

Extracellular Mg^{2+} - and Ca^{2+} -sensing in mouse distal convoluted tubule cells

BRIAN W. BAPTY, LONG-JUN DAI, GORDON RITCHIE, FRANK JIRIK, LUCIE CANAFF, GEOFFREY N. HENDY, and GARY A. QUAMME

Department of Medicine, University of British Columbia, University Hospital, Koerner Pavilion, Vancouver, British Columbia, and Departments of Medicine, Physiology and Human Genetics, McGill University and Royal Victoria Hospital, Montreal, Quebec, Canada

Extracellular Mg^{2+} and Ca^{2+} -sensing in mouse distal convoluted tubule cells. An immortalized cell line (designated MDCT) has been extensively used to investigate the cellular mechanisms of electrolyte transport within the mouse distal convoluted tubule. Mouse distal convoluted tubule cells possess many of the functional characteristics of the *in vivo* distal convoluted tubule. In the present study, we show that MDCT cells also possess a polyvalent cation-sensing mechanism that is responsive to extracellular magnesium and calcium. Southern hybridization of reverse transcribed-polymerase chain reaction (RT-PCR) products, sequence determination and Western analysis indicated that the calcium-sensing receptor (*Casr*) is expressed in MDCT cells. Using microfluorescence of single MDCT cells to determine cytosolic Ca^{2+} signaling, it was shown that the polyvalent cation-sensing mechanism is sensitive to extracellular magnesium concentration ($[Mg^{2+}]_o$) and extracellular calcium concentration ($[Ca^{2+}]_o$) in concentration ranges normally observed in the plasma. Moreover, both $[Mg^{2+}]_o$ and $[Ca^{2+}]_o$ were effective in generating intracellular Ca^{2+} transients in the presence of large concentrations of $[Ca^{2+}]_o$ and $[Mg^{2+}]_o$, respectively. These responses are unlike those observed for the *Casr* in the parathyroid gland. Finally, activation of the polycation-sensitive mechanism with either $[Mg^{2+}]_o$ or $[Ca^{2+}]_o$ inhibited parathyroid hormone-, calcitonin-, glucagon- and arginine vasopressin-stimulated cAMP release in MDCT cells. These studies indicate that immortalized MDCT cells possess a polyvalent cation-sensing mechanism and emphasize the important role this mechanism plays in modulating intracellular signals in response to changes in $[Mg^{2+}]_o$ as well as in $[Ca^{2+}]_o$.

An extracellular Ca^{2+} -sensing receptor (*Casr*), responsive to polyvalent cations such as Mg^{2+} , Gd^{3+} and neomycin, in addition to Ca^{2+} has been demonstrated in many tissues and many species [1]. This G protein coupled receptor was first cloned by Brown et al from the bovine parathyroid gland where it is involved with control of parathyroid hormone (PTH) secretion [2]. 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 carboxyl terminal domain of 222 amino acids that likely resides within the cytoplasm and is involved with intracellular signaling processes [1]. The evidence is that extracellular Ca^{2+} -concentration ($[Ca^{2+}]_o$) binds to the extracellular domain initiating a number of intracellular signals. Among other things, stimulation of G_i -proteins modulates adenylate cyclase activity and adenosine 3',5'-cyclic phosphate (cAMP) levels [3, 4] and G_q -proteins activate phospholipase C releasing inositol 1,4,5-trisphosphate and cytosolic Ca^{2+} [5–8]. *Casr*-mediated intracellular signaling pathways have important effects on cellular function [1].

A *Casr* similar to that found in the parathyroid gland has been shown to be present in bovine, rat, mouse, and human kidneys [2, 9–11]. Northern analysis and *in situ* hybridization revealed that the rat kidney *Casr* is expressed in renal outer medulla and cortical medullary rays, which are regions abundant in thick ascending limbs [11]. More recent studies using *in situ* hybridization and RT-PCR demonstrated that the rat kidney *Casr* is present in glomeruli, proximal tubules, cortical and medullary thick ascending limbs, distal convoluted tubules, cortical collecting ducts, and outer medullary collecting ducts [12, 13]. Immunocytochemical staining showed that the *Casr* located in the distal convoluted tubule is less abundant than other segments, particularly the thick ascending limbs and outer medullary collecting ducts [11]. The *Casr* appears to be preferentially located to the basolateral membrane of the distal convoluted tubule (presented at the 1996 American Society of Nephrology annual meeting); however, the functions of this basolateral *Casr* in the distal convoluted tubule are not understood.

The distal convoluted tubule plays an important role in salt conservation. This segment reabsorbs as much as 10% of the filtered magnesium and calcium that are under control of many hormones including PTH, calcitonin, glucagon, and vasopressin [14, 15]. Little is known about the cellular mechanisms of electrolyte absorption in the distal convoluted tubule because this segment is difficult to isolate for *in vitro* microperfusion studies. Accordingly, isolated cell lines have been used to study functional responses. An immortalized mouse distal convoluted tubule (MDCT) cell line has been extensively used to characterize cation transport in this nephron segment [16]. The MDCT cells possess amiloride-inhibitable Na^+ conductance, chlorothiazide-sensitive $NaCl$ cotransport, and hormone-stimulated magnesium and calcium transport [14–19]. Accordingly, this established cell line has proven to be very useful in describing functions of the intact distal

Key words: reverse transcriptase-polymerase chain reaction, intracellular Ca^{2+} , cAMP measurements, polyvalent cation sensing in DCT, transport of electrolytes, immortalized cell line.

Received for publication June 26, 1997
and in revised form September 25, 1997
Accepted for publication September 26, 1997

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convoluted tubule. In the present study, we show that a polyvalent cation-sensing mechanism is present in MDCT cells that responds sensitively and equipotently to extracellular magnesium concentration ($[Mg^{2+}]_o$) and $[Ca^{2+}]_o$. The presence of polyvalent cation receptors in this established cell line will facilitate study of their function in control of magnesium and calcium absorption within the distal convoluted tubule. Importantly, we demonstrate the presence of a *Casr* in MDCT cells that contributes, in part, to their ability to sense extracellular cations.

METHODS

Cell culture

The established mouse distal convoluted tubule cell line (MDCT) used in these studies has functional properties that are typical of the distal convoluted tubule such as chlorothiazide- and amiloride-enhanced calcium and magnesium transport, chlorothiazide-inhibitable NaCl uptake and amiloride-sensitive Na^+ entry [14–19]. It was kindly provided by Dr. Peter A. Friedman (Dartmouth Medical School, NH, USA). The MDCT cell line was cultured in Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 (GIBCO), 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 hybridization studies, MDCT cells were grown to confluence on plastic supports and harvested by scraping into buffered solution. For the fluorescent studies, confluent cells were washed three times with phosphate-buffered saline (PBS) containing 5 mM ethylene glycol-bis(β -aminoethyl ether) N,N,N',N'-tetraacetic acid (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 one to two days in supplemented media as described above.

RNA preparation

Total RNA was extracted from confluent MDCT cells using TRIzol (GIBCO BRL). Briefly, cells from a 150 cm^2 flask were rinsed in 5 ml PBS, pelleted and lysed with a five minutes incubation in 2 ml TRIzol reagent. Then, 0.4 ml chloroform was added and the mixture shaken vigorously for 15 seconds. After two minutes the mixture was centrifuged at $12,000 \times g$ for 15 minutes and the upper aqueous phase containing the extracted RNA aspirated. RNA was precipitated from the aqueous phase by an equal volume isopropanol and pelleted with centrifugation at $12,000 \times g$ for 10 minutes at 4°C. The RNA precipitate was washed $2 \times$ with 75% ice-cold ethanol, dried, and taken up in 200 μ l DEPC-treated distilled H_2O . Ten micrograms of RNA were incubated with RNase free DNase (134 U) in the presence of 5 mM $MgCl_2$ at 37°C for 10 minutes. The DNase was heat inactivated with a five minutes incubation at 99°C and the product stored at $-80^\circ C$. The same procedure was used to prepare total RNA from mouse cortical kidney.

Reverse-transcription-polymerase chain reaction analysis

Reverse-transcription-polymerase chain reaction (RT-PCR) was carried out as follows. First-strand cDNA was made using 3 μ g DNase I-treated total RNA in a total volume of 20 μ l as described [20]. Five microliters of the RT reaction were electrophoresed in ethidium-bromide stained 1% agarose gels to check

for quantity and quality of the cDNA. Five microliters were used for PCR amplification. A cDNA fragment of 509 bp corresponding to the mRNA sequence encoding the majority of the transmembrane-spanning regions was amplified using forward primer 5'-GTTCCGAAACACACCTATCGTCAAG-3' and reverse primer 5'-TGAACCTGGCTTCGTTGAAGTTCTC-3'. In each case, the PCR amplification (total volume 50 μ l) consisted of 32 cycles of denaturation at 94°C for 40 seconds, annealing at 55°C for 30 seconds, and polymerization at 72°C for 45 seconds using a GeneAmp PCR System thermocycler Model 9600 (Perkin-Elmer-Cetus, Branchburg, NJ, USA). Aliquots (4 μ l) of the PCR reaction taken after 20, 24, 28, 32 cycles were electrophoresed through ethidium-bromide stained 1% agarose gels.

Sequence analysis of PCR-amplified *Casr* product was performed after subcloning the amplified fragment into the pCRII TA cloning vector (Invitrogen, San Diego, CA, USA). Dideoxynucleotide sequencing using the T7/SP6 Sequencing Kit (Pharmacia LKB, Uppsala, Sweden) and [^{35}S] deoxycytidine ATP (1200 Ci/mmol; Amersham PLC, Oakville, ON, Canada) was carried out on miniprep DNA from at least three different subclones.

Southern hybridization

The probe was generated from 100 ng of full length HuKCaSR cDNA taken up in 9 μ l H_2O , boiled for five minutes and put on ice. After a quick spin 2.0 μ l $10 \times$ Klenow buffer, 2.0 μ l $10 \times$ ATG mix 1.0 μ l random hexamer (100 pmol/ μ l), 1.0 μ l Klenow, and 5.0 μ l 32-dCTP (50 μ Ci), were added and the reaction incubated at 37°C for 30 minutes. Unincorporated nucleotides were removed with a Sephadex G-50 spin column equilibrated with 10 mM Tris/HCl, 1 mM EDTA (pH 7.6).

The PCR products were displayed on a Southern blot by agarose gel (1%) electrophoresis and transferred by downward alkaline capillary transfer to GeneScreen. The blot was crosslinked in a UV Stratalinker (Stratagene, San Diego, CA, USA). The prehybridization solution was made from 0.3 g fat free BSA, 10.5 ml 1 M sodium phosphate, 9.0 ml formamide, and 10.5 ml 20% SDS. The boiled probe was added to the blot and allowed to hybridize at 50°C overnight. Two washes in 250 ml of 150 mM sodium phosphate and 0.1% SDS at room temperature were followed by successive washes at 50°C until the background radiation on the blot was minimal. The blots were exposed on Kodak X-OMAT film (Eastman Kodak, Rochester, NY, USA).

Western blots

Cells were lysed in triple detergent buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.02% NaN_3 , 0.1% SDS, 1 mM EDTA, 100 μ g/ml PMSF, 2 μ g/ml leupeptin, 2 μ g/ml aprotinin, 0.1% NP-40, 0.5% sodium deoxycholate) for five minutes at 0°C. The cell lysates were spun at $1200 \times g$, two minutes, 4°C and the supernatants were stored at $-80^\circ C$. Aliquots were electrophoresed through 8% SDS-polyacrylamide gels and blotted onto polyvinylidene difluoride (PVDF) membranes (BioRad). 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 one to two hours and incubated with an antiserum for the *Casr*. A mouse monoclonal antibody (ADD) raised against a peptide comprising residues 214 to 236 of *Casr* was used [21]. This antibody was provided by Drs. P.K. Goldsmith and A.M. Spiegel

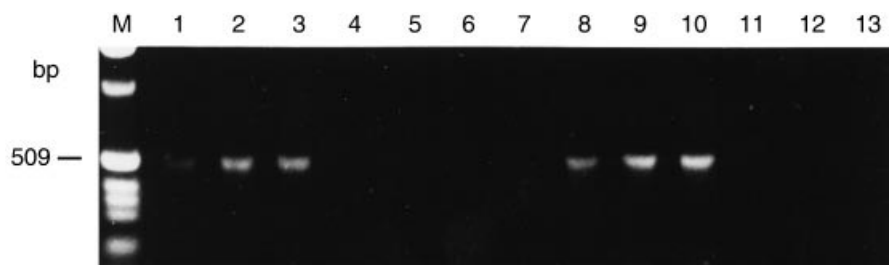


Fig. 1. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of calcium sensing receptor (*Casr*) mRNA expression.

Complementary DNA was synthesized from total RNA and used as template for PCR amplification. PCR products (4 μ l) after either 24 (lanes 1 to 7) or 32 (lanes 8 to 13) cycles were electrophoresed on an ethidium-bromide-stained 1% agarose-TBE gel. Lane M, 1 kb ladder; lanes 1 and 8, MDCT; lanes 2 and 9, AtT-20; lanes 3 and 10, TT; lanes 4 and 11, COS-7; lanes 5 and 12, MC3T3-E1; lanes 6 and 13, NIH3T3; lane 7, RT. After Southern blotting the PCR product was hybridized to a 32 P-labeled *Casr* DNA probe (data not shown).

(NIH, Bethesda, MD, USA) and K.V. Rogers (NPS Pharmaceuticals, UT, USA) and has been extensively characterized with respect to specificity for the parathyroid/kidney *Casr* [21]. As the control, immunoblotting was carried out as above with the antiserum preabsorbed for one hour with the peptide (10 μ g/ml) against which it was raised.

Determination of cytosolic free Ca^{2+}

All fluorescent studies were performed on single subconfluent MDCT cells within 10 days of isolation. Single cells were loaded with 10 μ M fura 2-AM according to previously described techniques [22]. The fluorescent dye, dissolved in dimethyl sulfoxide, was added directly to the medium with the aid of Pluronic F-127 acid (0.125%; Molecular Probes) and incubated for 30 minutes at 23°C. Loaded cells were washed twice with a buffered salt solution, which contained the following (in mM): 145 NaCl, 4.0 KCl, 1 $CaCl_2$, 0.5 $MgCl_2$, 0.8 Ka_2HPO_4 , 0.1 KH_2PO_4 , 5 glucose, and 20 HEPES-Tris, pH 7.4. The cells were incubated a further 20 minutes to ensure complete de-esterification and finally washed once with fresh buffer solution. Cover glasses, with cells loaded with fura 2, were mounted in a chamber containing 500 μ l buffer placed on the mechanical stage of a Nikon inverted microscope. The fluorescence signal was monitored at 500 nm with excitation wavelengths alternating between 335 nm and 385 nm, using a spectrofluorometer (Deltascan Photon Technologies, Santa Clara, CA, USA), and intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was calculated, assuming a dissociation constant of 224 nM for the fura 2 \cdot Ca^{2+} complex after correction for fluorescence from extracellular fura 2 and autofluorescence, according to previously described methods [22]. In all studies, rapid changes in magnesium ($[Mg^{2+}]_o$) and calcium ($[Ca^{2+}]_o$) extracellular concentrations of the bathing buffers were made by total replacement of the superfusion. In all experiments involving $[Ca^{2+}]_i$ analyses, single traces are shown, but similar results were obtained in at least four separate experiments from independent cell preparations.

cAMP measurements

3',5'-Adenosine monophosphate (cAMP) was determined in confluent MDCT cell monolayers cultured in 24-well plates in DMEM-Ham's F-12 media without serum. After addition of various hormones, MDCT cells were incubated at 37°C for five minutes in the presence of 0.1 mM isobutylmethylxanthine (IBMX). The cAMP was extracted with 5% trichloroacetic acid that was removed with ether acidified with 0.1 N HCl. The aqueous phase was dried, dissolved in Tris-EDTA buffer, and

cAMP was measured with a radioimmunoassay kit (Diagnostic Products Corporation, Los Angeles, CA, USA).

Statistical analysis

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

RESULTS

In a previous study with magnesium-depleted MDCT cells, we reported that 10 mM $[Ca^{2+}]_o$ was without effect on the Mg^{2+} refill, as determined with mag-fura-2, over 10 to 30 minutes of fluorescence determinations [17]. However, it is of interest in these studies that the addition of either $[Ca^{2+}]_o$ or $[Mg^{2+}]_o$ resulted in a rapid and transient intracellular increase in Ca^{2+} concentration. This prompted us to examine this cell line further for the expression of extracellular divalent cation-sensing receptors. In the present studies, a functional polyvalent cation-sensitive receptor was shown to be expressed in MDCT cells by two methods. First, the *Casr* sequence was obtained from RT-PCR amplified products of MDCT cell mRNA, and Western analysis showed that the message is expressed in protein form. Second, *Casr*-stimulated intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) release was determined by microfluorescence.

Reverse transcription-polymerase chain reaction analysis of *Casr* mRNA in immortalized cells

The primer set used in our study amplified a product from MDCT cell cDNA of 509 bp corresponding to a portion of the mRNA encoding the majority of the transmembrane spanning region of the *Casr* (Fig. 1). This was confirmed by subcloning and sequencing of the product. As the positive control, a similar PCR product was also obtained from AtT-20 and TT cell RNA, cell lines that have previously been reported to express the *Casr* [23–25]. As the negative control, under the identical conditions of reverse transcription and amplification, no PCR product was obtained from RNA of COS-7, MC3T3-E1 or NIH3T3 cells, all previously reported to be negative for the *Casr* [26]. The nucleotide sequence of the MDCT RT-PCR product shared greater than 92% identity with the corresponding rat *Casr* sequence (Fig. 2) [11].

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1   ttc cga aac aca cct atc gtc aag gcc acc aac cga gag
1   F   R   N   T   P   I   V   K   A   T   N   R   E

40  ctg tcc tac ctc ctg ctc ttc tca ctc ctc tgc tgc ttc
14  L   S   Y   L   L   L   F   S   L   L   C   C   F

79  tcc agc tcc ctg ttc ttc att ggg gag ccc cag gac tgg
27  S   S   S   L   F   F   I   G   E   P   Q   D   W

118 acc tgc cgc ctg cga cag ccc gcc ttc ggc atc agc ttc
40  T   C   R   L   R   Q   P   A   F   G   I   S   F

157 gtg ctt tgt atc tcg tgc atc ttg gtg aag acc aat cga
53  V   L   C   I   S   C   I   L   V   K   T   N   R

196 gtc ctc ctg gta ttt gag gcc aaa ata ccc acc agc ttc
66  V   L   L   V   F   E   A   K   I   P   T   S   F

235 cac cgg aag tgg tgg gga ctc aac ctg cag ttc ctg ctg
79  H   R   K   W   W   G   L   N   L   Q   F   L   L

274 gtt ttc ctc tgc acc ttc atg cag att gtc atc tgc atc
92  V   F   L   C   T   F   M   Q   I   V   I   C   I

313 atc tgg ctc tac acg gca ccc ccc tcc agc tac cgc aac
105 I   W   L   Y   T   A   P   P   S   S   Y   R   N

352 cac gag ctg gaa gac gaa atc ttc atc acg tgc cat gag
118 H   E   L   E   D   E   I   F   I   T   C   H   E

391 ggc tca ctc atg gcg ctc ggc tcc ctg atc ggc tac acc
131 G   S   L   M   A   L   G   S   L   I   G   Y   T

430 tgc ctc ctg gct gcc atc tgc ttc ttc ttt gcc ttc aag
144 C   L   L   A   A   I   C   F   F   F   A   F   K

469 tct cgg aag ctg cca gag aac ttc aac gaa gcc aag ttc
157 S   R   K   L   P   E   N   F   N   E   A   K   F

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Fig. 2. Nucleotide sequence of the immortalized mouse distal convoluted tubule (MDCT) cell reverse transcribed-polymerase chain reaction (RT-PCR) product shown in Figure 1. Primer sequences are underlined (with one nucleotide missing from each primer at the 5' and 3' ends of the actual PCR product). The sequence is greater than 92% homologous to the corresponding portion of the rat calcium sensing receptor (*Casr*).

Western analysis of the *Casr* in immortalized cells

Western analysis of cell extracts from MDCT cells revealed a similar pattern of bands to that seen in parathyroid (Fig. 3) and kidney (data not shown). The predominant species representing the nonglycosylated and glycosylated forms ranged from 120 to 160 kDa, with some additional higher molecular weight aggregates. The immunostaining of the bands was specifically abolished by preadsorption of the antibody with peptide (data not shown). Extracts of COS-7 kidney cells, mouse MC3T3-E1 osteoblast-like cells and mouse NIH3T3 fibroblasts were negative for specific *Casr* staining (Fig. 3).

Intracellular Ca^{2+} signaling in immortalized cells by *Casr* activation

The *Casr* has been shown to be coupled to a G_q -protein, which upon activation stimulates phospholipase C activity, leading to inositol 1,4,5- trisphosphate generation and in turn intracellular Ca^{2+} release [3, 5–8, 27]. To determine if the *Casr* expressed in the MDCT cells is associated with intracellular signaling, we determined $[Ca^{2+}]_i$ in response to extracellular polyvalent cations (Figs. 3 and 4). The MDCT cells were cultured in normal media containing 0.5 mM magnesium and 1.0 mM calcium. The cells were loaded with fura-2 for 30 minutes in buffer solutions containing

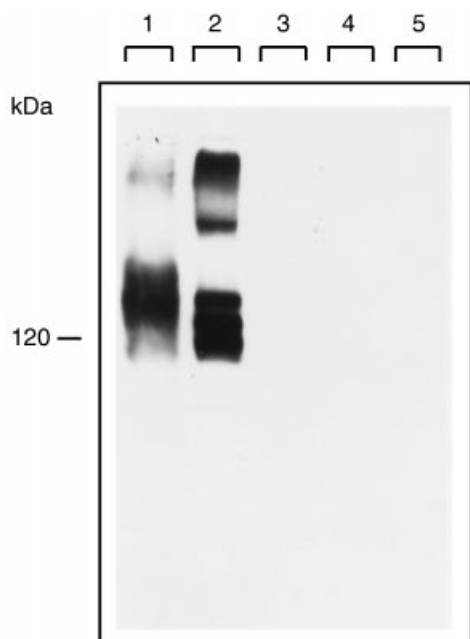


Fig. 3. Western analysis of the calcium sensing receptor (*Casr*). Cell or parathyroid gland extracts (10 μ g protein each) were subjected to SDS-PAGE on a 4 to 12% gradient gel. The blot was stained with *Casr* mouse monoclonal antibody (ADD [21]). Lane 1, MDCT cells; lane 2, rat parathyroid; lane 3, COS-7 cells; lane 4, MC3T3-E1; lane 5, NIH3T3 cells. The bands were demonstrated to be specific after staining the blot with the same antiserum preincubated with the peptide against which it was raised (data not shown).

0.5 mM magnesium and 1.0 mM calcium; the extracellular fura-2/AM was removed with two washes of nominally calcium- and magnesium-free buffer solutions and $[Ca^{2+}]_i$ was quantitated by fluorescence. The polyvalent cations (in mM), $MgCl_2$ 0.5, $CaCl_2$ 1.0, $GdCl_3$ 0.1, or neomycin 0.01, were added in buffer as indicated in Figure 3. The cations were changed by complete replacement of bathing solutions. $[Mg^{2+}]_o$ was tested in the presence of normal $[Ca^{2+}]_o$ in the buffer and the effects of $[Ca^{2+}]_o$ were determined in the presence of normal $[Mg^{2+}]_o$ (Fig. 3). The addition of external $[Mg^{2+}]_o$ increased $[Ca^{2+}]_i$ from basal levels of 107 ± 23 to peak concentrations of 1046 ± 121 nM ($N = 6$, $P < 0.05$; Table 1). $[Ca^{2+}]_o$ resulted in an increase in $[Ca^{2+}]_i$ to 732 ± 92 nM, Gd^{3+} to 1637 ± 161 nM, and neomycin to 1073 ± 155 nM from basal levels (Table 1). Replacement of bathing solution with equiosmolar salt solutions, containing either NaCl or KCl, did not elicit cytosolic Ca^{2+} transients. Of 108 individual cells studied in this report and others, a total of 24 cells failed to respond to extracellular polyvalent cations. There were no differences in the number of nonresponsive cells observed for each of the tested extracellular cations (Table 1). This appeared to be an all-or-none response, as subsequent additions of very high concentrations of extracellular cations did not stimulate $[Ca^{2+}]_i$ in those cells that initially did not respond. The reason for the nonresponsiveness in some of the MDCT cells is not known. In all cases, the change in cytosolic Ca^{2+} in the positive cells was transient, returning to near basal levels within one to two minutes. The majority of the cytosolic Ca^{2+} transients were monophasic. Moreover, the responses were not dependent on the presence of extracellular Ca^{2+} , indicating that the rise in $[Ca^{2+}]_i$ was from

intracellular sources. The profile of the *Casr*-mediated rise in cytosolic Ca^{2+} is different than the sustained rise in $[Ca^{2+}]_i$ observed following the addition of PTH or calcitonin to MDCT cells [19, 28, 29]. There was no change in the mean cytosolic Mg^{2+} concentration (0.52 ± 0.02 mM, $N = 5$) during these manipulations as determined with the Mg^{2+} -sensitive fluorescent probe, mag-fura-2 [26]. These results support the notion that the MDCT cell line possesses a functional *Casr* that elicits a transient increase in cytosolic Ca^{2+} in response to extracellular polyvalent cations.

The relative potencies of extracellular cations in stimulating *Casr*-sensitive cytosolic Ca^{2+} signaling for many renal and extra-renal cells have been reported to be in the order of 3 to 5 mM for $[Ca^{2+}]_o$ and about 5 to 20 mM for $[Mg^{2+}]_o$ [2, 6, 8, 10, 27, 30, 31]. Figure 5 summarizes the changes in cytosolic Ca^{2+} in response to $[Mg^{2+}]_o$ and $[Ca^{2+}]_o$ of MDCT cells in buffer solutions containing normal $MgCl_2$, 0.5 mM, and $CaCl_2$, 1.0 mM, concentrations. The addition of either 0.2 mM $[Mg^{2+}]_o$ or 0.2 mM $[Ca^{2+}]_o$ induced significant increases in cytosolic Ca^{2+} levels. Changing the bathing solution but not the $[Mg^{2+}]_o$ concentration by 0.5 mM had no effect on $[Ca^{2+}]_i$ (Fig. 5A). Replacement of bathing solutions containing 0.7 mM $MgCl_2$ (total) for normal buffer solutions containing 0.5 mM $MgCl_2$ and 1.0 mM $CaCl_2$ with one containing 1.2 mM $CaCl_2$ (total) resulted in an increase in $[Ca^{2+}]_i$ to 542 ± 73 nM, $N = 4$ (Fig. 5B). Note that the replacement of normal bathing solution containing 1.0 mM $[Ca^{2+}]_o$ did not elicit a response. Accordingly, the polyvalent cation-sensing mechanism in MDCT cells is sensitive to changes in both $[Mg^{2+}]_o$ and $[Ca^{2+}]_o$ within the physiological ranges of the divalent cations. Alternatively, there may be other receptors in the MDCT cell that are sensitive to $[Mg^{2+}]_o$ in the physiological range.

The *Casr* has been proposed to be a Mg^{2+} -sensing receptor in addition to its role in Ca^{2+} metabolism [1]. There is evidence of different binding sites for each polyvalent cation so that the receptor may respond in a selective manner to either $[Mg^{2+}]_o$ and $[Ca^{2+}]_o$ [31]. To test whether the MDCT *Casr* is responsive to $[Mg^{2+}]_o$ independently of $[Ca^{2+}]_o$, we determined the response to Mg^{2+}_o in the presence of large concentrations of $[Ca^{2+}]_o$ (Fig. 6). The MDCT cells were bathed in buffer solution containing 5 mM $CaCl_2$, no magnesium and challenged with 0.2 mM Mg^{2+}_o . The addition of 0.2 mM $[Mg^{2+}]_o$ stimulated a significant rise in $[Ca^{2+}]_i$ in the presence of 5 mM $[Ca^{2+}]_o$ (Fig. 6A). To determine the sensitivity of the *Casr* to $[Ca^{2+}]_o$ in the presence of a background of large concentrations of Mg^{2+}_o , MDCT cells were immersed in a buffer solution containing 5.0 mM $MgCl_2$, zero magnesium and 0.2 mM $CaCl_2$ was added (Fig. 6B). The addition of 0.2 mM $CaCl_2$ led to significant increases in $[Ca^{2+}]_i$. The polyvalent cation-sensitive mechanism present in MDCT cells is responsive to small changes in $[Mg^{2+}]_o$ or $[Ca^{2+}]_o$ in the presence of large background concentrations of extracellular calcium and magnesium, respectively. These results suggest that the *Casr* responds independently and equipotently to $[Ca^{2+}]_o$ and $[Mg^{2+}]_o$, or that there are separate receptors for the two cations.

Figure 7 illustrates the effect of repetitive stimulations of the *Casr* with sequential challenges of either $MgCl_2$ or $CaCl_2$. Both $[Mg^{2+}]_o$ and $[Ca^{2+}]_o$ stimulated transient increases in cytosolic Ca^{2+} that diminished after a number of repetitive additions of divalent cation (Fig. 7). The diminution of Ca^{2+} signaling after many repetitive stimulations may reflect desensitization of the

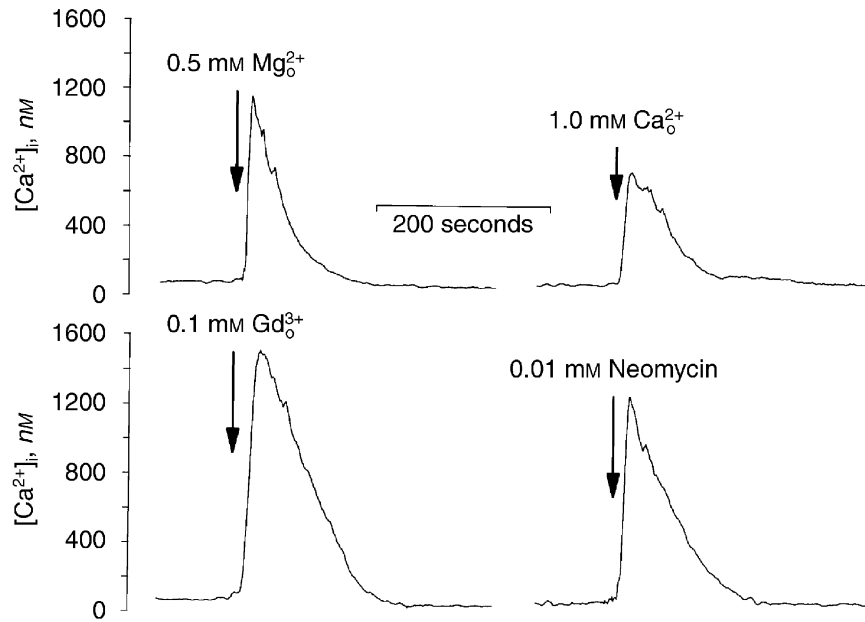


Fig. 4. External polyvalent cations transiently increase cytosolic Ca^{2+} in immortalized mouse distal convoluted tubule (MDCT) cells. MDCT cells were cultured in DMEM/Ham's F-12 (1:1) with 10% fetal calf serum containing 0.6 mM magnesium and 1.5 mM calcium. The cells were loaded with fura-2 for 30 minutes in a buffer solution containing (in mM): MgCl_2 0.5, CaCl_2 1.0, NaCl 145, KCl 4.0, K_2HPO_4 0.8, KH_2PO_4 0.2, glucose 5, and HEPES-Tris 20, pH 7.4. In order to test the effect of $[\text{Mg}^{2+}]_o$, the buffer solution was changed to one containing the above but without MgCl_2 . This bathing solution was replaced 1 to 2 minutes later with one containing 0.5 mM MgCl_2 . To test $[\text{Ca}^{2+}]_o$, the cells were initially bathed with the above solutions containing no CaCl_2 . This was replaced with one containing 1.0 mM CaCl_2 . The complete buffer solution, containing 0.5 mM MgCl_2 0.5, and 1.0 mM CaCl_2 , was used to determine the effects of external 0.1 mM GdCl_3 and 0.01 mM neomycin. Again, these were done through bath solution changes. Tracings are representative of 4 to 10 separate experiments.

Table 1. Polyvalent cations stimulate cytosolic Ca^{2+} transients in immortalized mouse distal convoluted tubule (MDCT) cells

Extracellular cation	Concentration mM	Response pos/total	Basal $[\text{Ca}^{2+}]_i$		$\Delta[\text{Ca}^{2+}]_i$ nM
			nM		
Mg^{2+}	0.5	(6/9)	107 ± 23 (6) ⁿ	1046 ± 121 ^a (6)	
Ca^{2+}	1.0	(10/13)	92 ± 15 (10)	732 ± 92 ^a (10)	
Gd^{3+}	0.1	(5/8)	93 ± 13 (5)	1637 ± 161 ^a (5)	
Neomycin	0.01	(7/11)	107 ± 8 (7)	1073 ± 155 ^a (7)	

Normal MDCT cells were loaded with fura-2 and MgCl_2 , CaCl_2 , GdCl_3 or neomycin added to buffer solutions at the indicated concentrations as given in legend to Fig. 3. $\Delta[\text{Ca}^{2+}]_i$ was the maximal change in cytosolic Ca^{2+} from basal Ca^{2+} levels in those that responded. The cells that responded to the addition of the polyvalent cations are indicated as a fraction of the total cells studied (pos/total). ()ⁿ is the number of separate observations and ^a indicates significance, $P < 0.05$ from basal $[\text{Ca}^{2+}]_i$.

Casr to $[\text{Mg}^{2+}]_o$ and $[\text{Ca}^{2+}]_o$ or depletion of intracellular Ca^{2+} stores as each cation challenge was applied. This phenomenon is distinct from the rapid desensitization observed in hormone-induced cytosolic Ca^{2+} transients observed in the thick ascending limb cell [22] or the sustained elevation seen in hormone-mediated increments in Ca^{2+} entry in distal convoluted tubule cells [14, 19].

***Casr* activation inhibits hormone-stimulated cAMP release**

Finally, we determined the effect of activation of *Casr* on hormone-stimulated release of cAMP. The MDCT cells possess parathyroid hormone (PTH), calcitonin, glucagon and arginine vasopressin (AVP) receptors, which are coupled to adenylate cyclase [14]. Parathyroid hormone, calcitonin, glucagon at 10^{-7} M, and AVP, 10^{-8} M, increase cellular cAMP levels by about threefold. The potent polyvalent cation, neomycin, was used to activate the *Casr* prior to the addition of hormones (Table 2). Pretreatment of the MDCT cells with neomycin inhibited hormone-stimulated cAMP accumulation. Next, we activated the Ca-SR with high levels of $[\text{Mg}^{2+}]_o$ or $[\text{Ca}^{2+}]_o$ (Table 2). Pretreat-

ment of MDCT cells with 10 mM $[\text{Mg}^{2+}]_o$ or $[\text{Ca}^{2+}]_o$ inhibited hormone-mediated cAMP release. Thus, the *Casr* present in MDCT cells may be coupled to G_i proteins leading to inhibition of hormone-responsive adenylate cyclase activity.

DISCUSSION

In this study, we show that the established MDCT cell line possesses a *Casr* that responds to $[\text{Mg}^{2+}]_o$ and $[\text{Ca}^{2+}]_o$, to transiently release Ca^{2+} from cytosolic stores. This immortalized cell line is representative of the intact distal convoluted tubule as it possesses many of the properties characteristic of this segment including chlorothiazide-sensitive NaCl cotransport, amiloride-inhibitable Na^+ conductance, and parathyroid hormone and calcitonin-stimulated calcium and magnesium transports [14, 17–19]. Furthermore, these cells possess vitamin D receptors, vitamin D metabolite-induced 24-hydroxylase activity, P-450 metabolites, and the calbindin 28K calcium-binding protein that are important in vitamin D actions [14, 32, 33]. Accordingly, it is of interest that this cell line also has an extracellular divalent cation-sensing receptor. Using RT-PCR and Southern blotting, we show that the MDCT cells possess transcripts for the *Casr*. The presence of the *Casr* protein in MDCT cells was documented by Western blot analysis using a specific *Casr* antiserum. Furthermore, determination of cytosolic Ca^{2+} with fluorescence showed that *Casr* in MDCT cells elicit intracellular signals in response to increasing extracellular concentrations of polyvalent cations, demonstrating that the receptor is functional. These studies with mouse distal convoluted cells are consonant with reports of *Casr* transcripts in the rat distal convoluted segment [11–13]. More recently, Riccardi et al showed that the rat *Casr* is localized to the basolateral membrane of the distal convoluted tubule (Riccardi et al, presented at the 1996 ASN). The presence of a *Casr* in the distal convoluted tubule may have important ramifications on cellular function within this segment.

The importance of the *Casr* in the control of $[\text{Mg}^{2+}]_o$ and $[\text{Ca}^{2+}]_o$ homeostasis is indicated by the fact that individuals with

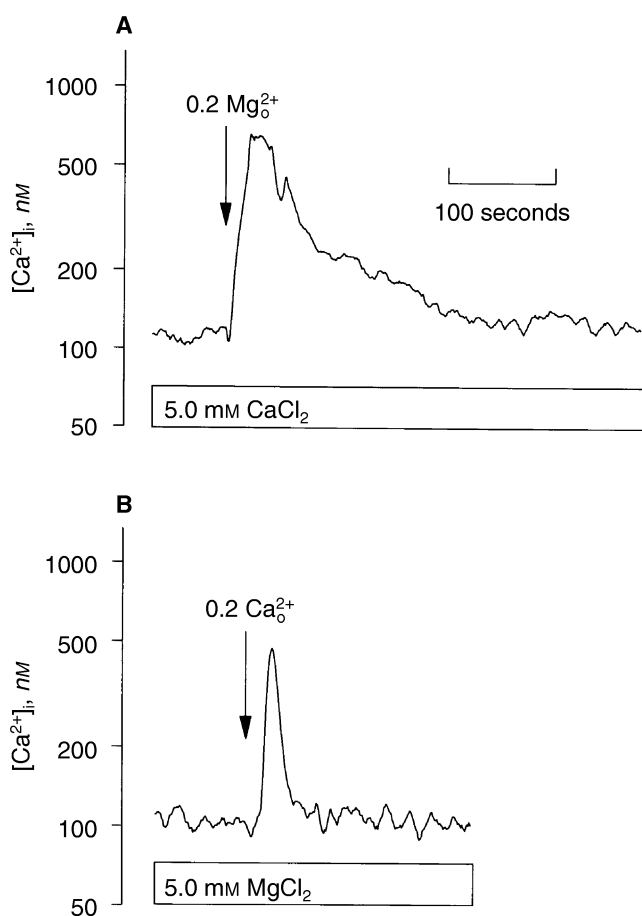


Fig. 5. The calcium sensing receptor (*Casr*) of immortalized mouse distal convoluted tubule (MDCT) cells responds sensitively to $[Mg^{2+}]_o$ and $[Ca^{2+}]_o$. MDCT cells were loaded with fura-2 according to methods given in legend to Figure 3, but the loading and test buffer solutions contained (in mM): $MgCl_2$ 0.5, $CaCl_2$ 1.0, $NaCl$ 145, KCl 4.0, Na_2HPO_4 0.8, KH_2PO_4 0.2, glucose 5, and HEPES-Tris 20, pH 7.4. $MgCl_2$ (A) or $CaCl_2$ (B) was added by changing buffer solutions containing the concentrations. Tracings are representative of 4 to 5 separate experiments.

familial hypocalciuric hypercalcemia (FHH) manifest hypermagnesemia and hypercalcemia, which is due in part to excessive renal reabsorption [34, 35, 37, 38]. Additionally, mice that are heterozygous or homozygous with inactivating mutations of the *Casr* show significant increases in $[Mg^{2+}]_o$ and $[Ca^{2+}]_o$ relative to normal mice [37]. Attie et al performed clearance studies on hypoparathyroid patients and concluded that inappropriate renal calcium conservation is due to an inherent abnormality of the kidney [38]. Conversely, they suggested that inappropriate magnesium reabsorption might be due predominantly to elevated circulating PTH levels. However, further experiments are required to determine the precise role of the *Casr* in distal tubular absorption of both magnesium and calcium.

In all studies to date, $[Ca^{2+}]_o$ has been found to be a more potent stimulator of Ca-SR-induced intracellular signaling than $[Mg^{2+}]_o$. The threshold value for $[Ca^{2+}]_o$ has been reported to be of the order of 1 to 5 mM for renal cells, whereas a similar cytosolic Ca^{2+} response requires the presence of 5 to 20 mM $[Mg^{2+}]_o$ [39]. These relative potencies of $[Ca^{2+}]_o$ and $[Mg^{2+}]_o$ recapitulates

their actions in bovine parathyroid cells and in *Xenopus* oocytes injected with cRNA of cloned *Casrs* [2, 6, 8]. Thus, it is interesting that the polyvalent cation-sensitive mechanism of MDCT cells was apparently as sensitive to $[Mg^{2+}]_o$ as it was to $[Ca^{2+}]_o$. This is particularly noteworthy as the $[Mg^{2+}]_o$ studies were performed in the presence of normal or elevated $[Ca^{2+}]_o$ (Fig. 4). The functional consequences of changes in the amino acid sequence of the *Casr* has been investigated by expressing a variety of mutated receptors in HEK 293 cells [40]. Some of the mutations diminish *Casr* signaling, others enhance the sensitivity to external $[Ca^{2+}]_o$, and still others are completely nonfunctional with no intracellular signaling as determined by changes in $[Ca^{2+}]_i$ [32, 39, 41, 42]. From these transfection studies of mutated *Casrs*, Bai et al suggested that discrete but interrelated cation binding sites may exist in this receptor [40]. Ca^{2+} binding to the *Casr* *per se* has yet to be examined. These discrete sites remain to be identified but it is apparent from these studies that changes in the extracellular domain of the *Casr* may alter sensitivity to the various ligands [40]. The present studies with the endogenous polyvalent cation-sensitive mechanism of the MDCT cell show that the intracellular signaling is responsive to both $[Mg^{2+}]_o$ and $[Ca^{2+}]_o$ and that there appears to be little interaction between these cations. Accordingly, the *Casr* may function as a $[Mg^{2+}]_o$ receptor (Mgsr) even in the presence of relatively high concentrations of $[Ca^{2+}]_o$ (Fig. 5). The sites involved in binding $[Mg^{2+}]_o$ and $[Ca^{2+}]_o$ and the cooperative association in intracellular signaling remain to be determined. Alternatively, the results of our studies may indicate the presence of separate receptors for $[Ca^{2+}]_o$ and $[Mg^{2+}]_o$ in MDCT cells.

Recently, Paulais, Baudouin-Legros and Teulon isolated renal tubules from mouse kidneys with the aid of collagenase and determined $[Ca^{2+}]_o$ -induced changes in $[Ca^{2+}]_i$ with fluorescence [10]. They showed that $[Ca^{2+}]_o$ elicited cytosolic Ca^{2+} transients in distal convoluted tubule fragments but required about 4 mM $[Ca^{2+}]_o$, and the response was significantly less than that observed in cortical thick ascending limb fragments. Riccardi et al have shown with immunocytochemistry that the apparent abundance of *Casr* in distal convoluted tubules is less than in the thick ascending limbs or the medullary collecting ducts (presented at the 1996 ASN). This is consistent with the functional observations of Paulais et al [10]. However, the EC_{50} for $[Ca^{2+}]_o$ of 3.5 mM reported in the latter study is much more than would normally be observed *in vivo*. It is possible that the use of collagenase to isolate these tubule fragments may have altered the response of the mouse *Casr* to $[Ca^{2+}]_o$. Alternatively, the receptor present in MDCT cells is more sensitive to $[Mg^{2+}]_o$ and $[Ca^{2+}]_o$ than isolated distal convoluted tubule segments.

Finally, activation of the polyvalent cation-sensing mechanism in MDCT cells with neomycin inhibited PTH-, calcitonin-, glucagon- and AVP-stimulated cAMP release (Table 2). 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 inhibit the PTH-mediated increase in cAMP in the proximal tubule and cortical thick ascending limb [43, 44]. The elevation of $[Ca^{2+}]_o$ also mitigates vasopressin-stimulated increases in cAMP production in the medullary thick ascending limb of Henle's loop [45, 46] and PTH-, calcitonin-, vasopressin- and glucagon-stimulated cAMP accumulation in the cortical thick ascending limb [44]. Jones, Frindt and Windhager have shown

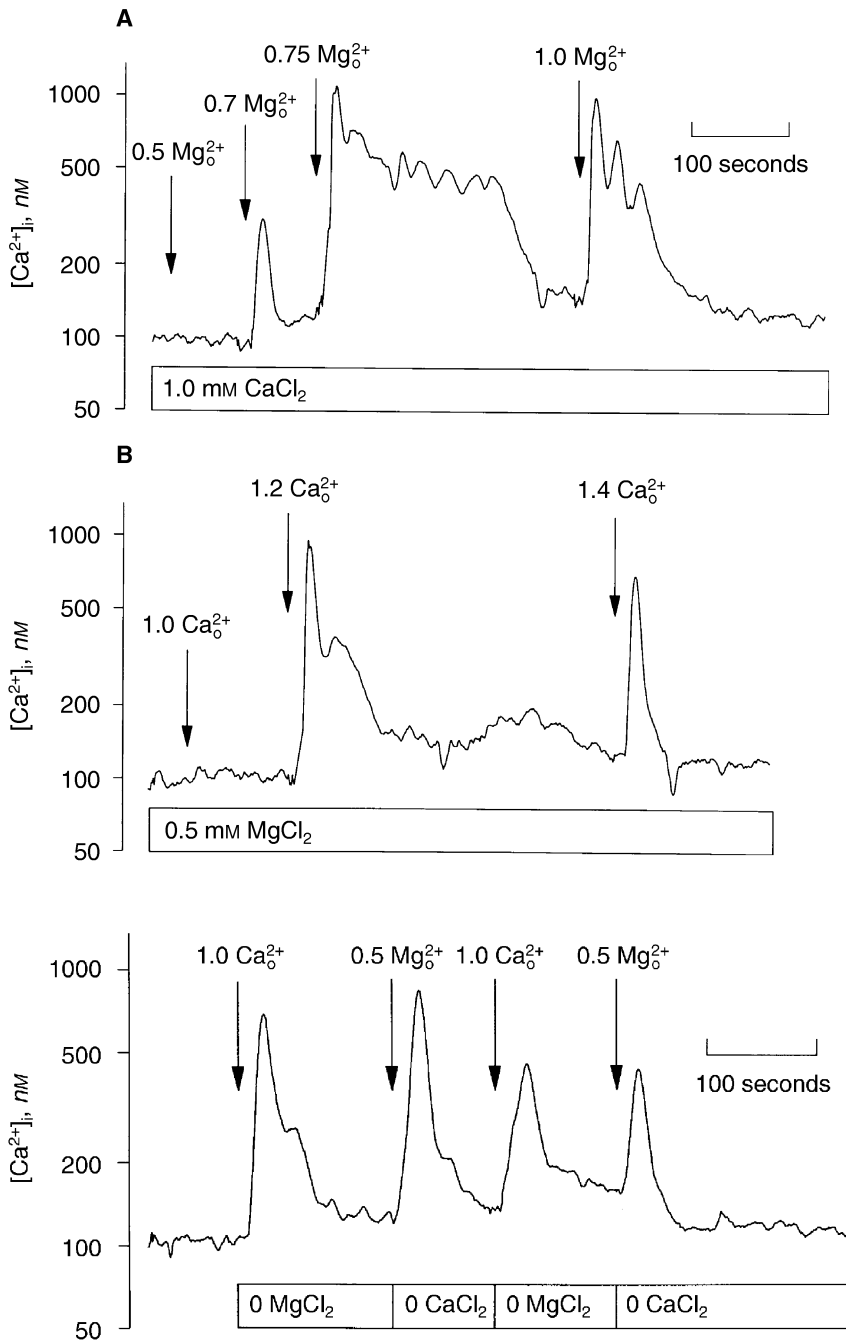


Fig. 6. The calcium sensing receptor (*Casr*) of immortalized mouse distal convoluted tubule (MDCT) cells is sensitive to small changes in $[Mg^{2+}]_o$ and $[Ca^{2+}]_o$ in the presence of elevated divalent cation concentrations. The MDCT cells were loaded with fura-2 as given in legend to Figure 4. The bathing solution was changed to one containing either 5.0 mM $CaCl_2$ (A) or 5.0 mM $MgCl_2$ (B). About five minutes later, the MDCT cells were challenged with bathing solutions containing either 0.2 mM $[Mg^{2+}]_o$ (A) or 0.2 mM $[Ca^{2+}]_o$ (B), in the presence of 5.0 mM $CaCl_2$ or 5.0 mM $MgCl_2$, respectively. Tracings are representative of four separate experiments.

Fig. 7. Repetitive stimulation of immortalized mouse distal convoluted tubule (MDCT)-calcium sensing receptor (*Casr*) with $[Mg^{2+}]_o$ and $[Ca^{2+}]_o$. MDCT cells were loaded with fura-2 in buffer solution (given in legend to Fig. 4) containing no magnesium and calcium. Buffer solution containing $[Mg^{2+}]_o$ 0.5 mM or $[Ca^{2+}]_o$ 1.0 mM, were changed where indicated. The given $[Mg^{2+}]_o$ and $[Ca^{2+}]_o$ concentrations are final concentrations. Tracings are representative of three separate experiments.

that $[Ca^{2+}]_o$ reduces the hydrosmotic response of cortical collecting ducts to AVP and cAMP [47]. Chen et al have shown that polyvalent cations inhibit agonist-stimulated cAMP accumulation in bovine parathyroid cells [4]. This inhibition was totally prevented following preincubation of the cells with pertussis toxin, which is known to ADP-ribosylate and uncouple the guanine nucleotide regulatory (G)-protein, G_i , from the cell surface receptors that are coupled to inhibition of adenylate cyclase [3]. Accordingly, the parathyroid gland *Casr* appears to be linked to adenylate cyclase via a G_i protein [4]. This also appears to be the case for MDCT cells. Glucagon and AVP stimulate magnesium

and calcium transport in the rat distal tubule and magnesium entry into MDCT cells [15, 17]. We postulate that activation of the polyvalent cation-sensing mechanism with high levels of $[Ca^{2+}]_o$ or $[Mg^{2+}]_o$ may inhibit glucagon- and AVP-stimulated magnesium and calcium transport. Further studies are needed to establish the functional roles of the polyvalent cation-sensing receptor(s) within the distal tubule.

In summary, a *Casr* is present in an immortalized mouse distal convoluted tubule (MDCT) cell line. This cell line has been extensively used to characterize magnesium and calcium transport in the distal convoluted segment of the nephron. The use of this

Table 2. Activation of calcium sensing receptor (*Ca sr*) inhibits hormone-stimulated cAMP release

	Control	PTH	Calcitonin	Glucagon	AVP
Control	19 ± 1 (11) ⁿ	56 ± 2* (5)	60 ± 0.1* (4)	105 ± 5* (7)	71 ± 2* (5)
Neomycin	22 ± 2 (3)	34 ± 1** (3)	33 ± 4** (3)	22 ± 1** (7)	24 ± 3** (4)
Ca ²⁺ _o	23 ± 2 (3)	—	—	32 ± 2** (3)	34 ± 3** (3)
Mg ²⁺ _o	24 ± 3 (3)	—	—	37 ± 3 (3)	27 ± 3** (3)

Where indicated, neomycin, 50 μM, extracellular Ca²⁺_o, 10 mM, or extracellular Mg²⁺_o, 10 mM, were added 5 minutes prior to the addition of parathyroid hormone (PTH), 10⁻⁷ M, calcitonin, 10⁻⁷ M, glucagon, 10⁻⁷ M, or arginine vasopressin (AVP), 10⁻⁸ M, and cAMP was measured 5 minutes later. Values are cAMP accumulation, pmol/mg protein · 5 min. ()ⁿ is the number of separate observations.

^a *P* < 0.01, from control values without hormone

^b Significance of neomycin or Ca²⁺_o or Mg²⁺_o values from those of the respective hormone treatments

cell line should allow insights into expression, control, and function of the *Casr* in distal convoluted tubule cells. This is particularly important as *in vivo* micropuncture and microperfusion approaches do not allow for study of cellular mechanisms of transport. *In vitro* microperfusions have not been performed in this portion of the tubule because of the difficulty in isolating intact segments.

ACKNOWLEDGMENTS

This work was supported by research grants from the Medical Research Council of Canada (MT-5793) to G.A.Q. and (MT-9315) to G.N.H. and from the Kidney Foundation of Canada to G.A.Q. and G.N.H. is a Scientist of the Medical Research Council of Canada. We thank Dr. Peter A. Friedman for providing the MDCT cell line, Drs. Kimberly V. Rogers (NPS Pharmaceuticals, UT, USA), Allen Spiegel and Paul Goldsmith (Metabolic Diseases Branch, NIDDK/NIH, Bethesda, MD, USA) for the ADD antibody, and Sem Kebache for technical assistance with these studies. We also acknowledge the secretarial assistance of Susanna Lau in the preparation of this manuscript.

Reprint requests to Dr. Gary A. Quamme, Department of Medicine, University Hospital, Koerner Pavilion, 2211 Wesbrook Mall, Vancouver, BC Canada, V6T 1Z3.

E-Mail: quamme@unixg.ubc.ca

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