Atrial natriuretic factor receptors and stimulation of cyclic GMP formation in normal and malignant osteoblasts

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Synthetic rat atrial natriuretic factor (Ile-ANF-26) stimulated cyclic GMP formation by up to several hundred-fold in osteoblast-rich cultures from newborn rat calvaria and in clonal osteogenic sarcoma cells (UMR 106-01) which are phenotypically osteoblast. ANF had no effect on the cyclic AMP response to parathyroid hormone in the same cells. Specific, high-affinity binding sites for ANF were identified in both cell types, with K_d and receptor numbers in normal osteoblasts of $1.2 \pm 0.1 \times 10^{-10}$ M and $42 \pm 4 \times 10^3$ per cell, and in UMR 106-01 cells of $1.4 \pm 0.1 \times 10^{-10}$ M and $22 \pm 4 \times 10^3$ per cell.

Atrial natriuretic factor *cyclic GMP* *Osteoblast*

1. INTRODUCTION

Atrial natriuretic factor (ANF) is a potent peptide hormone made in the cells of the cardiac atrium, stored there in granules, and acting upon vascular smooth muscle and other targets to influence vascular tone and fluid and electrolyte balance [1–3]. ANF actions include inhibition of hormone-stimulated aldosterone secretion [4], direct effects on the kidney to promote sodium and other electrolyte excretion [1,5], and inhibition of vasopressin release [6]. The major vascular effect of vasodilatation and lowering of blood pressure results from the ability to relax vascular smooth muscle directly [7] and to suppress elevated plasma renin levels [8].

ANF treatment results in a substantial increase in cyclic GMP production in several target cells in which this has been investigated [9–15]. The nature of the response suggests that this effect is mediated through a cell surface receptor, and specific receptors have been identified in rat aortic smooth muscle cells [9] in aortic and renal membranes [9,16], and in adrenal glomerulosa membranes [17]. In pituitary cells ANF increases cyclic GMP production by several hundred-fold [15]. Although this was originally suggested to be associated with inhibition of CRF-stimulated adenylate cyclase and ACTH secretion in those cells [18], this has not been confirmed in a recent careful study [15]. Thus the significance of ANF action upon anterior pituitary cells remains unknown.

The present work identifies specific binding sites of high affinity for ANF in normal and malignant osteoblasts, and a major effect of the hormone on cyclic GMP formation in these cells. The magnitude and specificity of this response suggests that the hormone is likely to have effects on functions of these cells.

2. MATERIALS AND METHODS

2.1. *Cell culture*

Osteoblast-rich cultures were prepared from newborn rat calvaria using methods described in detail in [19]. In the late digests after collagenase treatment of rat calvaria, osteoblast-rich cultures were chosen as rich in alkaline phosphatase and parathyroid hormone (PTH)-responsive adenylate

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cyclase. UMR 106-01 cells are clonal osteogenic sarcoma cells which are phenotypically osteoblast. The line is a subclone of the UMR 106 line [20] and its properties have been reported [21]. Cells were cultured as previously described [20,21] in 5% fetal calf serum and Eagle's minimal essential medium (MEM).

2.2. *Cyclic nucleotide responses*

Cyclic nucleotide responses to hormones were measured in cells growing as replicates in 12-place multiwell dishes (Costar). At or near confluence (0.4 to 0.8 $\times$ 10^6/well), cells were washed and medium replaced with 0.5 ml Eagle's MEM containing 0.1% bovine serum albumin and 1 mM isobutylmethylxanthine (IBMX). After 20 min at 37°C, hormones or carriers were added as indicated and incubations continued for a further 10 min or otherwise as shown. Reactions were stopped by aspirating media, washing once with cold phosphate-buffered saline (PBS), and adding 1 ml of 95% ethanol-HCl, pH 3.0, at 4°C [12]. After 4–6 h the ethanol was removed, and the extracts were dried under N_2 and reconstituted in cyclic GMP or cyclic AMP assay buffer. Samples were acetylated before radioimmunoassay as described [22]. [^{125}I]Iodotyrosine methyl esters of succinylated cyclic GMP and cyclic AMP were purchased from the Radiochemical Center, Amersham, England. Antisera to cyclic GMP and cyclic AMP were kindly provided by Dr N.H. Hunt (Canberra, Australia); their cross-reactivities have been reported [23].

2.3. *Binding studies*

^{125}I-ANF was prepared free of unlabelled peptide as follows. 1 mCi Na^{125}I was added to 20 μl ANF (20 μg) in 250 μl phosphate buffer, pH 7.4. Chloramine T (50 μg) was added in 100 μl of the same buffer, the contents mixed for 10 s, followed by addition of Na metabisulphite (50 μg in 100 μl). Purification was carried out on a Sephadex C25 (1 $\times$ 12 cm) column in a gradient of Na carbonate, pH 9.8 (50 ml of 5 mM to 50 ml of 200 mM). Mono[^{125}I]iodo-ANF (600 μCi/μg) elutes as a peak before the peak of unlabelled ANF. Binding of radiolabelled ANF to intact cells was studied with confluent replicate cultures growing in 12-place multiwell dishes. Medium was removed from cultured cells and replaced with 0.4 ml Eagle's MEM containing 0.1% bovine serum albumin at 22°C. ^{125}I-ANF was added in 50 μl of the same medium (30–50000 cpm), followed by 50 μl with or without increasing amounts of unlabelled ANF or other test substances. After incubation at 22°C for 60 min or otherwise as indicated, medium was rapidly aspirated, cells washed twice with excess cold PBS, and the cell layers removed in 0.5 ml of 0.5 M NaOH for counting in a Packard automatic gamma counter with 70% efficiency for ^{125}I. Kinetic calculations were based on Scatchard analysis.

2.4. *Hormones and chemicals*

Fetal calf serum was purchased from Flow Laboratories, Australasia, and culture media from Medos Company, Australia. IBMX was obtained from Aldrich Chemical, Milwaukee, WI, USA. Synthetic rat atrial natriuretic factor (Ile-ANF-26) was kindly provided by Dr D.F. Veber, Merck, Sharp and Dohme, West Point, Philadelphia, USA. Synthetic human PTH(1–34) was obtained from Beckman (Palo Alto, CA), synthetic salmon calcitonin (SCT) was a gift from Dr R.C. Orlowski, Armour Pharmaceutical, Kankakee, IL, USA. Synthetic rat calcitonin gene-related peptide (CGRP) was purchased from Bachem (Torrance, CA, USA).

3. RESULTS

3.1. *Effects of ANF on cyclic nucleotide production*

In osteoblast-rich calvarial cells, ANF treatment resulted in a 200-fold increase in cyclic GMP production, whereas no effect was obtained with PTH, SCT or rat CGRP (table 1). ANF had no effect on cyclic AMP formation, which was increased 30-fold by PTH, as expected, and 4-fold by CGRP. This effect of the latter hormone in osteoblast-like cells is being investigated further. Although the calvarial cultures are osteoblast-rich, they clearly are heterogeneous, representing osteoblasts at different stages of function, as well as containing other, non-osteoblast cells [19]. For that reason experiments were carried out in clonal osteogenic sarcoma cells which are phenotypically osteoblast. In UMR 106-01 cells ANF was the only treatment of those tested which increased cyclic GMP formation, and a specific response several

Table 1

Cyclic AMP and cyclic GMP responses to PTH, rat CGRP and ANF in osteoblast-rich calvarial cells

	Cyclic AMP (pmol/10^6 cells)	Cyclic GMP (fmol/10^6 cells)
Basal	7.5 ± 0.6	14.0 ± 1.0
PTH (10^{-8} M)	241 ± 24	14.0 ± 0.9
rCGRP (3×10^{-8} M)	26.0 ± 2.3	13.2 ± 1.1
ANF (3×10^{-8} M)	6.7 ± 0.8	945 ± 168

Replicate cultures (0.6×10^6 cells/well) were treated as described in section 2. Means ± SE of triplicates

Table 2

Cyclic AMP and cyclic GMP responses to ANF, PTH and forskolin in UMR 106-01 cells

	Cyclic AMP (pmol/10^6 cells)	Cyclic GMP (fmol/10^6 cells)
Basal	2.6 ± 0.4	20.9 ± 4.5
ANF (2.3×10^{-8} M)	2.1 ± 0.1	18841 ± 3672
PTH (10^{-8} M)	235 ± 27	64.6 ± 35
PTH + ANF	253 ± 29	27101 ± 1836
Forskolin (10^{-6} M)	53.1 ± 1.4	25.8 ± 4.3
Forskolin + ANF	53.3 ± 4.0	32706 ± 4537

Replicate cultures (0.75×10^6 cells/well) were treated as described in section 2. Means ± SE of triplicates

hundred-fold in magnitude was obtained (table 2). Since it has been proposed that ANF inhibits cyclic AMP response to hormones in some target cells [18], this possibility was tested in the clonal osteogenic sarcoma cells using PTH as a cyclic AMP stimulus. No evidence was obtained for any effect of ANF on cyclic AMP responses to PTH in repeated experiments (table 2). The cyclic GMP response to ANF in UMR 106-01 cells was dose-dependent (fig.1) with half-maximum effectiveness obtained at $0.5–3 \times 10^{-9}$ M in repeated experiments. A similar dose relationship occurred with osteoblast-rich calvarial cells (not shown).

3.2. *ANF binding to intact cells*

^{125}I-labelled ANF bound specifically to both normal osteoblast-rich cultures and clonal malignant osteoblasts (fig.2). Binding was saturable,

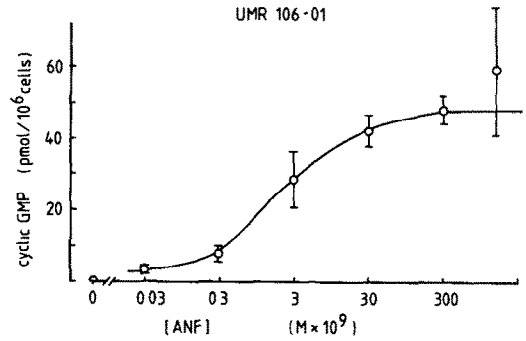

Fig.1. Effect of ANF on cyclic GMP formation in UMR 106-01 cells. Details in section 2. Points are means ± SE of triplicate measurements.

reaching a maximum after 50 min at 22°C (not shown), and no competition for binding was exerted by any of the following substances at concentrations of up to 1 μg/ml: PTH, SCT, rat CGRP, insulin, mouse epidermal growth factor, human transforming growth factor α, and human tumour necrosis factor α. Although established binding was found to be only poorly reversible (not shown), Scatchard analyses were carried out to obtain approximations of kinetic data. For osteoblast-rich cells the mean K_d in three experiments was $1.2 \pm 0.1 \times 10^{-10}$ M and receptor number $42 \pm 4 \times 10^3$ per cell. For UMR 106-01 cells K_d values were $1.4 \pm 0.1 \times 10^{-10}$ M and receptor number $22 \pm 4 \times 10^3$ per cell.

4. DISCUSSION

A new target cell response system is reported for ANF, in which osteoblast-like cells derived from newborn rat bones and from a characterized osteogenic sarcoma line have been found to show specific and substantial increases in cyclic GMP. Demonstration of this effect in calvarial osteoblast cultures alone would not be convincing evidence of an ANF effect on osteoblasts, since those cultures are heterogeneous. However, the finding in the clonal osteogenic sarcoma cells strongly suggests that some cells of the osteoblast series are a target for ANF action. The nature of ANF effect is obscure beyond the generation of cyclic GMP, but in the present experiments we have been able to demonstrate no effect on hormone-responsive cyclic AMP formation. Following the initial

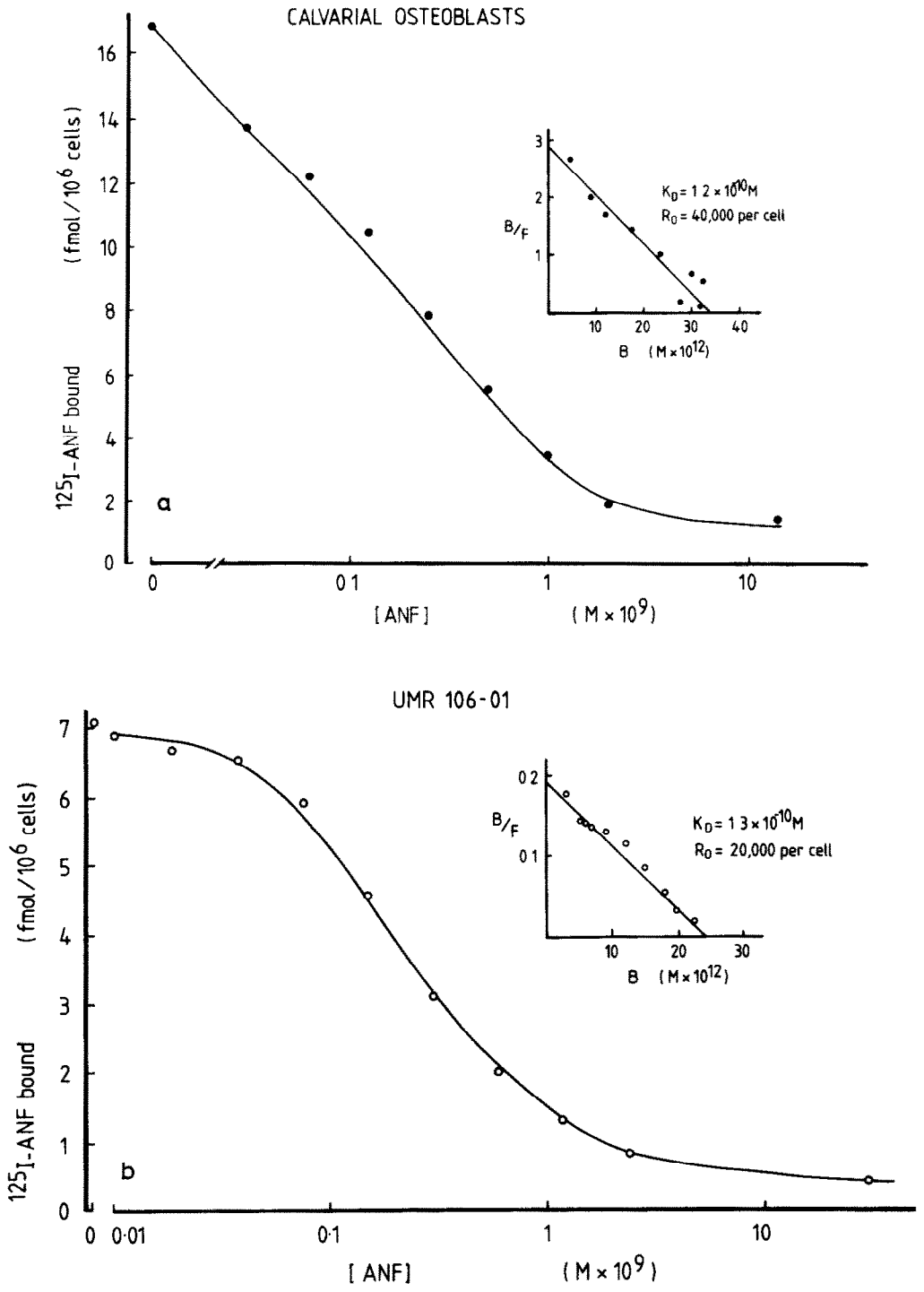

Fig.2. Specific binding of ^{125}I-ANF to intact calvarial osteoblasts (a) and to intact UMR 106-01 cells (b). Insets: Scatchard analysis of data. Details in section 2. (a) 25 fmol ^{125}I-ANF and 5×10^5 cells per incubation; (b) 20 fmol ^{125}I-ANF and 6×10^5 cells per incubation.

demonstration that ANF inhibited hormone-responsive adenylate cyclase in pituitary cells [18] there have been no further data to support this [15].

ANF is the most effective known hormonal promoter of cyclic GMP formation in cells. The increase in cyclic GMP formation in vascular smooth muscle cells [9,12] is probably related to the relaxant action of ANF on those cells, and implies a mediatory role for cyclic GMP in vascular smooth muscle relaxation, perhaps more important than that of cyclic AMP [12]. Generally, however, there is little available information on the intracellular effects of elevated cyclic GMP and cyclic GMP-dependent phosphorylation [24]. Especially in bone there is no hint of what role cyclic GMP might have. Early experiments with bone cell membranes [25] and with osteogenic sarcoma cells [26] failed to uncover any stimulus to elevated cyclic GMP formation. The present observation points to the possibility that cyclic GMP might mediate some specific function(s) of osteoblasts, especially since such a very large increase in cyclic GMP formation follows ANF treatment. It also indicates that ANF effects might be more pleiotropic than previously supposed, and we should seek a function for the hormone in bone metabolism.

Although bone is a substantial store of sodium it should not be assumed that ANF effects on bone cells resemble those in, for example, renal cells. By analogy with cyclic AMP, the nature of the cyclic GMP effect could be determined by the differentiated properties of the osteoblast target. Thus it will be important to determine what are the substrates for cyclic GMP-dependent protein phosphorylation in osteoblasts, and what effects ANF has on major processes in bone, such as bone formation, bone resorption, and the coupling between the two.

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