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Brain natriuretic factor

Augmentation of cellular cyclic GMP, activation of particulate guanylate cyclase and receptor binding

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The newly discovered peptide, brain natriuretic factor (BNF), caused a concentration-dependent increase (up to 400-fold) in intracellular cyclic GMP levels in cultured endothelial, smooth muscle and fibroblast cells. The extent of cGMP augmentation was comparable to that produced by atrial natriuretic factor (ANF). The activity of the membrane-bound guanylate cyclase of different rat tissues and cultured cells was markedly stimulated by the peptide and the addition of ATP potentiated the stimulation. As opposed to tissue particulate guanylate cyclase, the enzyme in cell membranes was slightly more sensitive to activation by BNF than to stimulation by ANF. On bovine aortic smooth muscle (BASM) cells, specific high-affinity binding sites (B_{max} = 398 fmol/10^6 cells, K_d = 0.52 nM) for BNF were observed for which ANF could compete with apparently equal affinity. These results suggest that activation of the cGMP pathway constitutes a common mechanism of action for both BNF and ANF.

Brain natriuretic factor; Atrial natriuretic factor; cyclic GMP; Guanylate cyclase; Receptor binding

1. INTRODUCTION

Many studies have demonstrated that atrial natriuretic factor (ANF) plays an important role in the regulation of blood pressure, electrolyte homeostasis, endocrine and other physiological functions [1]. The effects of ANF may be mediated by an increase in cellular cyclic guanosine monophosphate (cGMP) levels through the activation of particulate guanylate cyclase [2,3].

Recently, a new biologically active peptide has been discovered in porcine brain [4] which, in addition to a potent smooth muscle (chicken rectum) relaxing activity, can also produce hypotension, an increased diuresis and natriuresis in vivo. This new peptide, called brain natriuretic factor (BNF), shows a high degree of amino acid sequence homology (70%) to ANF. In order to examine the possibility that the biological actions of BNF may also be mediated by cGMP, we studied the influence of this new peptide on intracellular cGMP levels and the activity of guanylate cyclase as well as its binding properties in various tissues and cultured cells.

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2. MATERIALS AND METHODS

2.1. *Materials*

Synthetic porcine BNF, rat ANF(101–126), and atriopeptin I were obtained from Peninsula Labs (Belmont, CA). Radioiodinated peptides were prepared using the iodogen method [5], and purified by reversed-phase chromatography. Iodogen was purchased from Pierce (Rockford, IL). Rat lung fibroblasts (CCL192) were obtained from the American Type Cell Collection (Rockville, MD). Bovine aortic smooth muscle (BASM) and bovine aortic endothelial (BAE) cells were prepared as described [6]. Cells were grown and maintained in

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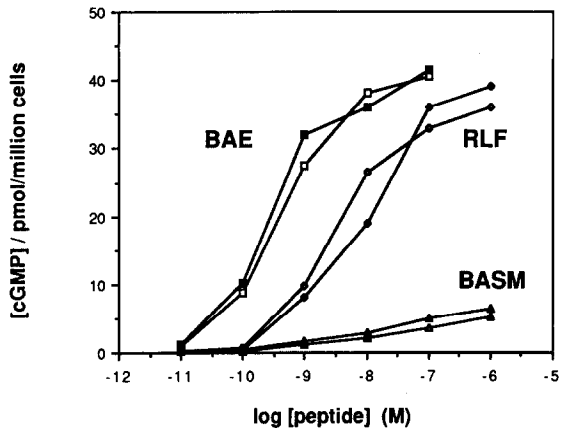


Fig.1. Augmentation of intracellular concentrations of cGMP induced by BNF (closed symbols) or ANF (open symbols) in bovine aortic endothelial (BAE, squares), rat lung fibroblast (RLF, diamonds) and bovine aortic smooth muscle (BASM, triangles) cells. Cells were incubated for 5 min with the indicated peptide concentrations in the presence of 0.5 mM isobutylmethylxanthine. Determination of cellular cGMP concentrations was performed as described in section 2.

culture using the same media and incubation conditions as in [7].

2.2. *Preparation of membranes from tissues and cultured cells*

Female Sprague-Dawley rats (body wt 250–300 g) were decapitated; tissues were obtained and homogenized at 4°C in 4 vols of 50 mM Tris containing 1 mM EDTA, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride and 20 μg/ml aprotinin (pH 7.4). For homogenization of cultured cells, this solution also contained 250 mM sucrose. The homogenates were centrifuged at 100000 × *g* for 60 min, particulate fractions being washed twice and resuspended in the same buffer.

2.3. *Cellular cGMP determination*

Cells grown to confluence in multiwell plates were washed twice with serum-free medium containing 10 mM Hepes, pH 7.3, and preincubated at 37°C for 10 min with the same buffer containing 0.5 mM isobutylmethylxanthine (IBMX). Various concentrations of ANF or BNF were added to the medium and cultures were incubated for a further 5 min. The medium was aspirated and cold 6% trichloroacetic acid was added to the plates. The amount of cGMP in the trichloroacetic acid extracts was determined by radioimmunoassay as in [6–9].

2.4. *Guanylate cyclase assay*

The activity of guanylate cyclase in washed particulate fractions was determined at 37°C in 50 mM Tris-HCl (pH 7.6) containing 1 mM GTP, 4 mM $MgCl_2$, a GTP-regenerating system (3.75 mM creatine phosphate, 10 U/ml creatine phosphokinase), and 0.5 mM IBMX. The reaction was stopped by the addition of ice-cold sodium acetate buffer (50 mM, pH 4.0) followed by immersion for 3 min in a boiling water bath. cGMP generated was quantified by radioimmunoassay.

2.5. *Binding studies*

Confluent cells were washed twice with serum-free Dulbecco's modified Eagle's medium and then incubated for 20 min at 37°C with medium containing 10 mM Hepes, pH 7.3, 1 mg/ml bovine serum albumin and the indicated concentrations of ^{125}I-BNF or ^{125}I-ANF (spec. act. 1600–1800 Ci/mmol). Following 5 washing steps with Hank's balanced salt solution containing 1 mg/ml bovine serum albumin, the cells were solubilized with 1 M NaOH and the radioactivity was determined. Non-specific binding was determined by incubating parallel culture dishes with ^{125}I-labeled peptides in the presence of 1 μM unlabeled peptides. All values presented are the means of duplicate or triplicate incubations from representative experiments that were performed two or three times.

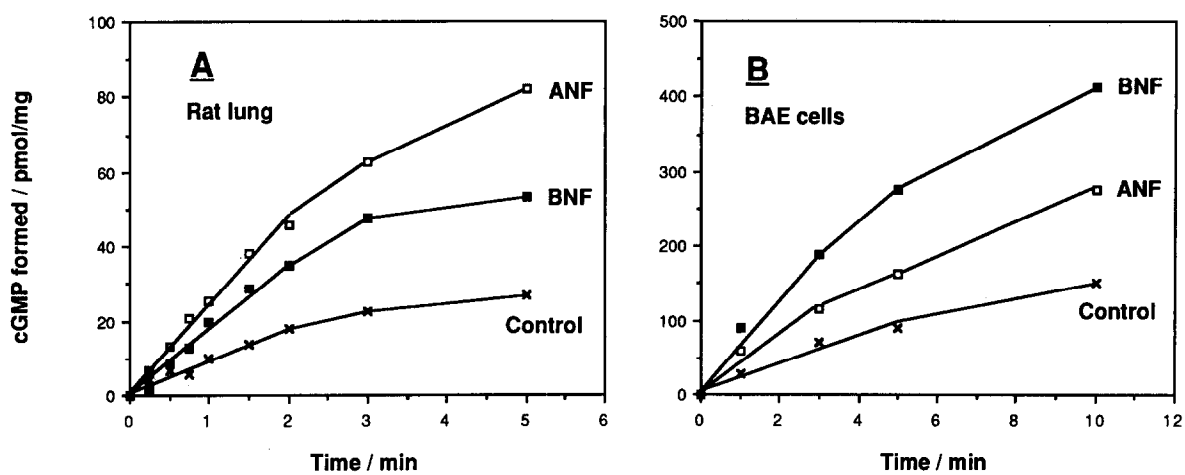


Fig.2. Time course of cGMP formation by rat lung (A) and bovine aortic endothelial cell (B) membrane-bound guanylate cyclase. Enzyme activity was monitored at 37°C in the absence (×) or presence of 1 μM each of BNF (closed symbols), or ANF (open symbols), as described in section 2.

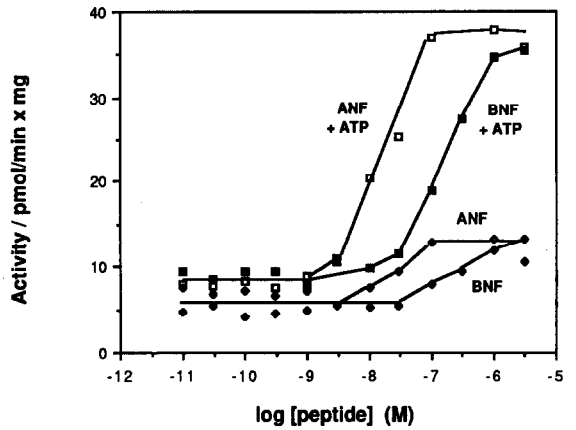


Fig.3. Stimulation of rat lung particulate guanylate cyclase activity by BNF (closed symbols) in comparison to ANF (open symbols). Peptides were added to the reaction mixtures for the enzyme assay at the indicated concentrations. Determination of enzyme activity was performed at 37°C in the absence (diamonds) or presence (squares) of 0.1 mM ATP.

3. RESULTS

The effect of BNF on the accumulation of intracellular cGMP was investigated using bovine aortic smooth muscle (BASM), bovine aortic endothelial (BAE), and rat lung fibroblast (RLF) cells. As shown in fig.1, incubation with BNF elevated the cGMP concentration in all cell lines in a dose-dependent fashion. The maximal increase in intracellular cGMP concentrations by BNF was found to be similar in BAE and RLF cells (~360- and 400-fold, respectively), while a lower response (~50-fold) was observed in BASM cells. In the latter cells, maximum stimulation apparently could not be reached up to the highest concentration of peptide examined. The effects of BNF and ANF on cGMP accumulation were comparable in each of the cell lines examined (fig.1).

In order to examine and compare the effects of BNF and ANF on particulate guanylate cyclase activity, membrane fractions were prepared from rat lung and BAE cells. In these preparations, the time course of guanylate cyclase activity was investigated in the presence of 1 μM BNF or ANF (fig.2). The enzymatic activity in the presence of BNF was significantly higher than in control incubations. Compared to the effect of ANF, the stimulation of enzyme activity by BNF was less marked in the lung membranes, whereas greater stimulation of guanylate cyclase by BNF was observed in the preparation derived from endothelial cells.

Fig.3 depicts the concentration dependence for stimulation of rat lung particulate guanylate cyclase activity by BNF and ANF. Both peptides activated the enzyme about 2.5-fold. However, the concentration required for half-maximal activation was about one order of magnitude higher for BNF (EC_{50} 100–200 nM) than that observed for ANF (EC_{50} 10–20 nM). The stimulation of membrane-bound guanylate cyclase activities by

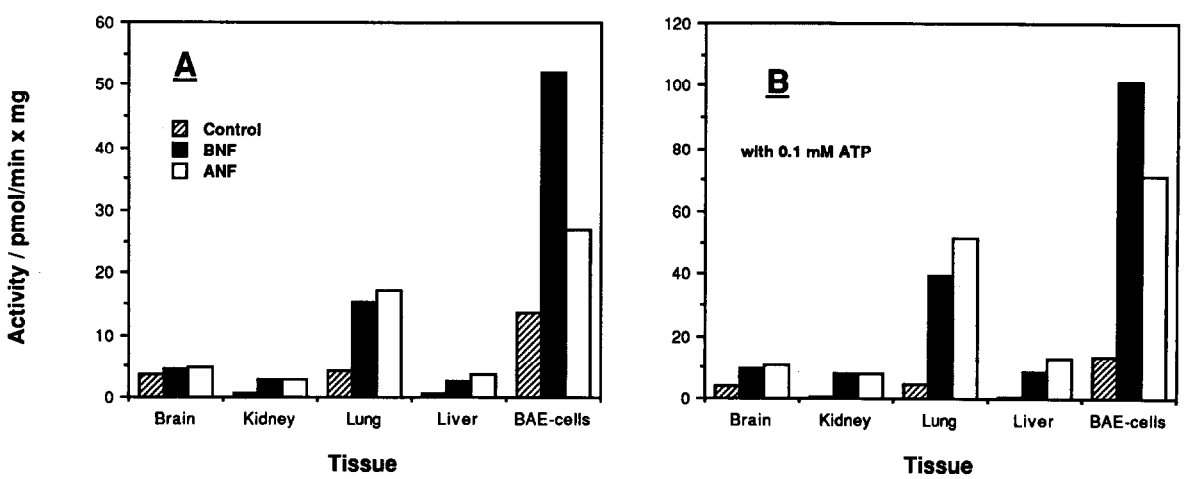


Fig.4. Activation of guanylate cyclase in rat tissue particulate fractions and BAE cell membranes by BNF (1 μM, closed columns) as compared to ANF (open columns). Basal enzyme activity in the membrane preparations is represented by the hatched columns. (A) Guanylate cyclase activity was determined as described in section 2; (B) enzyme activities after addition of 0.1 mM ATP.

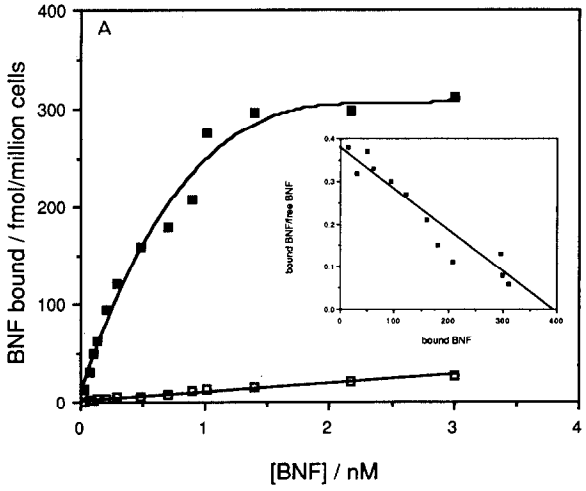
either BNF or ANF was markedly enhanced in the presence of ATP (0.1 mM) without a shift in the EC_{50} values. This has also been reported previously [10] for the stimulation of guanylate cyclase from other tissues by ANF.

The ability of BNF to stimulate the activity of particulate guanylate cyclase was further investigated in membrane preparations from a variety of rat tissues. As shown in fig.4, 1 μM BNF stimulated particulate guanylate cyclase activity in all tissues examined. The highest specific activity of the enzyme in tissue particulate fractions upon addition of BNF was observed in lung membranes, while the highest relative stimulation compared to basal activity was found in liver (5.2-fold) and kidney (4.8-fold) particulate fractions. Compared to the effects of 1 μM ANF on enzyme activity in these membrane preparations (fig.4), the extent of activation by BNF was nearly identical to that caused by ANF. For the particulate guanylate cyclase from cultured BAE cells, however, BNF was more effective than ANF.

The characteristics of BNF receptors were examined using BASM cells. Fig.5A demonstrates that a specific and saturable high-affinity binding of ^{125}I-BNF could be observed. Analysis of the binding data according to Scatchard (fig.5A, inset) resulted in a linear plot, suggesting the presence of a single class of receptors with an apparent equilibrium dissociation constant (K_d) of 0.52 nM and a maximum binding of 398 fmol/10^6 cells. As shown in fig.5B, unlabeled BNF as well as unlabeled ANF could compete with very similar affinity for the binding of radioactive BNF to these receptors while the truncated ANF analog, atriopeptin I, is slightly less potent. In the reverse experiment, unlabeled BNF could also displace ^{125}I-ANF from its binding sites on BASM cells.

4. DISCUSSION

At present, there is accumulating evidence that the biological functions of ANF are not restricted to peripheral tissues but are also of considerable importance in the central nervous system (CNS) [11]. The presence of ANF-like peptides has been demonstrated in hypothalamic regions [12]; however, their molecular structures apparently differ from the peripherally prevalent form in that three or four N-terminal amino acids are deleted [13]. Furthermore, an additional peptide, the recently identified BNF [4], is structurally related to ANF but has 8 amino acid substitutions and one insertion. This peptide family may contribute to



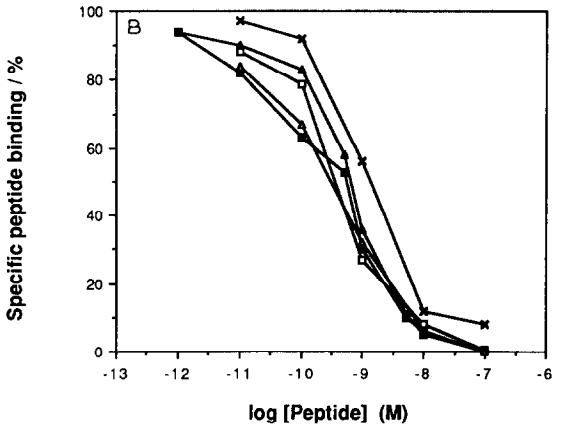


Fig.5. Characterization of BNF receptors on bovine aortic smooth muscle cells. (A) Equilibrium binding isotherm obtained after incubation of cells with increasing concentrations of ^{125}I-BNF for 30 min. Experimental conditions for determination of specific (closed squares) and nonspecific (open squares) binding are described in section 2. (Inset) Scatchard plot of the specific binding. (B) Displacement of radiolabeled BNF (closed symbols) from binding sites by unlabeled BNF (squares), or ANF (triangles). Open symbols represent competition of unlabeled BNF or ANF to the binding of ^{125}I-ANF. Additionally, the effect of different concentrations of AP-I on the binding of BNF is shown (×). ^{125}I-labeled peptides were used at 0.27 nM.

the central as well as the peripheral effects of these new hormones.

The present results show that BNF exerts some of its actions via an elevation of intracellular cGMP levels. This second messenger molecule can be produced by two isoforms of the enzyme, guanylate cyclase [14]. Only the particulate isoenzyme has been shown to be activated by the atrial peptide [2], and our experiments demonstrate that in tissues as well as in cell cultures, the brain peptide (BNF) also stimulates this enzyme. Surprisingly, no major differences in tissue specificity or potency can be observed for either peptide which suggests that the mechanism of guanylate cyclase activation is similar if not identical for BNF and ANF.

In brain, binding sites have been found which are believed to be specific for ANF [15]. However, the results of our investigations demonstrate that BNF has the ability to compete effectively for ANF binding in cultured cells; therefore, it is likely that the ANF receptors in the CNS can be occupied by BNF as well. Receptors for ANF can be classified into two subtypes, designated ANF-R1 and -R2, respectively, which may be distinguished by their molecular and functional characteristics [16]. Only the R1 receptor is coupled to the membrane-bound guanylate cyclase, while unique functions of the much more abundant R2 receptor remain unknown. The activation of tissue and cellular particulate guanylate cyclase by BNF demonstrated in this report shows that this peptide binds to the R1 receptor, while the direct binding experiments using radiolabeled BNF provide evidence for the occupation of the more abundant R2 receptor as well. Thus, BNF, as is the case with most ANF analogues, does not bind selectively to one of the two ANF receptors.

In conclusion, our results support the concept of a new class of biologically active peptides that may be part of a broader family of related peptides. The common feature of these peptides is the molecular mechanism underlying their action, i.e. the occupation of receptors coupled to the activation of membrane-bound guanylate cyclase and, subsequently, the elevation of intracellular cGMP. While investigations of cGMP metabolism may facilitate the discovery of other related peptides, this new class of peptide hormones should permit us to evaluate the biological functions of cGMP.

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REFERENCES

[1] Genest, J. and Cantin, M. (1987) Circulation 75 (suppl.I), I-118–I-124.
[2] Waldman, S.A., Rapoport, R.M. and Murad, F. (1984) J. Biol. Chem. 259, 14332–14334.
[3] Winquist, R.J., Faison, E.P., Waldman, S.A., Schwartz, K., Murad, F. and Rapoport, R.M. (1984) Proc. Natl. Acad. Sci. USA 81, 7661–7664.
[4] Sudoh, T., Kangawa, K., Minamino, N. and Matsuo, H. (1988) Nature 332, 78–81.
[5] Salacinski, P.R.P., McLean, C., Sykes, J.F.C., Clement-Jones, V.V. and Lowry, P. (1981) Anal. Biochem. 117, 136–146.
[6] Leitman, D.C. and Murad, F. (1986) Biochim. Biophys. Acta 885, 74–79.
[7] Leitman, D.C., Andresen, J.W., Catalano, R.M., Waldman, S.A., Tuan, J.J. and Murad, F. (1988) J. Biol. Chem., in press.
[8] Steiner, A.L., Parker, C.W. and Kipnis, D.M. (1972) J. Biol. Chem. 247, 1106–1120.
[9] Harper, J.F. and Brooker, G. (1973) J. Cyclic Nucleotide Res. 1, 207–218.
[10] Kurose, H., Inagami, T. and Ui, M. (1987) FEBS Lett. 219, 375–379.
[11] Saper, C.B., Standaert, D.G., Currie, M.G., Schwartz, D., Geller, D.M. and Needleman, P. (1985) Science 227, 1047–1049.
[12] Tanaka, I., Misono, K.S. and Inagami, T. (1984) Biochem. Biophys. Res. Commun. 124, 663–668.
[13] Imura, H. and Nakao, K. (1988) J. Cell Biochem. (Suppl.) 12A, 12.
[14] Waldman, S.A. and Murad, F. (1987) Pharmacol. Rev. 39, 163–196.
[15] Quirion, R., Dalpe, M. and Dam, T.V. (1986) Proc. Natl. Acad. Sci. USA 83, 174–178.
[16] Leitman, D.C. and Murad, F. (1987) Endocrinol. Metab. Clin. N. Am. 16, 79–105.