Increased effects of C-type natriuretic peptide on cardiac ventricular contractility and relaxation in guanylyl cyclase A-deficient mice

Melanie Pierkesa, Stepan Gambaryanb, Peter Bokníka, Suzanne M. Lohmannb, Wilhelm Schmitza, Regine Potthasta, Rita Holtwicka, Michaela Kuhna, *Wilhelm Schmitz, Regine Potthastc, Rita Holtwicka, Michaela Kuhn a, *

aInstitute of Pharmacology and Toxicology, University of Münster, Domagkstrasse 12, D-48129 Münster, Germany
bInstitute of Clinical Biochemistry and Pathobiology, University of Würzburg, Würzburg, Germany

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Abstract

Objective: The natriuretic peptides (NPs), atrial (ANP), B-type (BNP), and C-type (CNP) natriuretic peptides as well as their respective receptor-guanylyl cyclases (GC-A for ANP and BNP, and GC-B for CNP) are expressed in the heart. However, the local role of NPs in the regulation of cardiac contractility and the mutual interactions of NPs remain controversial. In the present study we evaluated the effects of ANP and CNP on cardiac function of wild-type (GC-A+/+) and GC-A-deficient (GC-A+/−) mice. Methods: The effects of NPs and their molecular mechanisms were assessed in the isolated perfused mouse working heart preparation. Results: In GC-A+/+ hearts, CNP exerted a biphasic action: an immediate increase in inotropy and lusitropy, followed by a slowly developing negative inotropic effect. These effects were mimicked by the cGMP-analogue, 8-pCPT-cGMP. In contrast, ANP did not affect cardiac function. In GC-A+/− hearts, the immediate contractile responses to CNP and 8-pCPT-cGMP were significantly enhanced. CNP increased cardiac cGMP levels and stimulated phospholamban (PLB) phosphorylation; the effect on PLB, but not cGMP, was enhanced in GC-A−/− hearts. In addition, cardiac expression of cGMP-dependent protein kinase (cGK I) was significantly increased in GC-A−/− mice. Conclusion: CNP exerts a biphasic, initially positive inotropic and lusitropic, then negative inotropic effect in isolated working mouse hearts. A putative mechanism contributing to the immediate contractile responses is cGMP/cGK I-dependent phosphorylation of PLB and subsequent activation of the sarcoplasmic reticulum Ca2+ -pump. ANP has no direct effects on cardiac contractility but chronic absence of its receptor, GC-A, results in increased responsiveness to CNP. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Natriuretic peptide; Hypertension; Hypertrophy; Contractile function; Gene expression

1. Introduction

The natriuretic peptide (NP) family contains at least three members: atrial (ANP), B-type (BNP), and C-type (CNP) natriuretic peptides. ANP and BNP are mainly released from the heart to act as circulating hormones (endocrine system), whereas CNP is an endothelial peptide presumably with local actions (paracrine system). The effects of these peptides are attributed to the activation of two specific receptor-guanylyl cyclases (GC): GC-A (with highest affinity for ANP and BNP) and GC-B (for CNP) [1]. Activation of these receptors results in an increase of intracellular cGMP levels which in turn mediates cellular responses leading to vasodilatation and increased natriuresis and diuresis [1]. Accordingly, deletion of GC-A in mice (GC-A−/−) prevents the vasorelaxant effects of ANP and BNP and leads to chronic hypertension and cardiac hypertrophy [2,3]. In contrast, the hypotensive and vasorelaxant effects of CNP are not changed in GC-A-deficient mice [4].

A third receptor which also triggers effects of NPs, the C-receptor (NPR-C), has a similar affinity for all three peptides. It is not linked to a guanylyl cyclase domain and is thought to function as a clearance receptor which

*Corresponding author. Tel.: +49-251-835-2597; fax: +49-251-835-5501.
E-mail address: mkuhn@uni-muenster.de (M. Kuhn).

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mediates the cellular internalization of NPs [5]. Recently it was demonstrated that the short cytoplasmic region of NPR-C activates GTP-binding proteins to inhibit adenyl cyclase, supporting a signal-transducing role for this receptor [6]. However, inactivation of the gene for NPR-C in mice indicated that at least within the cardiovascular system its main function is to remove NPs from the circulation, thereby modulating their local physiological effects [7].

All components of the NP system, the peptides and their receptors, are expressed within the heart. ANP and BNP are secreted from cardiomyocytes and CNP is synthesized and secreted by the coronary endothelium [8]. GC-A, GC-B and NPR-C are located on cardiac myocytes and fibroblasts as well as within coronary vessels [9]. It is postulated that besides their endocrine actions on distant tissues, the NPs modulate growth and contractility of cardiomyocytes and proliferation of cardiac fibroblasts in an autocrine/paracrine way [10,11]. However, direct effects of NPs on cardiac contractility remain controversial. For CNP, ‘acute’ positive inotropic effects on isolated canine atrial and ventricular preparations have been reported [12,13]. In isolated rat papillary muscles, CNP displays a positive lusitropic and delayed negative inotropic action [14]. The effects of ANP on cardiac contractility are even less clear. While some studies showed that ANP decreased contractility in isolated cardiomyocytes [15], other studies saw no effect of the peptide on cardiac mechanical functions [13,14].

To further characterize how NPs modulate cardiac function and to determine their mutual interactions, we studied responses to ANP and CNP in an in vitro isolated work-performing mouse heart preparation. In particular, CNP was found to have initial positive inotropic and lusitropic effects, followed by a delayed negative inotropic action. CNP/cGMP activation of cGMP-dependent protein kinase I (cGK I) and phospholamban (PLB) phosphorylation may lead to the early CNP contractile responses by facilitating pumping of Ca$^{2+}$ into the sarcoplasmic reticulum. In GC-A deficient (GC-A$^{-/-}$) mice, both CNP effects on early cardiac contractility, as well as cGK I expression and PLB phosphorylation are enhanced.

2. Methods

2.1. Animals

GC-A-deficient (GC-A$^{-/-}$) mice were provided by Dr. D.L. Garbers (HHMI, University of Texas Southwestern Medical Center, Dallas) [2]. Male GC-A$^{-/-}$ mice and their nontransgenic (GC-A$^{+/+}$) littermates, 6 months old, were used. Genotypes were confirmed by PCR. Blood pressure measurements were made in conscious mice using a tail cuff method (Softron, Tokyo) [4]. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the local animal care committee.

2.2. Measurement of cardiac contractile parameters in isolated work-performing heart preparations

Analysis of cardiac function was as described previously [16,17]. In brief, isolated hearts from GC-A$^{-/-}$ and GC-A$^{+/+}$ mice were perfused with Krebs–Henseleit (KH) buffer through the pulmonary vein and left atrium, in an anterograde, fluid-ejecting mode. Fluid ejected from an aortic cannula against a hydrostatic fluid column set at a height to yield a mean aortic pressure (afterload) of 50 mmHg. Venous return (preload) was measured using a flowmeter (Transonic Systems Inc., Ithaca, USA) and adjusted to $\pm$ 30 ml/min/g estimated dry heart weight. For this purpose, wet heart weights were directly measured prior to perfusion and dry weight was estimated on the basis of a wet-to-dry-weight curve plotted from previous observations in 15 hearts. Coronary flow (calculated as the difference between preload and aortic flow), heart rate, aortic pressure and left intraventricular pressure were continuously monitored, and the first derivatives of left intraventricular pressure, $+dP/dt$ and $-dP/dt$ (in mmHg/s), time to peak pressure and time to relaxation (in ms) were calculated (A Mon 2.1 program, Ingenieurbüro Jäckel, Hanau, Germany). These parameters have been shown to be good indices of cardiac contractility and relaxation in this experimental model [16–18].

2.3. Experimental protocols

After a 20-min equilibration period, the test agents were infused continuously for the duration of the experiment via the coronary arteries. Control hearts of each genotype were infused with KH-buffer for 5 min or 60 min ($n=5$).

(a) CNP was infused into GC-A$^{+/+}$ and GC-A$^{-/-}$ hearts for 60 min (100 nM CNP; $n=6$) and for 5 min (1 nM CNP, $n=3$; 10 and 100 nM CNP, $n=7$).

(b) cANP(4-23) (des[Gln$^{18}$,Ser$^{19}$,Gly$^{20}$,Leu$^{21}$,Gly$^{22}$]-ANP(4-23)-NH$^2_2$; rat), a specific NPR-C ligand [19], was infused into GC-A$^{-/-}$ hearts at 100 nM for 30 min ($n=4$).

(c) A71915 (Arg$^6$, β-cyclohexyl-Ala$^8$, D-Tic$^{16}$, Arg$^{17}$, Cys$^{18}$)-ANP(6-18)-NH$^2_2$, a specific antagonist which competitively binds to GC-A preventing its stimulation by ANP [20] was infused into GC-A$^{+/+}$ hearts at 300 nM. After testing for intrinsic activity (infused alone for 15 min), A71915 was combined with CNP (100 nM) and infused for an additional 5 min ($n=5$).

(d) ANP (100 nM) was infused into GC-A$^{+/+}$ hearts for 30 min ($n=6$).
8-pCPT-cGMP, a membrane permeable cGMP analogue, was infused into GC-A+/+ and GC-A−/− hearts at 100 μM for 10 min (n=6) and 60 min (n=2).

To study the effect of NPs on intracellular messenger systems, the isolated working hearts were quickly stopped by shock freezing both ventricles in liquid nitrogen. Untreated hearts infused with KH-buffer for the same time periods were used as controls. The frozen ventricles were then extracted for assay of cyclic nucleotide contents and for Western blot analysis.

2.4. Determination of intracellular cGMP and cAMP contents

Frozen ventricles were homogenized and cyclic nucleotides were extracted with ice-cold 70% (v/v) ethanol. After centrifugation (3000×g, 5 min, 4 °C) the supernatants were dried in a speed vacuum concentrator, resuspended in sodium acetate buffer (50 mM, pH 6.0) and acetylated, and then cGMP and cAMP contents were quantified by respective radioimmunoassays (Ref. [21] for cGMP; cAMP-RIA, Amersham, Freiburg, Germany). The pellets of the ethanol extracts were used for determination of protein content.

2.5. Quantitative real-time RT-PCR analysis of GC-B and NPR-C mRNA expression

Total RNA from control hearts perfused with KH-buffer was isolated by RNase Kit (Qiagen, Hilden, Germany) and 1 μg of RNA was reverse transcribed with random primers (Clontech, Heidelberg, Germany). A quantitative analysis of GC-B and NPR-C mRNA expression was performed by RT-PCR using the LightCycler™ Detection System (Roche, Mannheim, Germany) as described previously [22]. The system uses two fluorogenic probes to generate sequence-specific fluorescent signals during PCR. The following primers and fluorogenic probes were used (all designed and synthesized by Tib Molbiol, Berlin, Germany): for GC-B: forward primer, 5′-CCAGAACCACAACCTGAGCTATGC; reverse primer, 5′-GGGCAGCAGGATATAACAATGC (PCR product 599 bp), detection probe: 5′-LC Red640-CCCTGAGCAAGCCACCTTTCATC-p, anchor probe: 5′-CCAGGTGATGCCCCGAGAGGCAGATT-fluorescein; for NPR-C: forward primer, 5′-TCTGCTCATAATTTGACGAG; reverse primer, 5′-CAGAGGAAGTCCCCATACCG (PCR product 560 bp), detection probe: 5′-LC Red640-GTCACCCTACTGAGGACCGTGAAAC-p, anchor probe: 5′-AAACAAGCA-TACTCGTCCCTCCAAC-fluorescein. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for normalization [22]. Preliminary experiments showed that GAPDH mRNA expression is not different in GC-A+/+ and −/− hearts.

2.6. Western blot analysis

To determine the expression level of protein kinases, of phospholamban (PLB), and the effect of CNP and 8-pCPT-cGMP on PLB phosphorylation, frozen ventricles from hearts infused with these agents or with KH buffer (controls) were homogenized and analyzed by Western blot as described [23–25]. Antibodies used to study protein kinase expression were against cGMP-dependent protein kinase I (cGK I; diluted 1:3000) [23] and against the catalytic subunit of cAMP-dependent protein kinase (cAK, diluted 1:2000) [24]; detection was with an ECL system (Amersham-Pharmacia, Freiburg, Germany). To study PLB expression and phosphorylation, primary antibodies used were against total PLB (PLB A-1 antibody) and against PLB phosphorylated at serine-16 (PLB-PS-16). The pellets of the ethanol extracts were used for determination of protein content.

2.7. Agents

Rat ANP and human CNP were obtained from Calbiochem-Novabiochem (Bad Soden, Germany); 8-para-chlorophenylthio-cGMP (8-pCPT-cGMP) from Biolog (Bremen, Germany); A71915 and cANP from Bachem California Inc. (Torrance, CA, USA). All other agents were from Sigma (Deisenhofen, Germany).

2.8. Statistics

Results are presented as the means±S.E.M. and, where indicated, values were normalized by expression as % of baseline. Student’s t-test was used for comparison of data, except that the serial changes in cardiac function measured after peptide infusions within each study protocol were tested with a repeated-measures ANOVA followed by Bonferroni and Student–Newman–Keuls post hoc test for multiple comparisons (GraphPad InStat software). Results were considered statistically significant in all analyses at P<0.05.

3. Results

3.1. Baseline cardiovascular parameters

In agreement with previous observations [2–4], GC-A+/+ mice had markedly higher systolic (146±3.4 vs. 109±3.4 mmHg, *P<0.05) and diastolic blood pressures (88±4.7 vs. 69±3.2 mmHg, *P<0.05), and significantly...
Table 1  
Baseline parameters of isolated hearts from wild-type (GC-A $+/+$) and GC-A $+/−$ mice in the work-performing mode

<table>
<thead>
<tr>
<th>Baseline parameters</th>
<th>GC-A $+/+$</th>
<th>GC-A $+/−$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart weight, mg</td>
<td>181±4</td>
<td>251±18$^3$</td>
</tr>
<tr>
<td>Heart/body weight ratio, mg/g</td>
<td>6.2±0.2</td>
<td>8.4±0.2$^3$</td>
</tr>
<tr>
<td>CF, ml/min/g</td>
<td>16.3±1.1</td>
<td>15.5±0.8</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>426±8</td>
<td>399±12</td>
</tr>
<tr>
<td>Mean aortic pressure, mmHg</td>
<td>51.5±0.5</td>
<td>51.2±0.6</td>
</tr>
<tr>
<td>Intraventricular pressure, mmHg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic</td>
<td>95±2.3</td>
<td>96.3±2.1</td>
</tr>
<tr>
<td>Diastolic</td>
<td>−4.4±0.7</td>
<td>−3.2±0.7</td>
</tr>
<tr>
<td>Time to peak pressure, ms</td>
<td>34.2±0.9</td>
<td>35.1±0.7</td>
</tr>
<tr>
<td>Time to relaxation, ms</td>
<td>43.6±0.6</td>
<td>47.5±0.9$^3$</td>
</tr>
<tr>
<td>$+dP/dt$, mmHg/s</td>
<td>2690±60</td>
<td>2628±56</td>
</tr>
<tr>
<td>$−dP/dt$, mmHg/s</td>
<td>2790±93</td>
<td>2638±89</td>
</tr>
</tbody>
</table>

Basal conditions: preload of 30 ml/min/g, afterload of 50 mmHg aortic pressure. Values are means±S.E.M.,$^1P<0.05$ between $+/−$ and $+/+$ mice. CF, coronary flow; HR, heart rate; $+dP/dt$, maximal rate of contraction; $−dP/dt$, maximal rate of relaxation.

Increased heart weights and heart/body weight ratios as compared to wild-type mice (Table 1). Table 1 also shows the contractile parameters of isolated hearts from GC-A $+/+$ and GC-A $+/−$ mice in the work-performing preparation under basal conditions. The time to relaxation of the GC-A $+/−$ hearts was significantly prolonged as compared to GC-A $+/+$; no other basal parameters of cardiac function were significantly different between the two genotypes.

3.2. Effects of natriuretic peptides on cardiac contractile parameters

Infusion of 100 nM CNP into isolated working heart preparations during 60 min exerted a biphasic effect on the contractility of GC-A $+/+$ hearts. Firstly, an immediate increase in the maximal rates of contraction ($+dP/dt$) and relaxation ($−dP/dt$), a small increase in left ventricular systolic pressure and a shortened time of relaxation were observed (Fig. 1). These effects started immediately after addition of CNP, reached their maximum at 5 min and then, except for time to relaxation, reversed to baseline at about 15 min. Subsequently a progressive, slowly developing decrease in the contraction and relaxation rates, as well as maximal systolic pressure, was observed. The time of relaxation started to increase after 5 min, almost returning to baseline values after 60 min of treatment with CNP. Heart rate did not significantly change during 60 min of infusion with CNP. Coronary flow progressively increased to $≈128±5%$ of pretreatment values within 15 min ($^*P<0.05$ vs. baseline) and then remained stable for the rest of the CNP-infusion time.

The biphasic effects of CNP on cardiac contractility
were also observed in isolated hearts from GC-A−/− mice (Fig. 1). Remarkably, the initial changes (namely the positive inotropic and lusitropic actions) were significantly greater as compared to GC-A+/+ hearts. The CNP-induced increase in coronary flow (to 125±7% of baseline) was comparable to GC-A+/+ hearts (*P<0.05 vs. baseline). Heart rates did not significantly change during CNP treatment in GC-A−/− hearts either.

Control GC-A+/+ and GC-A−/− hearts infused with vehicle (KH buffer) revealed no significant changes in contractile function over a 60-min observation time, indicating that the delayed decrease in cardiac inotropy observed during infusion of CNP does not simply reflect a spontaneous deterioration of heart function but is indeed related to the specific actions of the peptide.

To elucidate whether the biphasic action of CNP is mediated by activation of the NPR-C receptor, the specific NPR-C ligand cANP [19], was tested on GC-Amediated by activation of the NPR-C receptor, the specific peaked at 10 min. Coronary flow was significantly increased to 125±6 (+/+) and 120±3% (GC-A−/−) (*P<0.05 versus baseline). No significant changes in heart rate were observed.

8-pCPT-cGMP mimicked the later phase of the CNP response, as observed in two experiments in which 8-pCPT-cGMP was continuously infused into GC-A−/− hearts for 60 min. The initial increase in inotropy was followed by a slowly developing decrease in both left ventricular pressure and maximal rate of contraction to values which were, respectively, 8±3% and 10±2% lower than baseline at 60 min. The positive lusitropic effect (increased maximal rate of relaxation and decreased time of relaxation) remained stable over 30 min and then slowly returned to baseline after 60 min of continuous 8-pCPT-cGMP infusion. No significant changes in heart rate were observed; coronary flow progressively increased to 140±8% of pretreatment values within 40 min, then remained stable for the remaining infusion time (*P<0.05 vs. baseline).

3.4. Effects of 8-pCPT-cGMP on cardiac contractile parameters

8-pCPT-cGMP, a membrane-permeable, phosphodiesterase-resistant, selective activator of cGMP-dependent kinases [27], mimicked the immediate (Fig. 3) and late (see below) effects of CNP on cardiac contractility and relaxation. As observed for CNP, the immediate inotropic and lusitropic responses to 8-pCPT-cGMP (100 µM), were more pronounced in GC-A−/− compared to GC-A+/+ hearts (Fig. 3). These cardiac contractile responses started immediately after addition of 8-pCPT-cGMP and peaked at 10 min. Coronary flow was significantly increased to 124±5 (+/+ ) and 120±3% (GC-A−/−) (*P<0.05 versus baseline). No significant changes in heart rate were observed.

To further characterize the immediate contractile responses to CNP, in an additional series of experiments CNP (1, 10 and 100 nM) was infused into isolated working hearts for 5 min. Each concentration was tested in a separate group of hearts. As shown in Fig. 2, lower concentrations of CNP (<10 nM) did not elicit any positive inotropic effect in GC-A+/+ and −/− hearts. The positive lusitropic effect was observed at a lower CNP concentration in GC-A−/− hearts (10 nM) as compared to GC-A+/+ hearts (100 nM CNP).

Next we demonstrated that the enhanced responsiveness of GC-A−/− hearts to CNP cannot be mimicked by ‘acute’ blockade of GC-A in GC-A+/+ hearts. The ANP antagonist A71915 [20], did not exert any intrinsic activity on GC-A+/+ hearts and additionally did not significantly alter the immediate effects of CNP on contractility and coronary flow in GC-A+/+ hearts (Table 2).

3.3. Characterization of the ‘acute’ effects of CNP on cardiac contractility

Table 2

<table>
<thead>
<tr>
<th>Agent</th>
<th>Mice GC-A</th>
<th>Time (min)</th>
<th>LVPmax</th>
<th>+ dP/dt</th>
<th>− dP/dt</th>
<th>HR</th>
<th>Time to relaxation</th>
<th>CF</th>
</tr>
</thead>
<tbody>
<tr>
<td>cANP 1-23</td>
<td>−/−</td>
<td>5</td>
<td>103±1.9</td>
<td>101.2±3.6</td>
<td>102.7±6.6</td>
<td>98±2.1</td>
<td>100±0.6</td>
<td>85±7.3</td>
</tr>
<tr>
<td>cANP 1-23</td>
<td>−/−</td>
<td>30</td>
<td>100±2.2</td>
<td>104±2.4</td>
<td>98±7.5</td>
<td>98±2.1</td>
<td>101±0.6</td>
<td>107±8.0</td>
</tr>
<tr>
<td>ANP</td>
<td>+/+</td>
<td>5</td>
<td>99±3.1</td>
<td>102±4.1</td>
<td>101±2.6</td>
<td>96±5.1</td>
<td>102±2.1</td>
<td>103±6</td>
</tr>
<tr>
<td>ANP</td>
<td>+/+</td>
<td>30</td>
<td>101±3.8</td>
<td>100±6.2</td>
<td>101±5.6</td>
<td>95±8.2</td>
<td>102±3.3</td>
<td>105±3</td>
</tr>
<tr>
<td>A71915</td>
<td>+/+</td>
<td>15</td>
<td>99±0.9</td>
<td>99±0.8</td>
<td>100±1.1</td>
<td>103±4.9</td>
<td>103±1.9</td>
<td>100±2</td>
</tr>
<tr>
<td>A71915+CNP</td>
<td>+/+</td>
<td>15±5</td>
<td>108±2.6</td>
<td>107±1.3</td>
<td>111±2.4</td>
<td>101±3.1</td>
<td>93±1.7</td>
<td>116±7.4*</td>
</tr>
<tr>
<td>Vehicle+CNP</td>
<td>+/+</td>
<td>15±5</td>
<td>112±1.1</td>
<td>109±0.8</td>
<td>115±2.5</td>
<td>98±3.5</td>
<td>90±0.7</td>
<td>111±4.1*</td>
</tr>
</tbody>
</table>

The NPR-C specific ligand, cANP 1-23 (100 nM), was infused into GC-A−/− hearts for 30 min (n=4); ANP (100 nM) was infused into GC-A−/− hearts for 30 min (n=6); the GC-A antagonist, A71915 (300 nM), or vehicle (KH) was infused into GC-A+/+ hearts for 15 min followed by CNP (100 nM) for an additional 5 min (n=5). Data are expressed as % of baseline (means±S.E.M.). *P<0.05 vs. baseline.

In order to elucidate the molecular mechanisms mediat-
Fig. 2. Acute effects of CNP (1, 10 and 100 nM, infusion for 5 min) on maximal rates of contraction ($+\frac{dP}{dt}$) and relaxation ($-\frac{dP}{dt}$), maximal left ventricular systolic pressure and time to relaxation in isolated working GC-A $+/-$ and GC-A $-/-$ hearts. Data are expressed as % of baseline and represent the mean±S.E.M. from 3–7 experiments. *$P<0.05$ vs. baseline, $\S$ $P<0.05$ vs. GC-A $+/-$.

Fig. 3. Acute effects of 8-pCPT-cGMP (100 $\mu$M, infusion for 10 min) on left ventricular systolic pressure (LVP$_{\text{max}}$), maximal rates of contraction ($+\frac{dP}{dt}$) and relaxation ($-\frac{dP}{dt}$) and time to relaxation in isolated working GC-A $+/-$ and GC-A $-/-$ hearts. Data are expressed as % of baseline and represent the mean±S.E.M. from six experiments. *$P<0.05$ vs. baseline, $\S$ $P<0.05$ vs. GC-A $+/-$.

GC-A $+/-$ and GC-A $-/-$ hearts perfused with KH-buffer (vehicle controls); 0.03±0.02 vs. 0.03±0.01 $\mu$mol/µg protein ($+/-$ vs. $-/-$, 5 min KH-infusion); 0.12±0.05 vs. 0.09±0.02 $\mu$mol/µg protein ($+/-$ vs. $-/-$, 60 min KH-infusion). ANP evoked a rather small, 2.5±0.3-fold increase in cGMP content of GC-A $+/-$ hearts and this effect was completely absent in GC-A $-/-$ hearts. Infusion of CNP induced marked increases of cGMP levels in both, GC-A $+/-$ and GC-A $-/-$ hearts (Fig. 4). The absolute cGMP levels measured after infusion of CNP for 5 min (10 and 100 nM CNP) or 60 min (100 nM CNP) were not significantly different between GC-A $+/-$ and GC-A $-/-$ hearts (Fig. 4). Also, the cGMP responses of GC-A $+/-$ hearts to CNP were not different in the presence or absence of the GC-A antagonist, A71915 (not shown).

Similar cAMP levels were detected in GC-A $+/-$ and GC-A $-/-$ hearts perfused with KH-buffer (controls); 1.56±0.33 vs. 1.55±0.4 $\mu$mol/µg protein ($+/-$ vs. $-/-$, 5 min KH-infusion); 1.45±0.05 vs. 2.15±0.48 $\mu$mol/µg protein ($+/-$ vs. $-/-$, 60 min KH-infusion). The cAMP contents of hearts treated with CNP or ANP at the indicated concentrations and time-periods were not different from these controls. Finally, the cAMP and cGMP...
contents of GC-A \(-/-\) hearts treated with the selective NPR-C ligand, cANP\(_{4-23}\), also were not significantly different from KH-perfused controls. These observations rule out the possibility that activation of NPR-C or modulation of cardiac cAMP levels mediate the cardiac contractile responses to CNP.

3.6. Expression of GC-B and NPR-C mRNA

To determine whether long-term absence of GC-A and subsequent abolition of the action of ANP modifies the expression of receptors for CNP, the cardiac mRNA expression of GC-B and NPR-C in control (KH-perfused) hearts was quantitated by real-time RT-PCR. As shown, the mRNA expression levels of GC-B and NPR-C were not significantly different in GC-A \(-/-\) versus GC-A \(+/+\) hearts (Fig. 5).

![Graph showing cyclic GMP content](image)

Fig. 4. Effect of CNP on cGMP content (in fmol/\(\mu\)g protein) in isolated working GC-A \(+/+\) and GC-A \(-/-\) hearts. Hearts were infused with 10 and 100 nM CNP for 5 min (left) and with 100 nM CNP for 60 min (right); control hearts were infused with vehicle (KH-buffer) for the same time periods. All data represent the mean \(\pm\) S.E.M. from six experiments. *\(P<0.05\) vs. vehicle controls.

3.7. Expression of cAMP- and cGMP-dependent protein kinases

In order to study potential intracellular targets of the CNP/GC-B system, the cardiac expression of cGMP-dependent protein kinase (cGK I) was studied by Western blot analysis. As shown in Fig. 6, cGK I expression was significantly increased in the ventricles from GC-A \(-/-\) as compared to GC-A \(+/+\) hearts by \(\approx 53 \pm 4\%\). The expression of the cAMP-dependent protein kinase (cAK) C subunit was not different between GC-A \(+/+\) and \(-/-\) hearts (Fig. 6). Infusion of CNP (100 nM, 60 min) did not affect ventricular cGK I expression levels (data not shown).

3.8. Expression and phosphorylation of phospholamban

To further investigate the mechanism of the inotropic and lusitropic effects of CNP, the expression and phosphorylation of the regulatory protein PLB were quantified by Western blot analysis. The expression levels of total PLB were not different in GC-A \(-/-\) as compared to \(+/+\) ventricles (Fig. 7, top). The phosphorylation-specific antibody detected almost no signal in the ventricles of vehicle-treated (control) hearts (Fig. 7, top). In contrast, a strong and concentration-dependent increase in PLB-PS-16 was detected in hearts treated with 10 and 100 nM CNP for 5 min (Fig. 7). After CNP infusion for 5 min, phosphorylation at Ser\(_{16}\) normalized to total PLB protein content, was significantly increased by both 10 nM and 100 nM in GC-A \(-/-\) compared to GC-A \(+/+\) ventricles (Fig. 7, bottom). Thus, the phosphorylation of PLB at Ser\(_{16}\) was increased by CNP and this effect was significantly enhanced in the GC-A \(-/-\) hearts. The levels of phosphorylated PLB in GC-A \(+/+\) and GC-A \(-/-\) hearts treated with 100 nM CNP for 60 min were slightly but not significantly lower (data not shown) than the phosphorylation levels in hearts infused with 100 nM CNP.

![Graph showing expression and phosphorylation of PLB](image)

Fig. 5. Quantitative RT-PCR analysis (LightCycler\(^{\text{TM}}\)) of GC-B and NPR-C mRNA levels in the ventricles of KH-perfused GC-A \(+/+\) and GC-A \(-/-\) hearts. Signal intensities were normalized to GAPDH signal intensity. Data represent the mean \(\pm\) S.E.M. from 10 experiments.

Fig. 6. Expression of cGMP-dependent protein kinase (cGK I) in isolated working GC-A \(+/+\) and GC-A \(-/-\) hearts. Hearts were infused with 10 and 100 nM CNP for 5 min (left) and with 100 nM CNP for 60 min (right); control hearts were infused with vehicle (KH-buffer) for the same time periods. All data represent the mean \(\pm\) S.E.M. from six experiments. *\(P<0.05\) vs. vehicle controls.
for only 5 min (above). The effects of CNP on PLB phosphorylation were mimicked by 8-pCPT-cGMP. After infusion of GC-A $-/-$ hearts with 100 μM 8-pCPT-cGMP for 60 min, phosphorylation at Ser$_{16}$, normalized to total PLB protein content, was 19±4.1 compared to 14±2.2 at 60 min of CNP infusion (in arbitrary units).

4. Discussion

The original findings of the present study are, firstly, that CNP exerts a biphasic effect on the performance of isolated mouse working hearts: an immediate increase in lusitropy, accompanied by a transient weak positive inotropic action, followed by a slowly developing decrease in inotropy. These effects on cardiac contractility are accompanied by a stable increase in coronary flow. Secondly, the immediate lusitropic effects of CNP correlate with activation of a cGMP-dependent pathway involving cGK I and phospholamban phosphorylation. Thirdly, ANP has no effect on cardiac function, but deletion of GC-A, a receptor for ANP, increases the cardiac responsiveness to CNP.

Our observations regarding the cardiac contractile effects of CNP are corroborated by published studies in isolated dog and rat heart preparations. CNP evoked a marked immediate increase in the contractile force of isolated canine atrial and ventricular preparations [12,13]. In these studies, only immediate effects of CNP were evaluated. Extending these observations, a more recent study in isolated rat papillary muscle demonstrated sequential actions of CNP on cardiac contractility, with an immediate positive lusitropic and a slowly developing negative inotropic effect [14]. The time course of these effects was nearly identical to that observed in our experiments. In both the previous [13,14] as well as present studies, ANP had no effect on any contractile parameter.

To further investigate the mechanisms mediating the
cardiac responses to CNP, potential downstream targets were studied. CNP markedly enhanced cardiac cGMP content of isolated mouse working hearts, while ANP only had a marginal effect, suggesting that GC-B, a specific CNP receptor, is involved in the responses to CNP. Moreover, cANP \(_{4-23}^{23}\), a selective ligand of the NPR-C receptor [19], did not affect cardiac contractility, ruling out CNP effects through this receptor. To further examine the receptor mediating CNP effects, we took advantage of a mouse model with targeted genetic deletion of the ANP receptor, GC-A [2-4]. The results of the experiments with GC-A-deficient \((-/-)\) hearts were surprising since the acute effects of CNP on cardiac contractility and relaxation were not only preserved but even increased in the absence of GC-A. Thus, although ANP has only very slight effects on cardiac cGMP content and clearly no effect on cardiac contractile parameters, the permanent abolition of its receptor, GC-A, increased the cardiac effects of the CNP/GC-B system. Since acute, short-term treatment with the GC-A antagonist, A71915 [20], did not increase the responsiveness of GC-A \(+/+\) hearts to CNP, we conclude that molecular changes leading to the increased responses to CNP in GC-A \(-/-\) hearts derive from long-term absence of the GC-A receptor.

ANP/GC-A might influence the CNP/GC-B transduction cascade in several ways, e.g. by affecting the expression and/or the activity of GC-B and downstream targets, or affecting the clearance of CNP via the NPR-C. However, RT-PCR analysis indicated that GC-B and NPR-C mRNA expression levels were similar in hearts from GC-A \(+/+\) and GC-A \(-/-\) mice. Consistent with this, cGMP responses to CNP were also similar in GC-A \(+/+\) and GC-A \(-/-\) hearts. However, other downstream targets of the CNP/GC-B system were found to be altered in GC-A \(-/-\) hearts. Physiologically relevant targets of cGMP in the mammalian heart include cGMP-inhibited PDE (type III PDE), which regulates intracellular cAMP levels, and type I cGMP-dependent protein kinase (cGK I) [28]. As shown, cardiac cAMP levels did not change in response to CNP, suggesting that cGK I is more likely than PDE to mediate the changes in cardiac contractility evoked by CNP. Activation of cGK I leads to phosphorylation/modulation of different regulatory proteins such as L-type calcium channels [29,30], phospholamban (PLB) and troponin I [14]. The complex pattern of CNP actions on mouse cardiac contractility suggests that several of these intracellular targets and effector systems may be involved. We focused our study on the mechanisms behind the most pronounced of the CNP actions in isolated mouse working hearts, namely the positive lusitropic effect. Brusq et al. [14] suggested recently that, in rat papillary muscle, the CNP-induced lusitropism is mediated by cGMP-dependent PLB phosphorylation and subsequent activation of the sarcoplasmic reticulum (SR) \(Ca^{2+}\) pump. Accelerated \(Ca^{2+}\) uptake by the SR enhances cardiac relaxation and increases availability of \(Ca^{2+}\) for contraction and thereby the maximal rate of contraction [31]. Consistent with this, in our studies with isolated working mouse GC-A \(+/-\) hearts, CNP had early positive lusitropic and inotropic effects and induced concentration-dependent accumulation of cGMP and concomitant phosphorylation of PLB at Ser\(_{16}\). Furthermore, in GC-A \(-/-\) hearts CNP-induced phosphorylation of PLB at Ser\(_{16}\) was markedly enhanced, whereas total PLB expression was unchanged.

Several observations support the hypothesis that cGK I is the target which is activated by the CNP/GC-B/cGMP system and mediates PLB phosphorylation: (1) the immediate effects of CNP on myocardial contractility as well as the increase in PLB phosphorylation are mimicked by 8-pCPT-cGMP, a direct activator of cGK I which lacks effects on phosphodiesterases [27]; (2) the expression levels of cGK I are markedly higher in GC-A \(-/-\) hearts; and (3) GC-A \(-/-\) hearts exhibit increased contractile responsiveness to both CNP and 8-pCPT-cGMP at early incubation times.

In summary, our study demonstrates that ANP itself has no effect on cardiac contractility, but that a permanent disruption of its receptor, GC-A, results in increased cardiac contractility and relaxation in response to CNP. These increased immediate positive inotropic and lusitropic effects of CNP on GC-A \(-/-\) hearts appeared to result from increased expression of cGK I mediating CNP/cGMP-dependent phosphorylation of PLB. The complex pattern of CNP-induced actions in the heart, i.e. the delayed negative inotropic action which is observed despite stable increases in cardiac cGMP and PLB-phosphorylation levels, clearly indicates that CNP has other effects on cardiac function independent of cGMP/cGK I-stimulated PLB phosphorylation. Further studies are needed to demonstrate the intracellular mechanisms of the negative inotropic effect of CNP and how other potential intracellular targets of cGMP are modulated by reciprocal interactions between the ANP/GC-A and CNP/GC-B systems.

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