Nitric Oxide, Atrial Natriuretic Peptide, and cyclic GMP Inhibit the Growth-promoting Effects of Norepinephrine in Cardiac Myocytes and Fibroblasts

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Abstract

This study tested the hypothesis that nitric oxide (NO) and atrial natriuretic peptide (ANP) can attenuate the effects of adrenergic agonists on the growth of cardiac myocytes and fibroblasts. In ventricular cells cultured from neonatal rat heart, ANP and the NO donor *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP) caused concentration-dependent decreases in the norepinephrine (NE)-stimulated incorporation of [^{3}H]leucine in myocytes and [^{3}H]thymidine in fibroblasts. In myocytes, the NO synthase inhibitor N^G-monomethyl-L-arginine potentiated NE-stimulated [^{3}H]leucine incorporation. In both cell types, ANP and SNAP increased intracellular cGMP levels, and their growth-suppressing effects were mimicked by the cGMP analogue 8-bromo-cGMP. Furthermore, in myocytes, 8-bromo-cGMP attenuated the α_1-adrenergic receptor–stimulated increases in c-*fos*. Likewise, ANP and 8-bromo-cGMP attenuated the α_1-adrenergic receptor–stimulated increase in prepro-ANP mRNA and the α_1-adrenergic receptor–stimulated decrease in sarcoplasmic reticulum calcium ATPase mRNA. The L-type Ca^{2+} channel blockers verapamil and nifedipine inhibited NE-stimulated incorporation of [^{3}H]leucine in myocytes and [^{3}H]thymidine in fibroblasts, and these effects were not additive with those of ANP, SNAP, or 8-bromo-cGMP. In myocytes, the Ca^{2+} channel agonist BAY K8644 caused an increase in [^{3}H]leucine incorporation which was inhibited by ANP. These findings indicate that NO and ANP can attenuate the effects of NE on the growth of cardiac myocytes and fibroblasts, most likely by a cGMP-mediated inhibition of NE-stimulated Ca^{2+} influx. (*J. Clin. Invest.* 1998. 101:812–818.) Key words: nitric oxide • atrial natriuretic peptide • cGMP • cardiac myocytes • cardiac fibroblasts

Introduction

Norepinephrine (NE)[1] induces myocardial hypertrophy associated with increased protein synthesis by myocytes, the induction of a fetal gene program, and the development of interstitial fibrosis (1–4). In cultured cardiac myocytes, NE causes myocyte hypertrophy and fetal gene expression primarily via activation of α_1-adrenergic receptors (2), whereas in cultured cardiac fibroblasts, the induction of DNA synthesis and proliferation by NE is mediated by β-adrenergic receptors (5). In both myocytes and fibroblasts, NE, acting via α_1-adrenergic or β-adrenergic receptors, respectively, causes the expression of peptide growth factors that can exert autocrine or paracrine effects on growth and gene expression (4, 5).

cGMP and substances that increase cellular cGMP, such as natriuretic peptides and nitric oxide (NO)–generating molecules, inhibit the stimulation of DNA synthesis by angiotensin, endothelin, and peptide growth factors in vascular smooth muscle cells, endothelial cells, mesangial cells, and cardiac fibroblasts (6–9, 10). There is no information about the effect of cGMP on protein synthesis in cardiac myocytes, or on the ability of cGMP-elevating substances to inhibit the growth-promoting effects of NE in either myocytes or fibroblasts from the heart. However, cardiac myocytes and fibroblasts have receptors for natriuretic peptides (10, 11), the myocardium can produce nitric oxide (12, 13), and a characteristic of the hypertrophic response is the induction of atrial natriuretic peptide (ANP) in cardiac myocytes (2, 14). Taken together, these observations raise the possibility that natriuretic peptides and NO could attenuate the effects of NE on the growth and phenotype of the myocardium.

Therefore, the primary purpose of this study was to test the hypothesis that cGMP and the cGMP-generating substances NO and ANP inhibit the ability of NE to cause growth of cardiac myocytes and fibroblasts. Our second, related goal was to test the hypothesis that cGMP inhibits the effect of NE on myocyte gene expression. These hypotheses were examined in cultures of neonatal rat cardiac myocytes and fibroblasts by determining the ability of cGMP, ANP, the NO donor *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP), and the NO synthase inhibitor N^G-monomethyl-L-arginine (L-NMMA) to modulate NE-stimulated protein synthesis and gene expression in myocytes, and DNA synthesis in fibroblasts. Finally, since NE increases Ca^{2+} influx via L-type Ca^{2+} channels (15), and cGMP inhibits L-type Ca^{2+} channels in cardiac myocytes (16–18), our third goal was to examine the possibility that the growth-inhibiting effects of cGMP are mediated via inhibition of Ca^{2+} influx.

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1. *Abbreviations used in this paper:* ANP, atrial natriuretic peptide; L-NMMA, N^G-monomethyl-L-arginine; NE, norepinephrine; NO, nitric oxide; SERCA2, sarcoplasmic reticulum calcium ATPase; SNAP, *S*-nitroso-*N*-acetyl-D,L-penicillamine.

Methods

Cultured neonatal rat cardiac myocytes and fibroblasts. Neonatal rat ventricular myocytes were isolated by a modification of the technique of Kasten (19), as described previously (4). Briefly, hearts were removed from 2-d-old neonatal rats anesthetized with ether and killed by decapitation. Ventricular tissues were digested with 0.1% trypsin (Worthington Biochemical Corp., Freehold, NJ) in HBSS (Ca^{2+} free; GIBCO BRL, Gaithersburg, MD) overnight at 4°C. Ventricular cells were then recovered by repeated digestions of the tissue in 10 ml of 0.1% collagenase in HBSS. The supernatants collected from each digestion were centrifuged at 100 *g* for 3 min (4°C). The pellets were resuspended in ice cold HBSS, pooled, and centrifuged at 100 *g* for 4 min (4°C). Cells were resuspended in DME containing 7% heat-inactivated FBS and preplated twice on T80 flasks (Nunc, Inc., Naperville, IL) for 75 min to enrich for myocytes and decrease contamination by nonmuscle cells. The nonadherent myocytes were then plated at a density of 100–200 cells/mm^2 for [^{3}H]leucine incorporation (24-well plate; Costar Corp., Cambridge, MA) and mRNA studies (100-mm plate; Fisher Scientific Co., Pittsburgh, PA). After 24 h, the culture medium was changed to serum-free DME containing insulin (5 μg/ml), transferrin (5 μg/ml), and sodium selenite (5 ng/ml); all experiments were performed 24 h later.

Cultures of neonatal rat ventricular nonmyocytes, which have been shown to be predominantly fibroblasts (20), were prepared as described previously by Sadoshima and Izumo (21). Briefly, hearts obtained from neonatal rats were enzyme-digested as described above for myocytes. The adherent nonmyocyte fractions obtained during preplating on T80 flasks were grown in DME containing 7% FBS until confluent (5–7 d), and passaged with trypsin-EDTA (0.25%, 0.53 mM; GIBCO BRL). All experiments were performed on cells from the first or second passages which were placed in serum-free DME containing insulin (5 μg/ml), transferrin (5 μg/ml), and sodium selenite (5 ng/ml) for 24 h before the experiment.

[^{3}H]leucine and [^{3}H]thymidine incorporation. Myocytes and fibroblasts were plated on 24-well plates (Costar Corp.) at a density of 100–200 cells/mm^2 (myocytes) or 50–100 cells/mm^2 (fibroblasts). Protein and DNA synthesis were measured as described previously (21, 22). Briefly, myocytes and fibroblasts were treated with ANP (0.1–100 nM; Calbiochem Corp., La Jolla, CA), SNAP (0.1–100 μM; Alexis Corp., San Diego, CA), 8-bromo-cGMP (1 mM; Sigma Chemical Co., St. Louis, MO), L-NMMA (1 mM, Sigma Chemical Co.), (±)-verapamil (10 μM; Sigma Chemical Co.), and/or nifedipine (1 μM, Sigma Chemical Co.) for 15–30 min before the addition of 1-norepinephrine (NE; 1 μM; Sigma Chemical Co.) and either 2.5 μCi/ml of [^{3}H]leucine (DuPont-NEN, Boston, MA) or 2 μCi/ml of [^{3}H]thymidine (DuPont-NEN) for 24 h. Cells were washed twice with 4°C PBS, and cold 5% TCA was added for 30 min to precipitate protein and DNA. The precipitates were washed twice with cold water and resuspended in 0.4 M NaOH. Aliquots were counted in a scintillation counter.

cGMP production. Myocytes and fibroblasts plated in 6-well plates (Costar Corp.) were pretreated with isobutylmethylxanthine (0.2 mM) for 15 min before the addition of ANP (100 nM) or SNAP (100 nM), and incubated at 37°C for an additional 30 min. The reaction was stopped by the addition of 0.1 M HCl, the plates were placed on ice for 30 min, and the supernatant was collected and titrated to pH 7.4 with 2.5 M sodium acetate (pH 5.8) and 5 M NaOH. cGMP levels were quantitated with a standard RIA kit (Biomedical Technologies, Inc., Stoughton, MA). NaOH (0.2 M) was subsequently added to the plates for 30 min, the cells were scraped, and the protein content was measured by the Bradford technique (Bio-Rad Laboratories, Hercules, CA) with BSA as a standard.

Assessment of mRNA levels. Myocytes were plated at a density of 200 cells/mm^2 in P100 plates (Falcon Labware, Cockeysville, MD). Myocyte mRNA levels were measured as described previously (4). Briefly, myocytes were treated with ANP (100 nM) or 8-bromo-cGMP (1 mM) for 5–10 min before the addition of 1 μM NE (in the

presence of 10 μM propranolol) and harvested 30 min or 24 h later to examine c-*fos* and prepro-ANP mRNA levels, respectively. Myocytes were resuspended in denaturing solution (4M guanidium thiocyanate, 25 mM sodium citrate, 0.5% laurylsarcosine, 0.1 M mercaptoethanol, pH 7). RNA was denatured with formaldehyde and formamide, and separated by size electrophoresis on a 1.3% agarose/4% formaldehyde gel (15 μg total RNA/lane). RNA was transferred to nylon membranes (Genescreen Plus; DuPont-NEN) by vacuum blotting (model 785; Bio-Rad Laboratories) and ultraviolet cross-linked (Stratalinker 1800; Stratagene Inc., La Jolla, CA).

Full-length cDNAs of rat prepro-ANP (courtesy of Dr. C. Seidman, Harvard Medical School) and mouse c-*fos* (courtesy of Dr. M. Greenberg, Harvard Medical School) were labeled with [^{32}P]dCTP (DuPont-NEN) to a specific activity of 1–2 × 10^6 cpm/ng cDNA by the random hexamer priming method, and hybridized to nylon blots for 18–24 h at 42°C. Blots were washed twice (15 min, room temperature) with 300 mM NaCl/30 mM trisodium citrate (pH 7) and 0.1% SDS, and twice (15 min, 50–55°C) with 30 mM NaCl/3 mM trisodium citrate (pH 7) and 0.1% SDS. Blots were exposed to XAR film (Eastman Kodak Co., Rochester, NY) with an intensifying screen at −70°C, and films were scanned with a laser densitometer (UltraScan 2202; LKB, Uppsala, Sweden). The sizes of the hybridized messages were estimated using 18S and 28S rRNA bands as standards. All blots were reprobed with a [^{32}P]ATP-labeled oligonucleotide complimentary to 18S rRNA (23), and all levels of mRNA reported in this manuscript are normalized to the level of 18S rRNA to correct for potential variations in the amount of RNA loaded or transferred.

Results

ANP and SNAP attenuate NE-stimulated protein synthesis in myocytes. The addition of NE (1 μM) to cultured myocytes for 24 h caused a 60±9% ($P < 0.001$; $n = 7$) increase in [^{3}H]leucine incorporation (Fig. 1). Preincubation with the α_1-adrenergic receptor–selective antagonist prazosin (1 μM) inhibited this effect of NE by 89±6% ($P < 0.001$; $n = 4$), confirming that the major effect of NE on protein synthesis in neonatal rat myocytes plated at low density is mediated by α_1-
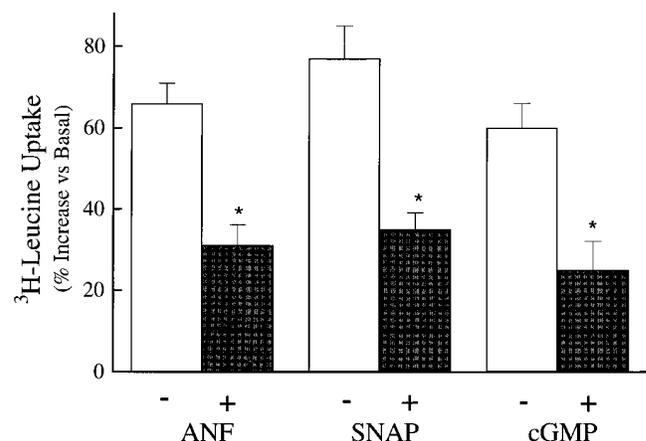
Figure 1. Effects of ANP (*ANF*), SNAP, and 8-bromo-cGMP on NE-stimulated protein synthesis in neonatal rat cardiac myocytes. Myocytes were incubated with NE (1 μM) and ANP (100 nM), SNAP (100 μM), or 8-bromo-cGMP (1 mM) for 24 h. Protein synthesis was assessed by measurement of [^{3}H]leucine uptake. ANP, SNAP, and 8-bromo-cGMP each inhibited NE-stimulated [^{3}H]leucine uptake by 53–57%. *$P < 0.002$ vs. basal uptake; $n = 15$ (ANP), 6 (SNAP), and 10 (cGMP) experiments, each performed in triplicate.

A

B
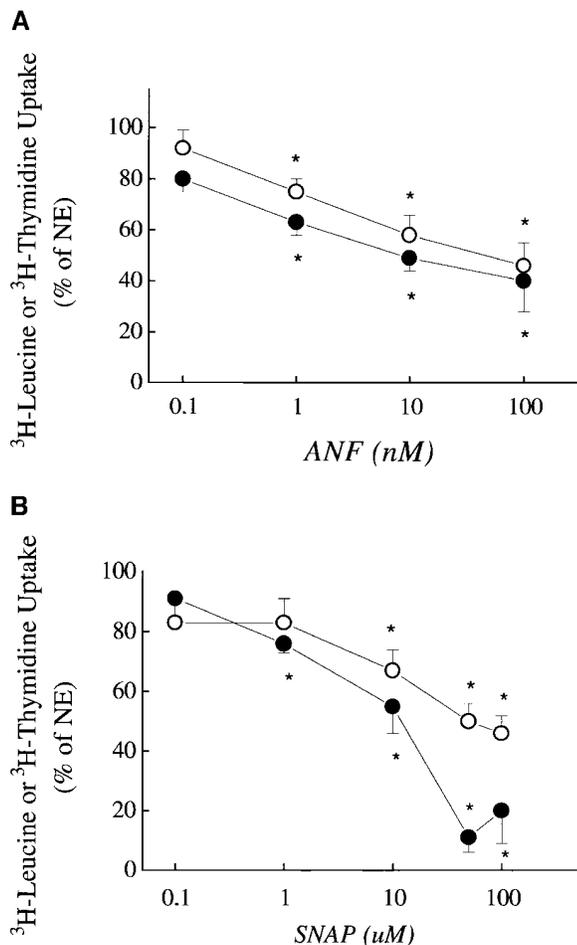
Figure 2. (*A*) Concentration dependence of ANP (*ANF*) inhibition of NE-stimulated protein synthesis in neonatal rat cardiac myocytes (○) or [³H]thymidine uptake in neonatal rat cardiac fibroblasts (●). (*B*) Concentration dependence of SNAP inhibition of NE-stimulated protein synthesis in neonatal rat cardiac myocytes (○) or neonatal rat cardiac fibroblasts (●). *$P < 0.01$ vs. basal uptake; $n = 4$–6 experiments, each performed in triplicate.

adrenergic receptors (1, 2). The addition of ANP for 24 h had no effect on basal [³H]leucine incorporation, but caused a concentration-dependent inhibition of the NE-stimulated increase (Fig. 2 *A*). At the highest concentration tested (100 nM), ANP attenuated the NE-mediated increase in [³H]leucine incorporation by 53±7% ($P = 0.001$; $n = 15$) (Fig. 1).

Similarly, addition of the NO-donor SNAP to myocytes for 24 h had no effect on basal [³H]leucine incorporation, but caused a concentration-dependent inhibition of NE-stimulated [³H]leucine incorporation (Fig. 2 *B*). At the highest concentration tested (100 μM), SNAP attenuated the NE-stimulated increase in [³H]leucine incorporation by 53±6% ($P = 0.001$; $n = 6$) (Fig. 1).
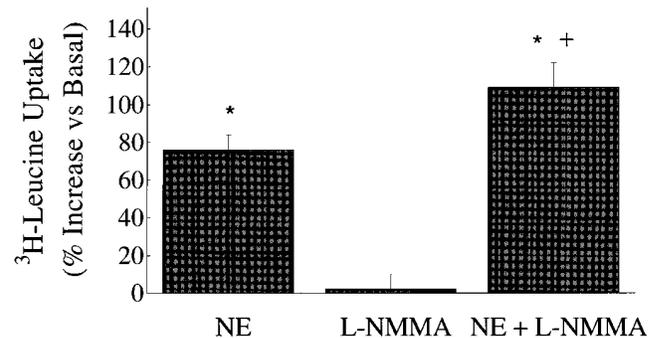
NO synthase inhibition potentiates NE-stimulated protein synthesis in myocytes. The addition of L-NMMA (1 mM) for 24 h had no effect on basal [³H]leucine incorporation in myocytes (2±8% increase vs. basal), but potentiated NE-stimulated [³H]leucine incorporation by 44±5% (NE alone, +76±8% vs. basal; NE plus L-NMMA, +109±13% vs. basal; $n = 6$; $P < 0.01$) (Fig. 3).

Role of cGMP in mediating the inhibitory effects of ANP and SNAP. In myocytes, the addition of ANP (100 nM) or SNAP (100 μM) for 30 min increased the cellular content of cGMP by two- to threefold (basal, 25±3 pmol/mg protein; ANP, 61±3 pmol/mg protein; SNAP, 81±12 pmol/mg protein; $n = 2$ for each), confirming that each is capable of stimulating cGMP production in neonatal cardiac myocytes. To determine whether cGMP can mimic the effects of ANP and SNAP, the cGMP analog 8-bromo-cGMP (1 mM) was added to cultures 15 min before NE. 8-Bromo-cGMP inhibited NE-stimulated [³H]leucine incorporation by 57±10% ($P = 0.002$; $n = 10$) (Fig. 1). In a separate set of experiments, NE-stimulated [³H]leucine incorporation was inhibited 48±9% by 8-bromo-cGMP, 59±8% by ANP, and 54±9 by the combination of ANP and cGMP ($P = $ NS; $n = 4$). Thus, the inhibitory effects of 8-bromo-cGMP and ANP were not additive.

The addition of 8-bromo-cGMP for 4 h followed by a washout immediately before the addition of NE for 24 h did not attenuate the NE-stimulated increase in [³H]leucine incorporation (NE alone, +61±24% vs. basal; NE plus 8-bromo-cGMP pretreatment, +81±34% vs. basal). Likewise, the addition of 8-bromo-cGMP during only the last 8 h of a 24 h exposure to NE did not inhibit [³H]leucine incorporation (NE alone, +47±12% vs. basal; NE plus 8-bromo-cGMP during last 8 h, +44±11% vs. basal; $P = $ NS; $n = 5$). In cardiac myocytes plated at high density (1,000 cells/mm²), 8-bromo-cGMP had no inhibitory effect on NE-stimulated [³H]leucine incorporation ($n = 2$; data not shown).

cGMP inhibits the NE-stimulated increase in c-fos mRNA in myocytes. The addition of NE (1 μM + 10 μM propranolol) to myocytes for 30 min caused a marked increase in the level of c-*fos* mRNA (Fig. 4 *A*). The addition of 8-bromo-cGMP (1 mM) for 30 min had no effect on the basal level of c-*fos* mRNA. However, the addition of 8-bromo-cGMP 15 min before the addition of NE attenuated the NE-stimulated increase in c-*fos* mRNA levels by 72±10% ($P = 0.002$; $n = 3$) (Fig. 4 *B*).

cGMP modulates the effects of NE on the levels of prepro-ANP and sarcoplasmic reticulum calcium ATPase (SERCA2) mRNA. The addition of NE (1 μM plus 10 μM propranolol) to myocytes for 24 h increased the level of prepro-ANP mRNA 11±3-fold ($n = 10$) (Fig. 5, *A* and *B*). The concurrent addition of 8-bromo-cGMP (1 mM) attenuated the NE-stimulated increase by 47±13% ($P < 0.004$ vs. NE-stimulated; $n = $

Figure 3. Potentiation of NE-stimulated protein synthesis in myocytes by inhibition of NO synthesis with L-NMMA. *$P < 0.01$ vs. basal uptake; [+]$P < 0.014$ vs. NE; $n = 6$ experiments, each performed in triplicate.
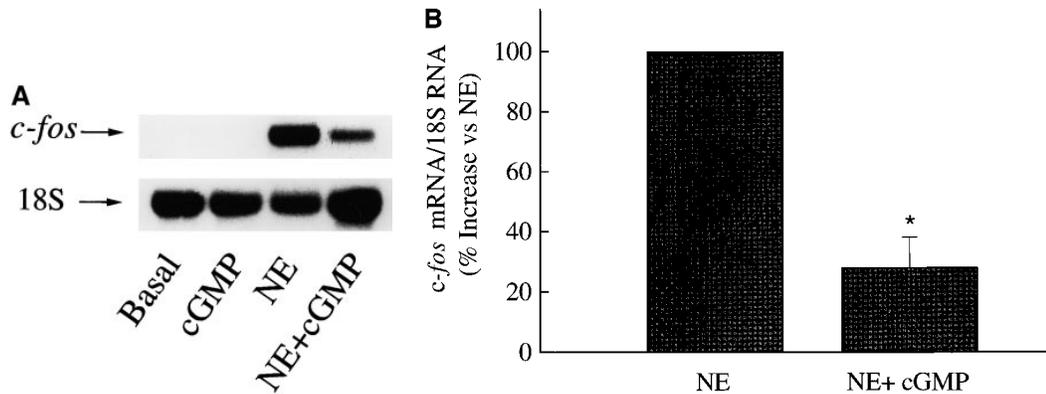
Figure 4. Inhibition of NE-stimulated c-*fos* mRNA induction by cGMP in neonatal rat cardiac myocytes. (*A*) Representative Northern hybridization depicting myocytes incubated with 8-bromo-cGMP (1 mM), NE (1 μM), or the combination for 30 min. (*B*) Mean data from three experiments as in *A*. cGMP inhibited the NE-stimulated induction of c-*fos* mRNA by 72±10%. *$P < 0.002$ vs. NE.

9) compared with cells not exposed to 8-bromo-cGMP (Fig. 5 *B*). Likewise, the concurrent addition of ANP (100 nM) attenuated the NE-stimulated increase in prepro-ANP mRNA by 42±4% compared with myocytes not exposed to ANP ($P = 0.002$ vs. NE-stimulated; $n = 3$).

The addition of NE (1 μM plus 10 μM propranolol) to myocytes for 24 h decreased the level of SERCA2 mRNA by 49±6% ($n = 6$). The concurrent addition of 8-bromo-cGMP (1 mM) (Fig. 5 *B*) or ANP (100 nM) for 24 h attenuated this effect by 60±17% ($P < 0.01$; $n = 6$) and 60% ($n = 2$), respectively.

ANP, NO, and cGMP inhibit NE-stimulated [³H]thymidine incorporation in cardiac fibroblasts. The addition of NE (1 μM) to fibroblasts for 24 h caused a 63±9% ($P < 0.001$; $n = 8$) increase in [³H]thymidine incorporation (Fig. 6). The addition of propranolol (10 μM) inhibited the NE-stimulated increase by 80±5% (12±3% increase vs. basal; $P < 0.001$; $n = 8$), confirming that under these conditions, NE-stimulated DNA synthesis is mediated predominantly by β-adrenergic receptors (5).

The addition of ANP (100 nM) had no effect on basal [³H]thymidine incorporation in fibroblasts, but the addition of

ANP 30 min before NE caused a concentration-dependent inhibition of NE-stimulated [³H]thymidine incorporation (Fig. 2 *A*). At the highest concentration tested, ANP (100 nM) attenuated NE-stimulated [³H]thymidine incorporation by 70± 8% ($P < 0.001$; $n = 11$) (Fig. 6). The addition of SNAP for 24 h caused a concentration-dependent decrease in basal [³H]thymidine incorporation by 12±4, 25±4, and 42±9% ($P = 0.02$; $n = 5$ for all) at 10, 50, and 100 μM, respectively. The addition of SNAP 15 min before NE (1 μM) likewise caused a further concentration-dependent inhibition of NE-stimulated [³H]thymidine incorporation (Fig. 2 *B*). At the highest concentration tested, SNAP (100 μM) attenuated the NE-stimulated increase in [³H]thymidine incorporation by 80±11% ($P = 0.001$ vs. SNAP alone; $n = 4$) (Fig. 6).

Role of cGMP in mediating the effects of ANP and NO in fibroblasts. ANP (100 nM) and SNAP (100 μM) increased cellular cGMP content in fibroblasts by ∼ 5- and 50-fold, respectively (basal, 51±9 pmol/mg protein/30 min; ANP, 240±6 pmol/mg protein/30 min; SNAP, 2,700±150 pmol/mg protein/30 min; $n = 2$ each). The addition of the cGMP analogue 8-bromo-cGMP (1 mM) alone for 24 h decreased basal
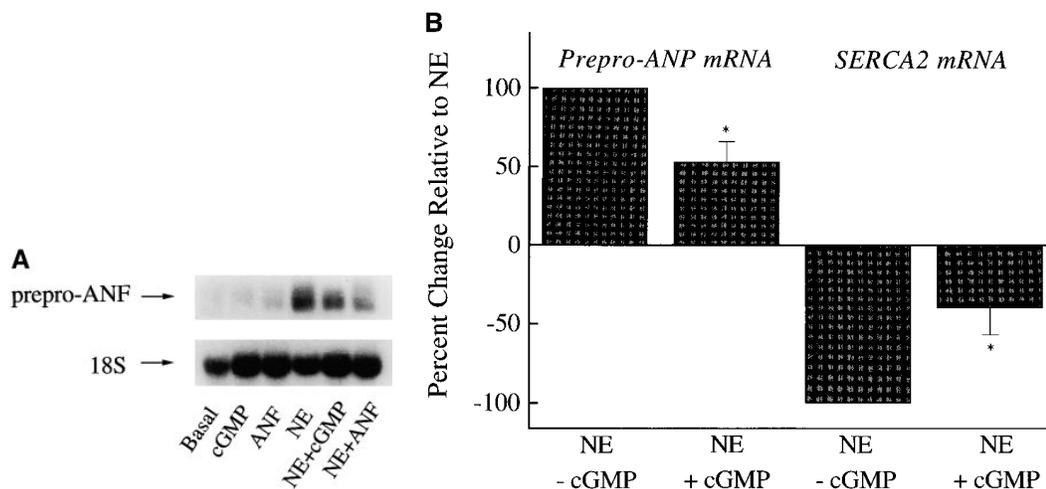
Figure 5. (*A*) Inhibition of NE-stimulated prepro-ANP (*ANF*) mRNA induction in neonatal rat cardiac myocytes by 8-bromo-cGMP and ANP (*ANF*). Northern hybridization showing the effects of a 24 h incubation with 8-bromo-cGMP (1 mM), ANP (100 nM), and NE (1 μM), alone and in combination, on the expression of prepro-ANP mRNA. (*B*) Average effects of cGMP (1 mM) on the regulation of prepro-ANP and SERCA2 mRNA by NE. Cells were incubated for 24 h with NE alone or in combination with 8-bromo-cGMP (*cGMP*). Data are presented as the percentage of the effect caused by NE alone. Depicted are the means of nine (ANP) or six (SERCA2) experiments. *$P < 0.01$ vs. NE alone.
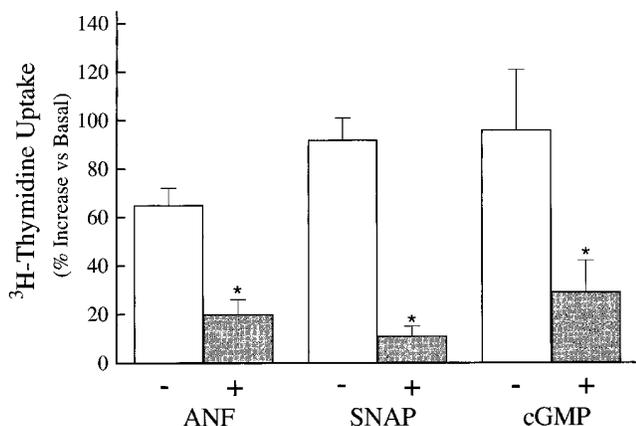
Figure 6. Inhibition of NE-stimulated DNA synthesis in neonatal rat cardiac myocytes by ANP (*ANF*), SNAP, and 8-bromo-cGMP. Fibroblasts were incubated with NE (1 μM) and ANP (100 nM), SNAP (100 μM), or cGMP (1 mM) for 24 h. DNA synthesis was assessed by the uptake of [³H]thymidine. *P < 0.001 vs. basal uptake. Depicted are the mean data from 11 (ANP), 4 (SNAP), and 5 (cGMP) experiments, each performed in triplicate.

[³H]thymidine incorporation by 35±4% ($P < 0.01$; $n = 7$). The addition of 8-bromo-cGMP 15 min before NE further inhibited the NE-stimulated increase in [³H]thymidine incorporation by 77±7% ($P = 0.001$ vs. 8-bromo-cGMP alone; $n = 5$) (Fig. 6).

Role of calcium influx in mediating the growth effects of cGMP, ANP, and NO in myocytes and fibroblasts. The addition of the L-type Ca^{2+} channel agonist BAY K8644 (1 μM) to myocytes for 24 h increased [³H]leucine incorporation by 28±6% ($n = 5$; $P = 0.01$). The addition of ANP (100 nM) inhibited BAY K8644–stimulated [³H]leucine incorporation by 70±8% ($n = 5$; $P = 0.014$). The addition of the L-type calcium channel inhibitors verapamil (10 μM) and nifedipine (10 μM) had no effect on basal [³H]leucine incorporation, but attenuated markedly the NE-stimulated increase, by 61±5% ($P < 0.001$; $n = 19$) (Fig. 7 A) and 63±4% ($P < 0.01$; $n = 3$), respectively. The inhibition of NE-stimulated [³H]leucine incorporation by verapamil was not additive with that caused by ANP (100 nM) (Fig. 7 B), SNAP (100 μM), or 8-bromo-cGMP (1 mM) (data not shown).

In fibroblasts, the addition of verapamil for 24 h decreased basal [³H]thymidine incorporation by 35±5% ($P = 0.05$; $n = 6$). The addition of verapamil or nifedipine 30 min before NE inhibited NE-stimulated [³H]thymidine incorporation by 84±4% ($P < 0.001$ vs. verapamil alone; $n = 12$; Fig. 7 A) and 65±9% ($P < 0.002$ vs. nifedipine alone; $n = 3$), respectively.

Discussion

The major new finding of this study is that ANP and NO exerted an antiadrenergic action by inhibiting the growth-promoting effects of NE in both cardiac myocytes and fibroblasts. NE stimulated protein synthesis in cardiac myocytes via activation of α_1-adrenergic receptors and stimulated DNA synthesis in cardiac fibroblasts via activation of β-adrenergic receptors. Both effects of NE were attenuated by ANP and NO. Con-

versely, inhibition of endogenous NO synthesis potentiated the magnitude of NE-stimulated protein synthesis. ANP and NO were coupled to the generation of cGMP in cardiac myocytes and fibroblasts, and cGMP mimicked the effects of ANP and NO in both cell types, suggesting that the growth-inhibiting effects of ANP and NO are mediated, at least in part, by cGMP. Consistent with these observations, we found that cGMP inhibited the reciprocal NE-stimulated changes in pre-pro-ANP (increase) and SERCA2 (decrease) mRNA levels that are characteristic of hypertrophied myocardium (24, 25). cGMP also attenuated the NE-stimulated induction of c-*fos* mRNA, indicating that its growth-inhibiting action may be mediated, at least in part, by inhibition of one or more early response genes.

There is precedent for a growth-inhibiting effect of cGMP and cGMP-generating compounds in vascular smooth muscle cells and cardiac fibroblasts. In vascular smooth muscle cells,
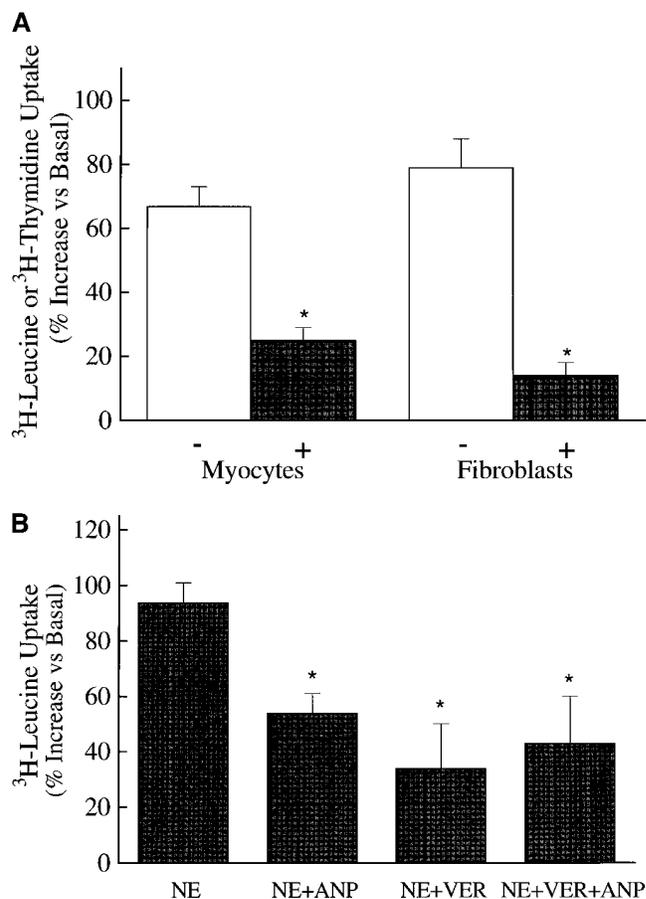

Figure 7. Effects of verapamil on NE-stimulated protein synthesis in neonatal rat cardiac myocytes and fibroblasts. (*A*) Verapamil inhibited NE-stimulated protein synthesis in myocytes and NE-stimulated DNA synthesis in fibroblasts. Cells were incubated with NE (*white bars*) or NE plus 10 μM verapamil (*black bars*) for 24 h. *P < 0.01 vs. NE alone. Depicted are the mean data from 19 experiments, each performed in triplicate. (*B*) Effects of a 24-h incubation with verapamil (*VER*; 10 μM), ANP (100 nM), and the combination, on NE-stimulated protein synthesis in neonatal rat cardiac myocytes. The combination of ANP plus verapamil had no greater effect than ANP or verapamil alone. *P < 0.001 vs. basal. Depicted are the mean data from three experiments, each performed in triplicate.

ANP, NO, and cGMP can inhibit serum-, epidermal growth factor–, or angiotensin-stimulated DNA synthesis and proliferation (6, 8, 10). NO has also been shown to inhibit serum-stimulated protein and collagen synthesis in vascular smooth muscle cells (26). In fibroblasts from neonatal rat heart, Long et al. (5) have shown that adrenergic agonists induce DNA synthesis via β- rather than α_1-adrenergic receptors, which are not expressed in this cell type (27). Our data indicate that in cardiac fibroblasts, β-adrenergic receptor–stimulated DNA synthesis is inhibited by ANP, NO, and cGMP. Recently, Cao and Gardner (9) have shown that ANP, NO, and cGMP inhibit peptide growth factor–, angiotensin-, and endothelin-stimulated DNA synthesis in cardiac fibroblasts. Therefore, our observations in cardiac fibroblasts extend the growth-inhibiting effect of cGMP-generating compounds to the β-adrenergic signaling pathway, and together with the findings in cardiac myocytes, provide the first evidence that cGMP-generating compounds exert an antiadrenergic effect on the hypertrophic actions of NE in the myocardium.

These data add to the known physiologic actions of ANP, NO, and cGMP in the myocardium. ANP acts via three receptor subtypes, termed A, B, and C, all of which have been identified in adult rat myocardium (28), and cardiac myocytes and fibroblasts isolated from adult rat heart (11). In neonatal rat cardiac myocytes, ANP inhibits both the amplitude of spontaneous contractions and Ca^{2+} influx (29). Likewise, NO, which couples to the generation of cGMP, can exert a direct negative inotropic effect on the myocardium (30). Further, it has been shown that cGMP and NO can inhibit the contractile responses to both α_1- and β-adrenergic receptor stimulation (13, 31). It has been suggested that the negative inotropic effects of cGMP, ANP, and NO are due to inhibition of Ca^{2+} influx via the L-type Ca^{2+} channel (16, 17). In contrast to amphibian cells, in which this effect is due to activation of a phosphodiesterase with a resultant decrease in cAMP, in mammalian cells, the effect of cGMP to inhibit Ca^{2+} influx does not depend on changes in cAMP, but rather appears to reflect a direct effect on the channel (16–18).

Our data suggest that the growth-inhibiting effects of cGMP, ANP, and NO in cardiac myocytes and fibroblasts were mediated in part by the inhibition of Ca^{2+} influx. First, in myocytes, protein synthesis stimulated by the Ca^{2+} channel agonist BAY K8644 was inhibited by ANP. Second, the growth-inhibiting effects of ANP and NO were mimicked both qualitatively and quantitatively by the Ca^{2+} channel antagonists verapamil and nifedipine. Finally, the effects of verapamil and nifedipine were not additive with those of ANP, NO, or cGMP in myocytes or fibroblasts.

The role of Ca^{2+} influx in mediating the myocyte growth response to NE is not clearly established, in part due to the technical difficulty of dissociating direct effects of Ca^{2+} from indirect effects due to cellular contraction (32). However, in neonatal rat cardiac myocytes, NE causes cellular growth and induction of prepro-ANP mRNA primarily via activation of α_1-adrenergic receptors of the pharmacological A subtype (2), which are coupled to an increase in L-type Ca^{2+} channel current in cardiac myocytes (15). Likewise, it has been shown that Ca^{2+} influx is essential for the α_1-adrenergic receptor–stimulated induction of prepro-ANP mRNA (14). In cardiac fibroblasts, β-adrenergic receptor stimulation, presumably acting via cAMP, also increases the L-type Ca^{2+} channel current (33). Booz et al. (34) observed that angiotensin-stimulated DNA

synthesis is mediated, at least in part, via a Ca^{2+}-dependent pathway in neonatal rat cardiac fibroblasts.

Since myocytes exhibit spontaneous contractions under the conditions of these experiments, we cannot determine whether the growth-inhibiting effects we observed were due to a direct decrease in intracellular Ca^{2+} or an indirect effect of reduced contractions. However, since fibroblasts do not contract spontaneously under these conditions, we can conclude that in these cells, the growth-promoting effect of Ca^{2+} influx is not related to contraction, and most likely reflects a direct biochemical action. We cannot exclude a role for non-cGMP–mediated effects of ANP or NO. In cardiac fibroblasts, ANP inhibition of serum-stimulated DNA synthesis appears to be mediated not only by the A and B subtypes, but also by the C subtype (9), which is not coupled to cGMP, and there is evidence for an antimitogenic effect of the C subtype in rat aortic smooth muscle and glial cells (35, 36).

Recently, Zheng et al. (37) observed that ATP inhibits hypertrophy stimulated by NE, basic fibroblast growth factor, and endothelin-1 in neonatal cardiac myocytes. It is noteworthy that the effects of ATP and cGMP on α_1-adrenergic receptor–stimulated hypertrophy differ in several ways. First, they exhibit markedly different temporal requirements. Whereas a transient preexposure to ATP inhibits the subsequent ability of α_1-adrenergic receptor stimulation to cause hypertrophy, cGMP must be present continuously with NE to exert its inhibitory action. Second, whereas ATP itself stimulates c-fos mRNA and does not inhibit the stimulation by NE, cGMP has no effect by itself and inhibits markedly the stimulation caused by NE. Third, cGMP but not ATP inhibits the ability of NE to downregulate the level of SERCA2 mRNA. These differences suggest that the two nucleotides inhibit α_1-adrenergic receptor–stimulated myocyte growth by distinct mechanisms.

In contrast to our findings, Harding et al. (38) found that phenylephrine-stimulated protein synthesis in neonatal rat cardiac myocytes was not inhibited by cGMP. Of note, we have found that the inhibitory effect of cGMP is absent in cells cultured at high density (1,000 cell/mm^2), suggesting that this discrepancy may reflect our use of low density (100–200 cells/mm^2) cultures as opposed to the high density (1,000 cells/mm^2) cultures used by Harding et al. (38). This thesis is supported by evidence that cell density can exert qualitative effects on the pathways that mediate NE-stimulated protein synthesis in neonatal rat cardiac myocytes (39).

The implications of these findings remain to be demonstrated. However, they raise the possibility that NO, ANP, and other cGMP-generating compounds could attenuate the effects of NE on the growth and phenotype of the myocardium. Such antiadrenergic actions might play an adaptive, counterregulatory role by acting in an autocrine or paracrine manner to limit the adverse effects (e.g., interstitial fibrosis and fetal gene induction) of increased sympathetic tone on the myocardium. This thesis is supported by a recent report from Wu et al. (40) that ANP stimulates apoptosis in neonatal rat cardiac myocytes and that this effect is abolished by norepinephrine, which by itself has no affect on apoptosis in this system.

Although ANP expression is minimal or absent in normal ventricular myocardium, several hypertrophic stimuli induce natriuretic peptides (23). Furthermore, expression of constitutive NO synthase (NOS3) and NO production are present in cardiac myocytes (13), and cardiac NO production may be increased due to induction of inducible NO synthase (NOS2)

in pathological states such as myocardial failure (41). Our finding that inhibition of NO synthesis by L-NMMA potentiated NE-stimulated protein synthesis in myocytes suggests that basal levels of endogenous NO production could modulate myocyte growth. Consistent with this view, myocardial hypertrophy and fetal gene expression were attenuated by chronic administration of the NO precursor L-arginine in the spontaneously hypertensive rat (42). Conversely, it is possible that excessive activation of ANP, NO, and other cGMP-generating pathways could be maladaptive by suppressing the requisite myocardial response to adrenergic or other hypertrophic stimuli. Understanding the physiologic and pathophysiologic effects of NO, natriuretic peptides, and other cGMP-generating compounds in the myocardium may allow the development of new strategies for modulating the growth and phenotype of the myocardium.

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