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ARTICLES

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

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Abstract There is evidence for a functionally important extracelluar 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). J. Cell. Biochem. 95: 1081–1092, 2005. © 2005 Wiley-Liss, Inc.

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

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 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

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.

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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 extracelluar 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-meditated 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 ($AlCl_3 \cdot 6\ H_2O$) 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; faction 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 a 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% CO_2. 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, Gαq(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 tranfected with wild-type SRE, or SREmutSRF or SREmutTCF.

All plasmid DNAs were prepared using the EndoFree^{TM} Plasmid Maxi Kit (Invitrogen). Transient transfections were preformed 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 TransFast^{TM} transfection reagent (Promega, Madison WI). The total plasmid DNA was equalized in each well by adjusting the total amount of DNA to 2 μg 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 μl of lysis buffer (25 mM HEPES pH 7.2, 5 mM $MgCl_2$, 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 anti-phospho-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 preformed 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 (800*g* 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 factions 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 μM aluminum, and 60 μM 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

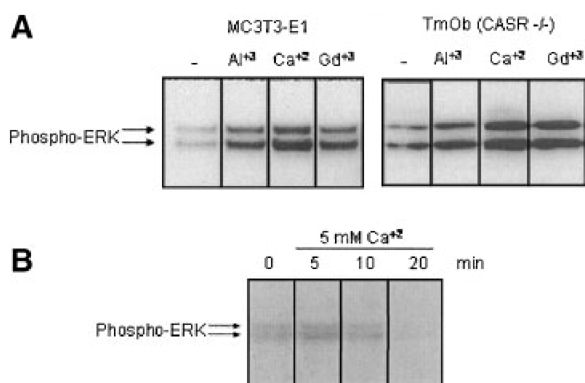

Fig. 1. Cation-stimulated ERK activation in mouse osteoblastic cells. **A**: ERK was activated by the addition of 25 μM Al^{+3}, 5 mM Ca^{+2}, and 60 μM Gd^{+3} in MC3T-E1, $TmOb^{+/+}$ and $TmOb^{-/-}$. Cells were incubated in D-MEM/F12 containing 0.1% BSA quiescence media and exposed to the various 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).

cations and NPS-568, a calcimimetic selective for CASR, were evaluated in MC3T3-E1 osteoblasts overexpressing wild-type SRE, SREmutSRF, 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-568R 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 response 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.

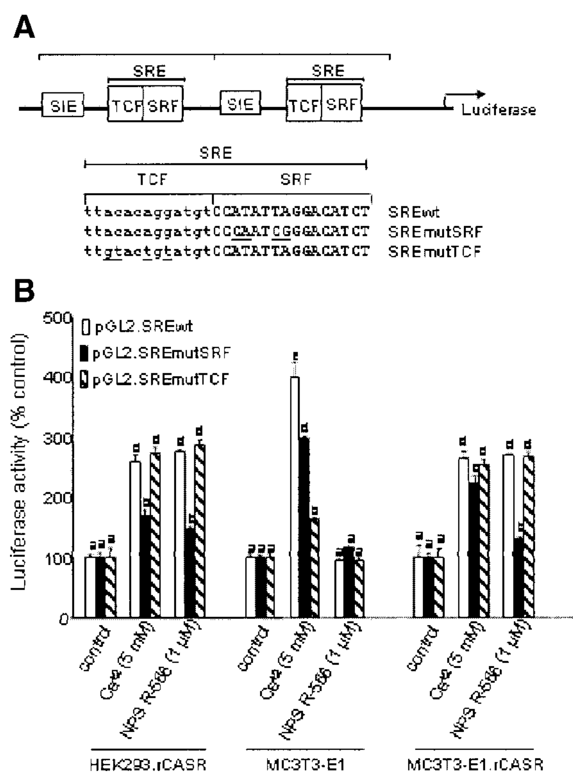

Fig. 2. TCF-dependent activation of SRE in MC3T3-E1 cells. **A**: Schematic representation of the wild-type and mutant SRE promoter constructs (SREmutSRF and SREmutTCF). The SRE promoter construct used in these studies has two copies of the SRE *cis*-acting element, which contains ternary complex factor (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 Ca^{+2} 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 $P < 0.05$.

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 Gαq(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 Gαq(305–359) inhibits extracellular cation stimulation of SRE-lucifierase activity in osteoblasts lacking known CASR as well as will inhibit the activity of known

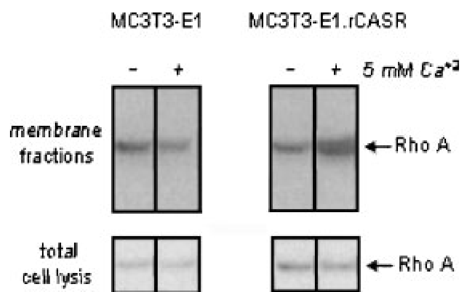

Fig. 3. Activation of RhoA in MC3T3-E1 cells. MC3T3-E1 transfected with vector or pcDNA3.rCASR were stimulated 5 mM Ca^{+2} for 5 min. Calcium (5 mM) induces membrane association of RhoA in as assessed by Western blot analysis of RhoA in membrane fractions prepared as described in Materials and Methods (**upper panel**). Total cell lysates were separated by SDS–PAGE and immunoblotted with anti-RhoA antibody (**lower panel**).

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 of 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, lignad-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 path-

ways (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 Gαq 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 Gαq 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

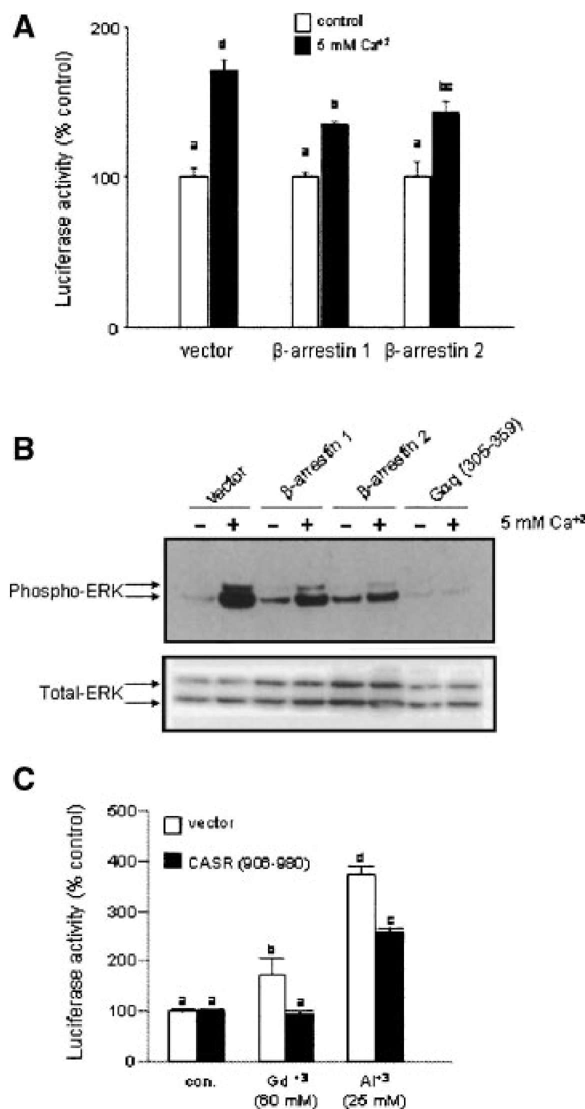

Fig. 4. Overexpression of β-arrestins and dominant negative Gαq mini-gene block cation-signaling in osteoblasts. **A**: Effect of β-arrestins on cation stimulation of SRE-luc. MC3T3-E1 cells were transiently co-transfected SRE-luciferase and pCMV-β-gal along with the construct directing the expression 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 ± 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 Gαq(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 Gαq(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 μM Gd^{+3} or 25 μM 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 ± SEM of triplicate samples from a typical experiment and repeated at least twice. Values sharing the same superscript are not significantly different at $P < 0.05$.

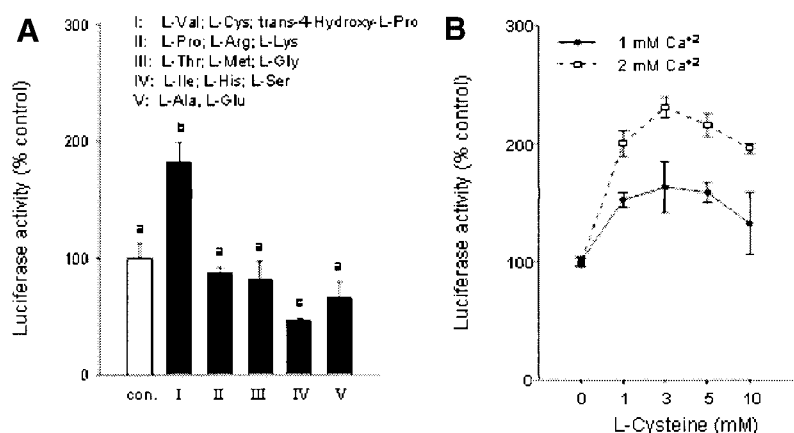

Fig. 5. L-Cysteine stimulation of SRE activation in MC3T3-E1 cells. **A**: Stimulation of SRE activation in MC3T3-E1 by L-amino acids. After overnight quiescent, MC3T3-E1 cells were treated by five groups of 3 mM mixture L-amino acids, respectively. Group I contains 5 mM L-Val, L-Cys, and trans-4-Hydroxy-L-Pro; 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 ± 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 ± SEM of triplicate samples.

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).

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	a	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	
Signaling	Gq-loop	+	+	Figure 4: Pi et al. [2002]; Pi and Quarles [2004]
	β-arrestin	+	+	Figure 4: Pi et al. [2005]
	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]
	Pertossis 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}.

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.

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