Differential Effects of cGMP Produced by Soluble and Particulate Guanylyl Cyclase on Mouse Ventricular Myocytes

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Particulate guanylyl cyclase (pGC) and soluble guanylyl cyclase (sGC) are cGMP-generation systems distributed in different intracellular locations. Our aim was to test the hypothesis that the functional effects of cGMP produced by pGC and sGC on contraction and Ca^{2+} transients would differ in ventricular myocytes. We measured myocyte shortening from adult mice using a video edge-detector and investigated the functional changes after stimulating pGC with C-type natriuretic peptide (CNP; 10^{-8} *M* and 10^{-7} *M*) or sGC with S-nitroso-*N*-acetyl-penicillamine (SNAP; nitric oxide donor; 10^{-6} *M* and 10^{-5} *M*). Significant concentration-dependent decreases in percentage shortening (PCS), maximal rate of shortening (RSmax), and relaxation (RRmax) were produced by CNP. To a similar degree, SNAP concentration-dependently reduced PCS, RSmax, and RRmax. The addition of Rp-8-[(4-chlorophenyl)thio]-cGMPS triethylamine (cGMP-dependent protein kinase inhibitor; 5×10^{-6} *M*) or erythro-9-(2-hydroxy-3-nonyl) adenine (cGMP-stimulated cAMP phosphodiesterase inhibitor; 10^{-5} *M*) reduced the responses induced by CNP or SNAP, suggesting that their actions were through cGMP-mediated pathways. While SNAP significantly increased intracellular cGMP concentration by 57%, CNP had little effect on cGMP production. We also found that CNP markedly decreased the amplitude of Ca^{2+} transients while SNAP had little effect, suggesting the cGMP generated by sGC may decrease myofilament Ca^{2+} sensitivity. The small amount of cGMP generated by pGC had a major effect in reducing Ca^{2+} level. This study suggested the existence of compartmentalization for cGMP in ventricular myocytes. Exp Biol Med 230:242–250, 2005

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An important intracellular second messenger that mediates many signaling events from phototransduction to contractile activity is cGMP. It has been established that increases in the intracellular concentration of cGMP usually exert negative metabolic and functional effects in both myocardium and isolated cardiac myocytes from many species (1–3) and cause reductions in local metabolism, force development, and inotropic and contractile duration (1, 3, 4). The specificity of these cellular responses to cytosolic cGMP is dictated by cGMP-binding motifs present in target proteins. In myocardium, there are at least two groups of proteins with allosteric sites for binding cGMP: the cGMP-dependent protein kinase and the cGMP-regulated phosphodiesterases (2, 3, 5). These effectors mediate almost all cardiac effects of cGMP in the heart (3, 5).

Intracellular cGMP is synthesized by guanylyl cyclases in response to both endogenous and exogenous compounds such as nitric oxide, natriuretic peptides or nonpeptide hormones, neurotransmitters, and toxins. There are only two forms of guanylyl cyclase in eukaryocytes: soluble guanylyl cyclase (sGC) and particulate guanylyl cyclase (pGC) (6). The existence of distinct particulate and cytosolic sources suggests that cGMP production may not be distributed uniformly within cells. In addition, diffusion of cGMP will likely be limited by the presence of a few different, ubiquitously expressed phosphodiesterases that have 12 subfamilies and may be able to hydrolyze cGMP. Some of the phosphodiesterases and their isomers are soluble, while others are bound to plasma and intracellular membrane (7). The separate subcellular sources of cGMP and the presence of cGMP phosphodiesterases support the possibility of localized elevation of cGMP within the cell.

It has been suggested that the different sources of cGMP have distinct mechanisms and differential effects in some cell types (8–10). In the present study, we tested the

hypothesis that the negative inotropic effects of cGMP elevation induced by activated pGC and sGC would have differential effects in cardiac myocytes. To test this hypothesis, we isolated mouse ventricular myocytes and used a video edge-detection technique to study the functional changes induced by the activation of these two cGMP-generation systems with C-type natriuretic peptide (CNP) and a nitric oxide donor, S-nitroso-*N*-acetyl-penicillamine (SNAP), respectively. We also examined whether the actions of CNP and SNAP were mediated by cGMP protein kinase or cGMP-affected cAMP phosphodiesterases through blocking their activity with their specific inhibitors. The effect of CNP and SNAP on intracellular cGMP production and Ca^{2+} transients on freshly isolated ventricular myocytes from adult mice was also investigated.

Materials and Methods

All experiments were conducted in accordance with the Guide for the Care of Laboratory Animals (DHHS Publications No. 85-23, revised 1996) and approved by our Institutional Animal Care and Use Committee. All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO), unless otherwise stated.

Myocyte Isolation. Ten adult mice (strain C57BL/6J, 6–8 weeks old, either gender) from Jackson Laboratory (Bar Harbor, ME) were used in the study. Freshly isolated ventricular myocytes were prepared from this laboratory by a standard protocol as previously described (11), with limited modifications. Briefly, the animals were anesthetized and the hearts were rapidly removed after ip injection of an overdose of pentobarbital (100 mg/kg). The aortas were rapidly cannulated and the hearts were subsequently mounted on a Langendorff perfusion apparatus. Aortic retrograde perfusion was initiated at 70 mm Hg constant pressure with modified minimal essential medium (MEM) supplemented with 10 m*M* taurine, 2 m*M* L-glutamic acid, and 20 m*M* HEPES, pH 7.35. After two mins, the hearts were perfused with MEM containing 0.1% type II collagenase (Worthington Biochemical Corp., Freehold, NJ) for 10 mins. The perfusion buffer was maintained at 37°C and equilibrated with a water-saturated gas mixture (95% O_2 and 5% CO_2). After collagenase digestion, the hearts were removed from the apparatus and the ventricles were cut into small pieces. The tissue was subsequently pipetted in the 0.1% collagenase solution until all of the large pieces were dispersed in the cell suspension. The supernatant containing isolated myocytes was then transferred to 15-ml centrifuge tubes. After washing three times in MEM supplemented with 0.5% bovine serum albumin (BSA), the cells were finally suspended in the media with 1 m*M* $CaCl_2$ at room temperature. Cell viability was assessed by a standard of maintenance of rod-shaped morphology and routinely is about 50%–70% in our laboratory.

Cell Contraction Measurement. Details of the analysis of myocyte function have been previously reported (12). Briefly, isolated cardiac myocytes were put into a chamber on the stage of an inverted microscope (Zeiss Axiovert 125; Carl Zeiss Inc. Thornwood, NY) in 2 ml MEM solution supplemented with 0.5% BSA and 2 m*M* $CaCl_2$. Two platinum wires placed in the chamber were used to pace the myocytes (1 Hz, 5-msec duration, voltage 10% above threshold, and polarity alternated with each pulse). The chamber was maintained at 37°C throughout the measurements. The contraction of individual myocytes was measured with a Myotrack system containing a camera and a video edge-detector (Matec Instruments, Northborough, MA). The contraction data were continuously obtained on a television monitor and a desktop computer. The parameters obtained during a single contraction included the diastolic cell length, percentage shortening (PCS), maximal rate of relaxation (RRmax), maximal rate of shortening (RSmax), time to peak shortening, time to 50% relaxation, and time to 90% relaxation.

Intracellular Ca^{2+} Transient Measurements. The ventricular myocytes were loaded with two μ*M* fura-2 acetoxymethyl ester (Molecular Probes, Eugene, OR) for 60 mins at 37°C in MEM buffer supplemented with 1 m*M* $CaCl_2$, 0.5% BSA. Then, cells were washed and resuspended in the same buffer. The fluorescence was measured with a dual-excitation fluorescence photomultiplier system (IonOptix Corp., Milton, MA). Myocytes were placed in a 37°C chamber on the stage of a Nikon inverted microscope (model TS100; Melville, NY) and imaged through a fluor 40× objective. The cells were exposed to light excitation at 360 or 380 nm wavelengths while being stimulated to contract at 1 Hz, 5-msec duration, voltage 10% above threshold, and polarity alternated with each pulse. Fluorescence emission was detected between 480 and 520 nm by a photomultiplier tube and qualitative changes in intracellular concentration were determined from the ratio of the fluorescence intensity at 360/380 wavelengths. Each experiment was performed in at least three myocytes from each animal, and nine animals were used.

Experimental Protocol. The following protocol was used for the myocyte shortening measurements. After the myocytes were placed in the measurement system, a stabilization period of 3 mins was allowed before the contraction data for the individual ventricular myocytes were recorded. A 3-min interval was assigned between reagent treatments, and 10 consecutive contractions were used for analysis. In the first group, CNP (a pGC activator) was added with a final concentration of 10^{-8} *M*, followed by a higher concentration of CNP (final concentration of 10^{-7} *M*). In the second group, the nitric oxide donor SNAP (an sGC activator) was added with the final concentration of 10^{-6} *M*, followed by a higher concentration of SNAP (final concentration of 10^{-5} *M*). In the third group, Rp-8-[(4-chlorophenyl)thio]-cGMPS triethylamine (Rp-8-pCPT-cGMP; a specific inhibitor of the cGMP protein kinase) was administered to the myocytes at a concentration of 5×10^{-6} *M*, and then CNP or SNAP was added at final

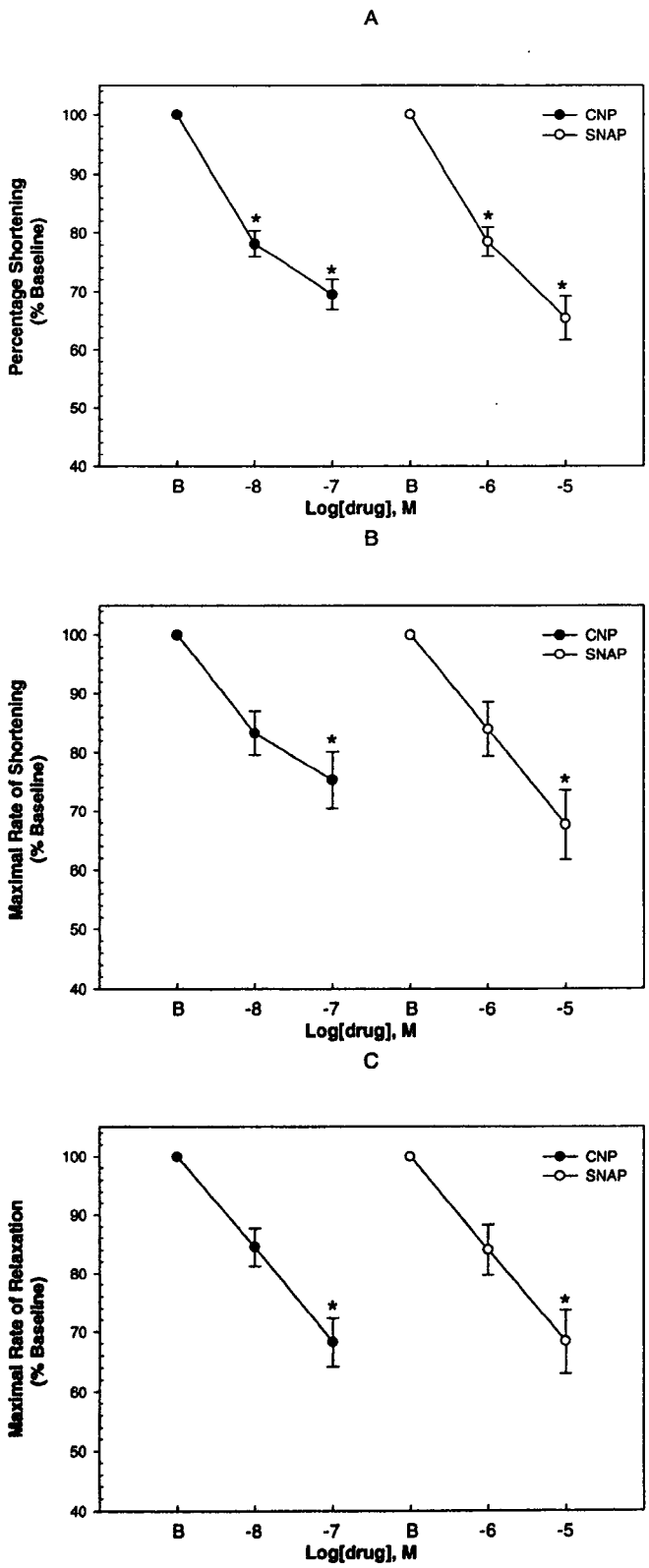

Figure 1. Effects of C-type natriuretic peptide (CNP) and S-nitroso-*N*-acetyl-penicillamine (SNAP) on myocyte percentage shortening (A), maximal rate of shortening (B), and maximal rate of relaxation (C) in freshly isolated ventricular myocytes ($N = 10$). Data are mean ± SEM. B, baseline, * $P < 0.05$ vs. baseline. There was no statistical difference between CNP and SNAP.

concentration of 10^{-7} *M* or 10^{-5} *M*, respectively. In the fourth group, erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA; an inhibitor of the cGMP-stimulated cAMP phosphodiesterase) was given at a concentration of 10^{-5} *M*, and then CNP or SNAP was added at a final concentration of 10^{-7} *M* or 10^{-5} *M*, respectively. In the fifth group, guanylin (a stimulator of particulate guanylyl cyclase) was added, with a final concentration of 10^{-7} *M*. In the sixth group, carbon monoxide (CO; a stimulator of soluble guanylyl cyclase) was added to produce a final concentration of 8×10^{-6} *M* (13). These concentrations were chosen in preliminary experiments to produce significantly reduced myocyte function. For all groups, a 3-min interval was allowed between treatments. Each group was repeated at least two more times for each animal ($N = 10$).

Measurement of Intracellular cGMP Concentration. The ventricular myocytes were used for the cGMP measurements. The same experimental manipulations described in the protocol were performed on the myocytes incubated in a 50-ml tube following the same treatments (e.g., concentration, temperature, electrical stimulation). After completion of each respective treatment, the myocytes were rapidly frozen in liquid nitrogen and then stored at –80°C for later cGMP determination. Three samples were collected from each mouse for the cGMP assay and included control, CNP 10^{-7} *M*, and SNAP 10^{-5} *M*. Samples were collected after a 5-min exposure. The samples were warmed to 0°C and the cells were homogenized in ethanol using a Brinkmann Polytron (Brinkmann Instruments, Westbury, NY) in an ice bath and then subjected to centrifugation at 30,000 *g* for 15 mins in a Sorvall RC-5B Centrifuge (Brinkmann Instruments). The supernatant was collected and the pellet was washed once. The combined supernatant was then evaporated to dryness in a 60°C bath under nitrogen gas. The residue was dissolved in 1.5 ml assay buffer (0.05 *M* sodium acetate; pH 5.8; containing sodium azide). Radioimmunoassay using a scintillation proximity assay determined cGMP (Amersham Biosciences, Piscataway, NJ). The assay measures the competitive binding of [^{125}I]labeled cGMP to a cGMP-specific antibody. After construction of a standard curve, cGMP levels were determined directly from the counts in pmols divided by the number of myocytes per tube times 100,000.

Statistical Analysis. A repeated-measure ANOVA using myocytes and treatments was used to compare variables measured in the experiment. Duncan's multiple range test was used to determine differences between baseline and various treatments. A value of $P < 0.05$ was used as the level of statistical significance. All values are expressed as mean ± SEM.

Results

Effects of CNP and SNAP on Myocyte Contraction. For cell contraction, the basal PCS (%) was 3.3 ± 0.1. When CNP was added, CNP decreased cell PCS in

Table 1. Effects of CNP and SNAP on Resting Cell Length, Time to Peak Shortening, Time to 50% Relaxation, and Time to 90% Relaxation[a]

	Resting cell length (μm)	TTP (secs)	T50 (secs)	T90 (secs)
Baseline	139.4 ± 3.1	0.39 ± 0.02	0.08 ± 0.01	0.36 ± 0.02
CNP				
10^{-8} *M*	140.9 ± 3.04	0.36 ± 0.02	0.10 ± 0.01	0.37 ± 0.02
10^{-7} *M*	141.1 ± 3.3	0.44 ± 0.03	0.12 ± 0.01	0.42 ± 0.03
Baseline	132.4 ± 4.1	0.38 ± 0.02	0.08 ± 0.01	0.35 ± 0.02
SNAP				
10^{-6} *M*	134.9 ± 4.2	0.38 ± 0.01	0.09 ± 0.01	0.35 ± 0.01
10^{-5} *M*	135.4 ± 4.2	0.39 ± 0.02	0.12 ± 0.01	0.39 ± 0.02

[a] CNP, C-type natriuretic peptide; SNAP, S-nitroso-*N*-acetyl-penicillamine; TTP, time to peak shortening; T50, time to 50% relaxation; T90, time to 90% relaxation.

a concentration-dependent pattern (Fig. 1A). In the presence of 10^{-5} *M* CNP, the PCS was depressed by 34%. The baseline PCS before SNAP treatment was 3.2 ± 0.1. To a similar degree, SNAP also dampened cell PCS concentration dependently, and the PCS was depressed by 33% at 10^{-5} *M*. The basal RSmax (μm/sec) for CNP and SNAP were 58.6 ± 2.2 and 56.1 ± 3.9, respectively. The basal RRmax (μm/sec) for CNP and SNAP were 57.6 ± 2.0 and 55.9 ± 3.8, respectively. Both reagents similarly decreased maximal rate of shortening and relaxation dose responsively in a parallel pattern (Fig. 1B and C). There was no significant difference between the effects of CNP and SNAP. Neither CNP nor SNAP significantly changed other cell contraction parameters such as diastolic cell length, time to peak shortening, time to 50% relaxation, and time to 90% relaxation (Table 1).

Stimulation of cGMP Synthesis in Ventricular Myocytes. To investigate whether the similar negative inotropic responses to CNP and SNAP were caused by a similar amount of cGMP produced endogenously, intracellular cGMP was measured (Fig. 2). A concentration of 10^{-5} *M* of SNAP significantly increased intracellular cGMP concentration by 57% above the basal level of 100.3 ± 22.5 pmol/10^8 myocytes. At a concentration of 10^{-7} *M*, CNP stimulation also promoted cGMP production by 12%, but the increment was not statistically significant.

Effects of Rp-8-pCPT-cGMPS on the Negative Cardiac Responses to CNP and SNAP. To investigate whether the negative inotropic effect of CNP and SNAP acted through the cGMP-dependent protein kinase, Rp-8-pCPT-cGMPS was used to specifically block enzyme activation. At a concentration of 5×10^{-6} *M*, Rp-8-pCPT-cGMPS abolished the negative inotropic effects of CNP in terms of PCS (Fig. 3A), RSmax, and RRmax (Table 2). In a similar pattern, Rp-8-pCPT-cGMPS also blocked the cellular responses to 10^{-5} *M* SNAP including PCS (Fig. 3B), RSmax, and RRmax (Table 2).

Effects of EHNA on the Negative Cardiac Responses to CNP and SNAP. To test whether the negative inotropic responses to CNP and SNAP were mediated by the cGMP-affected cAMP phosphodiesterases, EHNA, an inhibitor of the cGMP-stimulated cAMP phosphodiesterase, was used to treat myocytes. At the concentration of 10^{-5} *M*, EHNA blocked the myocyte responses to 10^{-7} *M* CNP in terms of PCS, RSmax, and RRmax (Table 3). In a similar pattern, EHNA also blocked the cellular contractile responses to 10^{-5} *M* SNAP including PCS, RSmax, and RRmax (Table 3). There were no significant differences between the effects of CNP and SNAP on cell contraction.

Effects of Guanylin and CO on Myocyte Contraction. Because guanylin and CO also stimulate pGC and sGC, respectively, we tested whether their effects were similar to CNP and SNAP. At the concentration of 10^{-7} *M*, guanylin significantly decreased cell PCS by 28% (Table 4) and RSmax by 21%. At the concentration of 8×10^{-6} *M*, CO also decreased myocyte PCS by 30% and RSmax by 28%. There was no significant difference between the effects of CO and guanylin.

Effects on Intracellular Ca^{2+} Transients. To examine the potential mechanism of action for pGC and sGC, we studied the effects of several agents that stimulate both on intracellular Ca^{2+} transients. Representative traces of Ca^{2+} transients are shown in Figure 4 (upper panel),

Table 2. Effects of Rp-8-pCPT-cGMPS on the RSmax and RRmax Induced by CNP and SNAP[a]

	RSmax (% baseline)		RRmax (% baseline)	
	CNP (10^{-7} *M*)	SNAP (10^{-5} *M*)	CNP (10^{-7} *M*)	SNAP (10^{-5} *M*)
Control	75.3 ± 4.8[b]	67.7 ± 5.9[b]	68.2 ± 4.1[b]	68.3 ± 5.4[b]
Rp-8-pCPT-cGMPS	99.2 ± 3.4	89.1 ± 4.8	98.8 ± 3.9	89.5 ± 4.0

[a] Values are mean ± SEM. RSmax, maximal rate of shortening; RRmax, maximal rate of relaxation; CNP, C-type natriuretic peptide; SNAP, S-nitroso-*N*-acetyl-penicillamine.
[b] Significantly different from baseline. There was no significant difference between CNP and SNAP.

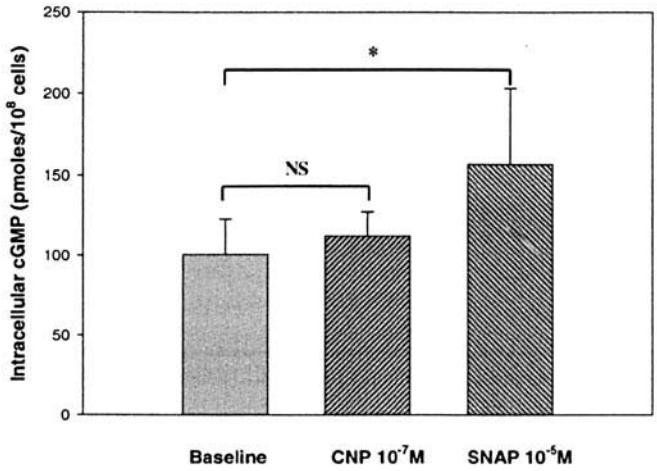

Figure 2. Effect of C-type natriuretic peptide and S-nitroso-*N*-acetyl-penicillamine on intracellular cGMP production in ventricular myocytes (N = 5). NS, not significant vs. baseline. *P < 0.05 vs. baseline.

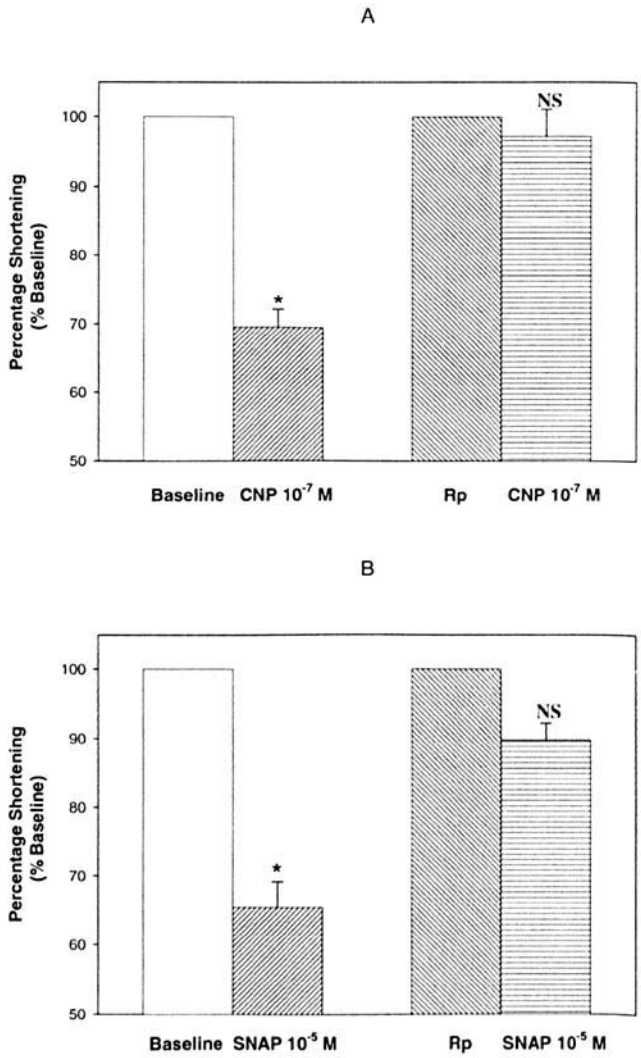

Figure 3. Effects of Rp-8-pCPT-cGMPS on the myocyte contractile responses to C-type natriuretic peptide (CNP) (A) and S-nitroso-*N*-acetyl-penicillamine (SNAP) (B) (N = 10). The baseline percentage shortening (PCS) in the absence of Rp-8-pCPT-cGMPS was 3.3 ± 0.1 (A). The PCS in the presence of Rp-8-pCPT-cGMPS was 3.0 ± 0.2. The baseline PCS in the absence of Rp-8-pCPT-cGMPS was 3.3 ± 0.1 (B). The baseline PCS in the presence of Rp-8-pCPT-cGMPS was 2.7 ± 0.2. NS, not significant vs. the value in the presence of Rp-8-pCPT-cGMPS. *P < 0.05 vs. the baseline value in the absence of Rp-8-pCPT-cGMPS. There was no statistically significant difference between CNP and SNAP.

demonstrating that only stimulating pGC (10^{-7} M CNP) decreased the amplitude of the Ca^{2+} transients, although stimulating both cyclases reduced cell shortening (Fig. 4, lower panel). Agents that stimulated pGC (i.e., CNP, guanylin), not sGC (i.e., SNAP, CO), decreased the percentage peak, the rate of development, and the return rate of the Ca^{2+} transients (Table 5). The resting fluorescence intensity ratio (360/380 nm) was not significantly changed across the experiment.

Discussion

The first major finding of the present study was that activation of both particulate and soluble guanylyl cyclase exerted similar negative inotropic effects on mouse ventricular myocytes. These effects on cell contraction were mediated by cGMP-dependent pathways involving the cGMP protein kinase and cGMP-affected cAMP phosphodiesterases. Blockage of either of these pathways abolished the cell responses to CNP or SNAP. Second, CNP and SNAP increased intracellular cGMP to different levels at concentrations that produced the same change in contraction, suggesting potential spatial distribution differences in cGMP production. Finally, CNP decreased Ca^{2+} transients, whereas SNAP had marginal effects on transients, further suggesting that compartmentalization of cGMP production may exist in ventricular myocytes.

Myocardial functional effects of both natriuretic peptides and nitric oxide have been observed in various studies. The administration of CNP has been recently reported to decrease cardiac contraction in the isolated, perfused, working mouse heart preparations (14) and cultured neonatal rat cardiac myocytes (15). Our observations of concentration-dependent negative inotropic effects of CNP are consistent with these studies. However, there have also been reports of positive inotropic effects in some studies (14, 16). These differences could be related to the preparations, cell types, concentrations, or factors present in coronary circulation that may modify the effect of CNP on the myocyte. Others have reported both positive and negative effects of nitric oxide (5, 17, 18). The positive effects may be related to increases in cAMP due to inhibition of phosphodiesterases, differential effects from NOS1 vs. NOS3 production, or nitrosylation-induced leakage at ryanodine receptors (5, 17, 18). We observed that SNAP, a nitric oxide donor, inhibited myocyte function in a dose-dependent pattern (11). In addition, the effects of both CNP and nitric oxide were verified by the use of

Table 3. Effects of EHNA on PCS, RSmax, and RRmax Induced by CNP and SNAP[a]

	PCS (% baseline)		RSmax (% baseline)		RRmax (% baseline)	
	CNP (10^{-7} *M*)	SNAP (10^{-5} *M*)	CNP (10^{-7} *M*)	SNAP (10^{-5} *M*)	CNP (10^{-7} *M*)	SNAP (10^{-5} *M*)
Control	69.5 ± 2.6[b]	65.4 ± 3.8[b]	75.3 ± 4.8[b]	67.7 ± 5.9[b]	68.2 ± 4.1[b]	68.3 ± 5.4[b]
EHNA	90.7 ± 2.2	92.1 ± 4.0	90.6 ± 1.8	90.1 ± 2.9	90.9 ± 2.0	92.5 ± 3.3

[a] Values are mean ± SEM. EHNA, erthro-9-(2-hydroxy-3-nonyl) adenine; PCS, percentage shortening; RSmax, maximal rate of shortening; RRmax, maximal rate of relaxation; CNP, C-type natriuretic peptide; SNAP, S-nitroso-*N*-acetyl-penicillamine.
[b] Significantly different from baseline. There was no significant difference between CNP and SNAP.

guanylin and CO, which stimulate pGC (6) and sGC (19), respectively, and increase cGMP levels in mammalian ventricular myocytes (13). These data suggest that CNP and nitric oxide similarly depress myocyte function through the guanylyl cyclase-cGMP signaling axis.

Cardiac function can be damped by cGMP through different pathways. In this study, we observed that the contractile responses to CNP and SNAP were mediated by cGMP-dependent pathways because the blockade of either the cGMP-dependent protein kinase or the cGMP-stimulated cAMP phosphodiesterase with specific inhibitors abolished their negative inotropic effects on cell contraction. Although the phosphodiesterase inhibitor EHNA may lead to the accumulation of adenosine because of its inhibition of adenosine deaminase (20), we do not think this would be important in a well-oxygenized environment. In addition, adenosine does not regulate intrinsic myocardial contraction in most animals, including mice (21). The observation here that CNP and nitric oxide act through cGMP-dependent pathways is concordant with many investigations in various animal models (5, 14–16).

Particulate and soluble guanylyl cyclase, although producing the same cyclic nucleotide, are located in different spatial subcellular locations, with one in subsarcolemmal areas and the other distributed in cytosol (6). In our study, we found that stimulation of sGC by SNAP produced a more than 50% increase of intracellular cGMP, whereas stimulation of pGC by CNP only has a marginal effect in increasing total cGMP levels. Figure 5 illustrates the potential difference between activation of pGC and sGC. Similar to our study, it had been reported that atrial natriuretic peptide (ANP) was markedly less effective in inducing cGMP synthesis than a nitric oxide donor in the kidney glomeruli (22). However, CNP was more potent in promoting cGMP formation than SNAP in cultured smooth muscle cells (23). Intracellular cGMP was increased by CNP in *ex vivo* mouse heart (14). These differences might be related to species, differential cell types, or drug concentrations. The concentration of intracellular cGMP (about 1 pmol/10^6 cells) in the isolated myocytes in our study was at the similar level to a previous report studying the effects of ANP in myocardium (24). The fact that CNP only marginally increased cGMP, yet produced the same negative functional effects as SNAP, suggests localization of the second messenger near its effectors.

At the concentrations used, stimulation of pGC and sGC with CNP and SNAP induced a similar negative inotropic response in the ventricular myocyte. It is clear that two cGMP-dependent pathways, cGMP protein kinase and cGMP-affected cAMP phosphodiesterases, mediated both actions of CNP and SNAP. These data exclude any major role for non-cGMP pathways in the process. These observations suggest the cGMP produced by pGC and sGC had differential effects in regulating myocyte function; namely, the small amount of cGMP produced by pGC near the sarcolemma was much more efficient in decreasing cell contraction than the cGMP produced by sGC, which was found throughout the cytosol. This suggests compartmentalization of cGMP production and/or effects (Fig. 5). The possibility of spatially distributed effects of cGMP was recently raised (10). Other evidence suggests that stimulation of these two cGMP-generation systems produced differential effects, including Ca^{2+} regulation and blockage of β-adrenergic signaling in various tissues (8–10).

Calcium levels and sensitivity are central to cardiac contraction, and cGMP can regulate cardiac myocyte contraction *via* several mechanisms that can be broadly classified as lowering either intracellular calcium level ($[Ca^{2+}]_i$) or the force developed for a given $[Ca^{2+}]_i$. A number of cGMP pathways have been found to decrease $[Ca^{2+}]_i$. One is reducing Ca^{2+} entry through L-type Ca^{2+} channel ($I_{Ca^{2+}}$) *via* cGMP protein kinase–dependent phos-

Table 4. Effects of Guanylin and CO on PCS and RSmax of Ventricular Myocyte[a]

	PCS (% baseline)	RSmax (% baseline)
Guanylin (10^{-7} *M*)	71.9 ± 3.2[b]	79.9 ± 2.6[b]
CO (7×10^{-6} *M*)	69.6 ± 4.2[b]	70.6 ± 3.0[b]

[a] Values are mean ± SEM. CO, carbon monoxide; PCS, percentage shortening; RSmax, maximal rate of shortening.
[b] Significantly different from baseline. There was no significant difference between guanylin and CO.

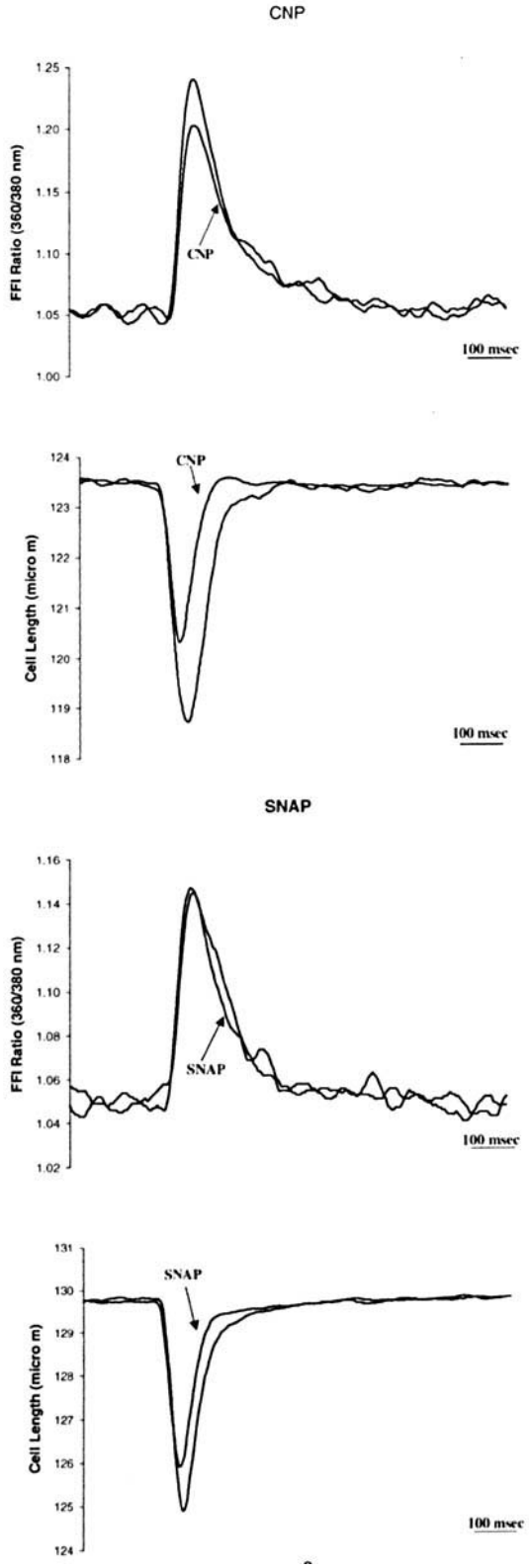

Figure 4. Representative traces of Ca^{2+} transients (upper panel) and cell motion (lower panel) at baseline and during response to a 3-min exposure to 10^{-7} *M* C-type natriuretic peptide (CNP; superimposed arrow) or 10^{-5} *M* S-nitroso-*N*-acetyl-penicillamine (superimposed arrow). Fura-2 fluorescence intensity ratio at 360 and 380 nm was used as an indicator of intracellular calcium level. Systolic myocyte shortening is directly displayed as a downward deflection of the myocyte motion trace. Both the reagents decreased the amplitude of cell shortening, but only CNP had an effect on the amplitude of the calcium transients.

phorylation (25, 26) and cGMP protein kinase–dependent phosphorylation of protein phosphatase 1 or 2A (27, 28). Interference with calcium release from the sarcoplasmic reticulum may also result from cGMP. Additionally, cGMP promotes Ca^{2+} sequestration into the sarcoplasmic reticulum by cGMP protein kinase–dependent phosphorylation of phospholamban (14, 29). The decrease of myofilament responsiveness to Ca^{2+} by phosphorylation of troponin I may also result from cGMP (30). There are other protein phosphorylation events leading to decreased $I_{Ca^{2+}}$ in smooth muscle, such as stimulation of Ca^{2+}-activated K^+ channels (31) and inhibition of receptor-mediated signal transduction (32), but their participation in cardiac contraction is currently unknown. In our study, CNP induced a substantial reduction in Ca^{2+} transients, an observation that may be explained by natriuretic peptide–induced effects on calcium release or reuptake (14, 33). It is also possible that some of the effects of CNP on calcium levels are related to its actions on the G-protein–activating domain reported for the natriuretic peptide clearance receptor (34). The nitric oxide donor SNAP had little effect in decreasing Ca^{2+} transient in ventricular myocytes. Similar observations have been reported using other species (35) or other nitric oxide donors (4, 36), suggesting that reducing the myofilament response to Ca^{2+}, rather than $[Ca^{2+}]_i$, may be a major pathway for the action of nitric oxide in regulating myocardium contraction. However, further studies are needed to identify the downstream protein targets mediating the effect.

Because we found differential effects on intracellular cGMP and Ca^{2+} transients at concentrations of CNP and SNAP that produced equal effects on cell contraction, the current data strongly indicate that the cGMP generated by the CNP/pGC-signaling axis works mainly to decrease intracellular calcium level, whereas cGMP produced by the SNAP/nitric oxide/sGC axis mainly decreases calcium sensitivity. This proposed spatial distribution effect of cGMP is consistent with a more recent report by Rho and colleagues that both forms of guanylyl cyclase can decrease $[Ca^{2+}]_i$, whereas sGC has an additional effect in blunting Ca^{2+} sensitivity (8). Another recent study (37) shows that following ANP, but not nitric oxide, the cGMP protein kinase is recruited to the plasma membrane in ventricular myocytes where many of its protein targets, including L-type Ca^{2+} channels, are concentrated (25, 26). Possibly, cGMP protein kinase translocation to the sarcolemma and inhibition of Ca^{2+} entry could occur in our model and, thus, mediate the effects of CNP on regulation of intracellular calcium level.

These spatial effects of cGMP may be due to localized formation and restricted diffusion in cytosol. It is known that the cytosol is full of cytoskeletal structures that may work as a physical barrier to cGMP diffusion. A high level of cGMP phosphodiesterase activity in cardiac myocytes (7) may serve as a functional barrier to cGMP movement in the cytosol. Thus, localized degradation and production could explain the

Table 5. Effects of CNP, SNAP, Guanylin, and CO on Peak High, Maximum Development Rate, and Return Rate of Calcium Transients[a]

	Peak high (%)	Maximum development rate (1/sec)	Return rate (1/sec)		Peak High (%)	Maximum development rate(1/sec)	Return rate (1/sec)
Baseline	16.7 ± 0.9	6.1 ± 1.3	1.6 ± 0.1	Baseline	1.35 ± 1.5	4.2 ± 0.6	1.4 ± 0.2
CNP (10^{-7} *M*)	14.0 ± 0.9[b]	4.3 ± 0.3[b]	1.4 ± 0.1[b]	Guanylin (10^{-7} *M*)	11.3 ± 1.6[b]	3.5 ± 0.6[b]	1.2 ± 0.2
Baseline	17.4 ± 1.1	5.3 ± 0.4	1.6 ± 0.2	Baseline	12.8 ± 1.4	3.9 ± 0.5	1.3 ± 0.2
SNAP (10^{-5} *M*)	16.4 ± 1.2	5.0 ± 0.4	1.7 ± 0.2	CO (8×10^{-6} *M*)	12.0 ± 1.7	3.7 ± 0.6	1.3 ± 0.2

[a] Values are mean ± SEM. CNP, C-type natriuretic peptide; SNAP, S-nitroso-*N*-acetyl-penicillamine; CO, carbon monoxide.
[b] Significantly different from corresponding baseline value.

differential effects of the two guanylyl cyclases. In addition, the compartmentalization of cGMP may be beneficial for signaling because it can be energetically efficient without accumulating cGMP throughout the bulk cytosol. This may explain the observations obtained in this study that even while producing very small amounts of cGMP, CNP still produced significant effects on cell contraction and Ca^{2+} transients. This may help to explain the beneficial effect of natriuretic peptides in congestive heart failure (38). The action of SNAP in myocytes produced a high level of cGMP in the cytosol and reduced calcium sensitivity. However, the local concentration of cGMP around the sarcolemma may not be as high as that generated by CNP.

In summary, we report differential effects of stimulation of the particulate and soluble forms of guanylyl cyclase on intracellular cGMP levels and Ca^{2+} transients and their similar effects on cell contraction in freshly isolated ventricular myocytes. The actions were mediated by cGMP-dependent pathways. The concept of spatial compartmentalization of cAMP has been recently raised (39, 40), but similar evidence for the sibling molecule of cAMP, cGMP, has been lacking. Our data indicate the existence of spatial compartmentalization for cGMP. Further study is needed to elucidate the full consequences of this uneven production and/or distribution of cGMP in the cytosol, the protein components responsible for this

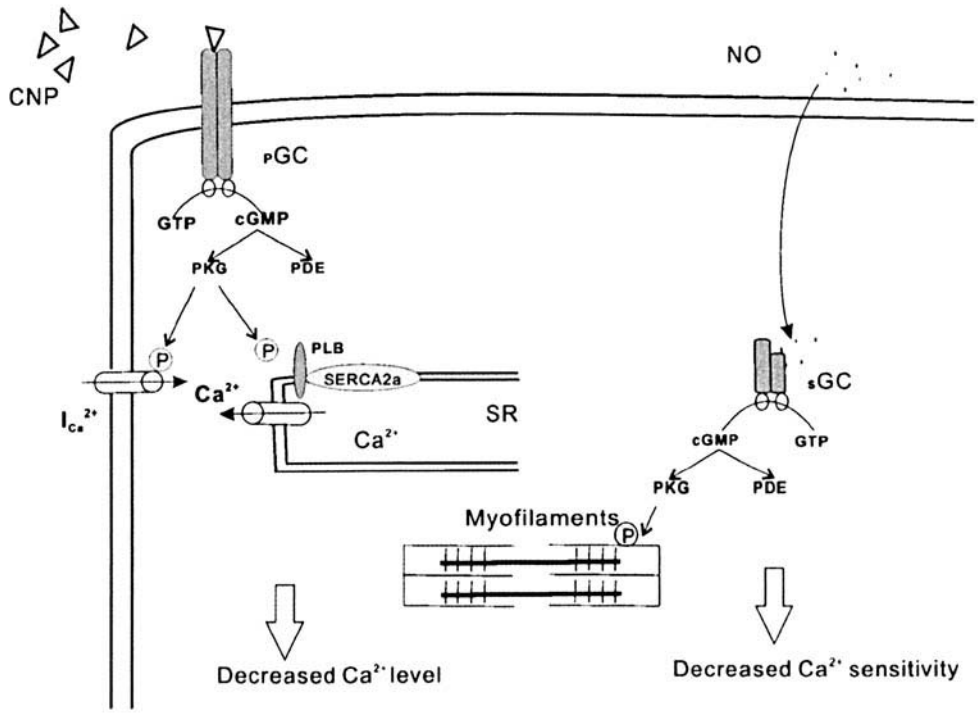

Figure 5. A model depicting differential signaling pathways during activation of the particulate and soluble guanylyl cyclases. After stimulation with C-type natriuretic peptide binding to its receptor, particulate guanylyl cyclase produced cGMP which accumulated near the sarcolemma because diffusion was restricted, perhaps in part by the cytosolic distribution of phosphodiesterases. Once activated by cGMP, the cGMP protein kinase phosphorylates an array of target proteins, such as L-type Ca^{2+} channel and phospholamban, which decreases the intracellular Ca^{2+} level. Soluble guanylyl cyclase produced cGMP in the presence of nitric oxide, resulting in the possible accumulation of cGMP near myofilaments, some of whose components (e.g., troponins) may be phosphorylated by activated cGMP protein kinase (PKG) to blunt Ca^{2+} sensitivity. Both signaling pathways lead to reduced cardiac function. Activation of cGMP-affected cAMP phosphodiesterases may also be involved.

compartmentalization and their effects on signaling near the sarcolemma.

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