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L.-J. Dai, H. S. Kang, D. Kerstan, G. Ritchie and G. A. Quamme
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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
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L.-J. Dai, G. Ritchie, D. Kerstan, H. S. Kang, D. E. C. Cole and G. A. Quamme
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Glucagon and arginine vasopressin stimulate Mg^{2+} uptake in mouse distal convoluted tubule cells

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Dai, Long-Jun, Brian Bapty, Gordon Ritchie, and Gary A. Quamme. Glucagon and arginine vasopressin stimulate Mg^{2+} uptake in mouse distal convoluted tubule cells. *Am. J. Physiol.* 274 (Renal Physiol. 43): F328–F335, 1998.— Glucagon and arginine vasopressin (AVP) enhance renal magnesium conservation through actions within the loop of Henle and the distal tubule. Studies were performed on an immortalized mouse distal convoluted tubule (MDCT) cell line to characterize the cellular actions of these hormones on Mg^{2+} transport in this segment of the distal tubule. Glucagon and AVP increased cellular cAMP concentrations by about fivefold above basal levels in normal and Mg^{2+} -depleted cells. Intracellular free Mg^{2+} concentration ($[Mg^{2+}]_i$) was determined on single MDCT cells using microfluorescence with mag-fura 2. To assess Mg^{2+} uptake, MDCT cells were first Mg^{2+} depleted (0.22 ± 0.01 mM) by culturing in Mg^{2+} -free media for 16 h and then placed in 1.5 mM $MgCl_2$, and the $[Mg^{2+}]_i$ was determined. $[Mg^{2+}]_i$ returned to basal levels, 0.53 ± 0.02 mM, with a mean refill rate, $d([Mg^{2+}]_i)/dt$, of 164 ± 5 nM/s. Both glucagon and AVP stimulated Mg^{2+} uptake into MDCT cells, 196 ± 11 and 189 ± 6 nM/s, respectively, at concentrations of 3×10^{-7} M and 10^{-7} M, respectively. Enhanced Mg^{2+} uptake for each of the hormones was concentration dependent and inhibited by the channel blocker, nifedipine. Hormone stimulation of Mg^{2+} entry was not dependent on protein synthesis. 8-Bromo-cAMP, 10^{-4} M, enhanced Mg^{2+} uptake (225 ± 13 nM/s), whereas phorbol esters were without effect. Finally, protein kinase A inhibition prevented glucagon and AVP stimulation of Mg^{2+} uptake, supporting the notion that the cAMP pathway is important as expected in the hormone action. These studies demonstrate that glucagon and AVP stimulate Mg^{2+} uptake in MDCT cells and suggest that these hormones act to control magnesium conservation in the convoluted segment of the distal tubule.

intracellular magnesium; fluorescence; channel blockers; intracellular adenosine 3',5'-cyclic monophosphate

THE DISTAL TUBULE REABSORBS significant amounts of magnesium and plays an important role in determining the final urinary excretion rate (34). In contrast to more proximal segments of the nephron, distal Mg^{2+} transport processes are postulated to be active and transcellular in nature (34). It is likely that hormonal control in this segment provides the fine tuning of renal magnesium conservation contributing to whole body magnesium balance. Knowledge of hormonal actions and interactions within the distal tubule is important to the understanding of overall renal magnesium conservation.

Glucagon has clearly been shown to increase magnesium conservation within both the loop of Henle and the distal tubule (2, 15). Bailly and colleagues (4, 5) have shown that glucagon administration markedly enhances magnesium reabsorption in the loop of Henle

and the superficial distal tubule of hormone-deprived rats lacking circulating parathyroid hormone (PTH), calcitonin, antidiuretic hormone, and glucagon (4, 5). Furthermore, using hormone-deprived rats, Elalouf et al. (14) demonstrated that glucagon may play an additive role with arginine vasopressin (AVP) in control of renal magnesium conservation (14). Although it is clear from these micropuncture studies that glucagon acts within the superficial distal tubule, the segment of the distal tubule remains unknown. The mammalian distal tubule is composed of a short post macula densa segment of the thick ascending limb, the distal convoluted tubule, the connecting tubule, and the initial collecting tubule (24). Also unknown are which cellular mechanisms are involved with glucagon stimulation of Mg^{2+} transport. Butlen and Jard (7) have shown that glucagon receptors are present in the rat distal tubule, and Bailly et al. (3) reported that glucagon stimulates adenylate cyclase in isolated rat distal convoluted tubules, so glucagon may act, in part, through release of the second messenger, cAMP.

On the other hand, the actions of AVP within the distal convoluted tubule are poorly understood (23). Morel and colleagues (8, 26, 27) reported that AVP receptors are present in the rat and mouse distal convoluted tubule. However, using hormone-deprived rats, Elalouf et al. (13) reported that AVP enhanced distal sodium and calcium reabsorption but did not significantly alter Mg^{2+} transport. Although it is clear that AVP enhances renal magnesium conservation, the micropuncture data suggest that the major effects appear to be within the thick ascending limb of the loop of Henle, with little effect on Mg^{2+} transport in the distal tubule (13, 33, 35).

The cellular mechanisms of magnesium absorption in the distal convoluted tubule are poorly understood. In vitro microperfusion studies have not been performed because of the difficulty in isolating intact tubule segments. In vivo micropuncture and microperfusion studies of superficial distal tubules do not allow for investigation of cellular mechanisms of Mg^{2+} transport (34). In the present studies, we use an immortalized mouse distal convoluted tubule (MDCT) cell line to determine the effects of glucagon and AVP on Mg^{2+} uptake within this segment (28). The MDCT cell line possesses many of the properties of the intact distal convoluted tubule. The MDCT cells exhibit amiloride-inhibitable sodium transport and chlorothiazide-sensitive NaCl cotransport (17, 18). Amiloride and chlorothiazide also stimulates Ca^{2+} and Mg^{2+} entry in these cells (10, 11, 17, 18). Furthermore, PTH and calcitonin stimulate calcium uptake in MDCT cells (20, 21). Accordingly, we used this cell line to investigate the

actions of glucagon and AVP on Mg^{2+} uptake in the distal convoluted tubule. Because there is not an available isotope for magnesium, we determined Mg^{2+} entry into MDCT cells in the present studies by first depleting the cells of intracellular Mg^{2+} by culturing in Mg^{2+} -free media for 16 h. The Mg^{2+} -depleted MDCT cells were then placed in medium containing 1.5 mM Mg^{2+} , and the refill rate, $d([Mg^{2+}]_i)/dt$ (where $[Mg^{2+}]_i$ is intracellular free Mg^{2+} concentration), was measured with microfluorescent studies using mag-fura 2 (29). Mg^{2+} uptake rate is concentration dependent and selective for magnesium (10). Moreover, the influx rate is rapid and reproducible so that it is possible to determine the effects of extracellular influences on transport rates. In the present study, we show that both glucagon and AVP stimulate Mg^{2+} entry in MDCT, possibly through cAMP-dependent mechanisms. Further studies are required to define the intracellular pathways involved in hormone stimulation of Mg^{2+} absorption in the distal tubule.

METHODS

Materials. Basal Dulbecco's minimal essential medium (DMEM) and Ham's F-12 media were purchased from GIBCO. Customized Mg^{2+} -free media were purchased from Stem Cell Technologies (Vancouver, BC, Canada). Fetal calf serum was from Flow Laboratories (McLean, VA). Mag-fura 2-AM was obtained from Molecular Probes (Eugene, OR). Glucagon, AVP, 8-bromo-cAMP, phorbol 12-myristate 13-acetate (PMA), and other materials were from Sigma Chemical (St. Louis, MO). The protein kinase A inhibitor, Rp-cAMPS, and protein kinase C inhibitor, R031-8220, were purchased from Calbiochem (San Diego, CA).

Cell culture. Distal convoluted tubule cells were isolated from mice by Pizzonia et al. (28). These cells were immortalized and functionally characterized as previously described by Friedman and Gesek (17, 20). The MDCT cell line was grown on 60-mm plastic culture dishes (Corning Glass Works, Corning Medical and Scientific, Corning, NY) in DMEM-F12 (1:1) media supplemented with 10% fetal calf serum, 1 mM glucose, 5 mM L-glutamine, 50 U/ml penicillin, and 50 μ g/ml streptomycin in a humidified environment of 5% CO_2 -95% air at 37°C. For the fluorescence studies, confluent cells were washed three times with phosphate-buffered saline containing 5 mM ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, 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 subconfluence over 1–2 days in supplemented media as described above. The normal media contained 0.6 mM Mg^{2+} and 1.0 mM Ca^{2+} . In the experiments indicated, MDCT cells were cultured in Mg^{2+} -free media (<0.01 mM), where indicated for 16–24 h prior to study. Other constituents of the Mg^{2+} -free culture media were similar to the complete media. These media contained 0.2% bovine serum albumin (BSA) rather than the fetal calf serum.

Determination of cAMP concentration. cAMP was determined in confluent MDCT cell monolayers cultured in 24-well plates in DMEM-F12 media without serum but with 0.1% BSA. The media contained 0.6 mM Mg^{2+} or zero Mg^{2+} where indicated. After addition of either glucagon or AVP, MDCT cells were incubated at 37°C for 5 min in the presence of 0.1 mM 3-isobutyl-1-methylxanthine. The cAMP was extracted with 5% trichloroacetic acid, which was removed with ether, and then the extract was acidified with 0.1 N HCl. The

aqueous phase was dried, dissolved in tris(hydroxymethyl)-aminomethane (Tris)-EDTA buffer, and cAMP was measured with a radioimmunoassay kit (Diagnostic Products, Los Angeles, CA).

Cytoplasmic Mg^{2+} measurements. Coverslips were mounted into a perfusion chamber, and intracellular free Mg^{2+} concentration ($[Mg^{2+}]_i$) was determined with the use of the Mg^{2+} -sensitive fluorescent dye, mag-fura 2. The cell-permeant acetoxymethyl ester (AM) form of the dye was dissolved in dimethyl sulfoxide to a stock concentration of 5 mM and then diluted to 5 μ M mag-fura 2-AM in media for 20 min at 23°C. Cells were subsequently washed three times with buffered salt solution containing (in mM) 145 NaCl, 4.0 KCl, 0.8 K_2HPO_4 , 0.2 KH_2PO_4 , 1.0 $CaCl_2$, 5.0 glucose, and 20 *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-Tris, at pH 7.4. The MDCT cells were incubated for a further 20 min to allow for complete deesterification and washed once with this buffer solution before measurement of fluorescence.

Epifluorescence microscopy was used to monitor changes in the mag-fura 2 fluorescence within single MDCT cells. The chamber (0.5 ml) was mounted on an inverted Nikon Diaphot-TMD microscope, with a Fluor $\times 100$ 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/s) and emission at 505 nm. All experiments were performed at 23°C with continuous change of bathing solution (1 ml/min). Media changes were made without interruption in recording.

The $[Mg^{2+}]_i$ was calculated from the ratio of the fluorescence at the two excitation wavelengths as previously described using a dissociation constant (K_d) of 1.4 mM for the mag-fura 2- Mg^{2+} complex (29). The minimum (R_{min}) and maximum (R_{max}) ratios were determined for the cells at the end of each experiment using 20 μ M digitonin. R_{max} for mag-fura 2 was found by the addition of 50 mM $MgCl_2$ in the absence of Ca^{2+} , and R_{min} was obtained by removal of Mg^{2+} and addition of 100 mM EDTA, pH 7.2. The change in $[Mg^{2+}]_i$ with time, $d([Mg^{2+}]_i)/dt$, was determined by linear regression analysis of the fluorescence tracing over the initial 500 s.

Statistical analysis. Representative tracings of fluorescence intensities are given, and significance was determined by Student's *t*-test or Tukey's analysis of variance as appropriate. A probability of $P < 0.05$ was taken to be statistically significant. All results are means \pm SE where indicated.

RESULTS

Glucagon and AVP stimulate cAMP release in Mg^{2+} -depleted MDCT cells. Bailly et al. (3) have reported that glucagon stimulates cAMP release in rat distal convoluted tubules but not rabbit distal segments. Morel and colleagues (26, 27) have shown that AVP-sensitive adenylate cyclase is present in the distal tubule, but, as with glucagon, there were some species differences. AVP stimulated cAMP release along the entire length of rat and mouse distal tubules, whereas it was observed only in the late segment of the rabbit distal tubule. In the support of these studies, Friedman and Gesek (18) showed that both glucagon and AVP stimulated cAMP release in MDCT cells. In the present study, we determined the concentration dependence of glucagon- and AVP-stimulated cAMP release in MDCT cells (Fig. 1). Both hormones elicited intracellular cAMP accumula-

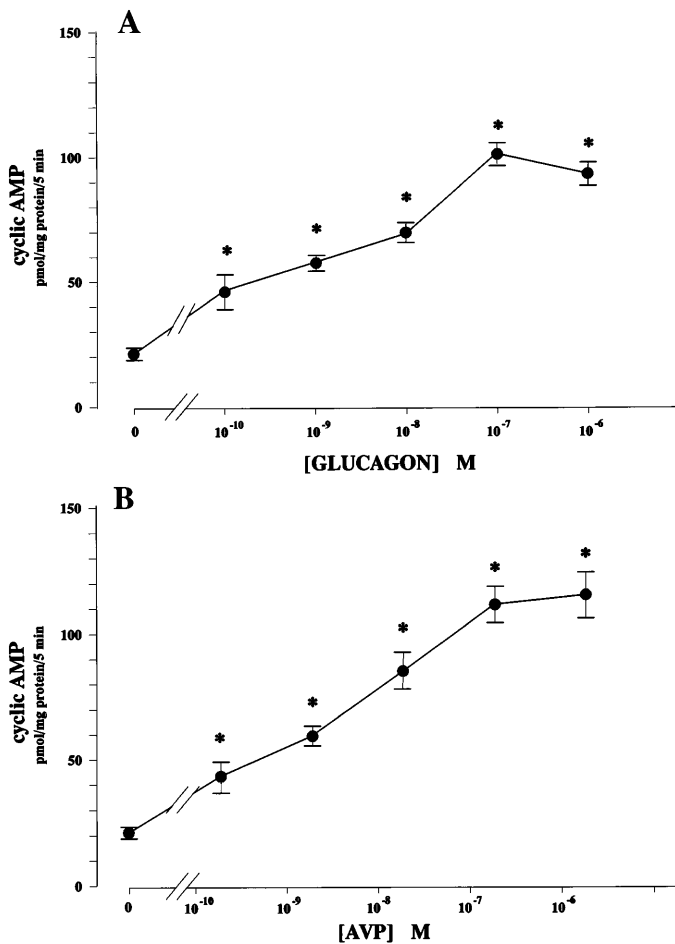


Fig. 1. Glucagon and arginine vasopressin (AVP) stimulate cAMP accumulation in MDCT cells. Glucagon (A) or AVP (B) were added, at the concentrations indicated, 5 min prior to measurement of cAMP. Values are means \pm SE for 3–9 observations, for 2 separate preparations. * $P < 0.05$, significantly different compared with control values.

tion in a concentration-dependent fashion. Second, we determined whether these hormones elicit cAMP accumulation in the Mg^{2+} -depleted MDCT cells used here. Glucagon or AVP at submaximal (10^{-9} M) and maximal (10^{-7} M) concentrations increased cAMP by similar amounts in normal and Mg^{2+} -depleted MDCT cells (Table 1). The concentration dependence of glucagon and AVP are far removed from the concentrations normally observed in vitro. A number of explanations may provide the basis for these discrepancies, including absence of normal bathing plasma, diminished circulation of hormone, or changes in membrane receptors due to cell culture conditions. Nevertheless, these responses indicate that hormone receptors are present in this cell line that activate intracellular signaling processes. These data confirm the observations of Friedman and Gesek (18) that receptors for these hormones are present in the MDCT cells, which, upon stimulation, release intracellular cAMP. Intracellular Mg^{2+} depletion did not alter hormone-responsive cAMP release in these cells (Table 1). Finally, glucagon or AVP did not induce rapid intracellular Ca^{2+} transients (data not shown), indicating that cytosolic Ca^{2+} signaling is

Table 1. Glucagon and AVP stimulate cAMP release in magnesium-depleted MDCT cells

	cAMP Release, pmol · mg protein ⁻¹ · 5 min ⁻¹	
	Normal cells	Mg ²⁺ -depleted cells
Control	22 ± 2 (6)	21 ± 1 (5)
Glucagon, 10 ⁻⁹ M	60 ± 5* (4)	60 ± 3* (4)
Glucagon, 10 ⁻⁷ M	92 ± 5* (4)	89 ± 3* (4)
AVP, 3 × 10 ⁻⁹ M	62 ± 4* (3)	61 ± 3* (4)
AVP, 3 × 10 ⁻⁷ M	89 ± 5* (4)	81 ± 7* (4)

Values are means \pm SE; number of experiments is in parentheses. Glucagon and arginine vasopressin (AVP) were added 5 min prior to the measurement of cAMP. * $P < 0.01$, significant vs. control values. There were no significant differences between normal and Mg^{2+} -depleted cells for each of the conditions tested. MDCT, mouse distal convoluted tubule.

not involved in the glucagon- or AVP-mediated pathways in MDCT cells (18).

Mg^{2+} uptake in MDCT cells. To determine Mg^{2+} uptake, subconfluent MDCT monolayers were cultured in Mg^{2+} -free medium for 16 h. These cells possessed a significantly lower $[Mg^{2+}]_i$ (0.22 ± 0.01 mM) than cells cultured in normal media (0.53 ± 0.02 mM). When the Mg^{2+} -depleted MDCT cells were placed in a bathing solution containing 1.5 mM $MgCl_2$, $[Mg^{2+}]_i$ increased with time and leveled at a $[Mg^{2+}]_i$ value of 0.52 ± 0.06 mM ($n = 9$), which was similar to basal levels observed in normal cells (Fig. 2). The average rate of refill, $d([Mg^{2+}]_i)/dt$, measured as the change in $[Mg^{2+}]_i$ with time, was 164 ± 5 nM/s ($n = 6$ cells), as determined over the first 500 s following addition of 1.5 mM $MgCl_2$.

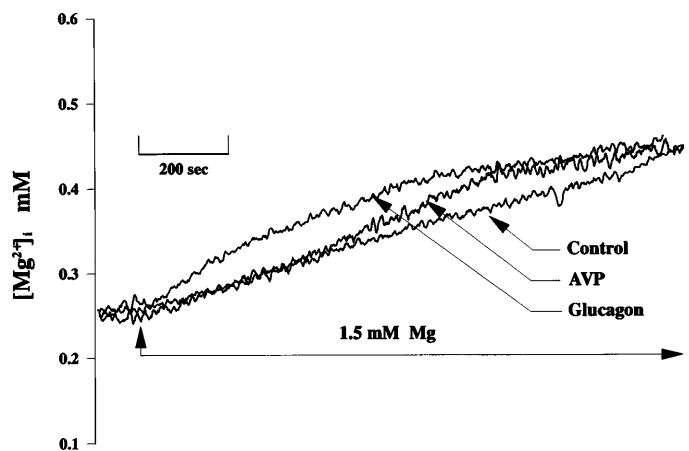


Fig. 2. Glucagon and AVP stimulate Mg^{2+} uptake in Mg^{2+} -depleted mouse distal convoluted tubule (MDCT) cells. Confluent MDCT cells were cultured in Mg^{2+} -free media (< 0.01 mM) for 16–20 h. Fluorescence studies were performed in buffer solutions in absence of Mg^{2+} , and where indicated $MgCl_2$ (1.5 mM final concentration) was added to observe changes in intracellular Mg^{2+} concentration ($[Mg^{2+}]_i$). Buffer solutions contained (in mM) 145 NaCl, 4.0 KCl, 0.8 K_2HPO_4 , 0.2 KH_2PO_4 , 1.0 $CaCl_2$, 5.0 glucose, and 10 HEPES-Tris, pH 7.4, with and without 1.5 mM $MgCl_2$. Glucagon, 10^{-7} M, or AVP, 3×10^{-7} M, were added with this buffer solution. Fluorescence was measured at 1 data point/s with 25-point signal averaging, and the tracing was smoothed according to methods previously described (29).

Glucagon stimulates Mg^{2+} uptake in MDCT cells. Glucagon, 10^{-7} M, added to the refill buffer solution increased the rate of Mg^{2+} entry into Mg^{2+} -depleted MDCT cells (Fig. 2). Glucagon, 10^{-7} M, increased the mean Mg^{2+} entry rate from 164 ± 5 to 196 ± 11 nM/s ($n = 5$), which represented a stimulation of $20 \pm 6\%$ above control values. In all cases where measured, $[Mg^{2+}]_i$ returned to basal levels of 0.52 ± 0.03 mM. The effect of glucagon on Mg^{2+} uptake was concentration dependent with maximal rate of stimulation at 10^{-6} M (273 ± 6 nM/s) and half-maximal stimulation at a concentration $\sim 10^{-7}$ M (Fig. 3). We have previously reported that dihydropyridines inhibit Mg^{2+} uptake into Mg^{2+} -depleted MDCT cells (10). To determine whether glucagon-induced Mg^{2+} entry is mediated through a dihydropyridine-sensitive pathway, we examined the effect of the channel blocker, nifedipine, on the changes in $[Mg^{2+}]_i$ following placement in the refill buffer solution containing 1.5 mM $MgCl_2$. The presence of 10^{-5} M nifedipine inhibited glucagon-stimulated Mg^{2+} uptake (24 ± 2 nM/s), indicating that this pathway is sensitive to the channel blocker and supporting the notion that glucagon-stimulated uptake is the same as the entry pathway observed in control cells (Table 2).

AVP stimulates Mg^{2+} entry. Figure 2 illustrates the effect of AVP, 3×10^{-7} M, on Mg^{2+} uptake in Mg^{2+} -depleted MDCT cells. The mean uptake rate, $d([Mg^{2+}]_i)/dt$, increased from control levels (164 ± 5 to 189 ± 6 nM/s, $n = 6$). Unlike the actions of glucagon, AVP responses were not immediate with the addition of the hormone to the refill solution. As shown, the response was initiated within 5–10 min following addition of the hormone to the superfusion solution. The reasons for this delay are not apparent at the present time. The following refill studies were uniformly performed 6 min after AVP was added to the MDCT cells. The refill rate, $d([Mg^{2+}]_i)/dt$, was determined over a standard 500-s time interval following the 6-min delay. AVP stimulates

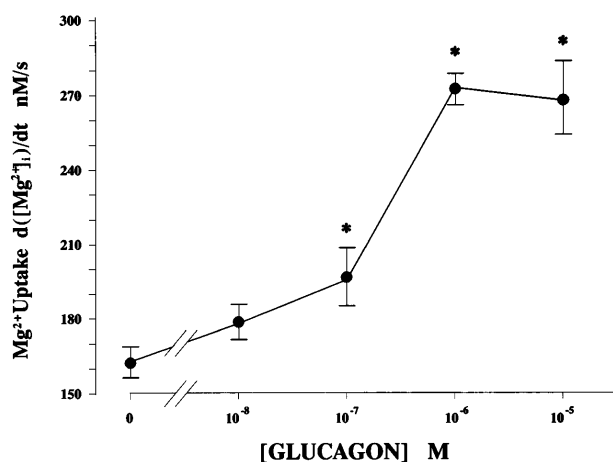


Fig. 3. Concentration dependence of glucagon stimulation of Mg^{2+} entry in MDCT cells. Rate of Mg^{2+} influx as determined by $d([Mg^{2+}]_i)/dt$ was measured with the given glucagon concentrations, using fluorescence techniques performed as described in legend to Fig. 1. $d([Mg^{2+}]_i)/dt$ values were determined over the first 500 s of fluorescence measurements. Values are means \pm SE for 3–6 cells. * $P < 0.05$, significantly different from control values.

Table 2. Hormone-stimulated Mg^{2+} uptake in MDCT cells is dihydropyridine sensitive

	Basal $[Mg^{2+}]_i$, mM	$d([Mg^{2+}]_i)/dt$, nM/s
Control	0.22 ± 0.02 (6)	164 ± 5 (6)
Control + nifedipine	0.22 ± 0.02 (4)	$42 \pm 21^*$ (4)
Glucagon	0.22 ± 0.01 (4)	$196 \pm 11^*$ (4)
Glucagon + nifedipine	0.21 ± 0.02 (3)	$24 \pm 2^*$ (3)
AVP	0.22 ± 0.2 (5)	$189 \pm 6^*$ (6)
AVP + nifedipine	0.21 ± 0.02 (3)	$75 \pm 9^*$ (3)

Values are means \pm SE; number of experiments is in parentheses. Measurements were performed in presence of 1.5 mM $MgCl_2$ with and without 10^{-7} M glucagon, or 3×10^{-7} M AVP according to the methods outlined in legend to Fig. 1. Nifedipine, 10^{-5} M, was added with the refill solution where indicated. * $P < 0.05$, significant vs. control values.

Mg^{2+} entry in a concentration-dependent manner (Fig. 4). The maximal effect was observed at $\sim 3 \times 10^{-6}$ M, which resulted in an increase of $d([Mg^{2+}]_i)/dt$ to 188 ± 2 nM/s. This change was significantly less than the response to maximal glucagon concentrations. AVP-stimulated Mg^{2+} uptake was inhibited by nifedipine, indicating that the hormonal response is through activation of channels responsible for Mg^{2+} entry in control MDCT cells (Table 2).

Glucagon- and AVP-stimulated Mg^{2+} entry does not require protein synthesis. Gesek and Friedman (20) have reported that PTH-stimulated calcium uptake in MDCT cells is sensitive to cycloheximide (1 μ g/min for 30 min), whereas calcitonin actions are insensitive to protein synthesis inhibitors. Accordingly, there may be diverse pathways involved with hormonal regulation of cation transport. Pretreatment of MDCT cells with cycloheximide, 1 μ g/ml for 30 min, did not alter glucagon or AVP stimulation of Mg^{2+} entry into MDCT cells (Table 3). It is apparent that de novo protein synthesis is not required for the acute actions of glucagon or AVP on Mg^{2+} uptake.

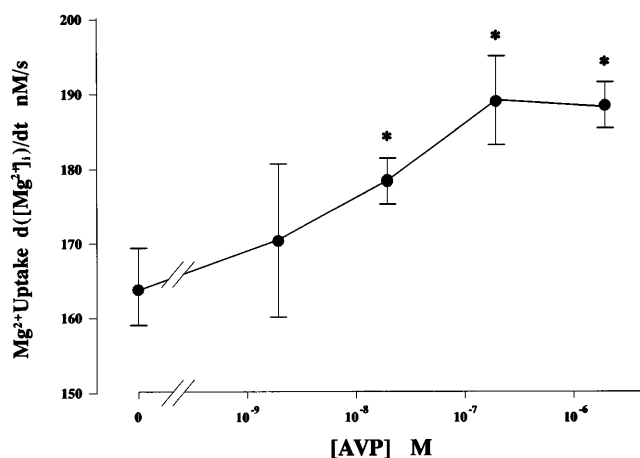


Fig. 4. Concentration dependence of AVP stimulation of Mg^{2+} uptake in MDCT cells. Rate of Mg^{2+} influx as determined by $d([Mg^{2+}]_i)/dt$ was measured with the given AVP concentrations using fluorescence techniques performed as described in legend to Fig. 1. Entry rate, $d([Mg^{2+}]_i)/dt$, was determined over 500 s, 5 min after addition of AVP. Values are means \pm SE for 3–6 cells. * $P < 0.05$, significantly different from control values.

Table 3. Role of protein synthesis on glucagon-stimulated Mg^{2+} uptake in MDCT cells

	Control, nM/s	Cycloheximide, nM/s
Control	164 ± 5 (6)	163 ± 6 (3)
Glucagon	196 ± 11 (4)	197 ± 11 (3)
AVP	189 ± 6 (6)	192 ± 2 (4)

Values are means ± SE; number of observations is in parentheses. Mg^{2+} entry was determined in presence of 10^{-7} M glucagon or 3×10^{-7} M AVP with and without pretreatment with 10^{-8} M cycloheximide. Protein synthesis inhibitor was applied 30 min prior to fluorescence studies. * $P < 0.05$, significant for cycloheximide vs. control values for each of the respective hormone treatments.

cAMP stimulates Mg^{2+} uptake in MDCT cells. Next, we determined whether activation of either protein kinase A or protein kinase C may have stimulated Mg^{2+} entry in Mg^{2+} -depleted MDCT cells. Friedman et al. (16) and Hilal et al. (22) have shown that activation of both pathways are necessary for PTH-mediated increases in calcium uptake in MDCT cells and distal tubule vesicles, respectively. The activations of these kinases with cAMP and phorbol esters were each without effect alone but significantly stimulated calcium entry when administered together. In the present studies, the addition of 8-bromo-cAMP, 10^{-4} M, 6 min prior to the fluorescence determinations stimulated Mg^{2+} uptake by $137 \pm 6\%$ above control values (Fig. 5). There was an apparent latent period prior to the effects of 8-bromo-cAMP of ~5–10 min, not unlike that observed for the responses of AVP. The addition of phorbol esters, on the other hand, had no apparent effect on Mg^{2+} entry at any time frame (Fig. 5). Moreover, cAMP, 10^{-4} M, stimulated Mg^{2+} uptake in the presence of the phorbol ester. We infer from these studies that hormone-stimulated Mg^{2+} uptake may involve intracellular cAMP accumulation.

Finally, we determined the effect of protein kinase A and protein kinase C inhibition on hormone-stimulated Mg^{2+} uptake. Rp-cAMPS, a protein kinase A inhibitor was applied 5 min prior to Mg^{2+} uptake measurements.

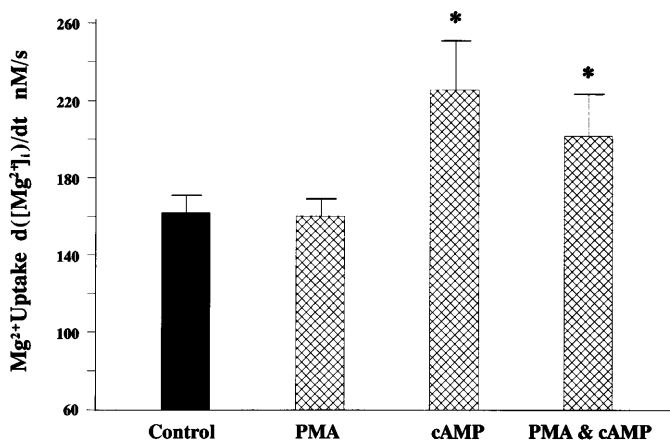


Fig. 5. cAMP stimulates Mg^{2+} entry in MDCT cells. Either 8-bromo-cAMP (10^{-4} M) or phorbol 12-myristate 13-acetate (PMA, 10^{-7} M) was added 6 min prior to determination of $d([Mg^{2+}]_i)/dt$ with microfluorescence according to the techniques illustrated in Fig. 1. Values are means ± SE for 3–6 cells. * $P < 0.01$, significantly different from control values.

Rp-cAMPS abolished the effects of glucagon and AVP (Table 4), suggesting that activation of protein kinase A is somehow involved with hormone actions. R031-8220, an inhibitor of protein kinase C, was without effect, supporting the notion that this signaling pathway is not involved with hormone stimulation of Mg^{2+} uptake.

DISCUSSION

Glucagon is a potent renal magnesium-conserving hormone (34). Bailly and Amiel (2) reported that the acute infusion of pharmacological concentrations of glucagon into parathyroid gland-intact rats led to a rapid fall in fractional magnesium excretion from $16 \pm 1\%$ to $9 \pm 2\%$. The response to glucagon is even greater in hormone-deprived animals (33). In these rats, fractional magnesium excretion markedly decreased by ~50% (from ~28% to basal levels) with glucagon administration (4, 5). This decrease in urinary excretion was attributed to a doubling of absolute magnesium reabsorption within the loop of Henle (increase from 6.5 ± 0.7 to 11.7 ± 0.7 pmol/min) and the distal tubule (increase from 0.85 ± 0.1 to 1.75 ± 0.3 pmol/min). In the loop, the increment in magnesium reabsorption was accompanied by an increase in calcium, sodium, potassium, and chloride, whereas in the distal tubule there was an increase in calcium transport but no change in fractional sodium, potassium, and chloride absorption (4, 32). Interestingly, Friedman and Gesek (18) failed to observe any effect of glucagon on $^{45}Ca^{2+}$ uptake into MDCT cells, even though they observed a significant increase in glucagon-stimulated cAMP formation. The micropuncture studies indicate that glucagon stimulates calcium as well as magnesium absorption in the distal tubule, although the effects on magnesium are relatively greater than those for calcium (5, 33). The results suggest that there may be quantitative differences in hormone action in the control of transport of the two divalent cations. Further studies are required to define the intracellular regulation of these cations within the nephron segments composing the distal tubule.

The micropuncture studies clearly indicated that glucagon stimulates Mg^{2+} transport in the superficial distal tubule (5). This segment comprises the distal convoluted tubule, connecting tubule, and initial cortical collecting tubule. Prior to the present studies, information was not available concerning which distal

Table 4. Glucagon and AVP act through protein kinase A-mediated pathway

	Control, nM/s	Glucagon, nM/s	AVP, nM/s
Control	164 ± 5 (6)	196 ± 11* (4)	189 ± 6* (6)
Rp-cAMPS	171 ± 3 (3)	176 ± 5 (3)	177 ± 8 (3)
R031-8220	167 ± 10 (3)	201 ± 17* (3)	197 ± 7* (3)

Values are means ± SE; number of observations is in parentheses. Mg^{2+} entry was determined in presence of 10^{-7} M glucagon or 3×10^{-7} M AVP with and without a 5-min pretreatment with either the protein kinase A inhibitor, Rp-cAMPS (10^{-6} M) or the protein kinase C inhibitor, R031-8220 (10^{-6} M). * $P < 0.05$, significantly different from control values.

segment was involved with hormone-stimulated distal tubular magnesium reabsorption. Of the segments composing the distal tubule, the evidence from the present study clearly indicates that the convoluted portion is involved with glucagon-induced magnesium conservation. Accordingly, it is inferred that glucagon enhances active Mg^{2+} transport in the distal convoluted tubule. Cellular mechanisms of Mg^{2+} transport in the other distal segments, connecting tubule and initial collecting tubule, have not been studied.

AVP has been shown to be an effective magnesium-conserving hormone in anesthetized and conscious hormone-deprived rats (1, 6, 13). Micropuncture studies of these animals have shown that AVP actions occur principally within Henle's loop (13, 30). Using micropuncture studies, Wittner and Di Stefano (35) demonstrated that AVP enhances magnesium absorption in mouse thick ascending limbs through changes in passive transport commensurate with increases in transepithelial voltage. In the micropuncture studies with hormone-deprived rats, Elalouf and colleagues (13) failed to discern any change in fractional magnesium absorption in the superficial distal tubule following physiological administration of AVP. In these studies, Elalouf et al. (13) reported that the fractional calcium absorption increased significantly from $42.0 \pm 5.8\%$ to $62.8 \pm 7.1\%$, whereas AVP increased fractional Mg^{2+} transport from $45.5 \pm 7.8\%$ to $55.3 \pm 15.5\%$, but this change was not statistically significant. Costanzo and Windhager (9) did not observe any change in calcium absorption in the microperfused rat distal tubule with administration of AVP. The animals used in this latter study were thyroparathyroidectomized but not hormone deprived as were those used by Elalouf et al. (13). In both studies, AVP enhanced sodium absorption in the distal tubule. In the present studies, AVP increased Mg^{2+} entry into an established distal convoluted tubule (MDCT) cell line, showing that this hormone acts within this segment of the distal tubule and is involved with renal magnesium conservation. Mg^{2+} transport in the distal convoluted tubule is probably transcellular and active in nature because of the high resistance and lumen-negative transepithelial voltage of this segment. Accordingly, AVP likely stimulates active Mg^{2+} transport in contrast to enhancing passive Mg^{2+} absorption in the thick ascending limb (35).

Interestingly, AVP stimulates Mg^{2+} entry 5–10 min following addition of the hormone (Fig. 1). This latency period was not observed with glucagon stimulation of Mg^{2+} uptake. The reasons for these differences are not known. Gesek and Friedman (20, 21) observed similar differences in action of PTH and calcitonin stimulation of Ca^{2+} uptake in MDCT cells. There was a latency period of 10–15 min prior to PTH stimulation of Ca^{2+} entry compared with calcitonin actions, which occurred immediately upon addition of the hormone (20, 21). Although the basis for this latency period is not known, it may have something to do with translational processes and protein synthesis, because Gesek and Friedman (20, 21) showed that incubation of MDCT cells with cycloheximide for 15 min inhibited PTH-stimu-

lated Ca^{2+} entry but had no effect on calcitonin actions. Furthermore, treatment of the cells with $1\alpha,25$ -dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] shortened the latency period associated with PTH-stimulated increases of $[Ca^{2+}]_i$ and Ca^{2+} entry but had no effect on Ca^{2+} entry by itself (19). These workers were unable to explain the origin of the latency period but concluded that it had a genomic mechanism that involved transcription because 5–6-dichloro-1- β -D-ribofuranosyl benzimidazole blocked the stimulatory response of $1,25(OH)_2D_3$ without inhibiting PTH actions (20). Friedman and Gesek (19) speculated that vitamin D may increase the number of PTH receptors expressed in the MDCT cells. These investigators were unable to show hormone-stimulated calcium entry with either AVP or glucagon (18). In the present studies, pretreatment of MDCT cells with cycloheximide did not inhibit either AVP- or glucagon-stimulated Mg^{2+} uptake, nor did it affect the latent period associated with AVP. It is apparent from the above-mentioned studies that peptide hormones act through different mechanisms to stimulate divalent cation transport in distal convoluted tubules. Further studies are needed to explain these diverse observations, but the hormonal control of calcium and magnesium in the distal convoluted tubule may involve unique intracellular signaling pathways.

Recent studies by Friedman et al. (16) indicate that PTH-mediated stimulation of calcium transport is through intermediary pathways involving activation of both protein kinase A and protein kinase C, as cAMP or phorbol esters had no effect on Ca^{2+} entry alone, but together they enhanced uptake. These studies are commensurate with the observations of Hilal et al. (22) using isolated membrane vesicles from rabbit distal tubules. Moreover, Lajeunesse et al. (25) have shown that $^{45}Ca^{2+}$ uptake in apical membrane vesicles harvested from rabbits pretreated with PTH is greater than those from control animals. Accordingly, PTH may have additional effects on the apical membrane to effect an increase in Ca^{2+} uptake. In the present studies, we show that addition of 8-bromo-cAMP stimulates Mg^{2+} uptake, but PMA does not increase entry rates (Fig. 4). We infer that activation of protein kinase A is involved in glucagon and/or AVP actions. However, it is not known how activation of protein kinase A stimulates Mg^{2+} uptake, nor is it known what other pathways may be involved. Glucagon appears to stimulate Mg^{2+} uptake to a greater degree than AVP, even though AVP generates similar amounts of cAMP (Figs. 1, 3, 4). On balance, the differences in time frame of hormone action and the disparity of cAMP release suggest that glucagon and AVP may operate through separate but interactive intracellular signaling pathways. More research is required to sort out these intracellular controlling mechanisms.

Although it is clear that glucagon and AVP stimulate Mg^{2+} transport in the distal tubule, it is not known what role these hormones play in orchestrating magnesium homeostasis. A protein meal markedly enhances glomerular filtration rate (GFR) and is a potent stimulus for glucagon secretion (1). Elevated circulating

glucagon concentrations contribute to the disposal of nitrogen metabolites of amino acids and excretion of phosphate and potassium (1). Conversely, glucagon enhances sodium, calcium, and magnesium reabsorption within the loop of Henle and distal tubule (4, 5, 12). Glucagon may be important in magnesium conservation following a protein ingestion with its associated increase in GFR and filtered magnesium (1). Stimulation of loop and distal magnesium conservation by AVP, on the other hand, may be necessary to maintain normal magnesium balance when salt and water flow to the distal tubule are reduced during antidiuresis. Evidence has been provided by de Rouffignac (31) that glucagon and AVP may regulate electrolyte homeostasis in concert with other hormones such as PTH, calcitonin, insulin and β -adrenergic agonists. These hormones act within the thick ascending limb and distal tubule to effect the complex regulation of magnesium balance.

In summary, glucagon and AVP stimulate Mg^{2+} uptake into MDCT cells, supporting the results of micropuncture reports that these hormones may act within the convoluted segment of the distal tubule. The cellular mechanisms by which glucagon and AVP stimulate uptake are unclear. The notable differences in hormone responses include the time frame of action and the disparity in maximally induced Mg^{2+} uptake, suggesting different intracellular pathways are involved with stimulation of Mg^{2+} uptake. Further studies are required to determine the intracellular signaling pathway mediated by these hormones.

We gratefully acknowledge the excellent secretarial assistance of Susanna Lau in the preparation of this manuscript.

This work was supported by Medical Research Council of Canada Research Grant MT-5793 (to G. A. Quamme).

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Received 25 April 1997; accepted in final form 23 October 1997.

REFERENCES

- Ahloulay, M., M. Déchaux, K. Laborde, and L. Bankir. Influence of glucagon on GFR and on urea and electrolyte excretion: direct and indirect effects. *Am. J. Physiol.* 269 (*Renal Fluid Electrolyte Physiol.* 38): F225–F235, 1995.
- Bailly, C., and C. Amiel. Effect of glucagon on magnesium renal reabsorption in the rat. *Pflügers Arch.* 392: 360–365, 1982.
- Bailly, C., M. Imbert-Teboul, D. Chabardés, A. Hus-Citharel, M. Montégut, A. Clique, and F. Morel. The distal nephron of rat kidney: a target site for glucagon. *Proc. Natl. Acad. Sci. USA* 77: 3422–3424, 1980.
- Bailly, C., N. Roinel, and C. Amiel. PTH-like glucagon stimulation of Ca and Mg reabsorption in Henle's loop of the rat. *Am. J. Physiol.* 246 (*Renal Fluid Electrolyte Physiol.* 15): F205–F212, 1984.
- Bailly, C., N. Roinel, and C. Amiel. Stimulation by glucagon and PTH of Ca and Mg reabsorption in the superficial distal tubule of the rat kidney. *Pflügers Arch.* 403: 28–34, 1985.
- Bouby, N., M.-M. Trinh-Trang-Tan, and L. Bankir. Stimulation of tubular reabsorption of magnesium and calcium by antidiuretic hormone in conscious rats: study in Brattleboro rats with hereditary hypothalamic diabetes insipidus. *Pflügers Arch.* 402: 458–464, 1984.
- Butlen, D., and S. Jard. Glucagon receptors along the nephron: (^{125}I) glucagon binding in rat tubules. *Pflügers Arch.* 404: 348–353, 1985.
- Chabardés, D., M. Imbert-Teboul, M. Gagnan-Brunette, and F. Morel. Different hormonal target sites along the mouse and rabbit nephron. In: *Biochemical Nephrology*, edited by W. G. Gader and U. Schmidt. Bern: Huber, 1978, p. 447–454.
- Costanzo, L. S., and E. E. Windhager. Effects of PTH, ADH and cyclic cAMP on distal tubular Ca and Na reabsorption. *Am. J. Physiol.* 239 (*Renal Fluid Electrolyte Physiol.* 8): F478–F485, 1980.
- Dai, L., P. A. Friedman, and G. A. Quamme. Cellular mechanisms of amiloride stimulation of Mg^{2+} uptake in mouse distal convoluted tubule cells. *Am. J. Physiol.* 272 (*Renal Physiol.* 41): F249–F256, 1997.
- Dai, L., P. A. Friedman, and G. A. Quamme. Cellular mechanisms of chlorothiazide and potassium depletion on Mg^{2+} uptake in mouse distal convoluted tubule cells. *Kidney Int.* 51: 1008–1017, 1997.
- Di Stefano, A., M. Wittner, R. Nischke, R. Braitsch, R. Greger, C. Bailly, C. Amiel, J. M. Elalouf, N. Roinel, and C. de Rouffignac. Effect of glucagon on Na^+ , Cl^- , K^+ , Mg^{2+} and Ca^{2+} transport in cortical and medullary thick ascending limbs of mouse kidney. *Pflügers Arch.* 414: 640–646, 1989.
- Elalouf, J. M., N. Roinel, and C. de Rouffignac. Effects of antidiuretic hormone on electrolyte reabsorption and secretion in distal tubules of rat kidney. *Pflügers Arch.* 401: 167–173, 1984.
- Elalouf, J. M., D. Chabane-Sari, and C. de Rouffignac. Additivity of the effects of glucagon and vasopressin on renal Mg reabsorption and urine concentration ability in the rat. *Pflügers Arch.* 407: 566–579, 1986.
- Elalouf, J. M., N. Roinel, and C. de Rouffignac. Effects of glucagon and PTH on the loop of Henle of rat juxtamedullary nephrons. *Kidney Int.* 29: 807–813, 1986.
- Friedman, P. A., B. A. Coutermarsh, S. M. Kennedy, and F. A. Gesek. Parathyroid hormone stimulation of calcium transport is mediated by dual signaling mechanisms involving protein kinase A and renal protein kinase C. *Endocrinology* 137: 13–20, 1996.
- Friedman, P. A., and F. A. Gesek. Mechanism of calcium transport stimulated by chlorothiazide in mouse distal convoluted tubule cells. *J. Clin. Invest.* 90: 429–438, 1992.
- Friedman, P. A., and F. A. Gesek. Calcium transport in renal epithelial cells. *Am. J. Physiol.* 264 (*Renal Fluid Electrolyte Physiol.* 33): F181–F198, 1993.
- Friedman, P. A., and F. A. Gesek. Vitamin D_3 accelerates PTH-dependent calcium transport in distal convoluted tubule cells. *Am. J. Physiol.* 265 (*Renal Fluid Electrolyte Physiol.* 34): F300–F308, 1993.
- Gesek, F. A., and P. A. Friedman. On the mechanism of parathyroid hormone stimulation of calcium uptake by mouse distal convoluted tubule cells. *J. Clin. Invest.* 90: 749–758, 1992.
- Gesek, F. A., and P. A. Friedman. Calcitonin stimulates calcium transport in distal convoluted tubule cells. *Am. J. Physiol.* 264 (*Renal Fluid Electrolyte Physiol.* 33): F744–F751, 1993.
- Hilal, G., D. Claveau, Q. Zuo, and M. G. Brunette. Interaction of second messengers on Ca^{2+} uptake by the renal distal luminal membranes (Abstract). *J. Am. Soc. Nephrol.* 6: 950, 1995.
- Koeppen, B. M., and B. A. Stanton. Sodium chloride transport: distal nephron. In: *The Kidney: Physiology and Pathophysiology*, edited by D. W. Seldin and G. Giebisch. New York: Raven, 1992, p. 2003–2040.
- Kriz, W., and L. Bankir. A standard nomenclature for structures of the kidney. *Am. J. Physiol.* 254 (*Renal Fluid Electrolyte Physiol.* 23): F1–F8, 1988.
- Lajeunesse, D., I. Bouhtiauy, and M. G. Brunette. Parathyroid hormone and hydrochlorothiazide increase calcium transport by the luminal membrane of rabbit distal nephron segments through different pathways. *Endocrinology* 134: 35–41, 1994.
- Morel, F. Sites of hormone action in the mammalian nephron. *Am. J. Physiol.* 240 (*Renal Fluid Electrolyte Physiol.* 9): F159–F164, 1981.
- Morel, F., M. Imbert-Teboul, and D. Chabardés. Receptors to vasopressin and other hormones in the mammalian kidney. *Kidney Int.* 31: 512–520, 1987.

28. **Pizzonia, J. H., F. A. Gesek, S. M. Kennedy, B. A. Coutermarsh, B. J. Bacskai, and P. A. Friedman.** Immunomagnetic separation, primary culture and characterization of cortical thick ascending limb plus distal convoluted tubule cells from mouse kidney. *In Vitro Cell. Dev. Biol.* 27: 409–416, 1991.
29. **Quamme, G. A., and L. Dai.** Presence of a novel influx pathway for Mg^{2+} in MDCK cells. *Am. J. Physiol.* 259 (*Cell Physiol.* 28): C521–C525, 1990.
30. **Rouffignac, C. de, B. Corman, and N. Roinel.** Stimulation by antidiuretic hormone of electrolyte tubular reabsorption in rat kidney. *Am. J. Physiol.* 244 (*Renal Fluid Electrolyte Physiol.* 13): F156–F164, 1983.
31. **Rouffignac, C. de.** Multihormonal regulation of nephron epithelia: achieved through combinational mode? *Am. J. Physiol.* 269 (*Regulatory Integrative Comp. Physiol.* 38): R739–R748, 1995.
32. **Rouffignac, C. de, J. M. Elalouf, and N. Roinel.** Glucagon inhibits water and NaCl transports in the proximal tubule of the rat kidney. *Pflügers Arch.* 419: 472–477, 1991.
33. **Rouffignac, C. de, J. M. Elalouf, N. Roinel, C. Bailly, and C. Amiel.** Similarity of the effects of antidiuretic hormone, parathyroid hormone, calcitonin and glucagon on rat kidney. In: *Nephrology*, edited by R. R. Robinson. Berlin: Springer, 1984, p. 340–357.
34. **Rouffignac, C. de, and G. A. Quamme.** Renal magnesium handling and its hormonal control. *Physiol. Rev.* 74: 305–322, 1994.
35. **Wittner, M., and A. Di Stefano.** Effects of antidiuretic hormone, parathyroid hormone and glucagon on the cortical and medullary thick ascending limb of Henle's loop of the mouse nephron. *Pflügers Arch.* 415: 707–712, 1990.

