated cells (25 ± 5 nM/s, *n* = 4) as in control cells (24 ± 5 nM/s, *n* = 5), indicating that the vitamin D metabolite 1,25(OH)₂D₃ stimulated Mg²⁺ entry and not intracellular Mg²⁺ release or inhibition of Mg²⁺ efflux. 1,25(OH)₂D₃ increased Mg²⁺ uptake in a concentration-dependent manner, with half-maximal responses (185 ± 3 nM/s) occurring at ~10^{–9} M (Fig. 2). This study indicates that 1,25(OH)₂D₃ stimulates Mg²⁺ entry into MDCT cells.

Steroid hormones may mediate cellular responses through nongenomic or genomic actions (10). Nongenomic effects of 1,25(OH)₂D₃ include rapid changes in phosphoinositide metabolism, increases in intracellular Ca²⁺ concentration, elevation of cAMP levels, and activation of protein kinase C (reviewed in Ref. 10). We have reported that many of these signaling pathways may influence hormone-mediated Mg²⁺ uptake in MDCT cells (16, 17). Accordingly, we tested the acute (<20 min) effects of vitamin D metabolites on the mean Mg²⁺ entry rate. The acute treatment of MDCT cells for 20 min with 1,25(OH)₂D₃ had no effect on Mg²⁺

Fig. 1. 1,25-Dihydroxyvitamin D [1,25(OH)₂D₃] stimulates Mg²⁺ uptake in Mg²⁺-depleted mouse distal convoluted tubule (MDCT) cells. MDCT cells were cultured in Mg²⁺-free media (<0.01 mM) for 16 h. Where indicated, 10^{–7} M 1,25(OH)₂D₃ was added to the buffer solution from a stock ethanol solution during the 16-h Mg²⁺-depletion period. Fluorescence studies were performed in buffer solutions in the absence of external magnesium, and, where indicated, MgCl₂ (1.5 mM final concentration) was added to observe changes in intracellular Mg²⁺ concentration ([Mg²⁺]_i). The buffer solutions contained (in mM) 145 NaCl, 4.0 KCl, 0.8 K₂HPO₄, 0.2 KH₂PO₄, 1.0 CaCl₂, 5.0 glucose, and 10 HEPES-Tris, pH 7.4, with and without 1.5 mM MgCl₂. Fluorescence was measured at 1 data point/s with 25-point signal averaging, and the tracing was smoothed according to methods previously described (17).



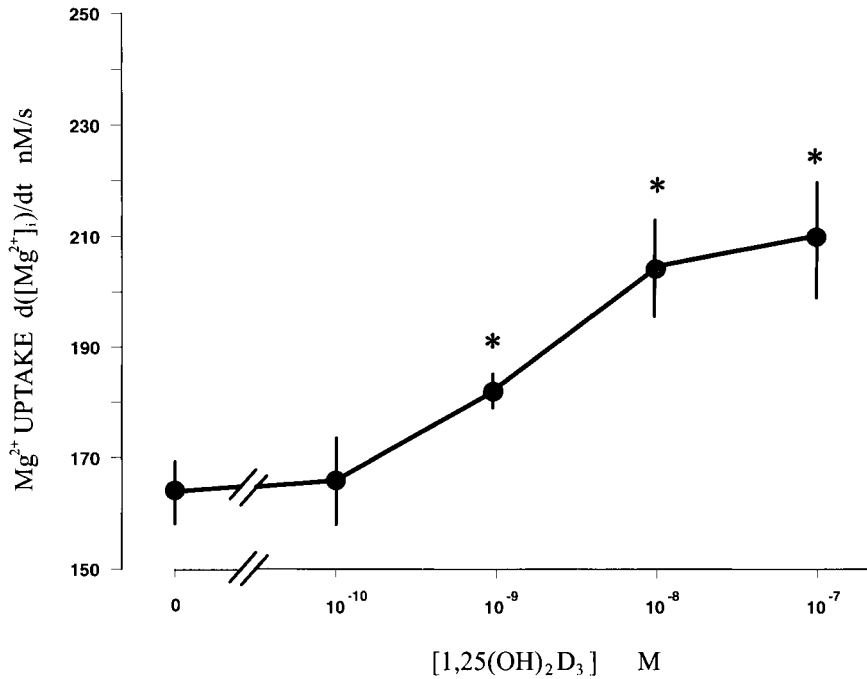


Fig. 2. Concentration dependence of 1,25(OH)₂D₃ stimulation of Mg²⁺ entry in MDCT cells. Values are means \pm SE for 3–6 cells. MDCT cells were treated with the given 1,25(OH)₂D₃ concentrations for 16 h before fluorescence determinations. The rate of Mg²⁺ influx, as determined by $d([Mg^{2+}]_i)/dt$, was measured with the given 1,25(OH)₂D₃ concentrations by using fluorescence techniques described in the legend for Fig. 1. $d([Mg^{2+}]_i)/dt$ values were determined over the first 500 s of fluorescence measurements. Significantly different from control values, * $P < 0.05$.

uptake (178 ± 17 nM/s, $n = 3$), which suggested that MDCT cells do not affect transport by nongenomic mechanisms. Genomic mechanisms involve changes in gene expression, requiring some hours before de novo protein synthesis. We determined this by using the transcriptional and translational inhibitors actinomycin D and cycloheximide, respectively. Because of the toxicity of actinomycin D, we measured the effect of the inhibitor for a 3- to 4-h incubation period. 1,25(OH)₂D₃ was equally effective in stimulating Mg²⁺ entry after 3 h of incubation as it was at 16 h (Fig. 3A). The MDCT cells were initially Mg²⁺ depleted by incubating them in magnesium-free media for 16 h before the addition of 1,25(OH)₂D₃, with and without actinomycin (5 μ g/ml). The 1,25(OH)₂D₃-treated Mg²⁺-depleted cells were incubated for a further 4–5 h; then, Mg²⁺ uptake was performed by microfluorescence. Actinomycin D did not alter basal Mg²⁺ uptake in cells that had been Mg²⁺ depleted for 16 h (18). In those cells treated with actinomycin D, 10⁻⁷ M 1,25(OH)₂D₃ failed to stimulate Mg²⁺ entry, indicating that the vitamin D metabolite increases uptake by mechanisms involving transcriptional processes (Fig. 3A). Next, we determined the effect of a translational inhibitor, cycloheximide. Cycloheximide (1.0 μ g/ml) was added with 10⁻⁷ M 1,25(OH)₂D₃ for 16 h before fluorescence studies. 1,25(OH)₂D₃ did not stimulate Mg²⁺ uptake (155 ± 18 nM/s, $n = 4$) in those cells treated with cycloheximide, indicating that translational processes were also involved in this response (Fig. 3B). Accordingly, these results indicate that 1,25(OH)₂D₃ stimulates Mg²⁺ entry by genomic mechanisms involving de novo synthesis of transport proteins.

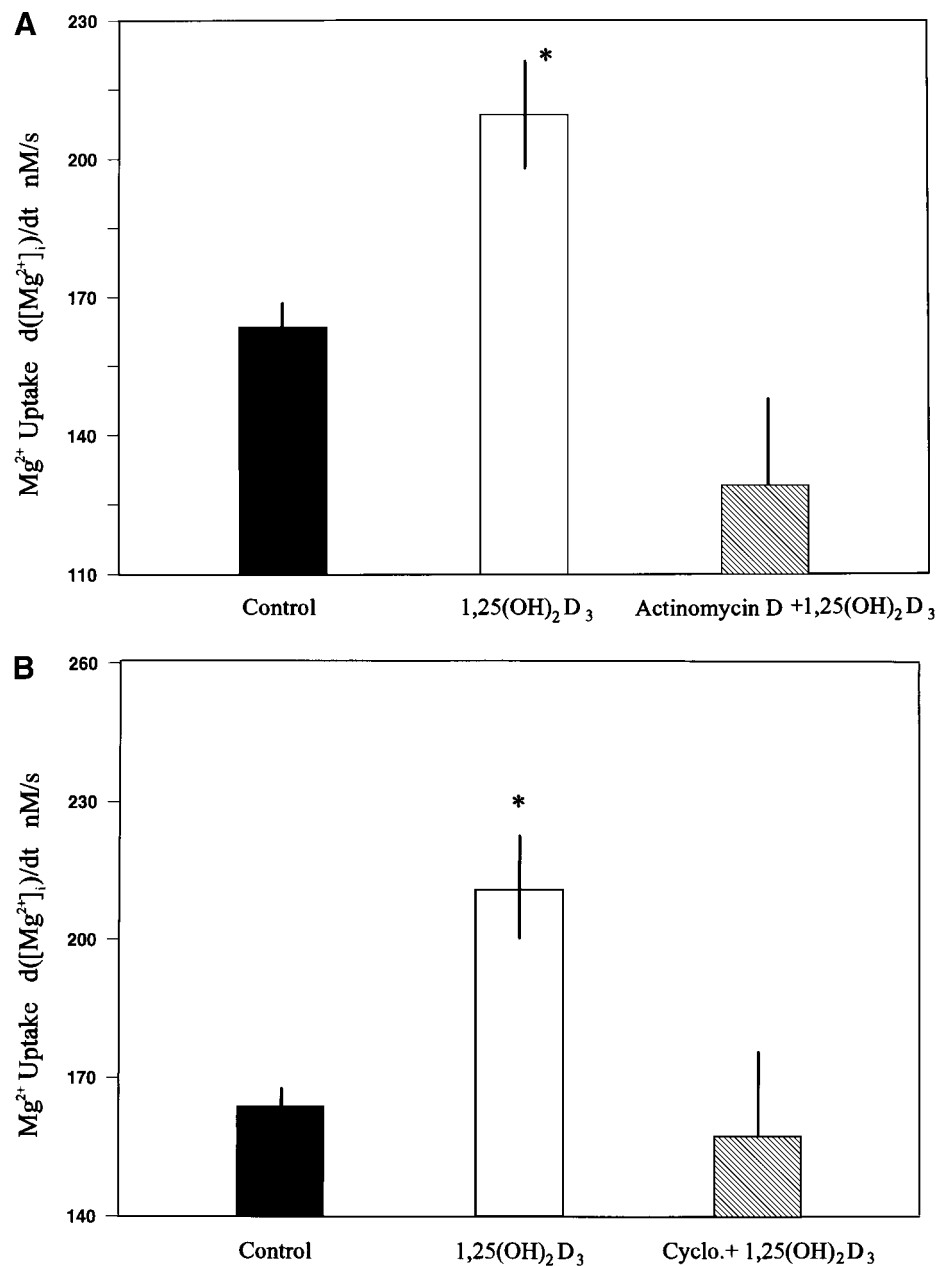
Acute effects of polyvalent cations on 1,25(OH)₂D₃-stimulated Mg²⁺ uptake. We have shown that elevation of extracellular polyvalent cations, neomycin,

Mg²⁺, and Ca²⁺ inhibit PTH-, glucagon-, and AVP-mediated cAMP formation and glucagon- and AVP-stimulated Mg²⁺ entry but not basal uptake rates in MDCT cells (3, 4). MDCT cells were treated with 10⁻⁷ M 1,25(OH)₂D₃ for 16 h before the addition of polyvalent cations and determination of Mg²⁺ uptake. Acute (5-min) addition of 50 μ M neomycin or elevation of 5.0 mM extracellular Mg²⁺ or 5.0 mM Ca²⁺ did not affect Mg²⁺ uptake into 1,25(OH)₂D₃-treated, Mg²⁺-depleted cells (Fig. 4). Accordingly, the acute activation of the CaSR does not alter 1,25(OH)₂D₃-stimulated Mg²⁺ entry in the cells pretreated with the vitamin D metabolite 16 h before experimentation.

Chronic effects of elevated extracellular Ca²⁺ on 1,25(OH)₂D₃-stimulated Mg²⁺ uptake. Next, we determined the effects of chronic elevation of extracellular Ca²⁺ on 1,25(OH)₂D₃-stimulated Mg²⁺ uptake. A buffer solution containing high CaCl₂ (5.0 mM) was added with 1,25(OH)₂D₃ for 16–20 h before fluorescence measurements were made. In support of our earlier findings, high extracellular Ca²⁺ for 16–20 h did not alter basal Mg²⁺ uptake (165 ± 7 nM/s, $n = 4$) (Fig. 5). However, high extracellular Ca²⁺ inhibited 1,25(OH)₂D₃-stimulated Mg²⁺ uptake (166 ± 6 nM/s, $n = 4$). Accordingly, the addition of high extracellular Ca²⁺ with 1,25(OH)₂D₃ diminished vitamin D-mediated Mg²⁺ entry. These findings suggest that signaling via the CaSR may alter 1,25(OH)₂D₃-induced synthesis of proteins involved in Mg²⁺ transport.

CaSR antibody blocks extracellular Ca²⁺ inhibition of 1,25(OH)₂D₃-stimulated Mg²⁺ uptake. If elevation of extracellular Ca²⁺ inhibits 1,25(OH)₂D₃-stimulated transport through CaSR-mediated transcriptional processes, then blocking this receptor should abolish the chronic effects of extracellular calcium. To do this, MDCT cells were incubated with a specific CaSR anti-

Fig. 3. 1,25(OH)₂D₃-stimulated Mg²⁺ uptake requires transcriptional/translational processes. Values are means ± SE. **A:** MDCT cells were Mg²⁺ depleted for 16 h, and 10⁻⁷ M 1,25(OH)₂D₃ was then added with and without actinomycin D (5.0 μg/ml) as indicated for 4–5 h before fluorescence determinations. Mg²⁺ uptake was determined with microfluorescence according to methods described in the legend for Fig. 1. **B:** MDCT cells were treated with 10⁻⁷ M 1,25(OH)₂D₃ for 16 h before the determination of Mg²⁺ uptake. In those cells indicated, cycloheximide (Cyclo; 1.0 μg/ml) was added with 1,25(OH)₂D₃ 16 h before Mg²⁺ uptake and cAMP determinations. Actinomycin D and cycloheximide were added from stock solutions of DMSO; the final DMSO concentration did not exceed 0.01% (vol/vol), which had no effect on Mg²⁺ uptake. Significantly different from control values, **P* < 0.01.



body. To substantiate that the CaSR was blocked, we measured the effect of extracellular Ca²⁺ and Mg²⁺ on PTH-mediated cAMP formation in control cells and in those pretreated with the CaSR antibody. PTH stimulated intracellular cAMP accumulation by about threefold (72 ± 8 pmol·mg protein⁻¹·5 min⁻¹) above control values (25 ± 1 pmol·mg protein⁻¹·5 min⁻¹). The presence of 5.0 mM Ca²⁺ or 5.0 mM Mg²⁺ abolished PTH-stimulated cAMP formation (33 ± 2 and 29 ± 1 pmol·mg protein⁻¹·5 min⁻¹, respectively) (Fig. 6). Preincubation of MDCT cells with the antibody prevented the effect of extracellular Ca²⁺ (60 ± 5 pmol·mg protein⁻¹·5 min⁻¹) or Mg²⁺ (72 ± 8 pmol·mg protein⁻¹·5 min⁻¹) on PTH-sensitive cAMP release (72 ± 8 pmol·mg protein⁻¹·5 min⁻¹). Accordingly, the antibody blocked the activation of the CaSR and the inhi-

biton of hormone-mediated cAMP formation. With this knowledge, we determined whether antibody blockade of the CaSR would inhibit the effect of elevated Ca²⁺ on 1,25(OH)₂D₃-stimulated Mg²⁺ uptake. We depleted MDCT cells of Mg²⁺ for 16 h before experimentation and incubated them with ADD antiserum to the CaSR for 2 h. The cells were then treated with 1,25(OH)₂D₃ with and without 5.0 mM CaCl₂ for an additional 4–5 h before microfluorescence studies were performed. MDCT cells treated with the antibody responded normally to 1,25(OH)₂D₃ (210 ± 11 nM/s, *n* = 5) regardless of the presence of elevated extracellular Ca²⁺ (202 ± 8 nM/s, *n* = 4) (Fig. 7). This study indicates that extracellular Ca²⁺ chronically inhibits 1,25(OH)₂D₃-mediated Mg²⁺ uptake through the activation of CaSR-mediated mechanisms.

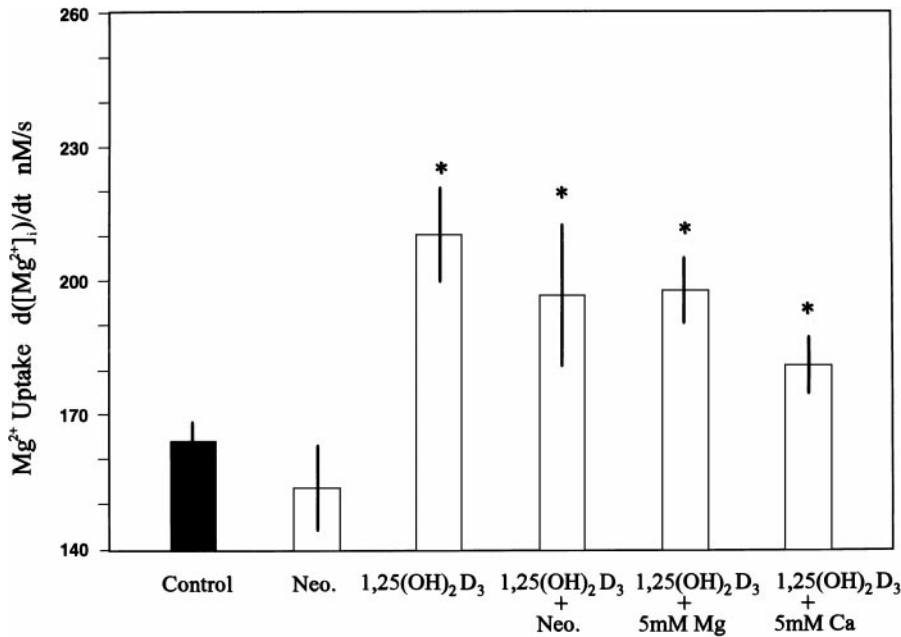


Fig. 4. Summary of the acute effects of cation-sensing receptor (CaSR) activation on 1,25(OH)₂D₃-stimulated Mg²⁺ uptake. Values are means \pm SE for 3–5 cells. Measurement $d([Mg^{2+}]_i)/dt$ was performed in Mg²⁺-depleted cells treated with or without 1,25(OH)₂D₃ for 16 h before performance of fluorescence. Mg²⁺ uptake was determined with 1.5 mM extracellular Mg²⁺ in the absence or presence of 50 μ M extracellular neomycin (Neo); 5.0 mM Mg²⁺ or 5.0 mM Ca²⁺ was added 5 min before determination of Mg²⁺ uptake. In the studies that tested high levels of extracellular Mg²⁺, 5.0 rather than 1.5 mM MgCl₂ was used. The studies were performed as described in the legend for Fig. 1. The Mg²⁺ uptake rate was determined over 500 s after addition of 1.5 or 5.0 mM Mg²⁺. Significantly different from control values, * $P < 0.05$.

Antisense ODN to the CaSR blocks extracellular Ca²⁺ inhibition of 1,25(OH)₂D₃-stimulated Mg²⁺ uptake. To substantiate the antibody studies, we used antisense ODN technology to block CaSR receptor expression and its function. The MDCT cells were loaded with sense or antisense CaSR ODNs. Cells with antisense ODN expressed little CaSR as determined by Western analysis (Fig. 8). In the representative experiment shown, the CaSR-to- β -tubulin densitometry ratio was 2.77 for no ODN; 2.6 for sense ODN; and 0.17, for antisense ODN, the last value representing 6.5% of the sense ODN ratio. Extracellular Ca²⁺ did not inhibit PTH-mediated cAMP formation in MDCT cells treated with antisense ODN to the CaSR (67 ± 1 pmol·mg protein⁻¹·5 min⁻¹), whereas the response in cells with

sense ODN was similar to that observed with control cells (32 ± 2 pmol·mg protein⁻¹·5 min⁻¹) (Fig. 9). Accordingly, antisense ODN blocks expression and function of the CaSR. We used this model to test whether extracellular Ca²⁺ diminishes 1,25(OH)₂D₃-stimulated Mg²⁺ uptake through the CaSR. Mg²⁺ uptake in sense ODN-treated cells was $115 \pm 2\%$ ($n = 5$) of control cells (Fig. 10). 1,25(OH)₂D₃ stimulated Mg²⁺ entry by $189 \pm 50\%$ ($n = 4$) in the sense CaSR ODN-treated cells in the presence of normal extracellular Ca²⁺ concentration (1.0 mM). Elevated extracellular Ca²⁺ (5.0 mM) inhibited 1,25(OH)₂D₃-stimulated Mg²⁺ uptake ($87 \pm 27\%$, $n = 6$) in these cells. In contrast, 1,25(OH)₂D₃-stimulated Mg²⁺ uptake was $172 \pm 31\%$ ($n = 6$) in cells loaded with antisense ODN in the pres-

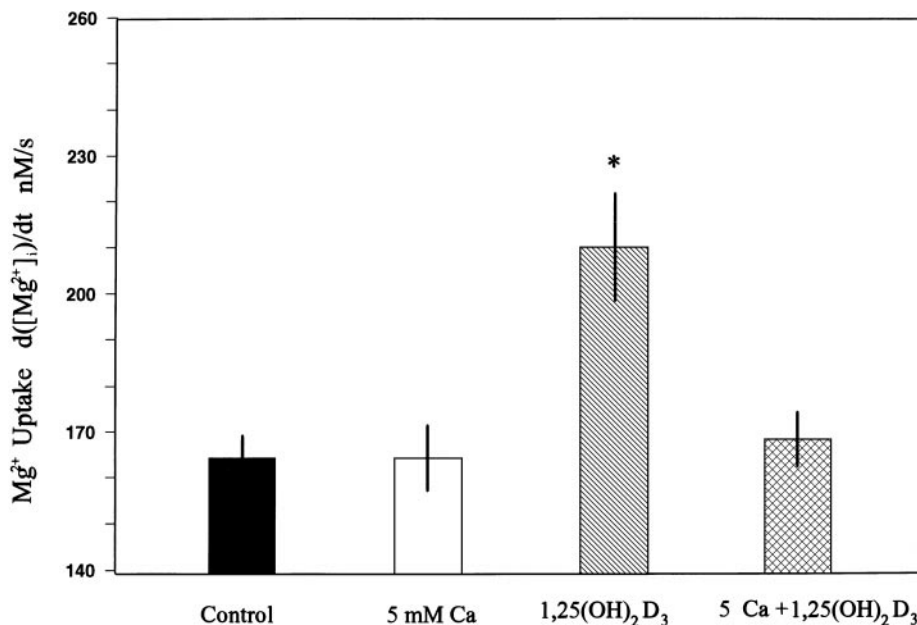
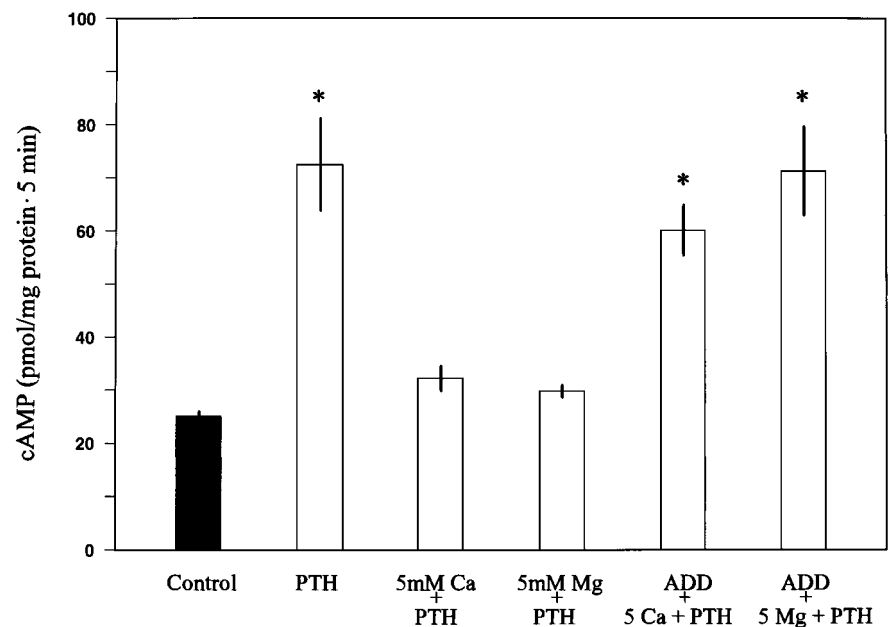


Fig. 5. Chronic activation of CaSR-sensing inhibits 1,25(OH)₂D₃-stimulated Mg²⁺ uptake. Values are means \pm SE for 3–5 cells. MDCT cells were treated with 10^{-7} M 1,25(OH)₂D₃ with and without 5.0 mM CaCl₂ for 16 h before microfluorescence measurements. Mg²⁺ uptake was determined with 1.5 mM extracellular Mg²⁺ in the absence or presence of 5.0 mM Ca²⁺ as indicated. We have previously shown that high extracellular Ca²⁺ does not affect basal Mg²⁺ uptake; i.e., the entry pathway is selective for Mg²⁺ (43). The studies were performed as described in the legend for Fig. 1. The Mg²⁺ uptake rate was determined over 500 s after addition of 1.5 mM MgCl₂. Significantly different from control values, * $P < 0.01$.

Fig. 6. CaSR antibody blocks extracellular polyvalent cation modulation of parathyroid hormone (PTH)-mediated cAMP formation. Values are means \pm SE for 10 observations. In the studies indicated, MDCT cells were preincubated with 1.6 μ g/ml of a mouse monoclonal antibody (ADD) or 1.6 μ g/ml mouse IgG in a buffer solution containing 0.02% BSA and (in mM) 0.5 MgCl₂, 1.0 CaCl₂, 145 NaCl, 4.0 KCl, 0.8 K₂HPO₄, 0.2 KH₂PO₄, 5.0 glucose, and 20 HEPES-Tris, pH 7.4, for 2 h before measurement of intracellular PTH-stimulated cAMP. Where indicated, either 5.0 mM CaCl₂ or 5.0 mM MgCl₂ was added for 5 min before PTH and cAMP concentrations were measured with radioimmunoassay after a further 5-min incubation period. Significantly different from control values, * P < 0.01.



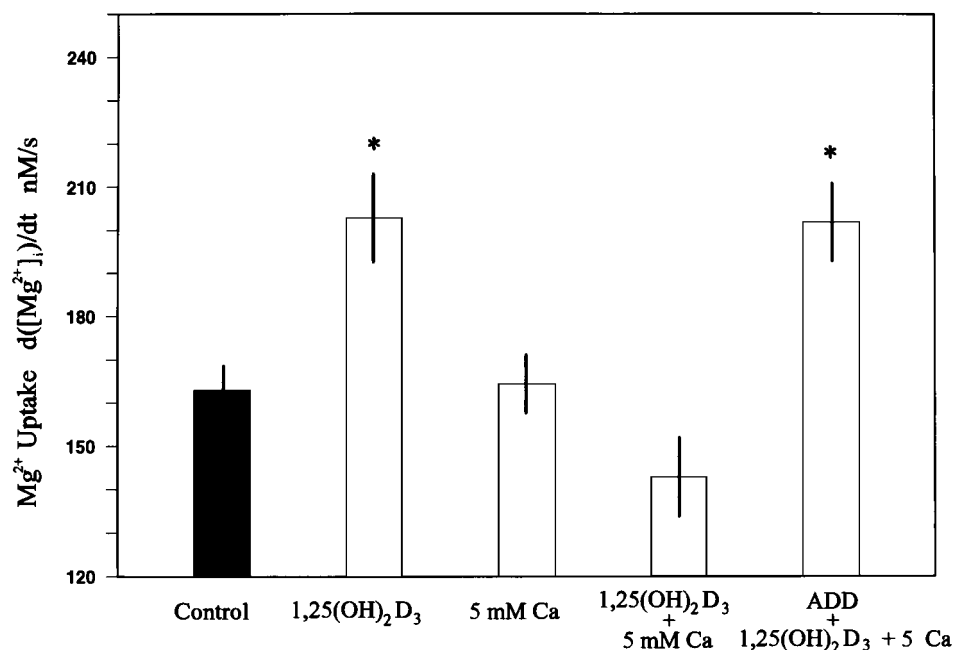
ence of normal Ca²⁺ and 181 \pm 26% (n = 9) in cells incubated with 5.0 mM Ca²⁺. Accordingly, the antisense CaSR ODN inhibits CaSR expression and mitigates the effect of extracellular Ca²⁺ on 1,25(OH)₂D₃-stimulated Mg²⁺ uptake. These studies, using antibody or antisense ODN technology, clearly indicate that extracellular Ca²⁺ chronically inhibits 1,25(OH)₂D₃-mediated Mg²⁺ uptake through activation of CaSR-mediated mechanisms.

DISCUSSION

The role of 1,25(OH)₂D₃ in renal magnesium reabsorption. Little is known about the effects of 1,25(OH)₂D₃ on tubular magnesium transport. On balance, urinary clearance data from clinical observations and experi-

mental studies indicate that 1,25(OH)₂D₃ may stimulate renal magnesium handling (1, 13, 25, 26, 30, 33, 36, 38, 39, 45). There have been no micropuncture studies performed to localize the actions of 1,25(OH)₂D₃ on magnesium transport. The data concerning the effects of 1,25(OH)₂D₃ on renal calcium absorption are more substantial but inconclusive (5, 7–9, 31, 49). The most convincing data demonstrating that 1,25(OH)₂D₃ may have a direct effect on calcium transport were obtained by using isolated cells. Bindels et al. (5) and Van Baal et al. (50) showed that 1,25(OH)₂D₃ increased calbindin D_{28k} protein levels and stimulated extracellular calcium absorption in primary cultures of rabbit distal connecting tubule and cortical collecting duct cells. The

Fig. 7. Specific antibody to the CaSR blocks extracellular Ca²⁺ inhibition of 1,25(OH)₂D₃-stimulated Mg²⁺ entry. Values are means \pm SE for 3–5 cells. MDCT cells were Mg²⁺ depleted for 16 h and incubated with CaSR mouse monoclonal antibody ADD (1.6 μ g/ml) for 2 h. The cells were then treated with 10⁻⁷ M 1,25(OH)₂D₃ with and without 5.0 mM CaCl₂ where indicated, and Mg²⁺ uptake measurement was performed with microfluorescence. The Mg²⁺ uptake rate was determined over 500 s after addition of 1.5 mM MgCl₂. Significantly different from control values, * P < 0.05.



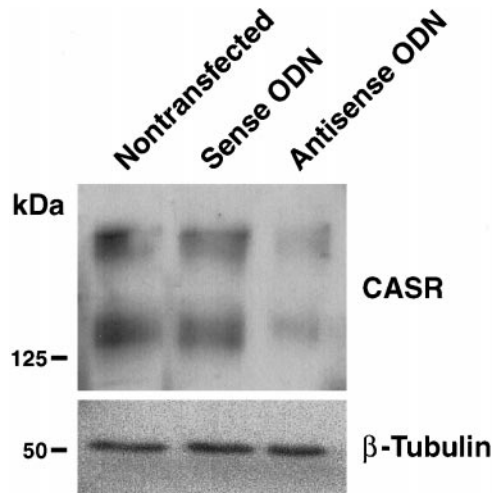


Fig. 8. Antisense oligodeoxynucleotides (ODN) to the CaSR diminish receptor expression in MDCT cells. Western analysis of the CaSR was performed in MDCT cells treated with sense or antisense ODNs at concentrations of 1 nmol/ml for 36–42 h before experimentation. Cell extracts (50 μ g protein) were subjected to SDS-PAGE on a 4–12% gradient gel. The blot was stained with CaSR mouse monoclonal antibody, and an identical blot was stained with β -tubulin antibody. Lane 1, control MDCT cells (no ODN); lane 2, sense ODN (CaSR sense); lane 3, antisense ODN (CaSR antisense). The CaSR bands were demonstrated to be specific after the blot was stained with the same antiserum preincubated with the peptide against which it was raised (data not shown). Western blots were performed twice with similar results.

maximal transport response occurred at \sim 48 h post-treatment, suggesting that the response involved initiation of transcriptional processes (5). The response to 1,25(OH)₂D₃ was independent of PTH and not additive to PTH-stimulated calcium transport. The above-mentioned authors also have biochemical evidence, as they showed that 1,25(OH)₂D₃ increased calbindin D_{28k}

mRNA and protein content without a change in Na⁺/Ca²⁺ exchange or Ca²⁺-ATPase RNA or protein in rabbit distal connecting tubule and cortical collecting duct cell preparations (50). Recently, Hoenderop et al. (29) have identified an apical Ca²⁺ channel in 1,25(OH)₂D₃-responsive cells that is responsible for calcium absorption in this segment. They have reported that 1,25(OH)₂D₃ stimulates Ca²⁺ transport in rabbit cortical collecting duct cells (29). The second study to clearly show effects of vitamin D metabolites on calcium transport was performed in MDCT cells by Friedman and Gesek (22). These co-workers reported that 1,25(OH)₂D₃ did not alter basal Ca²⁺ uptake but accelerated PTH-dependent calcium entry rates. This response was rapid, concentration dependent, significant at 2 h, maximal by 5 h, and mediated by translational processes because it was inhibited by cycloheximide (22). The reasons for the discrepancies between the above-mentioned two reports are not known; examples may be the different cell types or the techniques used to measure calcium transport in the two separate studies. Nevertheless, it is clear that 1,25(OH)₂D₃ increases calcium-binding protein in distal tubules, suggesting that it has significant actions on basal or hormone-mediated calcium transport. 1,25(OH)₂D₃ increases divalent cation-binding proteins, such as calbindin D_{9K} or calbindin D_{28k}, in distal tubule cells, including convoluted segment, connecting tubule, and collecting duct cells (34, 35, 46, 48). In a preliminary report, we have shown that 1,25(OH)₂D₃ stimulates calbindin D_{9K} in the MDCT cell line (14). The calbindin D group is thought to be involved, either directly or indirectly, in epithelial calcium transport (6–9, 21, 31, 49, 53). Experimental evidence also supports the notion that these proteins bind magnesium, and some investigators have postulated that they are involved in epithelial magnesium transport (2, 12, 28).

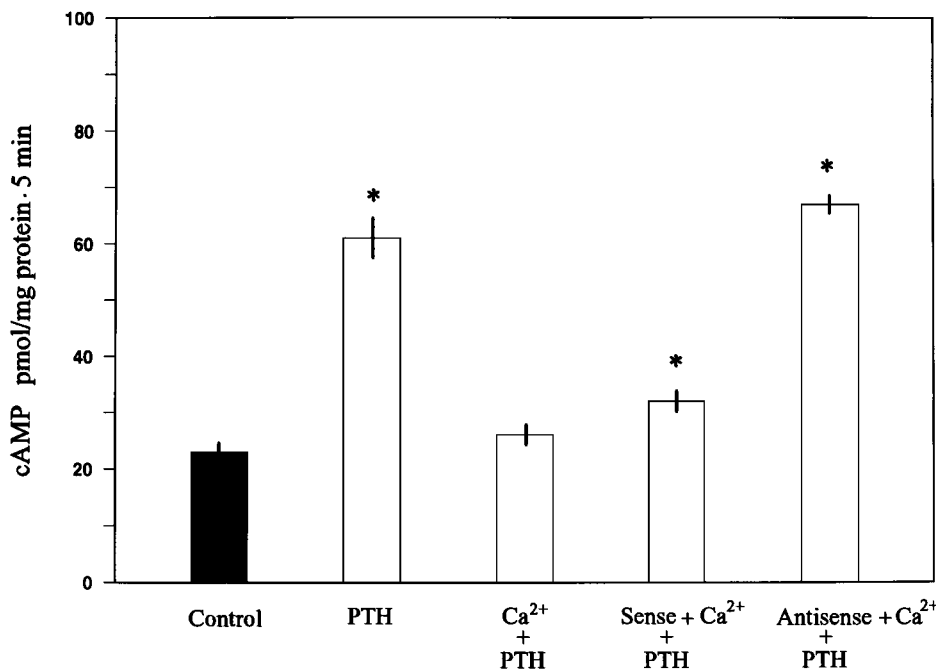
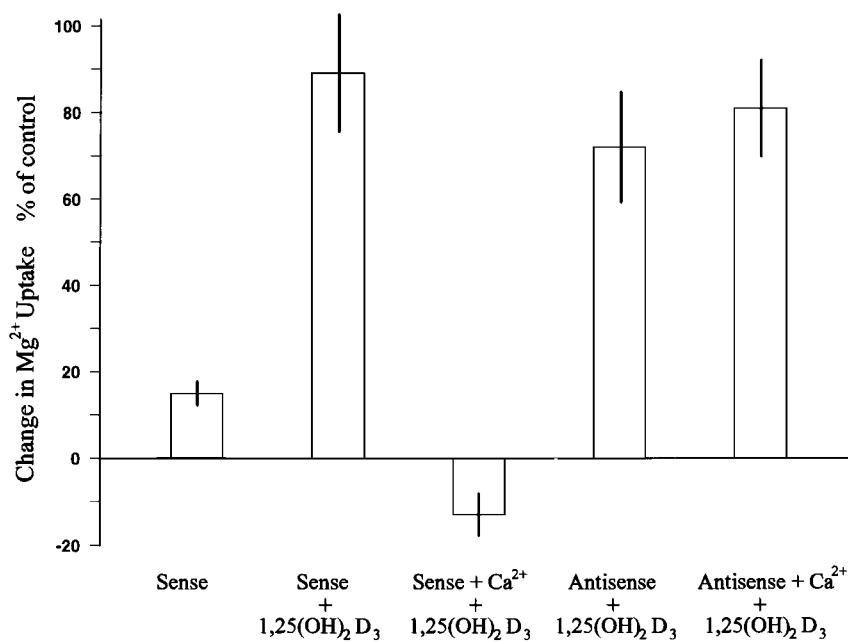


Fig. 9. Antisense CaSR ODN diminish CaSR inhibition of PTH-mediated cAMP formation. Values are means \pm SE for 6 individual observations. In the studies indicated, MDCT cells were loaded with sense or antisense ODN 72 h before measurement of intracellular PTH-stimulated cAMP. Where shown, 5.0 mM CaCl₂ was added for 5 min before PTH and cAMP concentrations were measured with radioimmunoassay after a further 5-min incubation period. Significantly different from control values, * P < 0.01.

Fig. 10. Antisense ODN to the CaSR diminishes extracellular Ca²⁺ inhibition of 1,25(OH)₂D₃-stimulated Mg²⁺ entry. Values are means \pm SE for 4–8 cells. MDCT cells were loaded with sense (S) or antisense (AS) ODN designed to the CaSR for 72 h before fluorescence studies. The cells were Mg²⁺ depleted for 16 h, treated with 10⁻⁷ M 1,25(OH)₂D₃ for 3–4 h with 5.0 mM CaCl₂. The Mg²⁺ uptake rate was determined over 500 s after addition of 1.5 mM MgCl₂.



The present studies with MDCT cells, a widely used model of the distal convoluted tubule, supports the notion that 1,25(OH)₂D₃ may affect renal magnesium conservation. The hormonally active vitamin D metabolite 1,25(OH)₂D₃ increases Mg²⁺ entry rates in MDCT cells in a concentration-dependent fashion (Fig. 2). The response involves de novo protein synthesis, as it was sensitive to actinomycin D and cycloheximide, inhibitors of transcription and translation, respectively (Fig. 3). The evidence supports the notion that 1,25(OH)₂D₃ stimulates the synthesis of proteins that are involved with Mg²⁺ entry, perhaps as yet uncharacterized Mg²⁺ channels (42). As the distal convoluted tubule is the terminal nephron segment providing final control of renal magnesium excretion, the influence of 1,25(OH)₂D₃ may play an important part in magnesium balance.

Effect of extracellular Mg²⁺/Ca²⁺ sensing on 1,25(OH)₂D₃-mediated Mg²⁺ uptake. The extracellular CaSR plays an important role in mineral balance by controlling, among other things, parathyroid gland function and renal calcium and magnesium reabsorption (11, 27, 42). The CaSR mediates a host of intracellular signaling pathways. The CaSR has been best characterized in parathyroid gland cells (11). In the parathyroid, CaSR regulates PTH secretion and activates multiple intracellular pathways involving activation of G α -coupled proteins and changes in gene expression among others (11). The ways that CaSR activation influences salt and water transport in the kidney are presently being addressed. Wang et al. (52) showed that the elevation of extracellular calcium inhibits NaCl transport in the thick ascending limb by release of cytochrome P-450 metabolites. We have demonstrated in immortalized MDCT cells that the CaSR initiates intracellular Ca²⁺ transients and inhibits PTH-, glucagon-, and AVP-mediated cAMP formation

and magnesium transport, probably by activating G α _i-proteins (3, 4, 17). Finally, Sands et al. (47) reported that extracellular calcium inhibits AVP-stimulated cAMP induction of water transport in the inner medullary collecting duct, again likely through G α _i-protein mediation. Accordingly, the CaSR may modulate epithelial cell functions in many cell types by different intracellular signaling pathways.

Elevation of extracellular Ca²⁺ inhibits 1,25(OH)₂D₃-stimulated Mg²⁺ uptake in MDCT cells (Fig. 5). The argument for modulation by the CaSR of 1,25(OH)₂D₃-induced transcriptional/translational processes involved with Mg²⁺ transport is persuasive. First, we show that 1,25(OH)₂D₃-mediated Mg²⁺ uptake is responsive to actinomycin D and cycloheximide (Fig. 3). The acute, short-term treatment of MDCT cells with 1,25(OH)₂D₃ does not elicit changes in Mg²⁺ entry rates, so that a minimum of 3 h was required; most of the present studies were performed after 16 h of 1,25(OH)₂D₃ treatment. Second, acute elevation of extracellular Ca²⁺ does not affect 1,25(OH)₂D₃-stimulated Mg²⁺ uptake (Fig. 4). Third, blockade of the CaSR with a specific antibody or prevention of CaSR expression with an antisense ODN abolished the action of high extracellular Ca²⁺ on 1,25(OH)₂D₃-responsive Mg²⁺ transport (Figs. 7 and 10). Taken together, these data indicate that 1,25(OH)₂D₃-stimulated Mg²⁺ entry into MDCT cells is modulated by extracellular polyvalent cations. Thus the presence and magnitude of any 1,25(OH)₂D₃ responses are dependent on the existing divalent cation concentration.

Role of 1,25(OH)₂D₃ in overall renal magnesium balance. 1,25(OH)₂D₃ increases intestinal calcium and magnesium absorption and renal conservation, leading to positive divalent cation balance. Elevation of serum calcium and magnesium concentrations, in turn, diminish renal absorption through the actions of the

CaSR on electrolytes in the loop and distal convoluted tubule and water absorption in the medullary collecting ducts (27, 41, 42, 44, 47). Activation of the CaSR modulates the excessive absorption of divalent cations, providing a negative-feedback control to effect normal mineral balance. This may also explain the discrepant reports in the literature concerning the effects of vitamin D metabolites on renal magnesium transport.

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