Natriuretic peptides increase β1-adrenoceptor signalling in failing hearts through phosphodiesterase 3 inhibition

Eirik Qvigstad1,2,4*, Lise R. Moltzau1,2, Jan Magnus Aronsen2,3, Cam H.T. Nguyen1,2, Karina Hougen2,3, Ivar Sjaastad2,3,4, Finn Olav Levy1,2*, Tor Skomedal1,2, and Jan-Bjørn Osnes1,2

1Department of Pharmacology, University of Oslo, PO Box 1057 Blindern, Oslo 0316, Norway; 2Center for Heart Failure Research, University of Oslo, Oslo, Norway; 3Institute for Experimental Medical Research, University of Oslo, Oslo, Norway; and 4Departement of Medicine, Ullevål University Hospital, Norway

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Aims

Whereas natriuretic peptides increase cGMP levels with beneficial cardiovascular effects through protein kinase G, we found an unexpected cardio-excitatory effect of C-type natriuretic peptide (CNP) through natriuretic peptide receptor B (NPR-B) stimulation in failing cardiac muscle and explored the mechanism.

Methods and results

Heart failure was induced in male Wistar rats by coronary artery ligation. Contraction studies were performed in left ventricular muscle strips. Cyclic nucleotides were measured by radio- and enzyme immunoassay. Apoptosis was determined in isolated cardiomyocytes by Annexin-V/propidium iodide staining and phosphorylation of phospholamban (PLB) and troponin I was measured by western blotting. Stimulation of NPR-B enhanced β1-adrenoceptor (β1-AR)-evoked contractile responses through cGMP-mediated inhibition of phosphodiesterase 3 (PDE3). CNP enhanced β1-AR-mediated increase of cAMP levels to the same extent as the selective PDE3 inhibitor cilostamide and increased β1-AR-stimulated protein kinase A activity, as demonstrated by increased PLB and troponin I phosphorylation. CNP promoted cardiomyocyte apoptosis similar to inhibition of PDE3 by cilostamide, indicative of adverse effects of NPR-B signalling in failing hearts.

Conclusion

An NPR-B-cGMP-PDE3 inhibitory pathway enhances β1-AR-mediated responses and may in the long term be detrimental to the failing heart through mechanisms similar to those operating during treatment with PDE3 inhibitors or during chronic beta-adrenergic stimulation.

Keywords

Natriuretic peptides • Phosphodiesterases • Beta-adrenoceptors • Heart failure • Cyclic nucleotides

1. Introduction

Atrial (ANP), B-type (BNP), and C-type (CNP) natriuretic peptide levels are increased in the myocardium of heart failure (HF) patients.1,2 Experimental HF models demonstrate functionally relevant cardiovascular effects elicited by elevated endogenous peptide levels.3,4 Natriuretic peptides affect cardiac and non-cardiac cells through receptors (NPRs) which are membrane-bound guanylyl cyclases (GCs), and their activation causes increased cGMP production.5,6 In the heart, cGMP can cause inhibitory effects through cGMP-dependent protein kinase (PKG).7 However, by competitive inhibition of cAMP degradation by phosphodiesterase 3 (PDE3),8 cGMP can theoretically also enhance cAMP-dependent signalling, e.g. following stimulation of β-adrenoceptors (β-AR). Existence of such a cGMP-mediated PDE3 inhibitory pathway has been proposed, as intracellular dialysis with cGMP in guinea-pig cardiomyocytes potentiated β-AR-mediated increase in Ca2+ currents9 and CNP increased cAMP efflux in rabbit atria through inhibition of PDE3.10 However, natriuretic peptide receptor A (NPR-A) stimulation

* Corresponding author. Tel: +47 228 40273/228 40237, Fax: +47 228 40202, Email: eirik.qvigstad@medisin.uio.no (E.Q.)/f.o.levy@medisin.uio.no (F.O.L.)

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with ANP was recently demonstrated to have no effect on \( \beta \)-AR-mediated contractility despite a robust increase in cGMP.\(^{11}\) Thus, the two NPRs may elicit different cardiac effects even though both receptors increase intracellular cGMP levels.

A possible cross-talk between the cGMP and cAMP signalling systems may have considerable impact on cardiac homeostasis in HF. Stimulation of \( G \)-coupled \( \beta \)-ARs activates protein kinase A (PKA) in the heart and elicits positive inotropic and lusitropic effects and increases apoptosis.\(^{12,13}\) The detrimental effects of long-term increased cAMP signalling through \( \beta \)-AR stimulation is the basis for the successful use of \( \beta \)-blockers in treatment of HF.\(^{14}\)

Cyclic GMP is produced by two main types of GCs that differ in their cellular localization and activation by specific ligands. Cytosolic soluble GC is activated by nitric oxide, whereas plasma membrane bound particulate GC is activated by natriuretic peptides.\(^5\) ANP and BNP elicit their effects by binding to and activation of the cell surface NPR-A, whereas CNP activates NPR-B.\(^5\)

Individual PDE subtypes regulate distinct cellular functions by selectively degrading different cAMP and cGMP pools and thus play crucial roles in regulating the amplitude, duration, and compartmentation of cyclic nucleotide signalling.\(^{15}\) Currently, five different PDE families have been described in cardiac muscle. PDE1, PDE2 and PDE3 display dual substrate specificity in vitro, whereas PDE4 and PDE5 selectively hydrolyze cAMP and cGMP, respectively.\(^{15}\) PDE3 shows high affinity for both cAMP and cGMP but has a lower \( V_{max} \) for cGMP which thus effectively inhibits the degradation of cAMP.\(^8\) PDE3-selective inhibitors such as milrinone have been used as positive inotropic agents to treat HF patients. Despite short-term beneficial haemodynamic effects, long-term effect of these cAMP-increasing drugs is harmful as they increase mortality.\(^{16}\)

In this paper, we demonstrate that selective NPR-B stimulation enhances \( \beta \)-AR-mediated responses in the failing heart through cGMP inhibition of PDE3. The effect is comparable to the enhancement obtained by the selective PDE3 inhibitor cilostamide and is able to overcome an inhibitory contractile component mediated through PKG activation. In contrast, selective NPR-A stimulation by BNP was unable to augment \( \beta \)-AR-mediated effects despite similar increase in cGMP levels.

### 2. Methods

For more detailed Methods, see Supplementary material online.

#### 2.1 Animals

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 project approval was granted by the Norwegian National Animal Research Committee.

Briefly, myocardial infarction was induced in male Wistar rats by proximal ligation of the left coronary artery.\(^{17}\) Six weeks later, HF rats were included if left ventricular end-diastolic pressure was \( \geq 15 \) mmHg and the rats had clinical signs of HF.

#### 2.2 Isolation of cardiomyocytes

Adult ventricular cardiomyocytes were isolated from excised failing rat hearts by aorta-perfusion with a nominally Ca\(^{2+}\)-free JOKLIK-MEM solution and enzymatic digestion using trypsin (60 U/mL) and collagenase (90 U/mL) as previously described.\(^{18}\)

### 2.3 Contraction studies

Muscle strips from non-infarcted left ventricle were prepared, stimulated at 1 Hz and the contraction–relaxation cycles were recorded and analysed as previously described.\(^{17}\) Maximal development of force (\( dF/dt \)\( _{max} \)) was used as an index of contractility and inotropic responses to agonists were expressed by increases in (\( dF/dt \)\( _{max} \)).

#### 2.4 PDE assay

PDE activity was assayed using a modification of a two-step procedure\(^{19}\) with mixture of \([\text{H}]\text{cAMP}\) and unlabelled cAMP to final concentration of 1 \( \mu \)mol/L.

##### 2.5 cAMP and cGMP measurements

CAMP was measured by radioimmunoassay as previously described\(^{20}\) and cGMP were measured by cGMP EIA kit (Cayman Chemicals).

#### 2.6 Annexin-V/propidium iodide staining

Detection and quantification of apoptotic cardiomyocytes was assessed by staining with phosphatidylserine binding Annexin-V-fluorescein (apoptotic cells; Annexin-V-Fluos staining kit, Roche Mannheim) and the nucleic acid binding propidium iodide (necrotic cells).

#### 2.7 Western blot

Phospholamban (PLB) and tropinin-I (TnI) phosphorylation was measured by western analysis using following antibodies: Ser16-phospho-PLB, anti-total PLB, Ser23/24-phospho-TnI, and anti-total TnI. Changes in phosphorylation were reported as relative changes to its own control value (non-stimulated).

#### 2.8 Drugs

Prazosin hydrochloride, (−)-noradrenaline bitartrate, and atropine sulphate were purchased from Sigma-Aldrich. Cilostamide, rolipram, and IC118 551 hydrochloride from Tocris Bioscience (UK), Rp-8-Br-PET-cGMPs and Sp-8-Br-PET-cGMPs from Biolog LSI (Germany) and ANP, BNP, and CNP from GenScript Corp (USA).

#### 2.9 Statistics

All results are expressed as mean \( \pm \) SEM unless otherwise indicated, and statistical significance was assessed with unpaired or paired Student’s t-test as appropriate. Bonferroni corrections were made when relevant. \( P < 0.05 \) was regarded as statistically significant.

### 3. Results

#### 3.1 Animal characteristics

Animal characteristics and haemodynamics data are given in Table 1.

#### 3.2 Effects of selective PDE inhibition in HF

To evaluate and interpret a possible PDE3-inhibitory effect of natriuretic peptide stimulation on \( \beta \)-AR-evoked functional responses, PDE activities, and the effects of selective PDE inhibitors, were explored in failing hearts.
3.2.1 PDE activity in HF
We studied total cardiac cAMP-PDE activities in HF and used cilostamide (1 μmol/L) and rolipram (10 μmol/L) to define the fractions of the total activity that were due to PDE3 (cilostamide-sensitive) and PDE4 (rolipram-sensitive), respectively. PDE3 and PDE4 provided ~80% of total cAMP-PDE activity in the failing heart with the activity of PDE4 being approximately twice that of PDE3 (Figure 1A).

3.2.2 PDE4 is the primary PDE regulating the increase in total camp levels elicited by β1-AR stimulation in HF
PDE inhibitors increase β1-AR-mediated elevation in cAMP by slowing down cAMP degradation. Failing left ventricular muscle strips were challenged with noradrenaline (1 μmol/L) and total cAMP levels were measured in the absence and presence of cilostamide (Cil; 1 μmol/L), rolipram (Rol; 10 μmol/L) or both inhibitors.

Noradrenaline increased cAMP levels significantly reaching a maximum level at 2 min (Figure 1B). As expected, preincubation with cilostamide or rolipram amplified the cAMP response elicited by noradrenaline and demonstrated that PDE4 is the main suppressor of β1-AR-mediated increase in total cAMP levels (Figure 1B). Noradrenaline in the presence of both cilostamide and rolipram increased total cAMP levels more than an additive effect of the two inhibitors implying a double PDE barrier controlling the β1-AR-mediated cAMP signal in failing hearts (Figure 1B).

3.2.3 PDE3 is the primary PDE regulating β1-AR-mediated inotropic responses in HF
β1-AR stimulation elicited a positive inotropic response in failing left ventricular strips of 108.3 ± 7.0% above control (EC50 = 6.99 ± 0.07, n = 21; Figure 1C). PDE3 inhibition (Cil; 1 μmol/L) sensitized the β1-AR-mediated inotropic response compared with control in HF (ΔEC50 = 0.66 ± 0.09 log units, n = 6, P < 0.005; Figure 1C). PDE4 inhibition (Rol; 10 μmol/L) did not significantly shift the β1-AR concentration curve compared with control (ΔEC50 = 0.13 ± 0.06, n = 7, P = 0.4; Figure 1C). The presence of both cilostamide and rolipram shifted the concentration-response curve to the left beyond that of an additive effect of the two inhibitors (ΔEC50 = 1.50 ± 0.04 log units, n = 6, P < 0.05; Figure 1C). Thus, PDE3 is the primary barrier for the cAMP signal eliciting the contractile response in HF and the effect of PDE4 inhibition is only revealed in the presence of concomitant PDE3 inhibition. The PDE inhibitors did not alter the maximal β1-AR-mediated responses (data not shown).

3.3 Effects of natriuretic peptides
3.3.1 Both BNP and CNP increase cGMP levels but only CNP reduces the contractile force
To evaluate NPR-A and NPR-B-coupled responsiveness, we measured cGMP levels in left ventricular muscle strips exposed to BNP or CNP. Both peptides increased cGMP levels concentration dependently (Figure 2A and B). NPR-B stimulation by CNP (300 nmol/L) elicited a negative inotropic response of 13.9 ± 1.5% (n = 8) which was attenuated by the selective PKG antagonist Rp-8-Br-PET-cGMPS (6.4 ± 2.0%, n = 6, P < 0.05; Figure 2C). NPR-A stimulation by BNP, however, elicited no negative inotropic response despite a robust increase in cGMP (Figure 2A, C, D). Similar results were obtained for ANP (data not shown). The discordance between cGMP levels and negative inotropic effects elicited by BNP and CNP indicates activation of different functional compartments by NPR-A and NPR-B stimulation. In cardiomyocytes BNP and CNP, each at 300 nmol/L, elicited a 3.5- and 5.7-fold increase in cGMP levels, respectively, (n = 5, P < 0.05; Figure 2D).

3.3.2 NPR-B but not NPR-A stimulation sensitizes β1-AR-mediated inotropic effects in HF
Selective activation of NPR-B by CNP enhanced a submaximal β1-AR-mediated inotropic response to noradrenaline concentration dependently (EC50 = 22 nmol/L, Figure 2E, i and F upper panel). The effect was reversed by timolol (Figure 2E, i) and was not present in the absence of β1-AR prestimulation (Figure 2E, ii) demonstrating that the positive inotropic response to CNP was indirectly mediated through β1-ARs. In the absence of β1-AR prestimulation, CNP dose dependently (EC50 = 55 nmol/L) elicited a negative inotropic response (Figure 2E, ii and F lower panel). CNP (preincubation: 300 nmol/L) sensitized the β1-AR-mediated inotropic response compared with control in HF (ΔEC50 = 0.41 ± 0.05 log units, n = 12, P < 0.05; Figure 3A). In the presence of rolipram, CNP shifted the concentration-response curve beyond that of an additive effect of the two drugs (ΔEC50 = 1.18 ± 0.10 log units, n = 7, P < 0.005; Figure 3A) mimicking the

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**Table 1** Animal and left ventricular muscle strip characteristics

<table>
<thead>
<tr>
<th></th>
<th>CHF rats (n = 59)</th>
<th>Sham rats (n = 14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt (g)</td>
<td>375.5 ± 3.2</td>
<td>389.0 ± 9.6</td>
</tr>
<tr>
<td>Heart wt (g)</td>
<td>2.65 ± 0.05</td>
<td>1.38 ± 0.04</td>
</tr>
<tr>
<td>Heart wt/body wt (g kg⁻¹ × 10³)</td>
<td>7.1 ± 0.1</td>
<td>3.5 ± 0.1</td>
</tr>
<tr>
<td>LVEDP (mmHg)</td>
<td>21.1 ± 1.0</td>
<td>2.50 ± 0.19</td>
</tr>
<tr>
<td>LVSP (mmHg)</td>
<td>101.6 ± 2.5</td>
<td>110.6 ± 3.9</td>
</tr>
<tr>
<td>Lung wt (g)</td>
<td>3.98 ± 0.14</td>
<td>1.74 ± 0.07</td>
</tr>
<tr>
<td>CSA (mm²)</td>
<td>0.51 ± 0.01 (n = 354)</td>
<td>0.51 ± 0.03 (n = 25)</td>
</tr>
<tr>
<td>CF (mN/mm²)</td>
<td>12.31 ± 0.47 (n = 354)</td>
<td>10.1 ± 1.03 (n = 25)</td>
</tr>
</tbody>
</table>

Characteristics of Sham animals shown for comparison. Data represent mean ± SEM. LVEDP, left ventricular end-diastolic pressure; LVSP, left ventricular systolic pressure; CSA, cross-sectional area of muscle strips; CF, basal contractile force of muscle strips.
Figure 1 PDE4 is the main PDE regulating total cAMP levels, whereas PDE3 is the main PDE regulating inotropic responses following β1-adrenoceptor stimulation in HF. (A) PDE3 and PDE4 activity was determined in failing left ventricular muscle strips as the fraction of total cAMP hydrolytic activity inhibited by cilostamide (Cil; 1 μmol/L) and rolipram (Rol; 10 μmol/L), respectively. Others: PDE activity not inhibited by cilostamide and rolipram. (B) The time-course of total cAMP levels in muscle strips stimulated by 1 μmol/L noradrenaline (NA) in the absence and presence of cilostamide, rolipram, or both inhibitors. (P < 0.05 vs. NA). (C) Concentration–response curves of inotropic responses to β1-AR stimulation in HF in the absence (Ctr) and presence of cilostamide, rolipram, or both inhibitors. \( * \)P < 0.05 vs. ctr.

pattern observed with the combination of rolipram and cilostamide (Figure 1C). To evaluate whether CNP sensitized the β1-AR-mediated inotropic response through inhibition of PDE3, we tested whether the effect of CNP was not detectable in the presence of cilostamide (1 μmol/L). Preincubation with both cilostamide and CNP did not cause further shift of the β1-AR-mediated concentration–response curve (ΔEC50 = 0.41 ± 0.10, n = 6, P < 0.05) than CNP alone, consistent with a PDE3-dependent mechanism (Figure 3A). Activation of PKA by β1-ARs results in phosphorylation of regulatory contractile proteins, including PLB and TnI.12 NPR-B stimulation by CNP also enhanced β1-AR-evoked PKA activation in failing hearts as noradrenaline (70 nmol/L) in the presence of CNP (300 nmol/L) increased phosphorylation of PLB and TnI more than the added increase by noradrenaline and CNP alone (Figure 3C and D). The possibility of a cAMP-independent mechanism was unlikely as CNP was not able to modulate α1-AR-mediated inotropic responses (data not shown).

In contrast to CNP, selective stimulation of NPR-A by BNP had no effect on β1-AR-mediated contractility despite an ~3-fold increase in cGMP (Figures 2E, iii and 3B). Similar results were obtained for ANP (data not shown). Submicromolar concentrations of BNP, however, seemed to elicit a minor enhancement of β1-AR responsiveness (Figure 2E, iii), probably reflecting a concentration of BNP able to activate both NPR-A and NPR-B due to loss of selectivity of the agonist.21 This was further tested by preincubation with a non-selective concentration of BNP (1 μmol/L),21 which significantly sensitized the β1-AR-mediated response compared with control in contrast to no effect by an NPR-A-selective dose of BNP (300 nmol/L) (Figure 3B). Similar to CNP, the presence of rolipram and BNP (1 μmol/L) together sensitized the β1-AR-evoked response beyond that of an additive effect of the two drugs (Figure 3B). BNP (1 μmol/L) also elicited a negative inotropic response of 7.6 ± 1.7% (n = 5) consistent with the activation of NPR-B at this concentration in contrast to 300 nmol/L. The presence of BNP or CNP did not change maximal β1-AR-mediated inotropic response (data not shown).

3.3.3 PKG activation inhibits β1-AR-mediated inotropic responses

Activation of PKG by cGMP can inhibit β-AR signalling.7,22 To address a possible involvement of PKG in the modulatory effect of CNP on β1-AR-mediated response, we performed experiments with a cGMP agonist and antagonist. The cGMP agonist and selective PKG activator Sp-8-Br-PET-cGMPs (25 μmol/L) elicited a negative inotropic effect on basal contractility (18.9 ± 4.7%, n = 4, P < 0.05) similar to CNP and shifted the noradrenaline concentration–response curve to higher concentration by 0.39 ± 0.04 log units (n = 4, P < 0.05; Figure 4). This is consistent with the expected inhibitory effect of PKG activation on β1-AR-mediated inotropic responses in failing hearts. PDE3 inhibition with cilostamide alone sensitized the β1-AR-mediated response more than the combination of CNP and cilostamide together (ΔEC50 = 0.65 ± 0.09, n = 10 vs. 0.41 ± 0.10 log units, n = 6, P = 0.05; Figure 4) indicating also a moderate inhibitory component of CNP in addition to the potentiating effect shown above. In the presence of Rp-8-Br-PET-cGMPs (cGMP/PKG-antagonist), CNP...
shifted the noradrenaline concentration–response curve to the same extent as cilostamide alone \((\Delta EC_{50} = 0.61 \pm 0.09, n = 9\) vs. \(0.65 \pm 0.09\) log units, \(n = 10\); Figure 4) revealing that the small inhibitory component of CNP was PKG dependent. Similarly, preincubation with both rolipram and CNP shifted the \(\beta_1\)-AR response less than the combination of rolipram and cilostamide \((\Delta EC_{50} = 1.18 \pm 0.09, n = 7\) vs. \(1.49 \pm 0.06\) log units, \(n = 5\), \(P < 0.05\) revealing an inhibitory component of CNP also during PDE4 inhibition. In the presence of Rp-8-Br-PET-cGMPs, however, the sensitizing effects of the two interventions were similar (Figure 4), again consistent with reversal of a PKG-dependent inhibitory component. Rp-8-Br-PET-cGMPs alone did not change basal or \(\beta_1\)-AR-mediated increase in contractility (data not shown).

### 3.3.4 CNP accentuates \(\beta_1\)-AR-mediated increase in cAMP levels to the same extent as cilostamide

To test whether CNP could accentuate the \(\beta_1\)-AR-mediated increase in total cAMP levels to the same extent as PDE3 inhibition with cilostamide, muscles were stimulated with noradrenaline (1 \(\mu\)mol/L) in the absence and presence of CNP (300 nmol/L) and cilostamide and total cAMP levels measured. Time–course
curves demonstrated that CNP did indeed accentuate the β1,-AR-mediated increase in cAMP levels to the same extent as the PDE3 inhibitor cilostamide, probably due to cGMP inhibition of PDE3 (Figure 5). PDE activity assays performed in muscles preincubated with CNP did not, however, reveal decreased PDE3 activity compared with control, probably reflecting dissociation of cGMP from PDE3 during dilution and homogenization (data not shown).

### 3.3.5 CNP increases apoptosis in HF

Chronic PDE3 inhibition and β1-AR stimulation induce apoptotic death of cardiomyocytes. Thus, we investigated the effect of selective PDE inhibitors and natriuretic peptides on cardiomyocyte apoptosis in the absence and presence of β1-AR stimulation. Under control conditions 6.0 ± 0.9% of the cardiomyocytes were apoptotic, as demonstrated by Annexin-V-fluos staining (Figure 6A). PDE4 inhibition (Rol; 10 μmol/L) did not increase apoptosis significantly, whereas PDE3 inhibition (Cil; 1 μmol/L) increased cardiomyocyte apoptosis to 16.9 ± 2.5% (n = 4, P < 0.05; Figure 6A). Stimulation of NPR-A by BNP (300 nmol/L) had no effect on apoptosis, whereas NPR-B stimulation by CNP (300 nmol/L) increased the number of apoptotic cells significantly to 15.1 ± 1.9% (n = 4, P < 0.05; Figure 6B). Noradrenaline (1 μmol/L) increased apoptosis to 18.0 ± 1.3% (n = 6, P < 0.05 compared with control) and the presence of rolipram or BNP...
did not cause further increase in number of apoptotic cells (Figure 6C). However, in the presence of cilostamide or CNP, noradrenaline increased the number of apoptotic cells to 28.6 ± 1.6% (n = 4, P < 0.05 vs. control) and 26.0 ± 1.3% (n = 4, P < 0.05 vs. control), respectively. Thus, CNP and cilostamide displayed similar effects on apoptosis (Figure 6C).

4. Discussion

We demonstrate for the first time that NPR-B stimulation enhances β₁-AR-mediated signalling in the failing heart. NPR-B stimulation increases the elevation of total cAMP levels, inotropic responses, and apoptosis mediated through β₁-ARs to the same extent as the selective PDE3 inhibitor cilostamide and also increases phosphorylation of PLB and TnI. The inhibitory effect of cGMP on PDE3 overcomes the ability of PKG to attenuate PKA-dependent effects on contractility. On the contrary, selective NPR-A stimulation did not augment β₁-AR-mediated signalling. Thus, different cGMP pools are generated by the two NPRs (Figure 7).

4.1 NPR-B promotes cardio-excitatory effects through PDE3 inhibition

NPR-B stimulation by CNP elicited a robust increase in cGMP. This is in agreement with Dickey et al., who demonstrated that NPR-B stimulation by CNP accounted for the majority of the natriuretic peptide-stimulated GC activity in failing hearts. The presence of CNP increased β₁-AR-mediated elevation of cAMP to similar levels as cilostamide, consistent with a reduced degradation of cAMP due to cGMP-dependent PDE3 inhibition. Furthermore, NPR-B stimulation by CNP (EC₅₀ = 22 nmol/L) significantly sensitized β₁-AR-mediated contractile responses and additional PDE3 inhibition did not promote further potentiation of the response. This indicates that CNP and cilostamide inhibit the same PDE subtype because an additive or synergistic effect on β₁-AR-evoked signalling would occur if the molecular target of the two interventions were different. This was supported by the data obtained in the presence of the PDE4 inhibitor rolipram, where CNP sensitized β₁-AR-mediated inotropic responses beyond the sum of the effects of the two drugs given separately, a similar pattern as observed in the presence of concomitant PDE3 and PDE4 inhibition with cilostamide and rolipram. Thus, our functional data support a pronounced inhibitory effect of NPR-B-generated cGMP on PDE3 in failing hearts which essentially mimics the effects of the selective PDE3 inhibitor cilostamide. Accordingly, NPR-B stimulation by CNP also enhanced β₁-AR-evoked PKA activity, the main downstream effector of cAMP, as demonstrated by an increase in phosphorylation of PLB and troponin I when compared with the added increase elicited by noradrenaline and CNP alone. CNP was unable to modulate a cAMP-independent α₁,-AR-mediated inotropic response, demonstrating a selective cardio-excitatory effect on cAMP-dependent responses.
We also demonstrate that chronic (20 h) NPR-B stimulation with CNP elicits cardiomyocyte apoptosis to a similar extent as PDE3 inhibition by cilostamide in the absence and presence of noradrenaline (20 h) stimulation. In support of our findings, Ding et al. demonstrated in primary cultured cardiomyocytes that chronic inhibition of PDE3 by cilostamide in the absence and presence of BNP, rolipram, CNP (10 μmol/L) or rolipram, CNP (10 μmol/L, *P < 0.05 vs. ctr) for 20 h and subjected to Annexin-V/propidium iodide staining. (C) β1-AR stimulation by noradrenaline (NA, 1 μmol/L, *P < 0.05 vs. ctr) in the absence and presence of BNP, rolipram, CNP (10 μmol/L vs. NA), or cilostamide (10 μmol/L vs. NA) for 20 h. (D) Illustration of Annexin-V-fluorescein (AN)/propidium iodide (PI) staining; ANpositive/PInegative apoptotic cardiomyocyte (solid arrow) and ANnegative/PIpositive necrotic/late apoptotic cells (dashed arrow) induced by NA. Ordinate: mean percentages of adherent cells with apoptotic labelling pattern (ANpositive/PInegative). Data are mean ± SEM of 4–7 experiments, each was performed in duplicate.

### 4.2 PKG-dependent inhibitory effects of CNP

CNP also exerted a small but significant PKG-dependent inhibitory effect on β1-AR-mediated contractility, effectively abolished by the selective PKG antagonist Rp-8-Br-PET-cGMPs, in addition to the potentiating effect described above. The selective PKG activator Sp-8-Br-PET-cGMPs effectively inhibited β1-AR-evoked contractility, demonstrating the ability of PKG to negatively modulate PKA-mediated responses. We demonstrate that PDE3 inhibition by cGMP following CNP stimulation overrides the inhibitory effect of PKG activation by cGMP on β1-AR-mediated inotropic responses.

Activation of the cGMP pathway has the potential to modulate cAMP-mediated signalling in the heart through PKG-independent and -dependent mechanisms. In ventricular myocytes, cGMP has been shown to activate PKG and inhibit cAMP-stimulated I_{Ca} independently of PDEs. Increased cGMP levels are also able to modulate cAMP responses through cGMP-dependent activation of PDE2. However, elevated levels of cGMP can inhibit PDE3 and promote cAMP-stimulated I_{Ca} in human atrial myocytes, and in human hypertrophic right ventricle the cGMP-PKG pathway is preferentially shifted towards inhibition of PDE3 due to decreased PKG activity. Accordingly, cGMP-dependent regulation of cAMP signalling is probably determined by the relative contribution and compartmentation of PDEs, PKG, and receptors activating cGMP pathways.

### 4.3 Cardiac PDEs

As previously reported we show that PDE4 constitutes above 50% of the total cardiac PDE activity and is the primary suppressor of β1-AR-mediated increase in total cAMP. We show that PDE3 is the main PDE subtype regulating β1-AR-mediated increase in contractility in the failing heart and a contribution of PDE4 is only revealed in the presence of concomitant PDE3 inhibition. Obviously, the regulatory role of various PDE isoforms may be different when based on the measurements of cAMP levels and on specific end-effects as increase in contractile response. Although FRET-based techniques are increasingly used to illustrate compartmentation of cAMP-mediated signalling, the utility of such techniques depends on the intracellular localization of the cAMP sensor. As there is no cAMP sensor selectively reflecting the inotropic effects, functional assays may better reflect the functional compartmentation of the cAMP signal most relevant for contractility. Thus, the apparent discordance between functional responses and increase in total cAMP levels may be explained by localization of PDE3 in close proximity to the pool of cAMP important for regulation of contractility in failing hearts. Thus, the failing rat heart seems similar to the failing human heart, where PDE3 is the most important regulatory PDE isoform.

Still, the combination of cilostamide and rolipram displayed a potentiating effect beyond an additive effect of the two drugs, suggesting the existence of a double PDE barrier modulating β1-AR responsiveness in failing rat myocardium.

We show that NPR-B, but not NPR-A, stimulation elicits PKG-dependent negative inotropic effects and increases β1,-AR-evoked signalling despite comparable increase in cGMP levels. This difference may be explained by an existence of parallel and spatially segregated cGMP signalling pathways coexisting within the cardiomyocyte. The modulatory effect of CNP on β1,-AR-evoked signalling was unknown until the present study, whereas NPR-A stimulation with ANP was recently demonstrated to have no effect on β-AR-mediated contractility despite a robust increase in cGMP. PDE3 activity is competitively inhibited by cGMP with a K_{i} of ~0.03 μmol/L, and may be inhibited through NPR stimulation as the subsarcolemmal...
cGMP concentration reached by maximal NPR activation is ~2.3 μmol/L.29

4.4 Natriuretic peptides in HF
CNP selectively stimulates NPR-B and is not able to increase cGMP in cells expressing only NPR-A.21 Thus, despite lack of selective NP-receptor antagonists, we consider the modulatory effects on β₁-AR signalling elicited by CNP (EC₅₀ = 22 nmol/L) to reflect selective NPR-B stimulation.6 BNP, however, has high affinity for NPR-A, but can activate NPR-B in micromolar concentrations due to limited receptor selectivity.21 Thus, we demonstrate that 1 μmol/L of BNP is able to sensitize β₁-AR-mediated inotropic responses similar to that observed by NPR-B stimulation by CNP. Increased levels of ANP, BNP, and CNP are produced directly within the myocardium in HF2 and parallels clinical severity and correlate negatively with indices of left ventricular function and correlate negatively with indices of left ventricular function such as ejection fraction and \(\frac{dP}{dt}_{\text{max}}\).1,30 However, the low endogenous NP-concentrations measured in tissue homogenates or plasma do not necessarily reflect the agonist concentration locally at the receptor. Nevertheless, increased peptide concentrations in HF elicit functional effects in vivo, effectively inhibited by the non-selective NPR antagonist HS-142-1,3,4 demonstrating the involvement of these peptides in cardiovascular homeostasis in HF. Unfortunately, no subtype selective NPR antagonist exists at the present time, making it difficult to differentiate between effects of the two receptors. NPR-B accounts for the majority of the natriuretic peptide-dependent activity in the failing heart.23 Thus, increased NPR-B-cGMP signalling may inhibit PDE3 in the failing cardiomyocyte. Chronic β-AR signalling is a harmful compensatory mechanism in the failing heart and PDE3 inhibition enhances β-AR-evoked signalling by slowing cAMP degradation.15,27 Clinical studies with milrinone (PDE3 inhibitor) demonstrate increased mortality, sudden death, and arrhythmias in HF patients.16 Similarly, detrimental effects of CNP mediated through a cGMP-PDE3 inhibitory pathway may be involved in the pathophysiology of HF. In contrast, CNP has been demonstrated to promote cardiovascular effects considered beneficial in HF, e.g. vascular smooth muscle relaxation and prevention of cardiac remodelling.6 Thus it is unclear whether CNP actually worsens cardiac function and long-term prognosis of HF analogous to PDE3 inhibitors. Complicating this picture, PDE3 inhibition increased survival and prevented cardiac remodelling in rodent models of HF in contrast to the detrimental effects observed in humans.16,31,32 Thus, further studies in appropriate HF models are needed to address this important question.

**Figure 7** Proposed mechanism for cardio-excitatory effects of natriuretic peptides in HF. NPR-B stimulation selectively increases a cGMP pool that is able to (i) inhibit cardiac PDE3, thus reducing cAMP degradation resulting in increased β₁-AR-evoked signalling and (ii) activate PKG that exert negative inotropic effects. PDE3 inhibition by cGMP following CNP stimulation overrides the inhibitory effect of PKG activation by cGMP on β₁-AR-mediated inotropic responses. In contrast, NPR-A stimulation seems to activate a different cGMP pool (separated by dotted line) not able to modulate β₁-AR-evoked effects.
In conclusion, we demonstrate that NPR-B stimulation by natriuretic peptides inhibits PDE3 and subsequently potentiates β1-AR-mediated signalling in the failing heart. Thus, the NPR-B-cGMP-PDE3 inhibitory pathway may be detrimental in HF. In contrast, selective NPR-A stimulation did not augment β1-AR-mediated signalling. Thus, apparently different functional cGMP pools are coupled to the two NPRs. Furthermore, PDE3 is the most important PDE subtype regulating β1-AR-mediated inotropic responses and apoptosis in HF, in contrast to its secondary role in the elevation of cAMP levels. Our findings are in substantial contradiction to the current view that natriuretic peptides are beneficial players in HF. Further investigation is needed to clarify the importance of NPR-B signalling in failing hearts.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

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