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Extracellular Calcium (Ca^{2+}_o)-Sensing Receptor in a Murine Bone Marrow-Derived Stromal Cell Line (ST2): Potential Mediator of the Actions of Ca^{2+}_o on the Function of ST2 Cells*

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ABSTRACT

The calcium-sensing receptor (CaR) is a G protein-coupled receptor that plays key roles in extracellular calcium ion (Ca^{2+}_o) homeostasis by mediating the actions of Ca^{2+}_o on parathyroid gland and kidney. Bone marrow stromal cells support the formation of osteoclasts from their progenitors as well as the growth of hematopoietic stem cells by secreting humoral factors and through cell to cell contact. Stromal cells also have the capacity to differentiate into bone-forming osteoblasts. Bone resorption by osteoclasts probably produces substantial local increases in Ca^{2+}_o that could provide a signal for stromal cells in the immediate vicinity, leading us to determine whether such stromal cells express the CaR. In this study, we used the murine bone marrow-derived, stromal cell line, ST2. Both immunocytochemistry and Western blot analysis, using an antiserum specific for the CaR, detected CaR protein in ST2 cells. We also identified CaR transcripts in ST2 cells by Northern analysis using a CaR-specific probe and by RT-PCR with CaR-specific primers, followed by nucleotide sequencing of the amplified products. Exposure of ST2 cells to high Ca^{2+}_o (4.8 mM) or to the polycationic CaR agonists, neomycin (300 μM) or gadolinium (100 μM), stimulated both chemotaxis and DNA synthesis in ST2 cells. Therefore, taken together, our data strongly suggest that the bone marrow-derived stromal cell line, ST2, possesses both CaR protein and messenger RNA that are very similar if not identical to those in parathyroid and kidney. Furthermore, as ST2 cells have the potential to differentiate into osteoblasts, the CaR in stromal cells could participate in bone turnover by stimulating the proliferation and migration of such cells to sites of bone resorption as a result of local, osteoclast-mediated release of Ca^{2+}_o and, thereafter, initiating bone formation after their differentiation into osteoblasts. (*Endocrinology* **139:** 3561–3568, 1998)

ACCUMULATING evidence suggests that hematopoiesis is supported by the specific microenvironment of the bone marrow, in which stromal cells play a central role by stimulating hematopoietic stem cell differentiation by secreting various humoral mediators as well as via cell surface molecules (1). A murine bone marrow-derived, stromal cell line, ST2, can support both myelopoiesis and B cell lymphopoiesis and has been used as a model that can reproduce an *in vitro* microenvironment supporting hematopoiesis (2). This cell line has also been shown to support the formation of osteoclasts in coculture with murine spleen cells or mononuclear cells derived from mouse bone marrow (3), suggesting that bone marrow stromal cells are involved in the control of bone turnover as well as hematopoiesis *in vivo*.

The resorption of bone can produce local increases in the extracellular calcium concentration (Ca^{2+}_o) beneath a resorbing osteoclast as high as 40 mM (4). Osteoclast formation can be activated by stromal cells through a mechanism involving cell to cell contact (1, 5). It is possible, therefore, that stromal cells that have participated in the process of osteoclast formation remain in close proximity to sites where active bone resorption is taking place and are capable of sensing the locally high levels of Ca^{2+}_o that result from osteoclast-mediated breakdown of bone. This high Ca^{2+}_o could, therefore, provide the stromal cells with a signal that modulates their subsequent physiological responses.

One possible mechanism by which stromal cells could respond to changes in Ca^{2+}_o would be via the Ca^{2+}_o-sensing receptor (CaR) that has been cloned from bovine and human parathyroid gland (6, 7), rat kidney (8) and thyroid C cells (9). The physiological relevance of the CaR has been documented in humans by showing that inactivating or activating mutations of the CaR gene cause inherited hyper- or hypocalcemic disorders (10, 11), respectively, rendering affected family members inappropriately resistant or sensitive, respectively, to the usual effects of Ca^{2+}_o on parathyroid and renal functions.

In a previous study using immunohistochemistry with CaR-specific antisera, we showed expression of this receptor in diverse cell types in human bone marrow, including alkaline phosphatase-positive, putative osteoblast precursors, nonspecific esterase-positive mononuclear cells, erythroid precursors, and megakaryocytes (12). These findings suggested that the CaR might be involved in the Ca^{2+}_o-sensing

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mechanism of these bone marrow-derived cells. In this study, we used ST2 cells as a model of stromal cells in bone marrow and examined them for the presence and possible role(s) of the CaR in regulating their function(s). We demonstrate herein clear expression of the CaR in ST2 cells, as assessed by immunocytochemical staining and Western blot analysis using a specific anti-CaR antiserum as well as by Northern analysis with a CaR-specific probe and RT-PCR with CaR-specific primers. We also confirm that CaR agonists stimulate both chemotaxis and DNA synthesis in the CaR-expressing ST2 cells examined in this study. These results show that both CaR protein and messenger RNA (mRNA) are expressed in the ST2 cell line; in addition, they suggest that the receptor could potentially play a pivotal role in regulating the function of stromal cells present within the marrow by sensing local changes in Ca^{2+}_o related to bone remodeling.

Materials and Methods

Materials

All routine culture media were obtained from Life Technologies (Grand Island, NY). Neomycin sulfate and anhydrous calcium chloride ($CaCl_2$) were purchased from Sigma Chemical Co. (St. Louis, MO), and Gd^{3+} (III) chloride hexahydrate was obtained from Aldrich Chemical Co. (Milwaukee, WI). [^{3}H]Methylthymidine was purchased from DuPont-New England Nuclear (Boston, MA).

Cell culture

ST2 cells were purchased from the RIKEN Cell Bank (Tsukuba, Japan). ST2 cells were grown in αMEM (Ca^{2+}, 1.8 mM; Mg^{2+}, 0.81 mM; $H_2PO_4^-$, 1.0 mM); supplemented with 10% FBS 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 flasks by detaching them gently with a cell scraper after reaching subconfluency. For morphological evaluation, ST2 cells were plated onto 12-mm circular glass coverslips in 24-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 PBS, fixed with 4% formaldehyde in PBS for 5 min, and washed with PBS once again. Each coverslip was stored at 4 C until assessment of the presence of the CaR as described below.

Immunocytochemistry for CaR in ST2 cells

A CaR-specific polyclonal antiserum (4637) was provided by Drs. Forrest Fuller and Karen Krapcho of NPS Pharmaceuticals (Salt Lake City, UT). This antiserum was raised against a peptide (FF-7; HNGFAKEFWEETFNC) corresponding to amino acids 345–359 of the bovine CaR (6) and 344–358 of the human and rat CaRs (7, 8), which resides within the predicted amino-terminal extracellular domain of the CaR. The antiserum has recently been fully characterized and used for studies of the endogenous CaRs expressed in various human and rat tissues in our laboratory (13–16). The antiserum was subjected to further purification using an affinity column conjugated with the FF-7 peptide, and the affinity-purified antiserum was used for immunocytochemistry and Western blot analysis as described below. Fixed ST2 cells were treated with Dako Protein Block Serum-Free Solution (Dako Corp., Carpinteria, CA) for 1 h and then incubated overnight at 4 C with primary antiserum (anti-CaR antiserum 4637) at a concentration of 5 μg/ml in blocking solution (Dako Corp.). Negative controls were carried out by performing the same procedure after preabsorption of the anti-CaR antiserum with 10 μg/ml of the synthetic CaR peptide against which it was raised. After washing the cells three times with 0.5% BSA in PBS for 10 min, alkaline phosphatase-coupled, goat antirabbit IgG (1:200; Life Technologies) was added and incubated for 1 h at room temperature. The cells were then washed three times with PBS for 10 min each time, and the color reaction was developed for 10–20 min using a solution consisting of 44 μl nitro blue tetrazolium chloride (75 mg/ml) and 33 μl 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt (50 mg/ml) in 10 ml 0.1 M Tris-HCl (pH 9.5), 0.1 M NaCl, 50 mM $MgCl_2$, and 1 mg/ml levamisole, which was included for inhibition of endogenous cellular alkaline phosphatase activity. The color reaction was stopped by washing twice in the above solution without nitro blue tetrazolium chloride or 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt and then twice in water.

Western analysis of CaR in ST2 cells

Monolayers of ST2 cells in 75-cm^2 flasks were rinsed twice with 1 mM EDTA in PBS and lysed with 1.0 ml of a lysis solution (1% SDS and 10 mM Tris-HCl, pH 7.4) heated to 65 C. The cells were scraped from the flasks, transferred to microcentrifuge tubes, and heated for an additional 5 min at 65 C. The viscosity of the sample was reduced by brief sonication, and insoluble material was removed by centrifugation for 5 min. The resultant whole cell lysate in the supernatant was stored at −20 C until Western blot analysis was performed.

Aliquots of 150 μg protein were dissolved in SDS-Laemmli gel loading buffer containing 100 mM dithiothreitol, incubated at 37 C for 15 min, and resolved electrophoretically on 6.5% SDS-polyacrylamide gels. Proteins were electrophoretically transferred to nitrocellulose at 240 mA for 40 min in transfer buffer containing 19 mM Tris-HCl, 150 mM glycine, 0.015% SDS, and 20% methanol. The blots were blocked for 2 h with 1% BSA in PBS containing 0.25% Triton X-100 (blocking solution) and then incubated overnight at 4 C with the affinity-purified antiserum (4637) or with peptide-blocked antiserum [the same amount of antiserum preincubated at room temperature for 60 min with twice the amount (wt/wt) of FF-7 peptide] at a concentration of 1 μg/ml in the blocking solution. The blots were washed three times with PBS containing 0.25% Triton X-100 (washing solution) at room temperature for 10 min each time. The blots were further incubated with a 1:2000 dilution of horseradish peroxidase-coupled, goat antirabbit IgG (Sigma Chemical Co.) in the blocking solution for 1 h at room temperature. The blots were then washed three times with the washing solution at room temperature for 40 min each time, and specific protein bands were detected using an enhanced chemiluminescence system (ECL, Amersham, Arlington Heights, IL).

Detection of CaR transcripts by Northern blot analysis

For the purpose of determining the sizes of the CaR transcripts in ST2 cells, Northern blot analysis was employed on aliquots of 5 μg polyadenylated [poly(A)$^+$] RNA obtained using oligo(deoxythymidine)-cellulose chromatography of total RNA. Poly(A$^+$)-enriched RNA samples were denatured and electrophoresed in 2.2 M formaldehyde-1% agarose gels along with an 0.24–9.5 kb RNA ladder (Life Technologies) and transferred overnight to nylon membranes (Duralon, Stratagene, La Jolla, CA). A 577-bp *Xho*I-*Sac*I fragment corresponding to nucleotides 721-1298 of the rat CaR complementary DNA (cDNA) was subcloned into the pBluescript SK$^+$ vector. The plasmid was then linearized with *Bgl*II, and a ^{32}P-labeled riboprobe was synthesized with the MAXIscript T3 kit (Pharmacia Biotech, Piscataway, NJ) using T_3 polymerase and [^{32}P]UTP. Nylon membranes were prehybridized for 2 h at 52 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), 5 × 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 transfer RNA, and 200 μg/ml calf thymus DNA. Labeled complementary RNA probe (2 × 10^6 cpm/ml) was then added, and the membranes were hybridized overnight at the same temperature. Washing was carried out at moderate stringency (0.3 × SSC [20 × SSC = 3 M NaCl and 0.3 M Na_3-citrate·2H_2O) and 0.5% SDS at 55 C] for 20 min. Membranes were then exposed to x-ray film (XAR-5, Eastman Kodak, Rochester, NY) for 4 days at −70 C.

PCR amplification of cDNA products from the mouse homolog of the CaR expressed in ST2 cells

Total RNA was prepared from ST2 cells using the TRIzol reagent (Life Technologies). One microgram of total RNA was used for the synthesis of single stranded cDNA (cDNA synthesis kit, Life Technologies). The resultant first strand cDNA was used for the PCR procedure. PCR was performed at a final concentration of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.8 mM $MgCl_2$, 0.2 mM deoxy-NTP, 0.4 μM forward primer, 0.4 μM

reverse primer, and 1 μl ELONGASE enzyme mix (a *Taq/Pyrococcus species* GB-D DNA polymerase mixture; Life Technologies). The primer sequences were: sense, 5′-AGAAGTTCCGAGAGGAAGCC-3′; and antisense, 5′-ACCTGTTGCCA-CCTTCTTCG-3′, designed from the extracellular domain of the rat CaR. To perform hot start PCR, the enzyme was added during the initial 3-min denaturation and was followed by 35 cycles of amplification (30-sec denaturation at 94 C, 30-sec annealing at 47 C, and 1-min extension at 72 C). The reaction was completed by 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 480-nucleotide-long amplified product was indicative of a positive PCR reaction.

Cloning and sequencing of RT-PCR products derived from the mouse homolog of the CaR

RT-PCR products were ligated into the pCR 2.1 vector of the TA cloning kit by incubation overnight at 14 C. Competent cells were transformed according to the manufacturer's instructions and placed on ampicillin-containing agar in the presence of X-galactosidase. Transformed cells were identified after overnight growth at 37 C as white colonies. The white colonies were used to inoculate Luria Bertoni medium, and plasmid DNA was extracted, digested with *Eco*RI, and fractionated by agarose gel electrophoresis. DNA from positive clones was further purified using the Qiagen Plasmid kit (Qiagen, Chatsworth, CA) and sequenced bidirectionally using M13 forward and M13 reverse primers with an automated sequencer (AB377, Applied Biosystems, Foster City, CA) in the DNA Sequence Facility of the University of Maine (Orono, ME), using dideoxy terminator *Taq* technology.

Chemotaxis assay of ST2 cells

Chemotaxis was evaluated using a Neuroprobe BW200S blindwell chamber (Neuro Probe, Gaithersburg, MD) as previously described (17, 18). $CaCl_2$ (1.8 or 4.8 mM), 300 μM neomycin sulfate, or 100 μM $GdCl_3 \cdot 6H_2O$ in serum-free DMEM were loaded into the lower chamber, which was separated from the upper well by a 5-mm membrane with 5-μm pores. ST2 cells (1×10^5 cells/ml) were dissociated briefly with trypsin-EDTA solution (Life Technologies), washed twice, suspended in serum-free αMEM, and added to the upper chamber. After a 5-h incubation at 37 C, cells on the upper surface of the membrane that had not migrated were scraped from the membrane, and cells that had migrated to the opposite side of the membrane were fixed with methanol and stained with Giemsa. The cells that had migrated were then counted in six high power fields (×400) using a light microscope. For the purpose of comparison between multiple assays, the data were normalized as the fold increase in cellular chemotaxis relative to that in the control.

DNA synthesis in ST2 cells

We assessed DNA synthesis in ST2 cells using [^{3}H]thymidine incorporation. ST2 cells were dissociated with trypsin-EDTA solution (Life Technologies) and seeded in 24-well (2.0-cm^2) plates at a density of 1000 cells/well in 500 μl αMEM containing 10% FBS as well as 1.8, 2.8, or 4.8 mM $CaCl_2$; 300 μM neomycin sulfate; or 100 μM $GdCl_3 \cdot 6H_2O$. After a 48-h incubation at 37 C, cells were pulsed with [^{3}H]thymidine (1 μCi/well). Incubations were terminated after overnight incubation by removal of the medium and addition of 5% trichloroacetic acid. Cells were then scraped and transferred to microcentrifuge tubes. After centrifugation at 15,000 × *g* and removal of the supernatant, the precipitate was washed with 75% ethanol and desiccated at room temperature. The residual pellet was dissolved in 20 mM NaOH and 1% SDS, and a scintillation cocktail was added. Samples were counted in a liquid scintillation counter.

Statistics

Experiments on chemotaxis and DNA synthesis were performed three times on separate occasions. Results are shown as the mean ± SEM for six determinations from a single representative experiment. Statistical evaluation for differences between groups was performed using one-way ANOVA followed by Fisher's protected least significant difference. For all statistical tests, $P < 0.05$ was considered significant.

Results

Immunoreactivity of CaR protein in ST2 cells using CaR-specific antiserum

To clarify whether the CaR is expressed by bone marrow-derived stromal cells, we investigated the presence of the receptor in the murine clonal stromal cell line, ST2. Immunocytochemistry of ST2 cells with a CaR-specific polyclonal antiserum revealed moderate CaR staining (Fig. 1A), which was eliminated by preincubating the primary antiserum with the peptide against which it was raised (Fig. 1B).

We also performed Western analysis on proteins isolated from ST2 cells (Fig. 2). A single major band was stained in the presence of specific antiserum (lane 1). The molecular mass corresponding to the position of the stained band (~150–160

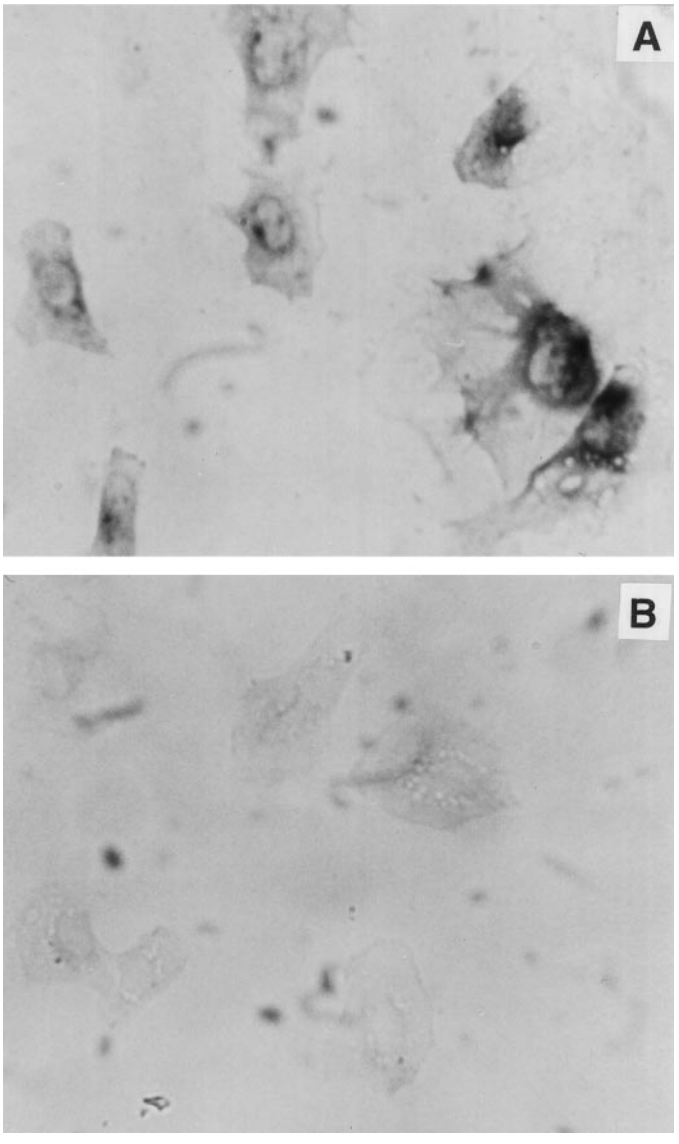

FIG. 1. Immunocytochemistry of ST2 cells was performed as described in *Materials and Methods* using a CaR-specific polyclonal antiserum (4637). Immunocytochemistry revealed moderate CaR staining (A), which was eliminated by preincubating the primary antiserum with the peptide against which it was raised (B). The photomicrographs were taken at a magnification of ×1000.

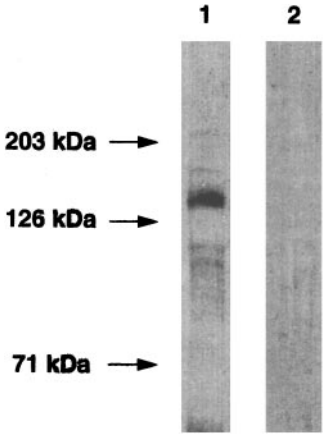

FIG. 2. Western analysis of whole cell lysates from ST2 cells was performed as described in *Materials and Methods*. A band at approximately 150–160 kDa that was stained in the presence of specific antiserum was consistent with the intact glycosylated CaR (lane 1). The specificity of the labeling by the antiserum used in this study to detect the CaR was confirmed by abolition of the bands in extracts of cells incubated with peptide-preabsorbed CaR antiserum (lane 2).

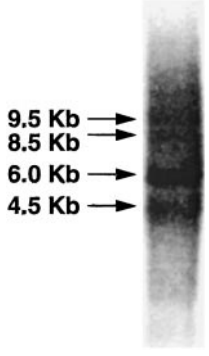

FIG. 3. Northern blot analysis of CaR transcripts in ST2 cells was performed as described in *Materials and Methods*. *Arrows* show the sizes of the CaR transcripts.

kDa) was of a size consistent with that of the intact, glycosylated CaR (19). The specificity of the anti-CaR antiserum used in this study was confirmed by abolition of the band after preabsorption of the anti-CaR antiserum with the peptide against which it was raised (lane 2).

Detection of CaR mRNA in ST2 cells by Northern blot analysis and RT-PCR

Figure 3 shows Northern blot analysis on poly(A)$^+$ RNA isolated from ST2 cells, which revealed four transcripts with sizes of 9.5, 8.5, 6.0, and 4.5 kb.

RT-PCR with CaR-specific primers amplified a product of the expected size (480 bp) for a product derived from CaR transcript(s) (Fig. 4). No products were observed when the reverse transcriptase was omitted during the synthesis of cDNA (data not shown). DNA sequence analysis of the PCR product revealed 95% sequence identity with the rat CaR cDNA (Fig. 5), showing a difference in its predicted protein sequence of one amino acid relative to that of the rat CaR at position 132. These results show that the PCR product corresponded to a CaR sequence, indicating the presence of *bona fide* CaR transcripts in these cells.

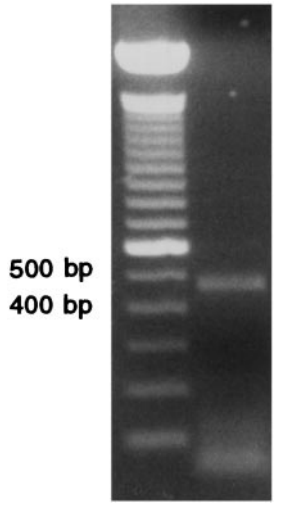

FIG. 4. Identification of CaR transcripts in ST2 cells by RT-PCR, performed as described in *Materials and Methods*. A product was amplified from reverse transcribed RNA isolated from ST2 cells, which was of the expected size (480 bp) for a CaR-derived product.

Chemotactic activity of ST2 cells toward high Ca^{2+}_o, neomycin sulfate, or Gd^{3+}_o

A chemotaxis assay was performed to determine the capacity of ST2 cells to migrate toward CaR agonists. As shown in Fig. 6, 4.8 mM Ca^{2+}_o induced a chemotactic response of ST2 cells over control values at 1.8 mM Ca^{2+}_o ($P < 0.05$). Both neomycin sulfate (300 μM) and extracellular gadolinium ion (Gd^{3+}_o; 100 μM) also induced significant chemotactic responses over the control level ($P < 0.05$).

DNA synthesis of ST2 cells stimulated by high Ca^{2+}_o, neomycin sulfate, or Gd^{3+}_o

We found that treatment of ST2 cells with increasing levels of Ca^{2+}_o up to 4.8 mM resulted in a dose-dependent stimulation of DNA synthesis over control values at 1.8 mM Ca^{2+}_o ($P < 0.05$ at 4.8 mM; Fig. 7). Neomycin sulfate (300 μM) and Gd^{3+}_o (100 μM) also significantly stimulated DNA synthesis over its control level ($P < 0.05$).

Discussion

In a previous study, we examined the expression of the CaR in primary cultures of human and murine bone marrow cells and showed that the CaR is present in low density mononuclear bone marrow cells as well as in cells of several hematopoietic lineages (12). As these cells were taken directly from bone marrow and may be similar to those present in marrow *in vivo*, this finding suggested that the CaR is expressed by various bone marrow-derived cells *in vivo*. However, these primary cultures contained mixed cell populations, and it is difficult to identify exactly which cell types express the CaR. To circumvent this problem, we used murine ST2 clonal cells in this study, which possess a bone marrow-derived stromal cell phenotype (2). Our results show that ST2 cells clearly expressed CaR protein by immunocytochemistry as well as by Western blot analysis, which revealed a specific band at a molecular mass consistent with that of the intact, glycosylated CaR (~150–160 kDa) (19). In

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Rat CaR   5' AGAAGTTCCGAGAGGAAGCC GAA GAG AGG GAT ATC TGC ATT GAT TTT AGC GAG CTC  56
                                  E   E   R   D   I   C   I   D   F   S   E   L   12
ST2                               --G --- --- --C --- --- --- --C --C --- --- ---

Rat CaR   ATC TCC CAG TAC TCT GAC GAG GAA GAG ATC CAG CAG GTG GTC GAA GTG ATC CAA 110
           I   S   Q   Y   S   D   E   E   E   I   Q   Q   V   V   E   V   I   Q  30
ST2       --- --- --- --- --- --T --- --- --- --- --- --- --- --G --- --- --- --G

Rat CaR   AAC TCT ACG GCC AAG GTC ATT GTC GTT TTC TCC AGC GGC CCG GAC CTA GAA CCT 164
           N   S   T   A   K   V   I   V   V   F   S   S   G   P   D   L   E   P  48
ST2       --- --- --A --- --- --- --- --- --- --- --- --- --- --A --- --- --- ---

Rat CaR   CTC ATC AAG GAG ATT GTG CGG CGT AAC ATC ACA GGC AGG ATC TGG CTG GCT AGC 218
           L   I   K   E   I   V   R   R   N   I   T   G   R   I   W   L   A   S  66
ST2       --- --- --- --- --- --- --C --- --- --- --- --- --- --- --- --- --C ---

Rat CaR   GAG GCC TGG GCC AGT TCC TCG CTG ATT GCT ATG CCT GAG TAT TTC CAT GTA GTC 272
           E   A   W   A   S   S   S   L   I   A   M   P   E   Y   F   H   V   V  84
ST2       --- --- --- --- --- --- --C --- --- --- --- --- --- --C --- --- --- ---

Rat CaR   GGG GGC ACC ATT GGG TTC GGT CTG AAG GCT GGG CAG ATT CCA GGC TTC AGA GAA 326
           G   G   T   I   G   F   G   L   K   A   G   Q   I   P   G   F   R   E  102
ST2       --- --T --- --- --- --- --- --- --A --- --- --- --- --- --- --- C-- ---

Rat CaR   TTC CTA CAG AAA GTT CAT CCT AGG AAG TCT GTC CAC AAT GGT TTT GCC AAA GAG 380
           F   L   Q   K   V   H   P   R   K   S   V   H   N   G   F   A   K   E  120
ST2       --- --- --- --- --C --- --C --- --- --- --- --- --- --- --- --- --- ---

Rat CaR   TTT TGG GAA GAA ACT TTT AAT TGC CAC CTC CAA GAA GGC GCA AAA GGA CCT TTA 434
           F   W   E   E   T   F   N   C   H   L   Q   E   G   A   K   G   P   L  138
ST2       --- --- --- --- --A --- --- --- --- --G --- --C --- --- --- --- --- ---
                                                        D

Rat CaR   CCT GTG GAC ACC TTC GTG AGA AGT CA CGAAGAAGGTGGCAACAGGT 3' 480
           P   V   D   T   F   V   R   S     146
ST2       --C --- --- --- --- --- --- --- --
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FIG. 5. Nucleotide and amino acid sequences of the 480-bp CaR RT-PCR fragment. DNA sequence analysis of the 480-bp PCR product amplified from reverse transcribed RNA isolated from murine ST2 cells revealed 95% sequence identity with the nucleotide sequence of the rat CaR cDNA. The *dashes* indicate the nucleotides of the CaR in ST2 cells identical to their counterparts of the rat CaR. The PCR product showed one difference in its amino acid sequence from that of the rat CaR at position 132 (shown in single letter code).

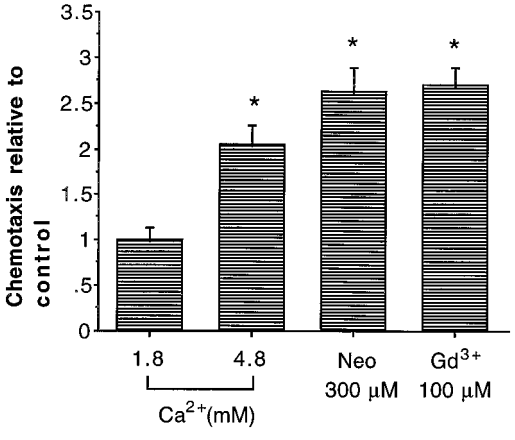

FIG. 6. Chemotactic activity of ST2 cells toward high $Ca^{2+}{}_o$, neomycin sulfate, or $Gd^{3+}{}_o$. The number of ST2 cells that migrated to the side of the membrane to which $CaCl_2$, neomycin sulfate, or $GdCl_3{\cdot}6H_2O$ had been added 5 h previously was counted as described in *Materials and Methods*. Each *bar* indicates the mean ± SEM for six determinations from a single representative experiment. *, $P < 0.05$ compared with cells exposed to 1.8 mM $Ca^{2+}{}_o$.

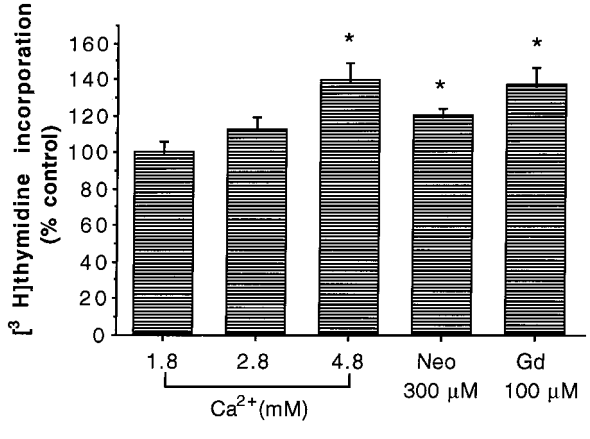

FIG. 7. Stimulation of DNA synthesis in ST2 cells by high $Ca^{2+}{}_o$, neomycin sulfate, or $Gd^{3+}{}_o$. ST2 cells were treated with high $Ca^{2+}{}_o$, neomycin sulfate, or $Gd^{3+}{}_o$ concentrations for 3 days before the addition of [^{3}H]thymidine as described in *Materials and Methods*. Values are expressed as a percentage of the value in controls (cells treated with 1.8 mM $Ca^{2+}{}_o$). Each *bar* represents the mean ± SEM for six determinations from a single representative experiment. *, $P < 0.05$ compared with cells treated with 1.8 mM $Ca^{2+}{}_o$.

addition, both Northern analysis performed on poly(A)$^+$ RNA from ST2 cells and RT-PCR performed on total RNA from these cells followed by sequence analysis of the PCR products indicated the presence of *bona fide* CaR transcripts. Thus, the present study shows that this stromal cell line expresses both CaR protein and mRNA.

The 9.5- and 4.5-kb transcripts in ST2 cells shown by Northern analysis are of sizes similar to those of transcripts

expressed in both mouse kidney and murine AtT-20 cells (20). However, ST2 cells expressed additional 8.5- and 6.0-kb transcripts instead of the 7.5-kb transcript observed in AtT-20 cells or mouse kidney (20). There are previous instances where the relative ratios of the abundance of CaR transcripts vary from organ to organ (reviewed in Ref. 21). Because the 4.5-kb transcript encodes the entire functional CaR protein, the significance of the larger transcripts remains uncertain. However, the possibility of organ/cell type-specific, post-transcriptional regulation of CaR expression as a result of variations in the stabilities of the various CaR transcripts cannot be ruled out.

Bone marrow stromal cells are known to originate from mesenchymal stem cells, which are also capable of differentiating into osteoblasts, chondrocytes, adipocytes, myoblasts, and fibroblasts (22). Stromal cells provide specific factors for hematopoietic stem cell survival and differentiation by cell to cell contact through cell surface molecules and by secreting various humoral factors, such as macrophage colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, and IL-7 (1). The ST2 cell line used in this study also retains such activities and supports both myelopoiesis and B cell lymphopoiesis under specific culture conditions (2). In addition, this cell line can support monocyte differentiation into osteoclasts in coculture with murine spleen cells or mononuclear cells of murine bone marrow in the presence of 1α,25-dihydroxyvitamin D_3 and dexamethasone (3). Recently, ST2 cells themselves have been used as a model for osteoblast precursors, because they have the capacity to differentiate into bone-forming osteoblasts in response to BMP-2 (23). Taken together, these data suggest that the ST2 cell line provides a useful *in vitro* model system capable of carrying out functions intimately related to the processes of both osteoclastic bone resorption and osteoblastic bone formation. Substantial release of calcium ions into the extracellular fluid of the bone/bone marrow compartment takes place during bone turnover (4). Therefore, our finding of the expression of the CaR in ST2 cells suggests that bone marrow stromal cells within the immediate vicinity of sites of resorptive activity could sense such changes in Ca^{2+}_o and modulate the processes of bone formation and resorption *in vivo* by cell to cell contact or secretion of humoral factors. Recently, Ca^{2+}_o was shown to induce the secretion of cytokines from human peripheral blood mononuclear cells (24, 25), suggesting that a similar process is possible in ST2 cells.

Our study also shows that high levels of Ca^{2+}_o and CaR agonists stimulate both chemotaxis and proliferation of ST2 cells. Although these actions could be mediated by the CaR, additional studies using approaches (*e.g.* antisense methodologies) that down-regulate the CaR and/or stromal cells derived from mice with targeted disruption of the CaR gene are needed to establish the role of CaR with certainty. Considering that ST2 cells have the potential to differentiate into bone-forming osteoblasts (23), our findings suggest a role for the CaR expressed in stromal cells in the reversal phase of bone remodeling. Bone formation is initiated by the migration of osteoblast precursors into resorption pits after the cessation of osteoclastic bone resorption (26). The CaR in stromal cells might sense the locally high levels of Ca^{2+}_o released from mineralized bone matrix during osteoclastic bone resorption (4). As a result, they might then proliferate, migrate into sites where new bone formation is required, and eventually initiate bone formation after their differentiation into mature osteoblasts.

Another possible implication of the CaR agonist-induced chemotaxis and proliferation of ST2 cells is that these responses might reflect the capacity of stromal cells to promote the differentiation of mononuclear precursors for formation of a subsequent generation(s) of new osteoclasts after the disappearance of earlier osteoclasts at a resorptive site. The fluid resorbed from bone by an active osteoclast that contains high levels of Ca^{2+}_o (4) is compartmentalized from the surrounding extracellular fluid during active bone resorption by the tight seal of the osteoclast to the underlying bone surface (4). Thus, the resorbed calcium ions are only released into the surrounding microenvironment, where they can potentially serve as a signal to cells within the surrounding bone marrow, if the resorbing osteoclast undergoes apoptotic degeneration or migrates elsewhere, and the concentrated Ca^{2+}_o within the solution beneath it is liberated. A previous study showed that Ca^{2+}_o could also stimulate the chemotaxis of monocytes (18) as well as the fusion of alveolar macrophages to form multinucleated giant cells (27). Hence, it is possible that stromal cells, by migrating into sites of active bone resorption along with monocytes in response to signals including high local levels of Ca^{2+}_o generated by the resorptive process, can first support the differentiation of mononuclear precursors into a new generation of osteoclasts and then later provide a source of osteoblast precursors for eventual initiation of bone formation (26). Clearly, the temporal duration and extent of resorption and the timely initiation of bone formation and full replacement of missing bone are crucial for skeletal integrity. Understanding the mechanisms underlying this highly orchestrated series of events is a challenge for ongoing research in this area.

Additional approaches that could potentially be taken to clarify these mechanisms is the investigation of bone turnover and metabolism in patients with autosomal dominant hypocalcemia and neonatal severe hyperparathyroidism (NSHPT), human disorders caused by activating and inactivating CaR mutations (10, 11), respectively, or using a homozygous CaR-deficient mouse, which is an animal model for NSHPT (28). The skeletal abnormalities found in NSHPT and the homozygous CaR-deficient mouse are thought to be caused secondarily by the associated severe hyperparathyroidism (29, 30), which makes it difficult to analyze the consequences of the CaR deficiency *per se* in these models. However, an *in vitro* study using primary cultures of bone marrow cells from homozygous CaR-deficient mice could circumvent this difficulty and provide insights into the role of the CaR in stromal cells in the control of bone turnover. Autosomal dominant hypocalcemia, in contrast, causes more modest changes in symptoms, bone metabolism, and circulating levels of calciotropic hormones and mineral ions (31); thus, investigating skeletal tissue and its metabolism in this disorder may also provide some clues to the biological importance of the CaR in bone turnover.

Recently, Quarles *et al.* reported that a Ca^{2+}_o-sensing mechanism in the mouse osteoblast-like MC3T3-E1 clonal cell line is functionally similar to but molecularly distinct

from the CaR (32). After failing to detect expression of the CaR by Northern analysis and RT-PCR in MC3T3-E1 cells, they identified nucleotide sequences of putative CaR-related receptors (*Casr-rs*) in mouse genomic libraries by PCR (33). The deduced protein sequence of one of these putative receptors (*Casr-rs1*) was 63% similar and 40% identical to the CaR over the available transmembrane region. This putative receptor might also exist in bone marrow stromal cells and could sense the local changes in Ca^{2+}_o caused by bone turnover. However, this CaR-related nucleotide sequence, initially identified in MC3T3-E1 cells by RT-PCR and used as a probe to screen mouse genomic libraries to identify other related sequences, could only be identified in subsequent analyses of mouse tissues, including MC3T3-E1 cells, by RT-PCR and not by Northern analysis (33), suggesting very low levels of expression of uncertain physiological significance. Thus, additional studies are necessary to determine whether these CaR-related nucleotide sequences are actually expressed as mature proteins in MC3T3-E1 cells using specific antisera raised to their predicted protein sequences and what, if any, function(s) they serve in these cells.

On the basis of our results in this study using the murine clonal ST2 cell line, we suggest that the CaR is expressed in bone marrow stromal cells *in vivo* and that in this cell type it could potentially play physiological roles in both bone formation and bone resorption. Recently, we also found that two other murine cell lines, the monocyte-macrophage-like J774 cell line and the osteoblast-like MC3T3-E1 cell line, express the CaR. Furthermore, CaR agonists stimulate chemotaxis and proliferation of both cell lines (34, 35). As stromal cells and osteoblasts both belong to the mesenchymal lineage, and monocytes-macrophages belong to the hematopoietic lineage (22), these findings suggest that the CaR is expressed in diverse bone marrow cells, confirming our previous observation with primary cultures of human and murine bone marrow (12). Both cell types are known to appear at sites of bone resorption at the end of the resorptive phase of bone remodeling and to initiate bone formation (26). Therefore, our findings are consistent with an important role for the CaR in the key reversal phase of bone remodeling through its sensing of calcium ions released by osteoclast-mediated bone resorption and ensuring the chemotaxis and proliferation of cell populations needed for the orderly transition from bone breakdown to its subsequent replacement by newly formed bone.

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