Am J Physiol Cell Physiol
280: C382–C393, 2001.

Expression of extracellular calcium-sensing receptor in human osteoblastic MG-63 cell line

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Received 12 May 2000; accepted in final form 15 September 2000

Yamaguchi, Toru, Naibedya Chattopadhyay, Olga Kifor, Chianping Ye, Peter M. Vassilev, Jennifer L. Sanders, and Edward M. Brown. Expression of extracellular calcium-sensing receptor in human osteoblastic MG-63 cell line. *Am J Physiol Cell Physiol* 280: C382–C393, 2001.—We have previously shown the expression of the extracellular calcium (Ca_o^{2+})-sensing receptor (CaR) in osteoblast-like cell lines, and others have documented its expression in sections of murine, bovine, and rat bone. The existence of the CaR in osteoblasts remains controversial, however, since some studies have failed to document its expression in the same osteoblast-like cell lines. The goals of the present study were twofold. *1*) We sought to determine whether the CaR is expressed in the human osteoblast-like cell line, MG-63, which has recently been reported by others not to express this receptor. *2*) We investigated whether the CaR, if present in MG-63 cells, is functionally active, since most previous studies have not proven the role of the CaR in mediating known actions of Ca_o^{2+} on osteoblast-like cells. We used immunocytochemistry and Western blotting with the specific, affinity-purified anti-CaR antiserum 4637 as well as Northern blot analysis and RT-PCR using a riboprobe and PCR primers specific for the human CaR, respectively, to show readily detectable CaR protein and mRNA expression in MG-63 cells. Finally, we employed the patch-clamp technique to show that an elevation in Ca_o^{2+} as well as the specific, allosteric CaR activator NPS R-467 (0.5 μM), but not its less active stereoisomer NPS S-467 (0.5 μM), activate an outward K^+ channel in MG-63 cells, strongly suggesting that the CaR in MG-63 cells is not only expressed but is functionally active.

G protein-coupled receptor; potassium channel; Northern analysis; reverse transcriptase-polymerase chain reaction; Western analysis; immunocytochemistry

MAINTAINING THE EXTRACELLULAR CALCIUM CONCENTRATION (Ca_o^{2+}) within a narrow physiological range is crucial for numerous cellular processes, including the maintenance of membrane potential as well as cellular proliferation, differentiation, and secretion (4). Precise regulation of Ca_o^{2+} is afforded by a G protein-coupled, Ca_o^{2+}-sensing receptor (CaR) that was originally cloned from bovine parathyroid gland and senses Ca_o^{2+} as an extracellular first messenger (6). The CaR was later isolated from rat C cells (18, 21) and kidney (38) and also shown to be present in the intestine (11), thereby implicating it in maintaining Ca_o^{2+} homeostasis not only through its actions on the secretion of Ca_o^{2+}-regulating hormones (i.e., parathyroid hormone and calcitonin) but also through its effects on tissues translocating Ca^{2+} into or out of the extracellular fluid (e.g., kidney and intestine).

Bone, like parathyroid, kidney, and intestine, participates in systemic Ca_o^{2+} homeostasis (5). Thus the CaR could also potentially play some role(s) within the skeleton by sensing local changes in Ca_o^{2+} owing to bone remodeling. Bone formation during skeletal turnover is preceded by the migration of macrophage-like mononuclear cells to sites of recent bone resorption during the "reversal" phase that precedes the laying down of new bone (3). Preosteoblasts subsequently migrate to the same sites, differentiate into mature osteoblasts, and eventually deposit and mineralize osteoid protein (36, 37). Bone resorption can produce local increases in Ca_o^{2+} beneath resorbing osteoclasts that reach levels as high as 8–40 mM (39). The latter could, therefore, provide both macrophage-like mononuclear cells and preosteoblasts in the local microenvironment with a signal that modulates their subsequent physiological responses, such as migration and proliferation. In fact, high Ca_o^{2+} induces chemotaxis of human peripheral blood monocytes (41) and both chemotaxis and proliferation of mouse osteoblastic MC3T3-E1 cells (44). These two cell types have the capacity, respectively, to differentiate into mature osteoclasts (19) and osteoblasts (40) under appropriate conditions in culture.

Whether the CaR mediates the known actions of Ca_o^{2+} on osteoblasts, however, remains controversial. We previously showed expression of this receptor in diverse cell types within human bone marrow, including alkaline phosphatase (ALP)-positive, putative osteoblast precursors (23). Using multiple detection methods (i.e., immunocytochemistry, Western and Northern analyses, and RT-PCR), we subsequently

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identified CaR protein and mRNA in the osteoblast-like cell lines UMR-106 and SAOS-2 (45). Recently, we also found that the murine ST-2 stromal cell line (43) and the murine MC3T3-E1 osteoblastic cell line (44) express the CaR. Others have likewise shown in recent studies that the CaR is expressed in the latter cell line (25) as well as in most osteoblasts in sections of murine, rat, and bovine bone (10). Furthermore, CaR agonists stimulate chemotaxis and proliferation of both ST-2 and MC3T3-E1 cells (43, 45), suggesting that the receptor could potentially represent the molecular mediator of some or even all of the previously documented actions of high Ca_o^{2+} on osteoblasts and/or their precursors.

However, some investigators have failed to detect CaR expression in osteoblast-like cells (33, 35). Indeed, Quarles and coworkers (35) have suggested that the effects of elevated levels of Ca_o^{2+} on MC3T3-E1 cells are mediated by a Ca_o^{2+}-sensing mechanism distinct from the CaR, based, in part, on their failure to detect the CaR by RT-PCR or Western blot analysis in U-2OS, SAOS-2, and MG-63 osteoblast-like cells in a recent study (33). Thus whether or not the CaR and/or other Ca_o^{2+} sensors are expressed in osteoblasts still remains controversial.

The goals of the present study were twofold: *1*) to determine whether the CaR is expressed in MG-63 cells, an osteoblast-like cell line that we have not studied previously; and *2*) to evaluate whether the CaR, if expressed in this cell line, is functionally active. Our results demonstrate readily detectable expression of a functionally active CaR on the cell surface of MG-63 cells, thereby further supporting our previous evidence that the CaR mediates as least some of the known actions of Ca_o^{2+} on osteoblast-like cells.

MATERIALS AND METHODS

Materials. All routine culture media were obtained from GIBCO BRL (Grand Island, NY). NPS R-467 and NPS S-467 were generous gifts of Dr. Edward F. Nemeth, NPS Pharmaceuticals, Salt Lake City, UT.

Preparation of bovine parathyroid cells. Parathyroid glands from calves were collected on ice, minced into small fragments, and digested at 37°C for 75 min with collagenase and DNase as described previously (7, 27). Dispersed cells were subsequently utilized for preparation of cellular proteins to be employed for Western blotting as described below.

Culture and maintenance of CaR transfected and untransfected HEK-293 cells. We obtained a clonal line of human embryonic kidney (HEK-293) cells stably transfected with the cDNA encoding the human parathyroid CaR [hPCaR4.0 (20)] (referred to here as HEKCaR cells) as well as untransfected HEK-293 cells (designated as HEK-293 cells) as generous gifts from Dr. Kimberly Rogers, NPS Pharmaceuticals. We have previously shown that untransfected HEK-293 cells do not express an endogenous CaR and are unresponsive to CaR agonists, whereas HEK-293 cells transiently or stably transfected with the CaR show robust, high Ca_o^{2+}-evoked increases in the cytosolic Ca^{2+} concentration (Ca_i^{2+}), accumulation of inositol phosphates, and regulation of other CaR-dependent signaling pathways (1, 26). Cells were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS) without pyruvate and with 200 μg/ml hygromycin.

Cell culture. MG-63 cells, established as an osteoblastic cell line from a human osteosarcoma, were obtained from Dr. Nancy Weigel, Baylor College of Medicine, Houston, TX, who obtained the cells originally from American Type Culture Collection (Manassas, VA). MG-63 cells were grown in α-Eagle's minimum essential medium (Ca^{2+} and 1.8 mM; Mg^{2+} and 0.81 mM; $H_2PO_4^-$, 1.0 mM) supplemented with 10% FBS (Hyclone, Logan, UT) and 1% penicillin/streptomycin in 5% CO_2 at 37°C. The medium was changed twice weekly, and the cells were subcultured into 25-cm^2 culture flasks by detaching them gently with a cell scraper after reaching subconfluency. For morphological evaluation, MG-63 cells were plated onto 12-mm circular glass coverslips in 12-well (2.0 cm^2) plates. After 24 h of culture, the medium was discarded, and each coverslip with adherent cells was washed once with phosphate-buffered saline (PBS), fixed with 4% formaldehyde in PBS for 5 min, and washed with PBS once again. Coverslips were stored for 1 to 7 days at 4°C before immunocytochemistry or immunofluorescence was performed, which had no effect on the results observed.

Immunocytochemistry for the CaR in MG-63 cells. Immunocytochemistry was performed using either mouse monoclonal anti-CaR antibody ADD, which was raised to a synthetic peptide within the CaR corresponding to residues 214–235, or polyclonal anti-CaR antiserum 4637, and the latter was affinity purified by us as described previously (27). Both antisera were generously provided by NPS Pharmaceuticals. Monoclonal antibody ADD (1, 2, 21) was raised to a peptide corresponding to amino acids 214–235 within the predicted extracellular domain of the human CaR, while antiserum 4637 was raised to a peptide corresponding to amino acids 345–359 of the bovine CaR (21, 27). Optimal stability of affinity-purified antiserum 4637 was obtained by storing it at −20°C in PBS, pH 7.4, that contained 50% glycerol, 1 mg/ml bovine serum albumin (BSA), and 1.5 mM sodium azide. Under these conditions, the titer of the antiserum was unchanged for at least 2 yr as assessed by Western blotting of proteins in crude plasma membranes prepared from CaR-transfected HEK-293 cells.

MG-63 cells fixed as described above were treated with Dako peroxidase blocking reagent (Dako, Carpenteria, CA) for 10 min to inhibit endogenous peroxidases and then with Dako protein block serum-free solution (Dako) for 1 h. They were subsequently incubated overnight at 4°C with either the monoclonal ADD antibody or the affinity-purified 4637 polyclonal antiserum at concentrations of 5 and 10 μg/ml, respectively, in blocking solution (23, 43). Negative controls were carried out by incubating the cells with the respective antisera after they had been preabsorbed with 10 μg/ml of the specific peptide against which each had been raised. After the cells were washed three times with 0.5% BSA in PBS for 10 min each, peroxidase-coupled sheep anti-mouse or goat anti-rabbit IgG (1:200; Sigma Chemical), respectively, were added and incubated for 1 h at room temperature. The cells were then washed with PBS three times, and the color reaction was developed using the Dako AEC substrate system (Dako) for about 10 min. The color reaction was stopped by washing the slides three times with PBS and once with water.

Detection of CaR by immunofluorescence and fluorescence immunolocalization by confocal microscopy. For detection of the CaR by immunofluorescence using antiserum 4637, formaldehyde-fixed MG-63 or HEKCaR cells were first treated for 10 min with PBS that contained 0.1 mM glycine to quench intrinsic fluorescence due to fixation. To block nonspecific

antibody binding sites, the cells were then incubated with PBS that contained 1% BSA and 1% normal goat serum for 30 min at room temperature. After blocking, the cells were incubated overnight at 4°C with 10 μg/ml of the antiserum in the blocking solution. On the following day, the cells were washed three times with 50% blocking solution in PBS and incubated with a secondary goat anti-rabbit antiserum tagged with the fluorophore Alexa 568 (Molecular Probes, Eugene, OR) for 3 h in the dark at room temperature. The cells were subsequently washed three times with PBS and three times with deionized water and mounted using antifading mounting fluid (Vector Laboratories, Burlingame, CA). For identification of plasma membrane localization of the CaR, some preparations of MG-63 cells were double immunostained for the CaR and for either of the two plasma membrane markers, Na^+-K^+-ATPase or alkaline phosphatase (ALP) (located on the cell surface of osteoblasts). After blocking the nonspecific background staining, the cells were incubated with a mixture of rabbit polyclonal anti-CaR antiserum 4637 and chicken anti-Na^+-K^+-ATPase (Chemicon International, Temecula, CA) or mouse monoclonal anti-alkaline phosphatase (Chemicon International) antibodies in blocking solution overnight at 4°C in a humidified chamber. The next day, after being washed as above, the cells were incubated with a mixture of secondary antibodies [i.e., Alexa Fluor 568-conjugated goat anti-rabbit IgG (Molecular Probes)/FITC-conjugated anti-chicken IgG (Sigma) or Alexa Fluor 568 goat anti-rabbit IgG/Alexa 488-conjugated goat anti-mouse IgG (Molecular Probes)] for 3 h in the dark at room temperature. Images of the resultant fluorescence were then collected on a Bio-Rad MRC 1024.2P multiphoton confocal microscope in the Brigham and Women's Hospital Confocal Microscope Core Facility. The system is equipped with krypton and argon lasers that can produce excitation wavelengths of 488, 568, and 647 nm. Alexa 568 was excited at 568 nm, which produces a red signal, while fluorescein or Alexa 488 was excited at 488 nm, which yields a green signal. The autofluorescence of the samples was minimal and was subtracted from the values obtained during measurements. Because peptide blocking of anti-CaR antiserum 4637 was performed for both immunoperoxidase staining and Western blotting to establish its specificity, this control was not repeated in the studies using immunofluorescence. Photomicrography was carried out at ×1,000.

Western analysis of the CaR in MG-63 cells. Dispersed bovine parathyroid cells or confluent monolayers of HEKCaR or MG-63 cells that had been cultured in six-well plastic cluster plates were rinsed with ice-cold PBS and scraped on ice into lysis buffer that contained 10 mM Tris·HCl, pH 7.4, 1 mM EGTA, 1 mM EDTA, 0.25 M sucrose, 1% Triton X-100, 1 mM dithiothreitol, and a cocktail of protease inhibitors (10 μg/ml each of aprotinin, leupeptin, and calpain inhibitor as well as 100 μg/ml Pefabloc). The cells were then passed though a 22-gauge needle 10 times. Nuclei and cell debris were removed by low-speed centrifugation (1,000 *g* for 10 min), and the resultant total cellular lysate in the supernatant was used either directly for SDS-PAGE or stored at −80°C.

Immunoblot analysis was performed essentially as described before (27, 28). Aliquots of supernatant fractions containing the total cellular lysate (20 μg of protein from HEKCaR and bovine parathyroid cells and 40 μg from MG-63 cells) were mixed with an equal volume of 2× SDS-Laemmli gel loading buffer containing 100 mM dithiothreitol, incubated at 37°C for 15 min, and resolved electrophoretically on linear 3–10% gradient gels. The separated proteins were then transferred to nitrocellulose filters (Schleicher and Schuel, Keene, NH) and incubated with blocking solution (PBS with 0.25% Triton X-100 and 5% dry milk) for 1 h at room temperature. The blots were subsequently incubated overnight at 4°C with affinity-purified polyclonal antiserum 4637 at 1 μg/ml with or without preincubation with twice the concentration (e.g., 2 μg/ml) of the peptide against which the antiserum was raised (as a control for nonspecific binding) in blocking solution with 1% dry milk. The blots were subsequently washed five times with PBS that contained 0.25% Triton X-100 and 0.1% dry milk (washing solution) at room temperature for 10 min each. The blots were further incubated with a 1:2,000 dilution of horseradish peroxidase-coupled goat anti-rabbit IgG (Sigma) in blocking solution with 1% dry milk for 1 h at room temperature. The blots were finally washed five times with the washing solution, and protein bands were detected using an enhanced chemiluminescence system (Renaissance kit, DuPont-NEN).

Detection of CaR transcripts in MG-63 cells by Northern blot analysis. To determine the size of the CaR transcript(s) in MG-63 cells, Northern blot analysis was employed on aliquots of 2 μg poly(A)$^+$ RNA obtained using oligo(dT) cellulose chromatography of total RNA (11). RNA samples were denatured and electrophoresed in 2.2 M formaldehyde-1% agarose gels along with a 0.24-kb to 9.5-kb RNA ladder (GIBCO BRL) and transferred overnight to nylon membranes (Duralon; Stratagene, La Jolla, CA). A 486-bp *Kpn* I-*Xba* I fragment corresponding to nucleotides 1745 to 2230 of the human parathyroid CaR cDNA (1, 20) was subcloned into the pBluescript SK$^+$ vector. The plasmid was then linearized with *Kpn* I, and a ^{32}P-labeled riboprobe was synthesized with the MAXIscript T3 kit (Pharmacia Biotech, Piscataway, NJ) using T3 polymerase and [^{32}P]UTP. Nylon membranes were prehybridized for 2 h at 55°C in a solution consisting of 50% formamide, 4× Denhardt's solution (50× Denhardt's = 5 g Ficoll, 5 g polyvinylpyrrolidone, and 5 g BSA/50 ml), 5× sodium chloride-sodium phosphate-EDTA (SSPE) (20× SSPE = 2.98 M NaCl and 0.02 M EDTA in 0.2 M phosphate buffer, pH 7.0), 0.5% SDS, 10% dextran sulfate, 250 μg/ml yeast tRNA, and 200 μg/ml calf thymus DNA. Labeled cRNA probe (2×10^6 cpm/ml) was then added, and the membranes were hybridized overnight at 68°C. Washing was carried out at high stringency [0.1× SSC (20× SSC = 3 M NaCl and 0.3 M Na_3-citrate·$2H_2O$) and 0.1% SDS at 68°C] for 30 min (6). The membranes were sealed in plastic and exposed to a PhosphorImager screen. The screens were analyzed on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) using the ImageQuant program.

RT-PCR of CaR transcripts in MG-63 cells. Total RNA was prepared from monolayers of MG-63 cells in 25-cm^2 culture flasks with the TRIzol reagent (GIBCO BRL). One microgram of total RNA was used for the synthesis of single-stranded cDNA (cDNA synthesis kit, GIBCO BRL). The resultant first-stranded cDNA was used for the PCR procedure (16, 45). PCR was performed in a buffer that contained the following final concentrations of the listed reagents: 20 mM Tris·HCl (pH 8.4), 50 mM KCl, 1.8 mM $MgCl_2$, 0.2 mM dNTP, 0.4 μM of forward primer, 0.4 μM of reverse primer, and 1 μl of ELONGASE enzyme mix (a *Taq*/*Pyrococcus* species GB-D DNA polymerase mixture; GIBCO BRL) (6, 10, 13–15). A human parathyroid CaR (20) sense primer, 5′-CGGGGTACCTTAAGCACCTACGGCATCTAA-3′, and antisense primer, 5′- GCTCTAGAGTTAACGCGATCCCAAAGGGCTC-3′, were employed for the reaction. This set of primers was designed to span two introns of the human CaR gene to avoid amplification of a similar-sized product from contaminating genomic DNA. To perform hot-start PCR, the enzyme was added during the initial 3-min denaturation and was

followed by 35 cycles of amplification (30-s denaturation at 94°C, 30-s annealing at 55°C, and 1-min extension at 72°C). The reaction was completed with an additional 10-min incubation at 72°C to allow completion of extension. PCR products were fractionated on 1.2% agarose gels. The presence of a 425-bp amplified product was consistent with a positive PCR reaction arising from CaR-related sequence within cDNA. The PCR product in the reaction mixture was then purified using the QIAquick PCR purification kit (Qiagen, Santa Clarita, CA) and subjected to direct, bidirectional sequencing employing the same primer pairs used for PCR by means of an automated sequencer (AB377; Applied Biosystems, Foster City, CA) in the DNA Sequence Faculty of the University of Maine (Orono, ME) using dideoxy terminator *Taq* technology.

Electrophysiological measurements. Channel activities were measured in cell-attached and inside-out patches in continuously superfused cells using the patch-clamp technique, as described previously (49). The extracellular bath solution contained, unless otherwise specified (in mM): 140 NaCl, 4.0 KCl, 0.75 $CaCl_2$, 1.0 $MgCl_2$, 10 glucose, and 10 HEPES, pH 7.4. Solutions containing CaR agonists, activators, or other agents were applied to the MG-63 cells by superfusion. The pipette solution contained, unless otherwise specified (in mM): 87.0 NaCl, 55.0 KCl, 1.0 $CaCl_2$, 1.0 $MgCl_2$, 10 glucose, and 10 HEPES, pH 7.4. When filled with this external solution, pipette tip resistances were 5–10 MΩ. Appropriate concentrations of EGTA were added to achieve final concentrations of 0.1 or 0.5 μM free Ca^{2+} in studies with inside-out patches. Currents were measured using an integrating patch-clamp amplifier, and single-channel currents were filtered at 3 kHz. Voltage stimuli were applied, and single-channel currents were digitized (200 μs per point) and analyzed using programs that were based on pCLAMP (Axon Instruments, Foster City, CA). The baseline current was monitored frequently to ensure proper analysis of single-channel currents.

The resting potential (V_r) of these cells had a mean value that averaged −70 mV in an extracellular solution that contained 5.4 mM K^+. V_r was measured in separate experiments or at the end of some single-channel recordings by breaking the membrane patch with negative pressure. The observed mean value of −70 mV was assumed for calculation of membrane potential (V_m) for experiments in which V_r was not measured. In experiments using cell-attached patches, V_m was expressed as V_r plus the voltage applied to the patch pipette (v_p). Upward deflections in the current records represent positive outward currents.

The probability of channel opening (P_o) was calculated from 20-s segments of current records using the equation $P_o = I/N \cdot i$, where I is the time-averaged current passing through the channels for a given period of time, N is the number of channels functioning independently within the membrane patch, and i is the single-channel current (49). Single-channel measurements were recorded at 0.75 mM Ca_o^{2+} or following an increase in the level of Ca_o^{2+} to 2.75 mM. Single-channel traces were also taken at various voltages before and after addition of the calcimimetic CaR activator, NPS R-467 (0.5 μM), or its less active stereoisomer, NPS S-467 (0.5 μM), at a level of Ca_o^{2+} of 1.0 mM. Statistical analyses of electrophysiological data were carried out using one-way analysis of variance (ANOVA). Significant treatment effects were further evaluated by Fisher's protected least-significant difference test of multiple comparisons utilizing one-way ANOVA. A P value of <0.05 was considered to indicate a statistically significant result.

RESULTS

Immunoreactivity of CaR protein in MG-63 cells using CaR-specific antibodies. To determine whether MG-63 cells express the CaR, we first performed immunocytochemistry using the immunoperoxidase technique with both affinity-purified anti-CaR antiserum 4637 and monoclonal anti-CaR antibody ADD. In both cases, there was readily detectable CaR immunoreactivity located around the periphery of the cell as well as over the cytoplasm of MG-63 cells (Fig. 1, *A* and *C*). The apparent intracellular staining exhibited a cytoplasmic

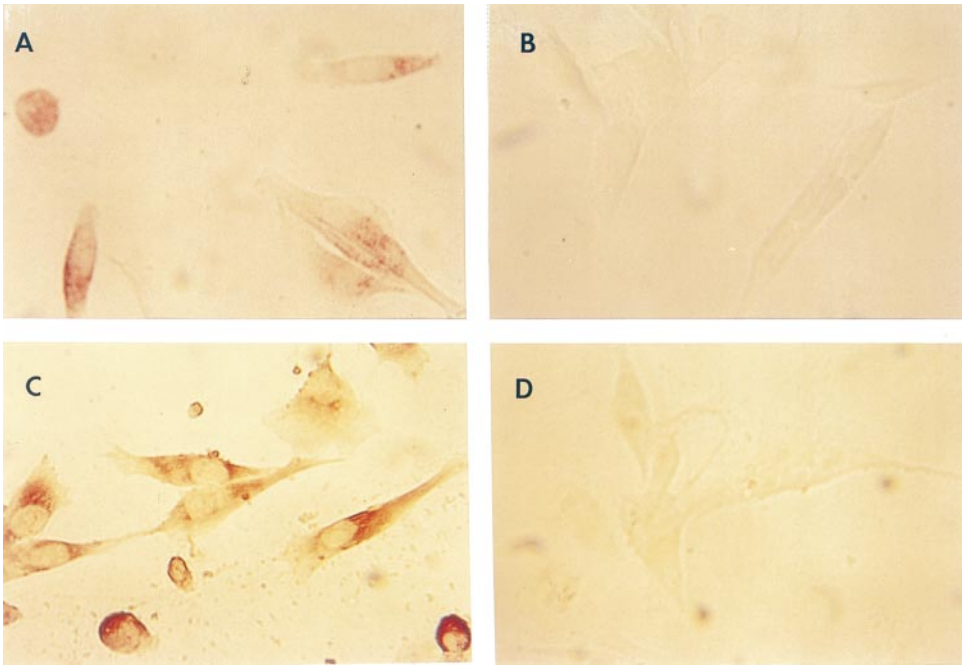

Fig. 1. Immunocytochemistry of MG-63 cells using monoclonal anti-Ca_o^{2+}-sensing receptor (CaR) antibody ADD and affinity-purified polyclonal anti-CaR antiserum 4637. *A* and *C*: immunocytochemistry using the immunoperoxidase technique with both antisera revealed strong CaR immunoreactivity in MG-63 cells, including staining of the cellular periphery as well as the cytoplasmic and perinuclear regions. *B* and *D*: staining was abolished by preincubating the 2 primary antisera with the respective peptides against which they were raised. Photomicrographs were taken at a magnification of ×400.

and perinuclear distribution. With both antisera, the immunoreactivity was eliminated by preincubating the respective primary antiserum with the peptide against which it was raised (Fig. 1, *B* and *D*). To further document the plasma membrane localization of the CaR in MG-63 cells, we performed confocal fluorescence immunocolocalization of the CaR using anti-CaR antiserum 4637 as well as antibodies to each of two plasma membrane markers, ALP (located in the plasma membrane of osteoblasts) and Na^+-K^+-ATPase (Fig. 2). There was clear colocalization of CaR immunoreactivity with each of the two plasma membrane markers, documenting expression of the receptor in the plasma membrane.

We next compared the pattern of CaR immunoreactivity in MG-63 cells with that in HEKCaR cells as a positive control. Examples of confocal images of CaR immunoreactivity in the two cell types using the immunofluorescence technique with anti-CaR antiserum 4637 are shown in Fig. 3. Similar to the results obtained above using the immunoperoxidase technique, there was prominent CaR immunoreactivity associated with the plasma membrane as well as a cytoplasmic and perinuclear distribution in MG-63 cells in a section passing through approximately the middle of several cells (Fig. 3). In contrast, in HEKCaR cells, the bulk of the immunoreactivity was associated with the plasma membrane (Fig. 3).

We next performed Western analysis on proteins isolated from total cellular lysates of MG-63 cells using the 4637 antiserum and compared the results with those observed using similar protein preparations from

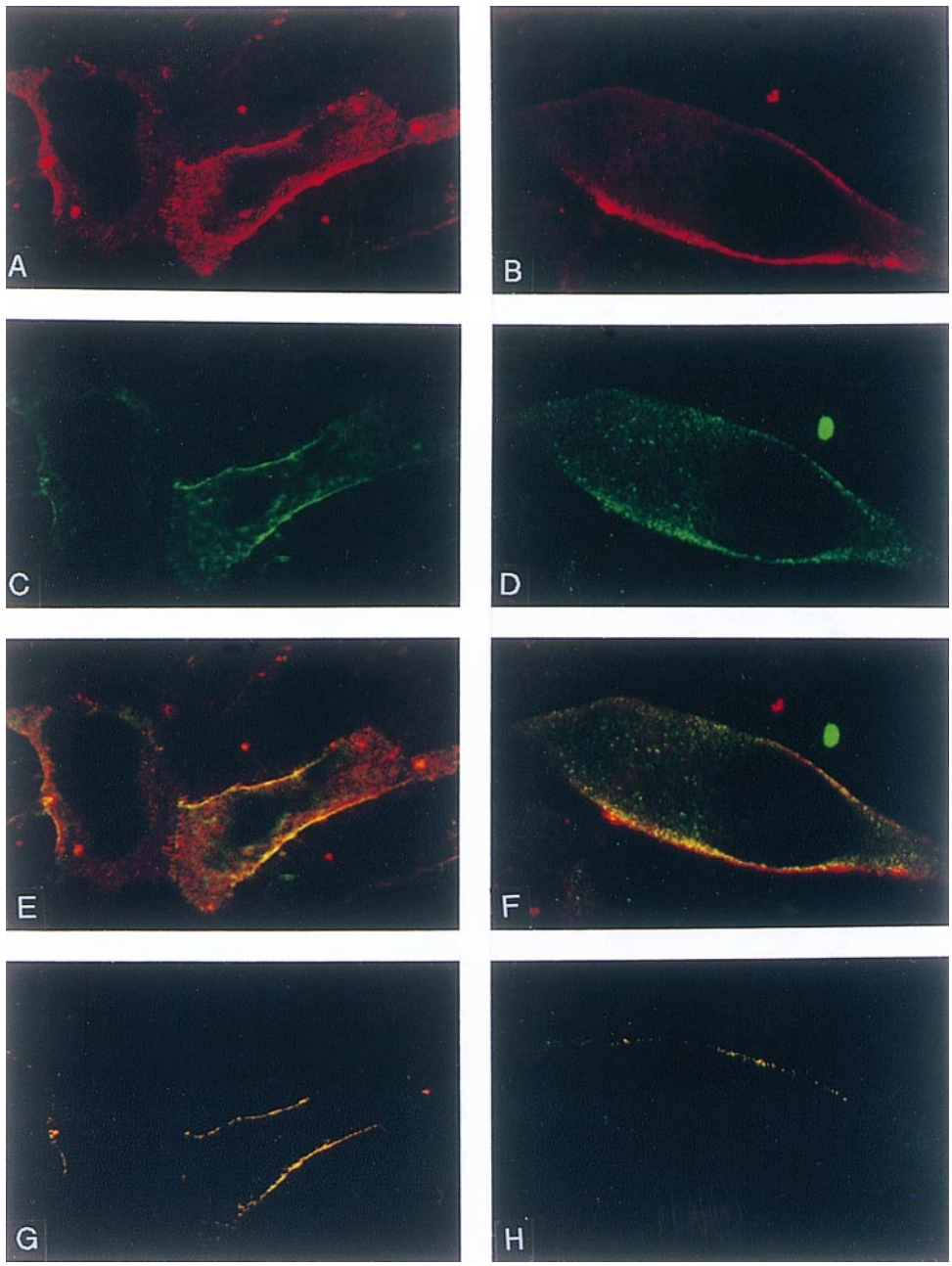

Fig. 2. Colocalization of the CaR with 2 plasma membrane markers in MG-63 cells. MG-63 cells were coimmunostained with anti-CaR antiserum (rabbit polyclonal antiserum 4637; red color in *A* and *B*) and either a chicken antibody to Na^+-K^+-ATPase (green color in *C*) or a mouse monoclonal antibody to alkaline phosphatase (green color in *D*), both of which are located in the osteoblast plasma membrane, as described in MATERIALS AND METHODS. The yellow color in *E–H* indicates regions of colocalization that are present within the plasma membrane. Photomicrographs were taken at a magnification of ×1,000.

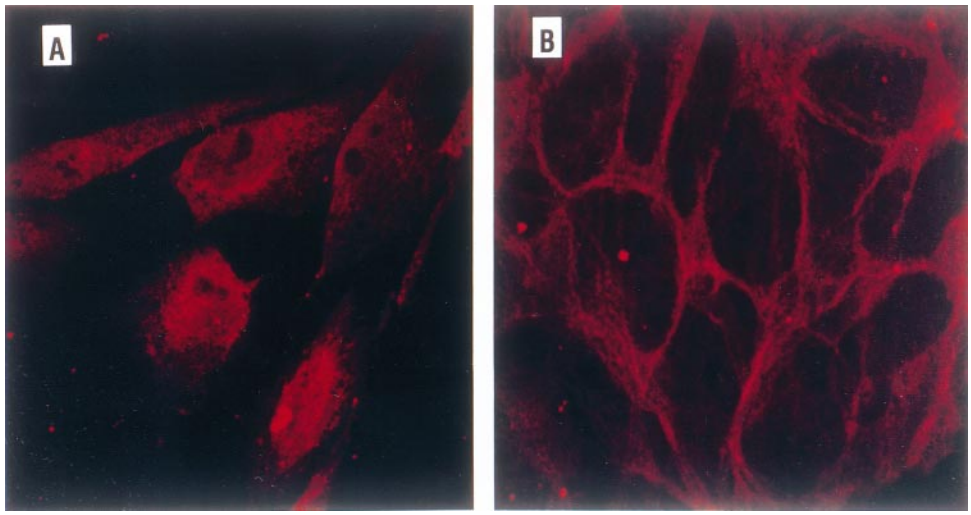

Fig. 3. Confocal images of CaR immunoreactivity in MG-63 cells (*A*) and CaR-transfected HEK (HEKCaR) cells (*B*) as assessed using immunofluorescence with affinity-purified anti-CaR antiserum 4637. Coverslips with attached MG-63 cells or HEKCaR were prepared, and the immunofluorescence technique and confocal imaging were carried out as described in MATERIALS AND METHODS. Photomicrographs were taken at a magnification of ×1,000.

bovine parathyroid and HEKCaR cells as positive controls (Fig. 4). Although the levels of CaR protein expression in parathyroid and HEKCaR cells were substantially higher than that in MG-63 cells, longer exposure times revealed that the immunoreactive bands in MG-63 cells, including the doublet at ~150 and ~170 kDa, were of sizes identical to those in one or both of the two positive controls. The specificity of the CaR-immunoreactive bands was confirmed by the marked reductions in their intensities following preabsorption of the 4637 antiserum with the specific peptide against which it was raised (Fig. 4).

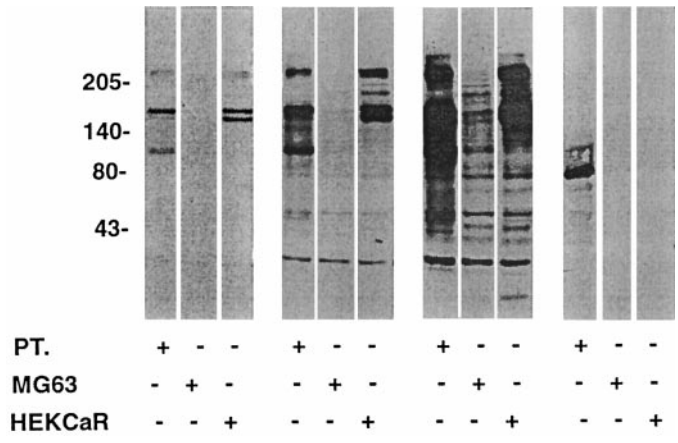

Fig. 4. Western blot analyses of CaR proteins in whole cell lysates isolated from MG-63 cells, bovine parathyroid (PT), or HEKCaR cells. Each protein sample, 20 μg for parathyroid (*left lanes*) and HEKCaR cells (*right lanes*) and 40 μg for MG-63 cells (*middle lanes*), was subjected to SDS-PAGE on a linear gradient running gel of 3–10%. The CaR-specific affinity-purified antiserum 4637 was then used as described in MATERIALS AND METHODS to identify expression of CaR protein in the resultant blots as indicated in the figure. The autoradiograms shown utilized 5- (*left*), 30- (*middle*), or 60-s (2 on *right*) exposures of the same blot using the enhanced chemiluminescence detection system (see MATERIALS AND METHODS). Results observed (farthest *right*) when the antiserum was preabsorbed with the peptide against which it was raised. Western blots shown are representative of 2 or more such blots for each cell type.

Detection of CaR mRNA in MG-63 cells by RT-PCR and Northern blot analysis. Figure 5*A* shows the results of RT-PCR performed with CaR-specific primers, which amplified a fragment of the expected size, 425 bp, for a product derived from an authentic CaR transcript (Fig. 5*B*, *middle lane*). No products were observed when the RT was omitted during synthesis of cDNA (Fig. 5*A*, *right lane*). Northern blot analysis carried out at high stringency using a CaR-specific riboprobe on poly(A)$^+$ RNA isolated from MG-63 cells revealed a single major transcript of ~5.2 kb (Fig. 5*B*), a size similar to that of the major CaR transcript in human parathyroid gland (20). DNA sequence analysis of the PCR product revealed a sequence that was 100% identical to the corresponding region of the human CaR cDNA (20) (Fig. 5*C*). These results show that the PCR product was amplified from authentic CaR transcript(s).

CaR-mediated activation of an outward K^+ channel in MG-63 cells. To determine whether the CaR expressed in MG-63 cells is functionally linked to a biological response, we examined the effects of CaR agonists on the activity of an outward K^+ channel. We have previously shown that similar K^+ channels in other types of cells are directly regulated by the CaR (42). The upward deflections of the current traces represent outward currents due to efflux of K^+. Raising Ca_o^{2+} from a low level (0.75 mM) at which the CaR is inactive to 2.75 mM evoked substantial activation of the channel (Fig. 6, *A* and *B*). Figure 6*C* shows pooled data from several such experiments, demonstrating that high Ca_o^{2+} promotes an approximate fivefold increase in the open state probability of the channel. Figure 6*D* shows changes in the current-voltage (*I-V*) relationships for the channel with alterations in the ratio of Na^+ to K^+ in the pipette solution. When the concentration of K^+ was reduced from 140 to 5.4 mM in the pipette by substituting KCl with an equimolar amount of NaCl, the *I-V* curve shifted substantially to the left, indicating that the channel is predominantly permeable to K^+.

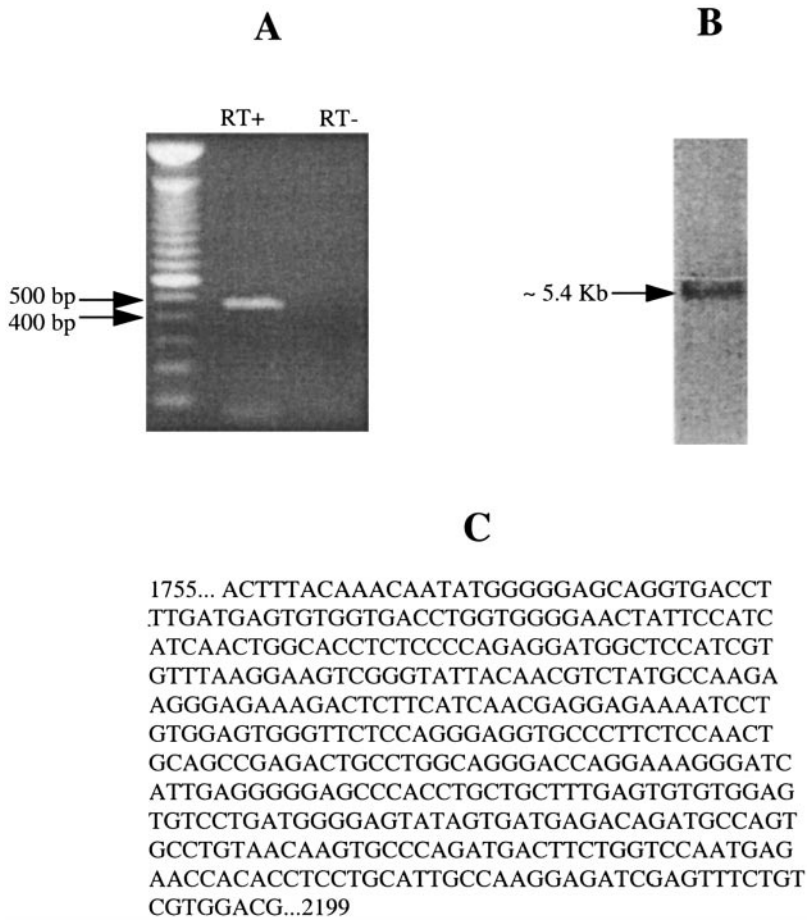

Fig. 5. Identification of CaR transcript in MG-63 cells using RT-PCR performed with an intron-spanning primer pair and sequencing of the amplified product or utilizing Northern blot analysis. *A*: RT-PCR, performed as described in MATERIALS AND METHODS, amplified a product of the expected size (425 bp) for having arisen from bona fide CaR transcript(s) (*middle lane*). The PCR reaction without RT showed no products (*right lane*). A 100-bp ladder (GIBCO BRL) was used as a size marker (*left lane*). *B*: Northern analysis was carried out as described in MATERIALS AND METHODS. Arrow shows the major CaR transcript (~5.2 kb), which is similar in size to the most abundant CaR transcript present in human parathyroid gland. *C*: nucleotide sequence of the PCR product amplified from MG-63 cells. This sequence is 100% identical to that of the corresponding region of the human parathyroid CaR. A similar result was obtained using another independent preparation of RNA from MG-63 cells.

We finally provided further documentation that the opening of this K^+ channel as a result of elevating Ca_o^{2+} is, in fact, mediated via the CaR by using a selective "calcimimetic" CaR activator, NPS R-467 (32). Addition of 0.5 μM NPS R-467 in the presence of 1 mM Ca_o^{2+} in the bath solution induced substantial activation of the channel (Fig. 7*A*). The activity of the channel at 1.0 mM Ca_o^{2+} (P_o ~0.2) is comparable to that observed with 0.75 mM Ca_o^{2+} in Fig. 6. In contrast, the same concentration of the less active stereoisomer of NPS R-467, NPS S-467, had little or no effect on channel activity (Fig. 7*B*). Figure 7*C* shows the *I-V* relationships of the channel under the control condition and after addition of NPS R-467 or NPS S-467, illustrating little change in the slope conductance. Therefore, the activation of the channel by NPS R-467 was principally the result of an increase in its open state probability without alterations in its selectivity or permeability for K^+. Statistical analysis showed a substantial, statistically significant increase in P_o after addition of NPS R-467, whereas NPS S-467 had no significant effect on P_o (Fig. 7*D*).

DISCUSSION

We decided to reevaluate the expression of the CaR in MG-63 osteoblastic cells, since in pilot studies performed before the publication of the report of Pi et al. (33), we observed robust CaR immunostaining in this cell line. Moreover, earlier studies had shown that high Ca_o^{2+} increases the secretion of osteocalcin by this cell line (29), indicating that MG-63 cells are capable of sensing Ca_o^{2+}, potentially doing so via the CaR or some other Ca_o^{2+}-sensing mechanism. Given the negative results of Pi et al. (33) on Western analysis as a method for detecting CaR protein in MG-63 cells using the 4637 antiserum, we repeated our earlier experiments utilizing immunostaining with this antiserum as well as with the ADD monoclonal anti-CaR antiserum used by these authors. The results of these studies indicated readily detectable CaR immunoreactivity in MG-63 cells using the immunoperoxidase technique with both antisera as well as immunofluorescence utilizing the 4637 antiserum.

It should be pointed out that a substantial amount of this CaR immunoreactivity was present intracellularly, as documented by confocal imaging, and exhibited a perinuclear and fine reticular cytoplasmic pattern of staining consistent with expression in the endoplasmic reticulum. Definitive plasma membrane localization could be documented, however, by the use of immunocolocalization via two-color confocal imaging of the CaR with two different plasma membrane markers, ALP and Na^+-K^+-ATPase. CaR immunoreactivity in CaR-transfected HEK-293 cells, in contrast, was

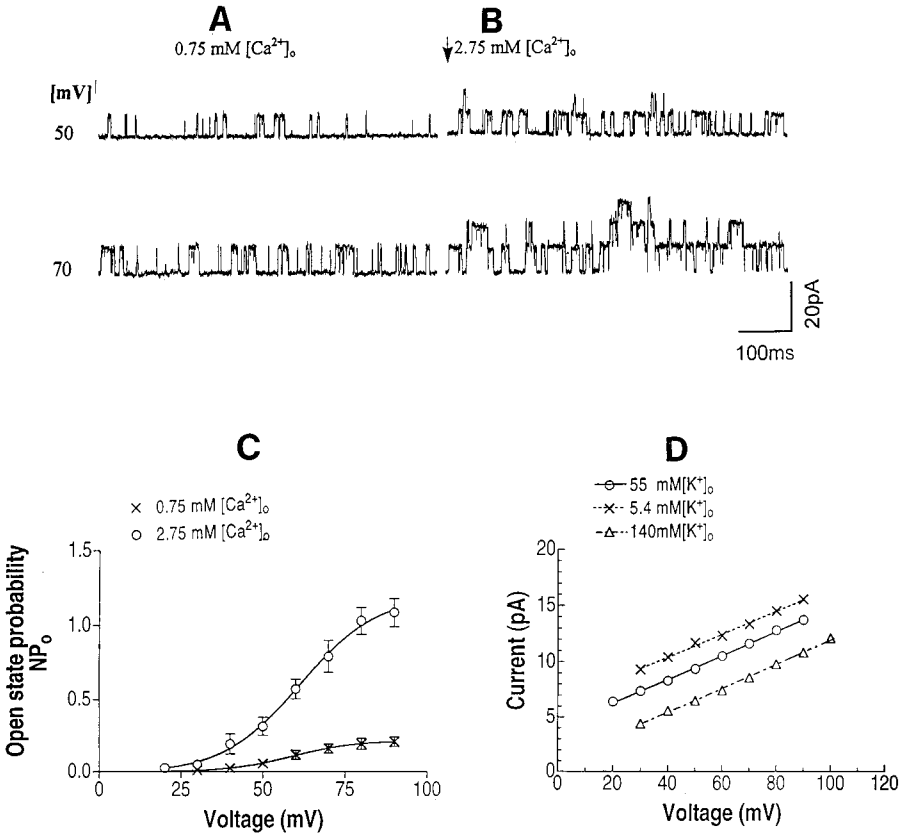

Fig. 6. Activation of K^+ channels in cell-attached patches of MG-63 cells by elevating extracellular Ca^{2+} (Ca_o^{2+}) from 0.75 to 2.75 mM. *A*: single-channel traces were taken at 2 different voltages [membrane potential (V_m)] as indicated on the traces (*left*) in the presence of 0.75 mM Ca_o^{2+} or (*B*) after elevating Ca_o^{2+} to 2.75 mM. *C*: the probability of channel opening (P_o) in cell-attached patches is plotted as a function of V_m at 2 different Ca_o^{2+}, 0.75 mM and 2.75 mM. Symbols and error bars are means ± SE of the mean, $n = 3$. *D*: current-voltage relationships in the presence of varying levels of K_o^+ and Na_o^+ (in millimolar) in the pipette solution: 140 KCl, no added NaCl (△); 55 KCl, 87 NaCl (○); and 5.4 KCl, 140 NaCl (x).

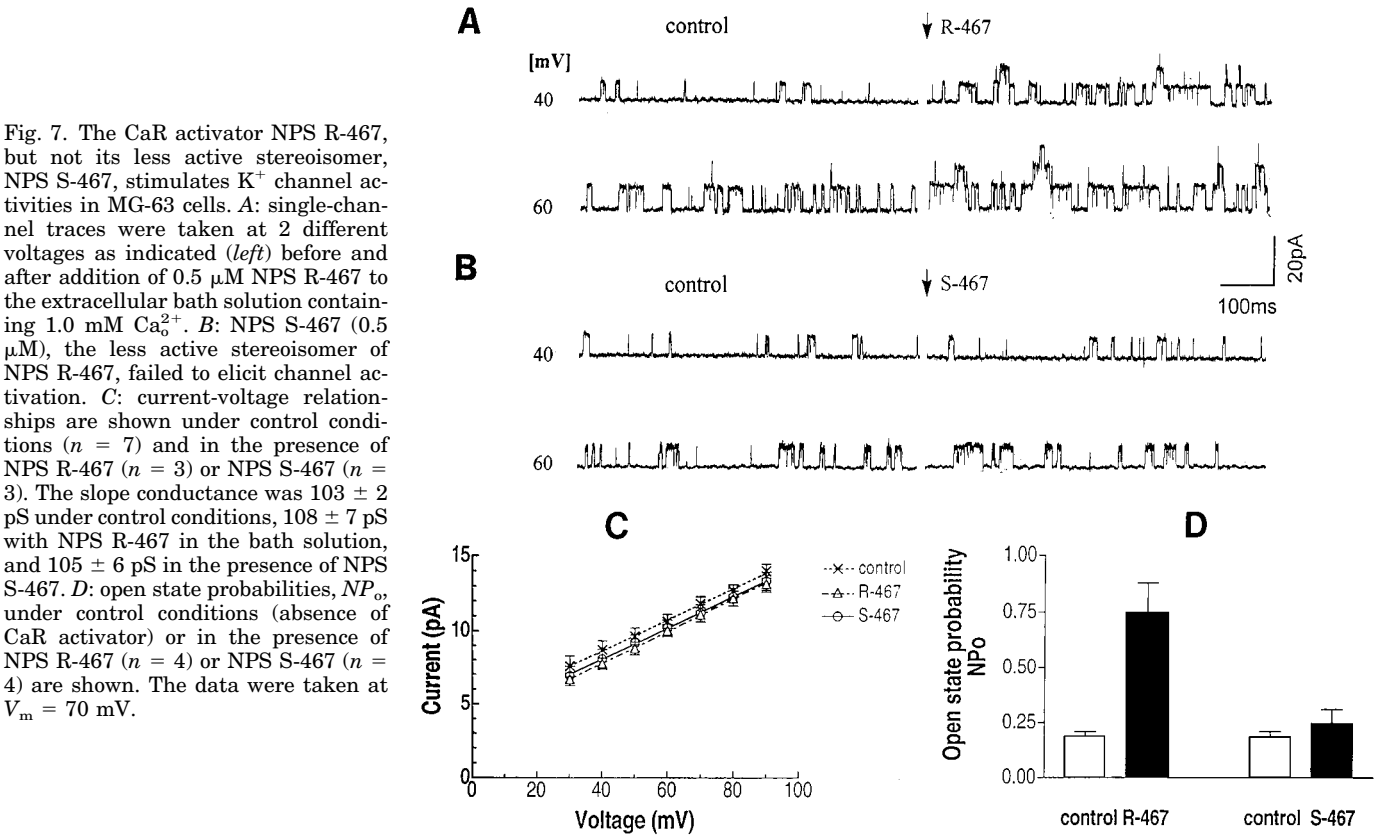

Fig. 7. The CaR activator NPS R-467, but not its less active stereoisomer, NPS S-467, stimulates K^+ channel activities in MG-63 cells. *A*: single-channel traces were taken at 2 different voltages as indicated (*left*) before and after addition of 0.5 μM NPS R-467 to the extracellular bath solution containing 1.0 mM Ca_o^{2+}. *B*: NPS S-467 (0.5 μM), the less active stereoisomer of NPS R-467, failed to elicit channel activation. *C*: current-voltage relationships are shown under control conditions ($n = 7$) and in the presence of NPS R-467 ($n = 3$) or NPS S-467 ($n = 3$). The slope conductance was 103 ± 2 pS under control conditions, 108 ± 7 pS with NPS R-467 in the bath solution, and 105 ± 6 pS in the presence of NPS S-467. *D*: open state probabilities, NP_o, under control conditions (absence of CaR activator) or in the presence of NPS R-467 ($n = 4$) or NPS S-467 ($n = 4$) are shown. The data were taken at $V_m = 70$ mV.

present principally in the region of the plasma membrane. Although the reason(s) underlying the different patterns of distribution of the receptor protein in the two cell types is (are) unknown, these results raise several issues. First, it is possible that the CaR subserves some intracellular function(s) in MG-63 cells and other cells in which we have previously shown that the receptor displays substantial intracellular immunoreactivity (43, 44). Second, these observations point out that it is very important, when performing Western blotting on MG-63 cells and other cells with prominent intracellular CaR immunoreactivity (see below), to prepare total cellular lysates to improve the sensitivity of the procedure for detecting receptor protein. In contrast, in parathyroid cells and HEKCaR cells, crude membrane preparations yield the bulk of CaR immunoreactivity for Western blotting.

We could also detect CaR protein expression in MG-63 cells using Western blotting with the affinity-purified polyclonal antiserum 4637. Although it was apparent that there was considerably less CaR protein expressed in MG-63 cells than in the two positive controls, bovine parathyroid cells and HEKCaR cells, the protein bands identified in MG-63 cells were essentially identical to those in one or both of the other two cell types. Moreover, both parathyroid and HEKCaR cells showed immunoreactive bands corresponding to various glycosylated forms of CaR monomers and dimers that were essentially identical to those observed in these cells in our previous studies (1, 2, 27). Thus we are confident that our preparation of affinity-purified anti-CaR antiserum 4637 binds to the same CaR proteins that are identified by other specific anti-CaR antisera. Moreover, given the results of our functional studies carried out using an electrophysiological approach (see below), it seems apparent that the level of authentic CaR protein expression in MG-63 cells is sufficient to activate a biological response, in this case stimulation of an outward K^+ channel. We have no explanation for the failure of Pi et al. (33) to detect CaR immunoreactivity in either MG-63 or CaR-transfected HEK-cells using a preparation of affinity-purified polyclonal anti-CaR antiserum 4637 that differed from ours, which we had affinity purified ourselves. It appears most likely that their preparation of affinity-purified antiserum had deteriorated during storage.

In addition, there are several reasons why Pi et al. (33) may have been unable to detect CaR protein by Western blotting in their MG-63 cells using the ADD antiserum, which clearly detected robust receptor expression in their CaR-transfected HEK-293 cells. First, by using crude membrane preparations, they may have lost most of the CaR immunoreactivity that we found to be present in the cytoplasm and perinuclear region of these cells. Indeed, we detected substantially less CaR immunoreactivity on Western blots of MG-63 cells when crude membrane preparations rather than whole cell lysates were utilized (Kifor and Brown, unpublished observations). Second, we have found that some cells contain substantial amounts of proteases capable of rapidly degrading the CaR. It is important to utilize optimal amounts and combinations of multiple protease inhibitors and to avoid excessively high concentrations of reducing agents to minimize degradation of the receptor. Pi et al. (33) scraped their cells off the culture dishes before adding protease inhibitors and used a different combination of inhibitors as well as a relatively high concentration (5%) of a reducing agent (2-mercaptoethanol) of which its use has been associated with degradation of the CaR in our hands (45) (Kifor and Brown, unpublished observations). Furthermore, we always compare results using several different titers of an antiserum for performing immunocytochemistry and/or Western blotting in a new cell type studied for CaR expression, since with low levels of receptor expression it may be necessary to increase the titer of the antiserum severalfold to obtain optimal results. Pi et al. (33) only report the results of Western blots of MG-63 cells performed with a single concentration of antisera ADD. Given that multiple parameters must be evaluated to optimize Western analysis of the CaR, we generally employ immunocytochemistry (combined with a method for detecting CaR transcripts, e.g., RT-PCR) as a screening method to study CaR expression in cells examined for the first time. Finally, it is, of course, possible that the CaR is not expressed in the line of MG-63 cells studied by Pi et al. (33).

Given our positive results for the detection of CaR protein expression in MG-63 cells, we then carried out both RT-PCR and Northern analysis to confirm the expression of CaR transcript(s) in this cell line. Indeed, RT-PCR amplified a band of the appropriate size for a product derived from bona fide CaR transcript(s), which was identical in its nucleotide sequence to that of the corresponding region of the human CaR cDNA. In addition, Northern analysis revealed a readily detectable transcript similar in size to a major CaR transcript previously observed in human parathyroid (20). It is of interest that Pi et al. (33) amplified a band of the appropriate size for a CaR-derived product by RT-PCR using RNA from their MG-63 cells. Moreover, Southern analysis on this PCR product using a CaR-specific probe revealed significant hybridization, although these workers were unable to confirm CaR-specific sequences on subsequent attempts to subclone and sequence this PCR product. It is unclear whether direct sequencing of this band without subcloning would have revealed CaR-specific sequence or, in fact, their line of MG-63 cells does not express CaR transcript(s).

To prove that the CaR was not only expressed in but was also functionally active in MG-63 cells, we utilized an approach that we have used in several previous studies (47–49) to document unequivocally that the receptor was functionally coupled to a biological response in various cell types using specific pharmacological probes. That is, we took advantage of the recently described selective calcimimetic CaR activator, NPS R-467, and its less active stereoisomer, NPS S-467 (32). We have previously shown that NPS R-467, but not NPS S-467 at the same concentration, activates nonselective cation channels and/or Ca^{2+}-activated K^+

channels in several cell lines (13–15, 42). Moreover, we validated this approach further by showing that these responses to CaR activators were not present in cells derived from mice homozygous for targeted disruption of the CaR gene (22, 46–49). Indeed, the results of our present studies indicate that in MG-63 cells raising Ca_o^{2+} or addition of NPS R-467 but not NPS S-467 activates an outward K^+ channel, similar to our earlier reports in other cells (12–15, 42). Thus the CaR present in the MG-63 cells employed by us is functional, at least as assessed by its coupling to activation of this K^+ channel.

Therefore, our results strongly support the expression of both CaR mRNA and functional CaR protein in the MG-63 cell line studied here. These data further support our earlier results (44, 45) and those of others (25) that the CaR is expressed in several osteoblastic cell lines. We recognize, however, that MG-63 cells were originally obtained from a human osteosarcoma and may not represent an optimal model for studying the functional relevance of the CaR in osteoblasts (17). The MG-63 osteosarcoma cell line shows increases in ALP and osteocalcin expression following treatment with 1α,25-dihydroxyvitamin D_3 (30), which are responses characteristic of relatively undifferentiated osteoblast precursors. In contrast, another human osteoblastic cell line, SAOS-2, shows high constitutive ALP expression but little or no osteocalcin expression, either with or without addition of 1α,25-dihydroxyvitamin D_3 (30). The mouse osteoblastic cell line, MC3T3-E1, is known to exhibit properties of osteoprogenitor cells and preosteoblasts in their actively growing stage. Following growth arrest, however, they differentiate and develop markers of mature osteoblasts, including the expression of high levels of ALP and the capacity to form mineralized bone matrix (44). Together, our present and prior studies, which show expression of the CaR in osteoblastic cell lines differing in their phenotypes and apparent stages of differentiation, suggest that this receptor is expressed in osteoblasts varying substantially in their developmental stages.

It should be pointed out that the MG-63 cell line also retains a fibroblast-like character, with its abundant expression of type III collagen (24) and low constitutive expression of ALP (17, 30). It is of interest in this regard that McNeil et al. (31) found that rat-1 fibroblasts express the CaR. Indeed, fibroblasts arise from the same mesenchymal stem cell (8) that gives rise to several types of CaR-expressing cells involved in bone growth and/or turnover, including chondrocytes (9), osteoblasts (44, 45), and stromal cells (43). Therefore, expression of the CaR appears to be characteristic of several cell types within this lineage.

Our present and prior studies (44, 45) have clearly shown that the CaR is expressed in clonal osteoblastic cell lines. Moreover, recent studies have documented the expression of CaR mRNA and protein in osteoblasts within sections of bovine, rat, and murine bone (10), providing additional evidence that bona fide osteoblasts express the CaR. Nevertheless, it is possible that other Ca^{2+} sensors molecularly distinct from the CaR (34, 35) are also present in osteoblasts and participate in their cation-sensing capacity. For instance, Pi et al. (32a) have recently isolated primary osteoblast-like cells from mice with targeted disruption of the CaR that still showed mitogenic and other biological responses to polyvalent cations. Additional studies are needed, therefore, utilizing genetic models of generalized (22) or tissue-selective "knock out" of the CaR as well as techniques that downregulate the function of the endogenous osteoblastic CaR, e.g., CaR antagonists or transfection with dominant negative CaR constructs (31), to establish definitively the CaR's role in modulating the full range of osteoblastic functions that are responsive to Ca_o^{2+}. The outcome of these experiments should answer the question of whether the CaR is the principal Ca_o^{2+} sensor in osteoblasts and/or whether they possess other Ca_o^{2+} sensors/receptors that also contribute to the regulation of osteoblast function by Ca_o^{2+}.

The authors gratefully acknowledge generous grant support from National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-41415, DK-48330, and DK-52005, NPS Pharmaceuticals, The St. Giles Foundation, and the National Space Bioscience Research Institute (to E. M. Brown), the Mochida Memorial Foundation Grant for Medical and Pharmaceutical Research (to T. Yamaguchi), and the Yamanouchi Foundation Grant for Research on Metabolic Disorders (to T. Yamaguchi).

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