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Review Article

Hylonome

Effects of amylin and adrenomedullin on the skeleton

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Abstract

Amylin and adrenomedullin are related peptides with some homology to both calcitonin and calcitonin gene-related peptide (CGRP). All these peptides have in common a 6-amino acid ring structure at the amino-terminus created by a disulfide bond. In addition, the carboxy-termini are amidated. Both amylin and adrenomedullin have recently been found to stimulate the proliferation of osteoblasts *in vitro*, and to increase indices of bone formation *in vivo* when administered either locally or systemically[1-4]. Both amylin and adrenomedullin have also been found to act on chondrocytes (Cornish et al., submitted for publication), stimulating their proliferation in culture and increasing tibial growth plate thickness when administered systemically to adult mice[2,4]. Studies of structure-activity relationships have demonstrated that osteotropic effects of amylin and adrenomedullin can be retained in peptide fragments of the molecules. The full-length peptide of amylin has known effects on fuel metabolism, and systemic administration of amylin is also associated with increased fat mass[2]. However, the octapeptide fragment of the molecule, amylin-(1-8), is osteotropic and yet has no activity on fuel metabolism[5]. Similar fragments of adrenomedullin have also been defined, which retain activity on bone but lack the parent peptide's vasodilator properties[3]. Both amylin-(1-8) and adrenomedullin-(27-52) act as anabolic agents on bone, increasing bone strength when administered systemically[3,4]. Thus, these small peptides, or analogues of it, are potential candidates as anabolic therapies for osteoporosis. Both amylin and adrenomedullin may have effects on bone metabolism. Amylin is secreted following eating and may direct calcium and protein absorbed from the meal into new bone synthesis. Amylin circulates in high concentrations in obese individuals, and might contribute to the association between bone mass and fat mass. Our recent findings demonstrating the co-expression of adrenomedullin and adrenomedullin receptors in osteoblasts[6], along with the findings that the peptide and its receptor are easily detectable during rodent embryogenesis[7], suggest that this peptide is a local regulator of bone growth. Thus, the findings reviewed in this paper illustrate that amylin and adrenomedullin may be relevant to the normal regulation of bone mass and to the design of agents for the treatment of osteoporosis.

Keywords: Bone, Osteoblasts, Osteoclasts, Osteoporosis, Peptide

Introduction

Amylin was originally isolated, approximately 15 years ago, from amyloid deposits in the pancreases of patients with insulinoma or diabetes mellitus[8,9]. It is co-secreted with insulin from the pancreatic β-cell and there is evidence that the insulin and amylin genes share transcriptional regulators[10]. Thus, hyperglycemia stimulates amylin secretion[11] and hypoglycemia reduces it[12]. The excursion in circulating insulin levels following a glucose challenge appears initially to be greater than that seen with amylin, however, amylin's secretion may be more sustained[13].

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Amylin may contribute to the relationship between body mass and bone density. Body mass, or more particularly fat mass, is a major determinant of bone density in women[14]. While this might be mediated to some extent by the effect of weight on skeletal load bearing, and by adipocyte production of estrogen, neither of these explanations is adequate to explain the published results[15]. Since both insulin and amylin are hypersecreted in obesity and since both may potentially act directly or indirectly to increase bone mass, they may contribute significantly to this relationship. Indeed, circulating insulin levels are directly related to bone density in normal postmenopausal women[14] and since amylin is co-secreted with insulin it would seem likely that a similar relationship for this peptide exists. In type I diabetic patients, amylin as well as insulin circulate at low levels and bone density is also reduced. We and others have demonstrated that amylin[1,16,17] and insulin[18] stimulate bone growth *in vitro* and *in vivo*.

Adrenomedullin was first described in 1993[19] where it was identified in a human phaeochromocytoma patient. This peptide has since been found to be present not only in the normal adrenal medulla but also in many other tissues[20]. Adrenomedullin is a potent vasodilator, acting directly on the renal, cerebral, mesenteric, pulmonary and systemic circulations, including the vascular supply of the skeleton[21]. Its haemodynamic effects are probably mediated via receptors on vascular smooth muscle cells and possibly endothelial cells. Binding to a number of other tissues, including lungs, heart, kidneys and hypothalamus has also been demonstrated[20]. We have recently shown specific binding of adrenomedullin to primary osteoblasts[6] and demonstrated that adrenomedullin stimulates bone growth *in vitro* and *in vivo*[3,4]. Bone strength was significantly increased when adrenomedullin was systemically administered to adult mice[4]. Thus, amylin and adrenomedullin may contribute to building stronger bones and we have investigated these related peptides in a number of skeletal models that are summarised in this review.

The peptides and their receptors

Amylin has an evolutionary relationship with insulin and IGF-1. The peptide is structurally related to calcitonin, Calcitonin gene-related peptide (CGRP) and adrenomedullin (sequence identities 13%, 43-49% and 20%, respectively)[22] (Fig. 1). All these peptides have an NH_2-terminal ring created by a disulfide bond, and are amidated at their carboxy-termini.

Amylin is a 37-amino acid peptide hormone that is produced principally in the β-cells of the pancreatic islets where its tissue content is less than 1% of that of insulin. Amylin has also been detected in tissues of the gastro-intestinal tract, with tissue concentrations about 1% of those in the pancreas being found in the pyloric antrum[23]. Amylin or its mRNA has also been found in lung, dorsal root ganglion, hypothalamus and neuro-endocrine tumors[24]. Circulating amylin levels are of the order of 5 pmol/liter rising to 10-20 pmol/liter following a meal. Amylin secretion is pulsatile, peaks occurring at about 5-minute intervals[25]. Levels are higher in obese subjects and those with type II diabetes[26], but appear to be decreased by leptin[27]. There is one report of amylin production from a human osteoblast-like cell line[28], raising the possibility of amylin production locally within the bone microenvironment, but we have been unable to confirm the presence of amylin mRNA in primary rat osteoblasts (Naot et al. unpublished data).

Adrenomedullin is a 52-amino acid peptide hormone and it differs from the other peptides in that it has a linear amino-terminal extension, consisting of 15 amino acids in the human and 13 in the rat. Adrenomedullin, similar to amylin, circulates at picomolar concentrations in both rats and man[20,29]. Adrenomedullin is present in normal adrenal medulla and in vascular beds throughout the body, including the atria, ventricles, endothelial cells, lungs, brain, kidneys and bone[7,20,30]. Adrenomedullin and its mRNA have been demonstrated by immunohistochemistry and *in situ* hybridization, respectively, to be expressed at high levels in the osteoblasts and chondrocytes of mouse and rat embryos[7]. These workers have identified adrenomedullin in maturing cartilage, hypertrophic cartilage and in osteoblasts of the developing bone. We have recently shown that adrenomedullin is also expressed in primary osteoblasts isolated from fetal rats[6]. Adrenomedullin mRNA was detected by reverse transciptase polymerase chain reaction (RT-PCR) and by northern blot analysis, and the cells also stained positive with anti-adrenomedullin antibodies in an immunocytochemical assay. We have also demonstrated by RT-PCR that adrenomedullin is present in human chondrocytes cultured from articular cartilage explants (Cornish et al., submitted for publication). The peptide has been shown to be produced in a macrophage-monocyte cell line[31], leading to speculation that adrenomedullin may be produced by the osteoclast, although to our knowledge this has not been investigated. An increasing number of conditions are being identified in which there are major perturbations of circulating adrenomedullin concentrations including acute sepsis[32], hyperthyroidism[33], and pregnancy[34], and high peptide concentrations have been reported in umbilical cord plasma, and amniotic fluid[34]. The increase in bone turnover seen in these conditions and in the fetus might be contributed to by adrenomedullin.

In non-osseous tissues, it has been demonstrated that separate specific receptors exist for amylin and CGRP, with some evidence pointing to more than one class of receptor

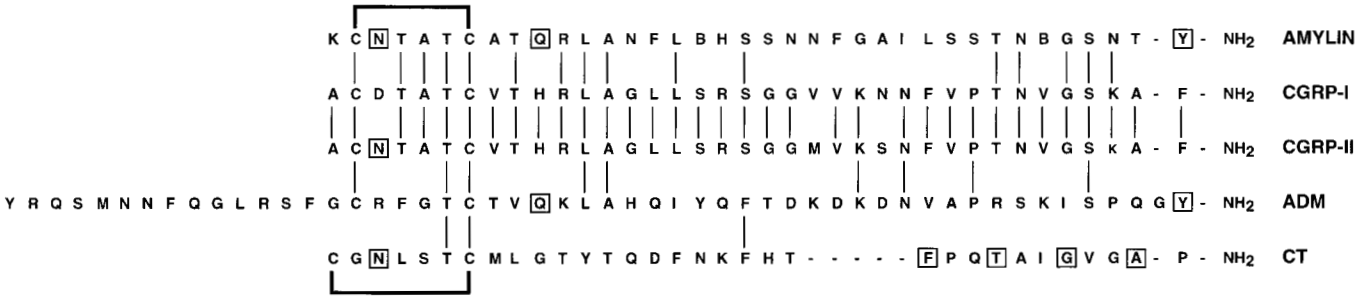

Figure 1. Amino acid sequences of amylin, adrenomedullin and related peptides. There is a disulfide bond between the two underlined cysteine residues in each molecule. All peptides are amidated at their carboxy-terminus. Amino acids are identified as follows: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P,proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; Y, tyrosine.

for the latter. Amylin, CGRP and calcitonin are able to displace each other from specific binding sites, implying significant cross-reactivity of each with the receptors of the other peptides. Whether this leads to significant biological effects at the physiological peptide concentrations is unknown.

Amylin's effects on osteoblast proliferation and osteoclastic bone resorption can be dissociated using amylin fragments and blockers (see below). This suggests that the peptide's actions on these two types of bone cells may be mediated by different receptors. The data available at the present time are consistent with the actions of amylin and CGRP on bone resorption being mediated by the calcitonin receptor. In the osteoblast, relative potencies of both agonists and antagonists for amylin, CGRP and adrenomedullin suggest that these peptides act through a common receptor on osteoblasts and that the receptor has a lower affinity for CGRP than for the other two peptides. Amylin is not only substantially more potent than CGRP in stimulating osteoblast proliferation, but the stimulation produced by CGRP 10^{-8}M, is completely blocked by the amylin receptor blocker amylin-(8-37) 10^{-10}M, whereas the CGRP receptor blocker CGRP-(8-37) 10^{-8}M is unable to completely block the effects of amylin in this model. This implies that CGRP is more likely to be acting through an amylin receptor than one specific for CGRP[35]. In a recent study in primary human osteoblast-like cells, amylin-(8-37) was able to block amylin's proliferative effects yet this antagonist was unable to block the proliferative effects of CGRP, although these workers were also unable to demonstrate that CGRP-(8-37) was able to block CGRP's proliferative effect either[36]. Recently, we have shown that the proliferative effects of amylin and adrenomedullin are dependent on the presence of the IGF-1 receptor[37,38], although neither of these peptides appear to compete for binding to this receptor, implying a less direct mechanism for its involvement.

Uncertainty still surrounds the identities of the receptors for this family of peptides in non-bone cells. A CGRP receptor was identified in 1995 by Kapas[39] but recent work by McLatchie et al. cast doubt upon the significance of this receptor[40]. They suggest that the CGRP receptor is in fact the so-called calcitonin receptor-like receptor (CRLR). Cells expressing this receptor alone are relatively unresponsive to CGRP, but in the presence of a molecule they have termed receptor activity modifying protein-1 (RAMP1), the CRLR is both glycosylated and translocated to the cell surface, conferring CGRP sensitivity. Separate proteins, RAMP2 and RAMP3, interact with this receptor to produce adrenomedullin receptors. Other groups have confirmed that these CRLR-based receptors account for most of the specific binding of adrenomedullin and CGRP in a variety of rat tissues[41]. When RAMPs 1 or 3 interact with the calcitonin receptor (CTR2), an amylin receptor is created[42,43]. Recently, we have demonstrated that primary rat osteoblasts and UMR-106.06 osteoblast-like cells express the mRNAs for all 3 RAMPs, CRLR, and for the putative adrenomedullin receptor identified by Kapas[6] (Fig. 2). In addition, we have shown that primary osteoblastic cells can bind ^{125}I-adrenomedullin with high affinity and analysis of competitive binding data suggested the existence of two types of binding sites for adrenomedullin on primary osteoblasts. However, the primary osteoblasts do not exhibit the calcitonin receptor[6]. This raises an interesting question, as to what receptor amylin is acting through. Thus, the receptor that binds and mediates amylin's effect in primary osteoblasts still needs to be identified. In all the findings establishing the importance of CRLR and CTR2 in association with the RAMPs as defining the receptors of this peptide family, the endpoint measured is a cAMP response which may not be the second messenger mediating the action of these peptides on osteoblast proliferation (Cornish, Callon and Reid, unpublished data). Therefore, the relevance to osteoblast biology of these findings remains to be determined. Neither the CGRP nor the adrenomedullin receptor show significant cross-reactivity with the other ligand, nor with amylin, as is demonstrated in proliferation experiments. Furthermore, the structure-activity-relationship for the action of adrenomedullin on the receptor of McLatchie et al. is quite different from that which we have found in osteoblasts. These workers find that adrenomedullin-(13-52) is the smallest active fragment whereas we find full activity in adrenomedullin-(27-52)[3]. Thus, the receptor complexes identified by McLatchie et al. appear to be different from the receptors functioning in primary osteoblast cultures.

The receptors referred to above are all 7-transmembrane G-protein coupled receptors, thought to act via adenylyl cyclase and/or calcium-inositol signaling pathways. It should be noted that the effects of these peptides on cyclic AMP concentrations in osteoblasts are modest (in comparison with parathyroid hormone, for instance) and that our recent work indicates that amylin and adrenomedullin activate mitogen-activated protein kinase in osteoblasts[38]. Further work is needed to delineate fully the second messenger pathways mediating their effects in bone and to define whether different second messenger systems are activated by the different amylin and adrenomedullin fragments.

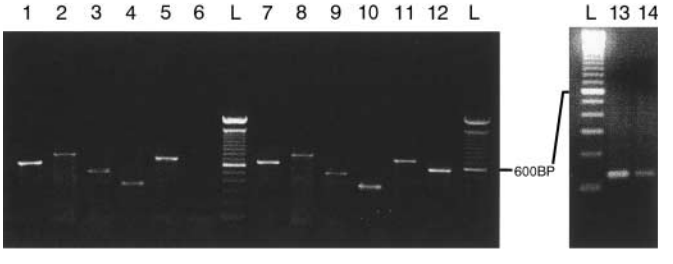

Figure 2. Expression of genes for adrenomedullin and its putative receptors in primary osteoblasts and UM106-06 cells. RT-PCR was carried out using specific primer pairs for the different cDNAs. The RT-PCR products were resolved on a 1% agarose gel, except for RAMP3 which was resolved on a 2% gel. All the amplified cDNA fragments were extracted from the gels and their DNA sequence determined. Lanes 1-6 and 13, RT-PCR products from primary osteoblasts; lanes 7-12 and 14, RT-PCR products from UMR106-06 cells. Lanes 1 and 7, adrenomedullin; lanes 2 and 8, L1; lanes 3 and 9, RAMP1; lanes 4 and 10, RAMP2; lanes 5 and 11, CRLR; lanes 6 and 12, CTR; lanes 13 and 14, RAMP3.

Effects on osteoclasts

Soon after the isolation of amylin and the recognition of its similarity to calcitonin, its effects on bone resorption were investigated. Similar to calcitonin and CGRP, amylin lowers plasma calcium levels when injected into man and rats although amylin and CGRP are several orders of magnitude less potent than calcitonin (Fig. 3a)[44]. A similar pattern of results is seen on isolated osteoclasts (Fig. 3b). Amylin inhibits osteoclast motility by way of increasing intracellular cyclic AMP concentration[45]. Amylin does not produce the osteoclast retraction seen with calcitonin. We have also studied the effect of amylin on osteoclast development in mouse bone marrow cultures[46]. As with calcitonin and CGRP, amylin inhibits both the mononuclear osteoclast-like cell formation and the fusion of these cells. In this system, calcitonin has a greater potency and amylin is more active at lower concentrations than CGRP[46].

Amylin has also been shown to reduce bone resorption in organ culture. It is approximately equipotent with CGRP in inhibiting calcitriol-stimulated resorption in fetal rat long bones[47]. Amylin reduces both basal and parathyroid hormone-stimulated bone resorption in neonatal mouse calvariae, and cyclic AMP production is also stimulated in this model[48,49]. In the studies of Cornish et al. (Fig. 4) inhibition of resorption was seen at concentrations as low as 10^{-9}M[49].

These results should be interpreted in the light of the marked propensity of amylin to adhere to the surfaces of laboratory plasticware[50], suggesting that the actual concentrations of amylin in all *in vitro* experiments may be one to two orders of magnitude less than the amount added to the media. Thus, both the osteoclast and calvarial studies imply that amylin may regulate bone resorption at physiological concentrations. This activity is dependent on the presence of the carboxy-terminal amide group. Amylin's inhibition of bone resorption in neonatal mouse calvariae only occurs with the intact molecule, in contrast to the situation with amylin action on osteoblasts. Amylin fragments, which act as antagonists in the osteoblast, do not affect amylin's effect on osteoclasts[35].

The effect of amylin on resorption *in vivo* has now been studied histomorphometrically in several different models. Cornish et al.[1] demonstrated 60-70% reductions in indices of bone resorption following daily local administration of amylin over the calvariae of adult mice (Fig. 5). Very similar changes in resorption indices were seen following systemic administration of amylin to adult male mice for 1 month[2]. Consistent with the results in the organ culture the amino-terminal octapeptide of amylin [amylin-(1-8)] is without effect on resorption in the systemic model[5]. In ovariectomized rats, intact amylin reduces urinary excretion of deoxypyridinoline and reduces bone loss[17] (Fig. 6). In contrast, the earlier

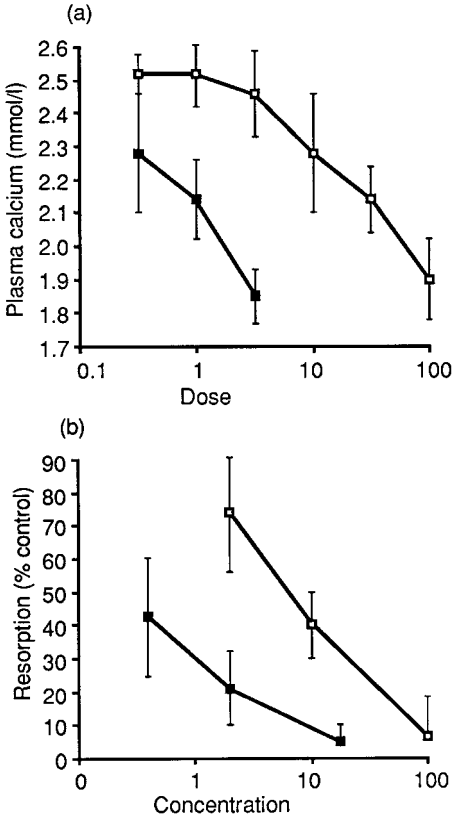

Figure 3. Effect of human amylin and human calcitonin on (a) plasma calcium in the rat, and (b) bone resorption by isolated osteoclasts *in vitro*. In (a), the doses are given in pmol/rat, and in (b), in pmol/l. Data are mean ± sem. (From MacIntyre 1989, used with permission). □: human amylin, ■: human calcitonin.

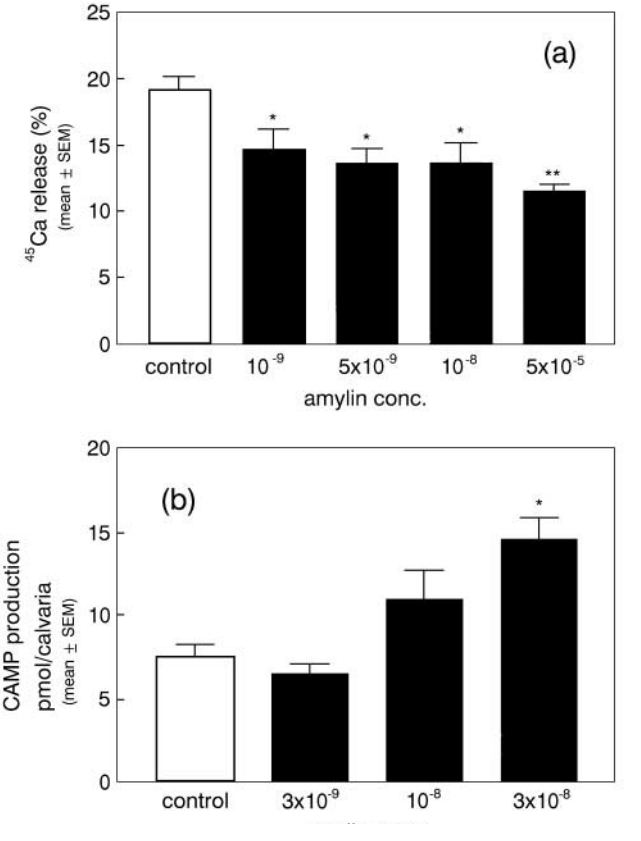

Figure 4. Effect of rat amylin on basal bone resorption in neonatal mouse calvariae. Based on data in Cornish et al. (1994).

experiment of Romero[51], in which amylin was administered systemically to rats, showed only a non-significant trend toward reduced resorption, and Borm et al. found no change in resorption markers in 23 diabetic patients receiving the amylin analogue, pramlintide, for 1 year[52]. The latter study needs to be interpreted with caution, since it used an amylin analog of unknown activity on bone, and it was uncontrolled.

Adrenomedullin, unlike the other members of this family, does not inhibit bone resorption as assessed by ^{45}Ca release in neonatal mouse calvarial organ cultures and there was no reduction in bone resorption indices seen when adrenomedullin is administered either locally or systemically[3,4].

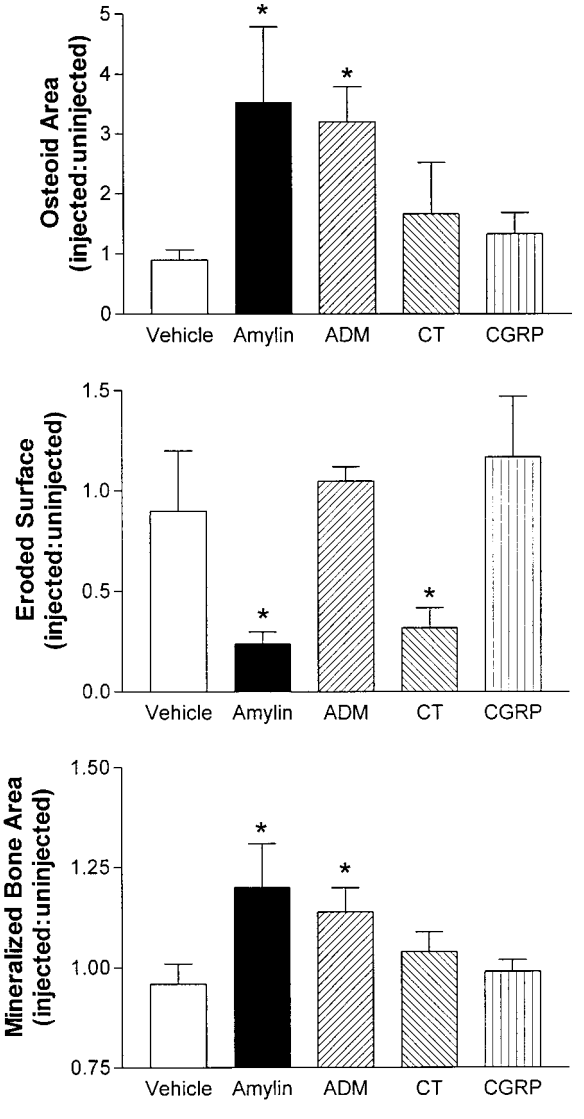

Figure 5. Comparison of the *in vivo* effects of rat amylin, human adrenomedullin (ADM), rat calcitonin (CT) and rat CGRP on bone histology in adult mice. Animals were injected daily with $4.1x10^{-9}$ mol of each peptide over the periosteum of one hemicalvaria for 5 days, then sacrificed 1 week later. 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 ($p<0.05$), Student's *t* test, between the injected and uninjected hemicalvariae are indicated by asterisks. (From Cornish et al. 1999, used with permission).

Effects on osteoblasts

The binding of amylin to osteoblast-like cells was demonstrated soon after the discovery of the peptide[53] and binding of amylin and stimulation of cyclic AMP production was also demonstrated in a pre-osteoblastic cell line[28]. However, these studies did not demonstrate cyclic AMP production in response to amylin in primary osteoblast cultures, although CGRP produced cyclic AMP in these cells. Cornish et al.[1] demonstrated stimulation of proliferation of

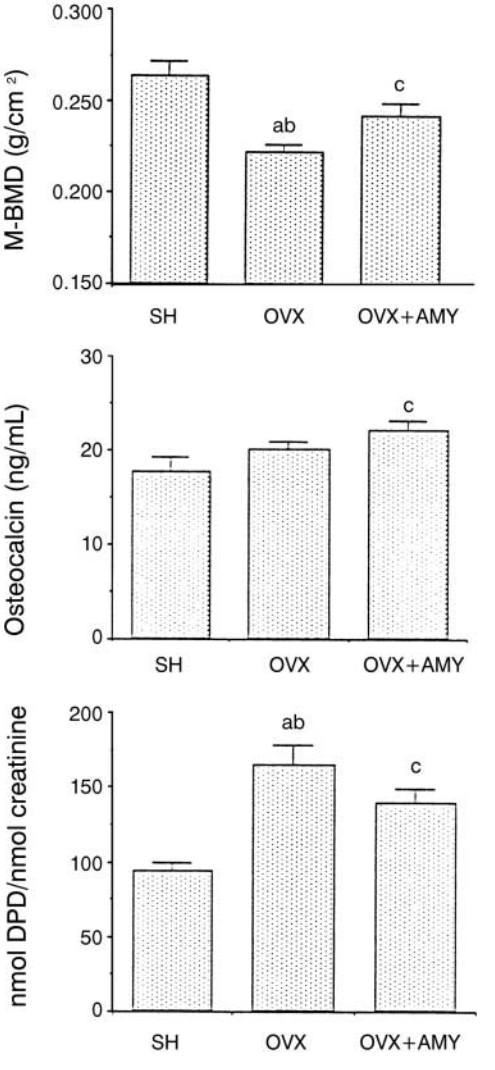

Figure 6. Effects of daily systemic administration of amylin (3μg/100g) to ovariectomized rats for 90 days. Indices assessed were distal metaphyseal bone mineral density of the femur (M-BMD), serum osteocalcin concentration, and urinary excretion of deoxypyridinoline (DPD). SH = sham operated, OVX = ovariectomized, AMY = amylin. a, significantly different from sham animals, $p<0.01$; b, significantly different from amylin-treated group, $p<0.05$; c, significantly different from sham, $p<0.05$. (From Horcajada-Molteni et al. 2000, used with permission).

fetal rat osteoblasts by amylin in concentrations as low as 10^{-11}M, (i.e. periphysiological concentrations) (Fig. 7). The maximal effects of amylin on cell proliferation in this culture system, are similar to those of other osteoblast mitogens[37], suggesting that these effects might be of physiological significance. Similar proliferative effects have been shown in human osteoblasts by Villa et al.[54] and by ourselves (unpublished data). The effects of amylin, CGRP and PTH on the development of mineralization in long-term osteoblast cultures have been compared, and amylin was found to be a more potent agent than either PTH or CGRP[16].

Amylin-(1-8) also stimulates osteoblast proliferation, though its half-maximally effective concentration is 10-fold higher than that of the intact peptide (ie 2.0 x 10^{-11}M for amylin and 2.4 x 10^{-10}M for the octapeptide)[35] (Fig. 7). This peptide also stimulates thymidine incorporation in neonatal mouse calvariae[55]. *In vivo* studies of amylin's effect on

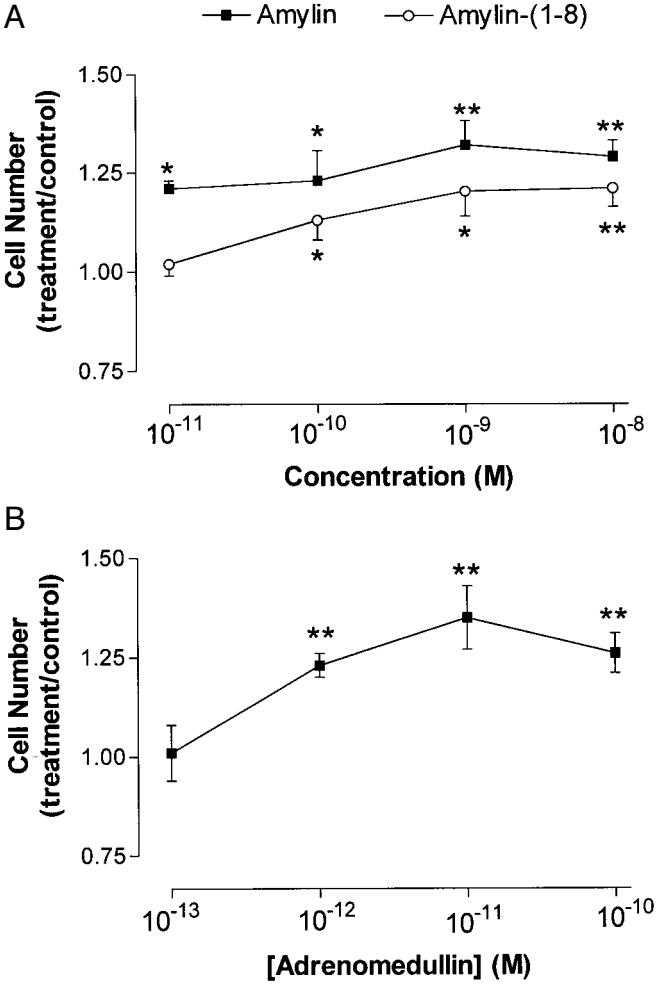

Figure 7. Dose-dependence of the effects of (A) rat amylin, rat amylin-(1-8), and (B) human adrenomedullin on numbers of fetal rat calvarial osteoblast-like cells in culture over 24 hours. Data are expressed as treatment to control ratios. n=6 at each data point. Data are mean ± sem. Statistical significance (by Student's *t* test) of differences from control: *p<0.03; **p<0.003. (From Cornish and Reid 1998, used with permission).

osteoblastic function are now available. Jacobs et al.[56] commented on an increase in cortical endosteal osteoblast numbers in normal rats treated with amylin, but not in diabetic rats similarly treated. Subsequently, the same group showed a transient elevation of serum osteocalcin in rats given daily injections of amylin[51]. Cornish et al. have shown 2- to 4-fold increases in histomorphometric indices of osteoblast activity in adult mice to which amylin was administered locally over the calvariae daily for five days[1] (Fig. 5). Similarly, we have now shown 30-100% increases in these indices following daily systemic administration of amylin over one month[2]. Horcajada-Molteni et al. have demonstrated increases in serum osteocalcin concentrations in ovariectomized rats treated systemically with amylin[17] (Fig. 6), although the small human study with pramlintide did not detect any changes in osteoblast function[52].

These *in vivo* studies have now been extended to amylin-(1-8). Daily systemic administration of this peptide to sexually mature male mice for 4 weeks almost doubled histomorphometric indices of osteoblast activity[5]. Thus, a number of studies have found evidence of an anabolic action of amylin and its amino-terminus in osteoblasts.

Adrenomedullin is a potent osteoblast mitogen, increasing cell numbers and thymidine incorporation into DNA at physiological concentrations of 10^{-12}M and greater[3] (Fig. 5). Structure-activity-relationship studies revealed that treatment for 24 hours with fragments of this protein, adrenomedullin -(15-52), -(22-52), or -(27-52), produced a similar stimulation of proliferation to that from the intact adrenomedullin molecule[3]. In vascular smooth muscle, loss of the ring structure in the adrenomedullin molecule, resulting from amino-terminal truncation or removal of the disulfide bond, eliminates specific binding and cyclic AMP formation[57]. In contrast, activity in osteoblasts is preserved in peptides as short as adrenomedullin-(27-52), suggesting that the disulfide bond and the ring structure created by it, are not necessary. This suggests that a different receptor mediates the effects of adrenomedullin in osteoblasts from that in vascular smooth muscle. The activity of these short fragments is also surprising when comparison is made with amylin. When the ring is removed from amylin, the resulting peptide [amylin-(8-37)] has no agonist activity on osteoblasts and is in fact an antagonist. The effects of the intact molecules of both adrenomedullin and amylin on osteoblast proliferation are similar in terms of maximal effect, although adrenomedullin produces detectable growth stimulation at lower concentrations than amylin. Like CGRP, the proliferative effects of adrenomedullin on osteoblasts are blocked by amylin antagonists, such as amylin-(8-37), again suggesting involvement of the same receptor.

Effects of peptides on bone volume

Considering that this family of peptides may be acting, in part, in an autocrine/paracrine manner, we used an *in vivo* model in which the local effects of factors on bone histomorphometry can be determined. The peptides were

administered by 5 daily subcutaneous injections over one of the hemicalvariae of adult mice and the uninjected contralateral hemicalvariae acted as controls. Amylin administration, at a dose of $4x10^{-9}$mol per injection, significantly increased bone formation, reduced resorption and led to a substantial increase in mineralized bone area[2] (Fig. 5). At equimolar doses, CGRP in the same model produced no significant effects in mineralized bone area and calcitonin caused a small, non-significant increase. Adrenomedullin increased indices of bone formation to a similar extent to amylin, although there was no effect on bone resorption[3] (Fig. 5). Adrenomedullin also significantly increased mineralized bone area in the calvaria[3].

The consistent effects of amylin on bone formation and resorption, suggested that amylin could contribute to the regulation of bone mass and have a potential therapeutic role in osteoporosis. However, to be used in this way it would be necessary that amylin should increase bone mass when administered systemically. Thus, we investigated amylin's effects in adult male mice injected subcutaneously with the peptide at a dose of 10 μg/day for 4 consecutive weeks (5 injections/week). This treatment also resulted in increases in bone formation and decreases in bone resorption[3]. There was a striking increase (70%) in trabecular bone volume in the tibiae of these animals which can be directly appreciated from the sections of bone, shown in Figure 8. Increases in trabecular thickness and number as well as a decrease in trabecular separation can be seen in this photomicrograph. The cortical thickness was also significantly increased in the tibial shafts.

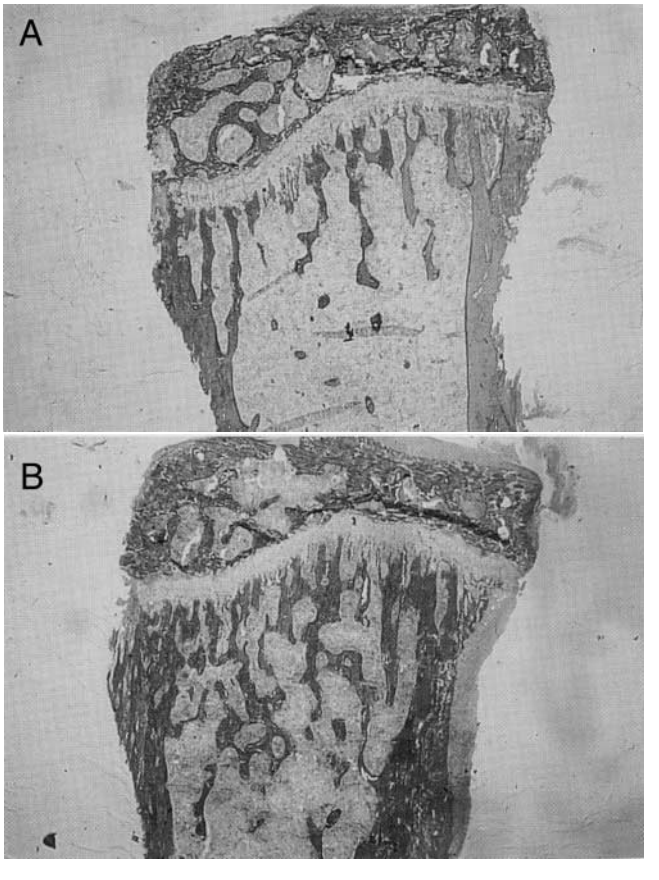

Figure 8. Photomicrographs of the proximal tibiae of mice treated systemically with (A) vehicle, or (B) amylin 10 μg/day for 4 weeks. Trabecular bone volume is increased 70% in the amylin-treated animals. (From Cornish et al. 1998, used with permission).

Amylin also increased the tibial length and growth plate width, implying that the chondrocyte is also an amylin-target cell. We have recently assessed this possibility directly in primary cultures of canine chondrocytes. These cells show increased thymidine incorporation and increased cell numbers after treatment for 24 hours with amylin in concentrations of 10^{-11}M and greater (Cornish et al., manuscript submitted). These changes in chondrocyte proliferation in response to amylin are comparable in magnitude to those seen in osteoblasts. A further significant change noted in these amylin-treated animals was an increase in the fat mass, which had been predicted from its effects on intermediary metabolism. Amylin causes insulin resistance in the liver and in muscle but not in adipocytes[24]. Thus, hyperamylinemia results in hyperinsulinemia that, in turn, stimulates lipogenesis. The effects of systemically-administered amylin have also been studied by Romero et al.[51] who found small increases in the bone mass of male rats in the absence of any changes in histomorphometric or biochemical indices of bone turnover. The more clear-cut result of our studies is possibly attributable to our use of a 100-fold larger dose of peptide. It is also possible that differences in peptide handling and dissolution may have contributed, since amylin passes only slowly into aqueous solution, is very adherent to plastic and glass surfaces, and (when in powder form) is readily lost as a result of its high electrostatic charge. Careful attention to these issues has permitted us to effect a ten-fold increase in the apparent potency of amylin in *in vitro* experiments, and these practical issues may have contributed to the magnitude of the effects demonstrated *in vivo* also. Other experimental differences such as the use of a different animal model from that used by Romero et al. may additionally have contributed to the more marked effects seen in our study.

Recently, we have assessed the effects of systemic administration of fragments of both amylin and adrenomedullin to determine whether their beneficial effects on bone can be dissociated from their potentially undesirable effects on fuel metabolism and blood pressure, respectively. Systemic administration of amylin-(1-8) at equimolar dosage used for the full molecule (2.2 μg) , almost doubled histomorphometric indices of osteoblast activity but did not change measures of bone resorption[5]. Trabecular bone volume increased by 36% (approximately half that seen for amylin entire molecule). On three-point bending tests of bone strength of the tibiae, displacement to fracture was increased by amylin-(1-8), from 0.302 ± 0.013 mm to 0.351 ± 0.017 mm ($p = 0.02$)[5]. In a separate experiment utilizing dynamic histomorphometry with bone-seeking fluorochrome labels, amylin-(1-8) was administered by local injection over the calvariae of female mice[5]. Amylin-(1-8) 40 nM increased the double-labeled surface three-fold. The effect was dose-dependent from 0.4 - 40 nM, and greater than that of an equimolar dose of

hPTH(1-34) (Fig. 9). Mineral apposition rate was increased by 40 nM amylin-(1-8) but not by hPTH(1-34)[5] (Fig. 9).

Adrenomedullin-(27-52), which is an osteoblast mitogen *in vitro* (but no longer vasoactive), produced similar effects. In the same systemic model and at an equimolar dosage to amylin (8.1 μg per injection), adrenomedullin-(27-52) produced increases in the indices of osteoblast activity, osteoid perimeter and osteoblast perimeter ($p<0.05$, for both). Osteoclast perimeter was not affected. There was a 21% increase in cortical width and a 45% increase in trabecular bone volume in animals treated with adrenomedullin-(27-52) ($p<0.002$ for both). Assessment of bone strength by three-point bending of the humerus showed both the maximal force and the displacement to the point of failure were increased in the animals treated with adrenomedullin-(27-52)[4] (Fig. 10). It is concluded that both amylin-(1-8) and adrenomedullin-(27-52) act as anabolic agents on bone. These findings may be relevant to the normal regulation of bone mass and to the design of agents for the treatment of osteoporosis.

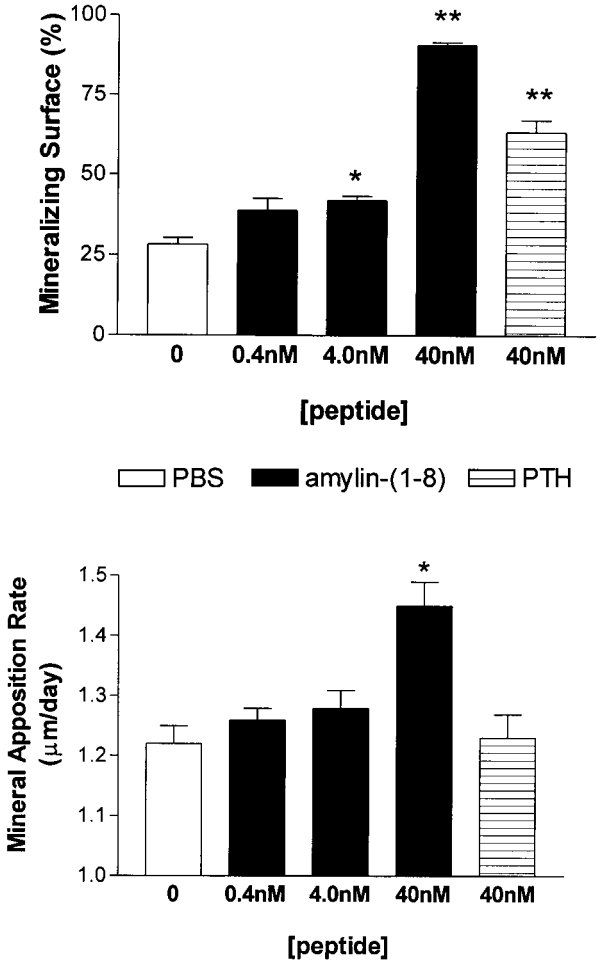

Figure 9. Effect of rat amylin-(1-8) and human PTH (1-34) on the extent of the fluorochrome double-labelled surface (upper panel) and mineral apposition rate (lower panel) in mice calvariae. Mice were treated with local subcutaneous injections over the central calvaria for 5 consecutive days and sacrificed 10 days later. (From Cornish et al. 2000, used with permission).

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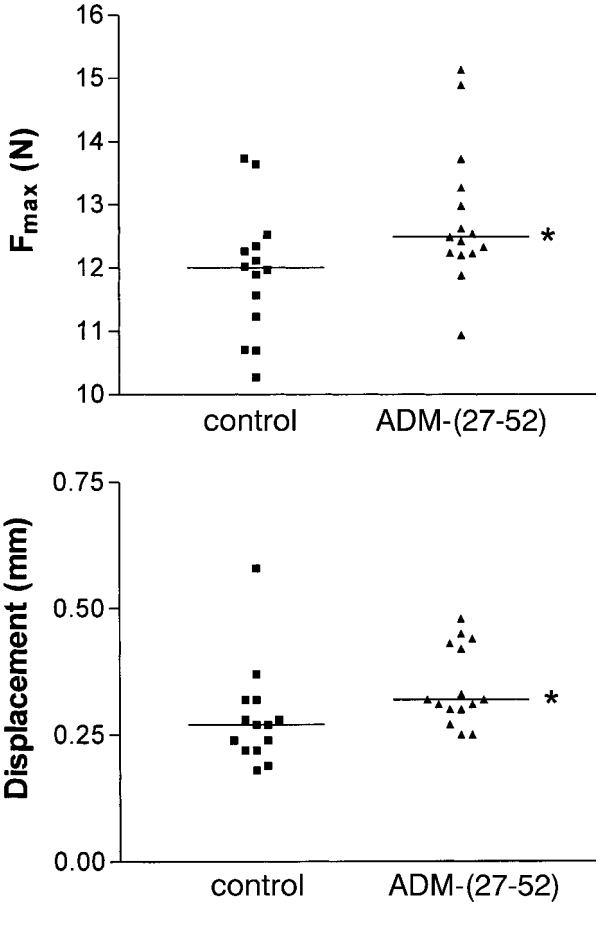

Figure 10. Effects of daily systemic administration of adrenomedullin-(27-52) for 4 weeks on mechanical strength of the humerus, assessed by maximal force (F_{max}) and displacement values from load-deformation curves obtained from three-point bending tests. n = 20 in each group. Data are mean ± sem. *, significantly different from control, $p<0.03$. (From Cornish et al. 2001, used with permission).

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