Naunyn-Schmiedeberg's Arch Pharmacol (2014) 387:407–417
DOI 10.1007/s00210-013-0953-1

ORIGINAL ARTICLE

Differential regulation of C-type natriuretic peptide-induced cGMP and functional responses by PDE2 and PDE3 in failing myocardium

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Received: 22 August 2013 / Accepted: 19 December 2013 / Published online: 15 January 2014

Abstract Recently, we showed C-type natriuretic peptide (CNP)-induced negative inotropic (NIR) and positive lusitropic response (LR) in failing rat heart. We wanted to study whether, and if so, how phosphodiesterases (PDEs) regulate CNP-induced cyclic 3′,5′-guanosine monophosphate (cGMP) elevation and functional responses. Inotropic and lusitropic responses were measured in left ventricular muscle strips and cyclic nucleotide levels, PDE activity and phospholamban (PLB) and troponin I (TnI) phosphorylation were measured in ventricular cardiomyocytes from Wistar rats with heart failure 6 weeks after myocardial infarction. CNP-mediated increase in global cGMP was mainly regulated by PDE2, as reflected by a marked amplification of the cGMP increase during PDE2 inhibition and by a high PDE2 activity in cardiomyocytes. PDE3 inhibition, on the other hand, caused no significant cGMP increase by CNP. The functional consequences did not correspond to the changes of cGMP. PDE3 inhibition increased the potency of the CNP-induced NIR and LR, while PDE2 inhibition desensitized the CNP-induced NIR, but not LR. A role for PDE2 on the maximal LR and PDE5 on the maximal NIR to CNP was revealed in the presence of PDE3 inhibition. CNP increased PLB phosphorylation about 25- to 30-fold and tended to increase TnI phosphorylation about twofold. As a whole, CNP-induced functional responses were only modestly regulated by PDEs compared to the cAMP-mediated functional responses to β_1-adrenoceptor stimulation, which are highly regulated by PDEs. There is a mismatch between the CNP-induced cGMP increase and functional responses. Global cGMP levels are mainly regulated by PDE2 after CNP stimulation, whereas the functional responses are modestly regulated by both PDE2 and PDE3, indicating cGMP compartmentation by PDEs affecting CNP-induced responses in failing hearts.

Keywords CNP · cGMP · Heart failure · Phosphodiesterase · Negative inotropic response · Lusitropic response

Electronic supplementary material The online version of this article (doi:10.1007/s00210-013-0953-1) contains supplementary material, which is available to authorized users.

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Introduction

C-type natriuretic peptide (CNP) is increased in heart failure (Del Ry et al. 2005; Kalra et al. 2003), and its receptor, natriuretic peptide receptor B (NPR-B), is mainly responsible for the particulate guanylyl cyclase (pGC) activity in failing hearts (Dickey et al. 2007). CNP also binds the NPR-C receptor; however, this receptor does not exhibit GC activity (Potter et al. 2009). Natriuretic peptides have been used as therapy in heart failure (Boerrigter et al. 2009), although recent data indicate that they are of limited value (O'Connor et al. 2011). Recently, we showed that CNP increases cyclic 3′,5′-guanosine monophosphate (cGMP) levels in cardiomyocytes and causes both a negative inotropic (NIR) and a positive lusitropic response (LR) in failing hearts (Qvigstad et al. 2010; Moltzau

et al. 2013). Further, we suggested that the concerted effects of PLB and TnI phosphorylation represent the main mechanisms explaining the PKG-mediated NIR and LR to CNP and that sarcoplasmatic reticulum Ca^{2+}-ATPase2 (SERCA2) activity was required for the responses (Moltzau et al. 2013).

Compartmentation, duration and amplitude of cGMP and cyclic 3′,5′-adenosine monophosphate (cAMP) signalling is regulated by phosphodiesterases (PDEs) (Omori and Kotera 2007). PDE1, 2, 3, 4, 5, 8 and 9 are described in the heart. PDE1, 2 and 3 can hydrolyse both cAMP and cGMP, whereas PDE4 and 8 are selective for cAMP, and PDE5 and 9 are selective for cGMP. However, cGMP can inhibit the cAMP-hydrolysing capability of PDE3 and possibly PDE1, whereas cGMP can activate PDE2 (Zaccolo and Movsesian 2007). The regulation and compartmentation of cAMP causing functional responses in the heart have been discussed for several years, and studies have shown an important role for PDEs in the regulation of cAMP-induced functional responses (Afzal et al. 2011a; Mika et al. 2012), but there are few studies on whether, and if so, how PDEs regulate both cGMP levels and cGMP-mediated functional responses in the heart. We wanted to investigate whether the CNP-induced NIR and LR we earlier found in the failing heart (Moltzau et al. 2013) were regulated by PDEs, which PDEs were involved and relate the changes of function to levels of cGMP and phosphorylated target proteins. In light of the important role of PDEs in regulating cAMP-induced functional responses, we wanted to compare the role of PDEs in the regulation of cGMP signalling to that in cAMP signalling resulting in changes in contractility.

We found a mismatch between changes of cGMP levels and functional responses in the presence of PDE inhibitors and NPR-B stimulation. The global cGMP increase after CNP stimulation is mainly and highly regulated by PDE2, whereas both PDE2 and PDE3 modestly regulate the cGMP mediating functional responses, but in opposite directions. The modest PDE regulation of cGMP-mediated functional responses to CNP is in sharp contrast to the prominent role of PDEs in regulating cAMP-mediated functional responses to, for example, β_1-adrenoceptor (β_1-AR) stimulation. Nevertheless, we demonstrate that PDEs are involved in the compartmentation of cGMP involved in CNP-induced functional responses in failing hearts.

Materials and methods

For more detailed materials and methods, see Online Resource.

CHF animal model

The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* by the U.S National Institutes of Health (NIH publication no. 85-23, revised 1996). Animal care was according to the Norwegian Animal Welfare Act which conforms with the *European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes* (Council of Europe no. 123, Strasbourg 1985) and was approved by the Norwegian Animal Research Authority. As described earlier (Sjaastad et al. 2000), myocardial infarction was induced by left coronary artery ligation in ~300-g male Wistar rats during anaesthesia (65 % N_2O, 32 % O_2 and 2–3 % isoflurane). Six weeks later, heart failure (HF) rats with left atrial diameter >5 mm and increased lung weight (>2.0 g) in the presence of a large anterolateral infarction were included in the study. In addition, echocardiographic analysis in a subset of animals revealed a marked left ventricular dilatation (left ventricular diameter in diastole (LVDd) 10.0± 0.1 mm) and increased LV end-diastolic blood pressure (EDP, 23±1 mmHg). See Table 1 for detailed animal characteristics (for comparison with sham-operated animals, see Birkeland et al. (2007)). The hearts were either used for isolation of cardiomyocytes or muscle strips. Inotropic and lusitropic responses, which are cardiomyocyte-specific effects, were measured in isolated left ventricular muscle strips, whereas measurements that would not be specific for cardiomyocytes (cAMP and cGMP assay, PDE activity assay and protein phosphorylation) were determined in isolated ventricular cardiomyocytes to ensure cardiomyocyte-specific effects.

Isolated muscle strips

HF left ventricular muscle strips were prepared and stimulated at 1 Hz. Every individual muscle was stretched to the

Table 1 Animal characteristics, echocardiographic and haemodynamic data of HF hearts

	Value
Animal characteristics (n=99)	
Body weight (g)	382±3
Heart weight (g)	2.7±0.1
Lung weight (g)	4.0±0.1
Echocardiographic data (n=10)	
LAD (mm)	6.8±0.2
LVDd (mm)	10.0±0.1
LVDs (mm)	8.8±0.2
FS (%)	12±1
Haemodynamic data (n=16)	
SBP	95±3
EDP	23±1

Data represent mean ± SEM. Echocardiographic and haemodynamic characteristics were measured in a subset of animals

LAD left atrial diameter, *LVDd* left ventricular diameter in diastole, *LVDs* left ventricular diameter in systole, *FS* left ventricular fractional shortening, *SBP* systolic blood pressure, *EDP* left ventricular end-diastolic blood pressure

maximum of the length-tension curve, typically to a tension of about 350 mN. As previously described (Sjaastad et al. 2003), maximal development of force ($(dF/dt)_{max}$), time to peak force (TPF), time to 80 % relaxation (TR80) and relaxation time (RT=TR80−TPF) were analysed from recorded contraction-relaxation cycles (CRCs). Inotropic responses were expressed as change of $(dF/dt)_{max}$ in percent of control levels, and NIR was defined as decrease in $(dF/dt)_{max}$. LR was expressed as decrease of RT in percent of basal; *n* represents the number of hearts used.

Isolation of cardiomyocytes

Hearts from HF rats were perfused in a Langendorff set-up with a Ca^{2+}-free Joklik-MEM buffer and digested enzymatically using collagenase type-II (90 U/ml final) (Worthington Biochem. Corp., 268 U/mg) as described in the Online Resource.

Cyclic AMP, cGMP assays and Western blot

Isolated cells were pre-incubated with PDE inhibitors for 15 min and stimulated for 10 min with an agonist as indicated. Cyclic AMP was measured by radioimmunoassay as previously described (Skomedal et al. 1980), and cyclic GMP was measured using the cyclic GMP enzyme immunoassay (EIA) kit from Cayman Chemical Company (USA). Western analysis for PLB-Ser16 and TnI-Ser23/24 phosphorylation was performed using antibodies for total and phosphorylated protein; *n* represents the number of experiments on isolated cardiomyocytes with one or two hearts in each experiment.

Phosphodiesterase assays

PDE activity was measured in isolated HF ventricular cardiomyocytes using a two-step procedure (Marchmont and Houslay 1980) with a mixture of [^{3}H]cAMP and unlabelled cAMP (or [^{3}H]cGMP and unlabelled cGMP) to a final concentration of 1 μM; *n* represents the number of hearts used.

Statistics

All results are presented as mean ± SEM. Statistical significance was calculated by unpaired Student's *t* test or one-sample *t* test where appropriate. Bonferroni corrections were made when relevant. For the statistics comparing results in the functional assays, one-sample *t* test was performed using the calculated difference (difference in log EC_{50}, NIR_{max} or LR_{max}) between the relevant control and the intervention in each experiment. In PDE activity assays, one-sample *t* test was performed comparing the PDE activity in each group (erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), cilostamide (Cil), sildenafil (Sfil) or rolipram (Rol)) from each experiment with a control value of 100 (100 % PDE activity). $P<0.05$ was considered to reflect significant differences (*$P<0.05$, **$P<0.01$, ***$P<0.001$).

Results

PDEs modestly regulate functional responses to CNP

We previously found CNP-induced NIR and LR in failing hearts (Qvigstad et al. 2010; Moltzau et al. 2013) and hypothesised that PDEs could regulate the cGMP-induced functional responses caused by CNP. Experiments were conducted in the absence and presence of separate PDE2, 3 and 5 inhibition or combinations thereof. Both time courses of the responses to CNP (Fig. 1a) and concentration-response relationships (Fig. 1b–e) of CNP were studied. The time course response to submaximal concentration of CNP (100 nM) showed a NIR (−9.6±1.2 % (average from all experiments; $n=13$, $P<0.005$, data not shown)) compared to a control value of −1.8±0.5 % ($n=13$). CNP alone caused a NIR with a $-\log EC_{50}$ value of 7.32±0.05 and a NIR to maximal CNP of 37.2±1.9 % below basal ($n=20$; Fig. 1b, d). Further, CNP alone caused a LR with a $-\log EC_{50}$ value of 7.14±0.07 and a LR to maximal CNP of 21±1 % ($n=20$; Fig. 1c, e). The presence of separate PDE2, 3 and 5 inhibitions did neither change the NIR nor the LR to maximal CNP concentration.

PDE2 inhibition desensitizes the NIR to CNP

In time-course studies, the PDE2 inhibitor EHNA (10 μM) tended to reduce the NIR to a submaximal concentration of CNP (100 nM) (−6.3±0.7 %, $n=4$ vs. −9.5±2.2 %, $n=4$; Fig. 1a part i). Studies on concentration-response relationship revealed that EHNA significantly shifted the concentration-response curve for the CNP-induced NIR to higher concentrations ($\Delta\log EC_{50}=-0.23\pm0.08$, $n=14$, $P<0.05$; Fig. 1b), whereas there was no significant shift in the LR ($\Delta\log EC_{50}=-0.16\pm0.13$, $n=14$; Fig. 1c). Thus, these results indicate regulation of the CNP-induced NIR by PDE2.

PDE3 inhibition sensitizes the functional responses to CNP

In contrast to PDE2 inhibition, the PDE3 inhibitor Cil (1 μM) tended to increase the NIR to a submaximal concentration of CNP (100 nM) (−12.2±2.0 %, $n=6$ vs. −6.5±1.4 %, $n=5$; Fig. 1a part ii). Studies on concentration-response relationship revealed that PDE3 inhibition by Cil increased the sensitivity to CNP significantly by shifting the concentration-response curves for NIR ($\Delta-\log EC_{50}=0.22\pm0.06$, $n=16$, $P<0.01$; Fig. 1b) and LR ($\Delta-\log EC_{50}=0.26\pm0.09$, $n=16$, $P<0.05$; Fig. 1c) to lower concentrations. Our results demonstrate that PDE3 regulates both the NIR and the LR to CNP.

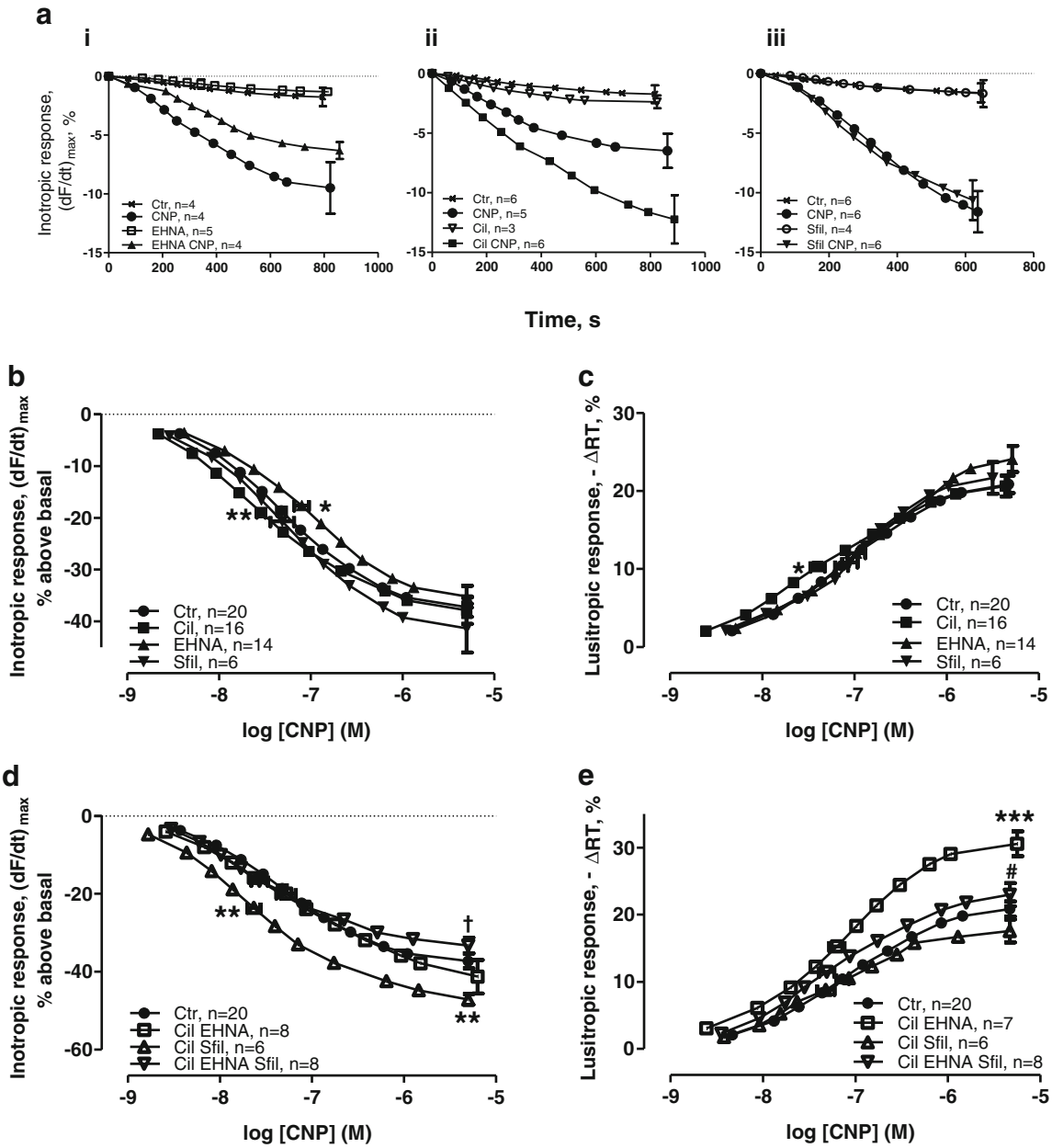

Fig. 1 PDE2 inhibition desensitized and PDE3 inhibition sensitized the CNP-induced negative inotropic response. **a** Time course curves of the inotropic response to submaximal NPR-B stimulation (CNP 100 nM) in HF left ventricular muscle strips in the absence and presence of (*i*) PDE2 inhibition (EHNA), (*ii*) PDE3 inhibition (Cil) and (*iii*) PDE5 inhibition (Sfil). Ctr: vehicle without CNP. **b**–**e** Concentration-response curves of the inotropic (**b**, **d**) and lusitropic (**c**, **e**) responses to NPR-B stimulation (CNP) in the absence and presence of: **b**, **c** PDE3 inhibition (Cil), PDE2 inhibition (EHNA) or PDE5 inhibition (Sfil); **d**, **e** combined PDE2/PDE3 inhibition (EHNA Cil), combined PDE3/PDE5 inhibition (Cil Sfil) or PDE2/PDE3/PDE5 inhibition (EHNA Cil Sfil). *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs. Ctr, One-sample *t* test. †$P<0.01$ Cil Sfil vs. Cil EHNA Sfil, #$P<0.05$ Cil EHNA vs Cil EHNA Sfil, unpaired Student's *t* test (Bonferroni-corrected). **a**-**e** Cil 1 μM, EHNA 10 μM and Sfil 0.1 μM

PDE5 inhibition does not affect the functional responses to CNP

The PDE5 inhibitor Sfil did not change the NIR to submaximal concentration of CNP (100 nM; Fig. 1a part iii) or the $-\log EC_{50}$ value for the NIR to CNP ($\Delta\log EC_{50}=0.01\pm0.14$, $n=6$; Fig. 1b) demonstrating no influence of PDE5 on the NIR to CNP. Nor did PDE5 inhibition by Sfil influence the sensitivity to CNP of the LR ($\Delta\log EC_{50}=-0.09\pm0.08$, $n=6$; Fig. 1c).

The effects of combined PDE inhibition on the functional responses to CNP

Inhibition of separate PDEs indicated a role both for PDE2 and PDE3, although in opposite directions, in the regulation of

the functional responses to CNP. To reveal possible cGMP PDE redundancy, we investigated the functional responses to CNP in the presence of combined PDE inhibition. The combined inhibition of PDE2 and PDE3 by EHNA and Cil, respectively, did not cause a shift in the concentration-response curve of CNP for NIR ($\Delta\log EC_{50}=-0.04\pm0.11$, $n=8$; Fig. 1d), which indicates that the opposite effects of Cil and EHNA on the CNP-induced NIR mutually eliminate each other. Further, there was no shift in the concentration-response curve of CNP for LR ($\Delta\log EC_{50}=0.06\pm0.19$, $n=7$; Fig. 1e) in the presence of Cil and EHNA compared to CNP alone. However, the LR to maximal CNP concentrations was significantly increased in the presence of both inhibitors ($\Delta LR_{max}=9.7\pm1.5$ %, $n=7$, $P<0.001$; Fig. 1e). This indicates dual control by PDE2 and PDE3 on the LR to CNP.

Combined PDE3 and PDE5 inhibition by Cil and Sfil significantly shifted the NIR to lower CNP concentrations ($\Delta\log EC_{50}=0.31\pm0.10$, $n=6$, $P<0.01$; Fig. 1d) in line with the results with PDE3 inhibition alone. Further, the combined PDE3 and PDE5 inhibition increased the maximum CNP-induced NIR ($\Delta NIR_{max}=-9.9\pm2.5$ %, $n=6$, $P<0.01$; Fig. 1d) compared to CNP alone, which was not the case with separate PDE3 or PDE5 inhibition (Fig. 1b). Combined PDE3 and PDE5 inhibition did not cause a significant shift of the concentration-LR curve of CNP ($\Delta\log EC_{50}=0.18\pm0.18$, $n=6$). PDE2 inhibition by EHNA abolished the effect of combined PDE3/5 inhibition on NIR_{max} ($P<0.01$; Fig. 1d). This is in line with the separate effect of EHNA. Thus, we revealed an additional effect on the NIR to maximal CNP in the presence of combined PDE3 and PDE5 inhibition not present with separate inhibition. The increase in LR_{max} to CNP in the presence of PDE2 and PDE3 inhibitors (EHNA and Cil, respectively) was abolished by further addition of the PDE5 inhibitor (Sfil) ($P<0.05$, Cil EHNA vs Cil EHNA Sfil; Fig. 1e).

To elucidate a possible role of PDE1 in the regulation of the functional responses to CNP, we used a high concentration of Sfil (10 μM) that will also inhibit PDE1 (Vandeput et al. 2009). There was neither any difference between the NIRs nor the LRs to CNP ($\Delta\log EC_{50}=0.1\pm0.1$ for both, $n=8$) in the presence of EHNA, Cil and a PDE5-selective concentration of Sfil (0.1 μM) compared to the presence of EHNA, Cil and a non-selective concentration of Sfil (10 μM). This indicated no role for PDE1 in the regulation of functional responses to CNP in the presence of PDE3 and PDE5 inhibition (data not shown).

PDE2 is the main regulator of global cGMP increase after CNP stimulation

In isolated cardiomyocytes, CNP significantly increased cGMP levels compared to the basal level, an effect which was amplified several fold in the presence of the PDE2 inhibitor EHNA (Fig. 2a). The presence of the PDE3 inhibitor Cil, the PDE5 inhibitor Sfil (0.1 μM) or the two combined caused no significant increase in CNP-stimulated cGMP elevation. In the presence of EHNA, the additional presence of Cil, Sfil or the two combined did not change the effect of CNP on cGMP levels, demonstrating that PDE2 is the major and dominating PDE regulating the global cGMP after CNP stimulation.

The total cGMP PDE activity in cardiomyocytes was 27.9 ±1.3 pmol cGMP hydrolysed×mg protein^{-1}×min^{-1} ($n=6$). PDE2 inhibition by EHNA caused a large and significant reduction in the total cGMP PDE activity, whereas PDE5 inhibition by Sfil caused a small but significant reduction (Fig. 2b). PDE3 inhibition by Cil did not cause a significant reduction in cGMP PDE activity (Fig. 2b). Thus, the PDE activity is mainly reduced by PDE2 inhibition, reflecting our observation that CNP-induced increase in global cGMP levels is mainly regulated by PDE2.

PDE inhibition does not significantly affect CNP-induced TnI-Ser23/24 and PLB-Ser16 phosphorylation

CNP significantly increased the PLB-Ser16 phosphorylation 25- to 30-fold compared to Ctr (Fig. 3a), but the effect was not

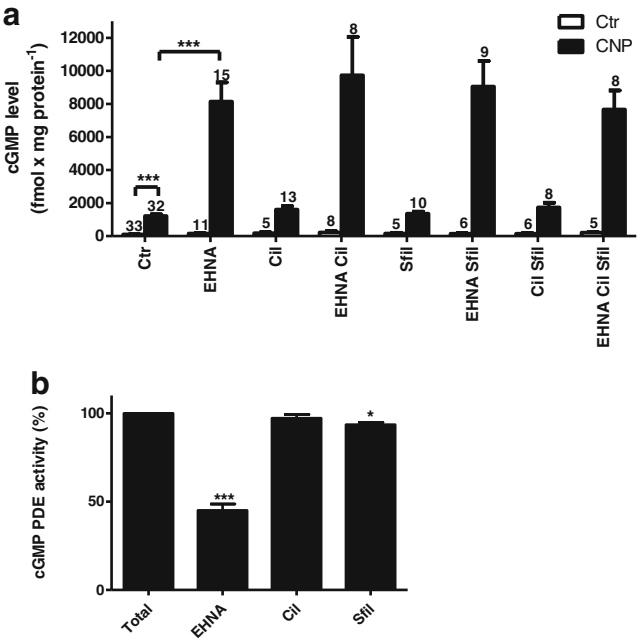

Fig. 2 PDE2 is the main regulator of global cGMP increase caused by NPR-B stimulation in cardiomyocytes. **a** Cyclic GMP levels were measured in HF isolated ventricular cardiomyocytes. Cells were pre-incubated without or with PDE inhibitors for 15 min before exposure to CNP (100 nM) for 10 min. Number of experiments is indicated *above bars*. ***$P<0.001$, unpaired Student's *t* test (Bonferroni-corrected). **b** Cyclic GMP PDE activities were measured in homogenates from isolated ventricular cardiomyocytes in the absence (*total*) and in the presence of PDE2 (*EHNA*), PDE3 (*Cil*) or PDE5 (*Sfil*) inhibition. Remaining PDE activity in the presence of inhibitors is expressed as percentage of total PDE activity. The total cGMP PDE activity was 27.9±1.3 pmol cGMP× mg protein^{-1}×min^{-1} ($n=6$). EHNA 10 μM, Cil 1 μM and Sfil 0.1 μM. *$P<0.05$, ***$P<0.001$, one-sample *t* test (Bonferroni-corrected)

significantly changed by EHNA, Cil or the combination. CNP tended to increase the TnI phosphorylation about twofold compared to Ctr (Fig. 3b) and was also not significantly changed by EHNA, Cil or the combination.

PDEs markedly regulate the functional responses and cAMP levels to β_1-AR stimulation in HF left ventricle

Our results showed that the cGMP-mediated functional responses to CNP were modestly regulated by PDE2 and PDE3 although in opposite directions. We have earlier found cAMP-mediated functional responses to be highly regulated (Afzal et al. 2011a). In light of this, for comparison with the cGMP signalling pathway, concentration-response relationships of noradrenaline (NA) in the absence and presence of different PDE inhibitors were examined in the same experimental system. β_1-AR stimulation caused positive inotropic response (PIR) and LR with $-\log EC_{50}$ values of 6.69±0.07 for both (n=19; Fig. 4a, b). PDE3 inhibition by Cil sensitized both the PIR (7.21±0.11, n=10, P<0.01; Fig. 4a) and the LR (7.22±0.13, n=10, P<0.01; Fig. 4b) to β_1-AR stimulation. PDE4 inhibition alone with Rol ($-\log EC_{50(PIR)}$=6.87±0.14, $-\log EC_{50(LR)}$=6.79±0.19, n=7; Fig. 4a, b) failed to sensitize the β_1-AR-stimulated functional responses. However, combined PDE3 and PDE4 inhibition caused a pronounced increase in the sensitivity to NA both on PIR ($-\log EC_{50(PIR)}$=8.20±0.12, n=4, P<0.001; Fig. 4a) and LR ($-\log EC_{50(LR)}$=7.80±0.09, n=3, P<0.001; Fig. 4b). In order to investigate the regulation by PDEs in cardiomyocytes, cAMP levels in this study were measured in isolated ventricular cardiomyocytes. β_1-AR stimulation by NA significantly increased cAMP levels compared to control (Fig. 4c). The presence of the PDE4 inhibitor Rol significantly increased NA-induced cAMP elevation. PDE3 inhibition by Cil caused no significant increase in NA-induced cAMP elevations. However, combined PDE3 and PDE4 inhibition by Cil and Rol significantly increased cAMP elevation by NA and beyond the added effect of Cil and Rol separately, demasking a secondary regulatory role of PDE3 by PDE4 inhibition, which is in agreement with earlier studies in ventricular muscle strips (Afzal et al. 2011a). The total cAMP PDE activity evaluated in homogenate from isolated ventricular cardiomyocytes was 40.5±6.0 pmol cAMP hydrolysed×mg protein^{-1}×min^{-1} (n=6). PDE3 inhibition by Cil significantly reduced the total cAMP PDE activity to 79.4±1.3 % (n=6, P<0.001). Rol (PDE4 inhibition) significantly reduced the total cAMP PDE activity to 42.2±1.5 % (n=6, P<0.001; Fig. 4d), which is consistent with PDE4 as the primary PDE regulating global cAMP levels. PDE2 inhibition by EHNA did not influence the effect of β_1-AR stimulation ($-\log EC_{50(PIR)}$=6.63±0.08, n=6; $-\log EC_{50(LR)}$=6.64±0.12, n=6; Fig. 4a, b) and caused no significant increase in NA-induced cAMP elevation (Fig. 4c) or reduction in cAMP PDE activity (Fig. 4d).

Discussion

This report is the first to show that both PDE2 and PDE3 regulate CNP-induced NIR and LR in failing cardiac ventricle. PDE3 inhibition sensitized both the NIR and LR to CNP despite no significant cGMP increase. PDE2 inhibition, which caused a large enhancement of CNP-induced cGMP increase, surprisingly desensitized the NIR to CNP. A role for PDE2 on the maximal LR and PDE5 on the maximal NIR to CNP was revealed in the presence of PDE3 inhibition. NPR-B-mediated increase in global cGMP was mainly regulated by PDE2, also

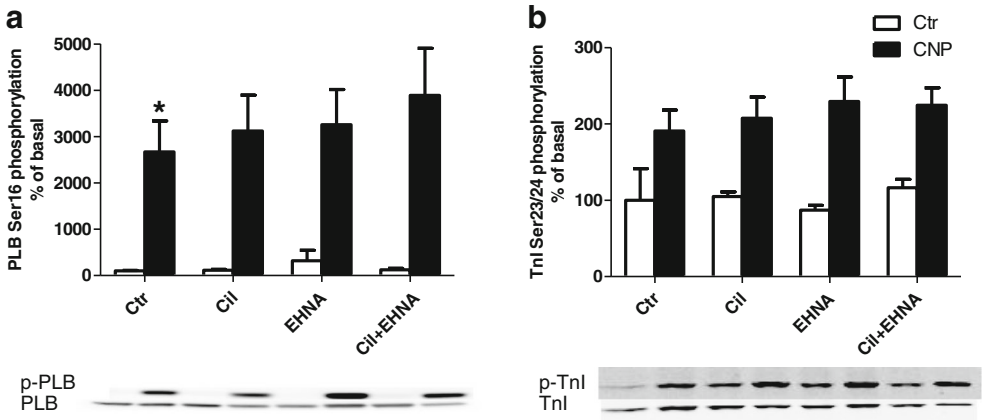

Fig. 3 PDE inhibition does not change the CNP-induced PLB-Ser16 and TnI-Ser23/24 phosphorylation in HF ventricular cardiomyocytes. Phosphorylation of **a** PLB at Ser16 (p-PLB, n=6) and **b** TnI at Ser23/24 (p-TnI, n=5) in isolated HF ventricular cardiomyocytes after NPR-B stimulation using CNP in the absence (Ctr) and in the presence of different PDE inhibitors. Representative Western blots are added showing the expression of total (PLB/TnI) and phosphorylated (p-PLB/p-TnI) protein. Monomeric PLB was detected at about 5 kDa and TnI at 28 kDa. CNP 100 nM, EHNA 10 μM and Cil 1 μM. *P<0.05, unpaired Student's t test

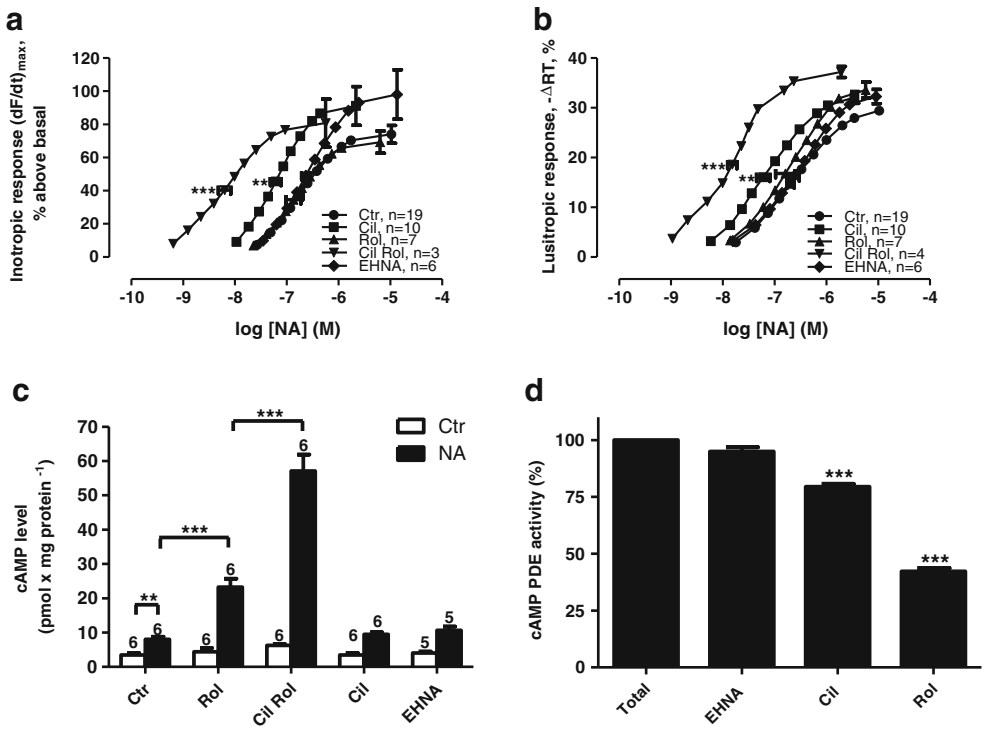

Fig. 4 PDE inhibition markedly potentiated functional responses and cAMP increase to β_1-AR stimulation in HF left ventricle. Concentration-response curves of the inotropic (**a**) and lusitropic (**b**) response to β_1-AR stimulation (*NA*) in the absence (Ctr, n=19) and presence of different PDE inhibitors: PDE2 inhibition (EHNA 10 μM; n=6), PDE3 inhibition (Cil 1 μM; n=10), PDE4 inhibition (Rol 10 μM; n=7), PDE3/4 inhibition (Cil Rol; n=3–4). **P<0.01, ***P<0.001, unpaired Student's t test. **c** Cyclic AMP levels were measured in HF isolated ventricular cardiomyocytes. Cells were pre-incubated without or with PDE inhibitors for 15 min before exposure to NA (100 nM) for 10 min. Number of experiments is indicated *above bars*. **P<0.01, ***P<0.001, unpaired Student's t test (Bonferroni-corrected). **d** cAMP PDE activities were measured in homogenate from isolated ventricular cardiomyocytes in the absence (*total*) and in the presence of PDE2 (*EHNA*), PDE3 (*Cil*) or PDE4 (*Rol*) inhibition. Remaining PDE activity in the presence of inhibitors is expressed as percentage of total PDE activity. The total cAMP PDE activity was 40.5± 6.0 pmol cAMP×mg protein^{-1}×min^{-1} (n=6). ***P<0.001, one-sample t test (Bonferroni-corrected). EHNA 10 μM, Cil 1 μM and Rol 10 μM

reflected by a high PDE2 activity in cardiomyocytes. Interestingly, the cGMP-mediated NIR and LR to CNP were only modestly regulated by PDEs compared to the extensive regulation by PDEs seen for cAMP-mediated PIR and LR to β_1-AR stimulation.

PDE3 suppresses the CNP-induced NIR and LR

Despite a significant enhancement of the NIR and LR to CNP, PDE3 inhibition caused no significant enhancement of CNP-stimulated global cGMP increase. However, a localised effect of PDE3 inhibition on cGMP levels might not be reflected in total cellular cGMP levels. Recently, CNP was shown to increase β_1-AR- and 5-HT$_4$-receptor-induced inotropic response via cGMP inhibition of PDE3 in failing rat left ventricle (Qvigstad et al. 2010; Afzal et al. 2011b). Related findings were presented in porcine atrium (Weninger et al. 2012, 2013; Levy 2013). Together, these data indicate the presence of NPR-B-induced cGMP increase and PDE3 activity in the same compartment. Consequently, cGMP will also be degraded by PDE3. Thus, a local cGMP increase in the presence of PDE3 inhibition by Cil could activate the PKG pathway causing increased NIR and LR to CNP (Fig. 5). We have earlier found that CNP increases SERCA2 activity, probably through PLB phosphorylation (Moltzau et al. 2013). PDE3 inhibition did not significantly increase CNP-induced PLB phosphorylation. However, there are indications that PDE3 is localised to the SR (Lugnier et al. 1993) with a similar or identical distribution as SERCA (Kauffman et al. 1986). Thus, a localised cGMP increase close to SERCA could explain the increased NIR and LR to CNP in the presence of Cil.

PDE2 enhances the CNP-induced NIR

We found that PDE2 contributes to an increase in the CNP-induced NIR in failing heart. This is in line with a study by Su et al. (2005), where EHNA inhibited the CNP-induced NIR in normal mouse hearts. We earlier found a PKG-mediated NIR to CNP (Moltzau et al. 2013). If the additional cGMP resulting from the large enhancement of CNP-stimulated cGMP increase by EHNA were activating the PKG pathway, we would expect an increased CNP-induced NIR. To the contrary, PDE2 inhibition desensitized the NIR to CNP. A clue to explain this may be that PDE2 is a cGMP-activated cAMP PDE (Francis

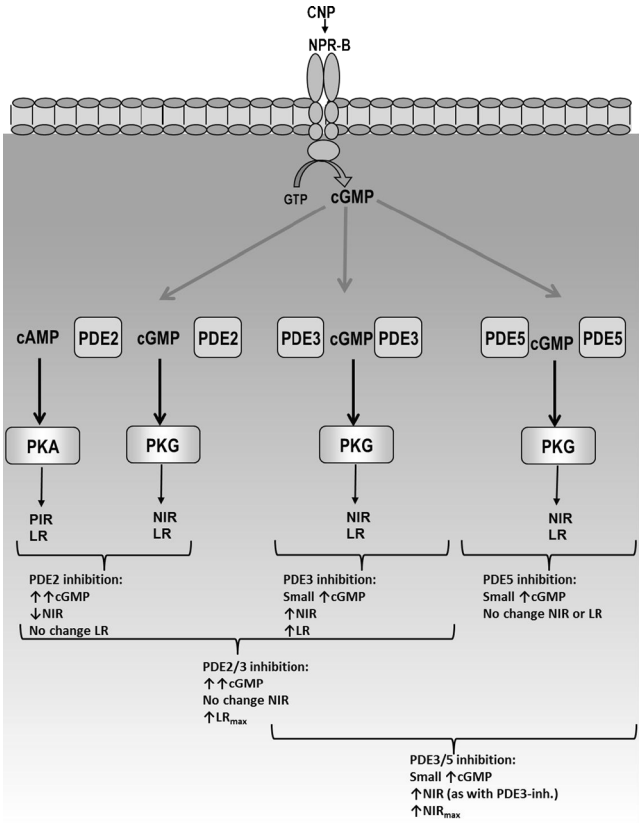

Fig. 5 Suggested regulation by PDEs of CNP-induced cGMP and functional consequences in failing rat hearts. PDE2 degrades both cGMP and cAMP but is stimulated by cGMP. PDE3 degrades both cGMP and cAMP but is inhibited by cGMP. PDE5 is selective for cGMP. PDE2 and PDE3 have different regulatory effects on the functional responses to CNP. PDE2 enhances the CNP-induced NIR possibly by its activation through cGMP, reducing cAMP levels, whereas PDE3 suppresses cGMP and thus the apparent PKG-mediated NIR and LR to CNP. The LR to maximal CNP is regulated by PDE2 and PDE3 combined, possibly through the suppressed activation of both the PKG and the PKA pathway

et al. 2011). Thus, a plausible explanation for our results could be that cGMP generated by NPR-B activates PDE2 and thereby decreases cAMP. This would potentially reduce a positive inotropic component through the cAMP-PKA pathway and thus cause an apparent increase in the CNP-induced NIR. In the presence of a PDE2 inhibitor, cGMP generated by NPR-B could in theory no longer cause a reduction in the cAMP, and the result would be an increased positive inotropic component reflected in a desensitized CNP-induced NIR (Fig. 5). Thus, our findings may reflect a crosstalk between the cGMP and cAMP system through PDE2. In support of this are studies showing cGMP activation of PDE2 by the activation of the soluble guanylyl cyclase pathway (Mery et al. 1993; Mongillo et al. 2006; Stangherlin et al. 2011; Mehel et al. 2013) and stimulation of the NPR-A receptor (Stangherlin et al. 2011) in different ventricular preparations. Recently, PDE2 was found to be up-regulated in failing hearts (Mehel et al. 2013). This might affect some cGMP-mediated effects in failing myocardium. The fact that PDE2 inhibition causes a major increase in the cGMP levels following NPR-B stimulation suggests that there is a substantial pool of cGMP close to PDE2 that could potentially activate it. However, this needs further studies.

Dual PDE2 and PDE3 inhibition increases LR to maximal CNP—cooperation between the cGMP and cAMP signalling pathways?

PDE3 inhibition significantly sensitized the CNP-induced LR, whereas PDE2 inhibition failed to affect the CNP-induced LR. In the presence of both PDE2 and PDE3 inhibition, however, the LR to maximal CNP was increased with no change of EC_{50}. This could be explained by a combined involvement of the PKA and PKG pathway as discussed. There was no significant increase in CNP-induced PLB phosphorylation by PDE inhibition. However, a tendency to increase CNP-induced PLB phosphorylation by combined PDE2 and PDE3 inhibition might still reflect a contribution from both PKA and PKG activation, resulting in increased LR to maximal CNP, not seen with separate PDE2 or PDE3 inhibition. However, further investigation is needed to clarify the role of a possible PDE2 activation by CNP.

Other PDEs involved in the regulation of CNP-induced functional responses?

Our results showed that the presence of combined PDE3 and PDE5 inhibition caused an increase in the NIR to maximal CNP which was not seen with PDE3 or PDE5 inhibition alone. This indicates that a role of PDE5 seems to be demasked when the PDE3 effect is abolished. PDE5 inhibition abolished the effect of combined PDE2 and PDE3 inhibition on the LR_{max} to CNP, further indicating a role for PDE5. The effect of PDE5 inhibition combined with PDE3 inhibition on the NIR_{max} to CNP could be explained by increased cGMP signalling through PKG. However, the abolished effect of PDE2 and PDE3 inhibition on the LR_{max} to CNP in the presence of PDE5 inhibition is hard to explain by increased cGMP signalling through PKG. A potential role for PDE5 in the regulation of various CNP-induced functional responses needs further investigation. A role for PDE1 was not revealed by combined PDE1, PDE2, PDE3 and PDE5 inhibition. Thus, our results show that the functional responses to CNP only seem to be under independent PDE2 and PDE3 regulation and to a small degree of PDE5 regulation.

Global cyclic nucleotide versus functional responses

There are only few studies investigating the PDE regulation of cGMP generated by pGC, whereas nothing is known about

the PDE regulation of functional responses to CNP. Castro et al. (2006) showed that cGMP generated by NPR-A is under exclusive control of PDE2. To the contrary, Doyle et al. (2002) showed that PDE3 limits the cGMP increase by NPR-B. Our results demonstrated that PDE2 inhibition caused a large global cGMP increase induced by CNP and a rather small, though statistically significant, effect on the functional responses to CNP. Further, PDE3 inhibition caused no significant accentuation of the CNP-stimulated increase in global cGMP levels but caused a moderate and statistically significant increase in the sensitivity to CNP-induced functional responses. An analogous mismatch was seen for the cAMP signalling pathway in cardiomyocytes, where PDE4 was the main regulator of β_1-AR-induced global cAMP increase, whereas PDE3 was the main regulator of functional responses. A further large increase of global cAMP level and functional response sensitivity to NA was demonstrated with combined PDE3 and PDE4 inhibition. These results confirm, in cardiomyocytes, earlier studies done in cardiac muscle strips ex vivo, where a mismatch between the total cyclic nucleotide level and functional response was shown for the 5-HT_4 receptor- (Afzal et al. 2008) as well as β_1- and β_2-AR-induced (Afzal et al. 2011a) functional responses. These mismatches between cyclic nucleotide levels and functional responses most likely reflect locally confined pools of cGMP and cAMP in the functional compartments.

The regulation by PDEs of cAMP versus cGMP signalling

Both the cAMP and the cGMP level are highly regulated by PDEs. However, in contrast to the cAMP system, the cGMP system seems to be only modestly affected at the functional level. The sensitivity (EC_{50}) of CNP-induced NIR is at the most shifted at about 0.3 log units (corresponding to about a twofold shift) in the presence of PDE inhibitors, whereas combined inhibition of PDE3 and PDE4 caused a major increase, a shift in the EC_{50} of 1.5 log units (corresponding to about a 30-fold shift), in the sensitivity of the β_1-AR-induced PIR. Thus, we found only modest regulation by PDEs of functional responses to CNP. Whether this is a general concept for the regulation by PDEs of cGMP-mediated functional responses needs further investigation.

Other possible signalling pathways of CNP?

The interpretation of our data is based on the assumption that CNP-induced cGMP is responsible for the effects we see in this study. Natriuretic peptides have, however, been shown to activate G_i through NPR-C (Anand-Srivastava and Cantin 1986; Anand-Srivastava et al. 1990; Rose et al. 2003); thus, CNP might also have cGMP-independent effects. However, our earlier finding of CNP-induced NIR and LR in failing hearts (Moltzau et al. 2013) does not support a major role for G_i-mediated effects as we did not see an effect of CNP in the presence of maximal β-adrenergic stimulation. Earlier studies show that ligands activating G_i markedly reduce β-adrenergic inotropic response (Skomedal et al. 1997). Further, as PDE2 and PDE3 hydrolyse both cAMP and cGMP, we cannot rule out that some of the functional responses to CNP in the presence of PDE inhibitors are due to cAMP-mediated effects not involving cGMP generated by NPR-B, even though all our studies with CNP were done in the presence of β-adrenoceptor antagonist. However, only some of our results in the presence of PDE inhibition could potentially be explained by increased cAMP-mediated signalling like the effects on NIR and LR seen with the PDE2 inhibitor EHNA (as discussed above).

Concluding remarks

We demonstrate that PDE2 and PDE3 play different regulatory roles on the NIR and LR to CNP. The regulation by PDEs of the functional responses to CNP is only modest compared to the extensive PDE regulation of the functional responses to β_1-AR stimulation. A mismatch between the cGMP levels and functional responses was found, as in the cAMP system. PDE2 enhances the CNP-induced NIR, whereas PDE3 suppresses CNP-induced NIR and LR (Fig. 5). A role for PDE2 on the LR and PDE5 on the NIR to maximal CNP was revealed in the presence of PDE3 inhibition. The cGMP increase after NPR-B stimulation is mainly regulated by PDE2. We conclude that CNP-induced cGMP elevation and the NIR and LR to CNP are differentially regulated by PDEs in the failing heart. This indicates cGMP compartmentation by PDEs affecting CNP-induced responses in failing hearts.

Acknowledgments We would like to thank Iwona Gutowska Schiander for isolation of cardiomyocytes and Cam HT Nguyen and Marie Dahl for performing Western blot experiments. This work was supported by The Norwegian Council on Cardiovascular Disease, The Research Council of Norway, Stiftelsen Kristian Gerhard Jebsen, Anders Jahre's Foundation for the Promotion of Science, The Family Blix Foundation, The Simon Fougner Hartmann Foundation, South-Eastern Norway Regional Health Authority, University of Oslo and the COST Action BM1005 (European Network on Gasotransmitters).

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