Atrial natriuretic factor and sodium nitroprusside increase cyclic GMP in cultured rat lung fibroblasts by activating different forms of guanylate cyclase

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We used cultured rat lung fibroblasts to evaluate the role of particulate and soluble guanylate cyclase in the atrial natriuretic factor (ANF)-induced stimulation of cyclic GMP. ANF receptors were identified by binding of ^{125}I -ANF to confluent cells at 37 °C. Specific ANF binding was rapid and saturable with increasing concentrations of ANF. The equilibrium dissociation constant (K_D) was 0.66 ± 0.077 nM and the B_{max} was 216 ± 33 fmol bound/ 10^6 cells, which corresponds to 130000 ± 20000 sites/cell. The molecular characteristics of ANF binding sites were examined by affinity cross-linking of ^{125}I -ANF to intact cells with disuccinimidyl suberate. ANF specifically labelled two sites with molecular sizes of 66 and 130 kDa, which we have identified in other cultured cells. ANF and sodium nitroprusside produced a time- and concentration-dependent increase in intracellular cyclic GMP. An increase in cyclic GMP by ANF was detected at 1 nM, and at 100 nM an approx. 100-fold increase in cyclic GMP was observed. Nitroprusside stimulated cyclic GMP at 10 nM and at 1 mM a 500–600-fold increase in cyclic GMP occurred. The simultaneous addition of 100 nM-ANF and 10 μM -nitroprusside to cells resulted in cyclic GMP levels that were additive. ANF increased the activity of particulate guanylate cyclase by about 10-fold, but had no effect on soluble guanylate cyclase. In contrast, nitroprusside did not alter the activity of particulate guanylate cyclase, but increased the activity of soluble guanylate cyclase by 17-fold. These results demonstrate that rat lung fibroblasts contain ANF receptors and suggest that the ANF-induced stimulation of cyclic GMP is mediated entirely by particulate guanylate cyclase.

INTRODUCTION

Extracts and purified peptides derived from mammalian atrial tissue produce natriuresis and diuresis *in vivo* (Cantin & Genest, 1985; Laragh, 1985; Ballermann & Brenner, 1985; Needleman & Greenwald, 1986). Because of their renal effects these peptides have been called atrial natriuretic factors (ANF). However, recently it has become clear that ANF also acts on tissues other than the kidney to regulate blood pressure and intravascular volume. ANF has been shown to produce vascular smooth muscle relaxation (Winkvist *et al.*, 1984), decrease cardiac output (Volpe *et al.*, 1986) and inhibit aldosterone (De Lean *et al.*, 1984), renin (Burnett *et al.*, 1984; Kurtz *et al.*, 1986) and vasopressin (Samson, 1985) secretion. In addition, ANF has been shown to inhibit water and saline intake after central administration of ANF (Antunes-Rodrigues *et al.*, 1985, 1986). The relative contribution of each of these effects of ANF to the overall role of ANF in blood pressure and volume homeostasis is unclear.

Specific receptors for ANF have been identified in membranes from aorta (Napier *et al.*, 1984), adrenal cortex (De Lean *et al.*, 1984) and brain (Quirion *et al.*, 1986) and in cultured smooth muscle (Hirata *et al.*, 1984; Leitman *et al.*, 1985; Schenk *et al.*, 1985), endothelial (Schenk *et al.*, 1985; Leitman & Murad, 1986) and

kidney (Ballermann *et al.*, 1985) cells. Binding of ANF to its receptors is associated with increased activity of particulate guanylate cyclase (Winkvist *et al.*, 1984; Waldman *et al.*, 1984, 1985; Tremblay *et al.*, 1985), elevated cyclic GMP (Winkvist *et al.*, 1984; for review see Leitman & Murad, 1987) and in some tissues an inhibition of adenylate cyclase activity (Anand-Srivastava *et al.*, 1984). Our laboratory has been using ANF as a pharmacological probe to elucidate the mechanism whereby hormones and other agents activate guanylate cyclase and increase intracellular cyclic GMP. Waldman and coworkers previously showed that ANF activated the particulate form of guanylate cyclase, but not the soluble form in a number of rat tissues (Winkvist *et al.*, 1984; Waldman *et al.*, 1984, 1985). However, because it has not been possible to activate the soluble enzyme by hormones or neurotransmitters in cell-free systems, ANF may also elevate cyclic GMP in intact cells by activating the soluble enzyme. To explore further the role of particulate and soluble guanylate cyclase in the ANF-induced stimulation of cyclic GMP, we have compared the effects of ANF and sodium nitroprusside on cyclic GMP accumulation and guanylate cyclase activity in cultured rat lung fibroblasts. In this study, we have identified ANF receptors in cultured rat lung fibroblasts. Furthermore, our results suggest that the increase in cyclic GMP by ANF is mediated entirely by particulate

Abbreviations used: ANF, atrial natriuretic factor; DMEM, Dulbecco's modified Eagle's medium; HBSS, Hanks' balanced salt solution; DSS, disuccinimidyl suberate; PAGE, polyacrylamide-gel electrophoresis.

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guanylate cyclase, whereas sodium nitroprusside increases cyclic GMP by activating only the soluble form of guanylate cyclase.

EXPERIMENTAL

Materials

Dulbecco's modified Eagle's medium (DMEM), Ham's F-12 medium, Hanks' balanced salt solution (HBSS), penicillin/streptomycin and glutamine were purchased from Grand Island Biological Co. Iodo-gen and DSS were obtained from Pierce. Rat ANF-(101-126) was from Peninsula Laboratories, Inc. Fetal calf serum was from Sterile Systems Inc. Rat lung fibroblasts (A.T.C.C. CCL-192) were obtained from the American Type Cell Culture. Sep-Pak C_{18} cartridges were from Millipore. All other reagents were obtained as previously described (Leitman *et al.*, 1985; Leitman & Murad, 1986).

Cell culture

Rat lung fibroblasts were maintained and subcultured in Ham's F-12 medium containing 10% fetal calf serum, 50 units of penicillin and 50 μ g of streptomycin/ml, and 584 μ g of glutamine/ml. The cells were grown with 5% CO_2 in a humidified incubator maintained at 37 °C.

^{125}I -ANF binding assay

ANF was iodinated by adding 0.2 nmol of ANF to two microfuge tubes coated with Iodo-gen (Salacinski *et al.*, 1981). Then 1 mCi of $Na^{125}I$ was added to each tube and the samples were incubated for 15 min at room temperature. Samples from both tubes were pooled and added to a Sep-Pak C_{18} cartridge to remove unbound radioactive iodide. The specific radioactivity, which ranged from 800 to 1300 Ci/mmol, was determined by self-displacement of unlabelled ANF in a binding assay using rat lung membranes.

Lung fibroblasts were grown to confluence in 24-well plates. The cells were washed 2 times with 1 ml of serum-free DMEM containing 10 mM-Hepes, pH 7.3. The cells were incubated for 20 min at 37 °C with various concentrations of ^{125}I -ANF. The cells were then rapidly washed four times with 1 ml of ice-cold HBSS containing 2 mg of bovine serum albumin/ml. The cells were solubilized with 1 ml of 1 M-NaOH and the amount of radioactivity was determined. Nonspecific binding was determined by the addition of 1 μ M-ANF to parallel dishes.

Cyclic GMP determination

Confluent fibroblasts in 35 mm culture dishes were washed twice with 2 ml of DMEM and then preincubated for 10 min at 37 °C in 990 μ l of DMEM containing 10 mM-Hepes and 0.5 mM-isobutylmethylxanthine. ANF or sodium nitroprusside (10 μ l) was added to the cells for various times. The medium was aspirated and then 750 μ l of ice-cold 6% (w/v) trichloroacetic acid was added to the cells. The intracellular levels of cyclic GMP were measured by radioimmunoassay as previously described (Leitman *et al.*, 1985; Leitman & Murad, 1986).

Guanylate cyclase assay

Confluent rat lung fibroblasts were washed and scraped off 150 mm culture dishes into ice-cold 50 mM-

Tris/HCl, pH 7.4, containing 150 mM-NaCl. The cells were centrifuged at 500 *g* for 5 min. The cell pellet was resuspended in 0.5 ml of 50 mM-Tris/HCl, pH 7.6, containing 1 mM-EDTA, 0.5 mM-phenylmethanesulphonyl fluoride and 0.01% bacitracin (buffer A) and then homogenized with a Polytron. The homogenate was centrifuged at 100 000 *g* for 1 h at 4 °C. The supernatant fraction was removed and assayed for soluble guanylate cyclase activity. In order to remove soluble guanylate cyclase from the particulate fraction the pellet was resuspended and homogenized twice in 1 ml of buffer A containing 100 mM-KCl and centrifuged at 100 000 *g* for 1 h. Finally, the pellet was homogenized in buffer A and centrifuged at 100 000 *g* for 1 h at 4 °C. The pellet was resuspended in 0.5 ml of 50 mM-Tris/HCl, pH 7.6, and assayed for particulate guanylate cyclase activity.

A 10 μ l aliquot of either the particulate or soluble fraction was added to 100 μ l of reaction mixture containing (final concentration) 50 mM-Tris/HCl, pH 7.6, 4 mM-MgCl₂, 1 mM-GTP, 1 mM-isobutylmethylxanthine, 0.01% bacitracin and a GTP-regenerating system comprising 15 mM-phosphocreatine and 20 μ g of creatine kinase. After incubation for various times at 37 °C the reaction was terminated by the addition of 900 μ l of 50 mM-sodium acetate, pH 4.0, and the samples were boiled for 3 min. The samples were acetylated and amount of cyclic GMP formed was measured by radioimmunoassay.

Affinity cross-linking

Confluent rat lung fibroblasts in 35 mm dishes were washed three times with 2 ml of HBSS containing 10 mM-Hepes, pH 7.3. The cells were incubated in 1 ml of HBSS containing 2 nM- ^{125}I -ANF for 1 h at room temperature. Then 1 ml of HBSS containing 0.4 mM-DSS (in dimethyl sulphoxide) was then added to the cells. After a 30 min incubation, the medium was aspirated, cells were washed with 2 ml of HBSS, 400 μ l of SDS sample buffer [62.5 mM-Tris/HCl, pH 6.8, 10% (v/v) glycerol and 2.3% (w/v) SDS] was added to the cells and the plates were placed in boiling water for 3 min. The samples were divided into two portions and 2-mercaptoethanol (5%, v/v) was added to one portion. The samples were boiled and then subjected to SDS/PAGE on a 7.5% separating gel (Laemmli, 1970). After electrophoresis, the gels were dried, stained for protein and then exposed to Dupont Cronex X-ray film at -70 °C.

RESULTS

ANF binding characteristics to rat lung fibroblasts

Fig. 1(a) shows the time course of ^{125}I -ANF binding to rat lung fibroblast cells at 37 °C. Binding of ANF was rapid and the maximal binding was reached at 20-30 min. Thereafter, the binding remained relatively constant until 40 min and then it began to decline. After 60 min the specific binding was approx. one-half of the binding that occurred at 30 min, suggesting that the fibroblasts were rapidly degrading ANF. The saturation curve shows that specific binding of ANF was saturable with increasing concentrations of ANF (Fig. 1b). A Scatchard plot of the binding data was linear, suggesting that rat lung fibroblasts contain a single class of ANF receptors (Fig. 1c). The equilibrium dissociation constant from four experiments was 0.66 ± 0.077 nM and the B_{max} was

Fig. 1. $^{125}\text{I-ANF}$ binding to confluent rat lung fibroblasts

(a) Time-course of specific ANF binding to fibroblasts. Confluent cells were incubated with 500 pM- $^{125}\text{I-ANF}$ for various times at 37 °C. (b) Saturation curve of ANF binding to fibroblasts. Confluent cells were incubated with increasing concentrations of $^{125}\text{I-ANF}$ for 20 min in the absence or presence of 1 μM unlabelled ANF. Total (●), specific (○), and nonspecific (▲) binding were determined as described in the Experimental section. (c) Scatchard plot of the equilibrium binding data. Each point represents the mean of triplicate samples. The s.e.m. of the triplicate samples was less than 10%. The data presented from one experiment are representative of four experiments with similar results. In (c), B_{max} = 216 fmol/10⁶ cells and K_D = 0.6 nM.

216 ± 33 fmol bound/10⁶ cells, which corresponds to 130000 ± 20000 sites/cell.

Molecular characteristics of ANF receptors in rat lung fibroblasts

The molecular characteristics of the ANF binding sites in rat lung fibroblasts were examined by covalently cross-linking $^{125}\text{I-ANF}$ to its receptors with DSS. Fig. 2(a) shows that under non-reducing conditions of SDS/PAGE two sites were specifically labelled. Approx. 70% of the binding sites had a molecular size of 66 kDa, while the remaining sites were of 130 kDa. In the presence of β -mercaptoethanol most of the 130 kDa sites were converted to 66 kDa (Fig. 2b). However, a low-abundance ANF-binding site of 130 kDa was still visible on the autoradiograms. Under the conditions used here, 66% of the total ANF receptors are cross-linked with $^{125}\text{I-ANF}$ (results not shown).

ANF and sodium nitroprusside increase cyclic GMP accumulation in rat lung fibroblasts

ANF produced a rapid rise in cyclic GMP by 1 min and a maximal 100-fold increase in cyclic GMP occurred at 5 min in the presence of isobutylmethylxanthine (Fig. 3a). The elevated levels of cyclic GMP were maintained for at least an additional 10 min. A 5-fold increase in cyclic GMP was detected at an ANF concentration of 1 nM and a 100-fold increase was observed at 100 nM (Fig. 3b). Sodium nitroprusside produced about a 350-fold increase in cyclic GMP by 1 min (Fig. 3a) and the maximal response occurred at 10 min. A 2-fold increase in cyclic GMP accumulation was observed at 10 nM, and 1 mM-sodium nitroprusside produced a 500–600-fold increase in cyclic GMP (Fig. 3c). The addition of sodium nitroprusside and a maximally effective concentration of ANF to rat lung fibroblasts resulted in levels of cyclic GMP that were additive (Table 1). This observation suggests that ANF and sodium nitroprusside were increasing different pools of cyclic GMP by activating different isoenzyme forms of guanylate cyclase.

Effect of ANF and sodium nitroprusside on particulate and soluble guanylate cyclase activity

Fig. 4(a) shows that 100 nM-ANF increased the activity of the particulate enzyme by 10-fold after 1 min. An increase in particulate guanylate cyclase activity by ANF was observed over a 15 min time course. The activity of the soluble enzyme was not altered by ANF (Fig. 4b). In contrast, sodium nitroprusside did not alter the activity of the particulate enzyme (Fig. 4a), but produced a maximal 17-fold activation of soluble guanylate cyclase (Fig. 4b).

DISCUSSION

ANF acts on a variety of tissues to elicit diverse physiological effects that serve to maintain blood pressure and intravascular volume. The precise cellular actions of ANF that mediate these effects are not known. In this study we demonstrated that rat lung fibroblasts possess ANF receptors that are coupled to guanylate cyclase and increased cyclic GMP formation. The lung fibroblast ANF receptors have a K_D of 0.66 nM. Surprisingly, rat lung fibroblasts have 130000 sites/cell, which is comparable with or greater than the number of ANF receptors reported for vascular (Hirata *et al.*, 1984; Leitman *et al.*, 1985; Schenk *et al.*, 1985; Leitman & Murad, 1986), adrenal cortical (D. C. Leitman & F. Murad, unpublished work) and kidney (Ballermann *et al.*, 1985) cells. At the present time these are thought to be the major target tissues for ANF.

Two forms of guanylate cyclase exist in most cells, and have different physicochemical properties (Murad, 1986). The membrane-bound form, known as the particulate enzyme, is composed of one peptide with a molecular size of 120–130 kDa (Kuno *et al.*, 1986), while the cytosolic or soluble form is composed of two subunits with molecular sizes of 70 and 82 kDa (Kamizaki *et al.*, 1986). The reason for the existence of two isoenzymes for guanylate cyclase is not clear. One potential advantage of having two isoenzymes is that the activation of each isoenzyme by various agents may lead to the elevation of

Fig. 2. Autoradiogram of SDS/PAGE of rat lung fibroblasts cross-linked with ^{125}I -ANF

Confluent cells were incubated for 60 min with 2 nM- ^{125}I -ANF in the absence (-) and presence (+) of unlabelled 1 μM -ANF. Samples were covalently cross-linked with 0.2 mM-DSS for 30 min. One-half of the sample was electrophoresed under non-reducing conditions (a), while the remaining portion was reduced with 2-mercaptoethanol prior to SDS/PAGE (b).

Table 1. Additive effect of ANF and sodium nitroprusside on cyclic GMP accumulation in rat lung fibroblast cells

Confluent rat lung fibroblasts were incubated for 10 min in the presence of either 10 μM -nitroprusside, 100 nM-ANF or the combination of both agents. The intracellular cyclic GMP levels were measured by radioimmunoassay as described in the Experimental section. Each value represents the mean $\pm$ S.E.M. from triplicate culture dishes from three different experiments.

Addition	Cyclic GMP (pmol/ 10^6 cells)
Control	0.246 ± 0.013
Nitroprusside (10 μM)	36.7 ± 3.1
ANF (100 nM)	41.6 ± 1.4
Nitroprusside + ANF	74.7 ± 2.8

different intracellular pools of cyclic GMP, which may mediate different cellular responses.

In the present study, we demonstrated that both ANF and sodium nitroprusside produce a large increase in intracellular cyclic GMP in rat lung fibroblasts. However, the increased cyclic GMP accumulation was clearly mediated by different isoenzymes of guanylate cyclase, since ANF activated only the particulate enzyme, while sodium nitroprusside activated only the soluble enzyme. When fibroblasts were exposed to ANF and sodium nitroprusside simultaneously there was an additive increase in cyclic GMP levels. These results suggest that ANF increases cyclic GMP formation in intact cells by activating only the particulate isoenzyme. Our findings also demonstrate that ANF and sodium nitroprusside

Fig. 3. Effect of ANF and sodium nitroprusside on cyclic GMP accumulation in rat lung fibroblasts

(a) Time course of ANF (○) and sodium nitroprusside (●)-induced stimulation of cyclic GMP accumulation. Confluent cells were incubated with either 100 nM-ANF or 1 mM-nitroprusside for various times at 37 °C. Concentration-response curves for ANF (b) and nitroprusside (c) on cyclic GMP accumulation. Cell cultures were incubated for 10 min at 37 °C and the intracellular cyclic GMP levels were measured by radioimmunoassay. Basal cyclic GMP levels were 213, 270 and 370 fmol/ 10^6 cells in (a), (b) and (c) respectively. Each point represents the mean of triplicate dishes. Data represent one experiment that was performed three times with similar results. S.E.M. values between triplicate samples were less than 10% of the mean.

Fig. 4. Effect of ANF and sodium nitroprusside on particulate and soluble guanylate cyclase activity in rat lung fibroblasts

Time course of the basal ($\blacktriangle$), and effect of ANF ($\circ$) and nitroprusside ($\bullet$) on particulate (a) and soluble (b) guanylate cyclase activity. Particulate and soluble fractions were exposed to either 100 nM-ANF or 100 μ M-nitroprusside for various times at 37 °C. The amount of cyclic GMP formed was measured by radioimmunoassay. Each point represents the mean of triplicate samples. Data represent values obtained in one experiment that was performed twice with similar results. S.E.M. values between triplicate samples were less than 5% of the mean.

increase two different pools of cyclic GMP in rat lung fibroblasts, establishing that these cells are an appropriate model to study whether these different pools of cyclic GMP promote different physiological responses.

We recently reported that, despite a linear Scatchard plot, cultured endothelial cells contain two ANF-binding sites, with molecular sizes of 66 and 130 kDa (Leitman & Murad, 1986; Leitman *et al.*, 1986). By comparing the ability of atrial natriuretic peptides to compete for these

two sites and elevate cyclic GMP, we have concluded that the 130 kDa site is the ANF receptor that is coupled to guanylate cyclase and mediates the increased cyclic GMP formation in endothelial cells (Leitman *et al.*, 1986). Rat lung fibroblasts also contain these two binding sites with a similar proportion as that found in endothelial (Leitman *et al.*, 1986) and smooth muscle and adrenal cortical (D. C. Leitman & F. Murad, unpublished work) cells. The recent discovery by Kuno *et al.* (1986) that particulate guanylate cyclase from rat lung tissue co-purifies with an ANF-binding site that has a molecular size of 120–130 kDa suggests that one ANF receptor subtype and particulate guanylate cyclase may reside in the same transmembrane glycoprotein complex. This observation also suggests that the 130 kDa ANF-binding site present in cultured rat lung fibroblasts is part of the particulate guanylate cyclase complex. However, further studies are needed to confirm this proposal.

Other investigators have used cross-linking techniques to characterize ANF receptors in a variety of tissues. It has been reported that rabbit aorta possess three ANF-binding sites with molecular sizes of 60, 70 and 120 kDa (Vandlen *et al.*, 1985), while the adrenal gland has two sites of 68 and 114 kDa (Meloche *et al.*, 1986). These findings are consistent with our studies suggesting that there are multiple ANF receptors in fibroblasts and other cell types. Our previous studies (Leitman *et al.*, 1986) provided evidence that cyclic GMP is the intracellular mediator of the 130 kDa ANF receptor and suggest that this receptor mediates the vascular relaxant and hypotensive effects of ANF. It has also been reported that ANF inhibits adenylate cyclase activity in a number of tissues (Anand-Srivastava *et al.*, 1984) and the accumulation of inositol monophosphate in response to noradrenaline in rat aorta (Rapoport, 1986). However, the ANF receptor subtype that mediates these effects and the effects of ANF on phosphoinositol metabolism and calcium mobilization have not been resolved (for review, see Leitman & Murad, 1987). Clearly, the development of specific ANF agonists and antagonists will be valuable probes to identify the intracellular messengers and physiological functions of these two ANF receptors.

The identification of a large number of receptors with high affinity in fibroblasts makes previous ANF binding studies with heterogenous tissues more difficult to interpret, since significant quantities of the ANF receptors in the tissues reported may have been located on the fibroblast cells. Furthermore, the contribution of the fibroblasts to the reported effects of ANF on cyclic GMP and guanylate cyclase activity is not known. In future studies it will be important to use homogenous populations of cells from tissues in order to determine the precise cell type that contains the ANF receptors and responses.

The role and significance of ANF receptors in rat lung fibroblasts is not known. We have also found specific, high-affinity ANF receptors that mediate an increase in cyclic GMP accumulation in cultured human fetal and adult lung fibroblasts (D. C. Leitman & F. Murad, unpublished work). It has been recently reported that lung tissue contains approx. 50 times more ANF than that found in the plasma (Sakamoto *et al.*, 1986). Our results, demonstrating that ANF binding declines dramatically with time in lung fibroblasts, suggest that these cells rapidly degrade ANF. Indeed, we find that radiolabelled ANF in the medium from cells incubated for 20 min has

a marked decreased binding when applied to fresh fibroblast cultures (D. C. Leitman & F. Murad, unpublished work). Since the lung is the first tissue with which ANF interacts after it has been secreted from the right atria, it may have a role in the processing of the ANF prohormone or metabolism of the active ANF peptide.

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