Published in final edited form as:

J Cell Biochem. 2005 August 15; 95(6): 1081–1092.

Osteoblast Calcium-Sensing Receptor Has Characteristics of ANF/7TM Receptors

Min Pi and L. Darryl Quarles*

Department of Internal Medicine, The Kidney Institute, University of Kansas Medical Center, Kansas City, Kansas

Abstract

There is evidence for a functionally important extracellular calcium-sensing receptor in osteoblasts, but there is disagreement regarding its identity. Candidates are CASR and a putative novel calcium-sensing receptor, called Ob.CASR. To further characterize Ob.CASR and to distinguish it from CASR, we examined the extracellular cation-sensing response in MC3T3-E1 osteoblasts and in osteoblasts derived from CASR null mice. We found that extracellular cations activate ERK and serum response element (SRE)-luciferase reporter activity in osteoblasts lacking CASR. Amino acids, but not the calcimimetic NPS-R568, an allosteric modulator of CASR, also stimulate Ob.CASR-dependent SRE-luciferase activation in MC3T3-E1 osteoblasts. In addition, we found that the dominant negative Gαq(305–359) construct inhibited cation-stimulated ERK activation, consistent with Ob.CASR coupling to Gαq-dependent pathways. Ob.CASR is also a target for classical GPCR desensitization mechanisms, since β-arrestins, which bind to and uncouple GRK phosphorylated GPCRs, attenuated cation-stimulated SRE-luciferase activity in CASR deficient osteoblasts. Finally, we found that Ob.CASR and CASR couple to SRE through distinct signaling pathways. Ob.CASR does not activate RhoA and C3 toxin fails to block Ob.CASR-induced SRE-luciferase activity. Mutational analysis of the serum response factor (SRF) and ternary complex factor (TCF) elements in SRE demonstrates that Ob.CASR predominantly activates TCF-dependent mechanisms, whereas CASR activates SRE-luciferase mainly through a RhoA and SRF-dependent mechanism. The ability of Ob.CASR to sense cations and amino acids and function like a G-protein coupled receptor suggests that it may belong to the family of receptors characterized by an evolutionarily conserved amino acid sensing motif (ANF) linked to an intramembranous 7 transmembrane loop region (7TM).

Keywords

G-protein coupled receptors; calcium-sensing; osteoblasts; β-arrestin; Gαq; ERK; SRE

Abbreviations used

CASR, calcium-sensing receptor; Ob.CASR, osteoblastic calcium-sensing receptor; GPCR, G-protein coupled receptor; ERK, extracellular signal-regulated kinase; SRE, serum response element; SRF, serum response factor; TCF, ternary complex factor

Osteoporosis is a disease of bone remodeling characterized by an imbalance between bone formation and resorption leading to low bone mass and the development of fractures as a consequence of the loss of skeletal architecture and mechanical strength. Recent efforts to

*Correspondence to: L. Darryl Quarles, MD, Summerfield Endowed Professor of Nephrology, University of Kansas Medical Center MS 3018, 3901 Rainbow Boulevard, 6018 Wahl Hall East, Kansas City, KS 66160. E-mail: dquarles@kumc.edu.

develop anabolic agents to increase trabecular bone volume and number have focused on targeting of G-protein coupled receptors in osteoblasts. For example, parathyroid hormone (PTH) that acts on the PTH receptor on cells within the osteoblastic lineage is the first anabolic agent to be approved for the treatment of osteoporosis [Seeman and Delmas, 2001]. Given the potential diversity of GPCRs in bone and osteoblasts, it is likely that other receptors might be exploited with greater anabolic and less bone resorptive potential.

There is compelling evidence for the presence of extracellular cation-sensing G-protein coupled receptors in bone that may have a physiological role in sensing the local release of calcium from osteoclast-mediated bone resorption. It is known, for example, that high ambient Ca^{2+} concentrations (in the range of 8 to 40 mM) are present at sites of bone resorption [Silver et al., 1988]. In addition, osteoclasts and osteoblasts display a functional response to extracellular calcium and other cations [Kanatani et al., 1991; Quarles et al., 1991, 1994, 1997; Chang et al., 1999; Pi et al., 2000; Shalhoub et al., 2003]. Ca^{2+} released by osteoclast-mediated bone resorption could potentially target calcium-sensing receptors located in osteoclasts and osteoblasts to, respectively, inhibit bone resorption and stimulate bone formation [Kellner, 1939; Quarles et al., 1988, 1990, 1991, 1994; Silver et al., 1988; Malgaroli et al., 1989; Zaidi et al., 1989, 1991, 1995; Kanatani et al., 1991, 1999; Brown et al., 1993; Ruat et al., 1995; Riccardi et al., 1996; Bowler et al., 1998].

The identities of the skeletal extracellular calcium-sensing mechanisms in osteoclasts and osteoblasts are uncertain [Quarles et al., 1988; Zaidi et al., 1989, 1991, 1995; Pollak et al., 1993; Sugimoto et al., 1993; Kameda et al., 1998; Kanatani et al., 1999; Seuwen et al., 1999; Seeman and Delmas, 2001; Yamaguchi et al., 1998b,d]. In osteoclasts, the prototypic calcium-sensing receptor, CASR, a channel, and a recently described cell surface Type II ryanodine receptor are proposed mechanisms for cation-sensing [Zaidi et al., 1989, 1991, 1995; Pollak et al., 1993; Sugimoto et al., 1993; Kameda et al., 1998; Kanatani et al., 1999; Seuwen et al., 1999]. In osteoblasts, some studies purport that CASR is responsible for the calcium-sensing in osteoblasts [Nemeth and Scarpa, 1987; Brown et al., 1993; House et al., 1997; Kameda et al., 1998; Yamaguchi et al., 1998a,c,e, 2001; Kanatani et al., 1999; Mathas et al., 2001; Brown, 2003; Chattopadhyay et al., 2004; Dvorak et al., 2004], whereas other studies provide evidence for the presence of a novel calcium-sensing receptor in osteoblasts, called Ob.CASR, and possible redundant calcium-sensing mechanisms in bone [Pi and Quarles, 2004].

The presence of Ob.CASR is supported by several observations. First, CASR knockout mice fail to display a bone phenotype in the absence of hyperparathyroidism [Garner et al., 2001; Tu et al., 2003] and the absence of CASR does not alter either the responsive of osteoblasts to extracellular calcium or their ability to undergo the typical maturational program leading to the formation of mineralized extracellular matrix *ex vivo* [Pi et al., 2000]. In addition, several laboratories have failed to confirm the expression of CASR in osteoblasts [Quarles et al., 1997; Pi et al., 1999; Shalhoub et al., 2003; Pi and Quarles, 2004]. It has been shown that CASR expression can be lost in cell cultures; consequently the absence of CASR in the clonal MC3T3-E1 cell lines does not preclude its presence in primary cultures [House et al., 1997; Kameda et al., 1998; Yamaguchi et al., 1998a,c,e; Kanatani et al., 1999]. However, distinct calcium-sensing responses have been detected in osteoblastic cultures lacking CASR [Demarest et al., 1997; Mailland et al., 1997; Quarles et al., 1997; Bapty et al., 1998; Pi et al., 1999; Shalhoub et al., 2003; Pi and Quarles, 2004], suggesting the presence of another receptor.

Defining the molecular targets for cation-sensing in osteoblasts is important. For example, the trivalent aluminum cation is a potent stimulus of osteoblastic function *in vitro* [Quarles et al., 1988, 1989, 1992, 1994, 1997] through the activation of Ob.CASR and under certain experimental conditions aluminum can uncouple bone formation from resorption leading to *de novo* bone formation [Quarles et al., 1988, 1990]. Also, the divalent strontium cation stimulates

osteoblast-mediated bone formation and inhibits osteoclast-mediated bone resorption in preclinical studies [Delannoy et al., 2002; Marie, 2003; Verberckmoes et al., 2003; Barbara et al., 2004] and the drug Strontium Ranelate is a recently approved treatment of osteoporosis [Meunier et al., 2004]. The predominant mechanism of strontium on bone is uncertain, since strontium can activate CASR [Brown, 2003; Coulombe et al., 2004], as well as Ob.CASR [Pi and Quarles, 2004] in vitro.

To further characterize the cation sensing mechanism in osteoblasts, we have performed a comparative analysis of extracellular ligand specificity and signal transduction pathways between CASR and Ob.CASR, the endogenous cation-sensing receptor in osteoblasts. Through this analysis, we have confirmed the presence of Ob.CASR and provided evidence that it is a G-protein coupled receptor, which senses extracellular cations and amino acids and is coupled to signal transduction pathways that overlap but are distinct from CASR.

MATERIALS AND METHODS

Materials

Aluminum chloride ($\text{AlCl}_3 \cdot 6 \text{H}_2\text{O}$) was obtained from Fisher (Springfield, NJ). Gadolinium chloride hexahydrate was purchased from Aldrich Chemical Co. (Milwaukee, WI). Calcium chloride, magnesium chloride, strontium chloride, strontium acetate, and L-amino acids were purchased from Sigma Chemical (St. Louis, MO). Bovine serum albumin (BSA; fraction V) was obtained from Roche Applied Science (Indianapolis, IN). The calcimimetic compound NPS-R568 *N*-(3-[2-chlorophenyl]-propyl)-(R)- α -methyl-3-methoxybenzylamine and its inactive isomer NPS-S568 were provided by Amgen (Thousand Oaks, CA).

Plasmids

Serum response element (SRE)-luciferase construct was a generous gift from Dr. Jeffrey E. Pessin [Yamauchi et al., 1993; Quarles et al., 1997]. The rat CASR cDNA was obtained from Dr. A.M. Snowman and Dr. S.H. Snyder, and subcloned in the mammalian expression vector pcDNA 3 (Invitrogen, Carlsbad, CA) as previously described [Spurney et al., 1999; Pi et al., 2002]. The Gαq(305–359) minigene construct that correspond to the COOH-terminal peptide sequence of Gαq residues 305 to 359, constructs of pcDNA3-Flag-rBarr1 and pcDNA3-Flag-rBarr2 were kindly provided by Dr. Louis M. Luttrell and Dr. Robert J. Lefkowitz from Duke University [Akhter et al., 1998]. pcDNA I-C3-toxin generously provided by Dr. J. Silvio Gutkind.

We used the previously described c-fos promoter luciferase reporter construct (SRE-luc) as a read out of extracellular cation sensing response in various cell lines [Yamauchi et al., 1993; Quarles et al., 1997]. This promoter reporter construct contains two growth factor-regulated promoter elements: the SRE, which binds a ternary complex comprising serum response factor (SRF) and a ternary complex factor (TCF) [Hill et al., 1995; Price et al., 1996; Johnson et al., 1997; Shaw and Saxton, 2003; Selvaraj and Prywes, 2004]. Both CASR and the putative Ob.CASR can activate the SRE-luc construct [Quarles et al., 1997; Pi et al., 2002]. To determine if TCF or SRF mediated the responses to CASR and Ob.CASR, we created inactivating mutations of TCF and SRF. Briefly, we synthesized oligomers containing inactivating mutations sequences for either the TCF binding domain (tacacagga → tgtactgta) or SRF binding domain (catattag → ccaatcgg), as previously described [Johnson et al., 1997]. The 3' end containing a *Bgl* II site was ligated to the 5' end containing a *Bcl* I site to create a tandem insert containing two copies of the mutated TCF or SRF regions, which were then subcloned into the *Bam*H I and *Bst*Y I site of the pGL2-Luciferase vector to replace the wild-type SRE. The correct orientation and sequence were confirmed by direct sequencing.

Cell Culture

All culture reagents were from Gibco Life Technologies, Inc. (Rockville, MD). Human embryonic kidney HEK-293 cells were obtained from American Type Culture Collection, Rockville, MD. HEK-293 cells and MC3T3-E1 cells stably expressing rat CASR were created as previously described [Spurney et al., 1999; Pi et al., 2002; Pi and Quarles, 2004]. TmOb (CASR^{+/+}) and TmOb (CASR^{-/-}) are mouse immortalized osteoblastic cell lines derived from wild-type and CASR gene knockout mouse calvaria, respectively, as previously described [Pi et al., 2000]. MC3T3-E1 cells are a previously characterized clonal calvaria-derived osteoblastic cell line [Quarles and Drezner, 1992] that are responsive to extracellular cations, but lack CASR expression [Quarles et al., 1997; Pi and Quarles, 2004].

MC3T3-E1, TmOb (CASR^{+/+}) and TmOb (CASR^{-/-}) cells were grown in α -MEM media and HEK-293 cells were grown in DMEM (Gibco Life Technologies, Inc.) supplemented with 10% fetal calf serum, at 37°C in a humidified atmosphere of 95% air and 5% CO₂. To evaluate cation responses, cells were induced to undergo growth arrest by overnight incubation in serum free media. Osteoblastic cell lines were quiesced by incubation in D-MEM/F12 (1 mM Ca⁺²) (Gibco Life Technologies, Inc.) containing 0.1% BSA and HEK293 cells quiesced in DMEM (Gibco Life Technologies, Inc.) containing 0.5 mM Ca⁺² and 0.1% BSA. Cations, calcimimetics, or amino acids were added at the concentrations stated.

Transfection

MC3T3-E1 cells were transiently transfected with the following plasmids β -arrestin 1, β -arrestin 2, G α q(305–355) or CASR (906–980) [Pi et al., 2002]. In studies assessing the involvement of RhoA, MC3T3-E1 cells were transiently co-transfected with SRE-luc and a plasmid containing C3 toxin. In studies assessing TCF- and SRF-dependent signaling pathway, MC3T3-E1, HEK-293 plus CASR, and MC3T3-E1 plus CASR were transfected with wild-type SRE, or SREmutSRF or SREmutTCF.

All plasmid DNAs were prepared using the EndoFreeTM Plasmid Maxi Kit (Invitrogen). Transient transfections were performed as follows: 1×10^5 MC3T3-E1 or HEK-293 were plated in the 6-well plate and incubated overnight at 37°C. A DNA-liposome complex was prepared by mixing DNA of the SRE-luciferase reporter plasmid, pCMV- β -gal and other expression vector as indicated with TransFastTM transfection reagent (Promega, Madison WI). The total plasmid DNA was equalized in each well by adjusting the total amount of DNA to 2 μ g per well with the empty vector. Quiescence of transfected cells was achieved in subconfluent cultures by removing the media and washing with HBSS (Gibco Life Technologies, Inc.) to remove residual serum, followed by incubation for an additional 24 h in serum-free quiescent media. Luciferase activity was assessed after 8 h of stimulation. The luciferase activity in cell extracts was measured using the luciferase assay system (Promega) following the manufacturer's protocol using a BG-luminometer (Gem Biomedical Inc., Hamden, CT).

Assay for ERK1/2 MAPK

After agonist treatment for the specified concentrations and duration, cells were washed twice with ice-cold PBS and scraped into 250 μ l of lysis buffer (25 mM HEPES pH 7.2, 5 mM MgCl₂, 5 mM EDTA, 1% Triton X-100, 0.02 tablet/ml of protease inhibitor cocktail). Equal amounts of lysates were subjected to 10% SDS-PAGE, and phospho-ERK1/2 levels were determined by immunoblotting using antiphospho-ERK1/2 MAP kinase antibody (Cell Signaling Technology, Beverly, MA). To confirm that variations in the amount of ERK did not contribute to cation-stimulation of ERK activity, in selected studies we used an anti-ERK1/2 MAP kinase antibody (Cell Signaling Technology) to measure ERK levels.

RhoA activation assay

Western blot analysis of RhoA was performed as previously published [Sauzeau et al., 2000] in total cell lysates and membrane fractions prepared from MC3T3-E1 cells transfected with empty vector and pcDNA.rCASR. Following incubation with and without calcium at the indicated concentrations, the cells were harvested in homogenization buffer A [50 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 1 mM EGTA, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.02 tablet/ml of protease inhibitor cocktail (Roche Applied Science)] at 4°C. Total cell lysates were sonicated and nuclei and cell debris were removed by low speed centrifugation (800g for 10 min) at 4°C. The resulting supernatant containing the membrane fraction was collected by centrifugation at 30,000 rpm for 30 min at 4°C. The pellet containing the crude membrane fractions was solubilized with buffer B containing 1% SDS, 10 mM Tris-HCl (pH 7.4) with freshly added protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 0.02 tablet/ml of protease inhibitor cocktail). Protein fractions were separated on 4%–12% SDS-PAGE, transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA), and the blots probed with a mouse monoclonal anti-RhoA antibody (clone 26C4, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The signal from immunoreactive bands was detected by ECL kit (Amersham Pharmacia Biotech, Piscataway, NJ).

Statistics

We evaluated differences between groups by one-way analysis of variance. Values sharing the same superscript are not significantly different at $P < 0.05$. All computations were performed using the Statgraphic statistical graphics system (STSC, Inc., Rockville, MD).

RESULTS

Cations Activate Extracellular Signal-Regulated Kinases (ERK) and a Serum Response Element (SRE) Reporter in Osteoblasts Lacking CASR

We assessed Ob.CASR function in CASR negative MC3T3-E1 osteoblasts and osteoblasts derived from CASR null mice, which possess characteristics of osteoblasts in culture and respond to extracellular cations [Quarles et al., 1997; Pi et al., 1999, 2000; Pi and Quarles, 2004]. Incubation of both MC3T3-E1 and osteoblasts derived from CASR^{-/-} mice with 5 mM calcium, 25 μ M aluminum, and 60 μ M gadolinium stimulated ERK activation (Fig. 1A), with maximal response at 5 min (Fig. 1B).

We have previously shown that both Ob.CASR and CASR activate an SRE-luciferase reporter construct in osteoblast cell lines [Pi and Quarles, 2004]. We mutated the TCF and SRF binding domains of SRE to evaluate if different elements are required for Ob.CASR and CASR activation (Fig. 2). The effects of extracellular cations and NPS-568, a calcimimetic selective for CASR, were evaluated in MC3T3-E1 osteoblasts overexpressing wild-type SRE, SRE-mutSRF, or SREmutTCF. To compare the response of Ob.CASR to CASR and to evaluate potential cell-type effects, we also examined the response of HEK-293 and MC3T3-E1 cells transfected with CASR and these reporter constructs. Exposure of MC3T3-E1 osteoblasts to 5 mM calcium stimulated the wild-type SRE-luc, but NPS-568 had no effect. In contrast, both calcium and NPS-568 stimulate wild-type SRE in CASR transfected HEK-293 cells and transfection of CASR into MC3T3-E1 cells imparted the response to NPS-568R. Mutations of the SRF region of the SRE-luc construct attenuated the response to CASR to a greater degree than Ob.CASR, whereas mutations of the TCF region decreased the response to Ob.CASR, but the TCF mutant constructs remained responsive to CASR. These differences were not due to cell type effects, since transfection of CASR into MC3T3-E1 imparted SRF-dependence to calcium and calcimimetic stimulation. These data support different coupling of CASR and Ob.CASR to signaling pathways linked to SRE, likely reflecting difference in the intracellular components of the receptor.

Endogenous Osteoblast Calcium-Sensing Receptor Is not Coupled to RhoA

The ability of Ob.CASR to preferentially activate TCF-dependent signaling (Fig. 2), suggests that this receptor might not be coupled to RhoA activation, since RhoA is known to activate SRE through a TCF-independent pathway [Hill and Treisman, 1995]. To investigate this possibility, we measured membrane-associated RhoA in MC3T3-E1 osteoblasts. Stimulation of the endogenous calcium receptor in MC3T3-E1 cells failed to activate RhoA, whereas MC3T3-E1 cells transfected with CASR resulted in a marked increase in the amount of RhoA in the membrane fraction after calcium stimulation (Fig. 3). To further examine the role of Rho in Ob.CASR signaling, we made use of a DNA construct that expresses the botulinum C3 exoenzyme. This toxin ADP ribosylates RhoA at asparagine 41, thereby preventing the exchange of GDP by GTP and retaining RhoA in its GDP-bound inactive form [Sauzeau et al., 2000]. Co-transfection with a plasmid expressing the C3 toxin failed to inhibit calcium stimulation of SRE activity in MC3T3-E1 osteoblasts (data not shown).

Ob.CASR Activation of ERK and SRE Are Mediated by G-Protein Coupled Receptor-Like Pathways

Activation of ERK and SRE can occur through multiple signaling pathways, including G-protein coupled receptors [Luttrell and Luttrell, 2003]. Since arrestins regulate almost all GPCR-mediated signaling, we assessed the ability of β -arrestins to inhibit cation-mediated signaling. Consistent with their effect to regulate receptor coupling to G proteins, both β -arrestin 1 and 2 inhibit cation-induced SRE (Fig. 4A) and ERK (Fig. 4B) activation. Additional evidence that this is through disruption of G-protein coupling is derived from the ability of the dominant negative G α q(305–355) to also block cation stimulation of ERK activation in osteoblasts (Fig. 4B). These results are consistent with previous studies showing that the dominant negative G α q(305–359) inhibits extracellular cation stimulation of SRE-luciferase activity in osteoblasts lacking known CASR as well as will inhibit the activity of known CASR transfected into HEK-293 cells (positive control) [Pi et al., 2002; Pi and Quarles, 2004]. We also found that a cDNA construct expressing the C-terminus of CASR can also inhibit cation stimulation of SRE-luc in osteoblasts (Fig. 4C). The fact that CASR is not expressed in these cells, suggest that the C-terminus of CASR is acting to sequester factors interacting with Ob.CASR that are necessary for activation of SRE.

Amino Acids Stimulate SRE-Luciferase by the Endogenous Cation-Sensing Receptor in MC3T3-E1 Osteoblasts

A characteristic of CASR is its ability to sense L-amino acids in the presence of extracellular calcium, which is mediated by the extracellular ANF motif [Conigrave et al., 2000]. Since our data suggest that Ob.CASR might be a related receptor, we examined if amino acids can stimulate SRE in MC3T3-E1 osteoblasts that lack CASR but have the functional Ob.CASR. Initially, we screened a various groups of L-amino acids for their ability to stimulate SRE-luciferase activity in MC3T3-E1 osteoblasts. We found that group I, which contained equal amounts (3 mM) of L-Val, L-Cysteine, and trans-4-Hydroxy-L-Proline, activated SRE in the presence of 1 mM calcium (Fig. 5A), whereas the remaining four groups did not stimulate Ob.CASR. Next, we tested individual amino acids for their ability to stimulate Ob.CASR. L-Cysteine > L-Glutamic acid > L-Isoleucine > trans-4-Hydroxy-L-proline individually stimulated SRE-luc activity in MC3T3-E1 osteoblasts (data not shown). Moreover, L-Cysteine resulted in a dose-dependent and calcium-dependent stimulation of SRE-luciferase activity in MC3T3-E1, achieving maximal stimulation at 3 mM in the presence of 2 mM calcium (Fig. 5B).

DISCUSSION

Using osteoblasts that lack CASR [Quarles et al., 1997; Pi et al., 1999; Pi and Quarles, 2004], we have extended our prior observations that Ob.CASR has overlapping but distinct

ligand specificity compared to CASR and further defined the signal transduction pathways coupled to Ob.CASR (Table I). Similar to CASR, ligand-mediated stimulation of Ob.CASR stimulates ERK activation (Fig. 1), as well as SRE activation (Fig. 2), which is a downstream readout for activation of several signaling pathways (vide infra). Moreover, in addition to confirming our prior observations that CASR and Ob.CASR are both activated by calcium and gadolinium [Pi and Quarles, 2004; Fig. 1], we now show that amino acids, which are reported to activate CASR [Conigrave et al., 2000], can also activate Ob.CASR (Fig. 5). The amino acid specificity, however, appears to be more restrictive than CASR. CASR can be activated by L-His, L-Phe, L-Tyr, L-Trp, L-Cys, L-Ala, L-Thr, L-Asn, L-Gln, L-Ser, L-Glu, Gly, L-Pro, L-Val, L-Met, L-Asp, L-Lys, L-Arg, but not L-Ile and L-Leu [Conigrave et al., 2000]; Ob.CASR is activated by L-Cysteine, L-Glutamic acid, L-Isoleucine, and trans-4-Hydroxy-L-Proline (Fig. 5). Also, whereas amino acids fail to activate CASR at extracellular calcium concentrations below 1.5 mM calcium, L-amino acid mixtures activated Ob.CASR activity at calcium concentrations of 1 mM (Fig. 5).

In addition, we extend the evidence that Ob.CASR is a G-protein coupled receptor [Quarles et al., 1994; Hartle et al., 1996; Arthur et al., 1997; Small et al., 1999]. In this regard, β -arrestins, which bind to and uncouple GRK phosphorylated GPCRs, attenuates the response of Ob.CASR in MC3T3-E1 osteoblasts as well as CASR transfected into HEK-293 cells (positive control), suggesting that both Ob.CASR and CASR are targets for classical GPCR desensitization mechanisms (Fig. 4). The finding that a dominant negative G α_q construct blocks Ob.CASR activation of ERK (Fig. 4) as well as SRE-luc [Pi and Quarles, 2004] provides evidence for G-protein coupling. Moreover, since both G α_q and β -arrestin bind to GPCRs, this might explain why the C-terminus of CASR can also inhibit the function of Ob.CASR in osteoblasts, by competing for common intracellular proteins required for activation of SRE.

In spite of the ability of CASR and Ob.CASR to active ERK and SRE-dependent pathways, the signal transduction pathways are not identical. For example, CASR stimulates RhoA (Fig. 3) and C3 toxin, an inhibitor of RhoA, blocks CASR-stimulated SRE-luciferase activity in HEK-293 cells transfected with CASR [Pi et al., 2002]. In contrast, Ob.CASR in MC3T3-E1 cells, which lack CASR, does not activate RhoA (Fig. 3) and C3 toxin fails to block Ob.CASR-induced luciferase activity (data not shown). These differential effects of CASR and Ob.CASR are not due to cell type differences, since the transfection of CASR into MC3T3-E1 osteoblasts imparts the ability to activate RhoA (Fig. 3).

The possibility that CASR and Ob.CASR have different coupling mechanisms is also supported by studies that determined which *cis*-acting elements in the SRE-luciferase reporter construct mediate CASR and Ob.CASR responses (Fig. 2). Recent studies indicate that RhoA selectively activates the SRF component through the activation of the myocardin-related MKL family of proteins that include MKL1 and MKL2 [Selvaraj and Prywes, 2004]. Consistent with CASR activation of RhoA, we found that mutations of the SRF domain of the SRE promoter construct inhibited CASR activation of SRE. On the other hand, the SRF mutation has no effect on Ob.CASR stimulation. Rather, Ob.CASR, which does not activate RhoA, activates SRE mostly through a TCF-dependent mechanism, as evidenced by the ability of mutation of the TCF binding site to abrogate cation stimulation of SRE in osteoblasts (Fig. 2).

Our current studies along with prior reports characterizing calcium-sensing responses in osteoblasts support the following conclusions, which are summarized in Table I. First, osteoblasts display a functional response to extracellular calcium and other cations via a G-protein coupled receptor-like mechanism. Second, the putative osteoblast cation sensing receptor, Ob.CASR, can be pharmacologically distinguished from CASR by differences in ligand specificity and by its failure to respond to calcimimetics. Third, Ob.CASR utilizes overlapping and distinct G-protein coupling and signal transduction cascades compared to

CASR. Fourth, CASR does not completely explain the response of osteoblasts to extracellular cations and another responsible molecule different from CASR, namely Ob.CASR, is necessary to fully account for the cation-sensing properties of osteoblasts. Indeed, the higher local calcium concentrations in bone as well as changes in pH and the release of matrix proteins that could bind to and modify calcium function may require a receptor with different affinity for calcium and/or responsiveness to other ligands/co-factors that are not required for sensing changes in serum calcium by CASR.

The identity of Ob.CASR remains unknown. The evidence for amino acid sensing and G-protein coupling, however, suggests that Ob.CASR may belong to the family of receptors characterized by an extracellular ANF domain, an evolutionarily conserved amino acid sensing motif (ANF) [Kuryatov et al., 1994] linked to a intramembranous 7 transmembrane loop region (7TM) that characterizes GPCRs. Currently, CDART identifies a total of 305 sequences with both the ANF and 7TM conserved domains [Marchler-Bauer et al., 2003]. Based on the current findings, it is possible that an orphan receptor in this large family of ANF/7TM receptor might represent Ob.CASR. Further studies will be necessary to establish this possibility.

References

- Akhter SA, Luttrell LM, Rockman HA, Iaccarino G, Lefkowitz RJ, Koch WJ. Targeting the receptor-Gq interface to inhibit in vivo pressure overload myocardial hypertrophy. *Science* 1998;280:574–577. [PubMed: 9554846]
- Arthur JM, Collinworth GP, Quarles LD, Gettys TW, Raymond JR. Specific coupling of a polycation-sensing receptor to G protein alpha subunits in MDCK cells. *Am J Physiol* 1997;273:F129–F135. [PubMed: 9249600]
- Arthur JM, Lawrence MS, Payne CR, Rane MJ, McLeish KR. The calcium-sensing receptor stimulates JNK in MDCK cells. *Biochem Biophys Res Commun* 2000;275(2):538–541. [PubMed: 10964699]
- Bai M, Trivedi S, Lane CR, Yang Y, Quinn SJ, Brown EM. Protein kinase C phosphorylation of threonine at position 888 in Ca^{2+} -sensing receptor (CaR) inhibits coupling to Ca^{2+} store release. *J Biol Chem* 1998;273(33):21267–21275. [PubMed: 9694886]
- Bapty BW, Dai LJ, Ritchie G, Jirik F, Canaff L, Hendy GN, Quamme GA. Extracellular Mg^{2+} and Ca^{2+} -sensing in mouse distal convoluted tubule cells. *Kidney Intl* 1998;53:583–592.
- Barbara A, Delannoy P, Denis BG, Marie PJ. Normal matrix mineralization induced by strontium ranelate in MC3T3-E1 osteogenic cells. *Metabolism* 2004;53(4):532–537. [PubMed: 15045704]
- Bowler WB, Gallagher JA, Bilbe G. G-protein coupled receptors in bone. *Front Biosci* 1998;3:d769–d780. [PubMed: 9682032]
- Brown EM. Is the calcium receptor a molecular target for the actions of strontium on bone? *Osteoporosis Int* 2003;14(Suppl 3):S25–S34.
- Brown EM, Butters R, Katz C, Kifor O. Neomycin mimics the effects of high extracellular calcium concentrations on parathyroid function in dispersed bovine parathyroid cells. *Endocrinology* 1991;128(6):3047–3054. [PubMed: 1645260]
- Brown EM, Gamba G, Riccardi D, Lombardi M, Butters R, Kifor O, Sun A, Hediger MA, Lytton J, Hebert SC. Cloning and characterization of an extracellular Ca^{2+} -sensing receptor from bovine parathyroid. *Nature* 1993;366:575–580. [PubMed: 8255296]
- Chang W, Tu C, Chen TH, Komuves L, Oda Y, Pratt SA, Miller S, Shoback D. Expression and signal transduction of calcium-sensing receptors in cartilage and bone. *Endocrinology* 1999;140:5883–5893. [PubMed: 10579354]
- Chang W, Chen TH, Pratt S, Shoback D. Amino acids in the second and third intracellular loops of the parathyroid Ca^{2+} -sensing receptor mediate efficient coupling to phospholipase C. *J Biol Chem* 2000;275(26):19955–19963. [PubMed: 10764812]
- Chattopadhyay N, Yano S, Tfelt-Hansen J, Rooney P, Kanuparthi D, Bandyopadhyay S, Ren X, Terwilliger E, Brown EM. Mitogenic action of calcium-sensing receptor on rat calvarial osteoblasts. *Endocrinology* 2004;145:3451–3462. [PubMed: 15084499]

- Conigrave AD, Quinn SJ, Brown EM. l-amino acid sensing by the extracellular Ca^{2+} -sensing receptor. *Proc Natl Acad Sci USA* 2000;97(9):4814–4819. [PubMed: 10781086]
- Coulombe J, Faure H, Robin B, Ruat M. In vitro effects of strontium ranelate on the extracellular calcium-sensing receptor. *Biochem Biophys Res Commun* 2004;323(4):1184–1190. [PubMed: 15451421]
- Dare E, Kifor O, Brown EM, Weber G. Characterization of the phosphatidylinositol-specific phospholipase C isozymes present in the bovine parathyroid and in human kidney HEK-293 cells stably transfected with the human parathyroid Ca^{2+} -sensing receptor. *J Mol Endocrinol* 1998;21(1):7–17. [PubMed: 9723859]
- de Jesus Ferreira MC, Helies-Toussaint C, Imbert-Teboul M, Bailly C, Verbavatz JM, Bellanger AC, Chabardes D. Co-expression of a Ca^{2+} -inhibitable adenylyl cyclase and of a Ca^{2+} -sensing receptor in the cortical thick ascending limb cell of the rat kidney. Inhibition of hormone-dependent cAMP accumulation by extracellular Ca^{2+} . *J Biol Chem* 1998;273(24):15192–15202. [PubMed: 9614133]
- Delannoy P, Bazot D, Marie PJ. Long-term treatment with strontium ranelate increases vertebral bone mass without deleterious effect in mice. *Metabolism* 2002;51(7):906–911. [PubMed: 12077740]
- Demarest KT, Minor LK, Gunnet JW. Calcium-sensing receptor mediated changes on adenylyl cyclase and intracellular calcium mobilization in SAOS-2 Osteosarcoma cells. *J Bone Miner Res* 1997;12 (Suppl 1):S413.
- Dvorak MM, Siddiqua A, Ward DT, Carter DH, Dallas SL, Nemeth EF, Riccardi D. Physiological changes in extracellular calcium concentration directly control osteoblast function in the absence of calciotropic hormones. *Proc Natl Acad Sci USA* 2004;101(14):5140–5145. [PubMed: 15051872]
- Garner SC, Pi M, Tu Q, Quarles LD. Rickets in cation-sensing receptor-deficient mice: An unexpected skeletal phenotype. *Endocrinology* 2001;142(9):3996–4005. [PubMed: 11517179]
- Hartle JE II, Pripic V, Siddhanti SR, Spurney RF, Quarles LD. Differential regulation of receptor-stimulated cyclic adenosine monophosphate production by polyvalent cations in MC3T3-E1 osteoblasts. *J Bone Miner Res* 1996;11:789–799. [PubMed: 8725176]
- Hill CS, Treisman R. Differential activation of c-fos promoter elements by serum, lysophosphatidic acid, G proteins and polypeptide growth factors. *EMBO J* 1995;14(20):5037–5047. [PubMed: 7588632]
- Hill CS, Wynne J, Treisman R. The Rho family GTPases RhoA, Rac1, and CDC42Hs regulate transcriptional activation by SRF. *Cell* 1995;81:1159–1170. [PubMed: 7600583]
- House MG, Kohlmeier L, Chattopadhyay N, Kifor O, Yamaguchi T, Leboff MS, Glowacki J, Brown EM. Expression of an extracellular calcium-sensing receptor in human and mouse bone marrow cells. *J Bone Miner Res* 1997;12:1959–1970. [PubMed: 9421228]
- Huang C, Hujer KM, Wu Z, Miller RT. The Ca^{2+} -sensing receptor couples to $\text{G}\alpha_{12/13}$ to activate phospholipase D in Madin-Darby canine kidney cells. *Am J Physiol Cell Physiol* 2004;286(1):C22–C30. [PubMed: 12954603]
- Johnson CM, Hill CS, Chawla S, Treisman R, Bading H. Calcium controls gene expression via three distinct pathways that can function independently of the Ras/mitogen-activated protein kinases (ERKs) signaling cascade. *J Neurosci* 1997;17(16):6189–6202. [PubMed: 9236230]
- Kameda T, Mano H, Yamada Y, Takai H, Amizuka N, Kobori M, Izuma N, Kawashima H, Ozawa H, Ikeda K, Kameda A, Hakeda Y, Kumegawa M. Calcium-sensing receptor in mature osteoclasts, which are bone resorbing cells. *Biochem Biophys Res Commun* 1998;245:419–422. [PubMed: 9571166]
- Kanatani M, Sugimoto T, Fukase M, Fujita T. Effect of elevated extracellular calcium on the proliferation of osteoblastic MC3T3-E1 cells: Its direct and indirect effects via monocytes. *Biochem Biophys Res Commun* 1991;181:1425–1430. [PubMed: 1764094]
- Kanatani M, Sugimoto T, Kanzawa M, Yano S, Chihara K. High extracellular calcium inhibits osteoclast-like cell formation by directly acting on the calcium-sensing receptor existing in osteoclast precursor cells. *Biochem Biophys Res Commun* 1999;261:144–148. [PubMed: 10405337]
- Kellner H. Zur histopathologie der knochen bei chronischer experimenteller fluroverobreichung. *Arch Exp Path Pharmacol* 1939;11:449–569.
- Kifor O, MacLeod RJ, Diaz R, Bai M, Yamaguchi T, Yao T, Kifor I, Brown EM. Regulation of MAP kinase by calcium-sensing receptor in bovine parathyroid and CaR-transfected HEK-293 cells. *Am J Physiol Renal Physiol* 2001;280:F291–F302. [PubMed: 11208605]

- Kuryatov A, Laube B, Betz H, Kuhse J. Mutational analysis of the glycine-binding site of the NMDA receptor: Structural similarity with bacterial amino acid-binding proteins. *Neuron* 1994;12:1291–1300. [PubMed: 8011339]
- Luttrell DK, Luttrell LM. Signaling in time and space: G protein-coupled receptors and mitogen-activated protein kinases. *Assay Drug Dev Technol* 2003;1(2):327–338. [PubMed: 15090198]
- Mailland M, Waelchli R, Ruat M, Boddeke HG, Seuwen K. Stimulation of cell proliferation by calcium and a calcimimetic compound. *Endocrinology* 1997;138:3601–3605. [PubMed: 9275041]
- Malgaroli A, Meldolesi J, Zallone AZ, Teti A. Control of cytosolic free calcium in rat and chicken osteoclasts. The role of extracellular calcium and calcitonin. *J Biol Chem* 1989;264:14342–14347. [PubMed: 2547794]
- Marchler-Bauer A, Anderson JB, DeWeese-Scott C, Fedorova ND, Geer LY, He S, Hurwitz DI, Jackson JD, Jacobs AR, Lanczycki CJ, Liebert CA, Liu C, Madej T, Marchler GH, Mazumder R, Nikolskaya AN, Panchenko AR, Rao BS, Shoemaker BA, Simonyan V, Song JS, Thiessen PA, Vasudevan S, Wang Y, Yamashita RA, Yin JJ, Bryant SH. CDD: A curated Entrez database of conserved domain alignments. *Nucleic Acids Res* 2003;31:383–387. [PubMed: 12520028]
- Marie PJ. Optimizing bone metabolism in osteoporosis: Insight into the pharmacologic profile of strontium ranelate. *Osteoporosis Int* 2003;14(Suppl 3):S9–S12.
- Mathas RS, Mathews CH, Machule C, Gao D, Li W, Denbesten PK. Identification of the calcium-sensing receptor in the developing tooth organ. *J Bone Miner Res* 2001;16(12):2238–2244. [PubMed: 11760837]
- Meunier PJ, Roux C, Seeman E, Ortolani S, Badurski JE, Spector TD, Cannata J, Balogh A, Lemmel EM, Pors-Nielsen S, Rizzoli R, Genant HK, Reginster JY. The effects of strontium ranelate on the risk of vertebral fracture in women with postmenopausal osteoporosis. *N Engl J Med* 2004;350(5):459–468. [PubMed: 14749454]
- Nemeth EF, Scarpa A. Rapid mobilization of cellular Ca^{2+} in bovine parathyroid cells evoked by extracellular divalent cations. Evidence for a cell surface calcium receptor. *J Biol Chem* 1987;262:5188–5196. [PubMed: 3558389]
- Nemeth EF, Steffey ME, Hammerland LG, Hung BC, Van Wagenen BC, DelMar EG, Balandrin MF. Calcimimetics with potent and selective activity on the parathyroid calcium receptor. *Proc Natl Acad Sci USA* 1998;95(7):4040–4045. [PubMed: 9520489]
- Pi M, Quarles LD. A novel cation-sensing mechanism in osteoblasts is a molecular target for strontium. *J Bone Miner Res* 2004;19(5):862–869. [PubMed: 15068510]
- Pi M, Hinson TK, Quarles LD. Failure to detect the extracellular calcium sensing receptor (CasR) in human osteoblast cell lines. *J Bone Miner Res* 1999;14:1310–1319. [PubMed: 10457263]
- Pi M, Garner SC, Flannery P, Spurney RF, Quarles LD. Sensing of extracellular cations in CasR-deficient osteoblasts: Evidence for a novel cation-sensing mechanism. *J Biol Chem* 2000;275(5):3256–3263. [PubMed: 10652312]
- Pi M, Spurney RF, Tu Q, Hinson TK, Quarles LD. Calcium-sensing receptor activation of Rho involves filamin and Rho-guanine nucleotide exchange factor. *Endocrinology* 2002;143:3830–3838. [PubMed: 12239094]
- Pi M, Oakley RH, Gesty-Palmer D, Cruickshank RD, Spurney RF, Luttrell LM, Quarles LD. {beta}-Arrestin- and GRK-Mediated CASR Desensitization. *Mol Endocrinol* 2005;19:1078–1087. [PubMed: 15637145]
- Pollak MR, Brown EM, Chou YYW, Hebert SC, Marx SJ, Steinmann B, Levi T, Seidman CE, Seidman JG. Mutations in the human Ca^{2+} -sensing receptor gene cause familial hypocalciuric hypercalcemia and neonatal severe hyperparathyroidism. *Cell* 1993;75:1297–1303. [PubMed: 7916660]
- Price MA, Cruzalegui FH, Treisman R. The p38 and ERK MAP kinase pathways cooperate to activate Ternary Complex Factors and c-fos transcription in response to UV light. *EMBO J* 1996;15(23):6552–6563. [PubMed: 8978682]
- Quarles LD, Drezner MK. Effects of etidronate-mediated suppression of bone remodeling on aluminum-induced de novo bone formation. *Endocrinology* 1992;131(1):122–126. [PubMed: 1611990]
- Quarles LD, Gitelman HJ, Drezner MK. Induction of de novo bone formation in the beagle. A novel effect of aluminum. *J Clin Invest* 1988;81(4):1056–1066. [PubMed: 3350964]

- Quarles LD, Gitelman HJ, Drezner MK. Aluminum-induced de novo bone formation in the beagle. A parathyroid hormone-dependent event. *J Clin Invest* 1989;83(5):1644–1650. [PubMed: 2708525]
- Quarles LD, Murphy G, Vogler JB, Drezner MK. Aluminum-induced neo-osteogenesis: A generalized process affecting trabecular networking in the axial skeleton. *J Bone Miner Metab* 1990;5:625–635.
- Quarles LD, Wenstrup RJ, Castillo SA, Drezner MK. Aluminum-induced mitogenesis in MC3T3-E1 osteoblasts: Potential mechanism underlying neo-osteogenesis. *Endocrinology* 1991;128:3144–3151. [PubMed: 1903700]
- Quarles LD, Hartle JE II, Middleton JP, Zhang J, Arthur JM, Raymond JR. Aluminum-induced DNA synthesis in osteoblasts: Mediation by a G-protein coupled cation sensing mechanism. *J Cell Biol* 1994;56:106–117.
- Quarles LD, Hartle JE, Siddhanti SR, Guo R, Hinson TK. A distinct cation-sensing mechanism in MC3T3-E1 osteoblasts functionally related to the calcium receptor. *J Bone Miner Res* 1997;12:393–402. [PubMed: 9076582]
- Quinn SJ, Kifor O, Trivedi S, Diaz R, Vassilev P, Brown E. Sodium and ionic strength sensing by the calcium receptor. *J Biol Chem* 1998;273(31):19579–19586. [PubMed: 9677383]
- Ray K, Fan GF, Goldsmith PK, Spiegel AM. The carboxyl terminus of the human calcium receptor. Requirements for cell-surface expression and signal transduction. *J Biol Chem* 1997;272:31355–31361. [PubMed: 9395465]
- Riccardi D, Lee WS, Lee K, Segre GV, Brown EM, Hebert SC. Localization of the extracellular Ca^{2+} -sensing receptor and PTH/PTHrP receptor in rat kidney. *Am J Physiol* 1996;271:F951–F956.
- Ruat M, Molliver ME, Snowman AM, Snyder SH. Molecular cloning in rat and localization to nerve terminals. *Proc Natl Acad Sci USA* 1995;92:3161–3165. [PubMed: 7724534]
- Ruat M, Snowman AM, Hester LD, Snyder SH. Cloned and expressed rat Ca^{2+} -sensing receptor. *J Biol Chem* 1996;271(11):5972–5975. [PubMed: 8626377]
- Sauzeau V, Le Jeune H, Cario-Toumaniantz C, Smolenski A, Lohmann SM, Bertoglio J, Chardin P, Pacaud P, Loirand G. Cyclic GMP-dependent protein kinase signaling pathway inhibits RhoA-induced Ca^{2+} sensitization of contraction in vascular smooth muscle. *J Biol Chem* 2000;275:21722–21729. [PubMed: 10783386]
- Seeman E, Delmas PD. Reconstructing the skeleton with intermittent parathyroid hormone. *Trends Endocrinol Metab* 2001;12:281–283. [PubMed: 11504661]
- Selvaraj A, Prywes R. Expression profiling of serum inducible genes identifies a subset of SRF target genes that are MKL dependent. *BMC Mol Biol* 2004;5(1):13. [PubMed: 15329155]
- Seuwen K, Boddeke HG, Migliaccio S, Perez M, Taranta A, Teti A. A novel calcium sensor stimulating inositol phosphate formation and $[\text{Ca}^{2+}]_i$ signaling expressed in GCT23 osteoclast-like cells. *Proc Assoc Am Physiol* 1999;111:70–81.
- Shalhoub V, Grisanti M, Padagas J, Scully S, Rattan A, Qi M, Varnum B, Vezina C, Lacey D, Martin D. In vitro studies with the calcimimetic, cinacalcet HCl, on normal human adult osteoblastic and osteoclastic cells. *Crit Rev Eukaryot Gene Expr* 2003;13(2–4):89–106. [PubMed: 14696958]
- Shaw PE, Saxton J. Ternary complex factors: prime nuclear targets for mitogen-activated protein kinases. *Int J Biochem Cell Biol* 2003;35:1210–1226. [PubMed: 12757758]
- Silver IA, Murrills RJ, Etherington DJ. Microelectrode studies on the acid microenvironment beneath adherent macrophages and osteoclasts. *Exp Cell Res* 1988;175:266–276. [PubMed: 3360056]
- Small K, Feng J, Lorenz J, Donnelly ET, Yu A, Im M, Dorn GW II, Liggett SB. Cardiac specific overexpression of transglutaminase II (Gh) results in a unique hypertrophy phenotype independent of phospholipase C activation. *J Biol Chem* 1999;274:21291–21296. [PubMed: 10409687]
- Spurney RF, Pi M, Flannery P, Quarles LD. Aluminum is a weak agonist for the calcium sensing receptor. *Kidney Int* 1999;55:1750–1758. [PubMed: 10231437]
- Sugimoto T, Kanatani M, Kano J, Kaji H, Tsukamoto T, Yamaguchi T, Fukase M, Chihara K. Effects of high calcium concentration on the functions and interactions of osteoblastic cells and monocytes and on the formation of osteoclast-like cells. *J Bone Miner Res* 1993;8:1445–1452. [PubMed: 8304045]
- Tu Q, Pi M, Karsenty G, Simpson L, Liu S, Quarles LD. Rescue of the skeletal phenotype in CasR-deficient mice by transfer onto the Gcm2 null background. *J Clin Invest* 2003;111:1029–1037. [PubMed: 12671052]

- Verberckmoes SC, De Broe ME, D'Haese PC. Dose-dependent effects of strontium on osteoblast function and mineralization. *Kidney Int* 2003;64:534–543. [PubMed: 12846748]
- Yamaguchi T, Kifor O, Chattopadhyay N, Brown EM. Expression of extracellular calcium (Ca^{2+}_o)-sensing receptor in the clonal osteoblast-like cell lines UMR-106 and SAOS-2. *Biochem Biophys Res Commun* 1998a;243:753–757. [PubMed: 9501005]
- Yamaguchi T, Olozak I, Chattopadhyay N, Butters RR, Kifor O, Scadden DT, Brown EM. Expression of extracellular calcium (Ca^{2+}_o)-sensing receptor expression in human peripheral blood monocytes. *Biochem Biophys Res Commun* 1998b;246:501–506. [PubMed: 9610391]
- Yamaguchi T, Chattopadhyay N, Kifor O, Brown EM. 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. *Endocrinology* 1998c;139:3561–3568. [PubMed: 9681508]
- Yamaguchi T, Kifor O, Chattopadhyay N, Bai M, Brown EM. Extracellular calcium (Ca^{2+}_o)-sensing receptor in a mouse monocyte-macrophage cell line (J774): Potential mediator of the actions of Ca^{2+}_o on the function of J774 cells. *J Bone Miner Res* 1998d;13:1390–1397. [PubMed: 9738511]
- Yamaguchi T, Chattopadhyay N, Kifor O, Butters RR, Sugimoto T, Brown EM. Mouse osteoblastic cell line (MC3T3-E1) expresses extracellular calcium (Ca^{2+}_o)-sensing receptor and its agonists stimulate chemotaxis and proliferation of MC3T3-E1 cells. *J Bone Miner Res* 1998e;13:1530–1538. [PubMed: 9783541]
- Yamaguchi T, Chattopadhyay N, Kifor O, Sanders JL, Brown EM. Activation of p42/44 and p38 mitogen-activated protein kinases by extracellular calcium-sensing receptor agonists induces mitogenic responses in the mouse osteoblastic MC3T3-E1 cell line. *Biochem Biophys Res Commun* 2000;279(2):363–368. [PubMed: 11118293]
- Yamaguchi T, Chattopadhyay N, Kifor O, Ye C, Vassilev PM, Sanders JL, Brown EM. Expression of extracellular calcium-sensing receptor in human osteoblastic MG-63 cell line. *Am J Physiol (Cell Physiol)* 2001;280(2):C382–93. [PubMed: 11208534]
- Yamauchi K, Holt K, Pessin JE. Phosphatidylinositol 3-kinase functions upstream of ras and raf in mediating insulin stimulation of c-fos transcription. *J Biol Chem* 1993;268:14597–14600. [PubMed: 8392056]
- Zaidi M, Datta HK, Patchell A, Moonga B, MacIntyre I. Calcium-activated intracellular calcium elevation: A novel mechanism of osteoclast regulation. *Biochem Biophys Res Commun* 1989;163:1461–1465. [PubMed: 2783143]
- Zaidi M, Kerby J, Huang CL, Alam T, Rathod H, Chambers TJ, Moonga BS. Divalent cations mimic the inhibitory effect of extracellular ionized calcium on bone resorption by isolated rat osteoclasts: Further evidence for a “calcium receptor”. *J Cell Physiol* 1991;149:422–427. [PubMed: 1660481]
- Zaidi M, Shankar VS, Tunwell R, Adebajo OA, Mackrill J, Pazianas M, O'Connell D, Simon BJ, Rifkin BR, Venkitaraman AR, Huang CL-H, Lai A. A ryanodine receptor-like molecule expressed in the osteoblast plasma membrane functions in extracellular Ca^{2+} sensing. *J Clin Invest* 1995;96:1582–1590. [PubMed: 7657829]

Fig. 1. Cation-stimulated ERK activation in mouse osteoblastic cells. **A:** ERK was activated by the addition of 25 μM Al^{3+} , 5 mM Ca^{2+} , and 60 μM Gd^{3+} in MC3T3-E1, TmOb^{+/+} and TmOb^{-/-}. Cells were incubated in D-MEM/F12 containing 0.1% BSA quiescence media and exposed to the various cations for 5 min and ERK activation determined as described in Materials and Methods. **B:** Time course of ERK activation by Ca^{2+} . MC3T3-E1 cells were exposed to 5 mM Ca^{2+} for 0, 5, 10, 20, and 30 min, and the whole cell lysates were subjected to Western Blot analysis using antibody specific for the phosphorylated forms of the ERK. We also performed Western Blot analysis of total ERK using an anti-ERK1/2 antibody in related experiments, which routinely showed equal loading (data not shown).

Fig. 2.

TCF-dependent activation of SRE in MC3T3-E1 cells. **A:** Schematic representation of the wild-type and mutant SRE promoter constructs (SREmutSRF and SREmutTCF). The SRE promoter construct used in these studies has two copies of the SRE *cis*-acting element, which contains ternary complex factor (TCF) and serum response factor (SRF) binding sites. The underlined letters indicates the mutated nucleotides that are known to inhibit activity of these elements. **B:** Mechanisms of calcium and NPS-R568 stimulation of SRE activity by CASR and Ob.CASR. HEK-293 cells transfected with CASR, MC3T3-E1 cells lacking CASR and MC3T3-E1 cells transfected with CASR were stimulated with 5 mM Ca²⁺ or 1 μM NPS-R568. Data are expressed as luciferase activity (mean ± SEM of triplicate samples) normalized by the β-gal activity expressed as percentage induction with respect to control cells. Values sharing the same superscript within each cell type are not significantly different at $P < 0.05$.

Fig. 3.

Activation of RhoA in MC3T3-E1 cells. MC3T3-E1 transfected with vector or pcDNA3.rCASR were stimulated 5 mM Ca^{+2} for 5 min. Calcium (5 mM) induces membrane association of RhoA in as assessed by Western blot analysis of RhoA in membrane fractions prepared as described in Materials and Methods (**upper panel**). Total cell lysates were separated by SDS-PAGE and immunoblotted with anti-RhoA antibody (**lower panel**).

Fig. 4.

Overexpression of β -arrestins and dominant negative G α q mini-gene block cation-signaling in osteoblasts. **A:** Effect of β -arrestins on cation stimulation of SRE-luc. MC3T3-E1 cells were transiently co-transfected SRE-luciferase and pCMV- β -gal along with the construct directing the expression of β -arrestin 1 or β -arrestin 2. SRE-luciferase activity was assessed after stimulation with 5 mM Ca^{2+} . Data represent the relative luciferase activity reported as the percent induction compared with the activity under nonstimulated conditions and normalized for β -galactosidase activity present in each cellular lysate. Values represent the mean $\pm$ SEM of triplicate samples from a typical experiment and repeated at least twice. Values sharing the same superscript are not significantly different at $P < 0.05$. **B:** Effect of β -arrestins and dominant negative G α q(305–359) on ERK activity in mouse osteoblasts MC3T3-E1. MC3T3-E1 cells were transiently co-transfected SRE-luciferase and pCMV- β -gal along with the expression constructs for β -arrestin 1, β -arrestin 2, or G α q(305–259), respectively. MC3T3-E1 osteoblasts were stimulated with 5 mM Ca^{2+} for 5 min, and the whole cell lysates were subjected to Western blot analysis using antibody specific for the phosphorylated forms of the ERK (**upper panel**) and total ERK (**lower panel**). **C:** C-terminus of CASR mini-gene inhibits cation stimulation

of SRE-luc in MC3T3-E1 osteoblasts. MC3T3-E1 cells were transiently co-transfected SRE-luciferase and pCMV- β -gal along with the construct directing the expression of C-terminus of CASR mini-gene. SRE-luciferase activity was assessed after stimulation with 60 μ M Gd^{+3} or 25 μ M Al^{+3} . Data represent the relative luciferase activity reported as the percent induction compared with the activity under nonstimulated conditions and normalized for β -galactosidase activity present in each cellular lysate. Values represent the mean $\pm$ SEM of triplicate samples from a typical experiment and repeated at least twice. Values sharing the same superscript are not significantly different at $P < 0.05$.

Pi and Quarles

Fig. 5.

L-Cysteine stimulation of SRE activation in MC3T3-E1 cells. **A:** Stimulation of SRE activation in MC3T3-E1 by L-amino acids. After overnight quiescent, MC3T3-E1 cells were treated by five groups of 3 mM mixture L-amino acids, respectively. Group I contains 5 mM L-Val, L-Cys, and trans-4-Hydroxy-L-Pro; group II contains 5 mM L-Pro, L-Arg, and L-Lys; group III contains 5 mM L-Thr, L-Met, and L-Gly; group IV contains 5 mM L-Ile, L-His, and L-Ser; group V contains 5 mM L-Ala and L-Glu. The data are expressed as percentage induction with respect to control cells and represent the mean $\pm$ SEM of triplicate samples. Values sharing the same superscript are not significantly different at $P < 0.05$. **B:** Dose-dependent effects of L-Cysteine. The effect of L-Cysteine at concentrations ranging from 1 to 10 mM on SRE-luciferase activity was assessed in the presence of 1 mM and 2 mM Ca^{+2} . The data are expressed as percentage induction with respect to control cells and represent the mean $\pm$ SEM of triplicate samples.

TABLE I

Comparison of CASR and Ob.CASR

	CASR	Ob.CASR	Reference
Agonist specificity	Ca	+	Figure 1: Brown et al. [1993]; Quarles et al. [1997]
	Gd	+	Figure 1: Brown et al. [1993]; Quarles et al. [1997]
	Sr	+	Coulombe et al. [2004]; Pi and Quarles [2004]
	Mg	+	Ruat et al. [1996]; Quarles et al. [1997]
	Al	+	Figure 1: Quarles et al. [1991]; Pi et al. [2000]
	Neomycin	+	Brown et al. [1991]; Quarles et al. [1997]
	Spermine	b	Quinn et al. [1998]; Yamaguchi et al. [2000]
	Amino acids	+	Figure 5: Chang et al. [2000]; Conigrave et al. [2000]
	Calcimimetics: NPS-R568	c	Figure 2: Nemeth et al. [1998]
	NPS-S568	c	
	Gq-loop	+	Figure 4: Pi et al. [2002]; Pi and Quarles [2004]
	β -arrestin	+	Figure 4: Pi et al. [2005]
Signaling	C-terminal peptide of CASR	+	Figure 4: Ray et al. [1997]; Pi et al. [2002]
	PKC inhibition	+	Quarles et al. [1994]; Bai et al. [1998]
	Pertussis toxin	+	Dare et al. [1998]; Arthur et al. [2000]
	PLD1 stimulation	+	Pi et al. [2000]; Huang et al. [2004]
	Inhibited against induced cAMP	+	de Jesus Ferreira et al. [1998]; Pi et al. [2000]
	Rho-dependent	+	Figure 3: Pi et al. [2002]; Huang et al. [2004]
	C3 Toxin	+	Pi et al. [2002]; Huang et al. [2004]; Pi et al. [2005]
	PI-PLC	d	Hartle et al. [1996]; Kifor et al. [2001]
	SRF-dependent	Weak	Figure 2: Pi et al. [2002]
	TCF-dependent	+	Figure 2

a, increases intracellular IP3 and Ca^{+2} ; b, stimulates ERK and p38 activation; c, stimulates activation of ERK, but not SRE luciferase, d, increases intracellular Ca^{+2} .