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Shared pathways of osteoblast mitogenesis induced by amylin, adrenomedullin, and IGF-1

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Abstract

Amylin and adrenomedullin, members of the calcitonin peptide family, are anabolic to bone. Here, we report overlapping molecular mechanisms by which amylin, adrenomedullin, and IGF-1 induce osteoblast proliferation. Co-treatment of osteoblastic cells with amylin or adrenomedullin and IGF-1 failed to induce an additive mitogenic effect. In osteoblastic cells, neutralization of the IGF-1 receptor blocked the proliferative effects of amylin and adrenomedullin, while neutralization of IGF-1 did not. Neither amylin- nor adrenomedullin-induced mitogenic signaling or cell proliferation in IGF-1 receptor-null fibroblasts. In addition, amylin and adrenomedullin receptor blockers inhibited the proliferative effects of IGF-1 in osteoblastic cells. These findings demonstrate overlap in the molecular mechanisms by which amylin, adrenomedullin, and IGF-1 induce mitogenesis in osteoblasts, and an important role for the IGF-1 receptor in the mitogenic actions of amylin and adrenomedullin. Our findings are potentially important in refining these peptides for the therapy of osteoporosis.

Keywords: Osteoblast; Calcitonin-like; In vitro; MAP-kinase; IGF-1 receptor

Bone is a dynamic organ that is continually remodeled by the complex coupling of the actions of the bone forming cells, osteoblasts, and bone resorbing cells, the osteoclasts. Bone remodeling is controlled by many systemic and locally produced growth factors and cytokines. Members of the calcitonin/amylin/adrenomedullin peptide family regulate bone cell activity and both amylin and adrenomedullin have anabolic effects on bone. In vitro and in vivo studies have shown amylin to be mitogenic to osteoblasts, to decrease the formation and activity of osteoclasts, and to increase bone mass after systemic administration in mice [1–3]. Adrenomedullin also increases the proliferation of osteoblast cells in vitro and in vivo, but it does not have any effect on the formation or function of osteoclasts [4,5].

The nature of the amylin receptor expressed by osteoblasts is uncertain. Primary rat osteoblasts express the putative amylin receptor components, receptor activity modifying proteins (RAMP) 1 and 3, but do not express calcitonin receptor (CTR) [5]. All the putative adrenomedullin receptor components including L1, calcitonin receptor-like receptor (CRLR), and (RAMP) 2 and 3 are expressed by primary rat osteoblasts and specific binding of ^{125}I-adrenomedullin to osteoblast membranes has been demonstrated [5].

Many anabolic agents promote bone growth in part by stimulating osteoblast proliferation. For example, transforming growth factor-beta (TGF-β) and insulin-like growth factor-I (IGF-1) are potent osteoblast mitogens [6,7]. Within the bone microenvironment these factors also appear to have additive effects that increase bone growth [8]. On the other hand, some factors interact and share similar pathways of action. Of note, IGF-1 appears to be required for the anabolic actions of parathyroid hormone and thyroid hormone on bone [9–11].

In the current study, we investigated the possible interactions between amylin and adrenomedullin and other anabolic factors in bone. We show that the mitogenic actions of amylin on osteoblasts are additive to those of peptides such as TGF-β and the C-terminal fragment of parathyroid hormone-related peptide

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(C-terminal PTHrP), but not to those of IGF-1 or insulin. The mitogenic actions of amylin in osteoblasts and fibroblasts require the presence of functional IGF-1 receptors, and in turn the proliferative response of osteoblasts to IGF-1 requires functioning amylin receptors. Experiments performed with adrenomedullin yielded similar results to those with amylin. These data suggest the existence of cross-talk between the mitogenic signaling pathways activated by IGF-1, amylin, and adrenomedullin in osteoblasts, and raise the possibility of a novel association between the IGF-1 receptor and the receptors for amylin and adrenomedullin that mediates the proliferative responses to each ligand.

Materials and methods

Reagents. Fetal calf serum, human TGF-β1, and culture media were from Invitrogen (Rockville, MD). IGF-1 was from Kabi Pharmacia (Gothenberg, Sweden). Rat amylin, rat amylin-(8-37), human adrenomedullin, and reduced-human adrenomedullin were from Bachem (Bubendorf, Switzerland). An IGF-1 neutralizing antibody was generously provided by Bernard Brier (Liggins Institute, University of Auckland). The IGF-1 receptor blocking antibody (Ab-1) was from Calbiochem/Novabiochem (Darmstadt, Germany). Antibodies to total and phosphorylated p42/44 MAP kinases were from Santa Cruz Biotechnology (Santa Cruz, CA). Pertussis toxin was from List Biological Laboratories (Campbell, CA). PD-98059 was from Biomol (Plymouth Meeting, PA).

Cell culture. Rat primary osteoblasts were isolated from 20-day fetal rat calvariae as previously described [4]. Mouse fibroblastic cell lines carrying either a null mutation for the IGF-1 receptor (IGF-1R$^-$) or expressing wild-type human IGF-1 receptor (IGF-1R$^+$) were gifted by Professor Stewart Gilmour (Liggins Institute, University of Auckland, New Zealand) [12]. Fibroblastic cells were cultured in 20% FCS/DMEM.

Cell proliferation assay. Primary rat osteoblasts or fibroblastic cells were seeded into 24-well plates, grown to semi-confluence overnight, growth arrested for 24 h, and treated with experimental compounds for 24 h, and proliferation was assessed by cell counts using a hematocytometer.

Immunoblotting. Primary rat osteoblasts or fibroblastic cells were grown to 80–90% confluence in 6-well tissue culture plates, serum-starved overnight and then treated with experimental compounds in 0.1% BSA/MEM at room temperature. Cell lysate collection and Western blotting were performed as previously described [13]. Immunoblots presented are representative of at least three separate experiments in each case.

Cell membrane preparation and receptor binding assay. Preparation of primary rat osteoblast cell membranes and receptor binding assays were performed as described previously [5]. The prepared membranes were thawed, aliquoted at 20 μg protein/tube, and resuspended in 250 μl binding buffer containing the ^{125}I-IGF-1 and unlabeled competitor. All binding data analyses were performed by nonlinear regression, using GraphPad 3.1 (GraphPad Software, San Diego, CA).

Results

Overlapping mitogenic actions of amylin and IGF-1 in osteoblastic cells

Amylin, TGF-β, C-terminal PTHrP, and IGF-1 each induce proliferation of primary rat osteoblasts. There is no additive effect of amylin with IGF-1 when maximally proliferative concentrations of each peptide are combined (Fig. 1A), similarly there was no additive effect on osteoblast proliferation in response to co-treatment with amylin and insulin. It is generally believed that insulin is acting on the osteoblasts via the IGF-1 receptor. However, the proliferative actions of amylin are synergistic with those of TGF-β and C-terminal PTHrP. Representative TGF-β data are shown in Fig. 1A. The above results suggest that the mitogenic actions of amylin and IGF-1 in osteoblasts involve a common effector pathway.

Stimulation of osteoblastic cell proliferation by amylin is not due to paracrine effects of osteoblast-derived IGF-1

To further explore the role of IGF-1 in the proliferative effects of amylin in osteoblasts, we treated osteoblasts in culture with a neutralizing antibody to IGF-1 (α-IGF-1). Neutralization of IGF-1 completely blocked IGF-1-stimulated osteoblast proliferation but did not inhibit the actions of amylin (Fig. 1B). Similarly TGF-β-stimulated osteoblast proliferation was not inhibited by addition of IGF-1 neutralizing antibody. These results suggest the proliferative actions of amylin are not due to an increase in IGF-1 production by primary rat osteoblasts.

Amylin-induced osteoblast proliferation involves the IGF-1 receptor and p42/44 MAP kinase signaling

The proliferative effects of amylin and IGF-1 on primary rat osteoblasts were blocked by the addition of an antibody to the IGF-1 receptor (Fig. 1C). Blockade of the IGF-1 receptor did not affect TGF-β-stimulated proliferation (Fig. 1C), indicating that the observed effect of the IGF-1 receptor antibody on amylin-induced osteoblast mitogenesis is not a non-specific effect.

We have previously demonstrated that p42/44 MAP kinase signaling is involved in transduction of the mitogenic signal induced by IGF-1 in osteoblasts [14]. Since a functional IGF-1 receptor is required for amylin-induced osteoblast proliferation, we determined whether p42/44 MAP kinase signaling is involved in the proliferative actions of amylin in osteoblastic cells. Amylin activates p42/44 MAP kinase signaling in osteoblastic cells, as demonstrated by immunoblotting for phosphorylated p42/44 MAP kinases (Fig. 2A). This effect is inhibited by pertussis toxin, an observation that implicates G_i proteins in the proliferative actions of amylin (Fig. 2B). As shown in Fig. 2C, PD-98059, a specific inhibitor of MEK, which phosphorylates and activates p42/44 MAP kinases, dose-dependently inhibits the mitogenic response of osteoblasts to amylin. Taken together, these data suggest that the ability of amylin to stimulate osteoblast proliferation requires a functional

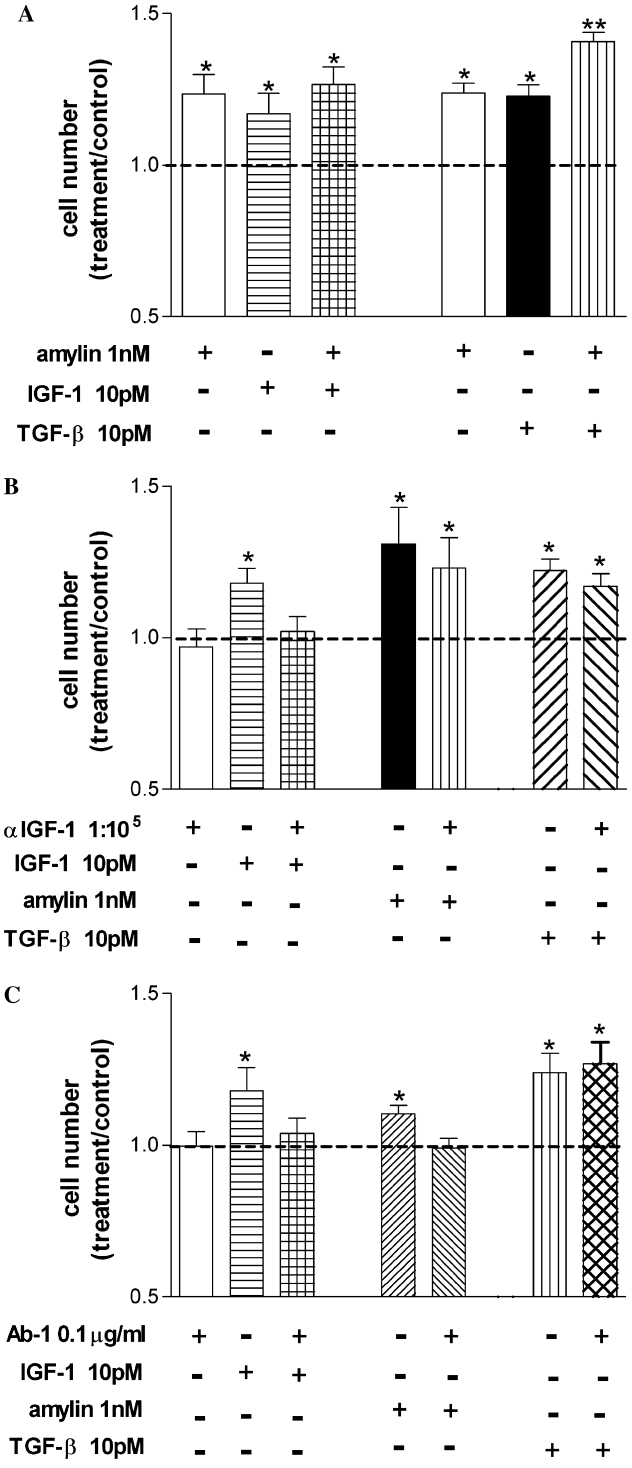

Fig. 1. Overlapping mitogenic actions of amylin, TGF-β, and IGF-1 in osteoblastic cells. Growth-arrested primary rat osteoblasts were treated for 24 h with maximally proliferative concentrations of amylin, TGF-β, and IGF-1. (A) Effects of peptides in combination: *, significantly different from control, $p < 0.001$; **, significant additivity with TGF-β and amylin, $p < 0.003$. (B) Effects of neutralizing IGF-1 antibody (αIGF-1) on amylin and IGF-1-stimulated proliferation: *, significantly different from control, $p < 0.001$. (C) Effects of anti-IGF-1 receptor antibody (Ab-1) on amylin and IGF-1-stimulated proliferation: *, significantly different from control, $p < 0.04$. Cell numbers are expressed as a ratio to that in untreated cells.

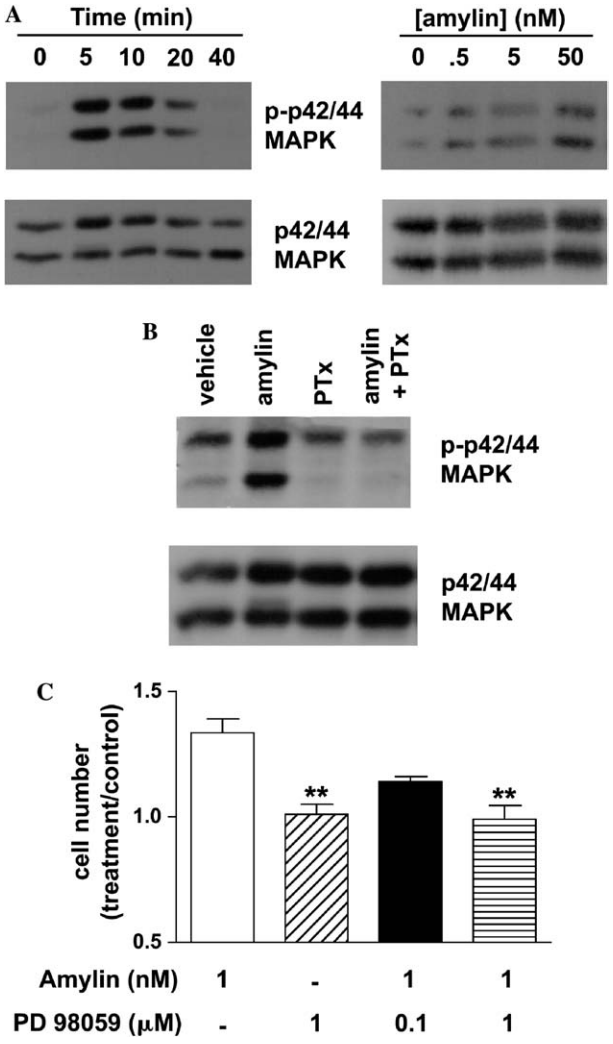

Fig. 2. Amylin signals osteoblast proliferation through G_i-dependent activation of p42/44 MAP kinases. (A) Whole cell lysates of primary rat osteoblasts treated with 50 nM amylin for the indicated times (left panel), or for 10 min with the indicated concentration of amylin (right panel), were sequentially immunoblotted with antibodies to phosphorylated p42/44 MAP kinases (top panel) and total p42/44 MAP kinases (lower panel). (B) Whole cell lysates of primary rat osteoblasts treated with vehicle (lanes 1 and 3) or 50 nM amylin (lanes 2 and 4) in the presence (lanes 3 and 4) or absence (lanes 1 and 2) of 200 ng/ml pertussis toxin (PTx) were sequentially immunoblotted with antibodies to phosphorylated p42/44 MAP kinases (top panel) and total p42/44 MAP kinases (lower panel). (C) Growth-arrested primary rat osteoblasts were treated for 24 h with 1 nM amylin in the absence or presence of the indicated concentrations of the MEK inhibitor, PD98059, and cell number determined using a hematocytometer. Cell numbers are expressed as a ratio to that in untreated cells and represent pooled data from three separate experiments. **, $p < 0.01$ vs amylin.

IGF-1 receptor, G_i proteins, and activation of p42/44 AP kinase signaling.

To directly examine the role of the IGF-1 receptor in the mitogenic actions of amylin, we studied murine fibroblastic cells lacking functional IGF-1 receptors [12]. IGF-1 and amylin stimulate proliferation of fibroblastic cells expressing wild-type IGF-1 receptor (IGF-1R)

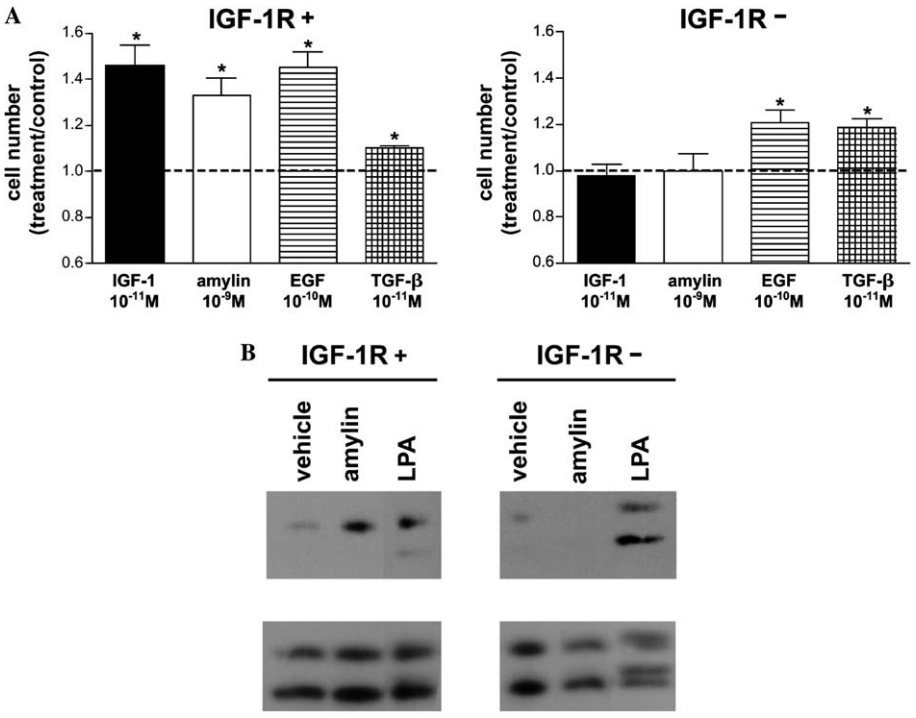

Fig. 3. The IGF-IR is required for amylin-induced cell proliferation and mitogenic signaling. (A) Fibroblastic cells expressing wild-type IGF-IR (IGF-IR$^+$) or lacking a functional IGF-IR (IGF-IR$^-$) were treated with the indicated growth factors and cell counts determined as described in Materials and methods. *, $p < 0.01$ vs untreated cells. (B) Whole cell lysates of IGF-IR$^+$ and IGF-IR$^-$ fibroblastic cells treated for 10 min with 50 nM amylin or 1 μM lysophosphatidic acid (LPA) were sequentially immunoblotted with antibodies to phosphorylated p42/44 MAP kinases (top panel) and total p42/44 MAP kinases (lower panel).

(Fig. 3A, left panel), but not of fibroblastic cells in which the IGF-1 receptor has been genetically inactivated (IGF-1R$^-$) (Fig. 3A, right panel). EGF and TGF-β-stimulated proliferation of both IGF-1R$^+$ and IGF-1R$^-$ cells (Fig. 3A). Amylin signaling to p42/44 MAP kinases also requires a functional IGF-1 receptor. Phosphorylation of p42/44 MAP kinases was observed in IGF-1R$^+$ cells, but not IGF-1R$^-$ cells, following treatment with amylin (Fig. 3B). Activation of p42/44 MAP kinases was observed in IGF-1R$^-$ cells following treatment with the phospholipid growth factor, lysophosphatidic acid (LPA, Fig. 3B). Densitometric analysis of three separate experiments demonstrated that amylin induced a 2.4-fold increase in phosphorylation of p42/44 MAP kinases in IGF-1R$^+$ cells, whereas p42/44 MAP kinase phosphorylation was not detected in IGF-1R$^-$ cells treated with amylin.

The mitogenic activity of adrenomedullin also involves the IGF-1 receptor

We have previously reported that amylin and adrenomedullin exert mitogenic actions in osteoblasts through a common pathway [4]. We therefore performed limited experiments to determine whether the IGF-1 receptor plays a role in the mitogenic actions of adrenomedullin. Like amylin, adrenomedullin rapidly activates phosphorylation of p42/44 MAP kinases in primary rat osteoblasts (Fig. 4A). The neutralizing antibody to the IGF-1 receptor inhibited the proliferative response to adrenomedullin in primary rat osteoblastic cells (cell numbers, expressed as treatment to control ratio, means ± SEM; adrenomedullin alone vs adrenomedullin + Ab-1: 1.11 ± 0.03 vs 0.98 ± 0.04, $p = 0.01$). Adrenomedullin stimulates mitogenesis in IGF-1R$^+$ fibroblastic cells but not in IGF-1R$^-$ cells (Fig. 4B), and adrenomedullin failed to activate p42/44 MAP kinase signaling in IGF-1R$^-$ cells (Fig. 4C). Analysis by densitometry of three separate experiments indicated that adrenomedullin exerted a 3.1-fold increase in p42/44 MAP kinase phosphorylation in IGF-1R$^+$ cells whereas p42/44 MAP kinase phosphorylation was not detectable in IGF-1R$^-$ cells treated with adrenomedullin. Collectively, these data suggest that the IGF-1 receptor is also required for the proliferative actions of adrenomedullin.

IGF-1-stimulated osteoblast proliferation is inhibited by blockade of the amylin receptor

The previous experiments indicate that a proliferative response of osteoblastic cells to amylin requires a functional IGF-1 receptor. We next determined whether inhibiting the osteoblast amylin receptor affected the mitogenic response to IGF-1. Addition of the amylin

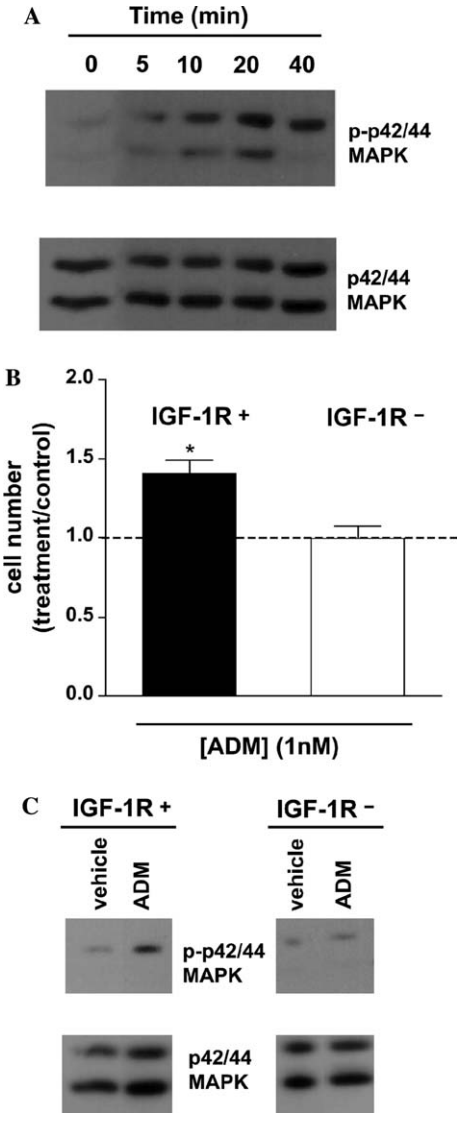

Fig. 4. Adrenomedullin activates cell proliferation and p42/44 MAP kinase signaling in an IGF-IR-dependent fashion. (A) Whole cell lysates of primary rat osteoblasts treated with 50 nM adrenomedullin for the indicated times were sequentially immunoblotted with antibodies to phosphorylated p42/44 MAP kinases (top panel) and total p42/44 MAP kinases (lower panel). (B) Fibroblastic cells expressing wild-type IGF-IR (IGF-IR$^+$) or lacking a functional IGF-IR (IGF-IR$^-$) were treated with 1 nM adrenomedullin and cell counts determined as described in Materials and methods. *, $p < 0.002$ vs untreated cells. (C) Whole cell lysates of IGF-IR$^+$ and IGF-IR$^-$ fibroblastic cells treated for 10 min with 50 nM adrenomedullin were sequentially immunoblotted with antibodies to phosphorylated p42/44 MAP kinases (top panel) and total p42/44 MAP kinases (lower panel).

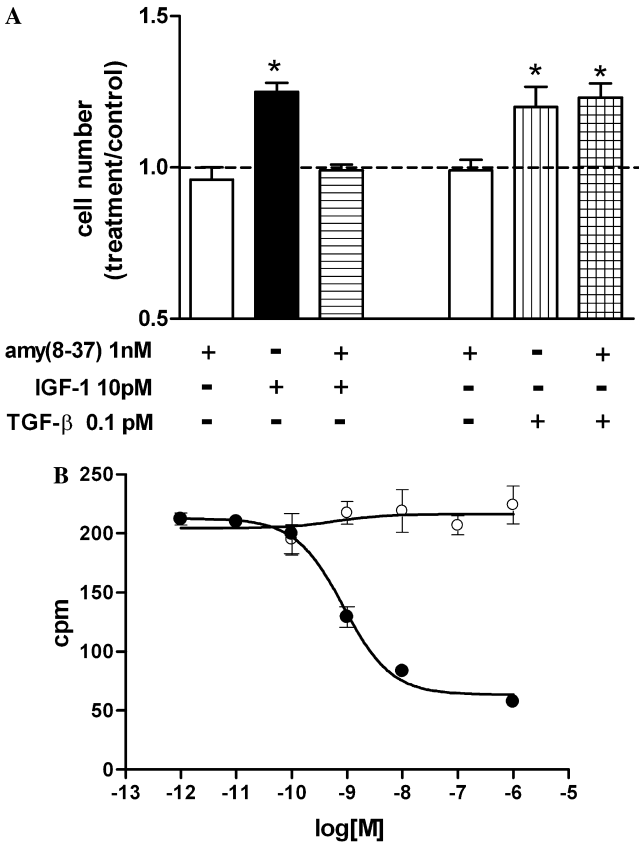

Fig. 5. IGF-1-stimulated osteoblast proliferation is inhibited by blockade of the amylin receptor. (A) Effects of amylin receptor blocker (amylin(8-37)) on IGF-1 and TGF-β-stimulated proliferation: *, significantly different from control, $p < 0.01$. (B) Competitive binding of ^{125}I-IGF-I and amylin-(8-37) to primary rat osteoblasts. Cellular membranes of primary osteoblasts were incubated with ^{125}I-IGF-I for 2 h at room temperature in the presence of increasing concentrations of unlabeled IGF-I (●) or amylin-(8-37) (○). The values are means ± SEM of triplicate determinations from a representative experiment. Similar results were observed in three separate experiments. The data were fitted to a single binding site model by nonlinear regression analysis.

receptor blocker, amylin-(8-37), inhibited IGF-1-stimulated osteoblast proliferation, but did not affect TGF-β-stimulated proliferation (Fig. 5A). We also observed that the adrenomedullin receptor blocker (reduced adrenomedullin) significantly inhibits the proliferative response of osteoblastic cells to IGF-1 (IGF-1 vs reduced adrenomedullin + IGF-1, means ± SEM; 1.21 ± 0.07 vs 1.10 ± 0.06, $p < 0.007$). These data suggest that the proliferative effect of IGF-1 in osteoblastic cells may involve the receptor(s) for amylin and adrenomedullin.

In light of the above findings, binding assays were conducted on cell membrane preparations from primary rat osteoblasts to determine whether amylin-(8-37) can competitively inhibit IGF-1 binding. We observed specific high affinity binding of ^{125}I-IGF-1 to primary rat osteoblast membranes with IC50 = 0.51 ± 0.34 nM. Addition of increasing concentrations of unlabeled amylin-(8-37) did not displace bound ^{125}I-IGF-1 (Fig. 5B). These results indicate that the inhibition by amylin-(8-37) of the mitogenic actions of IGF-1 in osteoblasts does not result from a direct competition for cellular binding sites.

Discussion

Adrenomedullin, amylin, and IGF-1 all promote the proliferation of osteoblast-like cells. The current

findings indicate that there is overlap in the mechanisms by which these three peptides stimulate osteoblast growth, and suggest that a functional IGF-1 receptor is required for the mitogenic actions of amylin and adrenomedullin. Thus, co-treatment of osteoblastic cells with amylin or adrenomedullin and IGF-1 does not produce additive effects on cell growth, and neutralization of the IGF-1 receptor inhibits the proliferative actions of both amylin and adrenomedullin. A member of the calcitonin family, calcitonin gene-related peptide, was previously reported to stimulate IGF-1 production by primary rat osteoblasts [15], so, we investigated whether the mitogenic actions of amylin or adrenomedullin in osteoblastic cells might be attributable to a paracrine action of osteoblast-derived IGF-1. However, a neutralizing antibody to IGF-1 did not inhibit the mitogenic actions of either amylin or adrenomedullin. Direct evidence for the involvement of the IGF-1 receptor in the mitogenic actions of amylin and adrenomedullin was provided by experiments in IGF-1 receptor-null fibroblastic cells, in which neither amylin nor adrenomedullin induced a proliferative response or activation of p42/44 MAP kinase signaling. Since our experiments were conducted in both osteoblasts and fibroblasts, the current data suggest a general requirement for the IGF-1 receptor in the mitogenic actions of amylin and adrenomedullin.

The amylin and adrenomedullin receptors are G-protein coupled receptors [16] and the IGF-1 receptor is a member of the tyrosine kinase receptor family [17]. The observation that the biological activities of peptides that signal through G-protein-coupled receptors (GPCR) may also require receptor tyrosine kinase activation has previously been reported for other GPCR agonists. Thrombin and angiotensin II bind to their own specific GPCRs, but are capable of inducing phosphorylation of the IGF-1 receptor and activation of downstream signaling molecules by activating cytoplasmic tyrosine kinases [18]. Neither thrombin nor angiotensin II binds to the IGF-1 receptor [18]. The IGF-1 receptor has been shown to directly interact with the $G_{\alpha i}$ subunit of the G-protein complex [19]. This "cross-talk" between GPCRs and tyrosine kinase receptors is a plausible explanation for the interactions we observed between the amylin/adrenomedullin receptors and IGF-1 receptor. We were unable to detect tyrosine phosphorylation of the IGF-1 receptor following treatment of osteoblastic cells with amylin (data not shown). Further investigation is required to determine the nature of the interaction between the actions of amylin/adrenomedullin and IGF-1 receptor signaling.

Surprisingly, peptide inhibitors of the amylin and adrenomedullin receptors also abrogated IGF-1-induced osteoblast proliferation. The explanation for this observation is not clear. It is not the result of an interaction between the inhibitory peptides and the IGF-1 receptor, because our binding studies indicated that the amylin receptor blocker does not inhibit binding of IGF-1 to osteoblastic cells. A non-specific action of the amylin/adrenomedullin receptor inhibitory peptides is not likely because they did not abrogate the mitogenic response to TGF-β. A possible explanation involves the formation of a cell-surface signaling complex that includes the IGF-1 receptor and components of the amylin/adrenomedullin receptor after liganding of either receptor. Interruption of the function of either receptor could thereby inhibit the biologic activity of ligands for either receptor. Further work is required to explore this possibility.

In summary, the current results suggest the existence of a novel interaction between the mitogenic signaling pathways activated by the peptide hormones amylin and adrenomedullin and the growth factor IGF-1. A functional IGF-1 receptor is required for amylin- and adrenomedullin-stimulated phosphorylation of p42/44 MAP kinases in, and proliferation of, osteoblasts and fibroblasts. In addition, the amylin and adrenomedullin receptors appear to be important for the IGF-1 mitogenic response in osteoblasts.

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