Human osteoblast-like cell proliferation induced by calcitonin-related peptides involves PKC activity

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Villa, I., C. Dal Fiume, A. Maestroni, A. Rubinacci, F. Ravasi, and F. Guidobono. Human osteoblast-like cell proliferation induced by calcitonin-related peptides involves PKC activity. Am J Physiol Endocrinol Metab 284:E627–E633, 2003. First published November 12, 2002; 10.1152/ajpendo.00307.2002.—The calcitonin peptides [calcitonin (CT), calcitonin gene-related peptide (CGRP), amylin] share many biological actions, including activity on bone cells. In the present study, CT (10⁻¹¹ to 10⁻⁹ M) stimulated [³H]thymidine incorporation in primary cultures of human osteoblasts (hOB), as already demonstrated for CGRP and amylin. RT-PCR analysis showed that the calcitonin receptor and the calcitonin receptor-like receptor are both expressed in hOB. In these cells, CT (10⁻¹⁰ M) and amylin (10⁻⁹ M), in contrast to CGRP (10⁻⁸ M), did not increase cAMP production. All three peptides stimulated protein kinase C (PKC) activity. To evaluate PKC involvement in hOB proliferation, cells were incubated with phorbol 12,13-dibutyrate, a stimulator of PKC activity; cell proliferation was increased in a dose-dependent manner (EC₅₀ = 3.4 × 10⁻⁹ M). Staurosporine (10⁻⁸ M), a PKC inhibitor, blocked phorbol 12,13-dibutyrate-induced PKC activity and cell proliferation. Inhibition of PKC by staurosporine also counteracted the stimulatory effect of CT, CGRP, and amylin on hOB proliferation. From these data, it is deduced that the activation of PKC is important for hOB proliferation and that it is involved in the anabolic effect of CT peptides on bone.

calcitonin gene-related peptide; amylin; bone cells; staurosporine; protein kinase C

CALCITONIN (CT), calcitonin gene-related peptide (CGRP), and amylin belong to the same family of peptides, the genes for which have a common ancestral origin. The first two peptides are generated by alternative splicing of the mRNA of the calcitonin gene (CALC 1) located on chromosome 11, whereas amylin is produced from mRNA of another CALC gene (CALC IV) located on chromosome 12 (5). The three peptides, which share substantial structural homology (36), produce similar biological effects in many tissues, including bone (29). It is generally acknowledged that CT plays an important role in skeletal homeostasis, being a key modulator of bone resorption through a direct effect on osteoclasts (22). The hormone, CT, inhibits bone resorption by inducing quiescence of cell motility and retraction of osteoclasts (24), and chronic administration of CT reduces the number of osteoclasts (5). There is also evidence that CT has a direct effect on osteoblasts (13), and, although the specificity of this effect is still controversial, CT appears to be involved in bone mass accrual as homozygous mice in a CALC1 knockout model develop osteopenia (19). A role on bone metabolism was also shown for CGRP and amylin as follows: CGRP inhibits osteoclast activity in vitro (39), prevents trabecular bone loss in ovariectomized rats (35), increases the number and size of bone colony development in vitro (32), stimulates the proliferation of osteoblast-like cells in vitro (37), and increases bone density in mice (4). Amylin decreases bone resorption by inhibiting the motility of osteoclasts (3), stimulates the proliferation of human and rat osteoblasts (8, 38), and increases bone mass in mice (7).

Each peptide of the CT gene family of peptides binds to its own specific receptor. Some of these receptors have been cloned: the CT receptor (CTR) and CT receptor-like receptor (CRLR), which achieve specificity for each peptide depending on the expression of accessory proteins or receptor activity-modifying proteins (RAMP1–3; see Ref. 23). The CTR belongs to a subfamily of the seven-transmembrane G protein-coupled receptor superfamily GPCR₁₂. Several isoforms of CTR have been identified so far (33). CTR is coupled to multiple signal transduction pathways through interaction with different G proteins. The binding of CT with its receptor activates, via the G protein Gs, the phospholipase C, which in turn leads to calcium mobilization and activation of protein kinase C (PKC). Although these signal pathways have been associated with specific effects of CT on osteoclasts, it is unclear which individual cascade is activated in osteoblasts.

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Heterogeneity of CGRP receptors has also been reported in that the CGRP-1 receptor subtype is sensitive to the antagonistic properties of the fragment peptide CGRP-(8–37), and the CGRP-2 receptor subtype is less sensitive (20). The main signal pathway used by CGRP-1 is the adenyl cyclase pathway, but CGRP has also been reported to increase intracellular calcium concentration through activation of phospholipase C (1). CGRP receptors are present in human osteoblast cells (hOB; see Ref. 34) and are coupled to the stimulation of osteoblast proliferation (8, 37). In early reports, it was suggested that amylin acts on a CGRP receptor to induce its biological effects, since in certain conditions the CGRP antagonist CGRP-(8–37) is able to block amylin activity (8); recent studies have clearly demonstrated the existence of distinct, highly specific receptors for amylin (20). The effects of amylin, CGRP, and CT in several cell types were shown to be mediated via activation of adenyl cyclase. However, in hOB, amylin does not induce any increase in cAMP production but does stimulate the proliferation of hOB (37). In addition, the CGRP-proliferating effect in hOB does not involve the accumulation of cAMP, although the peptide is able to stimulate adenyl cyclase in these cells (37).

The signal pathways involved in the anabolic effects of the CT gene family of peptides on osteoblasts are still a matter of debate; the variability of the data available may be dependent on the different cell lines or different experimental models employed. The present study was intended to assess the signaling pathways related to the proliferative effects of the CT gene peptides in hOB.

MATERIALS AND METHODS

Drugs. Salmon CT, phorbol 12,13-dibutyrate (PDB), and staurosporine were purchased from Sigma (St. Louis, MO); CGRP and amylin were purchased from the Peptides Institute (Osaka, Japan).

Cell culture. Bone cells were established in culture by a modification of the procedure by Gehron Robey and Termine (16). Cells were isolated from discarded trabecular bone samples obtained at osteotomy from patients who underwent orthopedic surgery for degenerative diseases or traumatic fractures of the femoral neck. None of the patients (51–73 yr old) submitted to surgery had any metabolic or malignant bone disease. Briefly, the trabecular bone taken from each subject was cut into small pieces (2 × 2 × 2 mm) and washed thoroughly with commercial standardized Joklik’s modified serum-free MEM (Sigma) to remove nonadherent marrow cells. The bone pieces were incubated with the same medium containing 0.5 mg/ml collagenase (type IV; Sigma) 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 10% FBS (HyClone, Logan, UT). The bone pieces were then placed in 25-cm² flasks and cultured in IMDM containing 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml gentamicin, and 0.25 µg/ml amphotericin B. Cells began to migrate within 1–2 wk and reached confluence after 1 mo. Culture medium was changed every 2–3 days. The cell population was tested for alkaline phosphatase and osteocalcin production after the addition of 10⁻⁸ M 1,25(OH)₂D₃ to ensure that the cells had these characteristic osteoblast responses. Alkaline phosphatase was determined by measuring the p-nitrophenol phosphate reduction (Roche Diagnostics, Basel, Switzerland) in the cell layer solubilized with 0.5 ml of 0.1% SDS. Osteocalcin was measured by immunoradiometric assay (Nichols, San Juan Capistrano, CA). All cells were used at the first passage to reduce the possibility of phenotype changes.

CAMP assay. Cell proliferation was evaluated using [³²P]thymidine incorporation in a semiconfluent cell monolayer. The culture medium for the semiconfluent cells was then changed to serum-free medium with 0.1% BSA, and the cells were incubated for 48 h. ¹²⁵I-BrdU was exposed for 24 h to the peptides and pulsed with 1 µCi [³²P]thymidine/well (sp act: 50.0 Ci/mmol) 4 h before the end of the experimental incubation. Cells were rinsed three times in 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 using a cell harvester (Canberra Packard Italia, Pero, Italy) and counted for radioactivity (counts/min (cpm)). Results are expressed as the ratio of the mean cpm of the treated group to the mean cpm of controls. Each experiment was performed in triplicate and repeated six times using cells from different donors each time.

PKC assay. After incubation with the peptides for different times (2, 5, 10, 30, and 60 min), cells were rinsed two times in cold PBS and solubilized in a lysis buffer containing 50 mM HEPES, 250 mM NaCl, 5 mM MgCl₂, 1% Triton, 10% glycerol, and 10 µg/ml aprotinin, leupeptin, and phenylmethylsulfonyl fluoride. The extracts were dried under vacuum at 60°C and then solubilized in 100 µl of water. PKC activity was determined by measuring the p-nitrophenol phosphate reduction (Roche Diagnostics, Basel, Switzerland) in the cell layer solubilized with 0.5 ml of 0.1% SDS. Osteocalcin was measured by immunoradiometric assay (Nichols, San Juan Capistrano, CA). All cells were used at the first passage to reduce the possibility of phenotype changes.
PKC activity and osteoblast proliferation

Fig. 1. Effect of different doses of calcitonin (10^{-12} to 10^{-9} M, filled bars), amylin (10^{-11} to 10^{-8} M, hatched bars), and calcitonin gene-related peptide (CGRP; 10^{-10} to 10^{-7} M, striped bars) on [3H]thyminidine incorporation in human osteoblast-like cells. Data are means ± SE of 4 experiments performed on cells from different donors. Each experiment was done with groups of 6–8 wells, and results are expressed as a ratio of the counts/min (cpm) of the treated cells to the cpm of controls (serum free). **P < 0.001 and *P < 0.01 vs. control.

Fig. 2. Expression of genes for calcitonin receptor (CTR) and calcitonin receptor-like receptor (CRLR) in human osteoblast-like cells obtained from 2 donors. RT-PCR was carried out using specific primer pairs for the different cDNAs. Lanes 1, DNA ladder (50-bp ladder). Lanes 2–5, CTR: lanes 2 and 4, PCR product obtained from cells from donor A cultured with 10% FBS (lane 2) and serum free for 48 h (lane 4); lanes 3 and 5, PCR product obtained from cells from donor B cultured with 10% FBS (lane 3) and serum free for 48 h (lane 5); lanes 6 and 11, PCR negative controls, samples with no RNA to test possible contamination of reagents. Lanes 7–10, CRLR: lanes 7 and 9, PCR product obtained from cells from donor A cultured with 10% FBS (lane 7) and serum free for 48 h (lane 9); lanes 8 and 10, PCR product obtained from cells from donor B cultured with 10% FBS (lane 8) and serum free for 48 h (lane 10).

Fig. 3. cAMP production in human osteoblast-like cells induced by calcitonin (CT; 10^{-10} M), amylin (AMY; 10^{-9} M), and CGRP (10^{-8} M). Data are means ± SE of 4 experiments performed on cells from 4 different donors. cAMP determination was done in triplicate, and results are expressed as fmol cAMP produced/well. **P < 0.001 vs. basal.

RESULTS

CT induced a significant increase in [3H]thyminidine incorporation in serum-free hOB culture at concentrations from 10^{-11} to 10^{-9} M, with the maximal effect (two times that of the control) at 10^{-10} M. The CT-proliferating effect was not antagonized by the CGRP-(8–37) or by the amylin-(8–37) fragments (10^{-8} M; data not shown). CGRP and amylin caused a proliferating effect similar to that of CT, with a maximal activity at 10^{-8} and 10^{-9} M, respectively. The stimulatory effects of CT, amylin, and CGRP treatment on hOB proliferation are reported in Fig. 1.

RT-PCR results show that hOB cells express the genes for both CTR and CRLR (Fig. 2). The mRNA for CTR was expressed at a relatively lower level than that of CRLR.

Of the three peptides, only CGRP was able to induce a significant increase in cAMP level in hOB (Fig. 3), whereas neither CT nor amylin was able to stimulate adenylyl cyclase activity in these cells. Conversely, all three peptides were able to induce a significant in-
crease of PKC activity with a comparable time course (Fig. 4, A-C). Maximal PKC activity was obtained 5 min after CT (10^{-9} M) or CGRP (10^{-8} M) and 2 min after exposure to amylin (10^{-9} M). To see whether or not the stimulation of PKC activity by the three peptides was involved in hOB proliferation, we tested the effect of PDB, a phorbol ester compound known to stimulate PKC activity. On hOB, PDB was able to increase cell proliferation in a dose-dependent manner between 10^{-8} and 10^{-6} M (Fig. 5). The stimulation of PKC activity induced by 10^{-7} M PDB was antagonized by the inhibitor of PKC activity staurosporine (10^{-9} M) at a concentration that, per se, did not change PKC basal activity (Fig. 6A). The higher concentration of staurosporine (10^{-8} M) significantly decreased basal

Fig. 4. Protein kinase C (PKC) activity induced by CT (10^{-10} M; n = 6; A), amylin (10^{-9} M; n = 10; B), CGRP (10^{-8} M; n = 12; C) in human osteoblast-like cells measured at 2, 5, 10, 30, and 60 min after treatment; n, no. of experiments performed with cells derived from different donors. Results are expressed as ratios of emitted fluorescence intensity of treated (I_{treated}) to emitted fluorescence intensity of untreated (I_{basal}) cells. Insets: typical gels obtained from cell lysates of human osteoblast-like cells treated with CT (A), amylin (B), and CGRP (C).

Fig. 5. Dose-response curve of phorbol 12,13-dibutyrate (PDB) treatment on [3H]thymidine incorporation in human osteoblast-like cells. Data are the means ± SE of 3 experiments performed with cells from 3 different donors. Each experiment was done with groups of 8 wells, and results are expressed as cpm/well. The calculated EC_{50} with nonlinear regression analysis is 3.4 × 10^{-8} M. *P < 0.001 vs. basal.

Fig. 6. A: effect of staurosporine (ST; 10^{-9} M) on PKC activity induced by PDB (10^{-7} M) in human osteoblast-like cells. Data are means ± SE of 4 experiments performed on cells from 4 different donors. Results are expressed as the ratio of I_{treated} to I_{basal}. *P < 0.05 vs. basal. §P < 0.01 vs. PDB (Kruskal-Wallis and Dunn’s test). B: effect of ST (10^{-9} M) on human osteoblast-like cell proliferation induced by PDB (10^{-7} M). Data are means ± SE of 3 experiments performed on cells from 3 different donors. Each experiment was done with groups of 6 wells, and results are expressed as a ratio of cpm of the treated cells to the cpm of controls (serum free). **P < 0.001 vs. basal. ***P < 0.001 vs. PDB (Kruskal-Wallis and Dunn’s test).
PKC activity, and 10^{-7} M had a toxic effect on the cells (data not shown). Staurosporine (10^{-9} M) significantly inhibited the PDB-induced hOB proliferation (Fig. 6B) as well as the proliferation induced by 10^{-10} M CT, 10^{-8} M CGRP, and 10^{-9} M amylin (Fig. 7).

**DISCUSSION**

The results of this study indicate that activation of PKC is involved in hOB proliferation induced by the family of CT peptides. Treatment with CT, amylin, and CGRP stimulated both [3H]thymidine incorporation and PKC activity in hOB, and inhibition of the latter activity by staurosporine also prevented the hOB proliferation induced by the three peptides. The involvement of PKC in hOB proliferation is further supported by the observation that staurosporine was able to counteract hOB proliferation induced by the phorbol ester derivative PDB, a known activator of PKC. However, the possibility that part of the stimulatory effect of the three peptides could be mediated by kinases other than PKC, such as cyclin-dependent kinases (17), cannot be ruled out, as it is known that staurosporine inhibits the activity of several kinases. In the present study, it is unlikely that staurosporine exposure could have induced apoptosis, since we used a very low concentration (10^{-9} M) that per se did not affect the viability of cells. It is known that staurosporine has a dual action on the cell growth cycle, with high concentrations stimulating apoptosis and low concentrations (2–6 × 10^{-8} M) blocking the transition from G0 to S phase (27).

The involvement of PKC in the proliferative effect exerted by the family of CT peptides on hOB is in line with the general view that PKC plays a pivotal role in cell proliferation (17). Activation of PKC was also shown to be involved in the mitogenic effect of lysophosphatic acid in rat osteoblasts through activation of a Gi protein and influx of extracellular calcium (18). Several PKC families of isoenzymes exist that are divided according to cofactor requirements for activity and responsiveness or nonresponsiveness to phorbol esters. Osteoblasts express both phorbol ester-sensitive and -insensitive isoenzymes (30). Because PDB is able to stimulate hOB proliferation and this effect is inhibited by staurosporine, it is likely that the phorbol ester-sensitive PKCs play an important role in hOB proliferation.

Activation of PKCs, with the exception of atypical PKCs, requires an increase in intracellular calcium concentrations and diacylglycerol and/or unsaturated fatty acids (6). CGRP was shown to increase calcium concentration in the human bone cell line OHS-4, which exhibits most of the osteoblast phenotype. The peptide causes an influx of calcium via voltage-gated calcium channels as well as a release of calcium from the endoplasmic reticulum (10). This effect of CGRP on intracellular calcium is not mediated via a G protein linked to the cAMP pathway (10). In human osteosarcoma cells, CT acutely increases net 45Ca uptake (12). On osteoclasts, CT-induced quiescence and cell retraction involve increased cytosolic free calcium concentrations (2, 40). The mitogenic action of CT on LNCaP, a cell line derived from prostate cancer, is thought to be mediated by CT-induced elevation of calcium levels (31).

Although the CTR is known to be coupled to stimulation of adenylyl cyclase activity in hOB, we did not detect any cAMP production induced by CT, thus excluding the possibility that the hOB-proliferating effect of CT is coupled to activation of the adenylyl cyclase-cAMP-PKA pathway. This possibility is also ruled out for amylin despite the previous study of Datta et al. (9), which demonstrated increased cAMP production after binding of amylin to osteoblast-like cells derived from osteosarcoma. This discrepancy might be because of the different type of cells that could activate different signaling cascades. In addition, the involvement of the adenylyl cyclase-cAMP-PKA pathway by CGRP could be excluded despite the significant enhancement of cAMP levels induced by the peptide in hOB. Although the CGRP-(8–37) peptide completely inhibits the CGRP-induced cAMP elevation, it is not able to block the proliferating effect of CGRP (37). In the same work, it was shown that CGRP and amylin act to stimulate hOB proliferation through different receptors, since amylin-(8–37) is able to antagonize the amylin proliferating effect but not that of CGRP. In the present study, the fact that CT induces hOB proliferation at low doses and that CTR is expressed in these cells suggests that CT acts through its own receptors. The activity of CT on osteoblasts is still controversial, although in 1988 Farley et al. (14) had shown that CT acts directly in vitro to increase bone formation and bone cell proliferation. Furthermore, CT is able to induce a dose-dependent increase in the secretion of insulin-like growth factor (IGF)-I and IGF-II in hOB (11).

The molecular pharmacology of CT, CGRP, and amylin is complex and far from being understood, since there are different CTR isoforms to which the CT gene family of peptides bind with different affinities. In addition, the affinity of the receptors for each peptide is determined by coexpression of one of the three RAMPs,
either with the CRLR or with the CTR (23). Because hOB are able to express both CRLR and CTR, further studies are needed to evaluate RAMPs expression in these cells. Expression of RAMP1 with CRLR binds CGRP with high affinity (20, 21). The CTR binds CT with high affinity, and the coexpression with RAMP1 or RAMP3 binds amyl with high affinity (29). In addition, when more than one RAMP is expressed in the same cell, a dominant or a competitive relationship exists between the different proteins (25). This extraordinarily elegant system allows for a diversification of receptor function, thus modulating the action of the CT gene products according to the local milieu (15). The cellular and molecular biology of the CT family of peptides and of their receptors has been extensively reviewed recently (28, 41).

In conclusion, this study has confirmed the anabolic potential of all three CT gene peptides on bone cells and has indicated that the activation of the PKC cascade is required for the CT peptides to induce hOB proliferation. The elucidation of the signaling pathway associated with osteoblast growth represents a step toward the assessment of the therapeutic potential of CT, amyl, and CGRP as attractive candidates for the treatment of osteoporosis.

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