Dissociation of the effects of amylin on osteoblast proliferation and bone resorption


Cornish, J., K. E. Callon, C. Q.-X. Lin, C. L. Xiao, T. B. Mulvey, D. H. Coy, G. J. S. Cooper, and I. R. Reid. Dissociation of the effects of amylin on osteoblast proliferation and bone resorption. Am. J. Physiol. 274 (Endocrinol. Metab. 37): E827–E833, 1998.—This study assesses the structure-activity relationships of the actions of amylin on bone. In fetal rat osteoblasts, only intact amylin and amylin-(1–8) stimulated cell proliferation (half-maximal concentrations 2.0 × 10^{-11} and 2.4 × 10^{-10} M, respectively). Amylin-(8–37), COOH terminally deamidated amylin, reduced amylin, and reduced amylin-(1–8) (reduction results in cleavage of the disulfide bond) were without agonist effect but acted as antagonists to the effects of both amylin and amylin-(1–8). Calcitonin gene-related peptide-(8–37) also antagonized the effects of amylin and amylin-(1–8) on osteoblasts but was substantially less potent in this regard than amylin-(8–37). In contrast, inhibition of bone resorption in neonatal mouse calvariae only occurred with the intact amylin molecule and was not antagonized by any of these peptides. The rate of catabolism of the peptides in calvarial cultures was not accelerated in comparison with that of intact amylin. This dissociation of the actions of amylin suggests that it acts through two separate receptors, one on the osteoblast (possibly the calcitonin receptor) and a second on the osteoblast.

AMYLIN IS A 37-amino acid peptide cosecreted with insulin from the β-cells of the pancreatic islets. It is structurally related to calcitonin gene-related peptide (CGRP) and more distantly to calcitonin. As a result of these similarities and the fact that calcitonin inhibits osteoclastic bone resorption, the actions of amylin in bone have been studied. Amylin mimics some of the effects of calcitonin on bone resorption, stimulating cAMP formation, inducing quiescence in osteoclasts, and reducing the amount of bone resorbed (1, 13). Similar effects are demonstrable in bone organ culture (5, 17, 24).

Recently, amylin has been found to stimulate the proliferation of osteoblasts in a dose-dependent manner in concentrations as low as 10^{-11} M, and histomorphometric indexes of bone formation are increased in vivo after the local injection of the peptide (6). Alam et al. (1) have shown that the antosteoclastic potency of amylin is substantially reduced in the absence of the carboxyl-terminal amide group, but more detailed studies of the structure-activity relationships for the bone effects of amylin are not available. Such data are important from at least two points of view. First, amylin belongs to a family of peptides, the members of which probably interact with a number of different receptors. The determination of the structural requirements for optimal action of a given peptide may give some insight into the receptor mediating a particular action. Second, the actions of amylin to stimulate bone formation and inhibit bone resorption make it an attractive candidate for the therapy of osteoporosis and possibly to promote the repair of local bone defects. The full peptide would be expensive to manufacture, not be active orally, and might have deleterious effects on carbohydrate metabolism (3). Therefore, a determination of whether the full molecule is necessary for its bone effects and whether it is likely that the skeletal actions can be dissociated from those on carbohydrate metabolism is important to a further evaluation of its therapeutic potential. The present study addresses these issues, using osteoblastic and calvarial cultures.

METHODS

Osteoblast-like cell culture. Osteoblasts were isolated by collagenase digestion from 20-day fetal rat calvariae. Calvariae were dissected aseptically, and the frontal and parietal bones were stripped of their periosteum. Only the central portions of the bones, free from suture tissue, were collected. The calvariae were treated two times with phosphate-buffered saline (PBS) containing 4 mM EDTA (pH 7.4) for 15 min at 37°C in a shaking water bath. After washing one time in PBS, the calvariae were treated two times with 3 ml of 1-mg/ml collagenase for 7 min at 37°C. After the supernatants from these two digestions were discarded, the calvariae were treated an additional two times with 3 ml of 2-mg/ml collagenase (30 min, 37°C). The supernatants of the latter two digestions were pooled and centrifuged, and the cells were washed in DMEM with 10% FCS, suspended in fresh medium, and then seeded at 5 × 10^{4} cells/ml in 24-well plates. The cells were subcultured after trypsinization using trypsin-EDTA (0.05%/0.53 mM), the cells were rinsed in MEM with 5% FCS, resuspended in fresh medium, and then seeded at 5 × 10^{4} cells/ml in 24-well plates (0.5 ml of cell suspension/ well, i.e., 2.5 × 10^{4} cells/well). The cells were cultivated under 5% CO₂ and 95% air at 37°C. Ascorbic acid in a concentration of 50 μM was added to the MEM used throughout. The osteoblast-like character of these cells has been established by demonstration of high levels of alkaline phosphatase activity and osteocalcin production (10) and a sensitive adenylyl cyclase response to parathyroid hormone and prostaglandin E₂ (12).

Proliferation studies (cell counts and thymidine incorporation) were performed in subconfluent cell populations; 24 h after subculturing, cells were changed to serum-free medium with 0.1% bovine serum albumin plus the experimental compounds. Cell numbers were analyzed 24 h after addition.
of the peptide or vehicle by detaching cells from the wells by exposure to trypsin-EDTA (0.05%/0.53 mM) for ~5 min at 37°C. Counting was performed in a hemocytometer chamber. Results are expressed per well. [3H]thymidine incorporation into actively growing and growth-arrested cells was assessed by pulsing the cells with [3H]thymidine (0.5 µCi/well) 2 h before the end of the experimental incubation. Experiments were terminated at 24 h by washing the cells in MEM, followed by the addition of 10% trichloroacetic acid. The precipitate was washed two times with ethanol-ether (3:1), and the wells were desiccated at room temperature. The residue was redisolved in 2 M KOH at 55°C for 30 min and neutralized with 1 M HCl, and an aliquot was counted for radioactivity. Results are expressed as disintegrations per minute per well. For cell counts and thymidine incorporation, each experiment was performed at least four times using experimental groups consisting of at least six wells.

Bone organ culture. Bone resorption studies were carried out in neonatal mouse calvariae as described previously (22). Mice were injected subcutaneously with 5 µCi of 45Ca at 2 days of age, and hemicalvariae were dissected out 4 days later. Hemicalvariae were preincubated for 24 h in medium-199 with 0.1% bovine serum albumin and then changed to fresh medium containing peptide or vehicle. Incubation was continued for a further 48 h. There were five to seven hemicalvariae in each group.

To determine whether the various peptide fragments underwent significant catabolism in organ culture, they were incubated with calvariae before being added to osteoblast cultures. The organ cultures were set up using hemicalvariae from animals that had not been pretreated with 45Ca. The fragments or vehicle was incubated with the calvariae for 2, 4, or 6 h. Extracts of the media were removed and then immediately added to osteoblast cultures for measurement of their effects on osteoblast proliferation, as described above.

Materials. The rat amylin-(1—8) and rat amylin-(8—37) used in this study were COOH-terminal amides synthesized on methylbenzhydrylamine resin by standard solid-phase techniques followed by hydrogen fluoride deprotection and cleavage from the resin. Amylin-(1—8) was cyclized in a dilute solution of 90% acetic acid by treatment with methanol and dried before the end of the experimental incubation. The other three peptides studied had no effect on osteoblast cell number or thymidine incorporation. Reduced amylin-(1—8), like the reduced full molecule, also had no proliferative effect on osteoblasts (data not shown).

The dose-response relationships for action of intact amylin and amylin-(1—8) on cell number are shown in Fig. 2. The intact molecule caused significant increases in cell number at concentrations of 10−12 M and greater, the half-maximally effective concentration being 2.0 × 10−11 M. The maximal effect of amylin-(1—8) was comparable with that of the intact molecule but was only produced at concentrations ≥10−9 M. Its half-

![Fig. 1](image)

Fig. 1. Effect of equimolar concentrations (10−9 M) of rat amylin fragments/analogs or vehicle on cell number and thymidine incorporation in cultures of fetal rat osteoblasts. Data are expressed as ratios of treatment to control and are means ± SE. *Significantly different from control, P = 0.002.
maximal concentration was $2.4 \times 10^{-10}$ M. Comparable results were seen in the actions of two peptides on thymidine incorporation into osteoblastic cells (data not shown).

Amylin-(8—37) functions as an amylin antagonist in other tissues, so its effects on amylin-stimulated osteoblast proliferation were examined (Fig. 3). Amylin-(8—37) blocked the proliferative effect of intact amylin with an inhibitory constant ($K_i$) of $5 \times 10^{-2}$ M. The comparable peptide fragment from CGRP, which is a CGRP-receptor blocker, also blocked the action of amylin, but this blockade was only complete at CGRP-(8—37) concentrations $>10^{-8}$ M, and the $K_i$ was $4 \times 10^{-10}$ M. Deamidated amylin and reduced amylin also blocked the action of the intact peptide on cell number and thymidine incorporation when present in equimolar concentrations (Fig. 4). In a similar manner, reduced amylin-(1—8) was an effective blocker of intact amylin (data not shown). Similar results with these blockers were seen for thymidine incorporation (data not shown).

The effects of these amylin antagonists on the actions of amylin-(1—8) were also assessed (Table 1). Amylin-(1—8) consistently stimulated both cell number and thymidine incorporation, and its effects were blocked by equimolar concentrations of all the antagonists. Similarly, reduced amylin-(1—8) was an effective blocker of amylin-(1—8) at equimolar concentrations (data not shown).

Bone resorption. The effects of intact amylin and its four derivatives on bone resorption were assessed in cultures of neonatal mouse calvariae (Fig. 5). Amylin inhibited basal bone resorption, which was not the case with the other fragments. To determine whether this apparent lack of effect of amylin-(1—8) was attributable to its being catabolized more rapidly than intact amylin in the organ culture, the effect of preincubation in the organ culture proliferative

Table 1. Interaction of amylin-(1—8) and amylin blockers on osteoblast proliferation

<table>
<thead>
<tr>
<th>Blocker</th>
<th>Cell Number ($\times 10^4$ cells/well)</th>
<th>Control</th>
<th>Amylin-(1—8)</th>
<th>Blocker</th>
<th>Amylin + blocker</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amylin-(8—37)</td>
<td>$4.83 \pm 0.06$</td>
<td>$5.85 \pm 0.21^*$</td>
<td>$4.80 \pm 0.08$</td>
<td>$4.83 \pm 0.12$</td>
<td></td>
</tr>
<tr>
<td>CGRP-(8—37)</td>
<td>$3.47 \pm 0.03$</td>
<td>$3.98 \pm 0.65^*$</td>
<td>$3.45 \pm 0.08$</td>
<td>$3.38 \pm 0.07$</td>
<td></td>
</tr>
<tr>
<td>Deamidated amylin</td>
<td>$3.68 \pm 0.11$</td>
<td>$4.19 \pm 0.08^*$</td>
<td>$3.62 \pm 0.10$</td>
<td>$3.68 \pm 0.06$</td>
<td></td>
</tr>
<tr>
<td>Reduced amylin</td>
<td>$3.97 \pm 0.11$</td>
<td>$4.68 \pm 0.08^*$</td>
<td>$3.90 \pm 0.16$</td>
<td>$3.98 \pm 0.10$</td>
<td></td>
</tr>
</tbody>
</table>

Data are expressed as means $\pm$ SE. CGRP, calcitonin gene-related peptide. All peptides were at $10^{-9}$ M except for CGRP-(8—37), which was at $10^{-8}$ M. *Significantly different from control, $P = 0.009$. 

Fig. 2. Dose response of rat amylin (○) or rat amylin-(1—8) (□) for 24 h on cell number in cultures of fetal rat osteoblasts. Data are expressed as ratios of treatment to control. Data are means $\pm$ SE. Significantly different from control, *$P < 0.03$ and **$P < 0.003$.

Fig. 3. Effect of rat amylin-(8—37) and rat calcitonin gene-related peptide (CGRP)-(8—37) on the effect of amylin ($10^{-9}$ M) on cell number in fetal rat osteoblasts. Data are expressed as ratios of treatment to control. Data are means $\pm$ SE. Significantly different from control, *$P \leq 0.01$ and **$P < 0.001$.

Fig. 4. Effect of reduced rat amylin and deamidated rat amylin on amylin action on cell number in fetal rat osteoblasts. All peptides were used at a concentration of $10^{-9}$ M. Data are means $\pm$ SE. *Significantly different from control, $P < 0.001$. 

Fig. 5. Effect of rat amylin-(8—37) and rat calcitonin gene-related peptide (CGRP)-(8—37) on the effect of amylin (10$^{-2}$ M) on cell number in fetal rat osteoblasts. Data are expressed as ratios of treatment to control. Data are means $\pm$ SE. Significantly different from control, *$P < 0.03$ and **$P < 0.003$.
activity of each peptide was assessed. In Fig. 6, it is shown that preincubation for 2 h had no effect on the osteoblastic activity of either intact amylin or its 1—8 fragment but that both peptides lost their activity if the preincubation lasted for four or more hours. This was true whether activity was assessed by cell number or by thymidine incorporation. Thus both peptides appeared to be inactivated at comparable rates, and their different effects on bone resorption cannot be accounted for by differential catabolism. This was consistent with the further finding that multiple additions of amylin-(1—8) (at 0, 8, and 30 h) did not influence bone resorption (data not shown).

The actions of the antagonist peptides on the inhibition by amylin of bone resorption are shown in Table 2. In contrast to the findings in osteoblasts, none of these peptides interfered with the reduction in bone resorption resulting from amylin treatment. The rate of catabolism of these peptides in calvarial culture was also assessed (Fig. 7). Preincubation of amylin-(8—37), deamidated amylin, or reduced amylin with calvariae for 6 h decreased their ability to block the stimulation of osteoblast proliferation by amylin. This indicates that the breakdown of these antagonists is, if anything, slower than that of the native peptide (see Fig. 6). Thus the failure of these peptides to block the effects of amylin on bone resorption is unlikely to result from their more rapid degradation in bone organ culture.

**DISCUSSION**

This study has examined the structure-activity relationships for the action of amylin on two different aspects of bone metabolism, osteoblast proliferation, and osteoclastic bone resorption. In osteoblasts, the intact peptide produces the greatest maximal effect. However, its NH₂-terminal fragment, amylin-(1—8), also stimulates osteoblast proliferation, although with a half-maximally effective concentration 10-fold higher than that of the parent molecule. In contrast, the COOH terminus of amylin has no effect on osteoblast proliferation, and removal of the COOH-terminus amide group or of the disulfide bond eliminates activity on osteoblasts. The interaction of amylin and its fragments with its receptor is clearly a complex event, since deamidated amylin lacks activity despite containing amylin-(1—8) within its structure. This implies that
the peptide containing the deamidated COOH terminus is able to block the stimulation of proliferation that would otherwise follow from the presence of the cyclic NH₂-terminal octapeptide. There is evidence that the amylin receptor belongs to the class of seven transmembrane segment receptors or G protein-coupled receptors (3). Recent analysis of ligand binding to members of this class of receptors, such as those for angiotensin II and neurokinin I, has shown a separation within the receptor molecule between binding sites for the peptide agonists and those for antagonists (2, 16). Our data for the relationship between ligand structure and activity are consistent with there being two such binding sites in the osteoblast amylin receptor, one for agonists such as amylin and amylin-(1—8) and a separate one for antagonists.

With respect to bone resorption, the structure-activity relationships are different. Intact amylin inhibits basal bone resorption by almost 50%, an outcome that would be expected from a potent antiresorptive agent interacting with this model in which basal resorption is high. However, none of the other fragments, including amylin-(1—8), have any effect on bone resorption. Our findings of reduced inhibition of bone resorption with deamidated amylin are consistent with the results of Alam et al. (1), who found that deamidated amylin was 10-fold less potent in its action on isolated rat osteoclasts than the parent molecule. Our results are apparently inconsistent with those of Datta et al. (8), who found comparable hypocalcemic effects in rats with amylin and deamidated amylin. It is possible that much of the hypocalcemic effects of these peptides are mediated by a direct effect on renal calcium excretion (14), so that this finding does not necessarily imply comparable effects at the osteoclast level.

A potential explanation for the lack of activity of the amylin fragments on bone resorption might be that they are more rapidly catabolized in organ culture than the parent molecule. However, the present studies of peptide incubation with calvariae indicate that the rates of breakdown of amylin-(1—8) and intact amylin are comparable, suggesting that the differences in activities of the octapeptide on osteoblasts and bone resorption reflect differences in the receptors mediating these effects of amylin. A similar observation suggesting multiple target receptors with differential binding has been made with respect to the related compound, CGRP. Thiebaud et al. (25) have shown that the action of that peptide on cAMP production in preosteoblasts is greatly reduced by NH₂- or COOH-terminal truncation, whereas the latter peptide retained its hypocalcemic effect.

A further potential explanation for the different findings with respect to osteoblast proliferation and bone resorption is that these studies were carried out in different species, the osteoblast studies in rat cells and the resorption studies in tissue from mice. As mentioned above, Alam et al. (1) found resorption effects to amylin peptides in rat osteoclasts to be similar to the present findings, and we have reported stimulation of bone formation in vivo by amylin in mice (6). This suggests that rats and mice respond similarly to these peptides and that species differences are unlikely to account for the different structure-activity relationships for the osteoblast and osteoclast actions of amylin.

The amylin effects on osteoblast proliferation are blocked by the amylin antagonist amylin-(8—37). At substantially higher concentrations, they are also blocked by the CGRP antagonist CGRP-(8—37). The differential sensitivity of the effects of amylin on osteoblasts to these two receptor blockers implies that the action is primarily through an amylin receptor rather than a CGRP receptor. The present studies have also defined new blockers of amylin action in osteoblasts, deamidated amylin, reduced amylin, and reduced amylin-(1—8). None of these appear to have been assessed as a potential amylin antagonist in other systems, and it will be of interest to determine what their effects are on amylin action in carbohydrate metabolism and on the actions of other related hormones such as CGRP. We have recently found that both are able to block the osteoblast-proliferative effects of the related peptide adrenomedullin (7). Their action on the vasodilator effects of adrenomedullin have not been studied. As shown in the present study, these antagonists also block the action of amylin-(1—8) on osteoblasts, but they do not interfere with the osteoblast proliferative effects of unrelated peptides, such as transforming growth factor-β and epidermal growth factor (J. Corshish, K. E. Callon, G. J. S. Cooper, and I. R. Reid, unpublished observation). Furthermore, they do not interfere with the action of amylin on bone resorption, reinforcing the suggestion that the receptor involved in this effect is different from that present in the osteoblasts. This possibility is further supported by the fact that the related peptide, adrenomedullin, is a potent stimulus to osteoblast proliferation but has no effect on basal or stimulated bone resorption (7). It is possible that the antiresorptive effects of this class of hormones are mediated by the calcitonin receptor, since the calcitonins are the most potent members of this family of peptides in this regard and since amylin has been shown to displace calcitonin from its receptor on osteoclasts (24). Studies of the binding of labeled amylin to isolated osteoblasts and osteoclasts will be needed to allow this possibility to be addressed. However, 125I-labeled amylin is not biologically active and does not appear to show specific binding to bone cells. Efforts are under way at the present time to produce a radiolabeled amylin that is biologically active, and further studies of the receptor interactions of these peptides will need to await this development. The isolation and sequencing of the amylin receptor(s) are also proving to be an elusive goal, but, when this is achieved, it will help address these questions.

The relevance of findings in cultured cells to in vivo physiology is always open to question, since differences in cell preparation and culture can substantially impact outcomes. In this regard, it should be noted that the proliferative effects of amylin have recently been confirmed in primary cultures of human osteoblast-like...
cells (26) and that these effects are demonstrable in vivo either after local injection of amylin over bone (6) or after its systemic administration (J. Cornish, K. E. Callon, A. R. King, G. J. S. Cooper, and I. R. Reid, unpublished observation). The congruence of the findings in these very different experimental systems suggests that osteoblast stimulation is a general property of amylin and not one confined to a specific set of experimental conditions.

What roles amylin plays in normal bone metabolism and bone pathology remain to be determined. It has been hypothesized that amylin secretion after a meal directs the absorbed calcium and protein from the meal into new bone synthesis by increasing bone growth at a time when substrates are available (13, 27). Amylin may also contribute to the relationship between body mass and bone density. Body mass, or more particularly fat mass (18, 23), is the principal determinant of bone mass and bone density. Body mass, or more particularly time when substrates are available (13, 27). Amylin directs the absorbed calcium and protein from the meal into new bone synthesis by increasing bone growth at a time when substrates are available (13, 27). Amylin may also contribute to the relationship between body mass and bone density.

The present studies have relevance to the development of therapies for osteoporosis. The amylin peptides show potential in this regard, since they are clearly potent stimulators of bone formation both in vitro and in vivo. Furthermore, they combine this activity with an inhibition of bone resorption. Amylin itself has been implicated in the pathogenesis of type 2 diabetes, in part as a result of amyloid deposition in the pancreatic islets and possibly also by contributing to the development of obesity. However, amylin actions on carbohydrate metabolism require the intact peptide, and its propensity to form amyloid is dependent, in part, on the amino acids between positions 25 and 29 (3). Thus the present demonstration that its osteoblast proliferative effects can be isolated to a smaller peptide, which has no effects on carbohydrate metabolism and no tendency to lead to amyloid formation, is a major step toward making the capacity of this peptide to stimulate bone formation available in the management of osteoporosis. The present data suggest that the preparation and assessment of amylin analogs hold promise for the development of a novel and clinically useful bone anabolic factor.

Wethank Usha Bava for technical assistance.

This research was supported by the Health Research Council of New Zealand.

Address for reprint requests: J. Cornish, Dept. of Medicine, Univ. of Auckland, Private Bag 92019, Auckland 1001, New Zealand.

Received 16 October 1997; accepted in final form 4 February 1998.

REFERENCES

17. Pietzschmann, P., K. H. Farsoudi, O. Hoffmann, K. Klaus- hofer, H. Horandner, and M. Peterlik. Inhibitory effect of amylin on basal and parathyroid hormone-stimulated bone