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Journal of Cellular Biochemistry 112:3300–3312 (2011)

Journal of Cellular Biochemistry

Comparisons Between the Effects of Calcitonin Receptor-Stimulating Peptide and Intermedin and Other Peptides in the Calcitonin Family on Bone Resorption and Osteoclastogenesis

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ABSTRACT

Calcitonin receptor-stimulating peptide (CRSP) and intermedin (IMD) are two recently discovered peptides in the calcitonin (CT) family of peptides. CRSP and IMD, similar to CT, calcitonin gene-related peptide (CGRP), and amylin (AMY), but in contrast to adrenomedullin (ADM), inhibited bone resorption in mouse calvarial bones. CRSP and IMD, similar to CT, CGRP, AMY, but in contrast to ADM, decreased formation of osteoclasts and number of pits in bone marrow macrophage cultures stimulated by M-CSF and RANKL, with no effect on the expression of a number of genes associated with osteoclast progenitor cell differentiation. CRSP and IMD inhibited osteoclastogenesis at a late stage but had no effect on DC-STAMP mRNA. IMD, similar to CGRP, AMY, and ADM stimulated cyclic AMP formation in M-CSF expanded osteoclast progenitor cells lacking CT receptors (CTRs). RANKL induced CTRs and a cyclic AMP response also to CT and CRSP, and increased the cyclic AMP response to CGRP, AMY, and IMD but decreased the response to ADM. Our data demonstrates that CRSP and IMD share several functional properties of peptides in the CT family of peptides, including inhibition of bone resorption and osteoclast formation. The data also show that the reason why ADM does not inhibit osteoclast activity or formation is related to the fact that RANKL decreases ADM receptor signaling through the adenylate cyclase-cyclic AMP pathway. Finally, the findings indicate that activation by CGRP, AMY, and IMD may include activation of both CT and CT receptor-like receptors. J. Cell. Biochem. 112: 3300–3312, 2011.

KEY WORDS: INTERMEDIN; CALCITONIN; CALCITONIN RECEPTOR-STIMLATING PEPTIDE; OSTEOCLASTS

The calcitonin (CT) family of peptides includes CT, two calcitonin gene-related peptides (α-CGRP, β-CGRP), amylin (AMY), and adrenomedullin (ADM). These peptides exhibit weak homologies at the amino acid sequence level, but stronger relationships at the secondary structure level [Findlay and Sexton, 2004]. In the amino terminal moiety, all peptides have a disulfide-bridged ring, followed by a potential amphipatic α-helix and a carboxy terminal proline amide group. Besides the amino terminal end, CT is almost entirely different from CGRP, AMY, and ADM. AMY and CGRP are very similar in their amino terminal parts and exhibit approximately 40% homology in the rest of the molecules. ADM exhibits 20% homology with

Abbreviations: AMY, amylin; ADM, adrenomedullin; BMM, bone marrow macrophage; CGRP, calcitonin gene-related peptide; CRLR, calcitonin receptor-related receptor; CRSP, calcitonin receptor-stimulating peptide; CT, calcitonin; CTR, calcitonin receptor; Epac, exchange protein directly activated by cyclic AMP; FACS, Fluorescence-activated cell sorting; FBS, fetal bovine serum; GPCR, G protein-coupled receptor; IMD, intermedin; M-CSF, macrophage colony-stimulating factor; MEM, minimal essential medium; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; OPG, osteoprotegerin; PKA, protein kinase A; PTH, parathyroid hormone; RAMP, receptor associated-modifying protein; RANK, receptor activator of NF-kB; RANKL, RANK ligand; TRAP, tartrate resistant acid phosphase; $TRAP^{+}$MuOCL, TRAP positive multinucleated osteoclasts.

Additional supporting information may be found in the online version of this article.

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

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Received 6 December 2010; Accepted 28 June 2011 • DOI 10.1002/jcb.23256 • © 2011 Wiley Periodicals, Inc.
Published online 11 July 2011 in Wiley Online Library (wileyonlinelibrary.com).

CGRP and AMY and considerably less with CT [Wimalawansa, 1997].

CT is a hypocalcemic hormone mainly due to its inhibitory effect on bone resorption. The acute decrease of serum calcium is caused by activation of CT receptors (CTRs) on mature osteoclasts; causing contraction, ceased motilitiy, and disruption of actin rings [Zaidi et al., 2002]. Less attention has been paid to the effect of CT on osteoclastogenesis although we and others have found that CT also inhibits formation of mature osteoclasts [Takahashi et al., 1982; Ibbotson et al., 1984; Roodman et al., 1985; Cornish et al., 2001; Granholm et al., 2007]. Inhibitory effects on bone resorption and osteoclast formation have also been observed with CGRP and AMY, but not with ADM [Cornish et al., 2001; Dacquin et al., 2004; reviewed in Naot and Cornish, 2008]. In addition, CGRP, AMY and ADM have been found to stimulate osteoblast proliferation in vitro and bone formation both in vitro and in vivo [reviewed in Lerner, 2006; Naot and Cornish, 2008; Lerner et al., 2009].

Effects of CT are mediated by the CTR, which belongs to the type II seven transmembrane G protein-coupled receptor (GPCR) superfamily [Poyner et al., 2002; Findlay and Sexton, 2004]. Activation by CGRP and ADM requires the presence of another type II GPCR called calcitonin receptor-like receptor (CLR or often abbreviated CRLR), which together with selective receptor activity-modifying proteins (RAMPs) functions as a ligand recognition and signaling complex [reviewed in Poyner et al., 2002; Udawela et al., 2004; Hay et al., 2006]. CRLR has large sequence homology with CTR, especially in the transmembrane domain, and is a receptor component recognized by CGRP and ADM. RAMPs are peptides in the family of type I single transmembrane proteins with a large extracellular amino terminal domain, a single transmembrane spanning domain and a short cytoplasmic domain [reviewed in Udawela et al., 2004; Hay et al., 2006]. Three RAMPs have been described so far, designated RAMP1-3, and these proteins are widely expressed and found also in cells not expressing CTR and CRLR. RAMPs can heterodimerize with both CRLR and CTR and these dimeric proteins make up different receptors for ligands in the CT family of peptides. Thus, CRLR and RAMP1 make up the receptor for CGRP, whereas CRLR and either RAMP2 or RAMP3 serve as receptors for ADM. Heterodimerization between CTR and one of the three RAMPs form receptors with high affinity to AMY, but also with some affinity to CGRP. At variance from this general view, enhanced bone resorption seen in *AMY* null mice ($Amylin^{-/-}$) can be observed also in the compound heterozygote *CTR* and *AMY* deficient mice ($Calcr^{+/-}/Amylin^{-/-}$), demonstrating that AMY inhibits bone resorption independently of the CTR [Dacquin et al., 2004]. In agreement with this observation, we have found that AMY can stimulate cyclic AMP formation in early osteoclast progenitor cells lacking CTR [Granholm et al., 2008].

Recently, two novel members in the CT family of peptides have been discovered. A novel peptide homologous to CGRP/ADM was identified from human and zebrafish expressed sequence tags and using a phylogenetic profiling approach also found in other mammals and teleosts [Roh et al., 2004]. The peptide was abundantly expressed in the intermediate loop of the pituitary gland and since intermedin (IMD) had been suggested as a hormone produced in this lobe already during the 1930s [Abramowitz et al., 1943], Roh et al. [2004] called the peptide IMD.

IMD can stimulate cyclic AMP formation in cells transfected with CRLR and either RAMP-1, -2 or -3 [Roh et al., 2004]. IMD/ADM2 has been found to exert several physiological/pharmacological responses similar to CGRP and ADM, including decreased blood pressure, inhibition of cardiac function [Roh et al., 2004; Pan et al., 2005] and suppression of gastric emptying and food intake [Roh et al., 2004]. IMD has also been found to cause renal effects similar to ADM [Fujisawa et al., 2004]. It is currently not known if IMD has any effect on bone cells.

Calcitonin receptor-stimulating peptide (CRSP) is the other novel member of the CT superfamily of peptides. It was identified from a porcine hypothalamus cDNA library and is a 38-amino acid peptide including two cysteine groups which form an intramolecular disulfide bond at the amino termial part, typical of peptides in the CT family and, in addition, exhibits a carboxy terminal amide group [Katafuchi et al., 2003]. The peptide enhanced cyclic AMP formation in cells expressing the CTR, but not in cells with receptors for AMY, CGRP, or ADM, and was therefore named CRSP. Analyses of CRSP mRNA and protein have shown that the highest expression can be found in the midbrain, hypothalamus, pituitary, and thyroid glands, but that some expression can also be observed in peripheral tissues such as lung, adrenal gland, liver, small intestine, and ovary.

Similar to CT, administration of CRSP to rats decreases serum calcium, but has no effect on blood pressure, in contrast to CGRP [Katafuchi et al., 2003]. The effect on serum calcium may be due to activation of CTR in osteoclasts since CRSP-1 decreases osteoclast formation in $1,25(OH)_2$-vitamin D3 stimulated co-cultures of spleen cells and stromal cells, as well as in M-CSF and RANKL stimulated bone marrow cells [Notoya et al., 2007]. It has been shown that CRSP-1 stimulated cyclic AMP formation, inhibited cell proliferation, and reduced uptake of Ca^+ in the renal epithelial cell line LLC-PK_1 [Katafuchi et al., 2003; Hamano et al., 2005].

In the present study, we have investigated the effects of IMD and CRSP on bone resorption, osteoclast formation, and cyclic AMP formation, using mouse bone organ and bone marrow macrophage (BMM) cultures. In addition, we have compared the effects of IMD and CRSP to those by all peptides in the CT superfamily of proteins with the aim to evaluate which receptors are involved.

MATERIALS AND METHODS

MATERIALS

Alpha-minimal essential medium (α-MEM), minimal essential medium (MEM) with Earle's/25 mM HEPES/L-Glutamine, L-glutamine, and all oligonucleotide primers were obtained from Invitrogen, Lidingö, Sweden; fetal bovine serum (FBS) from Bio Whittaker Europe, Verviers, Belgium; bensyl penicillin from AstraZeneca AB, Södertälje, Sweden; recombinant murine M-CSF and recombinant mouse RANKL from R&D Systems, Abdingdon, UK; rat ADM, rat amylin, rat CGRP, and synthetic bovine parathyroid hormone [PTH-(1-34)] from Bachem, Stockholm, Sweden; porcine CRSP-1 and mouse IMD from Phoenix Peptides, Karlsruhe, Germany; gentamycin sulfate, streptomycin sulphate, Acid Phosphatsase Leukocyte Staining kit, L-ascorbic acid,

essentially fatty acid free bovine serum albumin, forskolin, 8-bromoadenosine 5′-monophosphate free acid (8-bromo5-AMP), 3-isobutyl-1-methylxanthine (IBMX) and Cell Growth Determination Kit, MTT Based from Sigma Chemicals CO., St Louis, MO, USA; RNAqueous™-4PCR and DNA free from Ambion, Austin, TX; 1st Strand cDNA synthesis kit from Roche, Mannheim, Germany; Thermo Sequence-TM II DYEnamic ET™ from Amersham, Little Chalfont, Buckinghamshire, England; cAMP[^{125}I] Radioimmunoassay Kit (Adenosine 3′, 5′ cyclic monophosphate) from NEN™ Life Science Products; TaqMan Universal PCR Master Mix, fluorescent labelled probes and TaqMan® Gene Expression assays from Applied Biosystems, Warrington, UK. Rat monoclonal antibodies against mouse CD45R (B220) and mouse CD115 (c-Fms) and armenian hamster anti-mouse CD3e antibody were purchased from Nordic BioSite, Stockholm, Sweden. Antibodies against mouse CD3e, and CD45R were conjugated to fluorescein (FITC) and the rat anti-mouse CD115 antibody was conjugated to phycoerythrin (PE). N^6-monobutyric-cyclic AMP (6-MB-cAMP), 8-bromo-cAMP and para-methoxyphenylthio-2′-O-methyladenosine-3′,5′-cyclic monophosphate (8-pMeOPT-2′-O-Me.-cAMP) were purchased from BIOLOG Life Science Institute, Bremen, Germany; bone slices and RatLaps™ ELISA from Nordic Bioscience Diagnostics A/S, Herlev, Denmark; Ready Safe liquid scintillation cocktail from Beckman Coulter Inc, Fullerton, CS; (Fig. 4A upper panel and Fig. 4B); [^{45}Ca]$CaCl_2$ from Amersham, Little Chalfont, UK. Six-well plates, 24-well plates, 96-well plates, coverslips, 8-well chamber slides, and culture dishes were obtained from Nunc, Roskilde, Denmark and cell scrapers, culture dishes and 24-well plates from Costar Incorporated, Corning, NY. Indomethacin was a kind gift from Merck, Sharp & Dohme, Haarlem, the Netherlands; 1,25-dihydroxyvitmin D3 from Hoffmann-laRoche, Basel, Switzerland and rolipram by Dr. Sprzgala from Schering AG, Berlin, Germany.

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.

BONE RESORPTION

Neonatal mice were injected with 1.5 μCi ^{45}Ca 4 days prior to dissection to label the mineral part of the skeleton. At the age of 6–7 days, calvarial bones were microdissected and cultured as previously described [Lerner, 1987; Ljunggren et al., 1991]. The bones were preincubated for 24 h in α-MEM containing 0.1% albumin and 1 μM indomethacin [Lerner, 1987]. In order to prestimulate osteoclast formation and bone resorption before the effect by CT and its related peptides was assessed, bones were precultured for another 24 h with PTH (10 nmol/L) or 1,25$(OH)_2$-vitamin D3 (10 nmol/L). In addition, one group of bones were preincubated and then further cultured in the absence of stimulator as an unstimulated control group. Following the two preincubation periods, bones were cultured in the presence of either PTH or 1,25$(OH)_2$-vitamin D3, with and without one of the peptides in the CT family of peptides, or with and without cyclic AMP analogs, for 24 h. At the end of the experiments, the bones were dissolved in HCl and medium and bones were separately analyzed for radioactivity by liquid scintillation counting. Mobilization of isotope was expressed as the percentage release of the initial amount of isotope (calculated as the sum of radioactivity in medium and bone after culture) during the last 24 h [Ljunggren et al., 1991. To accumulate data from several experiments a 100%-transformation was made for each experiment; the ^{45}Ca-release in the hormone stimulated groups was referred to as maximum release and considered 100%. In the time-course experiments, the skeleton was prelabelled by injecting 12.5 μCi ^{45}Ca and the bones were preincubated for 24 h, and then prestimulated by PTH for 24 h and subsequently cultured for 72 h in the presence of PTH without and with one of the peptides in the CT family of peptides, as described above. The kinetics of the release of ^{45}Ca was analyzed by withdrawal of small amounts of medium at the stated time points.

Bone resorption was also studied by analyzing extracellular matrix breakdown, as assessed by the amount of collagen degradation fragments in culture media from bones cultured and exposed to test substances as descibed above. Collagen fragments in culture media were quantified using the RatLaps ELISA kit by following manufacturer's instruction.

OSTEOCLAST FORMATION

The femurs and tibiae from 5 to 7 weeks old male mice were dissected and cleaned from adhering tissues. The cartilage ends were cut-off and the cells in the marrow cavity were flushed out using α-MEM in a syringe with a sterile needle. The marrow cells were collected in α-MEM/10% FBS and the erythrocytes lysed in red blood cell lysis buffer (0.16 M NH_4Cl, 0.17 M Tris, pH 7.65). The remaining bone marrow cells were then washed and suspended in α-MEM/10% FBS containing L-glutamine (0.7 mM), antibiotics (100 U/ml bensylpenicillin, 100 μg/ml streptomycin, and 100 μg/ml gentamycin sulphate) and 100 ng/ml M-CSF. The cells were seeded at a density of 8×10^4 cells/cm^2 in a 60 cm^2 culture dish to which stromal cells and lymphoid cells cannot adhere. After 3 days, the cultures were vigorously washed with PBS twice and the cells attached to the bottom were then detached using 0.002% EDTA in PBS. These cells were resuspended in α-MEM/10% FBS with 100 ng/ml M-CSF and seeded at a density of 0.5×10^4 cells/cm^2 in 60 cm^2 dishes. After another 3 days, the cells were washed and detached as described for the initial 3 days culture period and used as BMMs. For further details, see Takeshita et al. [2000]. These cells did not express alkaline phosphatase, RANKL, osteoprotegerin (OPG) or CTR mRNA, but mRNA for RANK, c-Fms, cathepsin K, and tartrate-resistant acid phosphatase (TRAP), as assessed by quantitative real-time PCR [Granholm et al., 2007]. Using flow cytometry, we could demonstrate cell surface expression of the typical macrophage markers F4/80, Mac-1 (100% of cells positive), and c-Fms (75% of cells positive), but not the lymphoid cell markers CD3 and B 220 [Granholm et al., 2007].

For osteoclastogenesis experiments, BMM were seeded on 0.8 cm^2 glass chamber slides 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 and without different concentrations of one of the peptides in the CT family of peptides.

After 4 days, with a change of medium after 3 days, the cultures were harvested and the cells fixed with acetone in citrate buffer. Subsequently, cells were stained for TRAP and cells positive for TRAP and having three or more nuclei were considered osteoclasts ($TRAP^+$ MuOCL), and the number of osteoclasts counted. Osteoclasts formed in these cultures stimulated by M-CSF and RANKL were able to form pits when cultured on slices of bovine bone and osteoclast formation was associated with increased mRNA expression of CTR, TRAP, and cathepsin K (data not shown). Osteoclasts were not formed in the presence of M-CSF and either PTH or $1,25(OH)_2$-vitamin D3, indicating the lack of stromal cells in the cultures. Osteoclast formation caused by M-CSF and RANKL was abolished by OPG (data not shown).

FLUORESCENCE-ACTIVATED CELL SORTING (FACS)

BMM cells, obtained as described above, were washed with PBS/3% FBS and incubated with antibodies (0.4 μg/10^6 cells) against the macrophage marker mouse CD115 and the lymphoid cell markers CD3 and CD45R. $CD115^+$ $CD3^-$ $CD45R^-$ cells were sorted by flow cytometri in the flow cytometer cell sorter FACSVantageDiVa (BD, Biosciences) and seeded in 0.32 cm^2 96-well plates at a density of $10^4/cm^2$ 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 and without one of the peptides in the CT superfamily. After 4–5 days, with a change of medium after 3 days, the cultures were harvested and the cells fixed with acetone in citrate buffer/3% formaldehyde and subsequently stained for TRAP.

CELL PROLIFERATION ASSAY

Cell proliferation was assessed in M-CSF or M-CSF+ RANKL treated BMM, in the absence and presence of IMD or CRSP-1, by quantifying the amount of mitochondrial dehydrogenases able to release formazan from 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), at different time points, according to the instructions provided by the supplier.

PIT FORMATION

BMM were seeded (15,000 cells/well) on bovine bone slices in α-MEM/10%FCS with M-CSF (100 ng/ml) or M-CSF (100 ng/ml) and RANKL (100 ng/ml) with or without sCT, CRSP-1, or IMD. After 9 days, with medium changed day 3 and 6, the cells were stained for TRAP and $TRAP^+$ multinucleated osteoclasts counted. Subsequently, the cells were removed by sonication 2× in 0.25 M ammonium hydroxide followed by sonication in distilled water. Then, the slices were stained with toluidine blue to detect resorption pits. The resorptive activity of the osteoclasts was assessed by analyzing CTX fragments in culture media using ELISA and by following the instructions supplied by the manufacturer.

TRAP ACTIVITY ANALYSIS

BMM were seeded in 48-well plates at a cell density of 10^4 cells/cm^2 and incubated in the presence of M-CSF and RANKL without or with test substances for 96 h. The cells were then washed in PBS and lysed in Triton X-100 (0.2% in H_2O). After centrifugation, supernatant was collected and kept at −20°C until analyses. TRAP activity was determined using *p*-nitrophenyl phosphate as substrate at pH 4.9, in the presence of tartrate (0.17 M), according to the manufacturer's instructions (Sigma). The activity of the enzyme was assessed as the OD405 of liberated *p*-nitrophenol, and normalized to the amount of cell protein analyzed using the BCA protein assay kit (Pierce, Rockford, IL). The enzyme assays were performed under conditions where the reaction was proportional to amount of enzyme and reaction time.

GENE EXPRESSION

For quantitative real-time PCR analyses, BMM were seeded at a density of 10^4 cells/cm^2 in culture dishes and incubated in α-MEM/10% FBS in the presence of M-CSF (100 ng/ml; control), M-CSF (100 ng/ml)/RANKL (100 ng/ml), with or without the different peptides in the CT family for 96 h.

Total RNA was extracted from BMM by the RNAqueous®-4PCR kit by following the manufacturer's protocol. Extracted total RNA was treated with DNase I to eliminate genomic DNA according to the instructions supplied by the manufacturer. The RNA was quantified spectrophotometrically and the integrity of the RNA preparations was examined by agarose gel electrophoresis. One microgram of total RNA, following DNase I treatment, was reverse transcribed into single-stranded cDNA with a 1st Strand cDNA Synthesis Kit using oligo-p(dT)15 primers. After incubation at 25°C for 10 min and at 42°C for 60 min, the AMV reverse transcriptase was denaturated at 99°C for 5 min, followed by cooling to +4°C for 5 min. The cDNA was kept at −20°C until used for PCR.

Quantitative real-time RT-PCR analyses of *Acp5* (TRAP), *mmp9*, *tcirg1* (ATP6i), *oscar*, and β-*actin* mRNA expressions were performed using the TaqMan Universal PCR Master Mix kit and the ABI PRISM 7900 HT Sequence Detections System and software (Applied Biosystems, Foster City, CA) with fluorescence labeled probes (reporter fluorescent dye VIC at the 5′ end and quencher fluorescent dye TAMRA at the 3′ end) as described previously [Ahlen et al., 2002]. In each reaction, cDNA diluted 20-fold with nuclease-free water was amplified using a TaqMan Universal PCR Master Mix. Sequences for primers and probes recognizing *Acp5*, *mmp-9*, *oscar*, *tcirg1*, and β-*actin* have been described elsewhere [Granholm et al., 2007]. The reaction conditions were an initial step of 2 min at 50°C and 10 min at 95°C for 15 s, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. No amplification was detected in samples where the RT reaction had been omitted (data not shown). To control for 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 β-actin Ct values using the standard curve method (*User Bullentin #2*, Applied Biosystems).

The mRNA expression of DC-STAMP was assessed by semi-quantitative RT-PCR as previously described [Granholm et al., 2007].

CYCLIC AMP FORMATION

BMM were isolated as described above and then incubated at a density of 10^4 cells/cm^2 in 24 multi-well plates in the presence of α-MEM/10% FBS and M-CSF (100 ng/ml) overnight. Then, the cells were pre-stimulated with either M-CSF (100 ng/ml) or M-CSF and

RANKL (100 ng/ml) for 48–96 h. Calvarial halves were dissected and preincubated in α-MEM containing 0.1% albumin and indomethacin (1 μmol/L) for 24 h. In order to prestimulate osteoclast formation, bones were precultured for another 24 h with PTH (10 nmol/L). Cells or calvarial bones were then washed in serum-free medium and pre-incubated in HEPES-buffered α-MEM containing the cyclic AMP phosphodiesterase inhibitor rolipram (10 μmol/L) for 30 min at 37°C. Subsequently, the different peptides in CT family of peptides were added to the cells, or the calvarial bones, for 5 (cells) and 10 min (calvaria), respectively. Intracellular cyclic AMP was then extracated with 90% *n*-propanolol. The samples were evaporated, reconstituted in assay buffer and cyclic AMP analyzed using a commercially available RIA kit. The amount of cell protein was analyzed using the BCA protein assay kit.

STATISTICS

All statistical analyses were performed using one-way 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

EFFECTS ON BONE RESORPTION IN NEONATAL MOUSE CALVARIAL BONES

The effects by all known members of the CT family of peptides on ^{45}Ca release in mouse calvarial bones stimulated by PTH (10 nmol/L) was initially assessed using a wide range of concentrations of the peptides. As shown in Figure 1A, mouse IMD and porcine CRSP-1, similar to salmon CT, rat α-CGRP, rat β-CGRP, and rat AMY, concentration-dependently inhibited PTH stimulated ^{45}Ca release when assessed in bones prestimulated by PTH for 24 h and subsequently incubated with the hormone and the peptides for another 24 h. In contrast, rat ADM had no effect on PTH prestimulated ^{45}Ca release. Salmon CT was several orders of magnitude more potent than all the other peptides with half maximal inhibition (IC_{50}) at 3 pmol/L. The potency of α-CGRP, β-CGRP, and AMY were rather similar with IC_{50} at approximately 10–30 nmol/L, whereas IMD and CRSP were less potent with IC_{50} at approximately 0.3 μmol/L. IMD (1 μmol/L) and CRSP-1 (1 μmol/L), similar to CT (1 nmol/L), CGRP (1 μmol/L), and AMY (1 μmol/L), but not ADM (1 μmol/L), inhibited ^{45}Ca release in the calvarial bones prestimulated also by 1,25$(OH)_2$-vitamin D3 (10 nmol/L) and treated with the peptides for a subsequent 24-h period (Fig. 1B).

Detailed time-course experiments showed that the onset of inhibition of PTH stimulated ^{45}Ca release by IMD (1 μmol/L) and CRSP-1 (1 μmol/L) was rapid, with significant ($P < 0.05$) effects observed already at 3 h (first time point studied; Fig. 1C). Similarly, the effects by CT (1 nmol/L), CGRP (1 μmol/L), and AMY (1 μmol/L) were rapid with significant effects ($P < 0.05$) observed also at 3 h (Fig. 1D). The inhibitory effects of all five peptides progressively increased with time for 24 h, at which time point the inhibition levelled-off and bone gradually started to release increasing amounts of ^{45}Ca.

All the peptides that inhibited hormone stimulated ^{45}Ca release (IMD and CRSP-1 as well as CT, CGRP, and AMY,) significantly decreased also PTH prestimulated bone matrix degradation, as assessed by the release of collagen type 1 degradation product to the culture medium (Fig. 1E).

These data indicate that mature osteoclasts express functional receptors for CT, CGRP, AMY, CRSP, and IMD, but not for ADM.

EFFECTS ON CYCLIC AMP FORMATION IN NEONATAL MOUSE CALVARIAL BONES

Incubation of PTH prestimulated neonatal mouse calvarial bones with CT (1 nmol/L), CRSP-1 (1 μmol/L), CGRP (1 μmol/L), and AMY (1 μmol/L) for 10 min resulted in significant fourfold enhancement of cyclic AMP formation (Fig. 2A). IMD (1 μmol/L) and ADM (1 μmol/L) did not cause a statistically significant effect on cyclic AMP formation in the calvarial bones.

Addition of forskolin (10 μmol/L), which increases cyclic AMP formation through receptor independent activation of adenylate cyclase, abolished ^{45}Ca release in bone prestimulated by PTH (Fig. 2B). Cyclic AMP can bind to and activate the protein kinase A (PKA)/CREB pathway, as well as the exchange protein directly activated by cyclic AMP (Epac) pathway, causing phosphorylation of several transcription factors via Rap1/MEK/ERK pathway [Kawasaki et al., 1998; de Rooij et al., 1998; Holz et al., 2006]. We, therefore, added cyclic AMP analogs with affinity to either PKA or Epac to PTH prestimulated bones. 8-bromo-cyclic AMP (100 μmol/L), which is a non-selective analog, inhibited PTH stimulated ^{45}Ca release (Fig. 2B). In contrast, the PKA selective analog 6-monobutyryl-cyclic AMP (50 μmol/L), and the Epac selective analog 8-MeOPT-cyclic AMP (50 μmol/L), as well as the inactive control analog 8-bromo-5′-AMP (50 μmol/L), did not affect the response to PTH (Fig. 2B). Similar observations were made when the concentrations of the inactive analogs were increased to 100 μmol/L (data not shown).

EFFECTS ON OSTEOCLASTOGENESIS

IMD (1 μmol/L) and CRSP-1 (1 μmol/L) significantly inhibited the formation of $TRAP^+$ MuOCL in BMM cultures stimulated by M-CSF and RANKL (Fig. 3A,B). Similarly, CT (1 nmol/L), CGRP (1 μmol/L), and AMY (1 μmol/L) also significantly inhibited M-CSF/RANKL stimulated formation of $TRAP^+$ MuOCL (Fig. 3A,B). In contrast, ADM (1 μmol/L) did not affect $TRAP^+$ MuOCL formation. Inhibition by IMD and CRSP-1, as well as by CT, CGRP, and AMY, was associated with the presence of very many $TRAP^+$ mononucleated cells (Fig. 3B). The few $TRAP^+$ MuOCL still formed in M-CSF/RANKL treated BMM cultures additionally treated with IMD, CRSP-1, CT, CGRP, or AMY were clearly smaller than the large $TRAP^+$ MuOCL observed in M-CSF/RANKL or M-CSF/RANKL, and ADM treated cultures (Fig. 3B). There were no morphological signs of reduced number of cells or toxicity caused by IMD, CRSP-1, or the other peptides (Fig. 3B). In agreement with the morphology, IMD, CRSP-1, CT, CGRP, and AMY did not significantly affect total TRAP activity in BMM cultures (Fig. 3C), in which all of them significantly inhibited formation of $TRAP^+$ MuOCL (data not shown).

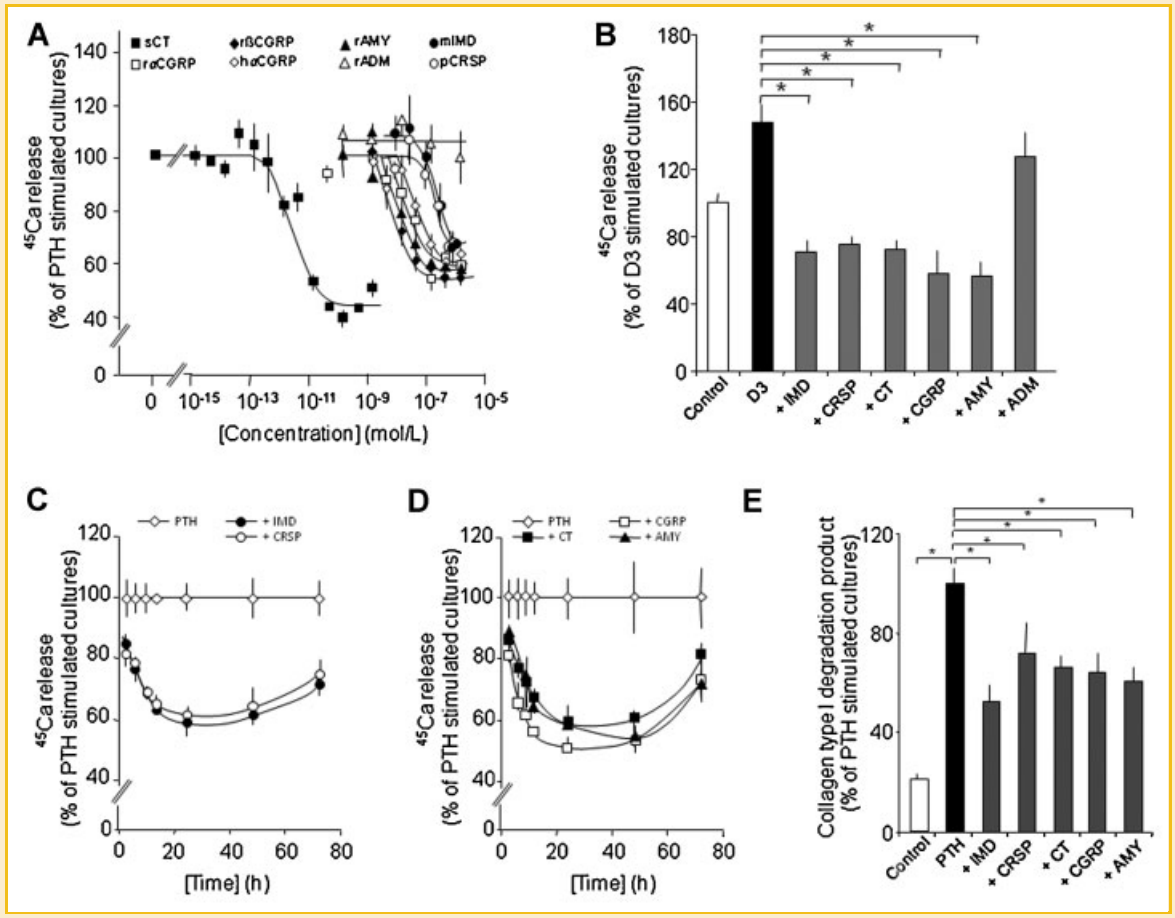

Fig. 1. Inhibitory effects by peptides in the CT family of peptides on mineral release and bone matrix degradation in neonatal mouse calvariae stimulated to resorb by parathyroid hormone or 1,25(OH)$_2$ vitamin D3. Bones were pre-stimulated by PTH (10 nmol/L) for 24 h and then sCT, rαCGRP, rβCGRP, hαCGRP, rAMY and rADM, mIMD, and pCRSP-1 were added for 24 h, at different concentrations, to the actively resorbing bones (A). After pre-stimulating bones with 1,25(OH)$_2$ vitamin D3 (10 nmol/L) for 24 h, 1 μmol/L (IMD, CRSP, CGRP and AMY) or 1 nmol/L (CT) were added for 24 h (B). In C and D bones were pre-stimulated by PTH (10 nmol/L) for 24 h and then 1 μmol/L (IMD, CRSP, CGRP, AMY, and ACM) or 1 nmol/L (CT) were added and release of ^{45}Ca was analyzed at different time points during a 72-h culture period. Mineral release was assessed by analyzing the percentage ^{45}Ca release (A–D). Matrix degradation was assessed by analyzing the concentration of collagen type I degradation fragment in culture media (E). The data shown are expressed as percent of PTH, or 1,25(OH)$_2$ vitamin D3, set to 100%. In A, dose–response curves are shown for the peptides on ^{45}Ca release, in (B–E) are shown the effects by sCT (1 nmol/L) and IMD, CRSP, CGRP, and AMY (all at 1 μmol/L) on ^{45}Ca release (B–D) and in E on bone matrix degradation. In all experiments, PTH or D3 were present also during the period when bones were treated with the peptides in the CT family. Symbols represent means for 5–12 bones and SEM is shown as vertical bars when larger than the height of the symbol. Statistically significant effects are shown by asterisks in B and E; $^*P < 0.05$.

M-CSF time-dependently enhanced the number of BMM, as assessed by MTT activity, an effect which was essentially unaffected by IMD (1 μmol/L) or CRSP-1 (1 μmol/L; Supporting Information 1A). Similarly, IMD and CRSP-1 did not affect MTT activity in M-CSF/RANKL stimulated BMM (Supporting Information 1B).

Time-course study showed that stimulation of BMM with M-CSF (100 ng/ml) and RANKL (100 ng/ml) resulted in formation of TRAP$^+$ MuOCL, which could be observed at 72 h and was further enhanced at 96 h (Fig. 3D). IMD (1 μmol/L) and CRSP-1 (1 μmol/L) caused an approximately 50% inhibition of the number of TRAP$^+$ MuOCL at both time points (Fig. 3D).

The inhibitory effect of CRSP-1 and IMD on formation of TRAP$^+$ MuOCL was concentration-dependent with IC_{50} at approximately 0.3 μmol/L (Fig. 3E,F).

The inhibitory effect of IMD and CRSP-1 on osteoclastogenesis could be observed also when adding the peptides during the last 24 h of M-CSF/RANKL stimulated formation of TRAP$^+$ MuOCL in BMM cultures (Fig. 3G).

The inhibitory effect by IMD and CRSP-1 could also be demonstrated using FACS sorted CD115$^+$ CD3$^-$ CD45R$^-$ BMM stimulated by M-CSF and RANKL (Supporting Information 2).

Formation of osteoclasts in RANKL stimulated BMM cultures is considerably slower when cells are cultured on bovine bone compared to BMM cultures on plastic. Therefore, the BMM cultures on bone slices were extended to 9 days. In addition, the osteoclasts have a different morphology with more cellular extensions when cultured on bone. The enhanced number of TRAP$^+$MuOCL observed in M-CSF/RANKL stimulated BMM on bone slices was substantially reduced by CT (1 nmol/L), IMD (1 μmol/L), and CRSP (1 μmol/L) (Fig. 4A upper panel). Toluidine blue stained excavations were abundant in M-CSF/RANKL stimulated BMM (higher magnification of these excavations is shown in Supporting Information 3) and very

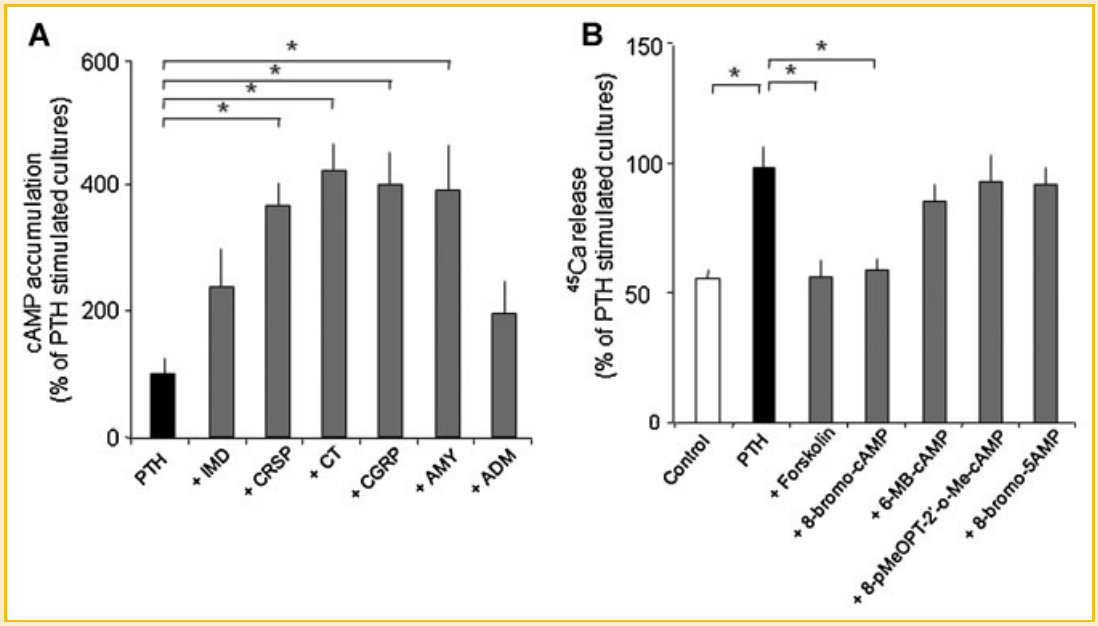

Fig. 2. Receptor activation was analyzed by measuring cAMP levels in neonatal calvarial bones (A). Bones were pre-stimulated by PTH (10 nmol/L) for 24 h and then incubated without or with CT (1 nmol/L), CGRP (1 μmol/L), AMY (1 μmol/L), ADM (1 μmol/L), IMD (1 μmol/L), and CRSP (1 μmol/L) (A) for 10 min. Cyclic AMP in the bones was analyzed using RIA. The role of PKA and Epac pathways in cyclic AMP signaling were analyzed in PTH-stimulated bones by adding specific and non-specific compounds activating either on one, or both, of these pathways (B). Inhibitory effects by forskolin and cyclic AMP analogs on mineral release were analyzed in neonatal calvarial bones. Bones were pre-stimulated by PTH (10 nmol/L) for 24 h after which forskolin (10 μmol/L), 8-bromo-cyclic AMP (100 μmol/L), 6-monobutyric-cyclic AMP (50 μmol/L), 8-MeOPT-cyclic AMP (50 μmol/L) and 8-bromo-5′-AMP (50 μmol/L) were added together with PTH, to the actively resorbing bones for 24 h (B). Mineral release was assessed by analyzing the percentage ^{45}Ca release. The data shown are expressed as percent of PTH, set to 100%. Columns represent means for seven bones and SEM is shown as vertical bars. Statistically significant effects are shown by asterisk; $^{*}P < 0.05$.

few such excavations were observed when cells in addition were treated with CT, IMD, or CRSP (Fig. 4A lower panel). In agreement with these observation, the release of CTX from M-CSF/RANKL stimulated BMM at days 7–9 was significantly inhibited by CT, IMD, and CRSP (Fig. 4C).

EFFECTS ON GENE EXPRESSION IN BMMs

The robust stimulation by RANKL of the mRNA expression of *Acp5*, *mmp9*, *tcirg1*, and *oscar* was unaffected by IMD (1 μmol/L), and CRSP-1 (1 μmol/L) (Fig. 5A–D). Similarly, CT (1 nmol/L), CGRP (1 μmol/L), AMY (1 μmol/L), and ADM (1 μmol/L) did not affect M-CSF/RANKL induced enhanced mRNA expression of *Acp5*, *mmp9*, *tcirg1*, and *oscar*. Nor did CRSP or IMD affect RANKL induced mRNA expression of DC-STAMP as assessed by semi-quantitative RT-PCR (Supporting Information 4).

EFFECTS ON CYCLIC AMP FORMATION IN BMMs

Our observations indicate that osteoclast formation and activity are sensitive to inhibition by CT, CRSP-1, CGRP, AMY, IMD but not to ADM. We have previously reported that CTR (assumed to be necessary for the response to CT and CRSP) is not expressed in early osteoclast progenitors but induced during osteoclast differentiation, whereas CRLR and RAMPs (assumed to be necessary for the response to CGRP, AMY, ADM, and IMD) are expressed in the osteoclast progenitors but not regulated during their differentiation [Granholm et al., 2008].

In BMM cultures stimulated by M-CSF, significant enhancement of cyclic AMP formation was caused by IMD (1 μmol/L), as well as by CGRP (1 μmol/L), AMY (1 μmol/L), and ADM (1 μmol/L) after both 48 and 96 h of M-CSF treatment (Fig. 6A). In contrast, CT (1 nmol/L) did not cause any cyclic AMP enhancement in the M-CSF treated BMM cells, whereas CRSP-1 (1 μmol/L) caused a small increase which was not statistically significant (Fig. 6A).

In cells stimulated by M-CSF and RANKL for 48 h, IMD, CRSP-1, CT, CGRP, and AMY stimulated cyclic AMP formation, a response, which was progressively increased with time (Fig. 6B). In contrast, the cyclic AMP response to ADM was not enhanced during osteoclast differentiation (Fig. 6B).

When the cyclic AMP response to all peptides at a wide range of concentrations was assessed it was observed that ADM, AMY, CGRP, and IMD, but not CT or CRSP-1, concentration dependently enhanced cyclic AMP in M-CSF stimulated BMM (Fig. 6C–H). Threshold for action was ≥0.1 μmol/L for ADM, AMY, and IMD, whereas CGRP was more potent with effects observed at and above 0.3 nmol/L. In M-CSF/RANKL stimulated BMM, CT, and CRSP-1 concentration dependently enhanced cyclic AMP formation with CT (≥7 pmol/L) being considerably more potent than CRSP-1 (≥30 nmol/L; Fig. 6F,G), as expected. Less expected was the observation that the cyclic AMP response to AMY and IMD was clearly up-regulated in M-CSF/RANKL stimulated BMM (Fig. 6D,H). At variance, the effect by CGRP at higher concentrations was also enhanced in M-CSF/RANKL stimulated osteoclast progenitors whereas the response at lower concentrations was decreased

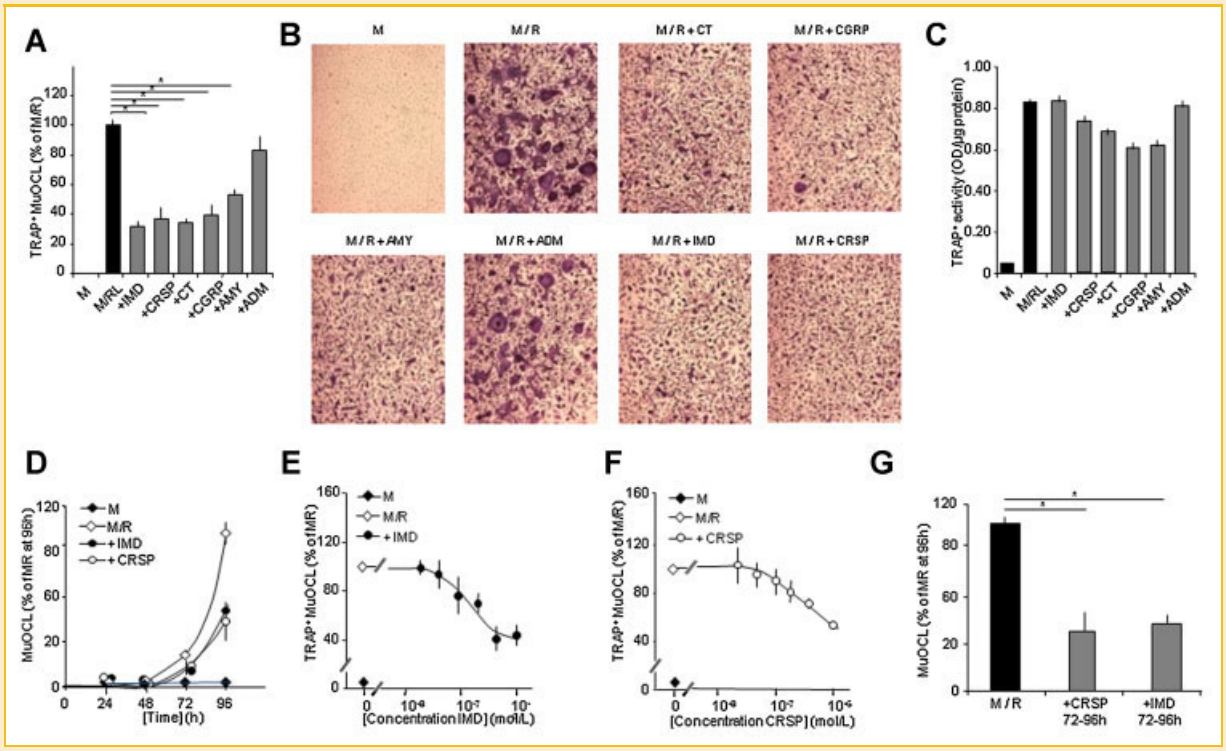

Fig. 3. IMD (1 μmol/L), CRSP (1 μmol/L), CGRP (1 μmol/L), AMY (1 μmol/L), and CT (1 nmol/L), but not ADM (1 μmol/L), inhibited the number of osteoclasts ($TRAP^+$MuOCL) formed in BMM cultures stimulated by M-CSF (100 ng/ml) and RANKL (100 ng/ml) for 96 h (A); in cultures treated with 1 μmol/L IMD, CRSP, CGRP, or AMY a large number of $TRAP^+$ mononucleated cells was formed (B). Neither IMD (1 μmol/L) or CRSP (1 μmol/L), nor CT (1 nmol/L), CGRP (1 μmol/L), AMY (1 μmol/L), or ADM (1 μmol/L) significantly affected total TRAP activity in M-CSF/RANKL stimulated BMM cultures (C). The inhibitory effects of IMD and CRSP on osteoclast formation were dependent on time (D) and concentration (E,F) and could be obtained by adding the peptides during the last 24 h of a 96 h culture (G). M-CSF is denoted M in the figure and M-CSF + RANKL is denoted as M/R. Symbols and columns represent the mean of six wells per group and SEM is given as vertical bars when larger than the height of the symbol. Statistically significant effects are shown by asterisks in A and G; $^*P < 0.05$.

(Fig. 6E). Most interestingly, the strong cyclic AMP response to ADM observed in M-CSF stimulated BMM was profoundly decreased in M-CSF/RANKL stimulated BMM (Fig. 6C).

DISCUSSION

Peptides in the CT superfamily of peptides, including CT, CGRP, AMY, IMD, and CRSP, but not ADM, inhibited bone resorption in organ cultured mouse calvarial bones as assessed either by mineral release or extracellular matrix degradation. This is the first report demonstrating inhibition of bone resorption in vitro by the most recently discovered members IMD and CRSP. The effects on periosteal bone resorption in calvariae were seen in bones stimulated by a peptide hormone (PTH) as well as by a steroid hormone (1,25(OH)2-vitamin D3), which stimulate bone resorption by different signal transduction pathways. The effects in PTH-stimulated bones were concentration dependent with the following rank order potency: salmon CT > > > > rat α-CGRP, rat β-CGRP, rat AMY > mouse IMD, porcine CRSP. It should be pointed out that the interpretation of the relative potencies should take into account that we used peptides from different species and that the target cells were from mice.

The effects by the peptides in the CT superfamily of peptides on bone resorption were assessed in bones prestimulated for 24 h with PTH or $1,25(OH)_2$-vitamin D3 in order to study their effects on active, preformed osteoclasts, rather than on osteoclast formation. The fact that inhibition of PTH stimulated ^{45}Ca release could be seen already 3 h after addition of the peptides indicates that the mechanism of action is due to reduced osteoclast activity. It has previously been shown that CT, CGRP, AMY, and CRSP can inhibit the activity of isolated mature osteoclasts [Zaidi et al., 2002; Notoya et al., 2007]. Taken together, the findings in the present study, together with previous data, indicate that IMD and CRSP, similar to CT, CGRP, and AMY, are all able to cause an acute effect on bone resorption due to a direct inhibitory effect on the activity of mature multinucleated osteoclasts. In contrast, ADM has no such effects (see further below).

Typically, the rapid inhibitory effect on PTH stimulated mineral release by IMD and CRSP was transient, as previously been reported for CT, CGRP, and AMY [reviewed in Zaidi et al., 2002; Lerner, 2006] and confirmed in the present study. This phenomenon has been described as "escape from CT induced inhibition of bone resorption" [Wener et al., 1972] and has been speculated to be due to down regulation of CTR induced by the hormone itself [Samura et al., 2000]. However, since not only peptides acting via the CTR, but also

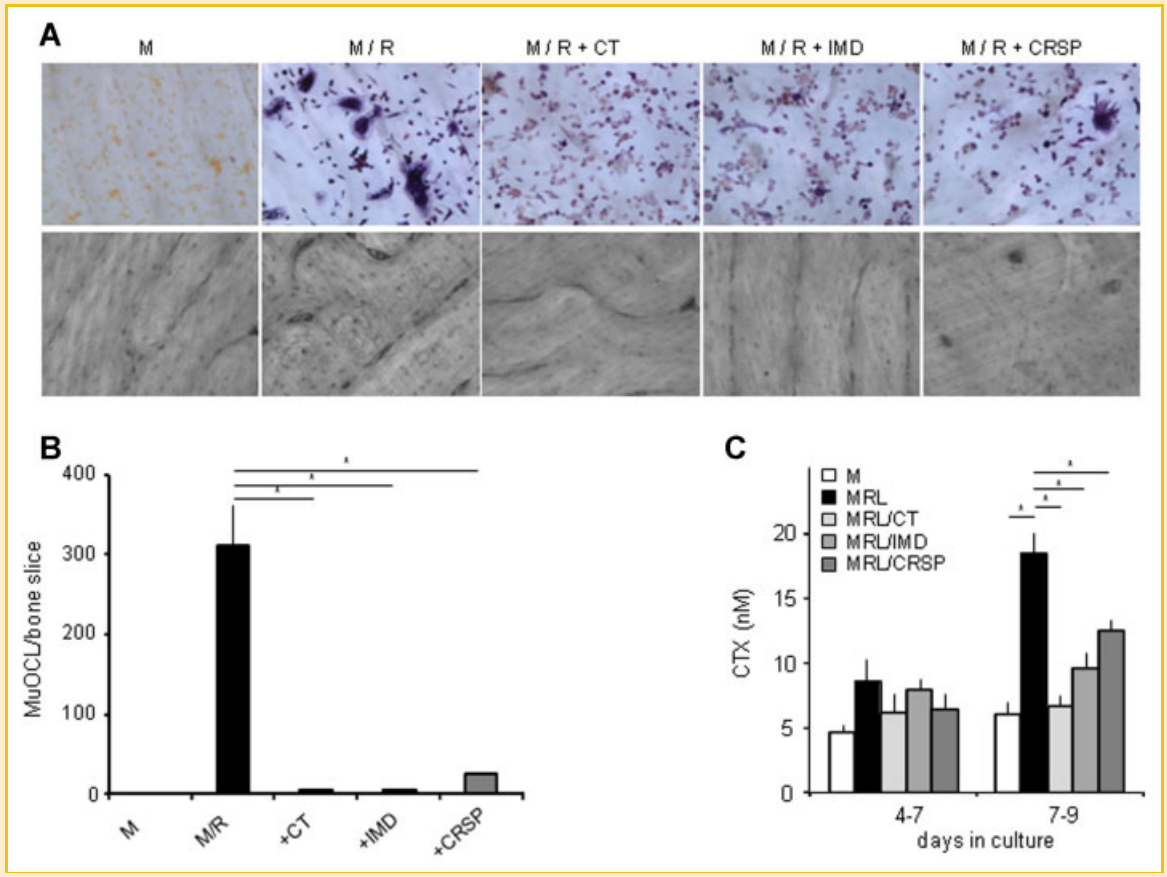

Fig. 4. RANKL (100 ng/ml) induced osteoclast formation on bovine bone slices as well as the appearance of resorption pits are inhibited by CT (1 nmol/L), IMD (1 μmol/L) and CRSP (1 μmol/L). TRAP staining of osteoclasts on bone (A, top). Toluidine blue staining of resorption pits (A, bottom). Quantification of TRAP positive multinucleated osteoclasts (MuOCL) on bone slices (B). Collagen type I fragments (CTX) in culture medium collected during days 4–7 and 7–9 (C). Columns represent the mean of four wells and statistically significant effects are shown by asterisks; $^{*}P < 0.05$.

peptides acting via CRLR/RAMPs (CGRP, IMD), cause this transient effect, it is not likely that CTR induced decreased *Calcr* expression is the mechanism. We have recently shown that CRLR and RAMP proteins in M-CSF/RANKL stimulated BMM are not regulated during osteoclastogenesis, nor is the expression of these molecules affected by challenge with any of the peptides in CT family of peptides [Granholm et al., 2008]. In contrast, *Calcr* was induced by RANKL and down-regulated by CT, as well as by CGRP, AMY, ADM, IMD, and CRSP-1 [Granholm et al., 2008], which indicates that signaling through both CTR and CLRL/RAMPs can down-regulate *Calcr* expression.

It has been shown in some studies that CT and CGRP can inhibit osteoclast formation in crude bone marrow cell cultures [Takahashi et al., 1982; Ibbotson et al., 1984; Roodman et al., 1985; Cornish et al., 2001] and recently, we have shown that this effect is due to a direct effect by CT on RANKL stimulated osteoclast precursor cells [Granholm et al., 2007]. Similarly, AMY also can inhibit osteoclastogenesis due to an effect on osteoclast progenitor cells [Dacquin et al., 2004]. It has recently been reported that also CRSP-1 can inhibit the formation of osteoclasts [Notoya et al., 2007]. In the present study, we show that IMD and CRSP, similar to CT, CGRP, and AMY, but not ADM, can inhibit osteoclastogenesis by a mechanism due to a direct effect on osteoclast progenitor cells. This was shown by demonstrating an inhibition by the peptides of the number of $TRAP^{+}$ multinucleated cells in RANKL stimulated BMM cultures on plastic and by showing that IMD and CRSP inhibit osteoclast formation also when RANKL stimulated BMM was cultured on bone slices. The effects on osteoclastogenesis by IMD and CRSP, the most recent members of the CT superfamily of peptides, were shown to be concentration dependent with apparent IC_{50} at approximately 0.3 μmol/L, similar to their potency in the mouse calvarial bone resorption bioassay. There were no morphological signs of cytotoxicity by IMD and CRSP in the BMM cultures, nor did these peptides affect cell proliferation as assessed by the MTT assay. Similar to CT [Granholm et al., 2007], IMD and CRSP could be added during the last 24 h of the osteoclastogenesis assay and still exert their effects, indicating that the mechanism is due an effect late during osteoclastogenesis unrelated to effects on osteoclast progenitor cell proliferation or survival. This is further indicated by the fact that very many $TRAP^{+}$ mononucleated cells were observed in the IMD and CRSP treated cells, as well as in CGRP and AMY treated cells, again similar to our observations in CT treated RANKL stimulated BMM cultures [Granholm et al., 2007]. Interestingly, the multinucleated osteoclasts still formed in IMD

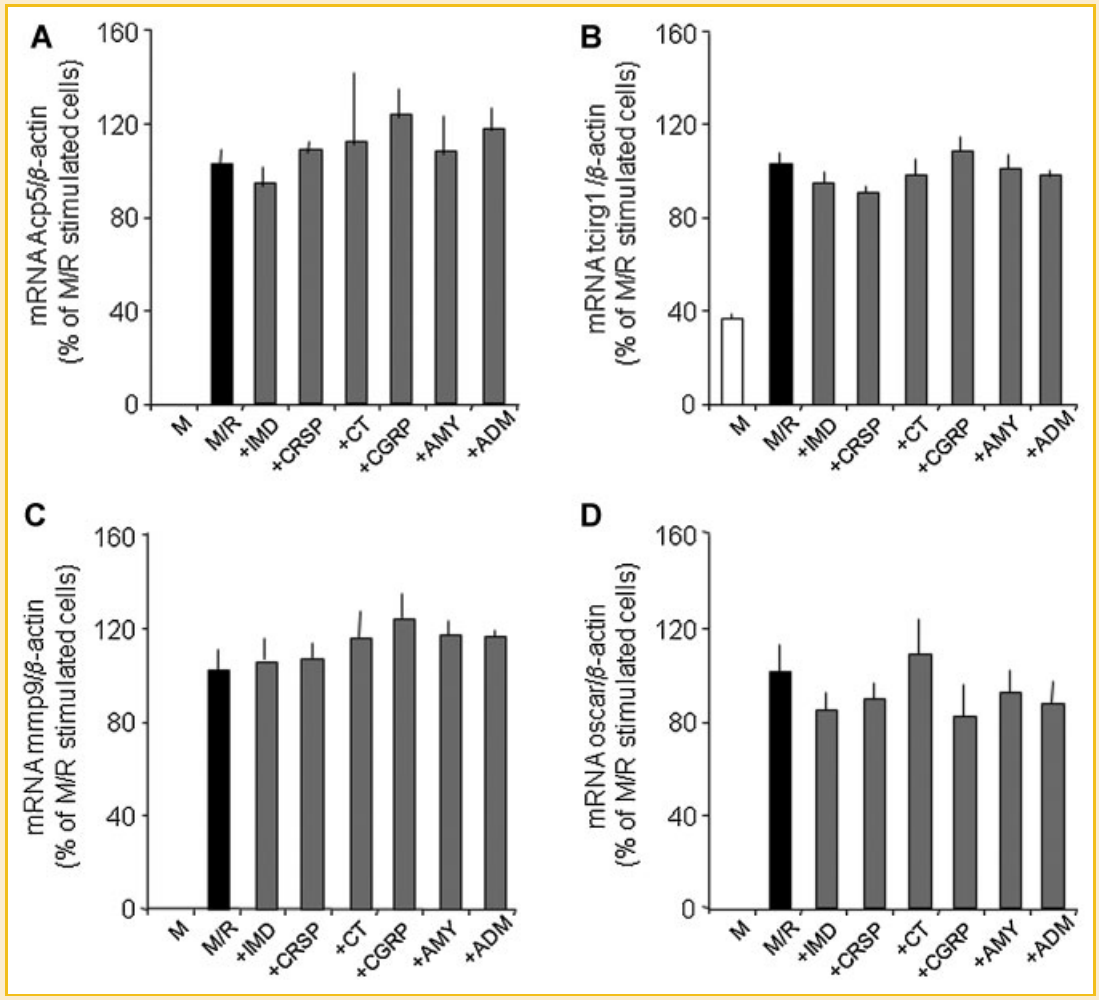

Fig. 5. Quantitative real-time PCR analysis of the effects of CT (1 nmol/L), CGRP (1 μmol/L), AMY (1 μmol/L), ADM (1 μmol/L), IMD (1 μmol/L), and CRSP (1 μmol/L) on the mRNA expression of *Acp5* (A), *tcirg1* (B), *mmp9* (C), and *oscar* (D) in BMM stimulated with M-CSF (100 ng/ml), and RANKL (100 ng/ml) for 96 h. As a control, cultures without RANKL were included in the analysis. M-CSF is denoted M in the figure and M-CSF + RANKL is denoted as M/R. Columns represent the mean of four wells and SEM is shown as vertical bars.

and CRSP treated BMM cultures were clearly smaller than those in M-CSF/RANKL control cultures.

We have previously shown that inhibition of osteoclast formation due to CTR signaling, caused by adding CT to BMM, is not associated with any effects of M-CSF/RANKL induced expression of very many genes known to be associated with differentiation, fusion or function of osteoclasts [Granholm et al., 2007]. In the present study, we confirm this observation by showing that AMY and CRSP, two other peptides assumed to signal through the CTR, also inhibit osteoclast formation without affecting the mRNA expression of *Acp5, mmp-9, tcirg1*, and *oscar*. We also show that CGRP and IMD, assumed to signal through CRLR/RAMPs, do not affect the mRNA expression of these RANKL induced osteoclastic genes. Thus, IMD and CRSP, similar to CT, CGRP, and AMY, seem to inhibit osteoclast formation independent on c-Fms and RANK signaling. The fact that the mononuclear BMM cells observed in cultures stimulated by RANKL together with either CRSP or IMD were $TRAP^+$, and that the peptides could be added late during osteoclastogenesis and still inhibit formation of mature osteoclasts, suggest the possibility that the peptides inhibited fusion of osteoclast progenitor cells. However, CRSP or IMD had no effect on the mRNA expression of DC-STAMP, which has been suggested to be important for the fusion process [Kukita et al., 2004].

The data in the present study show that ADM does not affect bone resorption or osteoclast formation, in contrast to the other members of the CT superfamily of peptides. Previously, Cornish et al. [1997] have reported that ADM does not affect basal or PTH stimulated bone resorption in mouse calvarial bones treated with ADM for 48 h, nor did local injection of ADM over the periosteum affect bone resorption or osteoclast number in the calvarial bones. The most likely explanation is that neither mature, nor osteoclast progenitor cells, express ADM receptors. In the calvarial bones, ADM enhanced cyclic AMP formation indicating the presence of ADM receptors. However, it is not possible to know in which cells this rise was caused since the calvarial periosteum contains several different hematopoetic and mesenchymal cell types. We, therefore, also

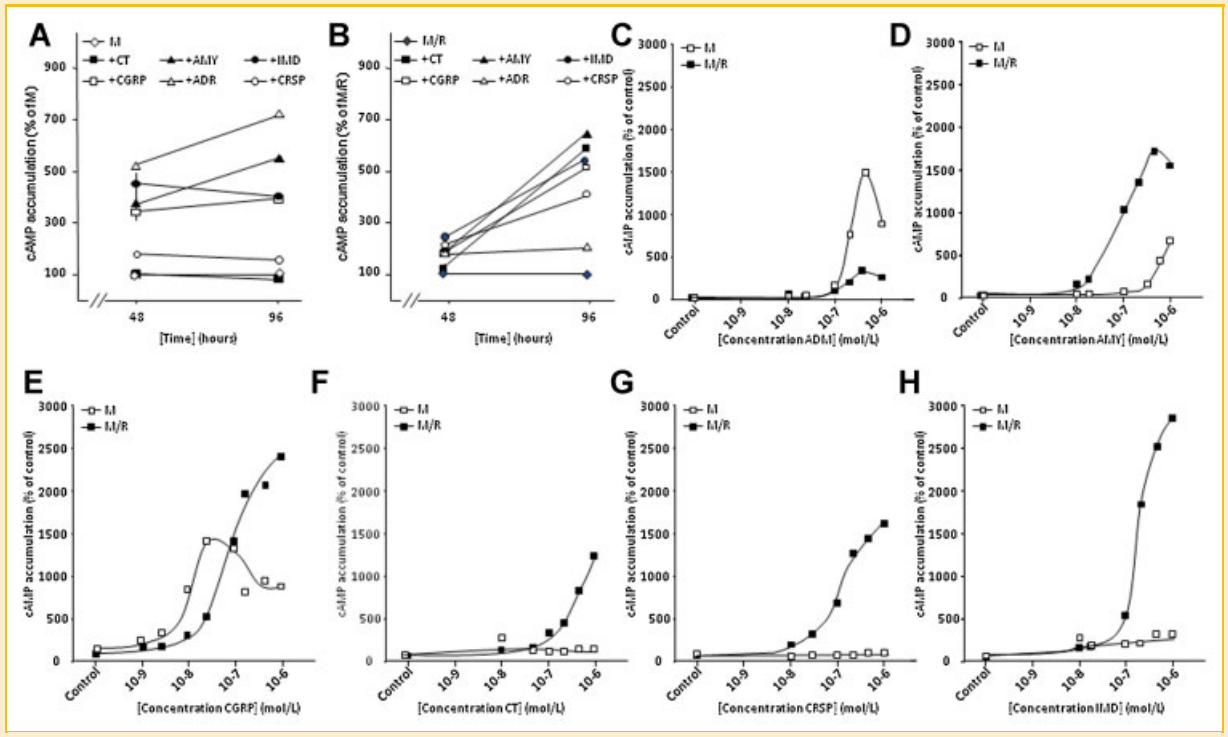

Fig. 6. Receptor activation was analyzed by measuring cyclic AMP levels in BMM cells, cultured for 48 and 96 h in the presence of 100 ng/ml M-CSF (A) or M-CSF in combination with 100 ng/ml RANKL (B). Cyclic AMP was measured 5 min after addition of CT (1 nmol/L), CGRP (1 μmol/L), AMY (1 μmol/L), ADM (1 μmol/L), IMD (1 μmol/L), and CRSP (1 μmol/L) and the concentration corrected for cell protein. In C–H is shown the effects of ADM (C), AMY (D), CGRP (E), CT (F), CRSP (G), and IMD (H) at different concentration in either M-CSF treated BMM or in M-CSF/RANKL pretreated BMM. M-CSF is denoted M in the figure and M-CSF + RANKL is denoted as M/R. Symbols represent the mean of four wells and SEM is shown as vertical bars, when larger than the radius of the symbol. Statistically significant effects ($P < 0.05$) in A, were observed at 2 and 4 days for CGRP, AMY, ADM, and IMD. Statistically significant effects ($P < 0.05$) in B, were observed at 2 days for CGRP, AMY, ADM, IMD, and CRSP, and at 4 days for CT, CGRP, AMY, IMD, and CRSP.

studied ADM signaling in M-CSF expanded osteoclast progenitors and could demonstrate a cyclic AMP response to ADM, as well as to CGRP, IMD, and AMY, but not to CT or CRSP-1. This shows that osteoclast progenitor cells express ADM receptors, a finding in agreement with our observation that these cells express CRLR and RAMP1-3 mRNA and protein [Granholm et al., 2008]. The fact that M-CSF stimulated BMM cells did not respond to CT or CRSP-1 is in line with the fact that these cells do not express CTR [Granholm et al., 2008]. In order to study the possibility that the lack of effect by ADM on osteoclast activity and formation may be due to that the responsiveness was affected during differentiation of the BMM along the osteoclastic lineage, we also studied ADM induced cyclic AMP formation in RANKL stimulated BMM. Our data demonstrate that the cyclic AMP rise induced by ADM was substantially deceased in M-CSF/RANKL stimulated BMM compared to that seen in BMM treated with M-CSF alone. In contrast, the responsiveness to CGRP, AMY, and IMD was enhanced by RANKL treatment and those to CT and CRSP-1 induced. What is the reason for the decreased ADM responsiveness? It is not likely that down-regulation of CRLR expression is the mechanism since we have not been able to demonstrate any down-regulation of CRLR mRNA and protein in RANKL stimulated BMM [Granholm et al., 2008]. As regards the RAMPs, we could demonstrate a decrease of RAMP1 mRNA during the initial stages of osteoclastogenesis, but no effect on RAMP1 protein. ADM is known to signal through CRLR and RAMP2 or RAMP3, but we could not observe any differences in RAMP2 or 3 mRNA or protein during osteoclast differentiation [Granholm et al., 2008]. This argues for that decreased receptor expression is not the reason for decreased ADM induced cyclic AMP response. Although the mechanism for decreased ADM signaling is not shown, our data strongly indicate that the lack of effect by ADM on osteoclast activity and formation is due to decreased ADM signaling and that ADM in this respect is different from the other peptides in the CT family of peptides. The observation might also indicate the possibility of the presence of unknown ADM receptors distinct from CRLR/RAMPs.

The data presented indicate that CTR is necessary for CT and CRSP-1 signaling, but not for AMY, IMD, CGRP, or ADM. This is in line with the view that IMD, CGRP, and ADM can act via CRLR/RAMPs, but at variance from the view that AMY is regarded to signal through CTR/RAMPs. The fact that enhanced bone resorption seen in *AMY* null mice can be observed in the compound heterozygote *CTR* and *AMY* deficient mice ($Calcr^{+/-}/Amylin^{-/-}$), also shows that AMY can inhibit bone resorption independently of the CTR [Dacquin et al., 2004]. The observation that the cyclic AMP response to AMY and IMD was clearly up-regulated in RANKL stimulated BMM at the same concentrations stimulating cyclic AMP in non-RANKL stimulated osteoclast progenitors, indicate that AMY and IMD not

only acts through CRLR but also through CTR. The cyclic AMP response to CGRP at low concentrations was decreased in RANKL stimulated BMM whereas that to higher concentrations was enhanced. We have no explanation for this different effect but it might be that CGRP at low concentrations and ADM share some receptor component or signal transduction pathway, which is decreased during osteoclastogenesis. At high concentrations, CGRP may signal also through CTR, similar to AMY and IMD. Final proof for which receptors are used for the different peptides must, however, await selective knockout or knockdown of CTR, CRLR, and RAMPs in osteoclasts.

Activation of adenylate cyclase and cyclic AMP leads either to activation of PKA in the canonical pathway or to activation of a non-canonical pathway involving Epac and Rap1/ERK/MEK [Kawasaki et al., 1998; de Rooij et al., 1998; Holz et al., 2006]. We have recently shown that osteoclast formation induced by M-CSF/RANKL stimulation of BMM can be inhibited not only by cyclic AMP analogues preferentially activating the canonical pathway but also by one analogue activating Epac, which indicates that the inhibitory effect by CT, CGRP, AMY, IMD, and CRSP-1 on differentiation of mononuclear osteoclast progenitor cells to mature osteoclasts can be regulated by signaling through either of these pathways. In the present study, we found that forskolin (increasing cyclic AMP independent on receptor activation) and a non-selective cyclic AMP analog inhibited PTH prestimulated bone resorption in neonatal mouse calvarial bones, but that cyclic AMP analogs selective for either the canonical or the Epac pathway were without effects. These findings indicate that parallel signaling through both pathways seems to be crucial for the inhibitory effect on osteoclast activity.

In summary, we have demonstrated that the newly discovered peptides IMD and CRSP, similar to CT, CGRP, and AMY, inhibit bone resorption and osteoclast formation. In contrast, ADM does not have any of these effects; most likely due to that ADM signaling was decreased during RANKL induced osteoclast differentiation. Our data further show that AMY, CGRP and IMD signaling include activation through both CTR and CRLR and that both the PKA and Epac pathways can independently of each other inhibit osteoclast formation but that both pathways are required to inhibit osteoclast activity.

ACKNOWLEDGMENTS

We are grateful for the excellent support by Mrs. Ingrid Boström for drawing the graphs.

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