Atrial Natriuretic Factor Reduces Cyclic Adenosine Monophosphate Content of Human Fibroblasts by Enhancing Phosphodiesterase Activity

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Abstract

Radioligand binding studies disclosed one class of high affinity atrial natriuretic factor (ANF) receptors on human fibroblast membranes (K_d = 66 pM; maximum number of binding sites [B_{max}] = 7,000 sites/cell). ANF increased cellular cyclic guanosine monophosphate (cGMP) content and suppressed isoproterenol- and PGE$_1$-elevated, but not basal, cAMP content. Pertussis toxin pretreatment, which maximally ADP-ribosylated G$_i$, the guanine nucleotide-binding protein that couples inhibitory receptors to adenylate cyclase and blocks receptor-mediated inhibition of adenylate cyclase, did not interfere with ANF suppression of isoproterenol- or PGE$_1$-elevated cellular cAMP content. Preliminary incubation of fibroblasts with 8-bromo cGMP or phosphodiesterase inhibitors, including 3-isobutyl-1-methylxanthine, Ro 20-1724, and cilostamide, however, prevented the ANF suppression of cAMP. MB 22948, an inhibitor that is partially selective for cGMP phosphodiesterase, did not block the effect of ANF. We conclude that in these cells, unlike other systems, ANF reduces cAMP content by activating a phosphodiesterase rather than by inhibiting adenylate cyclase.

Introduction

Atrial natriuretic factor (ANF),[1] a polypeptide hormone released by atrial myocytes (for reviews see references 1 and 2), causes sodium and water diuresis (3, 4), vascular smooth muscle relaxation (5, 6), and inhibition of renin (7) and aldosterone secretion (8–10). Although many of its physiologic effects are recognized, much remains to be learned about ANF at a molecular level.

Cyclic guanosine monophosphate (cGMP) may be an important mediator of ANF action. Atrial extracts injected into rats increased plasma and urine levels of cGMP; the latter increase was thought to result from renal production (11). ANF increased cGMP levels in isolated kidney cells (12), renal glomeruli and other defined nephron segments (13–15), and cultured vascular smooth muscle preparations (16). In vascular smooth muscle preparations, ANF-induced increases were

correlated with an apparently endothelium-independent vasorelaxation (17). ANF stimulation of particulate guanylate cyclase has been demonstrated in numerous tissues, including rat aorta, kidney, lung, liver, intestine, and testes (18). Based on a 20,000-fold copurification of ANF binding and guanylate cyclase activity from rat lung, at least one type of ANF-receptor apparently has intrinsic guanylate cyclase activity (19).

Modulation of cellular cAMP levels may be another mechanism of ANF action. ANF receptor-mediated inhibition of adenylate cyclase activity has been described in pituitary and adrenal cortical membranes and in homogenates of aorta and rat mesenteric and renal arteries (20–22). Reduced cAMP content has been reported to accompany elevated cGMP content in response to ANF treatment of isolated rat renal papillary cells (12). Reported effects of ANF on kidney membrane adenylate cyclase, however, have been mixed. No effect was seen with rat kidney membranes (18). In dog, ANF inhibited membrane adenylate cyclase activity of glomeruli, loops of Henle, and collecting ducts, but not proximal tubules (23). cAMP levels of aortic strips were unaffected by ANF treatment, even though ANF causes relaxation of the tissue (17).

One system in which receptor regulation of cyclic nucleotide levels has been well characterized is the human skin fibroblast. Adenylate cyclase activation and inhibition and guanylate cyclase activation have all been demonstrated in these cells (24–26). We now report in human skin fibroblasts a novel mechanism of ANF action: reduction of cAMP content through activation of a phosphodiesterase rather than inhibition of adenylate cyclase.

Methods

ANF (rat, Ser 99-Tyr 126 ANF and Arg 101-Tyr 126 ANF) was purchased from Peninsula Laboratories, Inc., Belmont, CA. ^{125}I-ANF (Ser 99-Tyr 126 ANF) and cAMP and cGMP RIA kits were purchased from New England Nuclear, Boston, MA; pertussis toxin from List Biological Laboratories, Campbell, CA; 8-bromo cGMP from P-L Biochemicals, Milwaukee, WI; isoproterenol from Sigma Chemical Co., St. Louis, MO; PGE$_1$ from Seragen Diagnostics, Inc., Indianapolis, IN; 3-isobutyl-1-methylxanthine (IBMX) from Aldrich Chemical Co., Milwaukee, WI; HBSS from Gibco, Grand Island, NY; MEM and 1-glutamine from Hazelton Research Products, Denver, PA; and FCS from Quality Biologicals, Inc., Gaithersburg, MD. Cilostamide was the kind gift of Dr. H. Hidaka (Mie University, Mie, Japan) and Otsuka Pharmaceutical Co., Osaka, Japan, and Ro 20-1724 was the gift of Dr. Michael Lin (National Institutes of Health) and Hoffmann-LaRoche Inc., Nutley, NJ. MB 22948 was obtained from May and Baker, Ltd., Dagenham, England.

Assay of cGMP and cAMP. Human fibroblasts were grown to confluence on 60-mm plates (Falcon Labware, Becton, Dickinson & Co., Oxnard, CA) in MEM with 10% FCS and 2 mM glutamine in a humidified 5% CO$_2$ atmosphere (24–26). Medium was changed 48 h before experiments. Cells were incubated overnight at 37°C with or without 0.4 μg/ml pertussis toxin, then washed twice with HBSS, pH 7.4, and incubated for 10 min at 37°C in HBSS with additions as indicated. IBMX, Ro 20-1724, MB 22948, and cilostamide were added 10 min

1. *Abbreviations used in this paper:* ANF, atrial natriuretic factor; B_{max}, maximum number of binding sites; EC$_{50}$, 50% effective concentration; G$_i$, inhibitory guanyl nucleotide-binding protein; IBMX, 3-isobutyl-1-methylxanthine; IC$_{50}$, 50% inhibitory concentration.

before addition of isoproterenol, PGE_1, and ANF as indicated. Stock solutions of IBMX, Ro 20-1724, and cilostamide were made in 2% DMSO, for an incubation concentration of 0.1% DMSO. Medium was rapidly replaced with 2.0 ml of cold 5% TCA and the plates were frozen on a dry ice-ethanol mixture. The plates were then thawed, and the supernatant was extracted with a Freon 11:tri-n-octylamine mixture (79:21, vol/vol) before RIA of cAMP in duplicate. Data reported are mean±SEM of values from quadruplicate plates in a representative experiment.

ANF(Ser 99-Tyr 126 ANF) membrane binding assay. Cells were harvested by scraping, sedimented at 3,000 g, and washed twice with 0.25 M sucrose/10 mM Tris-HCl (pH 7.5)/10 mM $MgCl_2$. After being suspended in 10 mM Tris-HCl (pH 7.5)/10 mM $MgCl_2$, cells were disrupted by a Polytron (Brinkmann Instruments, Co., Westbury, NY). Nuclei and cell debris were sedimented at 1,000 g. Membranes were recovered by centrifugation at 50,000 g. After being washed twice in 50 mM Tris-HCl, pH 7.5, membranes were stored frozen at $-70°C$.

Binding of ANF to 3–10 μg cell membranes was assayed using 50 pM ^{125}I-ANF(Ser 99-Tyr 126 ANF) (25,000 cpm/tube) with different concentrations of ANF(Ser 99-Tyr 126 ANF) in 50 mM Tris-HCl, pH 7.5/0.1% BSA/0.1% bacitracin, containing 0.25 μg each of pepstatin, leupeptin, soybean trypsin inhibitor, and chymotrypsin inhibitor (total volume, 0.25 ml). After incubation for 30 min at 30°C, samples were filtered according to Bruns et al. (27). Binding parameters were fitted with the LIGAND nonlinear least squares of curve-fitting program (28). Nonspecific binding was < 10% of total.

Miscellaneous. Protein was assayed according to Lowry et al. (29) using BSA as the standard for binding assays. For cAMP and cGMP determinations, protein was assayed with a dye-binding kit (Bio-Rad Laboratories, Richmond, CA) using gamma globulin as standard. Significance was determined by analysis of variance using paired t testing. Significance is defined as $P < 0.05$.

Results

Over the range of concentrations assayed, ANF(Ser 99-Tyr 126) apparently bound to a single class of high affinity sites in human fibroblast membranes (K_d = 66±18 pM; maximum number of binding sites [B_{max}] = 1,205±217 fmol/mg protein or 7,000 sites/cell) (data not shown).

Basal cAMP content of fibroblasts was unaffected by 1 μM ANF, whereas cAMP accumulation stimulated by isoproterenol or PGE_1 was significantly decreased (Table I). The inhibitory effect of ANF was concentration-dependent, with a 50% inhibitory concentration (IC_{50}) of ~ 10^{-10} M (Fig. 1). Some degree of inhibition occurred at all concentrations of isoproterenol (Fig. 2 A) and at PGE_1 concentrations > 0.03 μg/ml (Fig. 2 B). ANF inhibition was dependent on time of incubation. Maximal percentage inhibition with isoproterenol was observed at 10 min and with PGE_1 at 5–10 min (data not shown).

Incubation of cells with pertussis toxin, which blocks the function of G_i (30), the guanyl nucleotide-binding protein that couples inhibitory receptors to adenylate cyclase (31), decreased basal cAMP content and cAMP accumulation in response to isoproterenol and PGE_1, but did not interfere with the inhibitory effect of ANF (Table II). Toxin treatment, however, abolished the inhibitory effect on both PGE_1- and isoproterenol-stimulated cAMP of carbamylcholine, which acts through G_i to inhibit adenylate cyclase (Table III); thus, in this system, G_i was not involved in ANF action.

Incubation of fibroblasts with the phosphodiesterase inhibitor IBMX (10^{-4} M) elevated basal and isoproterenol- and PGE_1-stimulated cAMP content (Table I). In the presence of

Table I. Effect of IBMX on the ANF Inhibition of Agonist-stimulated cAMP

	Cell cAMP content		
Additions	Control	ANF	Change
	pmol/mg		*%*
None	8±0.6	8±0.8	NS
IBMX	34±1.6	34±2.2	NS
Isoproterenol	72±1.2	54±1.4	−24.2*
Isoproterenol plus IBMX	186±7.0	194±8.4	NS
PGE_1	560±16.4	374±20.0	−33.3*
PGE_1 plus IBMX	1,258±49.6	1,250±24.8	NS

Cells were incubated with or without 100 μM IBMX at 37°C for 10 min, then with 10 μM isoproterenol, 1 μg/ml PGE_1, and/or 1 μM ANF as indicated at 37°C for 10 min before assay of cAMP. The concentration of IBMX gave maximal effects on cAMP content. Data are the averages of quadruplicate determinations. Experiment was performed five times with similar results.
* $P < 0.0005$.

IBMX, the inhibitory effect of ANF on agonist-stimulated cAMP was abolished (Table I). Incubation with IBMX did not abolish the inhibitory effect of carbamylcholine on isoproterenol- and PGE_1-stimulated cAMP (data not shown). Treatment of the cells with Ro 20-1724, a more selective cAMP phosphodiesterase inhibitor, also elevated basal cAMP content (Table IV). Isoproterenol- and PGE_1-stimulated cAMP contents were enhanced; the inhibitory effect of ANF was substantially reduced. Cilostamide, another selective cAMP phosphodiesterase inhibitor, had relatively little effect on basal, isoproterenol-, or PGE_1-stimulated cAMP (Table IV). There was, however, marked attenuation of the ANF-dependent inhibition of agonist-stimulated cAMP (Table IV).

MB 22948, a somewhat selective inhibitor of cGMP phosphodiesterases, did not block the ANF response in an experiment in which IBMX inhibited the response (Table V). MB
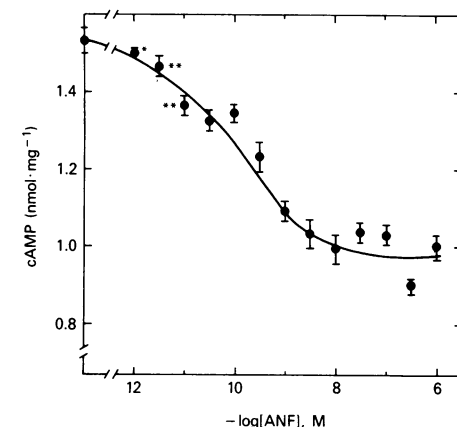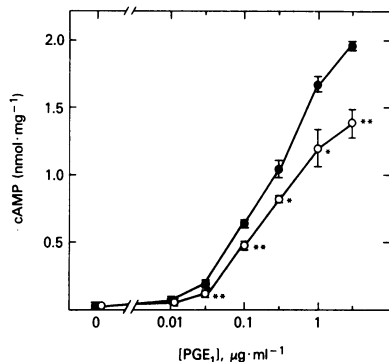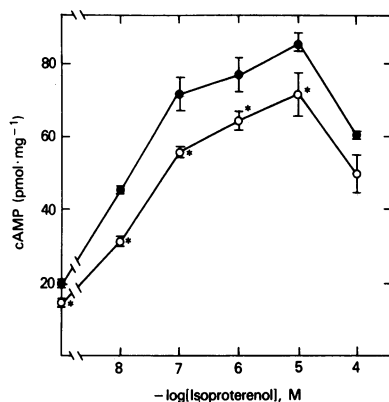

Figure 1. Effect of ANF on the PGE_1-stimulated cAMP content of fibroblasts. Cells were incubated for 10 min at 37°C with 1 μg/ml PGE_1 and ANF at the indicated concentration before determination of cAMP content. Data are mean±SEM (n = 4) of a representative experiment from a total of four. *, not significantly different from control; **, $P < 0.025$; no symbol indicates that $P < 0.0025$.

Figure 2. (*A*) Effect of ANF on isoproterenol-stimulated cAMP content of fibroblasts. Cells were incubated for 10 min at 37°C with isoproterenol as indicated, without (●) or with (○) 1 μM ANF before determination of cAMP content. Data are mean±SEM (*n* = 4) of a single experiment from a total of four. *, *P* < 0.05. This is the sole experiment in which ANF significantly decreased basal activity. (*B*) Effect of ANF on PGE₁-stimulated cAMP content of fibroblasts. Cells were incubated for 10 min at 37°C with PGE₁ as indicated without (●) or with (○) 1 μM ANF before determination of cAMP content. Data are mean±SEM (*n* = 4) of a representative experiment from a total of four. *, *P* < 0.025; **, *P* < 0.0025.

22948 at the concentration used in this experiment (1 μM) significantly enhanced ANF-stimulated cGMP, with minimal effects on PGE₁-stimulated cAMP. At 10 μM, stimulatory effects on agonist-enhanced cAMP and cGMP were observed (data not shown). No difference in cAMP or cGMP phosphodiesterase activity was observed in supernatant and particulate fractions prepared from control and ANF-treated fibroblasts

Table II. Effect of Pertussis Toxin on the ANF Responsiveness of Human Fibroblasts

Additions	Cell cAMP content		Change
	Control	ANF	
	pmol/mg		*%*
None	13±0.3	14±0.5	NS
Pertussis toxin	10±0.1	11±0.6	NS
Isoproterenol	143±2.6	117±2.5	−18.2*
Isoproterenol plus toxin	89±3.1	71±0.6	−19.6*
PGE₁	1,390±93.0	1,120±5.6	−19.3*
PGE₁ plus toxin	990±26.3	753±12.7	−23.9*

Cells were incubated overnight at 37°C with or without 0.4 μg/ml pertussis toxin (3 ml/dish), and then washed and incubated with or without 10 μM isoproterenol, 1 μg/ml PGE₁, and 1 μM ANF at 37°C for 10 min before assay of cAMP. All assays were performed in quadruplicate; experiment was repeated seven times.
* *P* < 0.0005.

Table III. Effect of Pertussis Toxin on Carbamylcholine Inhibition of Agonist-stimulated cAMP

Additions	Cell cAMP content		Change
	Control	Carbamylcholine	
	pmol/mg		*%*
Isoproterenol	129±5.2	59±3.0	−54.3* (7)
Isoproterenol plus pertussis toxin	97.4±5.28	92.0±4.68	−5.5‡ (4)
PGE₁	1,480±78	903±12.4	−39.0 (7)
PGE₁ plus pertussis toxin	1,210±25	1,220±30	0.8‡ (3)

Experiments (in quadruplicate) were performed as described in Table II except for the presence of 1 mM carbamylcholine as indicated. In parentheses, number of experiments.
* *P* < 0.0005; ‡ not significant.

(data not shown). Assays were performed under conditions used to assess basal, Ca²⁺-calmodulin-stimulated, and cGMP-stimulated phosphodiesterases. The effect of ANF on cAMP was inhibited by 8-bromo cGMP (Table VI), consistent with cGMP being an intermediate in these ANF-mediated effects on fibroblasts.

In these human fibroblasts, ANF mediated a rise in the cGMP content after a 30-s incubation at 37°C with a threshold response at 10⁻⁸ M and a 50% effective concentration (EC₅₀) of at least 10⁻⁶ M; cGMP content was still rising at 10⁻⁵ M ANF (the maximal concentration tested) (Fig. 3). There was no apparent effect of pertussis toxin upon the cGMP response to ANF (data not shown). The effect of ANF on cGMP content was time dependent (Fig. 4), with a rapid rise and fall within 2 min. IBMX exaggerated and prolonged the ANF effect. Untreated cells or those treated with IBMX alone did not exhibit any significant changes in cGMP.

Table IV. Effect of Ro 20-1724 and Cilostamide on the ANF-dependent Inhibition of Agonist-stimulated cAMP

Additions	Cell cAMP content		Change
	Control	ANF	
	pmol/mg		*%*
None	8.6±0.5	8.7±0.9	+2
Ro 20-1724	19.6±2.0	24.2±0.8	+23*§
Cilostamide	10.0±1.1	10.0±0.5	0
Isoproterenol	72.7±1.3	54.7±1.5	−24‡
Isoproterenol plus Ro 20-1724	169±4.3	154±27	−9*
Isoproterenol plus cilostamide	96.7±4.6	94.2±6.4	−3
PGE₁	561±16	374±20	−33‡
PGE₁ plus Ro 20-1724	740±27	711±26	−4
PGE₁ plus cilostamide	609±10	580±12	−5

Cells were incubated without or with 10⁻⁴ M Ro 20-1724 or 10⁻⁶ M cilostamide at 37°C for 10 min, then drugs were added as indicated: 10 μM isoproterenol, 1 μg/ml PGE₁, and 1 μM ANF. The incubation continued another 10 min before assay of cAMP. Experiment was performed five times with similar results.
* *P* < 0.05; ‡ *P* < 0.0005; § this response was not observed consistently (*n* = 7 experiments).

Table V. Effect of IBMX and MB 22948 on Inhibition of PGE₁-stimulated cAMP Accumulation by ANF

	Additions	Cell cAMP content		Change
		Control	ANF	
		pmol/mg		%
I*	None	37.5±8.62	46.5±12.28	+24.0[‡]
	+IBMX	175±18.1	180±29.4	+4.6[‡]
	+MB 22948	61.9±10.83	66.2±4.88	+6.9[‡]
II[§]	PGE₁	1,360±25.6	1,170±24.1	−14.1[‖]
	+IBMX	3,310±61.4	3,300±57.9	−0.1[‡]
	+MB 22948	1,640±23.9	1,469±23.7	−10.6[‖]

Cells were incubated with or without 100 μM IBMX or 1 μM [¶] MB 22948 at 37°C for 10 min; 1 μg/ml PGE₁ with or without 1 μM ANF was added and the incubation continued for another 10 min before assay of cAMP. The response of the fibroblasts to agonists (PGE₁, isoproterenol, and ANF) was dependent on passage number and freeze-down. The experiments in this table were performed with a different freeze-down of fibroblasts accounting for the quantitative differences in cAMP content. Experiment was performed three times with similar results.
* $n = 10$. [‡] NS; [§] $n = 4$. [‖] $P < 0.0005$; [¶] This concentration of MB 22948 resulted in increased cGMP content with minimal effects on cAMP levels.

Discussion

ANF reduced cAMP content in isoproterenol- or PGE₁-treated but not in untreated fibroblasts. As in other systems, ANF treatment increased cellular cGMP content in a dose-dependent manner. Although our findings are consistent with a single class of ANF receptors on fibroblast membranes, from the 1,000-fold difference in the potency of ANF to reduce cAMP content ($IC_{50} = 100$ pM) and elevate cGMP content ($EC_{50} > 100$ nM), one may infer that a second class of ANF receptors went undetected in the binding studies. Based on its affinity ($K_d = 66$ pM), the site we describe is presumably the one that mediates the effect of ANF on fibroblast cAMP levels. The very low potency of ANF to stimulate guanylate cyclase in

Table VI. Effect of 8-Bromo cGMP on ANF Inhibition of Isoproterenol-stimulated cAMP

Additions	Cell cAMP content		Change
	Control	ANF	
	pmol/mg		%
None	23.5±0.35	22.0±0.15	−6.4*
Isoproterenol	131±3.0	96.3±7.40	−18.2[‡]
8-bromo cGMP	40.8±1.45	42.5±2.00	+4.3[§]
8-bromo cGMP plus isoproterenol	109±1.0	115±11.2	+6.0[§]

Cells were incubated with or without 100 μM 8-bromo cGMP, then with 10 μM isoproterenol and/or 1 μM ANF as indicated at 37°C for 10 min before assay of cAMP. Experiment was repeated three times with similar results.
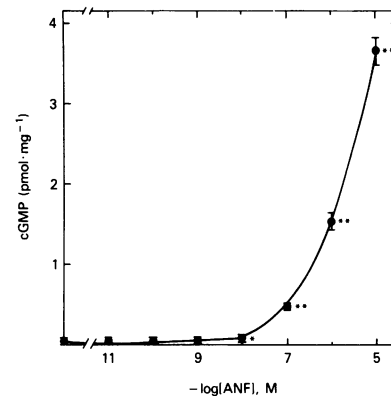* $P < 0.005$; [‡] $P < 0.001$; [§] NS.

Figure 3. Effect of ANF on the cGMP content of human fibroblasts. Cells were incubated at 37°C for 30 s with ANF at the concentrations noted. Data are mean±SEM ($n = 4$). Experiment was performed once. *, $P < 0.005$; **, $P < 0.0005$.

these fibroblasts ($EC_{50} > 100$ nM), however, would preclude detection of the relevant receptor, unless the receptor was present in large amounts. Because ANF does elevate cGMP in these fibroblasts, if only at relatively high doses, it is reasonable to conclude that a low affinity binding site was present but undetected.

One may explain the mechanism of ANF effect on cellular cAMP content in three ways: inhibition of adenylate cyclase, activation of a phosphodiesterase, or increased extrusion of cAMP into the culture medium. Our results are consistent with the conclusion that ANF treatment increases phosphodiesterase activity, as three phosphodiesterase inhibitors tested, IBMX, Ro 20-1724, and cilostamide, blocked or significantly reduced the effect of ANF. MB 22948, an inhibitor that was partially selective for the cGMP phosphodiesterases (32), did not block ANF inhibition of PGE₁-stimulated cAMP, which is consistent with the conclusion that the effect of ANF is mediated through a cAMP phosphodiesterase. No increase in phosphodiesterase activity was observed, however, in disrupted cell fractions. These data are similar to reports noted below of muscarinic cholinergic receptor-mediated activation of phosphodiesterase, in which effects are lost on cell lysis. Although ANF inhibition of adenylate cyclase activity has been described in vascular homogenates and pituitary, adrenal cortical, and renal membranes (20–23), our data do not support a similar mechanism in human fibroblasts. Inhibitory receptors, such as muscarinic receptors in the fibroblasts, are coupled to adenylate cyclase via G_i, a guanine nucleotide-binding protein, and pertussis toxin substrate (31); pertussis toxin ADP-ribosylates G_i and prevents receptor-mediated inhibition of adenylate cyclase (30). Pertussis toxin pretreat-
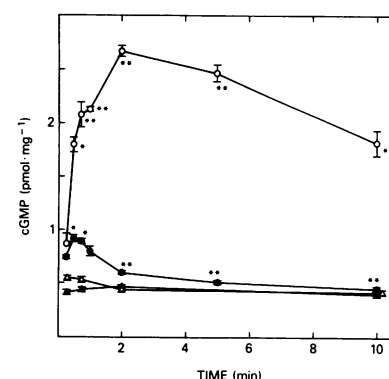
Figure 4. Effect of ANF and IBMX on the cGMP content of human fibroblasts. Cells were incubated at 37°C for the times indicated under basal (▲), 10⁻⁴ M IBMX (△), 1 μM ANF (●), and ANF plus IBMX (○) conditions. The experiment was performed once. *, $P < 0.025$; **, $P < 0.0005$.

ment, which ADP-ribosylated all available G_i and blocked muscarinic inhibition of adenylate cyclase, did not block ANF effects on cellular cAMP content. We conclude that the mechanism of ANF action in fibroblasts, unlike other tissues that have been studied, is to increase cAMP degradation rather than to inhibit its synthesis. The fact that 8-bromo cGMP inhibited the effects of ANF on isoproterenol-stimulated cAMP supports a role for the nucleotide as a possible intermediate. The possibilities for its action are diverse, including activation of a cAMP phosphodiesterase or cGMP-dependent protein kinase (33, 34). It is possible, however, that exogenous 8-bromo cGMP may elicit a response different from that found with endogenous cGMP. Further study of this pathway is clearly necessary.

Although the ANF and muscarinic mechanisms differ in the fibroblasts, muscarinic receptor modulation of cellular cAMP levels in another cell type may provide a model for ANF action in fibroblasts. In addition to the G_i-mediated muscarinic inhibition of adenylate cyclase that occurs in the fibroblasts and in NG108-15 neuroblastoma × glioma hybrid cells (35), muscarinic activation of a phosphodiesterase without inhibition of adenylate cyclase has been reported in the 1321N1 astrocytoma cell line. Like ANF in the fibroblasts, carbamylcholine had no effect on basal cAMP levels in the astrocytoma cells but reduced PGE_1- and isoproterenol-stimulated levels in a manner that was blocked by IBMX and not by pertussis toxin (36–38). That the carbamylcholine effect in the astrocytoma is a calcium-dependent phenomenon (37, 38) raises the possibility of a similar mechanism for ANF activation of a phosphodiesterase in the fibroblasts.

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