Vol. 207, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 6, 1995 Pages 133-139

# AMYLIN STIMULATES OSTEOBLAST PROLIFERATION AND INCREASES MINERALIZED BONE VOLUME IN ADULT MICE

Jill Cornish*, Karen E. Callon*, Garth J.S. Cooper†*, and Ian R. Reid*[1]

*Department of Medicine and †School of Biological Sciences, University of Auckland, Auckland, New Zealand

Received December 12, 1994

Amylin, a 37-amino-acid peptide co-secreted with insulin from the beta-cells of the pancreatic islets, has previously been demonstrated to inhibit bone resorption *in vitro*. However, its effects on bone formation and bone mass have not been assessed. We report that peri-physiological concentrations of amylin stimulate proliferation of fetal rat osteoblasts *in vitro*. When amylin is injected daily for 5 days over the calvariae of adult mice *in vivo*, there are substantial increases in histomorphometric indices of bone formation, a reduction in bone resorption, and a significant increase in mineralized bone area. Equimolar doses of calcitonin in this *in vivo* model produced an inhibition of bone resorption but no significant effect on bone area. These findings support a role for amylin as a physiological regulator of bone and suggest that it should also be evaluated as a potential treatment for osteoporosis. © 1995 Academic Press, Inc.

Body weight is one of the strongest correlates of bone density in man (1-3), and low body weight is a major risk factor for osteoporotic fractures (4, 5). These findings are often attributed to the skeletal hypertrophy in response to increased weight-bearing and, in women, to estrogen production by the adipocyte. These are not adequate explanations for much of the recent data in this field (6) so it is likely that other mechanisms are operative. Insulin circulates in higher concentrations in obesity, and peripheral concentrations of this peptide are related to bone density in normal postmenopausal women (7). Bone density is reduced in insulinopenic diabetes (8, 9). Thus, the anabolic actions of insulin on bone may contribute to the inter-dependence of weight and bone density . However, insulin is co-secreted with amylin from the pancreatic beta cell and so this latter peptide might also be involved in these relationships.

Amylin, a 37-amino acid peptide (10), circulates in higher concentrations in obesity (11, 12). It produces hypocalcaemia in the rat (13) and in man (14), and has been shown to reduce basal and parathyroid hormone-stimulated bone resorption in neonatal mouse calvariae (15).

[1] Correspondence to Dr. Reid. Fax: (64 9) 3737 006.

0006-291X/95 $5.00
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These findings can be accounted for by an inhibition of osteoclast function, probably via a direct effect on cell motility (16). However, its effects on osteoblast function and bone mass remain unknown and are assessed in the present studies.

## METHODS

Osteoblast-like cells were prepared by sequential collagenase digestion of calvariae from 20 day fetal rats, as described previously (17). The osteoblastic phenotype of these cells has been established by the demonstration of high levels of alkaline phosphatase and osteocalcin, and a sensitive adenylate cyclase response to parathyroid hormone and prostaglandins.

The effects of the peptides on bone histology *in vivo* were assessed by subcutaneously injecting the peptide daily for 5 days over the periosteum of the right hemicalvaria of adult, male Swiss-Webster mice. The animals were sacrificed 1 week following the last injection. Non-decalcified sections of the calvariae were prepared as described previously (18). Quantitative histomorphometry was performed by an operator blinded to the treatment each animal had received. Data are expressed as the ratio of each index measured in the injected hemicalvaria to that measured in the contralateral, uninjected hemicalvaria of the same animal and have been evaluated by paired t tests.

## RESULTS

### *Osteoblast Studies*

Exposure of growth-arrested cultures of fetal rat osteoblast-like cells to amylin for 24 hours resulted in a substantial, dose-related increase in cell number (Figure 1a). The related peptide, calcitonin gene-related peptide (CGRP), also increased osteoblast proliferation, but the effect was smaller and only significant at $10^{-8}$M. Rat calcitonin ($10^{-9}$-$10^{-7}$M) and salmon calcitonin ($10^{-7}$M) were without effect, and parathyroid hormone ($10^{-8}$M) reduced cell numbers 18% at 24 hours ($p=0.025$) (data not shown). Amylin's stimulation of osteoblast proliferation was not dependent on the basal growth rate of the cells and was also observed in actively growing cells (Figure 1b). The effect of amylin on osteoblast proliferation was sustained over time, as judged both by cell number which was increased in amylin-treated cells from 6 to 48 hours (Figure 2a), and by a sustained increase in DNA synthesis assessed by thymidine incorporation (Figure 2b).

### *In Vivo Calvarial Studies*

The effects of amylin on bone *in vivo* were compared with those of equimolar amounts of calcitonin and CGRP by injecting the peptides daily over the calvariae of live adult mice. Vehicle injection had no effect on any histomorphometric index (Figure 3) . In contrast, amylin produced two- to three-fold increases in the indices of bone formation and decreases in bone resorption of comparable magnitude. Mineralized bone area increased 20% after 5 days of treatment. Calcitonin and CGRP did not significantly change indices of bone

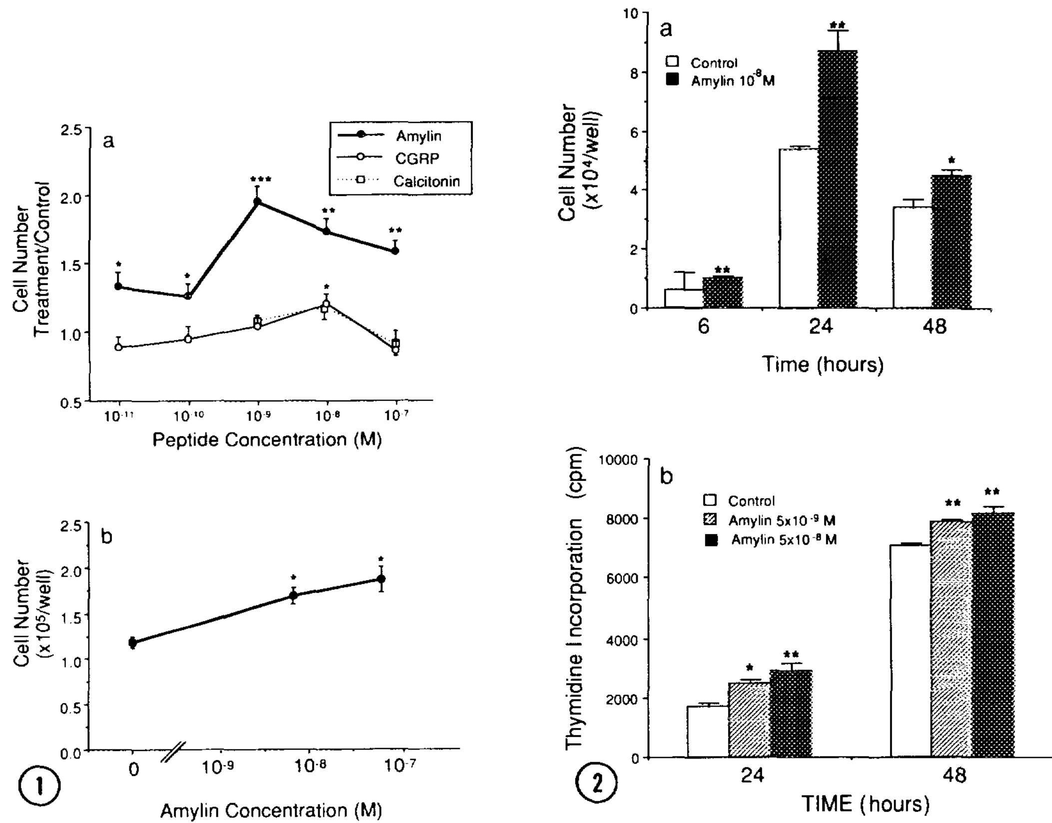


Figure 1. Dose-dependence of the effects of rat amylin, rat calcitonin gene-related peptide-1 (CGRP) and rat calcitonin on numbers of osteoblast-like cells in culture over 24 hours. Cells were either (a) growth-arrested as a result of being cultured in the presence of 0.1% bovine serum albumin or (b) were actively growing in culture medium containing 2% fetal calf serum. n=6 in each group. Data are mean ± sem. Statistical significance (by Student's t test) of differences from control: * $p \leq 0.05$; ** $p < 0.005$; *** $p < 0.001$.

Figure 2. (a) Time-course of the effect of rat amylin on proliferation of osteoblast-like cells from fetal rat calvariae. Statistical significance of differences from control at each time-point: * $p \leq 0.02$; ** $p < 0.005$.
(b) The effect of rat amylin on thymidine incorporation into osteoblast-like cells from fetal rat calvariae. Statistical significance of differences from control at each time-point: * $p = 0.002$; ** $p < 0.001$.
In both experiments the culture medium contained 0.1% bovine serum albumin. n=6 in each group. Data are mean ± sem.

formation but calcitonin reduced eroded surface to a similar extent to amylin. However, mineralized bone area was increased only 4% by calcitonin, a non-significant effect. The bone was histologically normal in all the treatment groups- there was no evidence of woven bone formation. Weight gain did not differ between treatment groups and the animals remained healthy, as judged by coat condition and behavior.

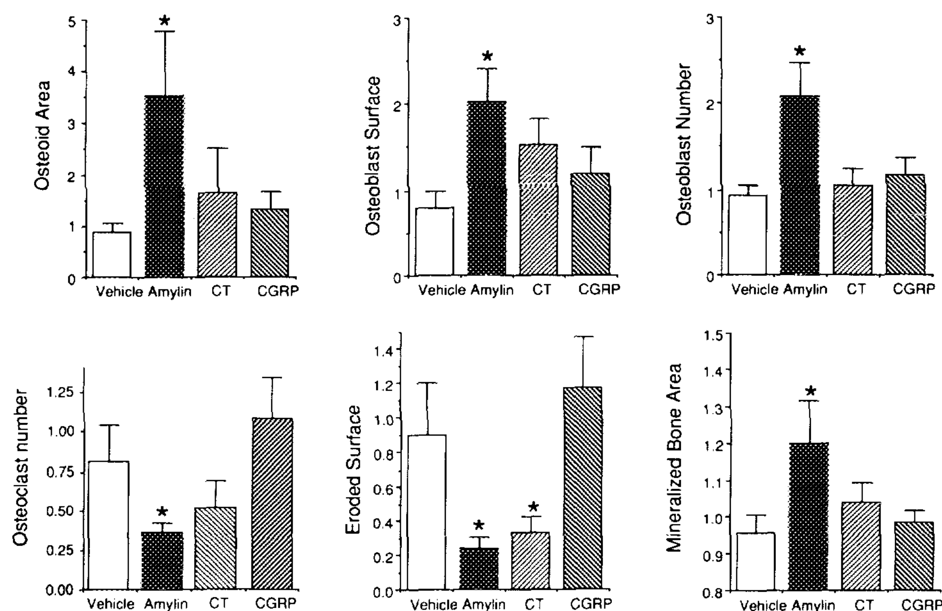


Figure 3. Comparison of the *in vivo* effects of rat amylin, rat calcitonin (CT) and rat CGRP in adult mice. The daily dose of each peptide was $4.1 \times 10^{-9}$ mol. Data are expressed as the ratio of each index measured in the injected hemicalvaria to that measured in the contralateral, uninjected hemicalvaria. n=5 in each group. Data are mean ± sem. Significant differences ($p<0.05$) between the injected and uninjected hemicalvariae are indicated by asterisks.

## DISCUSSION

These results establish amylin as a stimulator of osteoblast proliferation. *In vitro*, the effect of amylin on osteoblast numbers is similar to or greater than that of the established osteoblast growth factors, transforming growth factor-$\beta$, epidermal growth factor, and insulin-like growth factors 1 and 2, studied in comparable systems (19-23). The present data demonstrate stimulation of osteoblast proliferation at amylin concentrations comparable to those found in the circulation of normal subjects (24-26), even without making allowance for the high rates of loss of amylin onto the surfaces of laboratory plasticware (27). This suggests that amylin may be a physiological regulator of osteoblast function. There is some evidence for amylin production by osteoblasts (28), so its concentration in the bone microenvironment may be considerably higher than that found to be minimally active in these studies.

The *in vivo* model used in these studies is relatively new but has been used to assess the effects of a variety of hormones and cytokines on bone (18, 29-32). In general, the findings in this model have been consistent with those from other systems and are in accord with current understanding of the action of this wide range of factors in human bone physiology.

Indeed, the present findings with calcitonin closely parallel clinical experience with this peptide in the management of postmenopausal osteoporosis. Thus, the effects of amylin on bone formation and resorption found in the present studies and their synergism to increase mineralized bone area, might be expected to occur in humans also. This could be of potential therapeutic value in the treatment of local bone defects or delayed fracture healing, and amylin administration may have a role in the management of osteoporosis.

CGRP has significant sequence similarity to amylin, and micromolar concentrations of CGRP have been shown to increase the number of bone colonies formed in cultures of rat bone marrow (33). The present finding that amylin affects osteogenic cells at sub-nanomolar peptide concentrations, suggests that the CGRP effects on osteoblast proliferation and possibly on bone marrow cells may be mediated through the amylin receptor. The present results do not support a role for systemic CGRP influencing bone metabolism since circulating concentrations of this peptide are of the order of $10^{-12}$M (34).

The apparent involvement of a secretory product of the pancreatic beta cell in bone metabolism requires comment. Amylin is co-secreted with insulin in response to a meal. Thus, amylin may provide a post-prandial anabolic stimulus to bone (35), just as insulin does to protein synthesis in general. This makes physiological sense, since at this time the substrates for new bone are available from the diet. If amylin is a physiological regulator of bone formation then it could, with insulin (7), contribute to the dependence of bone density on body mass providing an indirect mechanism whereby skeletal strength responds to changes in skeletal load. Furthermore, amylin deficiency might contribute to the osteopenia observed in patients with type I diabetes mellitus.

Postmenopausal osteoporosis is an increasing health problem as a result of the aging of the population. Despite recent therapeutic advances there is a need for safer and more effective therapies. Most currently available treatments primarily inhibit bone resorption and are, therefore, limited in the extent to which they can reverse pre-existing bone loss. The discovery of a compound which combines anti-resorptive properties with stimulation of bone formation, and thus results in substantially greater increases in bone mass than are produced by calcitonin, offers the possibility of a novel therapy for this chronic disabling condition.

## ACKNOWLEDGMENTS

This work was supported by the Health Research Council of New Zealand. The authors are grateful to Prof. A.R.Bellamy and Prof. J. Kistler for their comments on the manuscript.

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