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Human Calcium-sensing Receptor Gene. VITAMIN D RESPONSE ELEMENTS IN PROMOTERS P1 AND P2 CONFER TRANSCRIPTIONAL RESPONSIVENESS TO 1,25-DIHYDROXYVITAMIN D

L. Canaff and G. N. Hendy
J. Biol. Chem., August 9, 2002; 277 (33): 30337-30350.

[Abstract] [Full Text] [PDF]

Adenosine modulates Mg²⁺ uptake in distal convoluted tubule cells via A1 and A2 purinoceptors

H. S. Kang, D. Kerstan, L.-J. Dai, G. Ritchie and G. A. Quamme
Am J Physiol Renal Physiol, December 1, 2001; 281 (6): F1141-F1147.

[Abstract] [Full Text] [PDF]

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

G. Ritchie, D. Kerstan, L.-J. Dai, H. S. Kang, L. Canaff, G. N. Hendy and G. A. Quamme
Am J Physiol Renal Physiol, May 1, 2001; 280 (5): F868-F878.

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Mg²⁺/Ca²⁺ sensing inhibits hormone-stimulated Mg²⁺ uptake in mouse distal convoluted tubule cells

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Bapty, Brian W., Long-jun Dai, Gordon Ritchie, Lucie Canaff, Geoffrey N. Hendy, and Gary A. Quamme. Mg²⁺/Ca²⁺ sensing inhibits hormone-stimulated Mg²⁺ uptake in mouse distal convoluted tubule cells. *Am. J. Physiol.* 275 (*Renal Physiol.* 44): F353–F360, 1998.—The distal convoluted tubule plays a significant role in renal magnesium conservation. An immortalized mouse distal convoluted tubule (MDCT) cell line has been extensively used to study the cellular mechanisms of magnesium transport in this nephron segment. MDCT cells possess an extracellular polyvalent cation-sensing mechanism responsive to Mg²⁺, Ca²⁺, and neomycin. The present studies determined the effect of Mg²⁺/Ca²⁺ sensing on hormone-mediated cAMP formation and Mg²⁺ uptake in MDCT cells. MDCT cells were Mg²⁺ depleted by culturing in Mg²⁺-free media for 16 h, and Mg²⁺ uptake was measured by microfluorescence after placing the depleted cells in 1.5 mM MgCl₂. The mean rate of Mg²⁺ uptake was 164 ± 5 nM/s in control MDCT cells. Activation of Mg²⁺/Ca²⁺ sensing with neomycin did not affect basal Mg²⁺ uptake (155 ± 5 nM/s). We have previously reported that treatment of MDCT cells with either glucagon or arginine vasopressin (AVP) stimulated Mg²⁺ entry. In the present studies, the addition of extracellular Mg²⁺ or Ca²⁺ inhibited glucagon- and AVP-stimulated cAMP formation and Mg²⁺ uptake in concentration-dependent manner with half-maximal concentrations of ~1.5 and 3.0 mM, respectively. Exogenous cAMP or forskolin stimulated Mg²⁺ uptake in the presence of Mg²⁺/Ca²⁺ sensing activation. We infer from these studies that Mg²⁺/Ca²⁺-sensing mechanisms located in the distal convoluted tubule may play a role in control of distal magnesium absorption.

intracellular magnesium; magnesium uptake; fluorescence; extracellular calcium; extracellular magnesium; neomycin; adenosine 3',5'-cyclic monophosphate measurements; glucagon; arginine vasopressin; neomycin

THE DISTAL TUBULE of the nephron plays a significant role in control of renal divalent cation absorption (21). About 10% of the filtered magnesium and calcium is reabsorbed in the distal segments including the distal convoluted tubule, connecting tubule, and initial collecting tubule (2, 13, 14). Absorption is transcellular and active in nature in this segment and as such is controlled by mechanisms acting within the cells comprising the distal tubule (21). This is also the site of action of hormones involved with control of divalent cation transport including parathyroid hormone (PTH), calcitonin, glucagon, and arginine vasopressin (AVP) (1, 9, 12, 13, 14). Using microfluorescence determinations, we have recently shown that glucagon and AVP stimu-

late Mg²⁺ entry into immortalized mouse distal convoluted tubule (MDCT) cells (9). As this cell line possesses many of the properties of intact distal convoluted tubule including sensitivity to the distal diuretics, amiloride and chlorothiazide, and hormone-stimulated calcium and magnesium transport, use of MDCT cells may allow us to determine the control of magnesium transport in this nephron segment (9, 14). Study of electrolyte transport in the intact distal convoluted tubule is not easy because of its inaccessibility and difficulty in isolation for in vitro microperfusion. Accordingly, study of this cell line may be useful in determining regulatory controls of Mg²⁺ uptake in the distal convoluted tubule. Glucagon and AVP enhance Mg²⁺ uptake in MDCT cells, in part, through cAMP-dependent pathways (9). These in vitro studies emphasize the importance of the convoluted segment of the distal tubule and the complexity of peptide hormone interactions in renal magnesium conservation. Control of distal magnesium transport involves the concerted actions of the various hormones, many of which are associated with changes in extracellular divalent cation concentrations (1, 12, 13, 21).

An extracellular Ca²⁺-sensing receptor (*Casr*), responsive to polyvalent cations such as Mg²⁺, Gd³⁺, and neomycin, in addition to Ca²⁺, has been demonstrated in many tissues and many species (6). This G protein-coupled receptor was first cloned by Brown et al. (3) from the bovine parathyroid gland, where it is involved with control of PTH secretion. The receptor is comprised of three major domains: 1) a large extracellular amino-terminal domain consisting of 613 amino acids, which is thought to possess the cation binding sites; 2) a 250-amino acid domain with seven predicted membrane-spanning segments characteristic of the superfamily of G protein-coupled receptors; and 3) a carboxy-terminal domain of 222 amino acids that likely resides within the cytoplasm and is involved with intracellular signaling processes (6). The evidence is that extracellular Ca²⁺ concentration ([Ca²⁺]_o) binds to the extracellular domain, initiating a number of intracellular signals; among other things, stimulation of G_i proteins modulates adenylate cyclase activity and cAMP levels (4, 8, 18), and G_q proteins activate phospholipase C releasing inositol 1,4,5-trisphosphate and cytosolic Ca²⁺ (7). *Casr*-mediated intracellular signaling pathways have been reported to have important effects on cellular function (15, 25, 30).

In a recent study, we showed that MDCT cells also possess a polyvalent cation-sensing mechanism that is responsive to extracellular Mg²⁺ and Ca²⁺ (3). Southern hybridization and sequence determination of RT-PCR products as well as Western analysis indicated that the *Casr* is expressed in MDCT cells. Using microfluorescence of single MDCT cells to determine cytosolic Ca²⁺ signaling, we have shown that the polyvalent cation-sensing mechanism is sensitive to extracellular Mg²⁺ and Ca²⁺ in concentration ranges normally observed in the plasma. Moreover, both extracellular Mg²⁺ and Ca²⁺ were effective in generating intracellular Ca²⁺ transients in the presence of large extracellular concentrations of Ca²⁺ and Mg²⁺, respectively (3). As these responses are unlike those observed for the parathyroid gland *Casr*, we postulated that different or additional Mg²⁺/Ca²⁺-sensing mechanisms may be present in MDCT cells (5, 7). We also showed that activation of the polycation-sensitive mechanism with either extracellular Mg²⁺ or Ca²⁺ inhibited PTH-, calcitonin-, glucagon-, and AVP-stimulated cAMP release in MDCT cells (3). These studies indicated that immortalized MDCT cells possess a polyvalent cation-sensing mechanism and emphasized the important role this mechanism plays in modulating intracellular signals in response to changes in extracellular Mg²⁺ as well as Ca²⁺. In the present study, we show that activation of Mg²⁺/Ca²⁺ sensing in MDCT cells inhibits hormone-stimulated Mg²⁺ entry in these cells. We infer from these observations that the Mg²⁺/Ca²⁺-sensing mechanism plays a significant role in controlling Mg²⁺ absorption within the distal convoluted tubule of the nephron.

METHODS

Materials. Basal DMEM and Ham's F-12 media were purchased from GIBCO Laboratories, Grand Island, NY. FCS was from Flow Laboratories (McLean, VA). Mag-fura-2-AM was obtained from Molecular Probes (Eugene, OR). Glucagon, AVP, aldosterone, and other materials were from Sigma, St. Louis, MO.

Cell culture. Immortalized MDCT cells were kindly provided to us by Dr. P. A. Friedman, Dartmouth Medical School. They have been extensively characterized by Drs. P. A. Friedman and F. A. Gesek (14). The MDCT cell line was cultured in DMEM-Ham's F-12, 1:1, media 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 the cAMP determinations, the MDCT cells were cultured to confluence in 24-well plastic dishes. Sixteen hours prior to the cAMP measurements, 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 prior to study. BSA, 0.2%, replaced FCS during this period. Other constitu-

ents 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 (3). After addition of various hormones, MDCT cells were incubated at 37°C for 5 min in the presence of 0.1 mM IBMX. The cAMP was extracted with 5% trichloroacetic acid, which was removed with ether acidified with 0.1 N HCl. The aqueous phase was dried and dissolved in Tris-EDTA buffer, and cAMP was measured with a radioimmunoassay kit (Diagnostic Products, Los Angeles, CA).

Cytoplasmic Mg²⁺ measurements. Coverslips with attached subconfluent cells were mounted into a perfusion chamber. For determination of intracellular Mg²⁺ concentration ([Mg²⁺]_i), they were incubated with 5 µM mag-fura-2-AM dissolved in Pluronic acid F-127 (0.125%; Molecular Probes) in media for 20 min at 37°C. The cells were subsequently washed three times with buffered salt solution containing (in mM) 145 NaCl, 4.0 KCl, 0.8 K₂HPO₄, 0.2 KH₂PO₄, 1.0 CaCl₂, and 20 HEPES-Tris, at pH 7.4. The MDCT cells were incubated for a further 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 fluorescence was monitored under oil immersion within a single cell over the course of study. Fluorescence was recorded at 1-s intervals 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 an interruption in recording.

The free [Mg²⁺]_i was calculated from the ratio of the fluorescence at the two excitation wavelengths as previously described (10) 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 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.

Transmembrane voltage measurements. Transmembrane voltage was measured with the use of the voltage-sensitive dye, 3,3'-dihexyloxycarbocyanine iodide, DiOC₆ (10). The intracellular dye was excited at 490 nm, and the emission was measured at 510 nm. The voltage-sensitive dye was calibrated by altering the transmembrane K⁺ gradient with sequential additions of small volumes of 1 M KCl in the presence of 5 µM valinomycin, and the transmembrane voltage was calculated from the fluorescent changes and the K⁺ distribution across the membrane.

Statistical analysis. Representative tracings of fluorescence intensity ratios are given, and significance was determined by Tukey's analysis of variance where indicated. Comparisons between groups of data were made using Student's *t*-test. *P* < 0.05 was taken to be statistically significant. All results are means ± SE where indicated.

RESULTS

Activation of the Mg²⁺/Ca²⁺-sensing mechanism inhibits hormone-stimulated cAMP release. We have reported that activation of the Mg²⁺/Ca²⁺-sensing mecha-

nism in MDCT cells with neomycin or high extracellular concentrations of Mg²⁺ or Ca²⁺ inhibited PTH-, calcitonin-, glucagon-, and AVP-stimulated cAMP accumulation in MDCT cells (3). Table 1 summarizes the effects of activation of the Mg²⁺/Ca²⁺-sensing mechanism on hormone-stimulated cAMP accumulation in normal MDCT cells. Glucagon, 10⁻⁷ M, and AVP, 10⁻⁸ M, increased cellular cAMP accumulation from control values of 19 ± 1 to 105 ± 5 and 71 ± 2 pmol·mg protein⁻¹·5 min⁻¹, respectively. Addition of neomycin, 50 μM, 5 min prior to the cAMP determinations abolished hormone-stimulated cAMP release (Table 1). Similarly, 10 mM [Mg²⁺]_o or 10 mM [Ca²⁺]_o completely inhibited glucagon- and AVP-dependent cAMP accumulation. Gd²⁺, Ni²⁺, Ba²⁺, and La³⁺ also inhibited glucagon-stimulated cAMP formation, indicating that polyvalent cation sensing in MDCT cells is similar to that observed in the parathyroid gland (4, 5, 8, 18).

Next, we determined the concentration dependence of extracellular Mg²⁺ or Ca²⁺ inhibition on glucagon-stimulated cAMP release. Figure 1 summarizes these results. The concentration of extracellular Mg²⁺ required for half-maximal inhibition was ~1.5 mM, and glucagon-stimulated cAMP release was completely inhibited at ~2.5 mM MgCl₂. These studies were performed with normal Ca²⁺ concentration, 1.0 mM, in the bathing solution. Extracellular Ca²⁺ was less potent, as it maximally inhibited hormone-related cAMP release at 5.0 mM with a half-maximal inhibition at ~3.0 mM. Accordingly, the Mg²⁺/Ca²⁺-sensing mechanism in MDCT cells is responsive within the physiological concentration range of these divalent cations, and the potencies of extracellular Mg²⁺ and Ca²⁺ are equivalent. It is of interest that these results were obtained in the presence of normal concentrations of either Ca²⁺, 1.0 mM, or Mg²⁺, 0.5 mM, in the respective experiments. Similar results were obtained with AVP-mediated cAMP release (data not shown).

Table 1. Activation of Mg²⁺/Ca²⁺ sensing inhibits hormone-stimulated cAMP accumulation

	cAMP Release, pmol·mg protein ⁻¹ ·5 min ⁻¹		
	Control	Glucagon	AVP
Control	19 ± 1 (11)	105 ± 5* (7)	71 ± 2* (5)
Neomycin	22 ± 2 (3)	22 ± 1† (7)	24 ± 3† (4)
Mg _o ²⁺	24 ± 3 (3)	37 ± 3*† (3)	27 ± 3† (3)
Ca _o ²⁺	23 ± 2 (3)	32 ± 2*† (3)	34 ± 3*† (3)
Gd _o ²⁺	22 ± 1 (5)	34 ± 1*† (5)	34 ± 2*† (5)
Ni _o ²⁺	25 ± 1 (5)	41 ± 3*† (5)	34 ± 2*† (5)
Ba _o ²⁺	24 ± 1 (5)	39 ± 2*† (5)	33 ± 1*† (5)
La _o ³⁺	24 ± 0.3 (5)	32 ± 1*† (5)	27 ± 1*† (5)

Values are means ± SE; number of observations is in parentheses. Where indicated, neomycin, 50 μM, or extracellular Mg²⁺ (Mg_o²⁺, 10 mM), Ca_o²⁺ (10 mM), Gd_o²⁺ (0.5 mM), Ni_o²⁺ (0.5 mM), Ba_o²⁺ (0.5 mM) or La_o³⁺ (0.5 mM), were added 5 min prior to the addition of either glucagon, 10⁻⁷ M, or arginine vasopressin, 10⁻⁸ M, and cAMP was measured 5 min after addition of the respective hormones. *P < 0.01, significance of hormone-treated vs. values without hormone treatment. †P < 0.01, significance of hormone-treated values in presence of neomycin or Ca²⁺ or Mg²⁺ vs. hormone-treated values in absence of pretreatment with the polyvalent cations. AVP, arginine vasopressin.

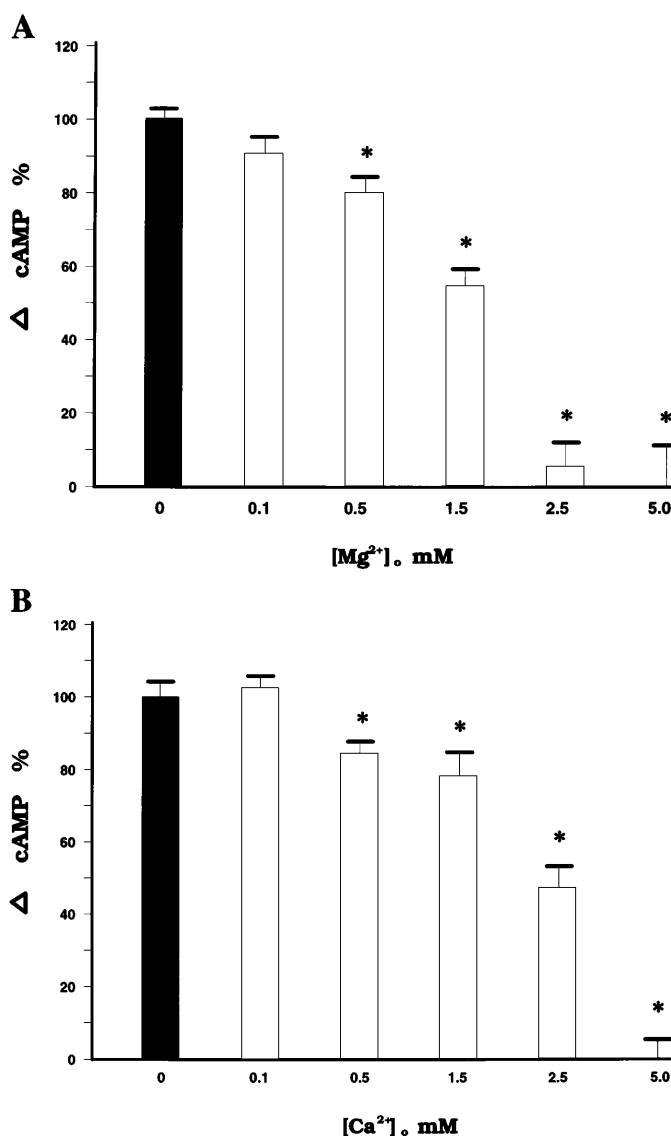


Fig. 1. Concentration dependence of extracellular Mg²⁺ or Ca²⁺ inhibition of glucagon-stimulated cAMP. Mouse distal convoluted tubule (MDCT) cells were cultured in DMEM-Ham's F-12 (1:1) with 0.2% BSA containing 0.6 mM magnesium and 1.5 mM calcium. At the time of experimentation, the cells were washed with a buffer solution containing (in mM) 0.5 MgCl₂, 1.0 CaCl₂, 145 NaCl, 4.0 KCl, 0.8 K₂HPO₄, 0.2 KH₂PO₄, 5 glucose, and 20 HEPES-Tris, pH 7.4. A: to test the effect of extracellular Mg²⁺ concentration ([Mg²⁺]_o), the buffer solution was changed to one identical to the above but without MgCl₂. This bathing solution was replaced 10 min later with one containing the indicated MgCl₂ concentrations. B: to test [Ca²⁺]_o, the cells were initially bathed with the above solutions containing no CaCl₂. This was replaced with one containing the indicated concentrations. Five minutes following the addition of either MgCl₂ or CaCl₂, 10⁻⁷ M glucagon was added, and cAMP was measured following a 5-min incubation period. Values are means ± SE for 2–3 experiments consisting of 5 individual observations each. *P < 0.01, significantly different from control values.

Activation of Mg²⁺/Ca²⁺ sensing diminishes hormone-stimulated Mg²⁺ uptake into Mg²⁺-depleted MDCT cells. Since there is not an appropriate radioisotope for Mg²⁺ to directly measure magnesium transport rates, we developed the following model to assess Mg²⁺ influx into single MDCT cells (10). Subconfluent MDCT mono-

layers were cultured in magnesium-free medium for 16 h. These cells possessed a significantly lower $[Mg^{2+}]_i$, 0.22 ± 0.01 mM, than that observed in normal MDCT cells, 0.53 ± 0.02 mM (Fig. 2). When the Mg²⁺-depleted MDCT cells were placed in a bathing solution containing 1.5 mM MgCl₂, the $[Mg^{2+}]_i$ increased with time and plateaued at 0.52 ± 0.06 mM ($n = 9$), which was similar to that observed for normal cells (10). The mean rate of refill, $d([Mg^{2+}]_i)/dt$, measured as the change in $[Mg^{2+}]_i$ with time, was 164 ± 5 nM/s ($n = 6$ experiments), as determined over the first 500 s following addition of magnesium. We have previously reported data that indicate the Mg²⁺ uptake is concentration dependent and selective for Mg²⁺ (10). We have further shown that glucagon and AVP stimulates Mg²⁺ entry into Mg²⁺-depleted MDCT cells by 15–20% over basal entry rates (9). Glucagon and AVP stimulates Mg²⁺ entry without changes in transmembrane voltage (-64.7 ± 0.9 mV, $n = 5$).

In the initial studies, we used neomycin to activate the Mg²⁺/Ca²⁺-sensing mechanism (3, 5, 30). Neomycin was added ~5 min prior to the addition of 1.5 mM MgCl₂ for measurement of Mg²⁺ uptake. The addition of neomycin, 50 μM, to the extracellular buffer solution had no effect on basal Mg²⁺ uptake (155 ± 5 nM/s, $n = 4$) into Mg²⁺-depleted MDCT cells (Fig. 2). However, pretreatment of cells with neomycin inhibited glucagon-stimulated Mg²⁺ entry. The mean Mg²⁺ uptake rate of glucagon-treated cells was 196 ± 11 nM/s ($n = 5$), and

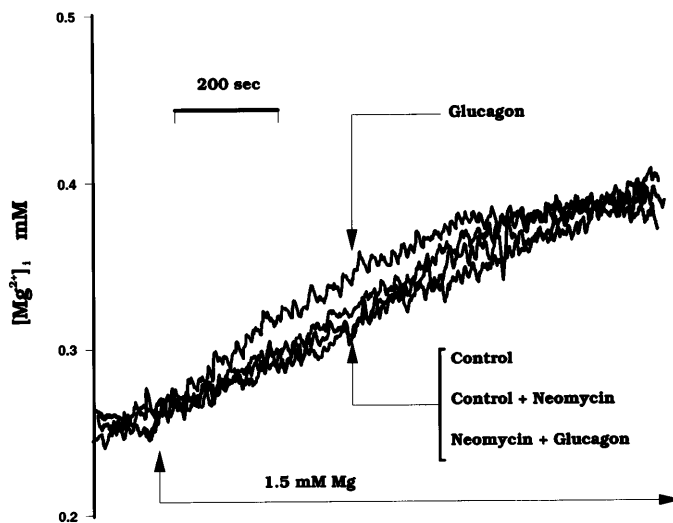


Fig. 2. Activation of the Mg²⁺/Ca²⁺-sensing mechanism diminishes hormone-stimulated Mg²⁺ uptake into MDCT cells. Intracellular magnesium concentration ($[Mg^{2+}]_i$) was determined with mag-fura-2. Cells were cultured in media containing no magnesium (magnesium concentration <0.01 mM) for 16 h. Basal $[Mg^{2+}]_i$ was determined, and the cells were subsequently placed in buffer solution containing 1.5 mM MgCl₂ at the time indicated. 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. Where indicated, 50 μM neomycin was added 5 min prior to the addition of hormone and 1.5 mM MgCl₂. Fluorescence was measured at 1 data point/s with 25 signal-point averaging and was smoothed according to methods previously reported (10). Magnesium uptake, $d([Mg^{2+}]_i)/dt$, was 196 nM/s with glucagon and 165 nM/s in the presence of neomycin. These fluorescence tracings are representative of 9 cells.

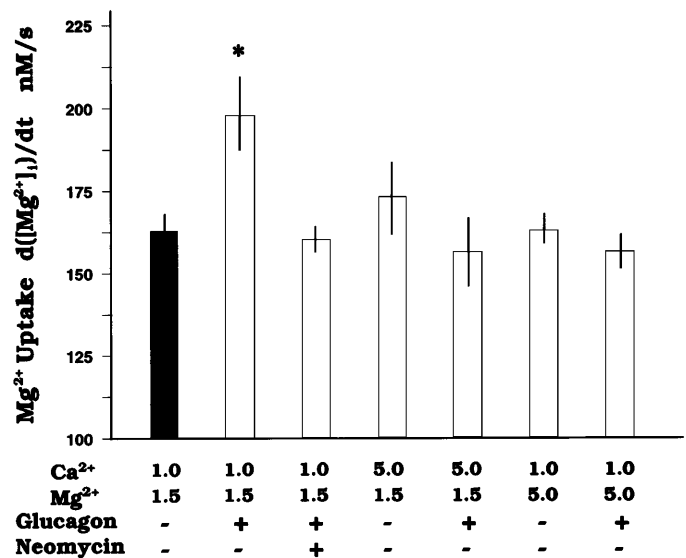


Fig. 3. Summary of the effects of Mg²⁺/Ca²⁺-sensing mechanism activation on hormone-stimulated Mg²⁺ uptake. Mg²⁺ uptake, $d([Mg^{2+}]_i)/dt$, was determined with 1.5 mM extracellular Mg²⁺ in absence (-) and presence (+) of 50 μM neomycin, 5.0 mM Ca²⁺, and 5.0 mM Mg²⁺, as indicated. Neomycin, Ca²⁺, or Mg²⁺ was added 5 min prior to the addition of 10⁻⁷ M glucagon and 1.5 mM MgCl₂. Studies were performed as given in legend to Fig. 2. Mg²⁺ uptake rate was determined over 500 s following addition of glucagon. Values are means \pm SE for 3–5 cells. * $P < 0.05$, significantly different from control values.

pretreatment with 50 μM neomycin diminished uptake to 162 ± 3 nM/s ($n = 3$), which was not different from control uptake rates (Fig. 3). Accordingly, activation of Mg²⁺/Ca²⁺-sensing mechanism with neomycin inhibits glucagon-stimulated Mg²⁺ entry. Neomycin, 50 μM, does not alter the membrane voltage either without (-61.3 ± 1.1 mV) or with glucagon (-62.0 ± 2.3 mV, $n = 3$). Activation of the Mg²⁺/Ca²⁺-sensing mechanism also inhibits AVP-stimulated Mg²⁺ uptake (data not shown). Addition of 50 μM neomycin at 5 min prior to the application of 3×10^{-7} M AVP diminished Mg²⁺ uptake from 189 ± 6 to 163 ± 4 nM/s ($n = 3$). Next, we tested whether elevated extracellular Ca²⁺ may affect Mg²⁺ entry. We have previously shown that addition of 10 mM extracellular Ca²⁺ does not alter basal Mg²⁺ entry into Mg²⁺-depleted MDCT cells (10). Extracellular Ca²⁺ was added 5 min prior to measurement of Mg²⁺ uptake. As with neomycin, extracellular Ca²⁺, 5 mM, did not change basal Mg²⁺ uptake rates but inhibited glucagon-stimulated Mg²⁺ entry (Fig. 3). As with extracellular Ca²⁺, large concentrations of extracellular Mg²⁺ inhibited glucagon-stimulated Mg²⁺ uptake into MDCT cells (Fig. 3). It should be kept in mind that the Mg²⁺ uptake rate, $d([Mg^{2+}]_i)/dt$, appears to saturate at ~5 mM (10). The basal $[Mg^{2+}]_i$ refill rate was 164 ± 5 nM/s in the presence of buffer containing no calcium and 5.0 mM MgCl₂. Glucagon failed to stimulate Mg²⁺ uptake (154 ± 6 nM/s), when determined in the presence of 5.0 mM MgCl₂. These results are consonant with the effects of the polyvalent cations on hormone-mediated cAMP accumulation (Table 1; Fig. 1).

Next, we measured glucagon-stimulated Mg²⁺ uptake rate below the apparent MgCl₂ concentration that

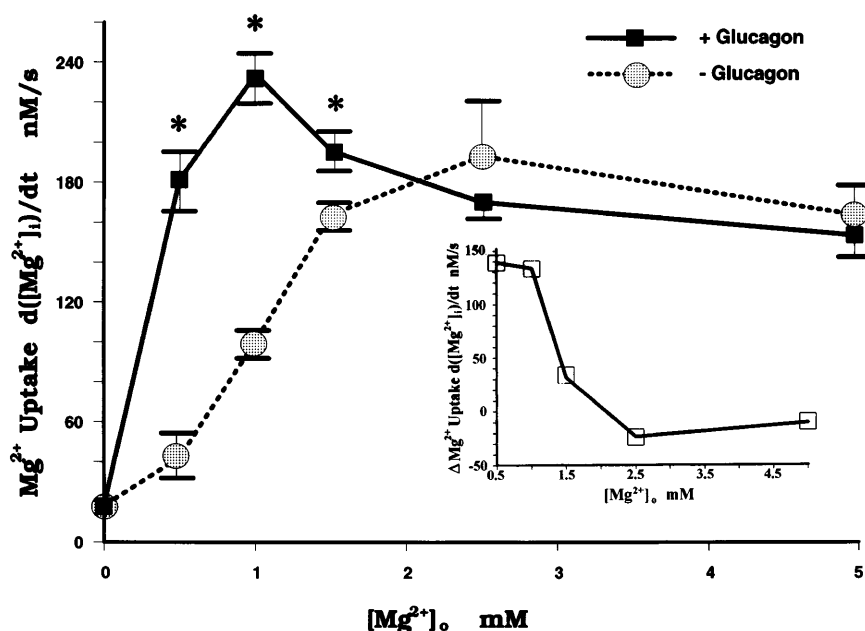


Fig. 4. Glucagon stimulation of Mg²⁺ uptake is dependent on the concentration of extracellular Mg²⁺ used to determine d([Mg²⁺]_i)/dt. Mg²⁺ uptake was measured with and without 10⁻⁷ M glucagon in presence of the extracellular MgCl₂ concentrations as indicated. *Inset*: change of hormone-stimulated Mg²⁺ uptake as a function of extracellular Mg²⁺ concentration used to perform the refill studies. Values are means ± SE for 3–6 observations. * *P* < 0.05, significantly different from control values not treated with glucagon.

produces saturation of d([Mg²⁺]_i)/dt. The refill rate, d([Mg²⁺]_i)/dt, normally saturates at ~5 mM MgCl₂ (10). This was also observed in the presence of glucagon (Fig. 4). However, uptake rates below this value were increased so that the hormone-stimulated d([Mg²⁺]_i)/dt was dependent on extracellular Mg²⁺ demonstrating greater fractional transport rates with the lower extracellular Mg²⁺ concentrations used to perform the uptake measurements (Fig. 4, *inset*).

Arthur (1) has recently shown that activation of the calcium-sensing receptor of Madin-Darby canine kidney cells, another distal tubule cell line, with high extracellular Ca²⁺ inhibited basal transepithelial calcium transport after 30 min but not after 5 min. We have tested whether the addition of 50 μM neomycin, for 30 min, might inhibit basal Mg²⁺ uptake in MDCT cells. The mean uptake rate, d([Mg²⁺]_i)/dt, was 153 ± 10 nM/s (*n* = 6) following 30 min of neomycin. Accordingly, in MDCT cells, we have no evidence that activation of Mg²⁺/Ca²⁺ sensing inhibits basal Mg²⁺ uptake. Further studies are necessary to determine whether this conclusion is valid for transepithelial Mg²⁺ transport.

Activation of Mg²⁺/Ca²⁺ sensing does not affect cAMP stimulation of Mg²⁺ uptake. We have shown that the addition of exogenous 8-bromo-cAMP stimulates Mg²⁺ entry into MDCT cells (9). Furthermore, protein kinase A inhibition diminishes hormone-stimulated Mg²⁺ uptake (9). Accordingly, glucagon and AVP act, in part, through a cAMP-dependent pathway. To determine whether activation of the Mg²⁺/Ca²⁺-sensing mechanism affects cAMP-mediated processes, we pretreated cells with neomycin to activate the receptor, then added 8-bromo-cAMP and measured Mg²⁺ uptake rate. Figure 5 summarizes these experiments. 8-Bromo-cAMP stimulated Mg²⁺ entry to a similar extent in the presence of neomycin as in the absence of receptor activation. Forskolin stimulates intracellular cAMP production in the presence of activation of Mg²⁺/Ca²⁺ sensing (8, 14). Accordingly, we performed Mg²⁺ uptake studies in the

presence of forskolin and neomycin to test whether forskolin alters transport despite activation of Mg²⁺/Ca²⁺ sensing. Again, forskolin stimulated Mg²⁺ uptake in the presence of activation of Mg²⁺/Ca²⁺ sensing by neomycin. These studies suggest that activation of the Mg²⁺/Ca²⁺-sensing mechanism inhibits hormone-stimulated Mg²⁺ uptake by inhibiting hormone-mediated cAMP generation.

Activation of Mg²⁺/Ca²⁺ sensing does not inhibit amiloride-stimulated Mg²⁺ uptake. We have shown that amiloride stimulates Mg²⁺ uptake rates by ~30–40% above basal levels in MDCT cells. Amiloride hyperpolarizes the plasma membrane by -28 ± 8 mV, thereby creating a more favorable electrical gradient

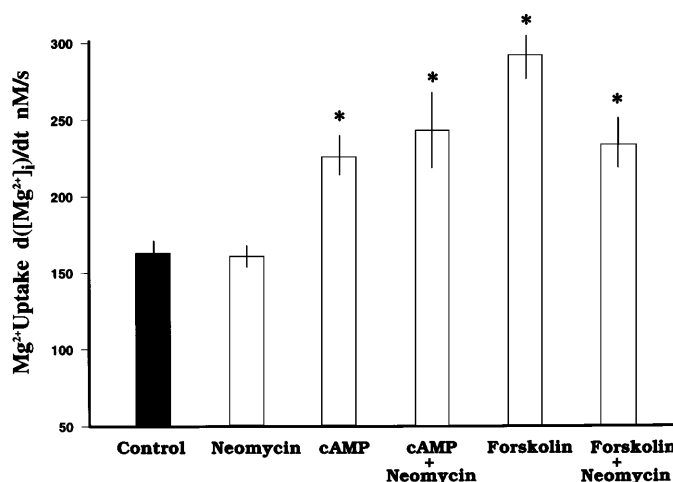


Fig. 5. Activation of Mg²⁺/Ca²⁺ sensing does not alter cAMP-mediated Mg²⁺ uptake. Either forskolin, 1 μM, to generate endogenous cAMP release, or 8-bromo-cAMP, 10⁻⁴ M, was added 6 min prior to determination of d([Mg²⁺]_i)/dt with microfluorescence according to the techniques illustrated in Fig. 3. Neomycin, 50 μM, was added 5 min prior to the addition of forskolin or 8-bromo-cAMP. Values are means ± SE for 3–6 cells. * *P* < 0.01, significantly different from control values.

for Mg²⁺ entry (10). We pretreated MDCT cells with neomycin, then determined the effect of amiloride on Mg²⁺ uptake. Amiloride increased Mg²⁺ entry (221 ± 12 nM/s) in the presence of neomycin, indicating that the activation of the Mg²⁺/Ca²⁺-sensing mechanism does not alter the cellular actions of this magnesium-conserving diuretic (Fig. 6). The increase in membrane voltage, from basal levels (−65.5 ± 2.0 mV), normally observed with amiloride (−75.3 ± 1.8 mV) was also not altered by neomycin (−74.7 ± 1.2 mV, *n* = 5).

DISCUSSION

We have reported that MDCT cells possess a Mg²⁺/Ca²⁺-sensing mechanism, which upon activation with polyvalent cations, such as neomycin, elicits cytosolic Ca²⁺ signals and diminishes hormone-mediated intracellular cAMP accumulation (3). In the present study, we show that activation of this Mg²⁺/Ca²⁺-sensing mechanism also inhibits glucagon- and AVP-stimulated Mg²⁺ uptake into MDCT cells. As this cell line possesses many of the transport properties of the intact distal convoluted tubule, we conclude that the Mg²⁺/Ca²⁺-sensing mechanism plays an important role in control of magnesium transport in this nephron segment.

These conclusions are consonant with the results of earlier micropuncture studies. Le Grimellec and colleagues (16, 17) used magnesium- or calcium-loaded rats having intact parathyroid glands and performed free-flow micropuncture experiments to determine the effects of hypermagnesemia and hypercalcemia on distal tubular absorption. They showed that magnesium delivery from the early distal tubule sampling site to the final urine relative to inulin delivery was markedly increased in hypermagnesemic and hypercalcemic rats. They interpreted this data to indicate “a drastic inhibition (by an unknown mechanism) of reabsorption taking place in the terminal segments of the nephron or a proportionately more important contribution of the

deeply located nephrons to magnesium excretion than expected from their number” (16, 17). In support of these early micropuncture experiments, our *in vivo* micropuncture studies indicated that the rate of magnesium absorption within the distal tubule was altered by extracellular Mg²⁺ and Ca²⁺ (19, 22). Distal tubules were perfused from a proximal site and tubule fluid sampled from early and late sites of superficial distal tubules. Magnesium absorption within the superficial distal tubule of thyroparathyroidectomized (TPTX) rats was highly dependent on delivery of magnesium to this segment (20, 22). The fraction of delivered magnesium absorbed in distal tubules amounted to 34 ± 10% in rats with normal plasma Mg²⁺ and Ca²⁺ concentrations of 0.78 ± 0.04 mM and 2.24 ± 0.04 mM, respectively (22). However, fractional magnesium absorption decreased significantly to 6 ± 3% when the animals were made hypermagnesemic (plasma magnesium, 3.58 ± 0.20 mM) and to 14 ± 7% in hypercalcemic rats (plasma calcium, 4.24 ± 0.36 mM) (19, 22). The animals used in these studies were TPTX but were intact with respect to other circulating hormones. The cellular basis for diminished fractional absorption in hypermagnesemia and hypercalcemia remained unexplained until a *Casr* was identified in the kidney and located to the distal tubule (4, 24).

We have previously shown that the MDCT possesses a *Casr* (3). The *Casr*, whether present in the parathyroid cell or expressed in *Xenopus* oocytes or HEK cells, responds differentially to extracellular Mg²⁺ and Ca²⁺. The sensitivity of the *Casr* as determined by inositol phosphates release, intracellular Ca²⁺ signaling, or modulation of cAMP accumulation was much more responsive to extracellular Ca²⁺ relative to Mg²⁺ (4, 5, 7, 18). It was of interest therefore that Mg²⁺/Ca²⁺ sensing in the MDCT cell line is as sensitive to extracellular Mg²⁺ as it is to Ca²⁺ (3). This suggested that either the nature of the *Casr* is different in these cells or that additional sensing mechanisms are present (3). The present results support (Fig. 1) these initial observations and show that Mg²⁺/Ca²⁺ sensing in the MDCT cell is responsive within the normal plasma concentration ranges for these divalent cations but also show that it is equally responsive to extracellular Mg²⁺ and Ca²⁺.

The *Casr* is localized along the length of the nephron from the proximal tubule to the collecting system with particular abundance in the basolateral membrane of the thick ascending limb and the apical membrane of the inner medullary collecting duct (23, 32). The functions of renal polyvalent cation-sensing receptor(s) are not fully understood. Hebert (15) has recently summarized the salient features of increases in extracellular Ca²⁺ and Mg²⁺ on loop and collecting duct function (15). Hypercalcemia and hypermagnesemia inhibit NaCl, calcium, and magnesium absorption in the thick ascending limb (19, 22, 31) and water permeability in the medullary collecting duct (25). Hebert (15) speculates that inhibition of salt reabsorption in the thick ascending limb and water transport in the medullary collecting duct together with diminished calcium absorption

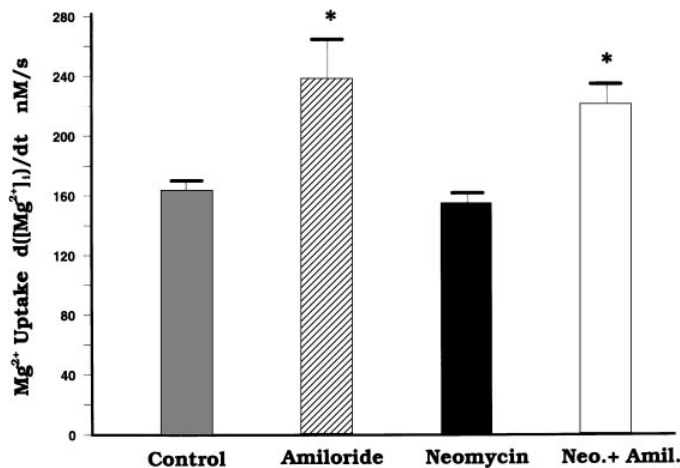


Fig. 6. Activation of Mg²⁺/Ca²⁺ sensing does not alter amiloride-stimulated Mg²⁺ uptake. Mg²⁺ uptake, d([Mg²⁺]_i)/dt, was performed in presence of 1.5 mM MgCl₂ and with or without 10 μM amiloride. Neomycin, 50 μM, was added where indicated 5 min prior to the addition of amiloride. Values are means ± SE for 3–6 cells. * *P* < 0.01, significantly different from control values.

in the thick ascending limb would wash out the calcium cations, minimizing the possibility of urinary stone formation. The functional roles of the *Casr* within the proximal tubule and segments of the distal tubule have not been determined. The present studies show that activation of the Mg²⁺/Ca²⁺-sensing mechanism inhibits hormone-stimulated Mg²⁺ uptake into MDCT cells. These results suggest that this receptor might play an important role in controlling magnesium absorption acting in at least two nephron segments; the thick ascending limb and the distal convoluted tubule.

The mechanisms by which the Mg²⁺/Ca²⁺-sensing mechanism inhibits magnesium transport in the thick ascending limb and distal tubule are becoming clearer. In the cortical thick ascending limb, magnesium and calcium transport is passive, dependent on the transepithelial voltage and the permeability of the paracellular pathway (11). Wang et al. (30) have reported that neomycin inhibits apical K⁺ channels and possibly apical Na-K-Cl cotransport through signaling pathways involving cytochrome *P*-450 metabolites. Accordingly, activation of the *Casr* would lead to a decrease in transepithelial voltage and diminished passive calcium and magnesium transport in the thick ascending limb (15, 30). Unlike the thick ascending limb, magnesium transport within the distal tubule is active and transcellular in nature so that the receptor must affect active magnesium absorption. Our results indicate that activation of the Mg²⁺/Ca²⁺-sensing mechanism inhibits glucagon- and AVP-stimulated Mg²⁺ uptake and hormone-mediated accumulation of cAMP in MDCT cells. As cAMP enhances Mg²⁺ entry into MDCT cells, we speculate that activation of the Mg²⁺/Ca²⁺-sensing mechanism may act, in part, through diminished hormone-responsive cAMP release. We were unable to show any effects of neomycin activation of Mg²⁺/Ca²⁺ sensing on membrane voltage either with glucagon or amiloride, suggesting that these effects were independent of voltage. We infer from these studies that the Mg²⁺/Ca²⁺-sensing mechanism in the distal convoluted tubule plays an important role in renal magnesium conservation in addition to its effects within the loop of Henle.

It has long been known that hypermagnesemia and hypercalcemia inhibit hormone-mediated cAMP accumulation in the proximal tubule, loop of Henle, and the collecting duct. Hypermagnesemia and hypercalcemia inhibits the PTH-mediated increase in cAMP in the proximal tubule and cortical thick ascending limb (26, 28). The elevation of extracellular Ca²⁺ also mitigates vasopressin-stimulated increases in cAMP production in the medullary thick ascending limb of Henle's loop (27, 29) and PTH-, calcitonin-, vasopressin-, and glucagon-stimulated cAMP accumulation in the cortical thick ascending limb (28). Finally, Sands et al. (25) have shown that AVP-elicited water permeability in rat kidney terminal inner medullary collecting ducts is inhibited with elevated plasma calcium. Accordingly, hypermagnesemia and hypercalcemia, probably through activation of the Mg²⁺/Ca²⁺-sensing mechanism, have significant effects on hormone-mediated cAMP generation along the length of the nephron. These actions are

also apparent on hormone-stimulated cAMP accumulation in MDCT cells. We have previously shown that cAMP, in part, mediates hormone-stimulated Mg²⁺ uptake (9). Accordingly, it is likely that functional responses within the distal convoluted tubule that are mediated by cAMP are also modulated by hypermagnesemia and hypercalciuria through Mg²⁺/Ca²⁺ sensing.

In summary, a Mg²⁺/Ca²⁺-sensing mechanism is present in MDCT cells which upon activation inhibits hormone-mediated cAMP accumulation and glucagon- and AVP-stimulated Mg²⁺ uptake in Mg²⁺-depleted cells. It is not known whether these responses or others are present in normal distal tubule cells. Also, the pathways by which the Mg²⁺/Ca²⁺-sensing mechanism alters these activities are yet to be fully elucidated. However, the functional responses observed in the present studies are in keeping with earlier microperfusion studies demonstrating that hypermagnesemia and hypercalcemia diminish magnesium absorption within the distal tubule (19, 20, 22). These studies show that the Mg²⁺/Ca²⁺-sensing mechanism is important in the regulation of renal magnesium transport at both the level of the distal convoluted tubule as well as the loop of Henle. It is envisioned that either hypermagnesemia or hypercalcemia could inhibit divalent cation absorption in the loop and hormone-mediated absorption in the distal convoluted tubule. The latter response would be appropriate to mitigate excessive magnesium and perhaps calcium reabsorption in the face of increased delivery to this segment.

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