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Effects of calcitonin gene-related peptide and amylin on human osteoblast-like cells proliferation

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Abstract

Expression of mRNA for calcitonin gene-related peptide (CGRP) and CGRP receptor has been detected in osteoblasts indicating that CGRP could play a role in bone metabolism. In the present study, we evaluated the effect of CGRP on primary culture of human osteoblast-like cells proliferation. The peptide was able to stimulate [^{3}H]thymidine incorporation in human osteoblast-like cells with a maximal effect at 10^{-8} M. The proliferating activity of CGRP was not inhibited by the two antagonists, CGRP-(8-37) or amylin-(8-37), whereas amylin fragment antagonized the proliferating activity of amylin. In human osteoblast-like cells CGRP, but not amylin, was able to stimulate adenylyl cyclase activity and this effect was completely antagonized only by CGRP-(8-37) and not by amylin-(8-37). These data suggest that the CGRP induced stimulation of cAMP is not involved in the peptide proliferating effect in human osteoblast-like cells and that in this cell population there are receptor subtypes for CGRP, distinct from that of amylin.

Keywords: Osteoblast, Human; CGRP (calcitonin gene-related peptide); CGRP-(8-37); Amylin, Amylin-(8-37); Proliferation; cAMP

1. Introduction

Calcitonin gene-related peptide (CGRP) is a 37-amino acid peptide generated by alternative tissue-specific splicing of the primary transcript of the calcitonin gene (Amara et al., 1982), and it is distributed in a variety of tissues (Van Rossum et al., 1997). In bone, CGRP is present in sensory nerve endings which innervate the bone metaphysisis, periosteum, (Kruger et al., 1989) and bone marrow (Bjurholm, 1991); recently, the expression of CGRP and CGRP receptor mRNA were demonstrated in human osteoblast-like cells (Drissi et al., 1997, 1999) implicating a role for the peptide in the local regulation of bone metabolism. Like calcitonin, CGRP inhibits osteoclast activity in vitro (Zaidi et al., 1987) and prevents trabecular bone loss in ovariectomized rats (Valentijn et al., 1997), but it has also been shown to have an osteogenic stimulating effect both in vivo and in vitro (Bernard and Shih, 1990; Shih and Bernard, 1997). Transgenic mice expressing CGRP in osteoblasts have an increased trabecular bone density compared with the wild type (Ballica et al., 1999). Furthermore, in rat osteoblasts, CGRP stimulates the production of insulin-like growth factor I (IGF-I) (Vignery and McCarthy, 1996), which is known to have an anabolic effect on bone (Machwate et al.,1994; Marie, 1999).

CGRP has considerable structural homology with amylin (Van Rossum et al., 1997), and both peptides produce similar biological effects in many tissues, including bone (Cornish et al., 1999). However, there is still uncertainty as to whether or not these peptides act through a common receptor in different type of cells. The two peptides are in fact able to displace each other from specific binding sites implying significant cross-reactivity with each other's receptors (Horne et al., 1994). The availability of selective pharmacological antagonists for the two peptides is a critical point for evaluating the role of each peptide in different type of cells. The two most widely used antagonists for CGRP and amylin, namely CGRP-(8-37) and amylin-(8-37), respectively, can indistinctly antagonize

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some of the actions shared by the two peptides, whereas they can selectively block other activities (Wimalawansa, 1996). These findings indicate that CGRP and amylin can act through the activation of a common receptor in some tissues, while in others, distinct receptors for either CGRP or amylin exist. On the basis of pharmacological evidence in the different tissues, the existence of at least three types of receptors for CGRP and one type for amylin has been proposed (Esfandyari et al., 2000; Poyner, 1997). The presence of mRNA for at least one type of CGRP receptor, demonstrated in human osteosarcoma cells and in normal human osteoblasts (Togari et al., 1997), would indicate that the bone anabolic effect of CGRP could be a receptor-mediated event.

We carried out the study reported here to examine whether or not CGRP has a proliferative effect on human osteoblast-like cells and to evaluate the receptor specificity by testing the effect of the receptor blockers, CGRP-(8-37) and amylin-(8-37), on CGRP and amylin induced activities. We used primary cultures of human osteoblast-like cells, since although they are a mixture of osteogenic cells, their proliferating activity in vitro may reflect bone formation in vivo (Marie, 1994).

2. Materials and methods

2.1. Drugs

αCGRP and amylin (Peptides Institute, Osaka, Japan), CGRP-(8-37) and amylin-(8-37) (Peninsula, St. Helen, UK).

2.2. Cell culture

Bone cells were established in culture by a modification of the Gehron Robey and Termine (1985) procedure. Cells were isolated from trabecular bone samples obtained from waste materials of patients (51–73 years old) who underwent to orthopaedic surgery for degenerative diseases or traumatic fractures of the femoral neck requiring osteotomy procedures. None of the patients submitted to surgery had any malignant bone disease.

Briefly, the trabecular bone taken from each subject was cut into small pieces ($2 \times 2 \times 2$ mm) and washed thoroughly with commercial standardized Joklik's modified minimal essential medium (Sigma, St. Louis, MO, USA) serum free to remove non-adherent marrow cells. The bone pieces were incubated with the same medium containing 0.5 mg/ml collagenase (type IV, Sigma, St. Louis, MO, USA) at 37°C for 30 min with rotation. The collagenase digestion was stopped by adding Iscove's modified Dulbecco's medium (IMDM) (Eurobio, Les Ulis, France) containing 20% fetal bovine serum (HyClone, Logan, UT, USA). The bone pieces (8–10 from each patient) were then placed in 25-cm^2 flasks and cultured in IMDM containing 20% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 U/ml mycostatin and 0.25 μg/ml amphotericin B. Cells began to migrate within 1–2 weeks and reached confluence after 1 month. Culture medium was changed every 2–3 days. The cell population was tested for alkaline phosphatase and osteocalcin production after $1,25(OH)_2D_3$ 10^{-8} M to ensure that the cells were endowed with osteoblast characteristics. Alkaline phosphatase was determined in the cell layer solubilized with 0.5 ml 0.1% sodium dodecyl sulphate (SDS) by measuring the *p*-nitrophenol phosphate reduction (Roche Diagnostics, Basel, Switzerland). Osteocalcin was measured by immunoradiometric assay (Nichols, San Juan Capistrano, CA, USA). All cells were used at the first passage to reduce the possibility of phenotype changes.

2.3. Cell proliferation assay

Cell proliferation was evaluated by [^{3}H]thymidine incorporation in semiconfluent cell monolayer. Semiconfluent cells were changed to serum-free medium with 0.1% bovine serum albumin and incubated for 48 h. Osteoblasts were then exposed for 24 h to the peptides and pulsed with 1 μCi of [^{3}H]thymidine/well (specific activity: 50 Ci/mM) 4 h before the end of the experimental incubation. Cells were rinsed three times in phosphate buffered saline (PBS) and solubilized in 100 μl of 0.1% SDS in 0.25 M NaOH. Cellular lysates were collected by aspiration on glass fiber-filters by a cell harvester (Canberra Packard Italia, Pero, Italy) and counted for radioactivity (cpm). Results are expressed as ratio of the mean cpm of treated group to the mean cpm of controls. Each experiment was performed using experimental groups consisting of at least 6 wells. Experiments were repeated six times each time using cells from different donors.

2.4. Cyclic AMP assay

cAMP production was determined in subconfluent cell monolayer. After a preincubation of 15 min with the phosphodiesterase inhibitor isobutylmetylxanthine 0.5 mM, cells were incubated for 10 min in presence or absence of the peptides. The reaction was stopped by aspirating the medium and cells were rinsed with cold PBS. One milliliter of ethanol 95% was added to each well and kept overnight at −20°C to extract cAMP from the cell layer. The extracts were dried under vacuum at 60°C and immediately assayed. cAMP was measured using a commercial radioimmunoassay kit ([^{125}I]cAMP, Amersham Italia, Milano, Italy).

2.5. Statistics

Statistical analysis was performed with the statistical package Prism vs. 2.01, (GraphPad Software, SanDiego, CA, USA). In the proliferation assays, the mean values for 6 wells of each group were used to calculate the ratio between treated groups and controls. Significance between

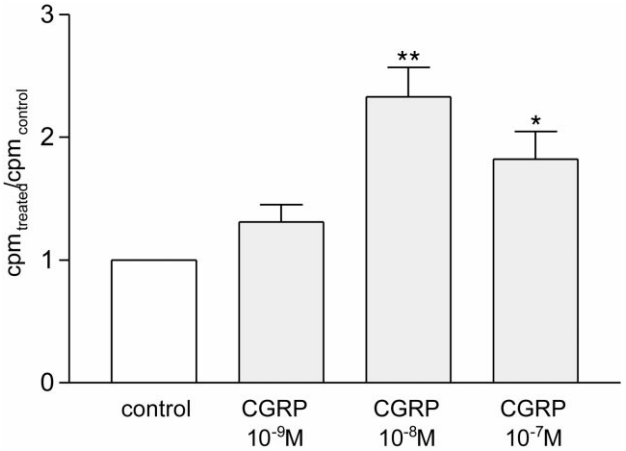

Fig. 1. Effects of different concentrations of CGRP on [^{3}H]thymidine incorporation in human osteoblast-like cells in culture. Data are the means ± S.E. of at least six experiments performed with groups of 6 wells and are expressed as a ratio of cpm of the treated cells to the cpm of the control (serum free). **: $P < 0.01$, *: $P < 0.05$ vs. controls, (Kruskal Wallis test).

groups was assessed analyzing the ratio obtained in the different experiments by means of a one way analysis of variance for non-parametric values (Kruskal Wallis test) and multiple comparison test (Dunn's test). Differences between groups in the cAMP determination were assessed by means of one way analysis of variance and Tukey's multiple comparison test. The concentration response curve of CGRP on cAMP production was analyzed using non-linear regression analysis.

3. Results

Cell culture responded to 1,25(OH)$_2$D$_3$ (10^{-8} M) with a significant increase of alkaline phosphatase and osteocalcin thus assuring that the culture were endowed with osteoblastic characteristic. Basal and stimulated alkaline phosphatase activity were 19.8 ± 2.8 and 38.7 ± 4.7 mU/mg proteins, respectively ($P < 0.01$) and osteocalcin values were 2.7 ± 0.6 and 26.4 ± 7.5 ng/mg proteins ($P < 0.02$). CGRP treatment of a serum-free human osteoblast-like cells culture for 24 h induced a significant increase in [^{3}H]thymidine incorporation (Fig. 1). The maximal effect, twofold that of controls, was obtained at 10^{-8} M; greater concentrations of the peptide did not further increase cell proliferation but on the contrary were less effective. Since amylin also induces human osteoblast-like cells proliferation (Villa et al., 1997) and, in order to

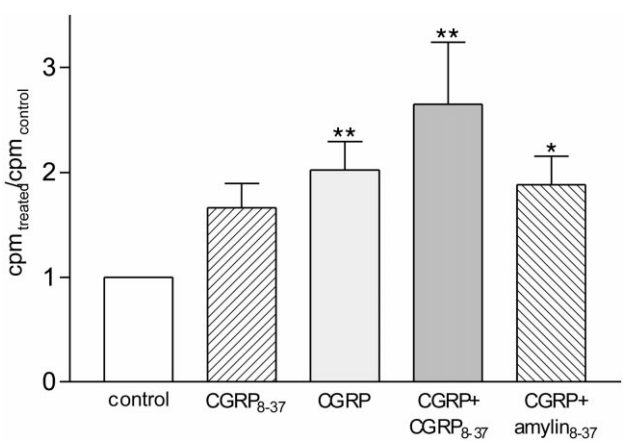

Fig. 2. Effects of the CGRP antagonist, CGRP-(8-37) (10^{-6} M) and amylin antagonist, amylin-(8-37) (10^{-6} M) on the proliferative action of CGRP (10^{-8} M) on human osteoblast-like cells measured as [^{3}H]thymidine incorporation. Data are the means ± S.E. of six experiments performed with groups of 6 wells and are expressed as a ratio of cpm of the treated cells to the cpm of the control (serum free). **: $P < 0.01$, *: $P < 0.05$ vs. controls, (Kruskal Wallis test).

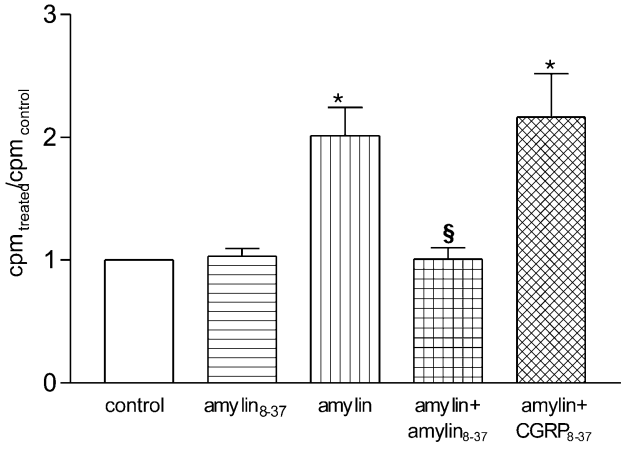

Fig. 3. Effects of the amylin antagonist, amylin-(8-37) (10^{-6} M) and CGRP antagonist, CGRP-(8-37) (10^{-6} M) on the proliferating action of amylin (10^{-9} M) on human osteoblast-like cells measured as [^{3}H]thymidine incorporation. Data are the means ± S.E. of six experiments performed with groups of 6 wells and are expressed as a ratio of cpm of the treated cells to the cpm of the control (serum free). **: $P < 0.01$, *: $P < 0.05$ vs. controls; §: $P < 0.05$ vs. amylin treated cells, (Kruskal Wallis test).

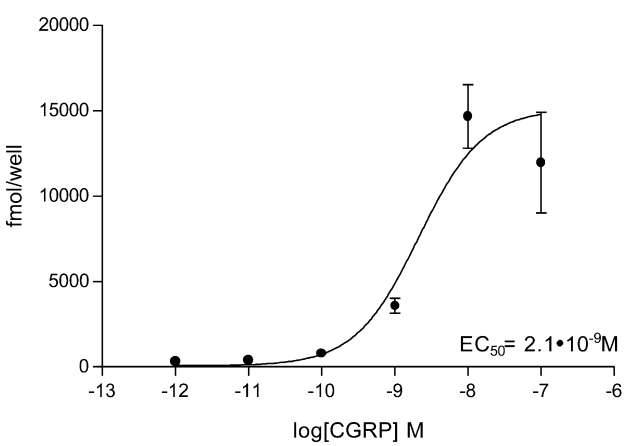

Fig. 4. Dose response of CGRP treatment on cAMP accumulation in human osteoblast-like cells. Data are the means ± S.E. of three experiments performed in triplicate and are expressed as fmol of cAMP produced per well. Dose–response curve was performed with nonlinear regression analysis.

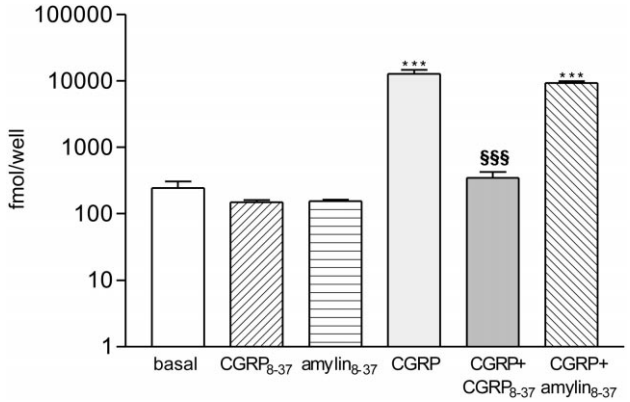

Fig. 5. Effects of the CGRP antagonist, CGRP-(8-37) (10^{-6} M) and amylin antagonist, amylin-(8-37) (10^{-6} M) on cAMP accumulation (fmol/well, log scale) in the absence or presence of CGRP (10^{-8} M). Data are the means ± S.E. of three experiments performed in triplicate and are expressed as fmol of cAMP produced per well. ***: $P < 0.001$ vs. controls; §§§: $P < 0.001$ vs. CGRP treated cells (one way analysis of variance).

explore the specificity of CGRP and amylin activities, the antagonists CGRP-(8-37) and amylin-(8-37) were used, and the results are reported in Figs. 2 and 3. The fragment CGRP-(8-37) (10^{-6} M) did not antagonize either CGRP (10^{-8} M) or amylin (10^{-9} M) effects on human osteoblast-like cells [^{3}H]thymidine incorporation but on the contrary produced an additional positive effect, which did not however reach statistical significance. Amylin-(8-37) (10^{-6} M) did not influence CGRP activity but was effective in antagonizing the proliferating effect of amylin. Lower concentration of the two fragments (10^{-8} M) gave the same results (data not shown). The fragments by themselves at 10^{-6} M had no effect on thymidine incorporation. Although CGRP-(8-37) showed a tendency to increase cell proliferation, this effect never reached statistical significance.

CGRP induced an increase in cAMP production in human osteoblast-like cells in a concentration related manner (Fig. 4). Maximal activation, 52-fold the basal activity, was observed at 10^{-8} M with a half maximal effective concentration (EC_{50}) of 2.1×10^{-9} M. Higher concentrations, 3×10^{-8} M, gave no further stimulation and 10^{-7} M was less effective. Amylin tested at the same range of concentrations, as for CGRP, did not increase cAMP production (data not reported). CGRP-(8-37) (10^{-6} M) was able to antagonize the CGRP (10^{-8} M) induced cAMP increment whereas the amylin antagonist (10^{-6} M) did not (Fig. 5). Both peptide fragments at a concentration of 10^{-6} M per se had no effect on cAMP production.

4. Discussion

The current results show that CGRP has a stimulating effect on [^{3}H]thymidine uptake in human osteoblast-like cells, which is not inhibited either by the antagonist peptide CGRP-(8-37) or by amylin-(8-37). In contrast, the CGRP stimulating activity on cAMP is selectively blocked by the CGRP fragment. This evidence suggests a heterogeneity of receptors for CGRP in human osteoblast-like cells. The existence of CGRP receptor subtypes was already proposed by several authors (Hall and Smith, 1998; Waugh et al., 1999) on the basis of the lack of antagonism by CGRP-(8-37) of some of the CGRP induced activities (Tomlinson and Poyner, 1996; Esfandyari et al., 2000). The best characterized receptor for CGRP is the $CGRP_1$ type which is effectively antagonized by CGRP-(8-37) (Aiyar et al., 1996).

The proliferative activity of CGRP on [^{3}H]thymidine incorporation has also been shown by Cornish et al. (1999) in primary culture of fetal rat osteoblasts; however, on rat osteoblasts, the effect of CGRP was antagonized by both CGRP-(8-37) and by amylin-(8-37), suggesting that CGRP and amylin act through a common receptor. Furthermore, in the same cells, CGRP induced only minimal changes of cAMP production (Cornish et al., 1999). In human osteoblast-like cells amylin fragment was ineffective in blocking CGRP induced proliferative activity and CGRP-(8-37), instead of blocking it, produced an increased positive effect (although this did not reach statistical significance). A possible explanation for this could be that the fragment, by blocking one type of CGRP receptor, could have a facilitatory effect on the others, and moreover, it could blunt the antiproliferative effect of cAMP on osteoblasts (Siddhanti and Quarles, 1994). The lack of antagonistic effect of amylin-(8-37) on CGRP proliferating activity and instead the antagonistic activity on amylin stimulated human osteoblast-like cells proliferation, strongly suggest that CGRP and amylin act through different receptors to stimulate human osteoblast-like cells proliferation. The different results obtained by Cornish et al. (1999) and by us might be due to the different type of osteoblasts used, rat vs. human. It is interesting to note that the expression of the mRNA for CGRP receptors was detected in human osteosarcoma cells and in human osteoblast-like cells (Drissi et al., 1999; Togari et al., 1997) while we have not found any reference so far indicating that CGRP receptor mRNA is also expressed in rat osteoblasts. Species differences between rat and human osteoblasts have been reported also for the effect of CGRP on intracellular calcium concentration. In a human osteoblast cell line, OHS-4, CGRP causes an influx of Ca^{2+} from the extracellular milieu via voltage-gated calcium channels (Drissi et al., 1998) whereas in rat osteoblastic cells, UMR106, the peptide inhibits uptake of calcium via ATP-sensitive K^+ channel mediated membrane hyperpolarization and subsequent inhibition of voltage-dependent Ca^{2+} channels (Kawase and Burns, 1998). In contrast, the increase of intracellular calcium concentration induced by CGRP from the endoplasmic reticulum is shared by both human and rat osteoblasts (Drissi et al.,

1998). These differences could be due to the presence in the cell of different types of receptor activity modifying proteins (RAMPs) that can regulate the ligand specificity of the calcitonin receptor-like receptor depending on which members of this new family of proteins are expressed in the cells (McLatchie et al., 1998; Foord and Marshall, 1999; Husmann et al., 2000).

The data obtained in our study showing that the stimulating effect of CGRP on cAMP production is blocked by CGRP-(8-37) but that nevertheless the peptide is still able to stimulate human osteoblast-like cells proliferation in presence of its fragment, are consistent with differential activation of second messenger systems to stimulate human osteoblast-like cells proliferation without involving cAMP activation. This evidence is also supported by the fact that amylin does not stimulate cAMP accumulation in these cells despite having a proliferating activity (Cornish et al., 1999; Villa et al., 1997). In general, studies of cultured osteoblasts have suggested that cAMP phosphokinase A dependent protein kinase cascade is involved in osteoblast differentiation rather than in proliferation (Siddhanti and Quarles, 1994). The significance of the remarkable stimulating effect of CGRP on cAMP in human osteoblast-like cells remains to be established. Since in osteoblasts, IGF-I production is induced by a rise of intracellular cAMP (McCarthy et al., 1995), it could be that CGRP stimulates this growth factor production through the activation of the adenylyl cyclase coupled receptor.

The peptide released from nerve fiber could have a potential role for the local regulation of bone remodeling and repair. It has been shown that the density of CGRP immunoreactive fibers increases near the sites of post fracture osteogenesis (Hukkanen et al., 1993). After bone destruction in arthritic rat, CGRP immunoreactive fibers in direct contact with the metaphyseal osteoblasts are increased (Imai et al., 1997). The increase in CGRP nerve fibers in pathological bone conditions suggests an anabolic role of the peptide in the fine neural adjustment of bone during bone reconstruction.

The present data provide further evidence to include CGRP among the peptides of the calcitonin family endowed with bone anabolic effect. Anabolic agents could produce a more significant enhancement of bone mass than antiresorptive drugs as the hierarchical order of bone remodeling sequence of events is such that any inhibitory effect on osteoclasts is transferred also to osteoblasts (Baron, 1996). Thus, the possible development of molecules promoting osteoblast activities could be relevant for the osteoporosis therapy particularly for the senile one that involves osteoblast senescence.

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