

# Systemic administration of a novel octapeptide, amylin-(1–8), increases bone volume in male mice

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**Jillian Cornish, Karen E. Callon, Juerg A. Gasser, Usha Bava, Edith M. Gardiner, David H. Coy, Garth J. S. Cooper and Ian R. Reid.** Systemic administration of a novel octapeptide, amylin-(1–8), increases bone volume in male mice. *Am J Physiol Endocrinol Metab* 279: E730–E735, 2000.—Amylin increases bone mass when administered systemically to mice. However, because of its size, the full peptide is not an ideal candidate for the therapy of osteoporosis. The fragment, amylin-(1–8), stimulates osteoblast proliferation *in vitro*, although it is without effect on carbohydrate metabolism. The present study assessed the effects of daily administration of this peptide on sexually mature male mice for 4 wk. Amylin-(1–8) almost doubled histomorphometric indices of osteoblast activity but did not change measures of bone resorption. Trabecular bone volume increased by 36% as a result of increases in both trabecular number and trabecular thickness, and tibial cortical width increased by 8%. On three-point bending tests of bone strength, displacement to fracture was increased by amylin-(1–8), from  $0.302 \pm 0.013$  to  $0.351 \pm 0.017$  mm ( $P = 0.02$ ). In a separate experiment using dynamic histomorphometry with bone-seeking fluorochrome labels, amylin-(1–8) was administered by local injection over the calvariae of female mice. Amylin-(1–8) (40 nM) increased the double-labeled surface threefold. The effect was dose dependent from 0.4 to 40 nM and was greater than that of an equimolar dose of human parathyroid hormone-(1–34) [hPTH-(1–34)]. Mineral apposition rate was increased by 40 nM amylin-(1–8) but not by hPTH-(1–34). Amylin-(1–8) thus has significant anabolic effects *in vivo*, suggesting that this peptide or analogs of it should be further evaluated as potential therapies for osteoporosis.

amylin; osteoporosis; peptide hormones; bone growth; osteoblast

THE PANCREATIC PEPTIDE amylin has previously been shown to stimulate proliferation of osteoblasts and to inhibit osteoclastic bone resorption, both *in vitro* and *in vivo* (4, 8, 13). These effects result in substantial increases in bone mass when the peptide is administered either locally or systemically to sexually mature mice (4, 6). The anabolic effects of amylin on osteoblasts are

shared by the related peptide adrenomedullin, although adrenomedullin does not modulate osteoclast activity (5). These findings suggest that this class of peptides might have a role in the therapy of osteoporosis, although because of its size the intact peptide is not an ideal therapeutic agent. One way of circumventing this problem is suggested by our recent observation that an octapeptide fragment of this hormone, amylin-(1–8), which is inactive on fuel metabolism (3), is still anabolic to osteoblasts (8). This small ring peptide is a more attractive candidate for pharmaceutical development and might be used as a model for the creation of orally active, nonpeptide analogs. However, this fragment has a lower potency than the intact molecule and lacks any inhibitory effects on osteoclastic bone resorption, so it is uncertain how substantial its effects on bone *in vivo* might be. We have now addressed this question by assessing the effects of 1 mo of daily systemic treatment with amylin-(1–8) on the histomorphometry and mechanical properties of the tibiae in sexually mature mice. In addition, we have studied its effects on dynamic bone histomorphometry after local injection over the calvariae of mice and compared these effects with those of human parathyroid hormone-(1–34) [hPTH-(1–34)].

## METHODS

### Systemic Study

**Experimental design.** Two groups of 20 sexually mature male Swiss mice aged between 40 and 50 days and weighing 25–32 g were given daily subcutaneous injections [2.2  $\mu$ g rat amylin-(1–8) in 50  $\mu$ l of water or water alone] in the loose skin at the nape of the neck for 5 days/wk over four consecutive weeks. This dose was chosen because the same molar dose (93 nmol/kg) of the intact peptide in this model produces substantial effects on bone turnover and bone area (6). Animals were housed in a room maintained at 20°C on 12:12-h light-dark cycles. They were fed a diet of 86 rodent pellets (New Zealand Stockfeed) *ad libitum* throughout the experiment. Each animal's weight was recorded at the beginning

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and end of the experiment. The study had the approval of the local institutional review board.

**Histomorphometry.** Indices of bone formation and resorption and bone volume were assessed in the proximal tibiae where the predominance of trabecular bone with its high surface-to-volume ratio results in larger changes in these measures than are seen elsewhere. One tibia from each animal was used for histomorphometric analyses. The tibiae were dissected free of adherent tissue, and bone lengths were recorded by measuring the distance between the proximal epiphysis and the distal tibio-fibular junction by use of an electronic micrometer (Digimatic Calipers, Mitutoyo, Japan). Bones were placed in 10% phosphate-buffered Formalin for 24 h, dehydrated in a graded series of ethanol solutions, and embedded, undecalcified, in methyl methacrylate resin (Acros Organics, Geel, Belgium). Tibiae were sectioned longitudinally through the frontal plane. Sections (4  $\mu\text{m}$  thick) were cut using a Leitz rotary microtome (Leica Instruments, Nussloch, Germany) and a tungsten-carbide knife (Microknife Sharpening) and then were mounted on gelatin-coated slides and air-dried. They were stained with Goldner's trichrome and examined using an Olympus BX 50 microscope (Olympus Optical, Tokyo, Japan) that was attached to an Osteomeasure Image Analyzer (Osteometrics, Atlanta, GA).

Tibial histomorphometric analyses were made from three adjacent sections one-third of the way through the anterior/posterior depth of the proximal tibiae. All trabecular bone tissue in the secondary spongiosa was quantified for bone volume in each section using a  $\times 10$  objective, and parameters were derived using the formulas of Parfitt et al. (10). Parameters of bone formation and resorption were measured using a  $\times 20$  objective in all trabecular bone tissue in the secondary spongiosa in the middle of the three adjacent sections. The surfaces measured were those immediately adjacent to unmineralized matrix, those adjacent to osteoblasts, and those adjacent to osteoclasts (osteoid, osteoblast, and osteoclast perimeters, respectively). The osteoclast index is the number of osteoclasts per millimeter trabecular perimeter. Perimeters and cell numbers were expressed per section. Cortical width was measured on both sides of the tibial shaft, 2.5 mm below the epiphyseal growth plate. Epiphyseal growth plate thickness was measured at three sites evenly spaced along its length. All measurements were made by one operator (J. Cornish) who was blinded to the treatment group of each bone. The precisions of these histomorphometric measurements in our laboratory (expressed as coefficients of variation of paired measurements) were as follows: mineralized bone area 1.3%, osteoid perimeter 6.9%, osteoblast perimeter 6.8%, osteoblast number 1.7%, osteoclast perimeter 7.9%, osteoclast number <1.0%, width measurements 1.7%.

**Mechanical strength of the tibia.** The remaining tibia from each animal was fixed in 70% ethanol and was used for mechanical strength estimations. The mechanical strength of the tibiae was determined by three-point bending tests. Each tibia was dissected free of soft tissue and tested on a MTS 858 Bionix Testing Machine (MTS Systems, Minneapolis, MN). Samples were tested at room temperature with a support span of 10 mm. Load was applied at a constant deformation rate of 2 mm/min with a force application at the upper anterior midpoint of the tibia. Load-deformation curves were recorded, and displacement values (a measurement of how much the bone bends from the time that the force is applied until its final failure point) were obtained directly from the curve and expressed in millimeters.

**Fat mass estimations.** Fat mass estimations were made from measurements of the animals' body densities calculated from water displacement. Immediately after death the mice

were submerged head first to the base of the tail in a 250-ml measuring cylinder containing 150 ml of water, and the displacement volume was recorded. The fraction of body weight that was fat mass was calculated using a modification of the Siri equation for use in rodents (9). The coefficient of variation for repeated measures of fat mass was 7%.

#### Local Injection Study

A further experiment was carried out using the local injection of peptide over the calvariae of female OF-1 mice to assess the effects of amylin-(1–8) on dynamic histomorphometry, to assess dose-response effects, and to allow comparison with an established anabolic agent, parathyroid hormone. Five groups of sexually mature mice ( $n = 5$  in each group) were allocated to receive 50- $\mu\text{l}$  injections two times a day over the central calvaria for 5 days. In the respective groups, the injections consisted of PBS, amylin-(1–8) in concentrations of 0.4, 4, or 40 nM, or 40 nM hPTH-(1–34). The mice were killed 14 days after the first injection, and the frontal and parietal bones were removed for histology. To permit dynamic histomorphometry, animals were labeled with alizarine (20 mg/kg sc injected at the lower back) at baseline. Further fluorochrome labels were administered at 10 days before necropsy (30 mg/kg sc calcine) and at 3 days before necropsy (20 mg/kg alizarine). Bones were fixed in Karnovski's fixative and embedded in HistoDur (Leica Instruments). The distribution of fluorescent labels was assessed using a Bioquant (R&M Biometrics, Nashville TN).

**Materials.** Rat amylin-(1–8) was synthesized as a COOH-terminal amide on methylbenzhydrylamine resin by standard solid-phase techniques, as described previously (8). It was cyclized in a dilute solution of 90% acetic acid by treatment with methanol solutions of iodine and was purified to >96% homogeneity by reverse-phase HPLC. To avoid losses when handling the peptide, an anti-static device (Zerostat 3; Discwasher, Reconton, Lake Mary, FL) was used to remove static electric charge from the peptide itself and from any containers in which it was placed. The hydrochloride salt of the peptide was produced by dissolving it in 3 mM hydrochloric acid (10  $\mu\text{mol}$  peptide in 50  $\mu\text{l}$ ) and leaving it for 1 h at room temperature before freeze-drying (model SVC 100H; Savant Instruments). Before use, it was redissolved in pure water with sonication (Soniprep 150, West Sussex, UK), cooled on ice for 15 s, and then stored at 4°C until required for injection. Amylin-(1–8) was dissolved for a minimum of 48 h before injection, because we have found that this increases the concentration of peptide in solution, as measured by HPLC. The molecule is very adherent to glass, so only plastic containers were used in handling it. hPTH-(1–34) was made using solid-phase synthesis (Novartis).

**Statistical analysis.** Data are presented as means  $\pm$  SE. Where parameters were measured more than one time in each animal (e.g., cortical thickness), these values were averaged to produce a single value for each animal before further analysis. The significance of treatment effects was evaluated using Student's *t*-tests for unpaired data. All tests were two tailed.

## RESULTS

### Systemic Study

Amylin-(1–8) produced substantial increases in all three indices of osteoblast activity assessed (osteoid perimeter from  $3.5 \pm 0.3$  to  $6.0 \pm 0.3$  mm, osteoblast perimeter from  $1.4 \pm 0.2$  to  $2.6 \pm 0.2$  mm, and number

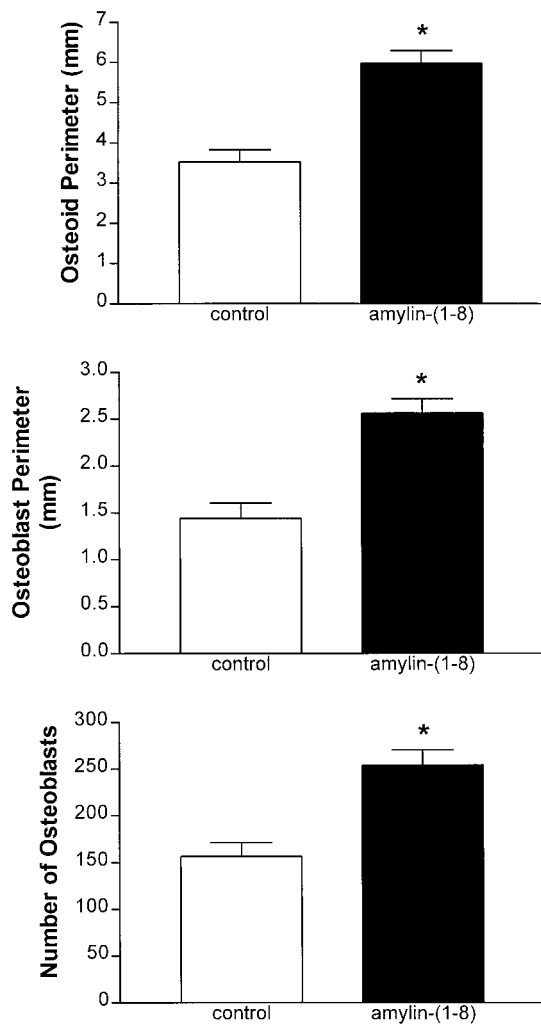


Fig. 1. Effects of daily systemic administration of amylin-(1-8) for 4 wk on histomorphometric indices of bone formation in the tibiae of normal sexually mature male mice ( $n = 20$  in each group). Data are means  $\pm$  SE. \*Significantly different from control,  $P < 0.0001$ .

of osteoblasts from  $157 \pm 15$  to  $254 \pm 17$ ,  $P < 0.0001$  for each; Fig. 1). In contrast, there was no effect on bone resorption (Fig. 2). These changes resulted in an 8.1% increase in cortical width [from  $0.160 \pm 0.005$  mm in control animals to  $0.173 \pm 0.004$  mm in those receiving amylin-(1-8)] and a 36% increase in trabecular bone volume [from  $13.7 \pm 0.8\%$  in the control animals to  $18.7 \pm 0.8\%$  in those treated with amylin-(1-8); Fig. 3]. The increase in trabecular volume was contributed to by increases in trabecular thickness and trabecular number and from a decline in trabecular separation (Fig. 4). These effects can be directly appreciated by comparing the sections of bone from control animals with those treated with amylin-(1-8), as shown in Fig. 5. When the contralateral tibiae were subjected to three-point bending to assess bone strength, the displacement to the point of failure was increased from  $0.302 \pm 0.013$  mm in control animals to  $0.351 \pm 0.017$  mm in those treated with amylin-(1-8) ( $P = 0.02$ ), suggesting that the bones of the treated animals were stronger (Fig. 6).

The effects of amylin-(1-8) were not confined to bone; there was also a near doubling of the thickness of the epiphyseal growth plate (Fig. 7). However, there was no significant difference in tibial lengths between the groups [control  $11.05 \pm 0.33$  mm, amylin-(1-8)  $11.15 \pm 0.25$  mm].

Body weight increased from  $27.8 \pm 0.4$  to  $32.8 \pm 0.5$  g in the control animals and from  $27.8 \pm 0.4$  to  $33.1 \pm 0.6$  g in those treated with amylin-(1-8). Fat masses at the end of the study were  $2.42 \pm 0.13$  and  $2.27 \pm 0.19$  g in the control and amylin-(1-8)-treated animals, respectively. These results were not significantly different between the groups.

#### Local Injection Study

Amylin-(1-8) injections increased the extent of the double-labeled surface (assessed using the second and third labels and expressed as a percentage of total bone surface) in a dose-dependent fashion (Fig. 8A). The effect was significant with 4 and 40 nM amylin-(1-8) and with 40 nM hPTH-(1-34). The increase in double-labeled surface observed with amylin-(1-8) was greater than with the same concentration of hPTH-(1-34). All of the labels showed sharp delineation, and no woven bone was observed with either peptide. Mineral apposition rate was also increased in mice receiving 40 nM amylin-(1-8) but not with hPTH-(1-34) (Fig. 8B).

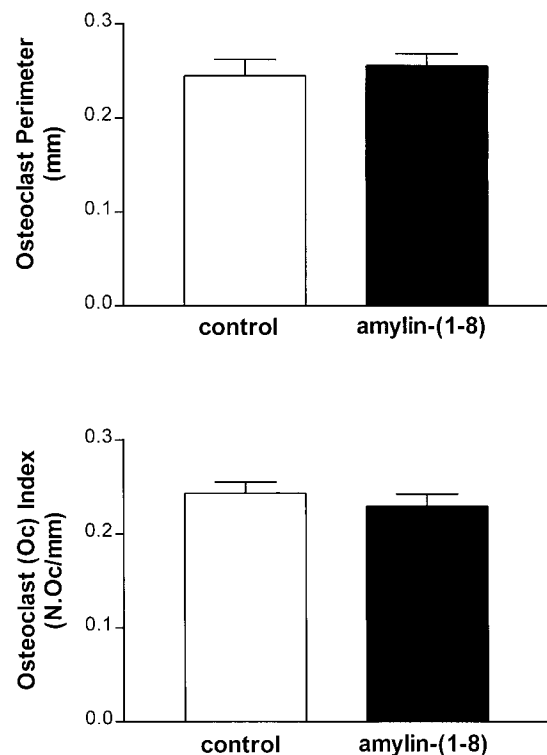


Fig. 2. Effects of daily systemic administration of amylin-(1-8) for 4 wk on histomorphometric indices of bone resorption in the tibiae of normal sexually mature male mice ( $n = 20$  in each group). Data are means  $\pm$  SE. There were no significant effects.

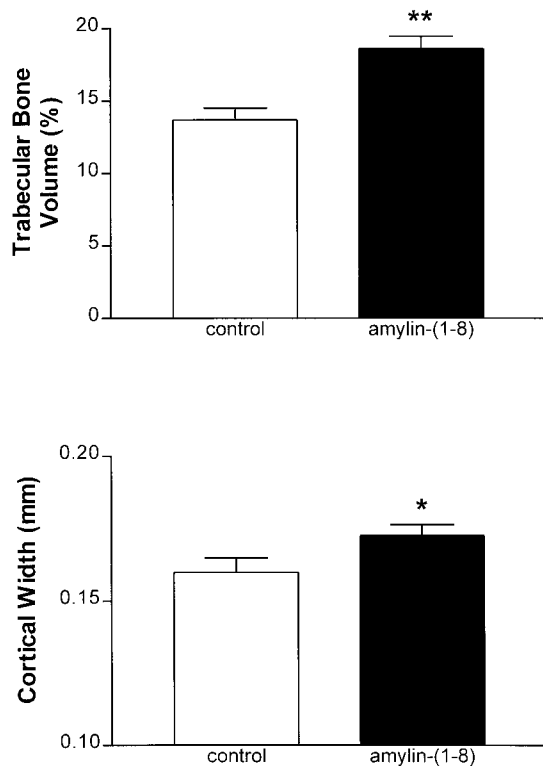


Fig. 3. Effects of daily systemic administration of amylin-(1-8) for 4 wk on histomorphometric indices of bone volume in the tibiae of normal sexually mature male mice ( $n = 20$  in each group). Data are means  $\pm$  SE. \*Significantly different from control,  $P = 0.04$ . \*\* $P < 0.0001$ .

## DISCUSSION

The present study demonstrates that the positive effects of intact amylin on both trabecular and cortical bone can be reproduced by the  $\text{NH}_2$ -terminal octapeptide of the molecule. The effect is less than what we found with equimolar doses of amylin itself (36% increase in trabecular bone volume compared with 70% with the intact peptide; see Ref. 6), consistent with the lower anabolic potency of the fragment in vitro and with its lack of effect on osteoclasts (8). The fact that positive effects on bone volume remain despite the absence of anti-osteoclast effects suggests that the increase in bone volume previously found with intact amylin is substantially attributable to its action on osteoblasts. The positive effects on double-labeled surfaces and mineral apposition rates in the local injection studies shown here are consistent with this. Although the systemic dose response of this effect requires further study, the effect on trabecular bone volume found here is substantial. It will be of interest to determine whether this activity can be increased in analogs of this small cyclic peptide and whether nonpeptide analogs that might be orally active can also be developed. However, the significance of the present data in the therapy of osteoporosis will ultimately be determined by the assessment of amylin-(1-8) or its analogs in human studies.

This study provides further evidence for the anabolic action on osteoblasts of the family of related peptides

consisting of amylin, adrenomedullin, and calcitonin gene-related peptide (CGRP), all of which have been shown to exert this action both in vitro and in vivo (2, 5, 11). Valentijn et al. (12) have recently shown partial prevention of postovariectomy bone loss in rats after daily injections of CGRP- $\alpha$  for 4 wk. The same group has shown that transgenic mice overexpressing the CGRP gene in bone have a 5% increase in distal femoral bone density at the age of 12 wk (1). Calcitonin does not stimulate bone formation in these models (4, 11). Amylin and adrenomedullin are approximately equipotent in their actions on osteoblasts, but CGRP is significantly less active (2, 4). Furthermore, CGRP activity on osteoblasts is completely blocked by amylin receptor blockers, whereas the converse is not true (7). These findings are consistent with the fact that the changes found in the present studies of amylin were

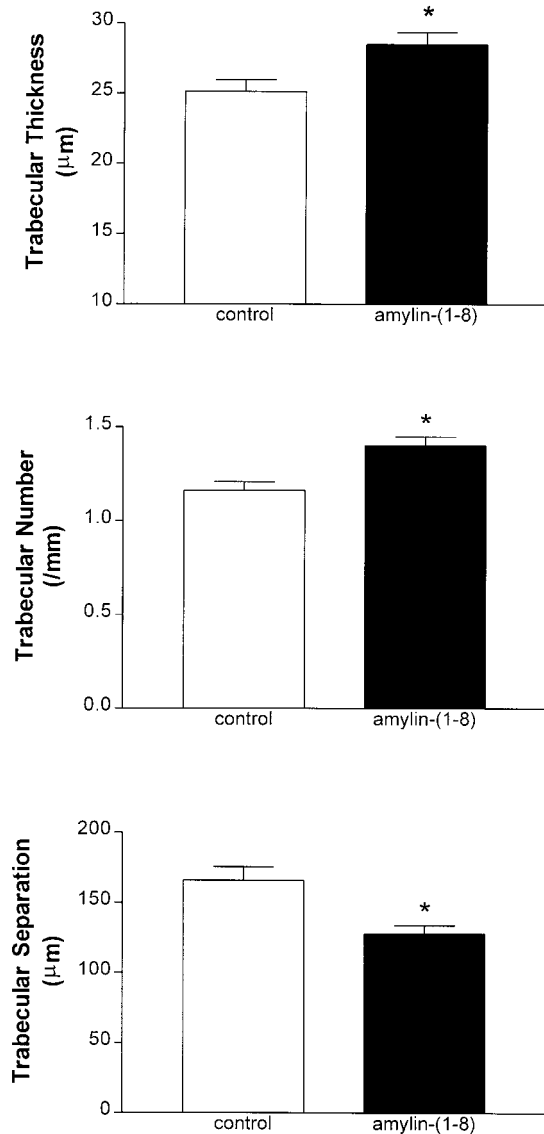


Fig. 4. Effects of daily systemic administration of amylin-(1-8) for 4 wk on histomorphometric indices of trabecular bone in the tibiae of normal sexually mature male mice ( $n = 20$  in each group). Data are means  $\pm$  SE. \*Significantly different from control,  $P < 0.008$ .

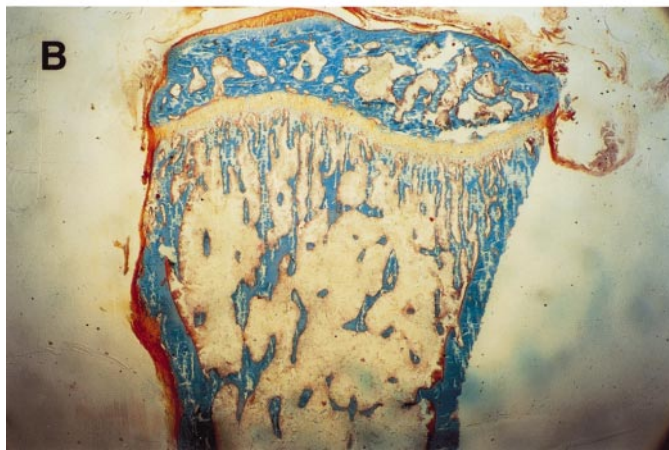
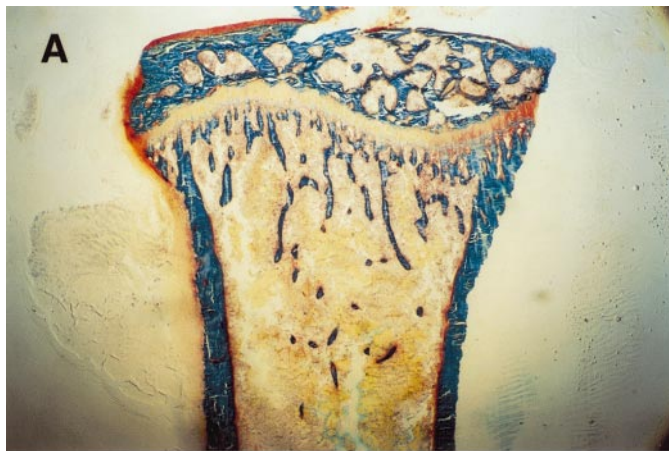


Fig. 5. Photomicrographs of the proximal tibiae of mice treated with vehicle (A) or amylin-(1-8) (B) demonstrating the increase in trabecular bone volume and cortical width associated with amylin-(1-8) treatment (magnification  $\times 80$ ). Sections have been stained with Goldner's trichrome, with mineralized bone appearing green.

achieved using lower doses of peptide than those of CGRP used by Valentijn et al. (12) in their study of ovariectomized rats. Thus the osteogenic effects demonstrated in the present study are likely to be mediated by a receptor with a higher affinity for amylin than for CGRP.

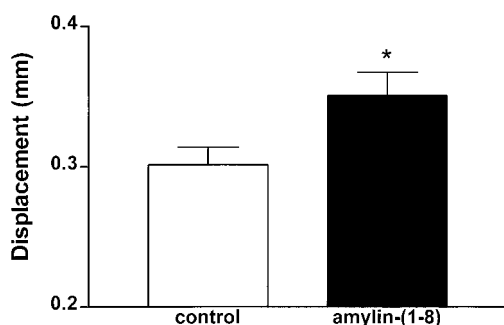


Fig. 6. Effects of daily systemic administration of amylin-(1-8) for 4 wk on mechanical strength of the tibiae, estimated using displacement values from load-deformation curves obtained from 3-point bending tests ( $n = 20$  in each group). Data are means  $\pm$  SE. \*Significantly different from control,  $P = 0.02$ .

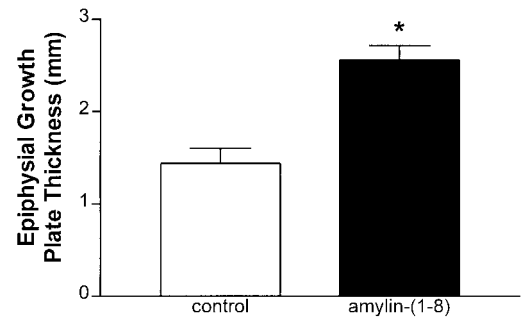


Fig. 7. Effects of daily systemic administration of amylin-(1-8) for 4 wk on the thickness of the epiphyseal growth plate in the tibiae of normal sexually mature male mice ( $n = 20$  in each group). Data are means  $\pm$  SE. \*Significantly different from control,  $P < 0.0001$ .

The effects of amylin-(1-8) are different from those of amylin itself with respect to the effects on fat mass in this model. The intact molecule increases fat mass in these mice when administered according to the schedule used in the present study (6). In contrast, the octapeptide has no effect on either body weight or fat

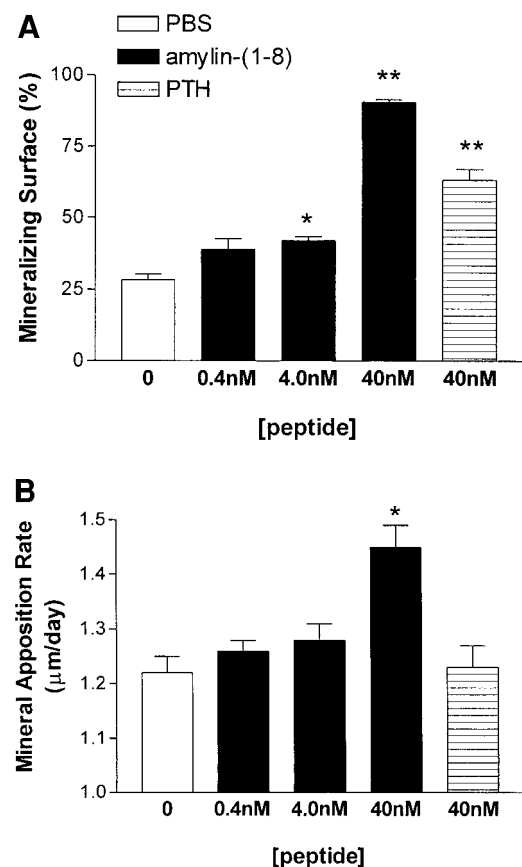


Fig. 8. Effect of amylin-(1-8) and human parathyroid hormone (PTH)-(1-34) on the extent of the fluorochrome double-labeled surface (A) and mineral apposition rate (B) in mice calvariae. Mice were treated with local subcutaneous injections over the central calvaria for 5 consecutive days and were killed 10 days later. [Peptide], peptide concentration. \*Significantly different from control,  $P < 0.04$ . \*\*Significantly different from control,  $P < 0.001$ . The groups receiving 40 nM amylin and 40 nM PTH were also significantly different from each other,  $P < 0.02$ .

mass in the present study. This is consistent with other evidence that any shortening of the parent molecule results in a loss of its activity on intermediary metabolism (3). Similarly, the formation of amyloid from amylin is dependent on residues in the COOH-terminal region of the molecule and would not be expected with this octapeptide (3).

A further effect of the systemic administration of intact amylin is an increase in the thickness of the tibial growth plate and an acceleration of linear growth of the tibia (6). This suggests that this peptide also affects chondrocyte activity, which is something we have now confirmed in cultures of isolated canine articular chondrocytes (unpublished observation). In the present study, a thickening of the tibial growth plate is again observed, although no significant effect on bone length was found. This is consistent with the findings of our *in vitro* studies that show that 10-fold higher concentrations of amylin-(1–8) than those of amylin itself are necessary to achieve comparable stimulation of chondrocyte proliferation. The chondrocyte actions of amylin-(1–8) may also have contributed to the increase in bone volume in the present study, but this is unlikely to be the major mechanism, because anabolic effects have been demonstrated in models that are free of chondrocytes (e.g., isolated osteoblast cultures and with local injection over calvariae *in vivo*; see Ref. 4). It will be of interest to determine whether the local or systemic administration of these peptides has a significant effect on articular cartilage *in vivo*, because this might have relevance to conditions such as osteoarthritis.

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