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Immature human osteoblastic MG63 cells predominantly express a subtype 1-like CGRP receptor that inactivates extracellular signal response kinase by a cAMP-dependent mechanism

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Abstract

Although accumulated data suggest that calcitonin gene-related peptide (CGRP) produces anabolic effects in skeletal tissue by directly acting on osteogenic cells, neither the distribution of CGRP receptor subtypes nor the associated cellular signaling pathways are well understood. In this study, we have pharmacologically and biochemically characterized CGRP-binding sites in immature human osteoblastic MG63 cells. In a [^{125}I]CGRP whole-cell-binding assay, nonlinear regression curve-fitting analysis demonstrated a single binding site ($K_D = 405 \pm 29$ pM; $13{,}100 \pm 223$ sites per cell). Immunocytochemical and Western blot analyses demonstrated that 48-, 52-, and 120-kDa forms of the calcitonin receptor-like receptor (CRLR) and a 15-kDa form of the receptor-activity-modifying protein-1 (RAMP-1) was expressed on the plasma membrane. CGRP strongly stimulated cellular cAMP production and this effect was antagonized not only by an antagonist of the subtype-1 CGRP ($CGRP_1$) receptor, CGRP-(8-37), but by an agonist of the putative subtype-2 CGRP ($CGRP_2$) receptor, [Cys(Acm)^{2,7}]-CGRP, that also itself acted as a weak agonist. In contrast to published data, CGRP dose- and time-dependently dephosphorylated and inactivated extracellular signal response kinase (ERK). This action was blocked by CGRP-(8-37), by an inhibitor of cAMP-dependent protein kinase (H-89), or by an inhibitor of protein phosphatases (vanadate). Prolonged CGRP treatments significantly suppressed DNA synthesis at 27 h, but up-regulated type I collagen. Both these actions were blocked by CGRP-(8-37) and mimicked by a specific inhibitor of ERK (PD98059). In summary, our data suggest that the CGRP receptors in MG63 cells meet many, but not all, of the classical criteria used to define $CGRP_1$ receptors. These receptors that functioned in a pharmacologically distinct manner could inhibit cell proliferation, and were substantially more sensitive to a $CGRP_2$ receptor agonist than are typical $CGRP_1$ receptors. These receptor proteins were not exactly matched with the known components of a $CGRP_1$ receptor that have been reported. Therefore, it is possible that the CGRP receptors expressed in immature osteoblastic human MG63 cells represent a variation of the known $CGRP_1$ receptor.
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Keywords: Calcitonin receptor-like receptor; CGRP (Calcitonin gene-related peptide); MAP (Mitogen-activated protein) kinase; Proliferation; Osteoblast

1. Introduction

Throughout skeletal tissue, prominent calcitonin gene-related peptide (CGRP)-positive peptidergic nerve fibers are found (Bjurholm et al., 1988, 1989; Deftos and Roos, 1989; Hill and Elde, 1991; Hares and Foster, 1991; Wimalawansa, 1996; Imai et al., 1997), and some of these osseal fibers form terminal structures that directly contact metaphyseal osteoblasts and osteoclasts (Bjurholm et al., 1989; Imai et al., 1997). Functional analyses have demonstrated that these fibers are associated with bone formation and repair (Bjurholm et al., 1990; Suva et al., 1993; Imai et al., 1997) and suggested that CGRP functions to up-regulate osteoblast recruitment and osteogenic activity (Bjurholm et al., 1989, 1990). In support of this concept, CGRP has been found to stimulate the formation of osteogenic cell colonies from

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cultured rat bone marrow stromal cells (Shih and Bernard, 1997) and to facilitate osteoblastic in vitro mineralization in confluent cultures (Burns and Kawase, 1997). CGRP also stimulated the production of insulin-like growth factor in subconfluent primary fetal rat osteoblasts (Vignery and McCarthy, 1996) while suppressing cytokine production (Millet and Vignery, 1997). Furthermore, targeted expression of CGRP to mouse osteoblasts increased bone density by 27–40%, stimulated cancellous bone formation, and stimulated osteoblastic parameters in vivo (Ballica et al., 1999). These data suggest that the osteoblast is a major target for CGRP in bone.

Despite this evidence, an anabolic role for CGRP in bone metabolism and bone remodeling has not been firmly established. One major reason for this long-standing skepticism stems from poor understanding of the mechanism of CGRP's intracellular signaling in the osteoblastic lineage. Historically, CGRP's osteoblastic response has been defined exclusively by stimulation of cellular cAMP levels; unfortunately, CGRP-induced changes in cAMP production have often been reported to vary dramatically between different osteoblast-like cell models (Bjurholm et al., 1992; Kawase and Burns, unpublished observations) and even between different preparations of primary osteoblastic cells (Michelangeli et al., 1989). To further complicate this issue, published reports clearly indicate that CGRP also signals through major cAMP-independent pathways (Kawase et al., 1995, 1996; Kawase and Burns, 1998; Aiyar et al., 1996; Drissi et al., 1998, 1999; Villa et al., 2002). A second major hindrance to understanding skeletal CGRP stems from the fact that osteoblastic CGRP receptors have never been completely characterized; only limited radioligand-binding studies have demonstrated CGRP receptors in a few types of osteogenic cell. Neither of the known protein components of a $CGRP_1$ receptor has yet been demonstrated in osteoblasts or preosteoblasts.

In rat osteoblastic UMR106 cells, thought to represent a relatively mature type of osteogenic cell, we found that CGRP stimulates cAMP formation little more than 1.5-fold, while robustly mobilizing intracellular Ca^{2+} and inducing membrane hyperpolarization (Kawase et al., 1995, 1996; Kawase and Burns, 1998). On studying expression of the mRNA encoding the calcitonin receptor-like receptor (CRLR) component of the $CGRP_1$ receptor in human cell-lines representing different stages of osteogenic maturation, Togari et al. (1997) suggested that the adenylate cyclase-associated $CGRP_1$ receptor is down-regulated during osteoblastic differentiation. These findings and interpretations of the associated data suggest that the bone anabolic actions of CGRP are mediated by different CGRP receptor subtypes and/or more diverse signaling pathways as an osteoblastic cell matures.

MG63 cells display a phenotype similar to immature osteoblastic cells (Jukkola et al., 1993); it is these cells that show the most pronounced cAMP response to CGRP among the many osteoblastic cell lines examined. To study the cAMP-associated CGRP receptor subtypes found in immature human osteoblastic cells, we have for the first time pharmacologically and biochemically characterized high-affinity CGRP-binding sites, CGRP-activated signaling pathways, and associated cellular functions in MG63 cells.

2. Materials and methods

2.1. Cell and cell culture

Human (MG63, SaOS-2) and rat (UMR106) osteosarcoma-derived osteoblastic cells were obtained from Dainippon Pharmaceutical (Osaka, Japan). These osteoblastic cells, as well as L6 cells (see below), were maintained in Dulbecco's modified Eagle Medium (DMEM; Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA) in humidified 5% CO_2, 95% air at 37 °C.

Compared to other osteoblastic cells (e.g., SaOS-2 or UMR106), MG63 cells are characterized by a pronounced cAMP response to CGRP and a poor response to parathyroid hormone-related protein (PTHrP) (Peptide Institute, Osaka, Japan) (Fig. 1). CGRP (1 pM to 100 nM) dose-dependently increased cAMP production at 15 min in serum-free Hank's balanced salt solution (HBSS) in the presence of 1 mM isobutyl methylxanthine (IBMX; Sigma, St. Louis, MO, USA), with a maximal 35-fold stimulation observed at 10 nM and an EC_{50} of approximately 150 pM. In either rat UMR106 or human SaOS-2 cells, CGRP elicited only 1.5- to 3-fold increases in cAMP production under the same conditions (data not shown).

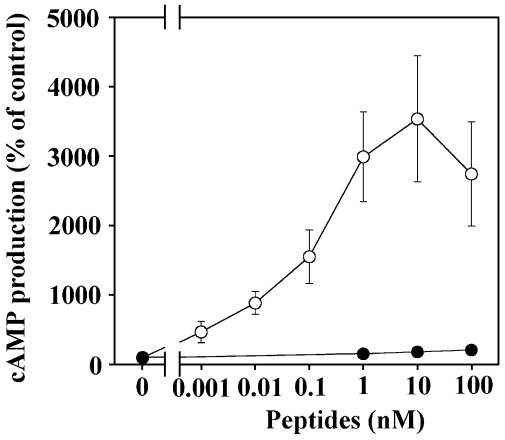

Fig. 1. The effects of CGRP on intracellular cAMP production in MG63 cells. Cells were treated with human CGRP (open circle) or PTHrP (closed circle) at the indicated concentrations for 15 min at 37 °C in serum-free HBSS containing 1 mM IBMX. Intracellular cAMP was then extracted and measured by specific EIA. The EC_{50} of CGRP is estimated as 150 pM. Each point and vertical bar represents the mean and S.D. of the data from five independent experiments, respectively. Significant effects were constantly observed from 0.001 nM or greater CGRP or from 1 nM or greater PTHrP ($P<0.05$ vs. control).

Rat skeletal muscle-derived L6 cells (Dainippon Pharmaceutical) have been demonstrated to express a $CGRP_1$ receptor that is composed of CRLR and RAMP-1 proteins (Conner et al., 2002). These cells were used as a positive control in the present study.

2.2. CGRP fragments

Human α-CGRP, CGRP-(8-37), CGRP-(19-37), CGRP-(23-37), and [$Cys(Acm)^{2,7}$]-CGRP were purchased from the Peptide Institute or Peninsula Laboratories (San Carlos, CA, USA). All other CGRP fragments were synthesized by solid phase methods and purified by reverse phase high-performance liquid chromatography. The structure of each of these peptides was verified by amino acid analysis and electrospray ionization–mass spectrometry.

2.3. [^{125}I]CGRP suspended whole-cell-binding assay

As described in our previous study (Kawase et al., 1999), a whole-cell saturation-binding assay was performed by a modification of the method of McGillis et al. (1993). In brief, cells from 40% confluent cultures were released by a nonenzymatic cell-releasing solution (JRH BioSciences, Lenexa, KS, USA) and suspended at 1×10^5 cells in 200 μl of chilled CGRP-binding buffer (20 mM HEPES, 10 mM KCl, 1 mg/ml bacitracin, 0.1 mg/ml aprotinin, 0.2% heat-denatured bovine serum albumin, pH 7.4). From 1.33 to 450 fmol of [^{125}I]CGRP-α tracer (Amersham-Pharmacia Biotech, IM.184, 2000 Ci/mmol, Buckinghamshire, UK) was added and the mixture was incubated at 4 °C over 120 min. After rapid centrifugation through a layer of binding buffer made 20% in glycerol, resulting cell pellets were dissolved in 50 μl of 0.05 N NaOH.

Nonspecific binding (background dpms) was estimated as the CGRP tracer binding obtained in the presence of 1 μM nonlabeled human CGRP. Background binding in all cases was less than 15% of total binding. Specific CGRP-binding was calculated by subtracting the nonspecific background from total binding. Values from six binding experiments (done in duplicate) were used to calculate the best-fit binding curve via nonlinear regression, and both one- and two-site binding models were evaluated (GraphPad Prism, San Diego, CA, USA). The best fit was obtained with a simple single-site binding model based on the formula for a rectilinear hyperbola [$y = ax/(b+x)$] with an R^2 correlation coefficient equal to 0.99781. Both the number of sites per cell (B_{max}) and apparent K_D were calculated by this computer model. These curve-fitted chart data were then graphed in PhotoShop 7.0 to produce the final figure.

A limited number of competitive binding assays were conducted with human CGRP-(8-37). The conditions of these assays were similar to those employed by Aiyar et al. (1999). A 0.2-ml volume of CGRP-binding buffer had 100 pM [^{125}I]CGRP tracer and 50 pM of cellular receptor sites. At the observed EC_{50} for CGRP-(8-37), 0.75 pM of receptor sites had bound tracer, and K_i was estimated from the formula $K_i = BIK_D/[(L_T R_T) + B(R_T - L_T)]$.

2.4. Immunocytochemical staining for CRLR, RAMP-1, and collagen type I

Cells cultured on cover slips for 24 h or cells freshly harvested and placed on glass slides coated with poly-L-lysine (Sigma) were fixed in 3.7% formaldehyde. After blocking with 3% bovine serum albumin-containing Tris-buffered saline (TBS), cells were probed with polyclonal anti-human RAMP-1 antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and polyclonal anti-rat CRLR antibody (1:2000; Alpha Diagnostics International, San Antonio, TX, USA). The localization of these proteins were then visualized with Alexa Fluor 546®-conjugated donkey anti-goat immunoglobulin G(H + L) [IgG(H + L)] antibody (1:100; Molecular Probes, Eugene, OR) and Alexa Fluor 488®-conjugated goat anti-rabbit IgG(H + L) antibody (1:100; Molecular Probes), essentially as described previously (Kawase et al., 2000, 2001).

In parallel experiments, cells grown on cover slips were fixed with cold methanol and directly subjected to immunocytochemical staining for collagen type I. After appropriate blocking, cells were probed with a monoclonal anti-human collagen type I antibody (CHEMICON International, Temecula, CA, USA) and visualized with Cy3™-conjugated anti-mouse IgG (Amersham-Pharmacia Biotech).

2.5. Visualization of CGRP-binding sites on living cells

Vials of dry fluorescein-conjugated human CGRP (Fluo-CGRP) (Advanced BioConcepts/New England Nuclear, Albany, MA, USA) were stored desiccated at −80 °C prior to use. Fluorescent ligand was dissolved to 5 μM in 100% fresh dimethyl sulfoxide (DMSO) and sonicated for 20 min at 22 °C immediately before use. MG63 cells were plated at varying densities ($1-5 \times 10^4$ cells/ml) onto sterile 1 mm circular glass cover slips held within 100 mm plastic tissue-culture dishes and grown to desired densities in 2% fetal bovine serum-containing medium. Cultures were changed to 0.2% fetal bovine serum-containing medium 4 h prior to the beginning of binding experiments were to start. Alternatively, cells were seeded on cover slips and incubated for 6 h in 0.2% fetal bovine serum-containing medium. Dishes containing cover slips of cells were incubated for 15 min on ice before one glass cover slip was placed into each well of a 6-well CoStar plate. To each well, 3 ml of CGRP-binding buffer containing 1 nM Fluo-CGRP was added. This combination was held at 22 °C for 10 min before starting a binding experiment. Plates were covered with foil and incubated for another 120 min at 22 °C on a rocker table set to provide continuous gentle mixing. At the end of incubations, the binding buffer was rapidly aspirated, and each well was washed with three changes of straight-binding buffer.

Prior to microscopy, cover slips were washed twice in 5 ml of fresh physiological saline solution (PSS; 130 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, 10 mM HEPES; pH 7.3). Each cover slip was then mounted into an Attofluor 38 mm cell-chamber (Molecular Probes) and covered with 1 ml of fresh PSS. The chamber was then examined with the 20× or 40× objective of an inverted Nikon TE-300 Eclipse microscope. Imaging was rapidly performed by laser-scanning confocal fluorescent microscopy using a computer-controlled Bio-Rad RadiancePlus 2000 confocal apparatus. The 488-nm line of an argon laser was used to excite the ligand and fluorescent emission was collected with a 530/60 nm filter. Iris diameters of 3.8–2.0 mm were used, and laser power was limited to 30% to minimize photo-bleaching of the fluorescein tag. There was no significant background fluorescence normally observed using this technique. Images were stored on the confocal system computer as digital Bio-Rad 'pic' files and were exported as 'tiff' files into Adobe PhotoShop (v 7.0) for image processing.

2.6. Measurement of intracellular cAMP

Cells were seeded into 24-multiwell plates at a density of 4×10^4 cells/well and cultured for 2 days. Cells were preincubated overnight in 1% fetal bovine serum-containing medium and then treated with CGRP and/or [Cys(Acm)^{2,7}]-CGRP for 15 min in the presence or absence of CGRP-(8-37) in serum-free-HBSS containing 1 mM IBMX at 37 °C, intracellular cAMP was extracted and subjected to the assessment using the cAMP enzyme immunoassay (EIA) system (Amersham-Pharmacia Biotech), as described previously (Kawase et al., 2001).

2.7. Western blot analysis for MAPK phosphorylation

As described previously (Kawase et al., 1999, 2001), cells were preincubated overnight in 1% fetal bovine serum-containing medium prior to the experiment. Cells were then treated with CGRP in combination with CGRP-(8-37) or sodium orthovanadate (Sigma-Aldrich). In some cases, cells pretreated with *N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoinoline sulfonamide (H-89;10 μM) (Calbiochem, San Diego, CA, USA) were used for CGRP stimulation. At the end of CGRP treatments, cells were lysed in Laemmli sample buffer and subjected to sodium dodecylsulfate-polyacrylamide gel (SDS-PAGE; self-made 9% linear-gel) and immunoblotting analysis using the following primary antibodies: polyclonal anti-phosphorylated-ERK antibody (1:1000; New England Biolabs, Beverly, MA, USA), polyclonal anti-ERK antibody (1:1000; New England Biolabs), polyclonal anti-phosphorylated-p38-mitogen-activated protein kinase (MAPK) antibody (1:1000; New England Biolabs), or polyclonal anti-p38-MAPK antibody (1:1000; New England Biolabs). Probed blots were then reacted with horseradish peroxidase-conjugated Protein A (1:10,000; Zymed, South San Francisco, CA, USA), followed by visualization with the ECL system (Amersham-Pharmacia Biotech). For re-probing with a different primary antibody, blots were stripped with Restore™ Western Blot Stripping Buffer (Pierce, Rockford, IL, USA).

2.8. Immunoprecipitation with an anti-phosphotyrosine antibody and Western blot with an anti-ERK antibody

As described above and previously (Kawase et al., 1999), cells of 70–90% confluency in 60-mm dishes (Falcon) were treated with CGRP, washed twice with ice-cold PBS (−) and harvested into cell lysis buffer A (25 mM Tris–HCl, 150 mM NaCl, 1% NP-40, 1 mM orthovanadate, plus 1 tablet/20 ml of Trypsin-Inhibitor Cocktail-Complete™ [Boehringer-Mannheim, Mannheim, Germany]; pH 7.6). After centrifugation (20,000 × *g*, 10 min, 4 °C), the supernatants were incubated with anti-phosphotyrosine antibody (clone PY20) (1:100) (Transduction Laboratories, Lexington, KY, USA) overnight and subsequently reacted with Agarose-Protein A (1:10) (Calbiochem) for 1.5 h at 4 °C. At the end of incubation, agarose beads were washed twice with ice-cold cell lysis buffer A and isolated target proteins were detached by boiling in Laemmli sample buffer. Any isolated proteins were then subjected to Western blot assay using anti-ERK antibody (1:5000) (Transduction Laboratories).

2.9. Assessment of ERK activity

As described previously (Kawase et al., 1999), cell cultures judged to be of 70–90% confluency in 60-mm dishes were treated with CGRP. According to the manufacturer's protocol, ERK activity was assessed using the MAPK activity assay kit (New England Biolabs): Protein samples were immunoprecipitated with anti-phosphorylated-p44/42-MAPK (=ERK) antibody (contained in the kit) in the presence of trypsin-inhibitors and subsequently with Agarose-Protein A (1:10). After washing twice with ice-cold cell lysis buffer (contained in the kit) and twice with kinase buffer (contained in the kit), the agarose beads were suspended in kinase buffer for the kinase assay in the presence of exogenous Elk-1 fusion protein and 1 mM ATP. Phosphorylated Elk-1 protein was separated by SDS-PAGE and detected by Western blot assay.

2.10. Western blot analysis for CRLR and RAMP-1

After washing three times with ice-cold PBS(−), early confluent cultures of cells were lysed on ice in Laemmli sample buffer and boiled for 1 min. Protein samples were here applied to pre-cast gradient gels (4–10% gel for CRLR or 10–20% gel for RAMP-1) (Daiichi Chemical, Tokyo) and electrotransferred to polyvinylidene difluoride (PVDF) membranes (Hybond-P; Amersham-Pharmacia Biotech). Fractionated proteins were probed with polyclonal anti-CRLR (1:5000) (Alpha Diagnostic International) or poly-

colnal anti-RAMP-1 antibodies (1:5000) (Santa Cruz) and visualized by the Amersham ECL system (Amersham-Pharmacia Biotech), essentially as described above.

Antibody specificity was demonstrated by antigen blocking. In brief, 1 μl of anti-RAMP-1 or anti-CRLR antibody was incubated for 3 h with 20 μg blocking peptide (Santa Cruz for RAMP-1; Alpha Diagnostic International for CRLR) in PBS(−) at 15 °C. The mixture was then centrifuged by 10,000 × *g* for 15 min at 4 °C to pellet any immune complexes. The resulting supernatant was taken and diluted by 1:5000 (for the antibody) with 3% bovine serum albumin-containing TBS. In the control, the antibody was processed as above without the blocking peptide. Protein-blotted membranes in duplicate were probed separately with these antibodies and at once visualized on a film.

2.11. Assessment of apoptotic cell death

As described previously (Kawase et al., 2000), apoptotic cell death in CGRP-treated cultures in 1% or 10% fetal bovine serum-containing medium was assessed with the Cellstain-Double staining kit (Dojin, Kumamoto, Japan), and the Annexin-V-FLUOS staining kit (Boehringer-Mannheim), in accordance with the recommended protocol by the manufacturer.

2.12. Measurement of DNA synthesis

Cells were seeded into 96-multiwell plates at a density of 2×10^4 cells/well and incubated with CGRP in 1% or 10% fetal bovine serum-containing medium for 24 h. For an additional 3 h, cells were labeled with bromo-deoxyuridine (BrdU). After fixation, the BrdU incorporated into cells was assessed using a microplate-reader by a standardized cell proliferation assay kit (Amersham-Pharmacia Biotech) in accordance with the manufacturer's protocol (Kawase et al., 2000).

2.13. Experimental protocol and preparation of MG63 cell total RNA

MG63 cells were plated at 9×10^4 cells/well into 6-well CoStar plates in 4 ml/well of DMEM supplemented with 1 mM glutamate and 2% fetal bovine serum. After covering approximately 70% of the well surface, cells were changed to DMEM supplemented with 0.2% fetal bovine serum and 150 μg/ml ascorbic acid and maintained for an additional 16 h. An experiment was initiated by addition of the indicated level of 2′-amino-3′-methoxyflavone (PD98059) in 4 μl of 0.1% DMSO as a 15-min pretreatment before appropriate CGRP concentrations were then dispensed in 4 μl of 0.01% trifluoroacetic acid. In all cases, appropriate amounts of 0.1% DMSO and/or 0.01% trifluoroacetic acid were added to otherwise untreated wells to serve as the appropriate control. Unless indicated otherwise, all treatments were performed in triplicate wells for 4 h at 37 °C. All medium was rapidly aspirated, and warmed TRI reagent (Sigma-Aldrich) was added at 0.7 ml/well with gentle mixing for 15 min. Each 1 ml of TRI cellular extract was combined with 0.2 ml chloroform for extraction. Each 0.5 ml of aqueous phase was mixed with 0.75 ml of isopropanol and centrifugation was used to obtain an RNA pellet. The RNA pellet was washed with 75% ethanol, air-dried, and then resuspended with 50 μl of molecular-grade water (Ambion, Austin, TX, USA). Prior to use, RNA was stored at −65 °C.

2.14. Evaluation of collagen I(α) mRNA levels

Fluorimetric quantitation of the RNA from each treatment group was performed using 1 μl of stock RNA in the Oligreen RNA dye quantitation kit (Molecular Probes) at a final Oligreen dilution of 1–400. Quantitation was performed with a Shimadzu RF-5301PC spectrofluorometer (excitation at 480 nm and emission at 520 nm, against a standard total RNA concentration curve). For Northern hybridization analysis, 5 μg of each treatment group sample was mixed with an equal volume of standard RNA loading buffer (Eppendorf, Hamburg, Germany), heated at 65 °C and loaded into the well of a 25 × 43 cm 1.25% agarose gel containing 40 mM 3-(*N*-morpholino) propanesulfonic acid (MOPS; pH 7.0), 10 mM sodium acetate, 2 mM EDTA, and 0.41 M formaldehyde. Upon separation of RNA species, the gel was rapidly imaged on a Molecular Dynamics Storm 860 phospho-fluoroimager workstation, so that the intensity of the 18S rRNA band could be used to normalize hybridization signals (see below). RNA was then completely transferred to a Nytran-Plus 0.2 μm membrane using a downward flow TurboBlotter apparatus (Schleicher & Schnell, Einbeck, Germany) with a buffer of 3M NaCl, 8 mM NaOH, and 2 mM sarkosyl. Following transfer, the RNA was fixed to the membrane by exposure to 110 mJ/cm^2 of 254 nm UV light.

Both pre-hybridization and hybridization of the Nytran-Plus membrane were performed at 42 °C in a solution containing 50% (v/v) deionized formamide, 20 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 6.5), 400 mM NaCl, 1 mM EDTA, and 0.5% SDS containing 0.5 μg/ml pre-boiled sheared salmon sperm DNA. Purified cDNA insert from ATCC clone #566032 (human 308919 2b70b08.rl) was labeled with [^{32}P]-dCTP by standard random-priming procedure (Prime-A-Prove, Ambion) to probe for Collagen I-α mRNA. Hybridization was performed for 16 h, and standard stringency washes of the membrane were performed, as recommended by Schleicher & Schnell. The blot was exposed to an 8 × 11 inch phosphorscreen for 48 h, and the latent image read on the phosphorimager workstation.

2.15. Statistical analysis

Unless otherwise stated, results are expressed as mean ± S.D. Statistical analyses were performed using Student's *t*-

test or analyzed by one-way analysis of variance (ANOVA). Comparisons between individual groups were made using Tukey's multiple comparison test; P values <0.05 were considered significant.

3. Results

3.1. CGRP receptors expressed in MG63 cells

As shown in Fig. 1, CGRP stimulated cAMP production in MG63 cells by 35-fold at a maximally effective concentration of 10 nM. Furthermore, this maximal response was substantially ($\sim$ 80%), but not completely, inhibited by the well-known antagonist for the $CGRP_1$ receptor subtype, CGRP-(8-37) (2 μM) (Chiba et al., 1989). These data raised the question of whether the response of these cells to CGRP was mediated by more than one type of CGRP receptor.

We therefore conducted multiple saturation-binding whole-cell experiments with radiolabeled human CGRP (Fig. 2). Detailed nonlinear regression curve-fitting analysis of saturation-binding data from multiple experiments (see Materials and methods) distinguishes only one class of high-affinity CGRP-binding site on MG-63 cells. There are approximately $13{,}100 \pm 223$ binding sites per MG63 cell with an apparent K_D of 407 ± 29 pM. This K_D value is comparable to the estimated EC_{50} (150 pM) for CGRP's stimulation of cAMP production in MG63 cells.

Limited numbers of competitive binding experiments demonstrated that 2–5 μM levels of CGRP-(8-37) could compete for a maximum of 78% of intact [^{125}I]-CGRP binding (data not shown). Analysis of CGRP-(8-37)'s displacement curve yielded an estimated K_i of approximately

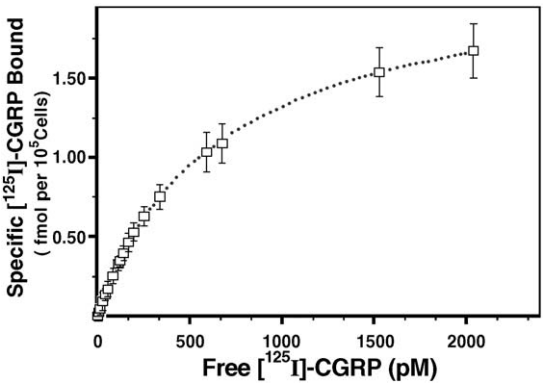

Fig. 2. Saturation binding analysis of [^{125}I]CGRP to intact MG63 cells. Binding in suspended whole cells was evaluated in triplicate at 4 °C in the presence of bacitracin and aprotinin, as described under Materials and methods. The results from four independent experiments are shown as the mean ± S.D. Nonlinear regression curve-fitting analysis was accomplished with GraphPad Prism, and the best-fit curve was accomplished with a single-site model having formula $y=ax/(b+x)$ and an R^2 correlation coefficient of 0.99781. This analysis indicates a K_D of 405 ± 29 pM and $13{,}100 \pm 223$ binding sites per MG63 cell. Similar results were obtained with classical Scatchard plot analysis (data not shown).

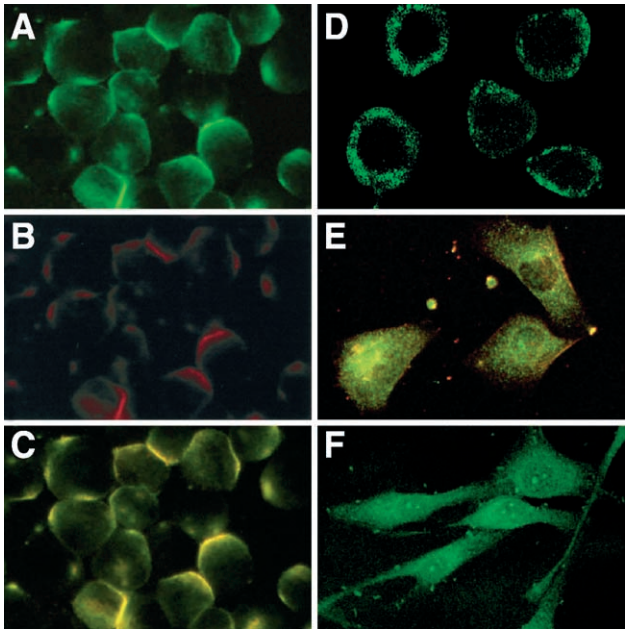

Fig. 3. Fluorescent photomicrographic demonstration of CGRP binding sites on MG63 cells. (A–C, E) Photomicrographs of fixed MG63 cells immunocytochemically stained for CRLR and/or RAMP-1. Cells plated on cover slips were incubated for 4 h (A–C) or 24 h (E), fixed with formaldehyde, and double-stained with anti-CRLR and anti-RAMP-1 antibodies without treatment with Triton X-100. The localization of these proteins was visualized with secondary antibodies conjugated to different fluorescent dyes. Photos A and B show the localization of CRLR and RAMP-1, respectively. These photos are superimposed to create Photo C. Photo E shows superimposed double staining. (D and F) Photomicrographs of Fluo-CGRP ligand bound to living MG63 cells. Cells plated on cover slips were incubated for 6 h (D) or 24 h (F) and then exposed to Fluo-CGRP in binding buffer. All experiments were repeated at least three times with similar results, and the results shown here are representative for all these data.

43 nM, in good agreement with published values associated with classical $CGRP_1$ receptors (Leuthauser et al., 2000). From these binding data, MG63 cells appear to predominantly express one subtype of the CGRP receptor.

The CGRP receptor with high potency for stimulating cAMP production has been classified as the $CGRP_1$ subtype (for review, see Wimalawansa, 1996). Therefore, specific antibodies were next employed to examine MG63 cells for the presence of the known components of a $CGRP_1$ receptor, the CRLR, and RAMP-1 proteins (McLatchie et al., 1998). Specific immunocytochemical staining of both adherent and detached cells demonstrated that both CRLR and RAMP-1 proteins were widely, if not uniformly, distributed on the plasma membrane. In detached cells fixed with diluted formaldehyde (Fig. 3A and B), superimposed photomicrographs clearly demonstrated multiple areas where both proteins were co-expressed (Fig. 3C). Fluo-CGRP binding visualized similar cell-surface binding sites in detached living cells (Fig. 3D). This binding of Fluo-CGRP (1 nM) was eliminated by competing with intact CGRP (3 μM) and inhibited approximately 70% by competing with CGRP-(8-37) (5 μM) (data not shown). The incomplete competition by

CGRP-(8-37) at 5 μM may well be due to the fact that its binding affinity for the $CGRP_1$ receptor is 5% or less than that of intact CGRP (Chiba et al., 1989).

When cells grown flattened on cover slips were examined by this method, before or after fixation (Fig. 3E and F), the definitive ring-like images of bound peptide typical of globular cells were not observed. Instead, the visualized binding sites and receptor components were widely distributed on the dorsal surface and edges of the plated cells. This result is due to the particular morphology of these cells, i.e., a swollen center housing the nucleus surrounded by extremely thin flattened cytoplasm firmly anchored to the cover slip. Thus, the dorsal surface of these cells fills the Z-plane of either a confocal or conventional microscopic image. With experience, it has been possible to determine that neither immunoreactivity nor binding was internalized during these binding experiments.

Western blot analyses demonstrated that CRLR-immunoreactive proteins detected in lysates from whole MG63 cells had molecular weights of approximately 48, 52, and 120 kDa (Fig. 4A), and that RAMP-1-immunoreactive protein was detected at 15 kDa (Fig. 4B). Immunostaining of each of these bands was strongly diminished or eliminated when the corresponding control peptide was added to compete for antibody. Very similar results were obtained from the positive control, L6 cells (Fig. 4A and B). Therefore, these findings in conjunction with the results of binding analysis and demonstration of a pronounced cAMP response suggest that the CGRP receptor subtype predominantly expressed in MG63 cells is probably a version of the $CGRP_1$ receptor commonly associated with a G_s-type regulatory protein and the stimulation of adenylate cyclase.

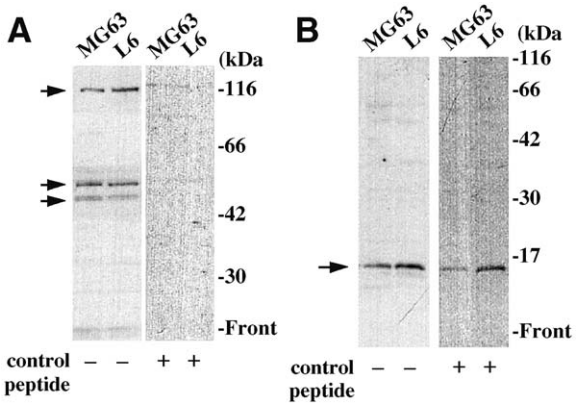

Fig. 4. Western blot analysis of CRLR and RAMP-1 proteins in whole cell samples from MG63 or L6 cells. Cells were directly lysed in the sample buffer and boiled for 1 min. After sonication and centrifugation to remove debris, cell lysates were applied to SDS-PAGE (A: 4–20% gradient gel, B: 10–20% gradient gel) and then immunoblotted using anti-human CRLR antibody (A) or anti-rat RAMP-1 antibody (B). Antibody specificity was verified by competing with the corresponding control peptide antigen, as recommended by the supplier and described under Materials and methods. Experiments were repeated three times, and the results shown here are representative for all these data.

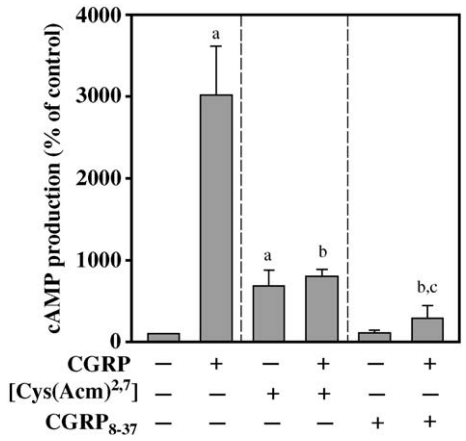

Fig. 5. Agonistic and antagonistic effects of CGRP-(8-37) and [Cys(Acm)^{2,7}]-CGRP in the production of cAMP by MG63 cells. As described under Materials and methods, cells were treated for 15 min with CGRP (1 nM), [Cys(Acm)^{2,7}]-CGRP (1 μM), or CGRP-(8-37) (1 μM) alone or in the indicated combinations. Each column and vertical bar represents the mean and S.D. of the data from five independent experiments, respectively. $^{a}P<0.001$ vs. control, $^{b}P<0.001$ vs. cultures treated only with CGRP, $^{c}P<0.01$ vs. cultures treated only with CGRP-(8-37).

3.2. CGRP-responsive intracellular signaling pathways in MG63 cells

To further characterize this receptor subtype in MG63 cells, we have obtained or prepared seven CGRP fragments [CGRP-(1-8), CGRP-(1-12), CGRP-(1-14), CGRP-(8-37), CGRP-(19-37), CGRP-(23-37), and [Cys(Acm)^{2,7}]-CGRP] to examine their potency in stimulating cAMP production. CGRP-(8-37) (1 μM) did not itself significantly increase intracellular cAMP, but inhibited CGRP-induced cAMP production by 93% (at 0.1 nM CGRP), by 92% (at 1 nM CGRP) (Fig. 5), and by 75% (at 10 nM CGRP) even in the presence of 1 mM IBMX. Interestingly, [Cys(Acm)^{2,7}]-CGRP (1 μM), a known agonist for the putative $CGRP_2$ receptor (Dennis et al., 1989), weakly stimulated cAMP production itself and also potently attenuated CGRP-induced cAMP production to the low levels characteristic of its own weakly agonistic action. The C-terminal fragments [CGRP-(19-37) or CGRP-(23-37)] less potently, but still significantly, antagonized CGRP action without showing any agonistic activity of their own. None of the N-terminal fragments [CGRP-(1-8), CGRP-(1-12), or CGRP-(1-14)] showed significant agonistic or antagonistic actions in our cAMP assays.

As previously reported (Kawase et al., 1999), ERK phosphorylation plays an important role in regulating the proliferation of CGRP-treated human gingival fibroblasts. Thus, the effects of CGRP on MAPK phosphorylations were examined in MG63 cells in serum-free HBSS. As expected, CGRP (0.1–100 nM) stimulated the phosphorylation of p38-MAPK in a dose- and time-dependent manner (data not shown). The maximal effects were observed with 10 nM at 5 min. It should also be noted that

very similar dose- and time-dependent relationships were observed in CGRP-stimulated phosphorylation of cyclic AMP-responsive element-binding protein (CREB) (data not shown).

Significantly, in complete contrast to our previous study in Gin-1 cells (Kawase et al., 1999), CGRP decreased the levels of phosphorylated ERK (Fig. 6A and B) in a dose- and time-dependent fashion. The inhibitory action was observed within 2 min and reached maximal effect at 15 min with 10 nM CGRP. This action was verified by an alternative method wherein an anti-phosphotyrosine antibody was used to precipitate corresponding proteins and an anti-pan-ERK antibody used to visualize ERK protein by Western blot (Fig. 6C). This unexpected action of CGRP would imply inactivation of ERK activity in MG63 cells. Thus, a third test was conducted to measure changes in the level of activated ERK through measuring the ability of cellular ERK to phosphorylate its downstream substrate, Elk-1 (Fig. 6D). Both tests verified the original result and demonstrated a dose-dependent inhibition of ERK activity after 15 min of CGRP treatment.

To identify the signaling pathway(s) responsible for mediating this effect of CGRP, specific pharmacological inhibitors and stimulators were applied. Agents known to amplify or promote cAMP levels and the function of cAMP-dependent signaling, IBMX (1 mM) and forskolin (1 μM), significantly enhanced CGRP-induced ERK dephosphorylation at 15 min (data not shown). Furthermore, both a cAMP-dependent protein kinase inhibitor (H-89; 10 μM), a nonspecific phosphatase inhibitor, (vanadate (1 mM)), and CGRP-(8-37) (1 μM) apparently blocked CGRP-induced ERK dephosphorylation at 15 min (Fig. 6E).

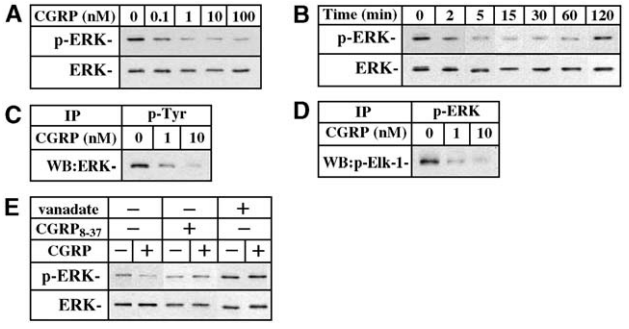

Fig. 6. The effects of CGRP on the phosphorylation and activity of ERK in MG63 cells. Cells were treated with the indicated concentrations of CGRP for 15 min (A, C, and D) or with CGRP (10 nM) for the indicated periods of time (B) in serum-free HBSS. (A, B) Fractionated cell samples were immunoblotted and probed as indicated with either anti-phosphorylated-ERK (p-ERK) or anti-pan-ERK (ERK) antibody. (C) In separate experiments, cells were lysed and immunoprecipitated with anti-phosphotyrosine antibody and Agarose-Protein A. Trapped proteins were then immunoblotted with anti-pan-ERK antibody. (D) Alternatively, lysed cells were immunoprecipitated with anti-phosphorylated ERK antibody and Agarose-Protein A, and isolated proteins were then subjected to a protein kinase assay in the presence of ATP using an Elk-1 fusion protein as substrate. Reaction products were then analyzed by immunoblotting using an anti-phosphorylated-Elk-1 antibody. (E) In these experiments, cells were treated with CGRP (1 nM) in the presence of either vanadate (1 mM) or CGRP-(8-37) (1 μM) for 15 min, and cell samples were subjected to Western blotting. All experiments were repeated at least three times, and the results shown here are representative for all these data.

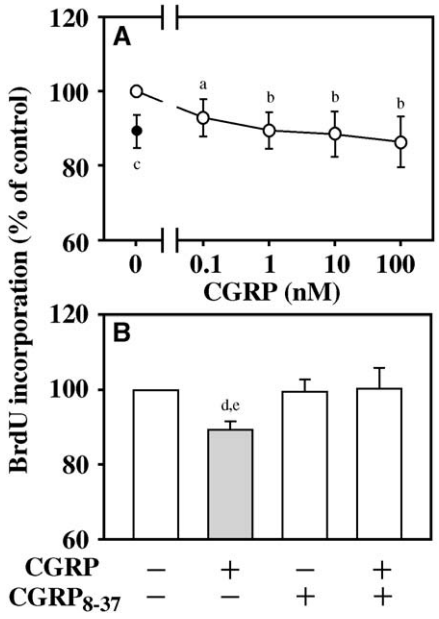

Fig. 7. The dose-dependent anti-proliferative effects of CGRP on MG63 cells. (A) Cells were seeded and treated with the indicated concentrations of CGRP (open circle), or with PD98059 (3 μM) (closed circle), for 24 h, before being labeled with BrdU for an additional 3 h in 10% fetal bovine serum-containing medium. An estimate of DNA synthesis was obtained by the amount of BrdU incorporated into DNA over 3 h. Each point and vertical bar represents the mean and S.D. of the data from five independent experiments, respectively. $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$ vs. control. (B) The specific inhibition by CGRP-(8-37) of CGRP's anti-proliferative effects in MG63 cells. Cells were treated CGRP (1 nM) in combination with CGRP-(8-37) (1 μM) and assayed as described above. Each column and vertical bar represents the mean and S.D. of the data from five independent experiments. $^{d}P<0.001$ vs. control, $^{e}P<0.01$ vs. the cultures treated with both CGRP and CGRP-(8-37).

3.3. Anti-proliferative action of CGRP on MG63 cells

ERK is thought to be involved in regulating cell proliferation in many different types of cells. Since CGRP decreased ERK phosphorylation and activity, we examined what effect CGRP has on the rate of DNA synthesis in MG63 cells. Treatment of freshly plated cells with CGRP (0.1–100 nM) slightly, but not significantly, increased DNA synthesis in 1% fetal bovine serum-containing medium, while CGRP significantly reduced the proliferative activity of MG63 cells grown in 10% fetal bovine serum-containing medium over 27 h (Fig. 7A). At 100 nM, CGRP inhibited DNA synthesis by approximately 10% versus nontreated cultures. The anti-proliferative effect of CGRP (1 nM) was completely blocked by co-treatment with CGRP-(8-37) (1 μM) (Fig. 7B). Similarly, a specific inhibitor of the MEK1/ERK pathway, PD98059 (3 μM), reduced DNA synthesis by approximately 10% versus nontreated cultures. Unlike

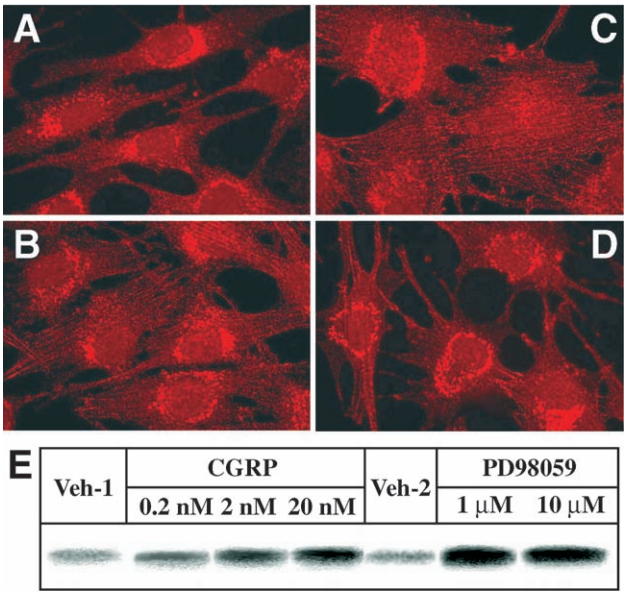

Fig. 8. The effects of CGRP on the expression of collagen type I protein and Col I(α) mRNA in MG63 cells. (A–D) Cells on cover slips were treated with CGRP (1 nM; B), PD98059 (3 μM; C) or CGRP plus CGRP-(8-37) (1 μM; D) for 24 h in 1% fetal bovine serum-containing medium (A, control). Cells were then fixed and immunocytochemically stained. (E) In a parallel set of experiments, cells were treated with the indicated concentration of CGRP or with PD98059 for 4 h before total cellular RNA was isolated and a specific cDNA probe for Col I(α) mRNA used in Northern blot analysis. Veh-1 represents addition of an equal volume of the vehicle for stock CGRP (0.01% trifluoroacetic acid), while Veh-2 represents addition of an equal volume of the vehicle for PD98059 (25% DMSO). (Final concentrations are 0.00001% TFA and 0.025% DMSO.) Experiments were repeated three times, and the results shown here are representative for all these data.

results from other cell types (Millet et al., 2000), however, CGRP did not appreciably increase the number of apoptotic MG63 cells, as assessed by assaying for leakage of the plasma membrane or for phosphatidylserine on the outer plasma membrane.

3.4. Up-regulation of collagen I biosynthesis in MG63 cells by CGRP

When freshly seeded cells were treated with CGRP (1 nM) for 24 h, production of collagen type I was stimulated substantially (Fig. 8A and B). This action was mimicked by PD98059 (3 μM) and inhibited by CGRP-(8-37) (1 μM) (Fig. 8C and D). This up-regulation at the protein level was reconfirmed by Northern blot analysis. CGRP (0.2–20 nM) and PD98059 (1–10 μM) convincingly up-regulated the expression of collagen type I α-chain mRNA after only 4 h of treatment (Fig. 8E).

4. Discussion

Although the $CGRP_1$ receptors, identified in different tissues and cell-types, possess components that vary somewhat in reported molecular weight and in reported signaling mechanisms, genes encoding the $CGRP_1$ receptor proteins have been successfully cloned from several species (Aiyar et al., 1996; Elshourbagy et al., 1998; Muff et al., 2001). In contrast, putative $CGRP_2$ receptors have so far only been classified by pharmacology; constitutive proteins have not yet been positively identified at the biochemical or genetic level. In the present study, we have found that (i) MG63 cells predominantly expressed one class of high-affinity membrane CGRP-binding sites that could be blocked by appropriate concentrations of CGRP-(8-37) and probably mediated CGRP's strong stimulation of cAMP formation, (ii) maximal CGRP-induced cAMP production was antagonized at least 78% by CGRP-(8-37), and (iii) both CRLR- and RAMP-1-immunoreactive proteins, the known components of a $CGRP_1$ receptor complex, were expressed on the cell surface. These three findings are consistent with the classical criteria used to define $CGRP_1$ receptors. The bulk of our data, taken together with published RT-PCR analysis of the mRNA for CRLR (Togari et al., 1997), suggest that the CGRP receptors predominantly expressed by MG63 cells are very similar to known $CGRP_1$ receptors.

However, some of our data are considerably less consistent with reports describing $CGRP_1$ receptors in other tissues or cell-types. (a) One of the CRLR-immunoreactive proteins was found to be 52 kDa in size rather than the predicted 60–70 kDa. (b) The anti-CRLR antibody used here also detected a third specific protein at 120 kDa. (c) A classical $CGRP_2$ receptor agonist acted as an antagonist on these CGRP receptors and proved itself to be a weak agonist. (d) CGRP-(8-37) produced 78% or 90% inhibition of CGRP binding or CGRP's stimulation of cAMP formation, respectively, instead of eliminating these actions. (e) Contrary to previous reports for CGRP signaling mediated by a $CGRP_1$ receptor in fibroblasts (Kawase et al., 1999), CGRP down-regulated ERK and inhibited cell proliferation in growing MG63 cells. To test our interpretations and conclusions, these unexpected findings need to be discussed in some detail.

Concerning point (a), the known human CRLR protein is composed of 461 amino acids (Muff et al., 2001) and nascent nonmodified protein should have a molecular weight of approximately 48 kDa, corresponding to the low-molecular-weight CRLR-immunoreactive protein detected in our experiments. Just as previously published, data suggest that the 48-kDa protein represents nonglycosylated CRLR, these reports would suggest that the 52-kDa CRLR we have detected is most probably a glycosylated-CRLR. Traditional affinity-labeling experiments have demonstrated CGRP-protein complexes of approximately 68 kDa (or heavier) in various native tissues or cells transfected with plasmids bearing CRLR and RAMP-1 cDNAs (Stangl et al., 1993; McLatchie et al., 1998; Aldecoa et al., 2000). In addition, Hagner et al. (2002) demonstrated using their recently developed anti-CRLR antibodies that two immunoreactive CRLR species of approximately 60 and 70 kDa are demonstrated in various human tissues. However, the

induced expression of Myc-tagged CRLR demonstrated immunoreactive proteins with Mr's ranging from 50- to 66-kDa (McLatchie et al., 1998; Aldecoa et al., 2000). To provide a positive control to aid in analyzing these complex issues, we have here employed skeletal muscle-derived L6 cells as a standard source of CRLR proteins. These cells were also found to express 48- and 52-kDa CRLR-immunoreactive proteins that appear identical to the corresponding proteins demonstrated in MG63 cells. Judging from our own and these published data, it is suggested that the immunoreactive protein (52 kDa) we have found is either a less glycosylated form of CRLR or a CRLR possessing high-mannose oligosaccharide rather than the more complex glycosyl side-chains that have been identified (McLatchie et al., 1998; Aldecoa et al., 2000; Leuthauser et al., 2000).

Concerning point (b), the 120-kDa immunoreactive protein was detected in every immunoblot, and it was present in roughly the same proportion to the 52-kDa form no matter which method was used to denature and reduce samples prior to gel electrophoresis were tested (e.g., 1 min boiling, 1 h heating at 60 °C, 2 h incubation at 37 °C, or overnight incubation at 4 °C in the sample buffer, or rapid sonication prior to loading sample application). Aldecoa et al. (2000) also showed a CRLR gene-derived protein of greater than 100 kDa but concluded that this form was due to sample aggregation during denaturation and reduction. From our data, we suggest that this protein is not due to simple nonspecific aggregation, but instead, may represent a tightly bound CRLR dimer. This dimer may form as an artifact during sample preparation, or it may represent an active form of CRLR that occurs to a greater or lesser extent in different cell-types. A more interesting explanation for this higher molecular weight form would be that it represents a splice-variant expressed by the CRLR gene. Further experiments will be necessary to distinguish between these possibilities.

Concerning point (c), [Cys(Acm)^{2,7}]-CGRP has generally been reported to be inactive at the $CGRP_1$ receptor and to possess selective affinity for the $CGRP_2$ receptor as a partial agonist (Dennis et al., 1989; Wisskirchen et al., 1998). In MG63 cells, this peptide potently antagonized CGRP's stimulation of cAMP formation and also showed weak agonistic activity on its own, a finding that seems inconsistent with other data suggesting that the MG63 cell CGRP receptor is a type of $CGRP_1$ receptor. Waugh et al. (1999) have demonstrated that the ability to observe agonistic actions by [Cys(Acm)^{2,7}]-CGRP depends on experimental conditions, in particular, receptor density and the efficiency of receptor–effector coupling in the target cell. At 13,200 receptors/cell, receptor density does not seem unusually high, but cell-specific differences in receptor coupling could explain our results. There may also be as-yet-unexplained mechanisms by which specific cellular components can further modulate the apparent binding characteristics of a $CGRP_1$ receptor. Generally consistent with published studies (Dennis et al., 1989; Maggi et al., 1990; Zaidi et al., 1990; Stangl et al., 1991; Rovero et al., 1992; O'connell et al., 1993; Wisskirchen et al., 2000), on the other hand, the results of our work with additional CGRP derivatives suggest that the C-terminal region (at a minimum residues 23–37) is necessary for receptor recognition, that the central region (probably residues 8–18 of α-helix) is important for maintaining high binding affinity, and that the N-terminal region (at least residues 1–8) is also needed to trigger receptor-mediated intracellular effects. The disulfide bond of this N-terminal region seems essential for high potency agonistic activity, but disulfide-containing N-terminal fragments, when used alone, had no effect on cAMP production.

Point (d) relates somewhat to points (a) and (c). CGRP-(8-37) is expected to block CGRP binding to a $CGRP_1$ receptor, but 20-fold or greater differences in affinity make such demonstration difficult in practice. Nonetheless, there is interesting parallel in the observations that this fragment could only block 78% of [^{125}I]CGRP tracer binding (at 5 μM) or 90% of CGRP's maximal stimulation of cAMP formation (at 1 μM). This raises the possibility that a small fraction of CGRP binding represents the existence of (an)other CGRP receptor subtype(s). If so, these additional CGRP receptor subtypes could not be distinguished in classical saturation binding experiments [or in binding assays based on fluorescent polarization assays of the binding of Fluo-CGRP to intact MG63 cells (Burns, unpublished observations)].

Furthermore, recent studies by several research groups have demonstrated that CGRP induces the mobilization of intracellular Ca^{2+} stores (Kawase et al., 1995; Drissi et al., 1998, 1999), induces membrane hyperpolarization (Kawase et al., 1996; Kawase and Burns, 1998), and stimulates cellular proliferation in relatively mature types of osteoblastic cells by cAMP-independent mechanisms (Villa et al., 2000, 2002); similar data are also available from human embryonic kidney (HEK)-293 cells (Aiyar et al., 1999). These data suggest that osteoblastic cells at certain differentiation stages may express a second putative CGRP receptor subtype, which is associated with a cAMP-independent signaling pathway(s), but pharmacologically indistinguishable by classical saturation binding studies from a $CGRP_1$ receptor subtype.

However, it must be noted that Rorabaugh et al. (2001), using radioligand binding and Northern blot analyses, have very recently provided data arguing against the existence of $CGRP_2$ receptors. They have instead proposed that various tissue- or cell-dependent factors can modulate a single CGRP receptor subtype to the extent that it can show different affinities for CGRP ligands in different tissues. If this proves true and is applicable to osteoblastic cells at different levels of maturity, it is also possible that a common receptor subtype (associated with default preferential signaling pathways) may be functionally modified by additional osteoblast-dependent factors to generate pharmacologically distinct intracellular effects. Such an adaptive biochemical mechanism could explain all details of our present data.

Point (e) is a complex issue that concerns not necessarily the receptor itself, but probably receptor-associated signaling pathways. Our previous study in human gingival fibroblasts using very similar methods (Kawase et al., 1999) demonstrated that CGRP stimulates the phosphorylation of ERK and consequently stimulates Gin-1 cell proliferation by a PD98059-sensitive mechanism; however, we have found nearly the opposite in MG63 cells. Although CGRP has frequently been reported to stimulate the proliferation of many cell-types thought to express a $CGRP_1$ receptor (Haegerstrand et al., 1990; Cheng et al., 1995; Kawase et al., 1999; Villa et al., 2000, 2002), CGRP has not been termed a general mitogen; there are a number of cell-types in which it has no significant proliferative effect (Kawase et al., unpublished observations). In addition, changes in cell proliferation reflect much more of a complex and summary cellular response than do simple acute cellular effects. Not only does cell proliferation involves complex regulation of the cell cycle, but it is also influenced by many endogenous factors/effects extending over a period of hours. Consequently, mitogenic or anti-proliferative responses may not directly reflect the initial response of a cell to pharmacological challenge. Furthermore, Cheng et al. (1995) demonstrated that there is a balance between the desensitization of CGRP receptors and the variable half-life of CGRP under different culture conditions [since CGRP is a good substrate for neutral endopeptidases (Cheng et al., 1995), metalloprotease-2 (Fernandez-Patron et al., 2000), and other cellular enzymes] that determines whether mitogenicity can be demonstrated. Thus, certain culture conditions or different levels of serum may mask the mitogenic action of CGRP. In the present study, prolonged CGRP treatments ($\sim$ 24 h) failed to significantly stimulate MG63 cell proliferation in the presence of 1% fetal bovine serum but did produce a significant reduction in the proliferation of MG63 cells growing in the presence of 10% fetal bovine serum. From these data, CGRP does not seem to be rapidly degraded to an inactive form in our experimental system, and we conclude that CGRP has stimulated (a) particular signaling pathway(s) to down-regulate cell proliferation.

In this study of MG63 cells, CGRP phosphorylated both CREB and p38-MAPK, the first demonstration of this in an osteogenic cell-type. Similar effects have previously been reported in other cells (Disa et al., 2000; Parameswaran et al., 2000). Unexpectedly, and contrary to what has been reported in the literature, CGRP induced the rapid dephosphorylation and apparent inactivation of ERK in osteogenic MG63 cells. To our knowledge, this is the first report that CGRP actively down-regulates ERK activity by dephosphorylation (probably through activation of cellular phosphatases, possibly by a cAMP-dependent mechanism), and this finding contrasts with the experimental results produced by Parameswaran et al. (2000) in HEK-293 cells transfected with recombinant $CGRP_1$ receptor. Since activated ERK has been demonstrated to positively regulate cell proliferation in IGF-I-treated MG63 cells via activation of cyclin-dependent kinase-2 (Cdk-2) (Zhang et al., 1999), it seems likely that down-regulation of ERK produces the inhibition of MG63 cell growth in our experiments conducted with 10% fetal bovine serum, just in the same way as does the administration of PD98059 to inhibit the MEK-1/ERK pathway.

The probable signaling cascade is illustrated in Fig. 9. In support of this explanation, CGRP significantly inhibited proliferation only in cultures exposed to 10% fetal bovine serum, a condition that is expected to supply a sufficient mixture of growth factors to stimulate mitogenic signaling pathways, e.g., ERK. Overall, the two findings addressed by point (e) again argue that the CGRP receptor expressed by MG63 cells is not producing all the same effects expected of a typical $CGRP_1$ receptor. However, since this anti-proliferative action is thought to be mediated by a cAMP-dependent mechanism, these findings do not necessarily indicate that the basic character of the CGRP receptor is fundamentally distinguishable from that of typical $CGRP_1$ receptors.

The ability of CGRP to attenuate the proliferation of rapidly growing MG63 cells may not seem consistent with its proposed role as an anabolic factor in bone; however, additional experiments demonstrated that CGRP stimulated the expression and secretion of collagen type I in MG63 cell cultures. Thus, one likely interpretation of these data is that CGRP stimulates the differentiation of immature osteoblas-

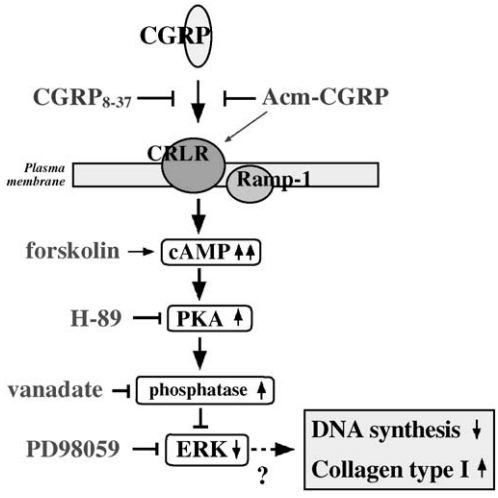

Fig. 9. Summary of CGRP receptor-mediated intracellular signaling pathways in immature osteoblastic human MG63 cells. CGRP binding activates the predominant CGRP receptor subtype expressed in MG63 cells, probably a version of $CGRP_1$, while CGRP-(8-37) antagonizes CGRP's binding to that site. $[Cys(Acm)^{2,7}]$-CGRP shows both agonistic and antagonistic actions at the receptor(s) expressed on these cells. The activated receptor(s) stimulate(s) cAMP production by adenylate cyclase, an effect mimicked by forskolin, and subsequently activate(s) the cAMP- dependent protein kinases (PKA), a step which is blocked by compound H-89. As a result of PKA activation, ERK is dephosphorylated and inactivated probably via activation of specific cellular phosphatases that are vanadate-sensitive. Substance PD98059 is an inhibitor of MEK-1, the major upstream kinase for ERK, and generally mimics the inactivation of ERK. As a result of activation of this cAMP-dependent signaling pathway, CGRP is able to suppress DNA synthesis and conversely up-regulate collagen synthesis in MG63 cells.

tic cells into more functional nonproliferating cells that begin to extrude the collagen-I-rich extracellular matrix expected from a maturing osteoblastic cell. Such an interpretation is supported by a recent report demonstrating that significant inhibition of MEK-1, the up-stream activator of ERK, promotes early osteogenic differentiation in both immature C2C12 mesenchymal cells and MC3T3-E1 pre-osteoblastic cells (Higuchi et al., 2002). Similarly, our own preliminary set of immunocytochemical experiments also showed that CGRP up-regulates the osteoblastic markers, alkaline phosphatase and osteocalcin, in MG63 cells (Kawase et al., unpublished observations). However, more quantitative additional studies are needed to reach firmer conclusions about how strongly CGRP promotes the osteogenic characteristics of MG63 cells and whether all these effects are produced primarily by inhibition of ERK activity.

In conclusion, our present data suggest that the CGRP receptors expressed in MG63 cells growing at log phase are very similar to typical $CGRP_1$ receptors, but that these CGRP receptors may function in a pharmacologically distinct manner.

Acknowledgements

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