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Procalcitonin's amino-terminal cleavage peptide is a bone-cell mitogen

(growth factors/osteoblasts/osteosarcoma/C cells/osteoporosis)

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ABSTRACT **The parafollicular-cell (C-cell) hormone calcitonin (CT) can preserve or even augment skeletal mass by inhibiting osteoclast-mediated bone resorption. The possibility of an additional anabolic skeletal influence has also been raised: C cells might, via CT or other secretory products, affect osteoblast-mediated bone formation. The 57-residue amino-terminal procalcitonin cleavage peptide, N-proCT, has recently been identified in human and rat C cells, where it is made and secreted in equimolar amounts with CT. The coelaboration of N-proCT and CT and N-proCT's sequence conservation during evolution prompted us to investigate the potential skeletal bioactivity of N-proCT. We found that synthetic human N-proCT, at nanomolar concentrations, stimulated proliferation of normal and neoplastic human osteoblasts. At maximally effective doses, human N-proCT caused more than a 100% increase above the control rate of DNA synthesis, an effect comparable to the maximal growth effect of insulin, a potent mitogen for osteoblasts. Human N-proCT exerted a similar maximal mitogenic effect in chicken osteoblast cultures but at 1000-fold greater concentrations than in human bone-cell cultures. The bone-cell action of N-proCT was potentiated with insulin with a >200% increase in DNA synthesis at high insulin concentrations. In sharp contrast to these findings for N-proCT, the other bioactive C-cell peptides, CT and somatostatin, showed no mitogenic effects in human or chicken osteoblast cultures. Our results indicate that the action of N-proCT on cultured bone cells is separate from and potentiated by insulin, a known growth factor. Unlike insulin and related growth factors such as insulin-like growth factor I, N-proCT is not mitogenic in skin fibroblast cultures. We propose that N-proCT is a C-cell hormone that promotes bone formation via stimulatory actions on osteoblasts and preosteoblasts.**

Calcitonin (CT) is a 32-amino-acid peptide hormone that in mammals is secreted mainly by thyroidal C cells (parafollicular cells). Although its precise role is disputed, CT's primary action is to inhibit bone resorption. This inhibitory action on osteoclasts has been considered the main mechanism for the protective and anabolic skeletal effects ascribed to CT (1–5). Several reports suggest that CT may also have a stimulatory effect on osteoblasts and their progenitors (6–8). While such diversity of CT effects could explain many of the anabolic skeletal effects associated with C-cell secretory activity, yet another explanation is suggested by recent findings in corticotropin (ACTH) biosynthesis. Non-ACTH peptides cogenerated during processing of the ACTH precursor show ACTH-related bioactivity that is distinct from but complementary to the role of ACTH in stress physiology (9, 10). In a similar manner, C cells could exert anabolic skeletal effects via the non-CT secretory peptides cogenerated with CT during processing of procalcitonin (proCT).

ProCT has been identified in fish, chickens, rats, and humans (11–13). In each species, proCT is ≈12 kDa with the CT sequence located internally and flanked by polybasic cleavage sites. Sequences of two non-CT secretory peptides are ≈2 and ≈7 kDa, respectively. Because of its smaller size, the 2-kDa peptide (C-proCT), derived from the carboxyl-terminal flanking region, was the first to be isolated, characterized, and synthesized (14–16). An early report on C-proCT claimed calcium-lowering and antiresorptive skeletal actions synergistic with CT (15). Other investigators and subsequently even the original reporting group found no CT-like actions for C-proCT (17, 18).

Recently, a 7-kDa 57-residue amino-terminal proCT cleavage peptide, N-proCT, was identified in rat and human C cells (19, 20). Like C-proCT, N-proCT is secreted in equimolar amounts with CT (19). Since CT and C-proCT circulate at roughly equimolar amounts, N-proCT is likely to circulate at significant concentrations. Unlike C-proCT, the N-proCT region has been highly conserved during evolution (Fig. 1). By testing partially purified rat N-proCT (from medullary thyroid carcinomas) we obtained preliminary evidence for N-proCT stimulation of osteoblast-like cells (21). However, because the rat medullary thyroid carcinomas also actively express the genes for insulin-like growth factors I and II (22), we could not rule out contamination by growth factors and therefore decided to investigate bone-cell regulatory effects with a synthetic N-proCT. The 57-amino-acid synthetic human N-proCT is mitogenic in human and chicken osteoblast cultures and this mitogenicity is both peptide and cell specific. We propose that N-proCT is a C-cell hormone with an anabolic osteoblastic effect distinct from and yet potentiated by insulin and insulin-like growth factors.

MATERIALS AND METHODS

Reagents and Synthetic Peptide Purification and Characterization. Unpurified synthetic human N-proCT was obtained from Applied Biosystems and then purified by HPLC (19). Structure and purity of isolated synthetic human N-proCT were verified by amino acid sequencing and by amino acid composition. Purified N-proCT recovery was 16% of the crude preparation; the purified peptide was lyophilized and stored at −20°C. Synthetic human CT was obtained from Ciba–Geigy, synthetic salmon CT was from Armour Pharmaceutical, and synthetic somatostatin was from Peninsula Laboratories. Purified bovine insulin was obtained from

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Abbreviations: CT, calcitonin; proCT, procalcitonin; N-proCT, amino-terminal procalcitonin cleavage peptide; C-proCT, carboxyl-terminal procalcitonin cleavage peptide; FBS, fetal bovine serum; TCA, trichloroacetic acid; ACTH, corticotropin.

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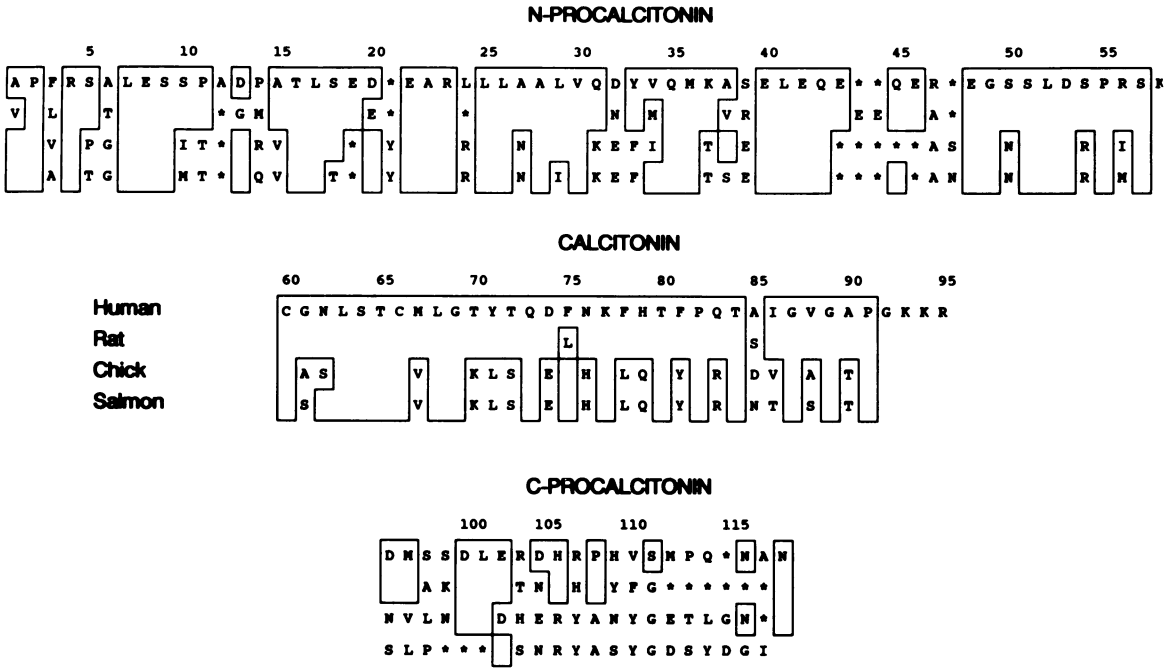

FIG. 1. The cDNA-predicted structures of human, rat, chicken, and salmon proCT. Sequences of rat, chicken, and salmon precursors are aligned below the sequence of the human peptide (adapted from ref. 19). An amino acid is not repeated in the subordinate sequences if it is the same amino acid in the human proCT sequence; such conserved regions are boxed. Substitutions are indicated by the standard single-letter abbreviations. An asterisk indicates a skip introduced in the rat, chicken, or salmon sequence to maximize matches with the human sequence. The regions corresponding to CT, N-proCT, and C-proCT are labeled.

Collaborative Research. Tissue culture media, buffered saline solutions, collagenase, and trypsin/EDTA were obtained from GIBCO. [*methyl*-^{3}H]Thymidine (6.7 Ci/mmol; 1 Ci = 37 GBq) was from New England Nuclear. Other reagents were from J. T. Baker or Sigma.

Cell Culture and *in Vitro* Cell Proliferation Assays. *In vitro* mitogenic assays used [^{3}H]thymidine incorporation into trichloroacetic acid (TCA)-insoluble material as an index of DNA synthesis and cell proliferation (23–25). [^{3}H]Thymidine-containing samples were analyzed with 2% or less counting error.

Human osteoblasts were isolated from femur fragments obtained during hip replacement in normal adults (ages, 48–62 yr) with osteoarthritis (26). The cells were collected and plated into tissue culture flasks in calcium-free minimal essential medium (MEM) with 15% fetal bovine serum (FBS) (27). At confluence, osteoblast-enriched cultures were trypsin treated, rinsed in phosphate-buffered saline (PBS), resuspended in BGJ (Fitton–Jackson modified BGJ_b) medium containing 5% FBS, and plated in 48-well plates (50,000 cells per well). After 16 hr, the medium was aspirated, the cultures were rinsed twice with PBS, and serum-free BGJ medium was added. After an additional 6 hr, medium was withdrawn and replaced by 165 μl of BGJ medium containing the desired test agent(s). After 16–18 hr, [^{3}H]thymidine (1 or 2 μCi per well) in 50 μl of BGJ medium was added to each well, and the incubation was continued for 4 hr. Incorporation of [^{3}H]thymidine into TCA-precipitable material was then quantified (23, 24). One group of cultures in each experiment was treated with a maximally effective concentration of bovine insulin (10 μg/ml, or 2 μM) as a positive control; this high concentration of insulin is as mitogenic as insulin-like growth factor I or II (25, 28, 29).

Chicken primary osteoblast cultures were prepared by digestion with collagenase (2 mg/ml in MEM) of calvaria from 16-day-old embryos and then plated into 48-well plates (50,000 cells per well) in serum-free BGJ medium (23, 24). After 24 hr, the medium was replaced with fresh BGJ medium containing the test agent(s). Test incubations and determinations of DNA synthesis were as described above.

Human U-2 OS osteosarcoma cells were obtained from American Type Culture Collection (ATCC no. HTB 96) and maintained in BGJ medium plus 1% FBS in T flasks. Confluent cells were treated with trypsin and plated into 48-well plates (20,000 cells per well) in BGJ medium containing 1% FBS. The time course for subsequent medium changes, as well as test incubations and [^{3}H]thymidine incorporation, were as described for normal human bone cells.

Primary skin cell cultures were prepared from both embryonic chicken and neonatal rat skin through collagenase treatments as described above. Skin cell suspensions were plated in BGJ medium with 1% or 5% FBS and switched to serum-free medium at least 6 hr before experiments.

Cell Counting Experiments. Although previous studies established a direct correlation between [^{3}H]thymidine incorporation and increases in cell number (23, 24), we confirmed our inference of increased cell numbers after N-proCT treatment. We extended treatment times to 44 hr to ensure enough cell division to detect increased cell number. We added trypsin/EDTA to release the cells, terminated the trypsin treatment with FBS (final concentration, 20%), dispersed the cells by pipetting, and then counted them with a hemocytometer.

Statistical Analyses. For statistical analyses and experimental comparisons we averaged normalized data from multiple independent experiments. Experimental means were determined for the data from each treatment group (six to eight replicate cultures for each treatment group in each experiment); these values were then normalized versus the mean of the control (no additions) group. The normalized data for each treatment group from four or more experiments were then averaged and compared by unpaired Student's t test (30). Significance was based on $P < 0.05$. Half-maximal effective doses were ascertained by interpolation of curves drawn through all treatment means up to the initial maximal effect.

RESULTS

Mitogenic Action of Human N-proCT on Human U-2 OS Osteosarcoma Cells. Based on [^{3}H]thymidine incorporation into DNA, N-proCT was mitogenic in U-2 OS cultures, with a half-maximal effect at ≈5 nM; maximally effective doses (10 nM or greater) doubled cell proliferation (Fig. 2). A comparable mitogenic effect was obtained with 2 μM insulin.

Mitogenic Action of Human N-proCT on Osteoblast Cultures Derived from Adult Human Bone. As in the U-2 OS cultures, insulin doubled DNA synthesis rates in these cultures. N-proCT showed virtually the same maximal effect as insulin in primary cultures of normal human bone cells; the effect (doubling) was half-maximal at ≈0.3 nM and maximal at ≈1 nM (Fig. 3).

Mitogenic Action of Human N-proCT on Cultured Embryonic Chicken Osteoblasts. We assessed the human N-proCT response by using embryonic chicken calvarial cells, which have been used in the detection and assay of factors regulating osteoblasts and progenitors (23–25). Maximally effective doses of N-proCT doubled DNA synthesis in these cultures (Fig. 4), comparable to the effect on human bone cells. The half-maximal response was observed at a dose (≈2 μM) 1000 times greater than required in human bone-cell cultures. The high doses needed to stimulate chicken cells indicate that sequence differences between chicken and human N-proCT may be biologically significant. Despite this 1000-fold difference in dose–response to human N-proCT, the chicken and human bone-cell cultures did not differ significantly in dose–response to insulin (data not shown). To rule out nonspecific peptide/protein effects as the basis for the effects of high concentrations of human N-proCT on chicken cells, we tested two C-cell peptide hormones, CT and somatostatin. Neither somatostatin nor CT (human or salmon) over a range of 0.1 nM to 100 μM significantly affected chicken bone-cell proliferation. Since the mitogenic effect of CT on chicken calvarial cells is only ≈50% (6), the lack of a CT effect in our experiments could reflect insensitivity due to cell density or other culture conditions. In any case, the effects of N-proCT or high doses of insulin clearly exceed that of CT.

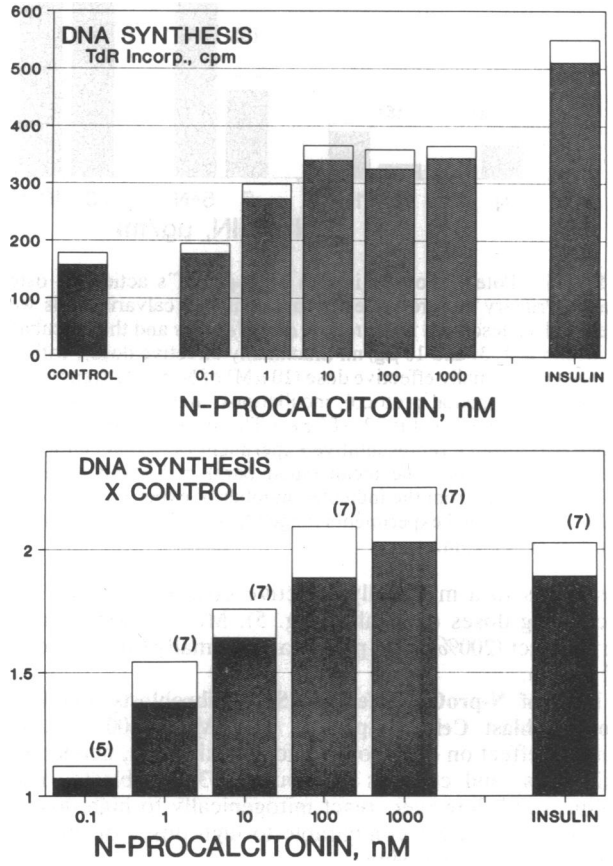

FIG. 2. Mitogenic action of synthetic human N-proCT in human osteosarcoma cell cultures. Serum-free cultures of human U-2 OS cells were incubated as indicated for 22 hr, and [^{3}H]thymidine uptake into TCA-insoluble material was determined during the last 4 hr. Unmodified culture medium served as the control treatment, and the maximally effective concentration (2 μM, or 10 μg/ml) of insulin served as the positive control. (*Upper*) A representative experiment showing the mitogenic action of human N-proCT. Shown are the mean (solid bar) ± SE (open bar) for each treatment (n = 6–8 cultures per treatment). (*Lower*) Mean (±SE) of normalized data. For each experiment, results were normalized to the mean value of untreated cultures. The number of replicate experiments for each treatment is indicated in parentheses. The mean values at 1 nM or greater differed significantly from control. The mean of control values in individual experiments ranged from 139 to 883 cpm with an average of 424 cpm.

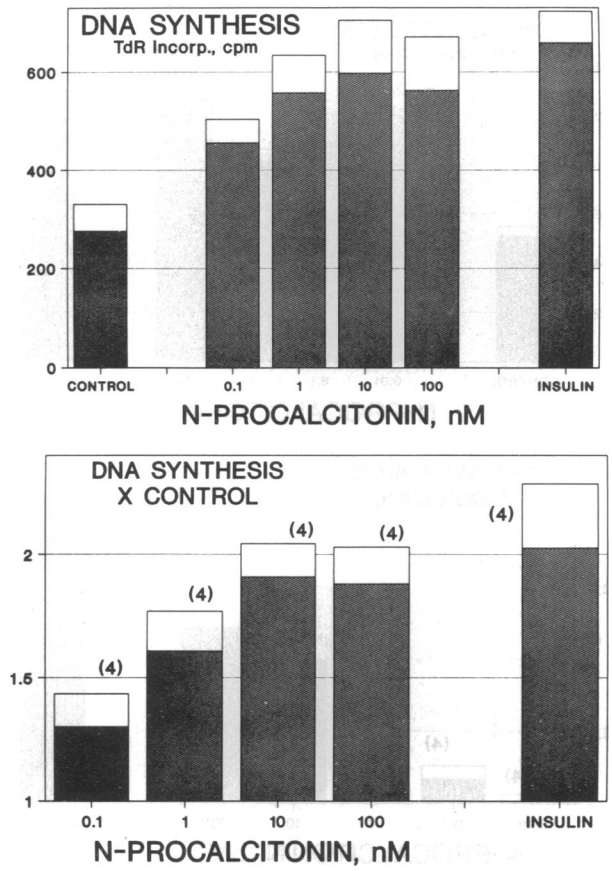

FIG. 3. Mitogenic action of N-proCT in human osteoblast cultures. Serum-free primary cultures of human bone cells were incubated for 22 hr as indicated, and [^{3}H]thymidine uptake was determined during the last 4 hr. Cell cultures were prepared as described in *Materials and Methods*; experiments were performed as described in Fig. 2. (*Upper*) Data from a representative experiment (n = 6–8 cultures per treatment); mean (solid bars) ± SE (open bars) are shown. (*Lower*) Mean ± SE of normalized data from the indicated number of experiments. The data were calculated and analyzed as described in Fig. 2. The mean of control values in individual experiments ranged from 55 to 296 cpm with an average of 176 cpm.

Cell Number Increased by Human N-proCT in Osteosarcoma and Osteoblast Cultures. It has been previously documented in osteoblastic cultures that DNA synthesis estimated by incorporation of [^{3}H]thymidine into TCA-insoluble macromolecules correlates directly with cell proliferation estimated by cell counting (23, 24). N-proCT caused a doubling of cell number within 44 hr in both human U-2 OS

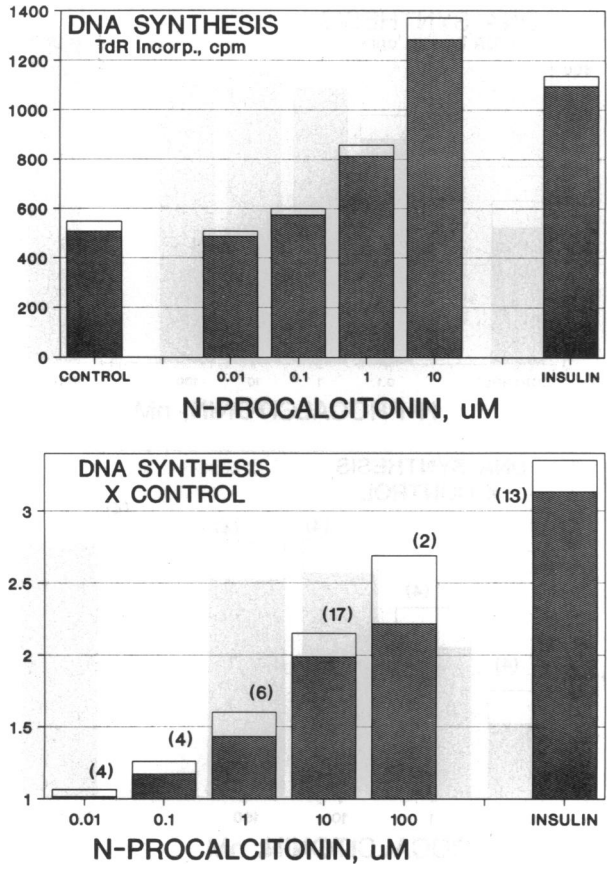

FIG. 4. Dose–response for N-proCT's effect on chicken osteoblasts. Primary cultures of chicken calvarial cells were prepared as described in *Materials and Methods*. Experiments were performed as described in Fig. 2. (*Upper*) The mean (solid bars) ± SE (open bars) from a representative experiment (n = 6–8 cultures per treatment). (*Lower*) Mean ± SE of normalized data. Data analysis was as described in Fig. 2. Means for effects with N-proCT doses of 1 μM or greater are significantly different from control. Control values in individual experiments ranged from 300 to 986 cpm with an average of 560 cpm.

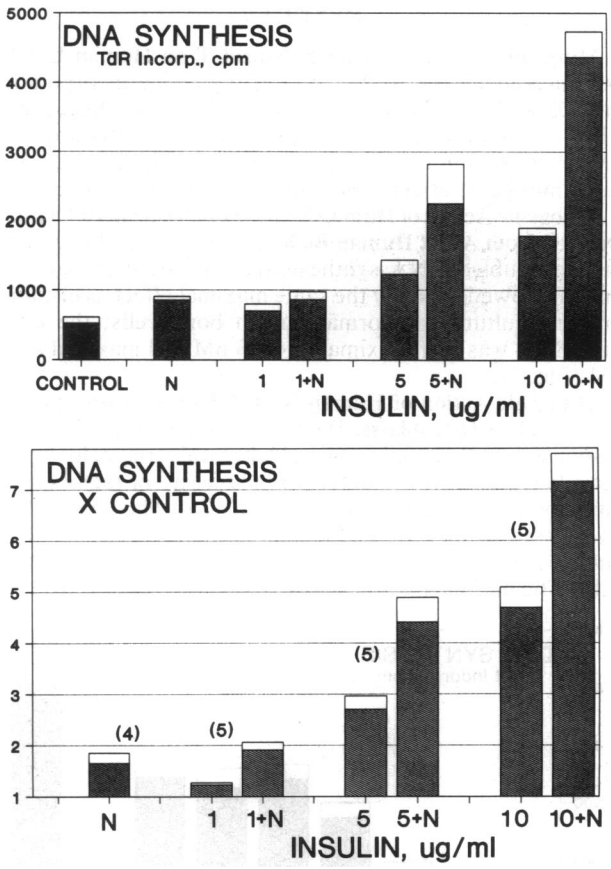

FIG. 5. Potentiation by insulin of N-proCT's action on osteoblasts. Primary cultures of embryonic chicken calvarial cells were prepared as described in *Materials and Methods* and then incubated in insulin at 1, 3, and 10 μg/ml (maximally effective dose), with and without a maximally effective dose (10 μM) of N-proCT (represented by N). Experimental protocol, incubations, and data analysis were done as described in Fig. 2. (*Upper*) The mean (solid bars) ± SE (open bars) from a representative experiment (n = 6–8 cultures per treatment). (*Lower*) The mean (solid bars) ± SE (open bars) of normalized data from the indicated number of experiments. Control values in individual experiments ranged from 400 to 593 cpm with an average of 506 cpm.

cells and chicken calvarial cells (Table 1), an increase that agreed with our estimations of DNA synthesis rates via [^{3}H]thymidine incorporation.

Potentiation of the Mitogenic Effects of N-proCT by Insulin. The somewhat greater maximal mitogenic effect of insulin and the limited availability of a standard bioactive insulin-like growth factor I preparation led us to use high doses of bovine insulin as a positive mitogenic control (25, 28, 29). Dose–response studies indicated maximal proliferative effects on human and chicken bone cells at 1–2 μM bovine insulin (data not shown). The actions of N-proCT appeared somewhat distinct from those of insulin; we found progressively larger responses to a maximally effective dose of N-proCT with increasing doses of insulin (Fig. 5). Most striking was the large effect (200%) of N-proCT at maximally effective doses of insulin.

Lack of N-proCT Effect on Skin Fibroblasts and Other Nonosteoblast Cells. N-proCT, 0.1 nM to 100 μM, was without effect on embryonic chicken skin cells, neonatal rat skin cells, and cultures of mouse 3T3 fibroblasts. These cultures did, however, react mitogenically to high doses of insulin to an extent comparable to high doses of insulin in bone cells (data not shown).

Table 1. Comparison of the growth effects of N-proCT and insulin in two types of osteoblast-like cell cultures

	Chicken calvarial cells		Human U-2 OS cells	
Treatment	Cells per well	× control	Cells per well	× control
Control (BGJ)	25,900 (±3,100)	1.00 (±0.12)	4,800 (±800)	1.00 (±0.16)
N-proCT*	69,600 (±9,500)	2.70 (±0.37)	13,200 (±1460)	2.72 (±0.30)
Insulin (2 μM)	126,000 (±15,700)	4.87 (±0.61)	12,900 (±800)	2.62 (±0.16)

Chicken osteoblasts and human osteosarcoma cells were plated in 48-well microtiter plates and incubated for 2 days. Cells were then released from each well by treatment with trypsin and were dispersed for counting in a hemocytometer. Means and SEM of each treatment group (n = 6 or 8) are presented. Both treatments (insulin and N-proCT) significantly increased cell number.
*Chicken calvarial cells, 10 μM; human U-2 OS cells, 1 μM.

DISCUSSION

The elucidation of CT biosynthesis revealed two proCT-derived peptides produced and secreted in amounts equimolar with CT (19). C-proCT and CT are known to be cosecreted *in vivo*. A preliminary report has identified N-proCT in the plasma of normal humans and in medullary thyroid carcinoma patients, suggesting that N-proCT is also secreted *in vivo* (31). Our present *in vitro* results with bone cells suggest that N-proCT derived from C cells is a skeletal-regulating peptide hormone that exerts anabolic effects related to but distinct from those of CT. These two proCT-derived peptides differ in primary actions and skeletal cell target. CT acts mainly to inhibit osteoclasts, whereas N-proCT stimulates osteoblasts and/or preosteoblasts. Thus, the skeletal preservation and growth frequently associated with C-cell hyperplasia in several animal species could reflect the concurrent secretion and concerted, distinct skeletal actions of these two complementary proCT-derived peptides. Such a multifactorial scheme for preservation and growth of the skeleton strengthens the concept that functional interrelationships may exist between peptides arising from a common precursor, an idea first suggested by the discovery of the various stress-related roles played by the peptide hormones derived from proopiomelanocortin processing in the pituitary (9, 10). Based on the structures and activities of the multiple proopiomelanocortin-derived peptides and those of the multiple proCT-derived peptides (CT, N-proCT, and C-proCT), we suggest that sequence conservation of "cryptic" prohormone regions during evolution is an important criterion for identifying other potentially important secretory peptides.

Despite the frequent reports of a correlation between changes in C-cell activity and bone mass (2, 4, 32, 33) and even reports of *in vitro* stimulatory effects of CT on osteoblasts (6, 7), it has been difficult to prove that CT is an effective bone-forming agent, much less a critical physiologic regulator of bone formation (1, 34). Perhaps the role of the C cell and the proCT-derived peptides it elaborates can now be understood by considering the distinct skeletal actions of CT and N-proCT. The *in vitro* N-proCT effects we report here suggest physiological and pathophysiological significance that can only be proven by *in vivo* studies. Our *in vitro* studies show efficacy of N-proCT at nanomolar concentrations, equipotency with high doses of insulin, and relative specificity for osteoblasts, indicating that this peptide could have pathogenetic and therapeutic importance in osteopenic states characterized by deficient osteoblasts. N-proCT is the only systemic factor yet found that specifically stimulates osteoblast proliferation as potently as insulin and the insulin-like growth factors (29). The *in vitro* proliferative effect of N-proCT on osteoblasts is greater than the effect of known skeletal-regulating hormones such as CT, parathyroid hormone, growth hormone, and estradiol (6, 7, 35, 36). This finding might mean that bone formation is controlled more by N-proCT than by CT, which would explain why the increased bone formation associated with C-cell hyperplasia cannot be replicated by chronic administration of CT alone and why CT treatment alone has only limited benefit in human osteoporosis (1). By promoting proliferation of preosteoblasts and osteoblasts, N-proCT might offset the putative coupled inhibition of bone formation attendant on CT inhibition of osteoclast-mediated bone resorption (1). Treatment with a combination of the two proCT-derived C-cell peptides might be far more effective than treatment with either agent alone.

We must still systematically test many cell types to define the target-cell specificity for N-proCT. Such specificity is clearly narrower than the effects of insulin, insulin-like growth factors, and other growth factors being considered for treatment in osteopenic states (1, 28, 29, 37). The dilemma of generalized growth effects—on soft as well as hard tissues—which could possibly deter treatment of bone loss with insulin-like and other growth factors, might be circumvented by administration of N-proCT.

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