Journal of Cellular Biochemistry 104:920–933 (2008)

Expression of the Calcitonin Receptor, Calcitonin Receptor-Like Receptor, and Receptor Activity Modifying Proteins During Osteoclast Differentiation

Susanne Granholm,* Pernilla Lundberg, and Ulf H. Lerner

Department of Oral Cell Biology, Umeå University, Umeå, Sweden

Abstract The expressions of the calcitonin receptor (CTR), the calcitonin receptor-like receptor (CLR), the receptor activity-modifying proteins (RAMP) 1–3, and of the receptor component protein (RCP) have been studied in mouse bone marrow macrophages (BMM) during osteoclast differentiation, induced by treatment with M-CSF and RANKL. Analyses of mRNA showed that CLR and RAMP1–3, but not CTR, were expressed in M-CSF stimulated BMM. RANKL gradually increased CTR mRNA, transiently enhanced CLR and transiently decreased RAMP1 mRNA, but did not affect RAMP2, RAMP3, or RCP mRNA. However, RANKL did not affect protein levels of CLR or RAMP1–3 as assessed by Western blots or FACS analyses, whereas immunocytochemistry showed enhanced CTR protein. Analyses of cAMP production showed that BMM cells expressed functional receptors for calcitonin gene-related peptide (CGRP), amylin, adrenomedullin, and intermedin, but not for calcitonin and calcitonin receptor stimulating peptide (CRSP), but that RANKL induced the expression of receptors for calcitonin and CRSP as well. Calcitonin, CGRP, amylin, adrenomedullin, intermedin, and CRSP all down regulated the CTR mRNA, but none of the peptides caused any effects on the expression of CLR or any of the RAMPs. Our data show that BMM cells express receptors for CGRP, amylin, adrenomedullin, and intermedin and that RANKL induces the formation of receptors for calcitonin and CRSP in these cells. We also show, for the first time, that the CTR is not only down regulated by signaling through the CTR but also by the peptides signaling through CLR/RAMPs. J. Cell. Biochem. 104: 920–933, 2008. © 2008 Wiley-Liss, Inc.

Key words: bone; osteoclast; calcitonin receptor; calcitonin receptor-like receptor; receptor activity modifying protein

Calcitonin (CT) was discovered by Copp and Cheney [1962] as a hormone produced in the parafollicular cells (C-cells) of the thyroid gland. CT belongs to a family of peptides, consisting of two calcitonin gene-related peptides (α-CGRP, β-CGRP), amylin (AMY), adrenomedullin (ADM), and the recently discovered intermedin/adrenomedullin2 (IMD) and calcitonin receptor activating peptides 1–3 (CRSP1–3) [Wimalawansa, 1997; Katafuchi et al., 2003a,b; Chang et al., 2004; Roh et al., 2004]. CT, the CGRPs and AMY, but not ADM, inhibit

Abbreviations used: ADM, adrenomedullin; AMY, amylin; AMV, avian myeloblastosis virus; BMM, bone marrow macrophages; cAMP, cyclic 3′,5′ adenosine monophosphate; CT, calcitonin; sCT, salmon calcitonin; CTR, calcitonin receptor; CGRP, calcitonin gene-related peptide; CLR, calcitonin receptor-like receptor; CRSP, calcitonin receptor-stimulating peptide; D3, $1,25(OH)_2$-vitamin D3; Epac, exchange protein directly activated by cAMP; FBS, fetal bovine serum; GPCR, G-protein coupled receptor; IBMX, 3-isobutyl-1-methylxanthine; IMD, intermedin; M-CSF, macrophage colony-stimulating factor; α-MEM, α-Minimal Essential Medium; OPG, osteoprotegerin; PKA, protein kinase A; PTH, parathyroid hormone; RAMP, receptor activity modifying protein; RANK, receptor activator of NF-κB; RANKL, receptor activator of NF-κB ligand; RCP, receptor component protein; TRAP, tartrate resistant acid phosphatase; $TRAP^+$ MuOCL, $TRAP^+$ multinucleated osteoclast.

© 2008 Wiley-Liss, Inc.

Grant sponsor: Swedish Research Council; Grant number: 07525; Grant sponsor: Swedish Rheumatism Association; Grant sponsor: Royal 80 Year Found of King Gustav V; Grant sponsor: SalusAnsvar; Grant sponsor: County Council of Västerbotten.

*Correspondence to: Susanne Granholm, Department of Oral Cell Biology, Umeå University, S-901 87 Umeå, Sweden. E-mail: susanne.granholm@odont.umu.se

Received 26 October 2007; Accepted 27 November 2007

DOI 10.1002/jcb.21674

osteoclast-mediated bone resorption [Lerner, 2006], and we have shown that this is also the case for IMD and CRSP-1 [Granholm and Lerner, 2006]. These findings indicate that the receptors for CT, CGRPs, AMY, IMD, and CRSP-1, but not those for ADM, are expressed on the mature multinucleated osteoclast. We have also shown that CT, as well as CGRP, AMY, IMD, and CRSP can act on mononucleated osteoclast progenitor cells, preventing formation of multinucleated, terminally differentiated osteoclasts, without affecting a number of genes known to be important for osteoclast formation and activity [Granholm and Lerner, 2006; Granholm et al., 2007].

The calcitonin receptor (CTR) was first cloned by Lin et al. [1991] and shown to belong to a new group of G protein-coupled receptors (GPCR), which also included the parathyroid hormone/parathyroid hormone related peptide (PTH/PTHrP) receptor, and receptors for secretin, vasoactive intestinal peptide (VIP), growth hormone releasing hormone (GHRH), and glucagone-like peptide 1 [Lin et al., 1991; Goldring et al., 1993].

The receptors for CGRP, ADM, and IMD consist of another GPCR, the calcitonin receptor-like receptor (CLR), in complex with one of three receptor activity modifying peptides (RAMP1–3). The CLR was first discovered as an orphan receptor with large sequence homologies with the CTR. Aiyar et al. [1996] showed that HEK293 cells, transfected with CLR cDNA, could bind ^{125}I-CGRP with high affinity. The cellular response to CGRP includes activation of adenylyl cyclase and led to a 60-fold increase of cyclic AMP (cAMP) production [Aiyar et al., 1996]. McLatchie et al. [1998] discovered a 148-amino acid protein and were able to show that the functional receptor for CGRP was not the CLR alone, but a complex formed by the CLR and this protein, which was denoted receptor activity modifying protein 1 (RAMP1). Searches in databases led to the discovery of two additional RAMP1-like proteins; RAMP2 and RAMP3 [McLatchie et al., 1998]. When CLR was co-expressed with RAMP2, it functioned as a receptor for ADM but not for CGRP [McLatchie et al., 1998]. All three RAMPs can form heterodimers with both CTR and CLR, and the different combinations determine the affinity of the receptor for different ligands, that is, CTR in complex with either of the RAMPs functions as a receptor for AMY, CLR in combination with either RAMP2 or RAMP3 functions as a receptor for ADM, whereas CLR in combination with RAMP1 is a receptor for CGRP. The CLR alone does not function as a receptor. IMD seems to be recognized by both CTR and CLR in combination with either of the RAMP proteins [Roh et al., 2004; Takei et al., 2004]. Finally, the CTR recognizes CT and CRSP. The nomenclature for the receptors for CT, CGRP, AMY, and ADM has been ratified by the International Union of Pharmacology [Poyner et al., 2002]. The different receptor complexes are summarized in Table I.

The three RAMPs share about 30% sequence identity and have a short intracellular C-terminus, a single-transmembrane spanning α-helix and an extracellular N-terminus. In the N-terminus there are four highly conserved cysteine residues [McLatchie et al., 1998; reviewed by Udawela et al., 2004; Hay et al., 2006; Sexton et al., 2006]. The functions of the different domains are not fully known, but the N-terminal seems to be involved in ligand recognition [Udawela et al., 2006], the transmembrane region is important for forming a stable complex with the receptor [Steiner et al., 2002], whereas the C-terminal may influence intracellular signaling upon ligand binding [Udawela et al., 2006]. RAMP interaction with CLR leads to modifications of the terminal glycosylation of the receptor and heterodimerization of CLR with one of the RAMP proteins is essential for efficient transport of the complex to the cell surface [reviewed by Sexton et al., 2006]. More recently, the RAMP proteins have also been shown to influence post-endocytotic receptor recycling/degradation pathways [Bomberger et al., 2005; Cottrell et al., 2007].

The CLR receptor complex, mediating the CGRP and ADM signaling, requires yet another component for full efficiency. Receptor

TABLE I. Receptors for the Different Members of the Calcitonin Family of Peptides

Receptor	Receptor complex
ADM1	CLR + RAMP2
ADM2	CLR + RAMP3
AMY1	CTR + RAMP1
AMY2	CTR + RAMP2
AMY3	CTR + RAMP3
CGRP	CLR + RAMP1
CRSP	CTR
CT	CTR
IMD	CTR/CLR + RAMP1–3

component protein (RCP) is a 148-amino-acid intracellular protein that co-immunoprecipitates with RAMP1 and CLR in Western blot analyses. In cells expressing RCP antisense constructs, responsiveness to CGRP and ADM was reduced. No effects on ligand affinity or receptor density were seen and therefore, it is suggested that the role of RCP is to couple the receptor to the intracellular signaling pathways [Evans et al., 2000; Prado et al., 2002].

RAMP proteins are expressed in various tissues throughout the body and the distribution of RAMPs overlap, but are not consistent with expression of CTR and CLR. This suggests that RAMP protein play a further more important role, interacting with other receptors as well. CLR and CTR are both GPCRs and several other members of this receptor family have in fact been shown to interact with at least one of the three RAMPs [Christopoulus et al., 2003; reviewed by Sexton et al., 2006]. Recently, RAMP2 and RAMP3 have also been shown to interact with the calcium-sensing receptor (CaSR), which belongs to another class of the GPCRs [Bouschet et al., 2005].

We have recently found that all the peptides of the CT family, except for ADM, can affect the formation of multinucleated osteoclasts in bone marrow macrophage (BMM) cultures [Granholm and Lerner, 2006]. In this study, we have examined the expression of the different receptor components in BMM cultures during osteoclast differentiation, as well as in mature osteoclasts. We have, for the first time, detected the CTR, CLR as well as all three RAMPs at both mRNA and protein levels in osteoclast-like cells. We have also examined whether the peptides of the CT family have any effects on these expressions.

MATERIALS AND METHODS

Experimental Animals

CsA mice from our own inbred colony were used in all experiments. Animal care and experiments were approved and conducted in accordance with accepted standards of humane animal care and use as deemed appropriate by the Animal Care and Use Committee of Umeå University, Umeå, Sweden.

Materials

Alpha-Minimal Essential Medium (α-MEM), HEPES-buffered α-MEM, L-glutamine, and all oligonucleotide primers were purchased from Invitrogen, Lidingö, Sweden; fetal bovine serum (FBS) from Bio Whittaker Europe, Verviers, Belgium (BMM); bensyl penicillin from AstraZeneca AB, Södertälje, Sweden; recombinant murine M-CSF and recombinant mouse RANKL from R & D Systems, Abdingdon, UK; rat adrenomedullin, rat amylin and rat CGRP from Bachem, Stockholm, Sweden; porcine CRSP-1 and mouse intermedin from Phoenix Peptides (Karlsruhe, Germany); Triton X-100 from Calbiochem/Merck (Darmstadt, Germany); gentamycin sulfate, streptomycin sulphate, Acid Phosphatsase Leukocyte Staining kit and SIGMA 104 Phosphatase Substrate from Sigma Chemicals CO. (St Louis, MO); IBMX from Sigma–Aldrich (Stockholm, Sweden); RNAqueous™-4PCR and DNA free from Ambion (Austin, TX); 1st Strand cDNA Synthesis kit and PCR Core kit from Roche (Mannheim, Germany); Thermo Sequence-TM II DYEnamic ET™ from Amersham (Little Chalfont, Buckinghamshire, England); QIAquick PCR purification kit and HotStar Taq polymerase kit from Qiagen Ltd. (Crawley, West Sussex, England); BCA Protein Assay Kit and SuperSignal chemiluminescense kit from Pierce Biotechnology, Inc. (Rockford, IL); BD Cytofix/Cytoperm™ Kit from BD Bioscience (San Diego, CA); cAMP [^{125}I] Radioimmunoassay Kit from NEN™ Life Science Products (Boston, MA) and TaqMan Universal PCR Master Mix, TaqMan® gene expression assays from Applied Biosystems (Warrington, UK); rabbit polyclonal antibody against mouse CLR and RAMP1, rabbit polyclonal antibody against human/mouse RAMP2 and RAMP3, goat polyclonal antibody against human actin, normal goat IgG, normal rabbit IgG and normal rat IgG2 from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); FITC-conjugated polyclonal swine anti-rabbit Ig from DAKO (Glostrup, Denmark); 6-well plates, 24-well plates, 96-well plates, coverslips, 8-well chamber slides, and culture dishes from Nunc (Roskilde, Denmark) and cell scrapers, culture dishes and 24-well plates from Costar Incorporated (Corning, NY). Synthetic salmon calcitonin (sCT) was a kind gift from Alejandra Martinez (Novartis Pharma AG, Switzerland) and rabbit polyclonal antibody against rat CTR was a kind gift from Dr P. Sexton, Monash University, Clayton, Melbourne, Australia.

Bone Marrow Macrophage Cultures

Highly purified bone marrow macrophages (BMM) were isolated according to Takeshita et al. [2000] and Granholm et al. [2007]. These cells did not express alkaline phosphatase, RANKL, OPG, or CTR mRNA, but mRNA for RANK, c-fms, cathepsin K and TRAP, as assessed by quantitative real-time PCR (data not shown). FACS analysis has shown that these BMM are devoid of T- and B-cells expressing CD45R and CD3 and that 75% of the cells express CD115/c-Fms and all cells express CD11b/Mac-1 [Granholm et al., 2007].

For osteoclastogenesis experiments, BMM were seeded either on 0.8 cm^2 glass Chamber slides or 0.32 cm^2 96-well plates at a density of 10^4 cells/cm^2 in α-MEM/10% FBS containing either 100 ng/ml of M-CSF (controls) or 100 ng/ml of M-CSF + 100 ng/ml of RANKL, with or with out test substances. After 1–6 days, with a change of medium after 3 and 5 days, the cultures were harvested and the cells fixed with acetone in citrate buffer and subsequently stained for TRAP. The TRAP positive cells with three or more nuclei were considered osteoclasts and the number of multinucleated osteoclasts was counted ($TRAP^+$ MuOCL). Osteoclasts formed in these cultures stimulated by M-CSF and RANKL were able to form pits when cultured on slices of bovine bone. Osteoclasts were not formed in the presence of PTH or D3, indicating the lack of stromal cells in the cultures. Osteoclast formation caused by M-CSF and RANKL was abolished by osteoprotegerin (OPG) (data not shown).

Fluorescence-Activated Cell Sorting (FACS)

BMM cells were seeded at a density of 2×10^4/cm^2 in 60 cm^2 culture dishes and cultured in the presence of M-CSF (100 ng/ml) or M-CSF (100 ng/ml) and RANKL (100 ng/ml). At 48 h, cells were washed with PBS and harvested using EDTA. The cells were then either fixed and permeabilized using BD Cytofix/Cytoperm™ Kit or suspended in FACS buffer (PBS, 3% FBS), and stained with antibodies (0.4 μg/10^6 cells) against either mouse CLR or RAMP1, or human/mouse RAMP2 or RAMP3 and subsequently with appropriate secondary antibodies (1.7 μg/10^5 cells) against either goat IgG or rabbit IgG, conjugated to FITC. As a negative control, cells incubated with normal goat IgG and normal rabbit IgG were also analyzed. Ten thousand cells were analyzed from each sample. Debris and cell fragments were excluded by a threshold value of approximately 50% of the FSC of the mean population FSC. The cells were analyzed using a flow cytometer (FACSCalibur; Becton Dickinson, San Jose, CA).

Western Blot

BMM cells were seeded at a density of 2×10^4/cm^2 in 60 cm^2 culture dishes and cultured in the presence of M-CSF (100 ng/ml) or M-CSF (100 ng/ml) and RANKL (100 ng/ml). Cells were harvested at 48 h and washed three times with ice-cold PBS (pH 7.2). The cells were suspended in 0.6 ml RIPA buffer (1% Igepal CA-630, 0.1% SDS, 2 mM EDTA, 50 mM NaF, in PBS) containing protease inhibitors (0.1 mg/ml PMSF, 1 mM natriumorthvanadat, 10 μg/ml pepstatin A, and 10 μg/ml leupeptin) and incubated at 4°C for 15 min. The cells lysates were transferred to 1.5 ml tubes and incubated on ice for an additional 60 min and thereafter centrifuged (10,000*g* at 4°C, 5 min). The protein concentration was determined using the BCA protein assay kit with bovine albumin as standard. If necessary, the samples were concentrated using centrifugal filter devices, according to manufacturers' instructions. The proteins were separated in SDS-polyacrylamide gel electrophoresis (7.5% polyacrylamide for CTR and CLR and 12% for RAMP1, RAMP2, RAMP3, and actin detection), thereafter blotted onto a nitrocellulose filter. The membranes were incubated in blocking solution of TBS (150 mM NaCl, 20 mM Tris, pH 7.2) with 1% dry-milk and 1% BSA for 60 min at room temperature before incubation with the primary antibody over night at 4°C. The primary antibody (0.2 mg/ml) was diluted 1:1,000 in 5 ml of TBST (150 mM NaCl, 20 mM Tris, 0.05% Tween, pH 7.2) with 1% dry-milk and 1% BSA. The membranes were washed three times in TBST to remove unbound antibodies and then incubated with the secondary, peroxidase-linked antibody (0.3 mg/ml, diluted 1:2,000 in 5 ml of TBST) for 60 min at room temperature. After washing three times in TBST, proteins were detected using a chemiluminescense kit according to manufacturer's instructions and detected using Chemi Doc™ XRS (BIORAD Laboratories AB, Sundbyberg, Sweden).

Immunocytochemistry

BMM cells were seeded at a density of 10^4 cells/cm^2 in 8-well chamber slides and cultured

in α-MEM/10% FBS containing either 100 ng/ml of M-CSF (controls) or 100 ng/ml of M-CSF + 100 ng/ml of RANKL. Medium was changed at day 3. At 48, 72, and 96 h, cultures were washed in PBS, air dried in room temperature and thereafter fixed with cold acetone (−20°C) for 20 min. Unspecific binding was blocked with PBS/4% BSA and the cultures were thereafter incubated with antibodies recognizing mouse CTR (0.2 mg/ml) in PBS/4% BSA, 200 μl/well. After a washing step, the cultures were incubated with a swine-anti rabbit IgG antibody (0.5 mg/ml), conjugated to FITC, diluted 1:200 in 200 μl. Controls included cells incubated with normal rat IgG as an isotype control, as well as cells incubated with only the secondary antibody. The cells were then analyzed using a Leica DMRBE microscope (Leica Mikroskopie und Systeme GmbH, Wetzlar, Germany) together with a Leica DC200 digital camera and Leica DC200 software (Leica Microsystems AG, Wetzlar, Germany). Another set of cultures were fixed with acetone in citrate buffer and subsequently stained for TRAP.

Cyclic AMP Formation

BMM were seeded in 24-well plates at a density of 10^4 cells/cm^2 in α-MEM/10% FBS containing either 100 ng/ml of M-CSF (controls) or 100 ng/ml of M-CSF + 100 ng/ml of RANKL. After 72 h, cells were washed in serum-free α-MEM, and thereafter incubated in HEPES-buffered α-MEM containing 100 μM IBMX for 30 min, after which CT (1 nM), CGRP (1 μM), AMY (1 μM), ADM (1 μM), IMD (1 μM), or CRSP (1 μM) was added to examine the stimulatory effect of each peptide on intracellular signaling. Concentrations of CT, CGRP, AMY, ADM, IMD, and CRSP were determined by concentration-response experiments [Granholm et al., 2007; Granholm and Lerner, in preparation] and set to induce maximal inhibitory effect on osteoclast activity and osteoclastogenesis without causing toxicity to the cells. After 5 min, cells were harvested and cAMP was extracted from the cells, using 90% *n*-propanol. Propanol was removed by evaporation and the remaining sample was resuspended in assay buffer and analyzed using a cAMP [^{125}I] Radioimmunoassay Kit according to manufacturers' instructions.

Gene Expression in BMM Cultures

BMM were seeded at a density of 10^4 cells/cm^2 in 10 cm^2 6-well plates and cultured in α-MEM/10% FBS containing either M-CSF (100 ng/ml) or M-CSF (100 ng/ml) + RANKL (100 ng/ml), in the absence or presence of sCT (1 nM), CGRP (1 μM), AMY (1 μM), ADM (1 μM), IMD (1 μM), or CRSP (1 μM) to evaluate the effect of each peptide on RNA expression. Concentrations of peptides were set as described above. At 24, 48, 72, and 96 h, total RNA was extracted.

RNA Isolation and First Strand cDNA Synthesis

Total RNA was extracted by using the RNAqueous™–4PCR kit (Ambion) according to the manufacturer's instructions. Samples were subsequently digested with DNA-free. The quality of the RNA preparations was analyzed in 1.5% agarose gel electrophoreses and visualized using ethidium bromide. Single-stranded cDNA were synthesized from 0.5 μg of total RNA using a 1st strand cDNA synthesis kit and oligo(dT)$_{15}$ primers, according to the manufacturer's protocol. To ensure that there was no genomic DNA in the samples, reactions without AMV reverse transcriptase was included as a negative control. The mRNA expression was then analyzed, using semi-quantitative reverese transcriptase-polymerase chain reaction (RT-PCR), or quantitative real-time PCR.

Semi-Quantitative Reverse Transcriptase-Polymerase Chain Reaction

First strand cDNA was amplified by PCR using a PCR core kit and PC690G Gradient Thermal Cycler (Corbett Research, Australia) or Mastercycler Gradient (Eppendorf, Hamburg, Germany). The PCR reactions for GAPDH, RAMP2, and RAMP3 were performed using PCR standard protocol. The conditions for PCR were: denaturing at 94°C for 2 min, annealing at various temperatures for 35 s, followed by elongation at 72°C for 40 s; in subsequent cycles denaturing was performed at 94°C for 35 s. The PCR reactions for RCP, CLR, CTR, and RAMP1 were initiated with hot start, using HotStar Taq polymerase kit. Annealing temperatures were 55°C (RCP, RAMP1), 57°C (GAPDH, RAMP3), 59°C (RAMP2), 61°C (CLR), and 67°C (CTR). The sequences of primers and GenBank accession numbers are listed in Table II. The expressions of these factors were compared at the logarithmic phase of the PCR reaction, the products separated in electrophoreses on a 1.5% agarose gel, and visualized using ethidium bromide. Primers were designed using ABI Prism™ Primer

TABLE II. Sequences of Primers Used in Semi-Quantitative RT-PCR

mRNA specis	Sequences	GenBank	Base-pair
RCP		NM007761	361
Sense primer	5′-GTGTTCCAGTTACTAACG-3′		
Antisense primer	5′-CCTACTTGTCTCGTTTTCGT-3′		
CLR		AB015595	309
Sense primer	5′-CTCATCTCCTCTACATTCATCC-3′		
Antisense primer	5′-CGAAGAAATTACCTCTCC-3′		
CTR		U185421	168
Sense primer	5′-TGCTGGCTGAGTGCAGAAACC-3′		
Antisense primer	5′-GGCCTTCACAGCCTTCAGGTAC-3′		
RAMP1		NM016894	226
Sense primer	5′-TGGAGACTATTGGGAAGACG-3′		
Antisense primer	5′-CGTAGGAGACGGGAAAGT-3′		
RAMP2		NM019444	334
Sense primer	5′-AAAGGGAAGATGGAAGACTACG-3′		
Antisense primer	5′-GGAACAATGAGAACACCACACC-3′		
RAMP3		NM019511	495
Sense primer	5′-ATGAAGACCCCAGCACAGC-3′		
Antisense primer	5′-GACCCTCTTACAGCAACT-3′		
GAPDH		M32599	267
Sense primer	5′-ACTTTGTCAAGCTCATTTCC-3′		
Antisense primer	5′-TGCAGCGAACTTTATTGATG-3′		

GenBank accession number and size for the predicted PCR products.

express™ (Applied Biosystems, Foster City, CA) or Omiga™ (Genetic Computer Group, Inc., Madison, WI). The identity of the PCR products was confirmed using a QIAquick purification kit (Qiagen Ltd.) and a Thermo Sequence-TM II DYEnamic ET™ terminator cycle sequencing kit (Amersham) with sequences analyzed on an ABI377 XL DNA sequencer.

Quantitative Real-Time Polymerase Chain Reaction

Quantitative real-time PCR analyses of CLR, RAMP1, RAMP2, and RAMP3 were performed using TaqMan® gene expression assays and primers and probe for detection of the CTR and cathepsin K were designed using ABI Prism™ Primer express™ (Applied Biosystems). Sequences of primers and probes and GenBank accession numbers have been described previously [Granholm et al., 2007]. To control variability in amplification due to differences in starting mRNA concentrations, β-actin was used as an internal standard. The relative expression of target mRNA was computed from the target Ct values and the β-actin Ct value using the standard curve method (*User Bulletin #2*, Applied Biosystems).

Statistical Analyses

All statistical analyses were performed using one-way analysis of variance (ANOVA) with Levene's homogenicity test, and post-hoc Bonferroni's, or where appropriate, Dunnett's $T3$ test or using the Independent-Sample T-test (SPSS for Windows, Apache Software Foundation). Statistical level was set to 0.05. All experiments have been performed at least twice with comparable results and all data are presented as the means ± SEM.

RESULTS

The Effects of RANKL on the mRNA Expression of CTR, CLR, RAMPs, and CGRP-RCP in BMM

Treatment of mouse BMM with M-CSF (100 ng/ml) and RANKL (100 ng/ml) resulted in an increased number of TRAP$^+$-MuOCL, which was maximal after 96 h, after which time point the number declined (Fig. 1A,B). Semi-quantitative RT-PCR analyses of the expression of the CTR, CLR, RAMP1–3, and RCP mRNA at 96 h showed that M-CSF treated BMM cells express CLR, RAMP1–3 as well as RCP mRNA (Fig. 1C). As expected [Takeshita et al., 2000; Granholm et al., 2007], no CTR mRNA was detected (Fig. 1C). In BMM treated with M-CSF (100 ng/ml) and RANKL (100 ng/ml), the CTR was clearly detected after 96 h, as was the CLR, RAMP2–3, and RCP mRNAs. In contrast to the CTR, the expression of RAMP1 mRNA was down regulated (Fig. 1C). At 96 h, RANKL had no effect on mRNA expression of CLR nor RAMP2 and RAMP3. In order to be able to semi-quantitatively detect the RAMP2 and RAMP3 mRNA we had to run the PCR reactions for 38 cycles. The levels of receptor components

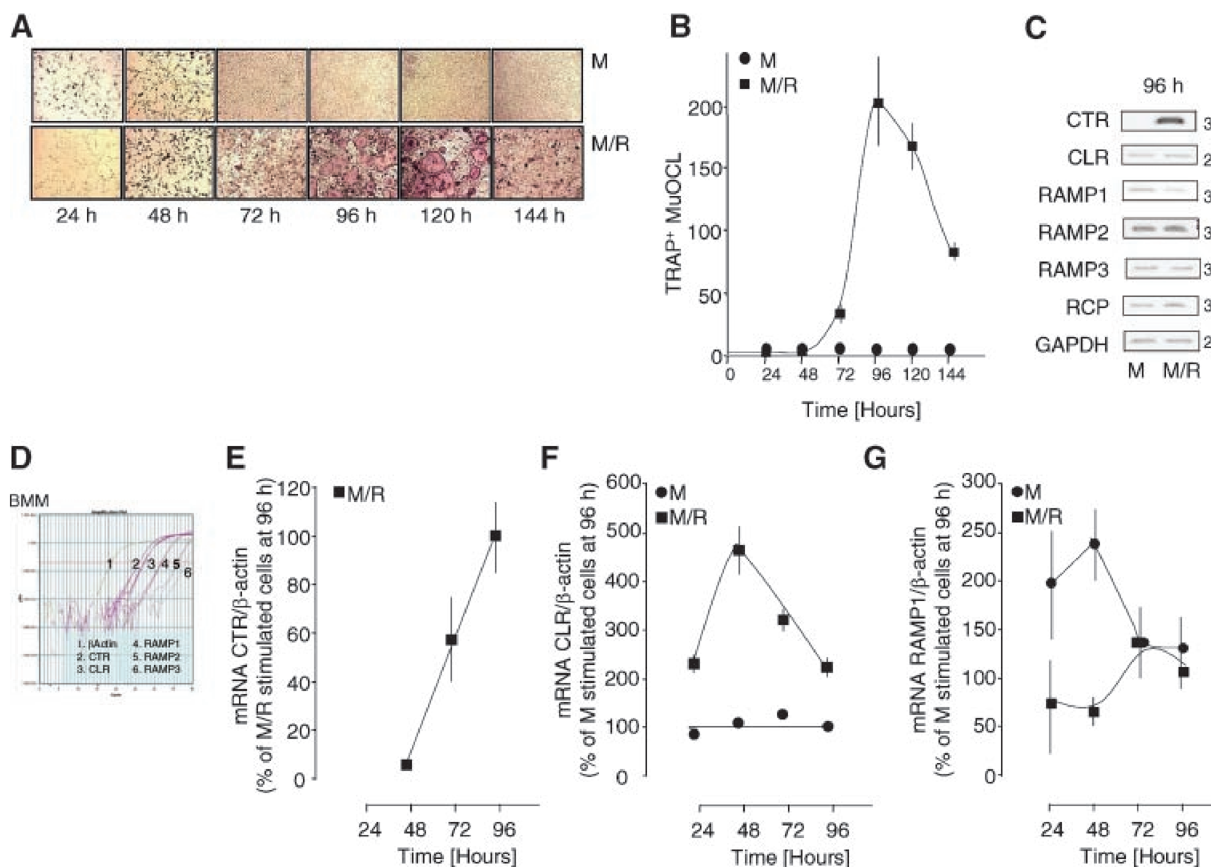

Fig. 1. TRAP staining shows time-dependent formation of multinucleated TRAP$^+$ cells in BMM cultures stimulated by M-CSF (100 ng/ml) and RANKL (100 ng/ml), but not in cultures stimulated with only M-CSF (**A**). The formation of TRAP$^+$ MuOCL in BMM cultures stimulated with M-CSF and RANKL is time-dependent and reaches a maximum after 96 h (**B**). Semi-quantitative RT-PCR analysis of the mRNA expression of receptor complex components CTR, CLR, RAMP1–3, and RCP in BMM stimulated with M-CSF or MCSF and RANKL for 96 h. Samples were calibrated using the house keeping gene GAPDH (**C**). Quantitative real-time PCR analysis of the relative mRNA expression of β-actin, CTR, CLR, and RAMP1–3 in BMM stimulated with M-CSF or M-CSF and RANKL for 96 h (**D**). In (**E–G**) is shown the quantitative real-time PCR analysis of CTR (E), CLR (F), and RAMP1 (G) in BMM cultures, incubated as in (C) for 24–96 h. M-CSF is denoted M in the figure and MCSF+ RANKL is denoted as M/R. Symbols represent the mean of four wells.

relative the house-keeping gene β-actin and to each others, in BMM cells treated with M-CSF and RANKL for 96 h, were also analyzed using quantitative real-time PCR. CLR, CTR, and RAMP1 mRNA were expressed at comparatively high levels, whereas RAMP2 and RAMP3 m RNA were expressed at lower levels (Fig. 1D). As a comparison, we analyzed the mRNA expressions in mouse brain extracts and found that all receptor components were expressed at relatively high levels compared to the expression of β-actin (not shown). Since there were such low levels of RAMP2 and RAMP3 mRNA expression in the M-CSF/RANKL treated BMM we excluded them from further mRNA analysis. Treatment of BMM with M-CSF and RANKL resulted in an increased mRNA level of the CTR which could be observed after 48 h, was clearly evident after 72 h and even higher after 96 h (Fig. 1E). CLR mRNA expression was transiently increased by M-CSF/RANKL with a peak at 48 h, after which time point the expression decreased to levels close to those in M-CSF treated BMMs (Fig. 1F), in agreement with the semi-quantitative analysis (Fig. 1C). RAMP1 mRNA was reduced by M-CSF/RANKL at 48 h. However, in the experiments used for quantitative PCR, the expression of RAMP1 mRNA at 96 h was equal with those in M-CSF treated cells.

The Effects of RANKL on the Protein Expression of CTR, CLR, RAMP1, RAMP2, and RAMP3 in BMM

FACS analyses of permeabilized BMM cells, cultured for 48 h in M-CSF, showed that BMM cells stimulated by M-CSF expressed all three

RAMP proteins, as well as CLR protein (Fig. 2A). CLR and all three RAMPs were also detectable at the cell surface on non-permeabilized cells cultured in M-CSF (Fig. 2B). Total protein levels of CLR and RAMP1–3 were unaffected by the addition of RANKL to the cultures (Fig. 2C). Neither did RANKL cause any changes in the surface expression on non-permeabilized cells (not shown).

Western blot analyses of BMM, cultured for 48 h in M-CSF, showed the presence of fragments corresponding to different glycosylated forms of the CLR (74, 60 kDa), the RAMP1 homodimer (37 kDa), and RAMP2 monomer (37 kDa), respectively (Fig. 2D, left panel). Addition of RANKL to the cultures did not affect the level of protein. Samples were calibrated using actin. Western blot analyses of RAMP3 showed the presence of a weak band at 27 kDa and a more dense band at 37 kDa. The reported size of the RAMP3 protein is 27 kDa and is most likely represented by the weak band. The weak protein expression is in agreement with the very low mRNA expression of RAMP3. The denser band at 37 kDa might be a modified form of RAMP3, but since we are uncertain if this is RAMP3 we have excluded it from our analyses. These considerations also have implication for the interpretation of the RAMP3 FACS analysis.

Immunocytochemical analyses of the CTR expression in non-permeabilized BMM cells showed that no CTR-like antigen was expressed in M-CSF treated cells but that when adding RANKL to the cultures, the receptor starts to appear on mononucleated cells at 48–72 h (not shown) and after 96 h, both mono- and multinucleated cells expressed the receptor (Fig. 2E). In parallel, we stained cells for TRAP$^+$ and could see both mono- and multinucleated stained cells (not shown). We also tested the antibodies against CLR, RAMP1, RAMP2, and RAMP3 for immunocytochemistry but were unable to detect any specific binding.

The Effects of the Peptides in the CT Family of Peptides on cAMP Production in BMM Cultures Stimulated With M-CSF and RANKL

Analysis of cAMP formation in BMM stimulated by M-CSF for 72 h showed that CGRP

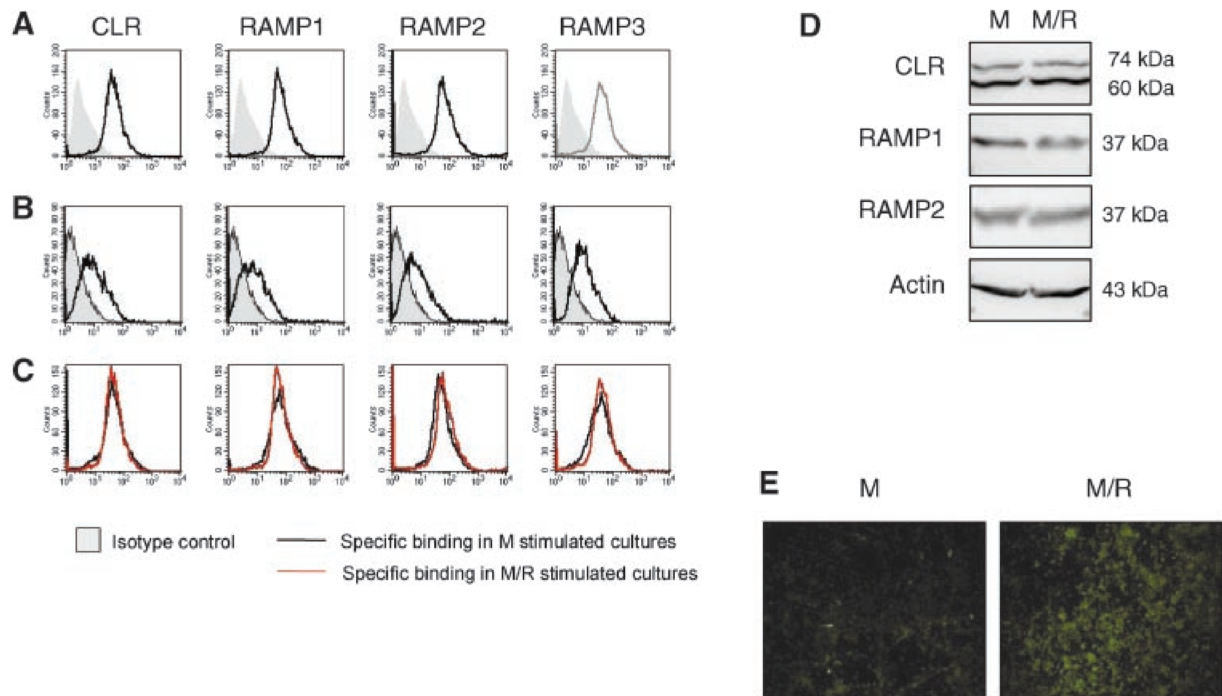

Fig. 2. FACS analyses of CLR, RAMP1, RAMP2, and RAMP3 expression in BMM cells, stimulated for 48 h with M-CSF (100 ng/ml); total protein (**A**) and cell surface expression (**B**). Appropriate isotypes were used as negative controls. Addition of RANKL (100 ng/ml) did not affect the total protein levels (**C**). Western blot analyses of CLR, RAMP1, and RAMP2 in BMM cells cultured for 48 h identified proteins corresponding to CLR, RAMP1, and RAMP2 (**D**). Immunocytochemical analysis shows CTR expression in cultures stimulated for 72 h with M-CSF (100 ng/ml) in combination with RANKL (100 ng/ml) but not in cultures stimulated with M-CSF alone (**E**). M-CSF is denoted M in the figure and MCSF + RANKL is denoted as M/R.

(1 μM), AMY (1 μM), ADM (1 μM), and IMD (1 μM) stimulated cAMP production at 72 h (Fig. 3A). In contrast, CT (1 nM) and CRSP (1 μM) did not affect the cAMP levels (Fig. 3A). In BMM cells stimulated with M-CSF and RANKL for 72 h, CT (1 nM) and CRSP (1 μM), both caused an accumulation of cAMP similarly to CGRP, AMY, and ADM (Fig. 3B).

The Effects of the Peptides in the CT Family of Peptides on mRNA Expression of CTR, CLR, and RAMPs in BMM Cultures Stimulated With M-CSF and RANKL

In line with previous observations, RANKL time-dependently induced the mRNA expression of the CTR and cathepsin K in BMM cells cultured for 96 h (Fig. 4A–F). As expected, CT (1 nM) abolished RANKL induced CTR mRNA expression (Fig. 4A). Most interestingly, CGRP (1 μM), AMY (1 μM), ADM (1 μM), IMD (1 μM), and CRSP (1 μM) also significantly ($P < 0.05$) inhibited RANKL induced CTR mRNA (Fig. 4B–F). In contrast, none of the peptides affected RANKL induced cathepsinK mRNA expression induced by RANKL (Fig. 4A–F).

In contrast to the down-regulation of CTR, the peptides in the CT family did not affect the transient induction of CLR mRNA (Fig. 5A–F). Nor did the peptides affect the initial decrease of RAMP1 mRNA caused by RANKL treatment (Fig. 6A–F).

DISCUSSION

The discovery of the RAMP proteins led to a new understanding of the regulation of differential cellular responses. The initial studies made by McLatchie et al. [1998] showed, for the first time that, not only were these proteins acting as chaperones for the CLR, RAMP proteins were also able to alter the receptor specificity for different ligands. The CT family of peptides signal via the CTR or the CLR. All three RAMPs can form heterodimers with both CTR and CLR, and RAMP heterodimerization is necessary for CLR translocation to the cell surface [reviewed by Hay et al., 2006; Sexton et al., 2006].

Notoya et al. [2007] have shown that CRSP can inhibit the formation of osteoclasts in spleen cells co-cultured with stromal cells derived from bone marrow (ST2) as well as in bone marrow cells stimulated with M-CSF and RANKL. We have recently shown that in BMM cultures, in which osteoclast differentiation was induced by RANKL, all members of the CT family of peptides, except for ADM, can act directly on osteoclast mononucleated progenitor cells and inhibit the formation of multinucleated

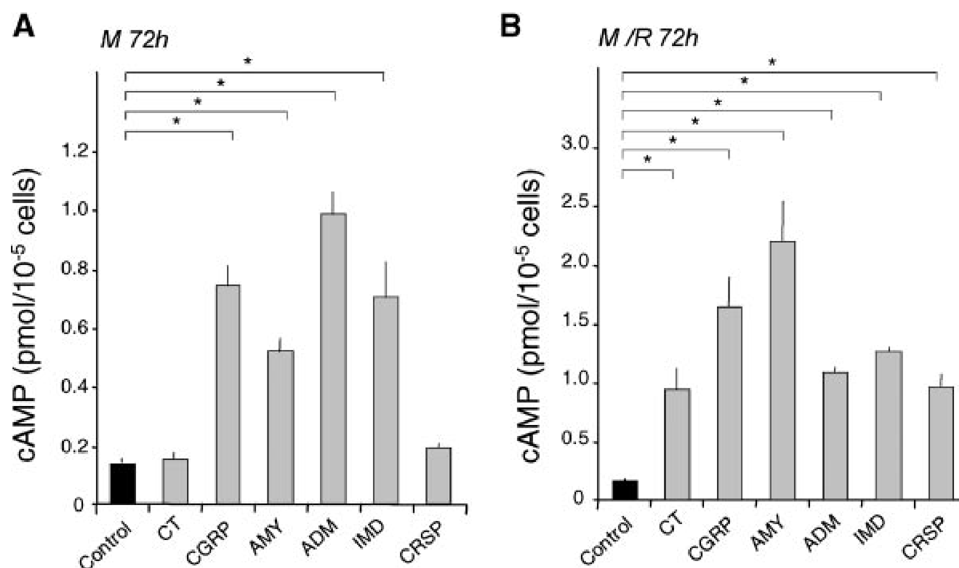

Fig. 3. Analysis of cAMP production after addition of CT (1 nM), CGRP (1 μM), AMY (1 μM), ADM (1 μM), IMD (1 μM), and CRSP (1 μM), respectively, in BMM stimulated with 100 ng/ml of M-CSF (**A**) or 100 ng/ml of M-CSF and 100 ng/ml of RANKL (**B**) for 72 h. MCSF is denoted M in the figure and is denoted as M-CSF + RANKL as M/R. Symbols represent the mean of four wells and SEM is shown as vertical bars. *Statistical significance at $P < 0.05$.

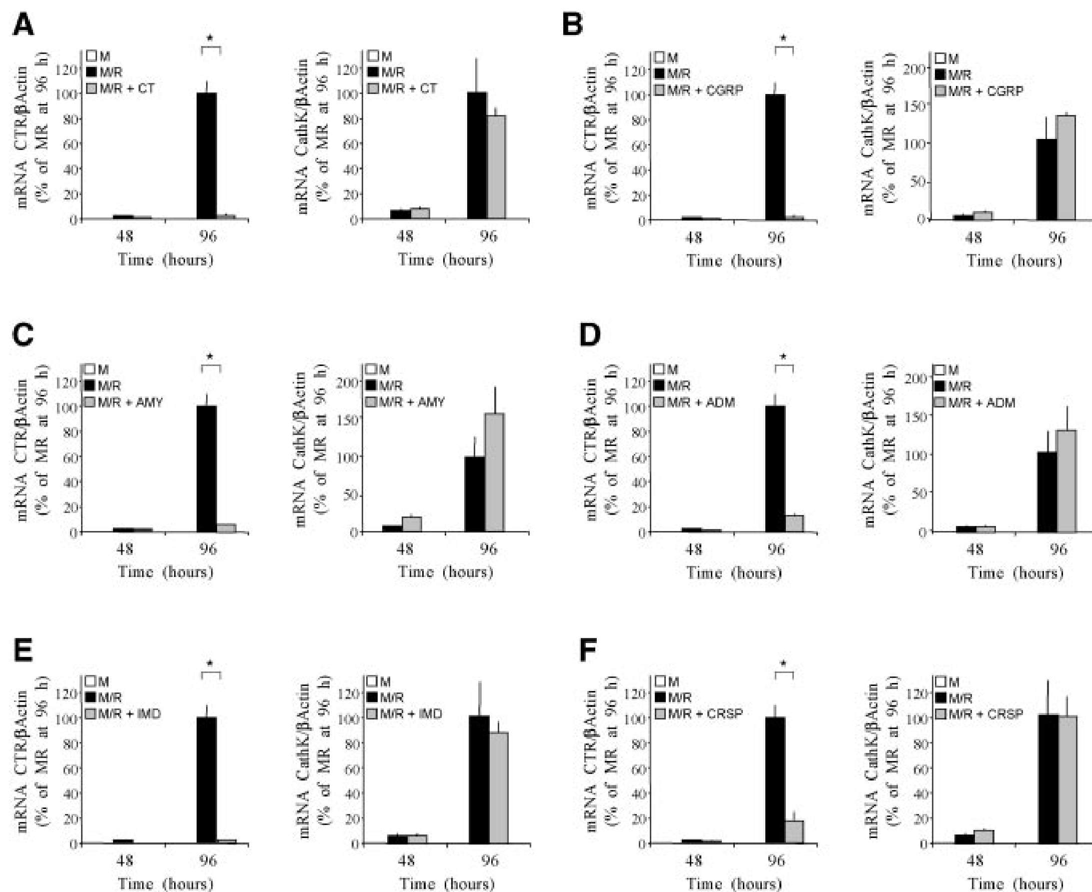

Fig. 4. Analysis of the effects of CT (**A**), CGRP (**B**), AMY (**C**), ADM (**D**), IMD (**E**), and CRSP (**F**) on CTR and cathepsin K mRNA expression in BMM cultures stimulated with 100 ng/ml of M-CSF or 100 ng/ml of M-CSF and 100 ng/ml of RANKL for 48 and 96 h. M-CSF is denoted M in the figure and is denoted as M-CSF + RANKL as M/R. Symbols represent the mean of four wells and SEM is shown as vertical bars. *Statistical significance at $P < 0.05$.

osteoclasts [Granholm and Lerner, 2006]. Therefore, we attempted to examine the expression of the components needed to form the receptors for the peptides in the CT family in BMM cells. The BMM cells used were devoid of T- and B-cells, indicated by the lack of cells expressing CD45R and CD3, and all cells expressed the macrophage marker CD11b/Mac-1 [Granholm et al., 2007].

We found that BMM cells, cultured in the presence of M-CSF express CLR, RAMP1–3, but no CTR mRNA. Western blot and FACS analyses showed that BMM cells express CLR, RAMP1, RAMP2, and probably also RAMP3 proteins and that these receptor components also were expressed on the cell surface.

When adding RANKL to the cultures, TRAP positive mononucleated cells started to appear at 48 h, and at 96 h most of them had fused to form multinucleated TRAP positive osteoclast-like cells. We have recently shown that RANKL time-dependently induce mRNA expression of a number of genes needed for osteoclast differentiation and function in these cells [Granholm et al., 2007]. The CLR mRNA was transiently upregulated by the addition of RANKL at 48 h, whereas the RAMP1 was transiently down-regulated. At the protein level, however, RANKL did not affect the level of CLR or that of the three RAMPs. The CTR mRNA started to appear after 48 h of RANKL stimulation and RANKL induced the expression of CTR-like immunoreactivity on the cell surface of both mono- and multinucleated cells.

Nakamura et al. [2005] have reported, using laser capture microdissection, that multinucleated osteoclast-like cells derived from co-cultures of mouse spleen and bone marrow cells, express mRNA for CTR, CLR, and RAMP2, but not for RAMP-1 and -3. If the receptors for the

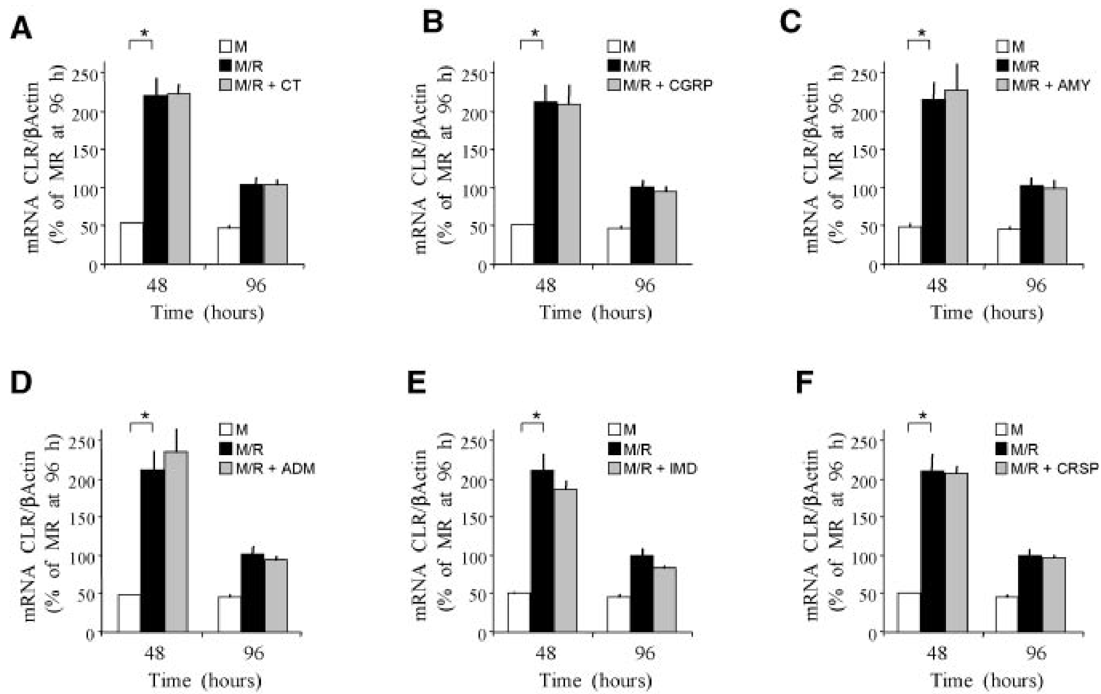

Fig. 5. Analysis of the effects of CT (**A**), CGRP (**B**), AMY (**C**), ADM (**D**), IMD (**E**), and CRSP (**F**) on CLR mRNA expression in BMM cultures stimulated with 100 ng/ml of M-CSF or 100 ng/ml of M-CSF and 100 ng/ml of RANKL for 48 and 96 h. M-CSF is denoted M in the figure and is denoted as M-CSF + RANKL as M/R. Symbols represent the mean of four wells and SEM is shown as vertical bars. *Statistical significance at $P < 0.05$.

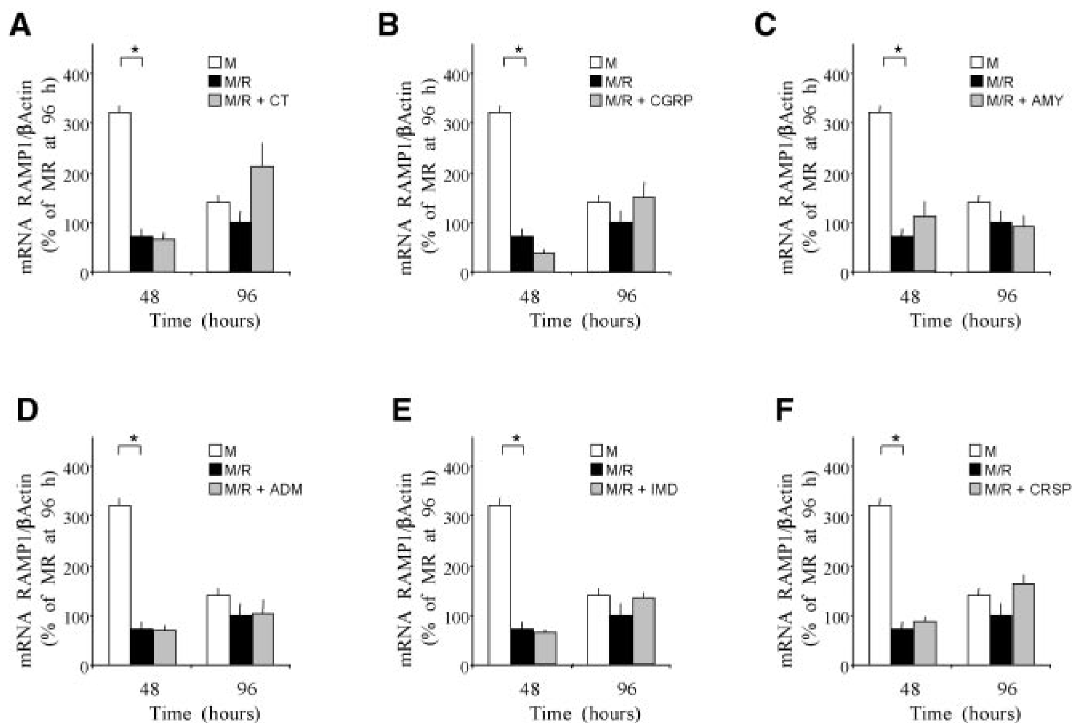

Fig. 6. Analysis of the effects of CT (**A**), CGRP (**B**), AMY (**C**), ADM (**D**), IMD (**E**), and CRSP (**F**) on RAMP1 mRNA expression in BMM cultures stimulated with 100 ng/ml of MCSF or 100 ng/ml of M-CSF and 100 ng/ml of RANKL for 48 and 96 h. M-CSF is denoted M in figure and is denoted as M-CSF + RANKL as M/R. Symbols represent the mean of four wells and SEM is shown as vertical bars. *Statistical significance at $P < 0.05$.

peptides in the CT family are made up in accordance with the current general view, the report by Nakamura et al. makes it difficult to explain the inhibition of OCL activity caused by CGRP, and hard to understand why not ADM inhibits bone resorption. Our findings show, for the first time, that both mononucleated undifferentiated osteoclast progenitor cells and differentiated osteoclasts express the receptor components needed to form receptors for CGRP, ADM, and IMD. We also confirm that RANKL induces the expression of CTR needed for CT, CRSP, and AMY signaling. Although the receptor components, needed for signaling by all members in the CT family of peptides, are expressed by osteoclasts, one of the members, ADM, does not affect osteoclast formation or osteoclast activity. Thus, the lack of effect by ADM is most likely due to differences in downstream signaling (see further below).

The intracellular signaling following GPCRs activation often includes activation of adenylate cyclase and formation of cAMP. In order to see if the receptor components expressed in BMM could make up functional receptors linked to adenylate cylcase, we measured intracellular cAMP levels following stimulation of the cells with the different peptides of the CT family. In mononucleated osteoclast progenitor cells cultured in M-CSF, addition of CGRP, AMY, ADM, and IMD caused an elevation of intracellular cAMP, whereas addition of CT or CRSP had no effect. These results are to large extent in line with the data of both mRNA and protein analysis since CGRP, ADM, and IMD signal via complexes formed by the CLR and the different RAMPs, whereas CTR is recognized by CT and CRSP. However, AMY also caused an increase in cAMP levels which indicates that AMY, at variance from the general view, also may signal through CLR and RAMPs in BMM. The finding in the present study, that AMY signaling can be observed in cells lacking CTR, is in line with the observation that the decreased bone mass and enhanced bone resorption seen in Amy-deficient mice can also be seen in compound heterozygote mice for Ctr and Amy inactivation [Dacquin et al., 2004]. RANKL induces the expression of the CTR, and stimulation of BMM cells cultured in M-CSF and RANKL by CT, CGRP, AMY, ADM, IMD, and CRSP, respectively, caused an elevated level of intracellular cAMP, indicating that these cells express receptors which can be activated by all of these peptides.

According to earlier studies, ADM, in contrast to the other peptides in the CT family, does not inhibit bone resorption and nor does ADM inhibit osteoclast formation [Granholm and Lerner, 2006]. This cannot be explained by a lack of receptor components, or by the absence of cAMP signaling. Cyclic AMP functions as a second messenger activating at least two pathways; the protein kinase A signaling (PKA) and the exchange protein directly activated by cAMP (Epac) signaling [Kawasaki et al., 1998; de Rooij et al., 1998]. We have recently shown that two different cAMP analogues, activating either PKA or Epac signaling, can inhibit the formation of multinucleated osteoclasts [Granholm et al., 2007] indicating that both these pathways are important in negative regulation of osteoclast formation. Christopoulus et al. [2003] reported that when the VIP/PACAP receptor 1 (VPAC1) receptor was expressed in combination with RAMP2, the intracellular response following activation of the receptor was affected. When analyzing the response to VIP in COS-7 expressing VPAC1 receptor alone or in combination with RAMP2, the presence of RAMP2 did not affect the accumulation of cAMP. However, although the potency of VIP was the same, in COS-7 cells co-expressing VPAC1 and RAMP2 the maximal effect on phosphoinositide hydrolysis was significantly higher than in cells expressing only VPAC1 [Christopoulus et al., 2003]. Not much have been done to study the possible effects of RAMP proteins on receptor complex compartmentalization and the intracellular signaling following receptor activation, but one explanation to our results may be that the intracellular signaling following ADM stimulation of cAMP in osteoclast precursor cells is not coupled to the PKA/Epac signaling pathways.

It is well known that CT causes a down regulation of the CTR. It is, however, not known if the other peptides in the CT family are able to regulate CTR, nor is it known if the peptides can regulate the expressions of CLR and RAMP1–3. We, therefore, examined the effects of CT, CGRP, AMY, ADM, IMD, and CRSP, on the mRNA expression of the different receptor components. We found that none of the peptides had any effect on the mRNA levels of either CLR or the RAMPs. Surprisingly, not only peptides acting via the CTR (CT and CRSP and maybe

also AMY in these cells), but also those acting via CLR/RAMPs (CGRP, ADM, IMD) abolished RANKL induced CTR mRNA. It is interesting to note that also ADM, which does not affect osteoclast differentiation or activity, affects RANKL induced enhancement of CTR. These observations suggest the existence of not only homologous but also heterologous down regulation of the CTR. In line with this view, we have observed that also the neuropeptide VIP can abolish RANKL induced CTR expression (unpublished results).

Our data show that BMM express CLR, RAMP1, RAMP2, and possibly also RAMP3, making up functional receptors linked to cAMP for CGRP, AMY, ADM, and IMD and that RANKL induces the formation of the CTR and functional receptors for CT and CRSP in these cells. We also show that the CTR expression can be both homologously and heterologously down regulated and demonstrate a novel uni-lateral, cross-talk between CLR and CTR in regulation of the sensitivity to peptides in the CT family.

ACKNOWLEDGMENTS

We are thankful for the antibodies, provided by Dr. P. Sexton. We are grateful for the excellent technical support by Mrs. Inger Lundgren.

REFERENCES

Aiyar N, Rand K, Elshourbagy NA, Zeng Z, Adamou JE, Bergsma DJ, Li Y. 1996. A cDNA encoding the calcitonin gene-related peptide type 1 receptor. J Biol Chem 271: 11325–11329.

Bomberger JM, Parameswaran N, Hall CS, Aiyar N, Spielman WS. 2005. Novel function for receptor activity-modifying proteins (RAMPs) in post-endocytotic receptor trafficking. J Biol Chem 280:9297–9307.

Bouschet T, Martin S, Henley JM. 2005. Receptor-activity-modifying proteins are required för forward trafficking of the calsium-sensing receptor to the plasma membrane. J Cell Sci 118:4709–4720.

Chang CL, Roh J, Hsu SYT. 2004. Intermedin, a novel calcitonin family peptide that exists in teleosts as well as in mammals: A comparison with other calcitonin/intermedin family peptides in vertebrates. Peptides 25: 1633–1642.

Christopoulus A, Christopoulus G, Morfis M, Udawela M, Laburthe M, Couvineau A, Kuwasako K, Tilakaratnes N, Sexton PM. 2003. Novel receptor partners and function of receptor activity-modifying proteins. J Biol Chem 278: 3293–3297.

Copp DH, Cheney BA. 1962. Calcitonin: A hormone from the parathyroid that lowers calcium level of the blood. Nature 193:381–382.

Cottrell GS, Padilla B, Pikios S, Roosterman D, Steinhoff M, Grady EF, Bunnett NW. 2007. Post-endocytotic sorting of calcitonin receptor-like receptor and receptor activity-modifying protein 1. J Biol Chem 282:12260–12271.

Dacquin R, Davey RA, Laplace C, Levasseur R, Morris HA, Goldring SR, Gebre-Medhin S, Galson DL, Zajac JD, Karsenty G. 2004. Amylin inhibits bone resorption while the calcitonin receptor controls bone formation in vivo. J Cell Biol 164:509–514.

de Rooij J, Zwartkruis FJT, Verheijen MHG, Cool RH, Nijman SMB, Wittinghofer A, Bos JL. 1998. Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. Nature 396:474–477.

Evans BN, Rosenblatt MI, Mnayers LO, Oliver KR, Dickerson IM. 2000. CGRP-RCP, a novel protein required for signal transduction at calcitonin gene-related peptide and adrenomedullin receptors. J Biol Chem 275:31438–31443.

Goldring SR, Gorn AH, Yamin M, Krane SM, Want JT. 1993. Characterization of the structural and functional properties of cloned calcitonin receptor cDNAs. Horm Metab Res 25:477–480.

Granholm S, Lerner UH. 2006. The members of the calcitonin gene family of peptides, including recently discovered intermedin and CRSP, inhibit both osteoclast activity and formation. J Bone Min Res 21 (Suppl 1): S164.

Granholm S, Lundberg P, Lerner UH. 2007. Calcitonin inhibits osteoclast formation in mouse hematopoetic cells independently of transcriptional regulation by RANK and c-Fms. J Endocrinol 195:415–427.

Hay DL, Poyner DR, Sexton PM. 2006. GPCR modulation by RAMPs. Pharmacol Ther 109:173–197.

Katafuchi T, Kikumoto K, Hamano K, Kangawa K, Matsuo H, Minamino N. 2003a. Calcitonin receptor-stimulating peptide, a new member of the calcitonin gene-related peptide family. J Biol Chem 278:12046–12054.

Katafuchi T, Hamano K, Kikumoto K, Minamino N. 2003b. Identification of second and third calcitonin receptor-stimulating peptides in porcine and brain. Biochem Biophys Res Commun 308:445–451.

Kawasaki H, Springett GM, Mochizuki N, Toki S, Nakaya M, Matsuda M, Housman DE, Grabiel AM. 1998. A family of cAMP-binding proteins that directly activate Rap1. Science 282:2275–2279.

Lerner UH. 2006. Deletions of genes encoding calcitonin/α-CGRP, amylin and calcitonin receptor have given new and unexpected insights into the function of calcitonin receptors and calcitonin receptor-like receptors in bone. J Musculoskelet Neuronal Interact 6:87–95.

Lin HY, Harris TL, Flannery MS, Aruffo A, Kaji EH, Gorn A, Kolakowski LF, Jr., Lodish HF, Goldring SR. 1991. Expression cloning of an adenylate cyclase-coupled calcitonin receptor. Science 254:1022–1024.

McLatchie LM, Fraser NJ, Main MJ, Wise A, Brown J, Thompson N, Solari R, Lee MG, Foord SM. 1998. RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor. Nature 393:333–339.

Nakamura M, Morimoto S, Yang Q, Hisamatsu T, Hanai N, Nakamura Y, Mori I, Kakudo K. 2005. Osteoclast-like cells express receptor activity modifying protein 2: Application of laser capture microdissection. J Mol Endocrinol 34:257–261.

Notoya M, Arai R, Katafuchi T, Minamino N, Hagiwara H. 2007. A novel member of the calcitonin gene-related peptide family, calcitonin receptor-stimulating peptide,

inhibits the formation and activity of osteoclasts. Eur J Pharmacol 560:234–239.

Poyner DR, Sexton PM, Marshall I, Smith DM, Quirion R, Born W, Muff R, Fischer JA, Foord SM. 2002. International union of pharmacology. XXXII. The mammalian calcitonin gene-related peptides, adrenomedullin, amylin, and calcitonin receptors. Pharmacol Rev 54:233–246.

Prado MA, Evans-Bain B, Dickerson I. 2002. Receptor component protein (RCP): A member of a multi-protein complex required for G-protein-coupled signal transduction. Biochem Soc Trans 30:460–464.

Roh J, Chang CL, Bhalla A, Klein C, Hsu SYT. 2004. Intermedin is a calcitonin/calcitonin gene-related peptide family peptide acting through the calcitonin receptor-like receptor/receptor activity-modifying protein receptor complexes. J Biol Chem 279:7264–7274.

Sexton PM, Morfis M, Tilakaratne N, Hay DL, Udawela M, Christopoulos G, Christopoulos A. 2006. Complexing receptor pharmacology Modulation of family B G protein-coupled receptor function by RAMPs. Ann NY Acad Sci 1070:90–104.

Steiner S, Muff R, Gujer R, Fischer JA, Born W. 2002. The transmembrane domain of receptor-activity-modifying protein 1 is essential for the functional expression of a calcitonin gene-related peptide receptor. Biochemistry 41:11398–11404.

Takei Y, Hyodo S, Katafuchi T, Minamino N. 2004. Novel fish-derived adrenomedullin in mammals: Structure and possible function. Peptides 25:1643–1656.

Takeshita S, Kaji K, Kudo A. 2000. Identification and characterization of the new osteoclast progenitor with macrophage phenotype being able to differentiate into mature osteoclasts. J Bone Miner Res 15:1477–1488.

Udawela M, Hay DL, Sexton PM. 2004. The receptor activity modifying protein family of G protein coupled receptor accessory proteins. Semin Cell Dev Biol 15:299–308.

Udawela M, Christopoulos G, Tilakaratne N, Christopoulos A, Albiston A, Sexton PM. 2006. Distinct receptor activity-modifying protein domains differentially modulate interaction with calcitonin receptors. Mol Pharmacol 69:1984–1989.

Wimalawansa SJ. 1997. Amylin, calcitonin gene related peptide, calcitonin, and adrenomedullin: A peptide superfamily. Crit Rev Neurobiol 11:167–239.