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RESEARCH PAPER

SERCA2 activity is involved in the CNP-mediated functional responses in failing rat myocardium

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BACKGROUND AND PURPOSES

Myocardial C-type natriuretic peptide (CNP) levels are increased in heart failure. CNP can induce negative inotropic (NIR) and positive lusitropic responses (LR) in normal hearts, but its effects in failing hearts are not known. We studied the mechanism of CNP-induced NIR and LR in failing hearts and determined whether sarcoplasmatic reticulum Ca^{2+} ATPase2 (SERCA2) activity is essential for these responses.

EXPERIMENTAL APPROACH

Contractility, cGMP levels, Ca^{2+} transient amplitudes and protein phosphorylation were measured in left ventricular muscle strips or ventricular cardiomyocytes from failing hearts of Wistar rats 6 weeks after myocardial infarction.

KEY RESULTS

CNP increased cGMP levels, evoked a NIR and LR in muscle strips, and caused phospholamban (PLB) Ser^{16} and troponin I (TnI) $Ser^{23/24}$ phosphorylation in cardiomyocytes. Both the NIR and LR induced by CNP were reduced in the presence of a PKG blocker/cGMP analogue (Rp-8-Br-Pet-cGMPS) and the SERCA inhibitor thapsigargin. CNP increased the amplitude of the Ca^{2+} transient and increased SERCA2 activity in cardiomyocytes. The CNP-elicited NIR and LR were not affected by the L-type Ca^{2+} channel activator BAY-K8644, but were abolished in the presence of isoprenaline (induces maximal activation of cAMP pathway). This suggests that phosphorylation of PLB and TnI by CNP causes both a NIR and LR. The NIR to CNP in mouse heart was abolished 8 weeks after cardiomyocyte-specific inactivation of the *SERCA2* gene.

CONCLUSIONS AND IMPLICATIONS

We conclude that CNP-induced PLB and TnI phosphorylation by PKG in concert mediate both a predictable LR as well as the less expected NIR in failing hearts.

Abbreviations

CNP, C-type natriuretic peptide; CRC, contraction–relaxation cycle; Iso, isoprenaline; LR, lusitropic response; LTCC, L-type Ca^{2+} channel; NCX, Na^{+}–Ca^{2+} exchanger; NIR, negative inotropic response; PLB, phospholamban; Rp, Rp-8-Br-Pet-cGMPS; RyR, ryanodine receptor/SR release channel; SERCA2, sarcoplasmatic reticulum Ca^{2+} ATPase2; SR, sarcoplasmatic reticulum; TG, thapsigargin

Introduction

Natriuretic peptides (NPs) exert diverse effects in the cardiovascular system, but their direct effects on cardiomyocytes have not been elucidated. Atrial and brain NPs signal through natriuretic peptide receptor A (NPR-A), whereas C-type natriuretic peptide (CNP) signals through NPR-B. NPR-A and NPR-B are particulate guanylyl cyclases, and stimulation of these receptors results in increased cGMP production. All three NPs can also bind to NPR-C, which does not have guanylyl cyclase activity (Potter *et al.*, 2009).

CNP has been reported to cause both positive (Beaulieu *et al.*, 1997; Hirose *et al.*, 1998; Wollert *et al.*, 2003) and negative inotropic responses (NIR) (Brusq *et al.*, 1999; Nir *et al.*, 2001; Pierkes *et al.*, 2002; Su *et al.*, 2005; Zhang *et al.*, 2005a) in normal hearts, as well as positive lusitropic responses (LR) in normal rat and mouse hearts (Brusq *et al.*, 1999; Pierkes *et al.*, 2002). In a previous study, we showed that cGMP generated by NPR-B increases β_1-adrenoceptor-mediated positive inotropic responses through inhibition of PDE3, and also found that stimulation of NPR-B alone (by CNP) in failing rat hearts induced a NIR (Qvigstad *et al.*, 2010). Myocardial levels of CNP are increased in heart failure (HF) (Kalra *et al.*, 2003; Del Ry *et al.*, 2005). CNP is known to cause phosphorylation of both phospholamban (PLB) (Brusq *et al.*, 1999; Wollert *et al.*, 2003) and troponin I (TnI) (Brusq *et al.*, 1999) in normal hearts, but whether there is more than one mechanism involved in the CNP-induced functional responses (NIR and LR) or whether the same mechanisms are responsible for both the NIR and LR induced by CNP has not been clarified. Furthermore, the functional responses to CNP in failing hearts have largely been unexplored. Both NPs (Boerrigter *et al.*, 2009) and increased sarcoplasmatic reticulum Ca^{2+} ATPase2 (SERCA2) activity (Gwathmey *et al.*, 2011) have been suggested as therapies for HF. Thus, we aimed to investigate the NIR and the LR induced by CNP in failing hearts, to clarify whether PLB and TnI phosphorylation are involved in these CNP-induced functional responses, and also to determine whether SERCA2 activity is essential for these responses to CNP.

We found that CNP induced a relatively slowly developing, concentration-dependent PKG-mediated NIR and LR in failing cardiac ventricle. Furthermore, CNP increased PLB phosphorylation and SERCA2 activity, indicating that increased SERCA2 activity has a role in the functional effects of CNP in the failing myocardium. This result was substantiated using a mouse model with cardiomyocyte-specific inactivation of the SR Ca^{2+}-ATPase gene (SERCA2-KO), where it was found that CNP was unable to elicit a NIR.

Our results support the hypothesis that the concerted effects of PLB and TnI phosphorylation are the main mechanisms involved in the PKG-mediated functional responses to CNP in failing myocardium.

Methods

For more detailed methods, see Supporting Information. Nomenclature of drugs and molecular targets conforms to *British Journal of Pharmacology*'s Guide to Receptors and Channels (Alexander *et al.*, 2011).

Animal models

All experimental procedures conformed to the *Guide for the Care and Use of Laboratory Animals* by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), and all studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). Animal care was according to the Norwegian Animal Welfare Act that conforms to the *European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes* (Council of Europe No. 123, Strasbourg 1985) and the experiments were approved by the Norwegian Animal Research Authority. Myocardial infarction (MI) was induced in ~300 g male Wistar rats during anaesthesia (65% N_2O, 32% O_2 and 2–3% isoflurane through endotracheal intubation) by left coronary artery ligation, as described previously (Sjaastad *et al.*, 2000). Six weeks later, rats with left atrial dilatation (>5 mm) and increased lung weight (>2.0 g) in the presence of a large anterolateral MI and clinical signs of HF were included in the study (Sjaastad *et al.*, 2000). Echocardiographic and haemodynamic analyses were performed in a subset of rats as previously described (Sjaastad *et al.*, 2000). In experiments with SERCA2-KO mice, inactivation of the *Serca2* gene was obtained by administration of a single dose of 40 $mg \cdot kg^{-1}$ tamoxifen i.p. (Hougen *et al.*, 2010). The mice were killed 4 and 8 weeks after gene inactivation. See Supporting Information Table S1 for detailed animal characteristics (HF rats; Sham data are included for comparison).

Isolation of cardiomyocytes

Failing hearts were perfused (Langendorff set-up) with a Ca^{2+}-free Joklik-MEM (Sigma-Aldrich, St. Louis, MO, USA) buffer and digested enzymatically by using collagenase type II (90 $U \cdot mL^{-1}$ final) (Worthington Biochemical Corp., Freehold, NJ, USA; 268 $U \cdot mg^{-1}$), as described in the Supporting Information. Cardiomyocytes used in cell contraction and Ca^{2+} transient measurements were isolated by a slightly different protocol, as previously described (Bøkenes *et al.*, 2008).

Isolated muscle strips

HF left ventricular muscle strips were taken distantly from the infarction area, prepared and stimulated electrically at 1 Hz. Contraction–relaxation cycles (CRCs) were recorded and analysed as previously described (Qvigstad *et al.*, 2010) with respect to maximal development of force $[(dF/dt)_{max}]$, time to peak force (TPF), time to 80% relaxation (TR80) and relaxation time (RT = TR80 – TPF). Inotropic responses are expressed as change (Δ) of $(dF/dt)_{max}$ as % of basal and NIR defined as a decrease in $(dF/dt)_{max}$. LR was expressed as a decrease in RT as % of basal. *n* represents the number of hearts used.

Cell contraction and Ca^{2+} transient measurements

Cardiomyocytes were loaded with fluo-4 and field stimulated electrically at 1 Hz. Ca^{2+} transients were recorded as previously described (Bøkenes *et al.*, 2008). Cell contractions were further recorded in unloaded cardiomyocytes. Three hearts were used in each dataset on cell contraction and Ca^{2+} transient measurements.

Cyclic GMP assays and Western blot

Muscle strips were freeze-clamped 15 min after the addition of agonist and kept at −70°C until used in the cGMP assay. Isolated cells were stimulated for 10 min with agonist as indicated. cGMP was measured using the cyclic GMP enzyme immuno assay kit (Cat#581021) from Cayman Chemical Company (Ann Arbor, MI, USA). PLB Ser^{16} and TnI $Ser^{23/24}$ phosphorylation were measured by Western analysis using antibodies for total and phosphorylated protein. *n* represents the number of experiments on isolated cardiomyocytes with one or two hearts in each experiment.

Statistics

All results are presented as mean ± SEM. Statistical significance was calculated by Student's *t*-test or one-sampled *t*-test. $P < 0.05$ was considered to reflect significant differences (*$P < 0.05$, **$P < 0.01$, ***$P < 0.005$).

Results

CNP causes NIR and LR in both Sham and HF muscle strips

To evaluate the CNP-induced NIR and LR in left ventricular muscle strips from Sham-operated (Sham) and HF rats, we studied the concentration–response relationship to cumulative doses of CNP. The –log EC_{50} of CNP was 7.1 ± 0.1 ($n = 4$) for the NIR in Sham (not shown) and 7.1 ± 0.1 ($n = 6$) in HF respectively (Figure 1E). To evaluate the maximal responses of CNP in myocardium from the Sham and HF groups, 300 nM CNP was added to muscle strips in each group. The maximal NIR was 11.5 ± 1.8% in Sham and 17.6 ± 1.0% in HF muscle strips
($P < 0.05$; Figure 1A), and the maximal LR was 6.3 ± 0.4% and 15.9 ± 1.4% ($P < 0.05$; Figure 1B). Overall, the functional response to CNP was increased in HF muscle strips compared with Sham (Figure 1A,B). As CNP has been proposed as a therapeutic for HF, we further explored the functional role of CNP in HF muscle strips and cardiomyocytes.

CNP increases cGMP levels in muscle strips

To clarify the presence of functional NPR-B receptors, the HF left ventricular muscle strips were stimulated with increasing concentrations of CNP. As shown previously (Qvigstad *et al.*, 2010), we confirmed and substantiated that CNP induced a concentration-dependent increase in cGMP with a –log EC_{50} of 6.9 ± 0.2 (n = 5–11, Figure 1C). One micromolar CNP caused a three- to fourfold increase in cGMP.

CNP causes NIR and LR through PKG in HF muscle strips

The overall response to CNP in HF left ventricular muscle strips was a NIR, measured as a reduction in maximal development of contractile force, $(dF/dt)_{max}$, and a (positive) LR, measured as a shortening of both the CRC and RT compared with basal (Figure 1D). However, the response to CNP was biphasic in 5 out of 12 muscle preparations with an initial transient small positive inotropic response followed by a slowly developing NIR (Figure 4Aii). The NIR to CNP was present in all muscle strips. To further evaluate the CNP-induced NIR and LR, respectively, we studied the concentration–response relationship by addition of cumulative doses of CNP. The –log EC_{50} of CNP was 7.1 ± 0.1 ($n = 6$) and 7.0 ± 0.2 ($n = 6$) for the NIR and LR, respectively (Figure 1E,F). The maximum NIR was a 35.2 ± 2.9% reduction in $(dF/dt)_{max}$ and maximum LR (LR_{max}) was 21.2 ± 3.7% shortening of RT, both were compared to basal. To investigate whether the CNP-induced functional responses were mediated through PKG, muscle strips were pre-incubated with the PKG blocker/cGMP analogue Rp-8-Br-Pet-cGMPS (Rp; 3 μM) before adding increasing concentrations of CNP. Rp significantly reduced the maximal CNP-induced NIR (ΔNIR_{max} = 13.9 ± 3.3%, $n = 6$, $P < 0.01$) and LR (ΔLR_{max} = −11.6 ± 2.0%, $n = 6$, $P < 0.005$) (Figure 1E,F). The sensitivity to CNP in the presence of the PKG blocker was nominally reduced for NIR (Δ–log EC_{50} = 0.3 ± 0.1, $n = 6$, $P = 0.06$) (Figure 1E). These results are consistent with CNP-induced functional responses being mediated through PKG. Contractions in isolated cardiomyocytes were measured by use of a video-edge technique to verify cardiomyocyte-specific CNP effects. The CNP-induced effect was biphasic with an initial increase in maximal fractional shortening (FS) in all cells. However, CNP concentration-dependently reduced FS in the stable phase [–5.3 ± 1.7% with 100 nM CNP ($n = 9$) and –14.6 ± 2.3% with 300 nM CNP ($n = 8$), both $P < 0.05$ vs. Ctr; Supporting Information Figure S2], and decreased the time to 50% relaxation from peak shortening [–10.2 ± 1.8% with 100 nM CNP ($n = 9$) and –16.5 ± 1.8% with 300 nM CNP ($n = 8$, $P < 0.05$, results not shown)].

CNP induced phosphorylation of PLB and TnI

Earlier studies found that CNP increases the phosphorylation of PLB (Brusq *et al.*, 1999; Wollert *et al.*, 2003) and TnI (Brusq *et al.*, 1999) in normal hearts. We wanted to clarify whether this also occurs in failing hearts and is mediated through PKG. We found that in failing hearts CNP significantly increased phosphorylation of both PLB Ser^{16} and TnI $Ser^{23/24}$ ($P < 0.05$ for both 0.1 and 1.0 μM CNP). Both effects were reduced in the presence of the PKG inhibitor Rp showing that this response is dependent on PKG (Figure 2A,B).

CNP increases Ca^{2+} transient amplitude

To assess the effects of CNP on cytosolic Ca^{2+}, we studied the Ca^{2+} transients in isolated cardiomyocytes. After the addition of CNP the Ca^{2+} transients showed a biphasic increase, where the initial increase in amplitude was followed by a slow and minor decrease until stabilization (Figure 2C). In the stable phase, the CNP-induced increase in amplitude was 26 ± 6% (n = 9, $P < 0.01$; Figure 2D).

CNP-induced NIR and LR are preserved during Ca^{2+} channel activation, but not during maximal β-adrenoceptor stimulation

As shown earlier, CNP increased phosphorylation of PLB Ser^{16} and TnI $Ser^{23/24}$. To further elucidate the mechanism of action, we determined the effects of CNP during two different regulatory states of target proteins: (i) maximal β-adrenoceptor stimulation, obtained by addition of Iso in the presence of

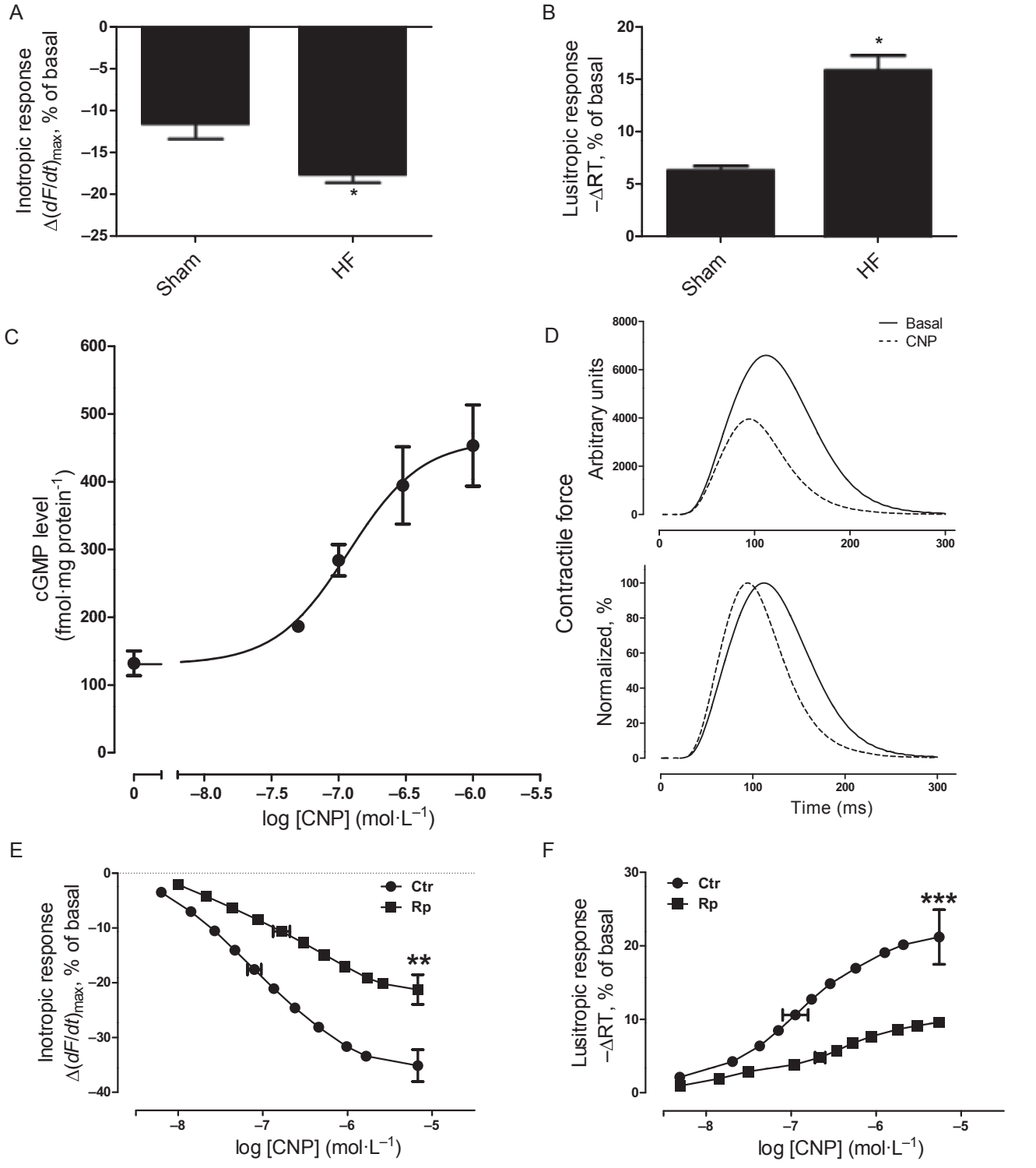

Figure 1

CNP increases cGMP and causes functional responses in Sham and HF left ventricular muscle strips. (A, B) Maximal inotropic response (A) and lusitropic response (B) to CNP in Sham and HF muscle strips. (C) cGMP levels in the presence of increasing concentrations of CNP (15 min of incubation) in HF left ventricular muscle strips (n = 5–11). (D) Illustration of contraction–relaxation cycles (signal averaged across about 25 cycles), arbitrary units (upper) and normalized (lower), basal and in the presence of CNP [(CNP) = $10^{-5.66}$ M (2.2 μM)] in one HF left ventricular muscle strip, representative of six experiments. After NPR-B stimulation with CNP, there is a reduced contractile force (negative inotropic response) and a shortening of relaxation phase of the cycle (positive lusitropic response) compared with basal. (E, F) Concentration–response curves of inotropic response (E) and lusitropic response (F) to CNP stimulation in the absence and presence of the cGMP/PKG antagonist Rp-8-Br-PET-cGMPS (Rp, 3 μM) in HF left ventricular strips. Vertical error bars: SEM. n = 6, $^{**}P < 0.01$, $^{***}P < 0.005$ Ctr versus Rp.

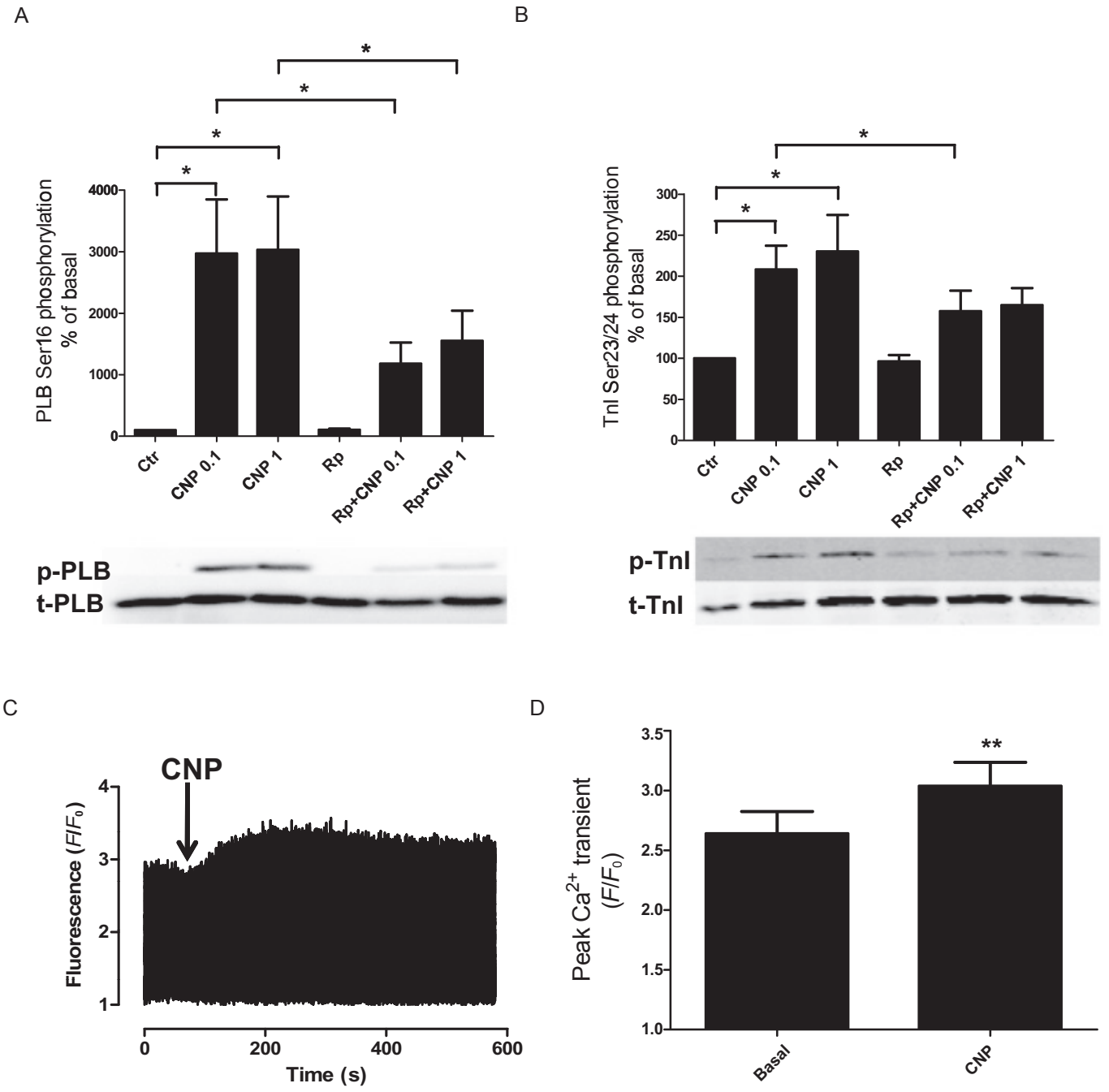

Figure 2

CNP causes PLB and TnI phosphorylation and increases the amplitude of the Ca^{2+} transient. (A, B) Phosphorylation of PLB on Ser^{16} (A) and TnI on $Ser^{23/24}$ (B) in HF ventricular cardiomyocytes stimulated by CNP [0.1 μM (0.1) and 1 μM (1.0)] in the absence and presence of the cGMP/PKG antagonist Rp-8-Br-PET-cGMPS (Rp, 3 μM). Representative blots of $n = 8$. *$P < 0.05$. p-PLB, phosphorylated PLB; p-TnI, phosphorylated TnI; t-PLB, total PLB; t-TnI, total TnI. The excised bands show monomeric PLB at about 5 kDa and TnI at 28 kDa. (C) Representative tracings of the Ca^{2+} transient after stimulation with CNP (300 nM). (D) The peak Ca^{2+} transient (F/F_0) in the absence and presence of CNP (300 nM) in isolated HF ventricular cardiomyocytes. $n = 9$, ***$P < 0.005$ basal versus CNP.

the non-selective phosphodiesterase inhibitor IBMX to ensure maximal cAMP production, which will activate PKA and induce phosphorylation of downstream targets such as LTCC, PLB and TnI, causing both inotropic and lusitropic responses; (ii) Ca^{2+} channel activation by BAY-K8644 (Kokubun and Reuter, 1984), which was previously shown not to cause LR (Skomedal *et al.*, 1988) but to induce an inotropic response quantitatively comparable to that induced by β-adrenoceptor stimulation. In the presence of Iso + IBMX, CNP was unable to induce either a NIR

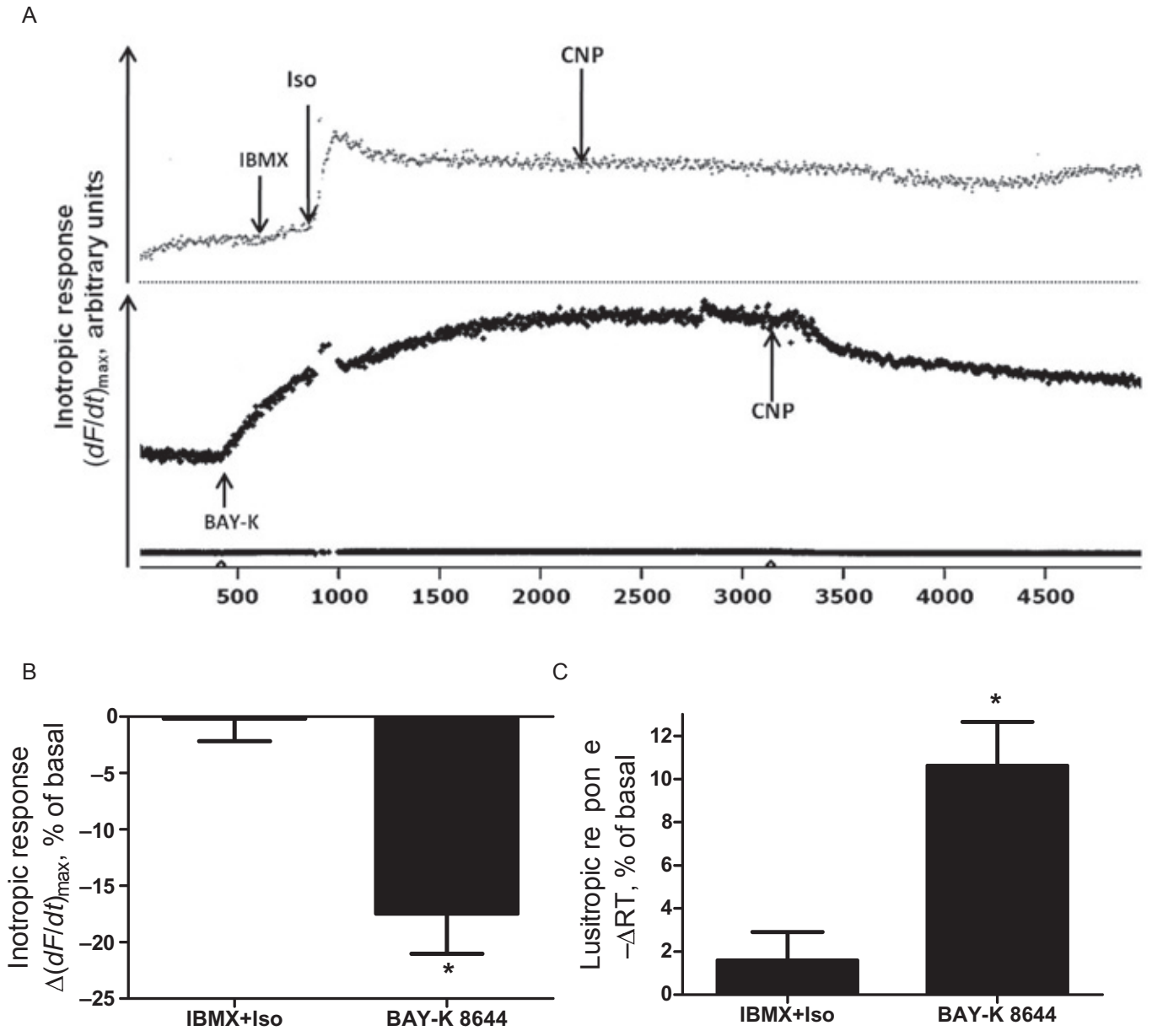

Figure 3

CNP causes a NIR and a LR in the presence of a Ca^{2+} channel activator but not during β-adrenoceptor stimulation. (A) Original tracings of contracting HF left ventricular muscle strips demonstrating the effect of CNP in the presence of the LTCC activator BAY-K8644 or β-adrenoceptor stimulation with Iso and IBMX. (B, C) The maximal inotropic (B) and lusitropic (C) response to CNP (16 min after addition) as % of basal in the presence of IBMX and Iso ($n = 4$) or BAY-K8644 ($n = 4$). BAY-K8644: 1.7 μM; CNP: 300 nM; IBMX: 100 μM; Iso: 500 μM, *$P < 0.05$ basal versus CNP.

$[\Delta(dF/dt)_{max} = 0.2 \pm 2.0\%$ of basal, $n = 4]$ or a LR ($LR_{max} = 1.6 \pm 1.3\%$, $n = 4$). These effects were observed both at a high concentration of Iso (500 μM) in the presence of timolol (Figure 3) and at a lower concentration of Iso (1 μM) in the absence of timolol in the perfusate (NIR = 0.6 ± 2% and LR = 0.5 ± 0.1%, $n = 6$; Supporting Information Figure S3). In contrast, in the presence of BAY-K8644, CNP was able to induce a NIR (17.5 ± 3.6%, $n = 4$, $P < 0.05$; Figure 3A,B) and a LR (10.6 ± 2.0%, $n = 4$, $P < 0.05$; Figure 3C). BAY-K8644 itself induced an inotropic response (228 ± 25% of basal, $n = 4$) and extended the CRC ($LR_{max} = -10.0 \pm 1.0\%$ of basal, $n = 4$); an effect that was eliminated by CNP ($LR_{max} = 0.6 \pm 1.4\%$ of basal, $n = 4$). The lack of a NIR and a LR to CNP in the presence of IBMX and Iso is consistent with common target proteins, such as PLB and TnI, being involved in the lusitropic effects of CNP (cGMP pathway) and Iso (cAMP pathway).

CNP increases SERCA2 activity in failing cardiomyocytes

The phosphorylation of PLB induced by CNP could increase SERCA2 activity. The SERCA inhibitor thapsigargin (TG; 1 μM) caused a transient negative followed by a positive ino-

tropic response in muscle strips (Figure 4A and 4Ai) and a negative effect on the Ca^{2+} transient (6.45 ± 0.38 vs. 2.26 ± 0.21, $P < 0.05$, not shown). These opposite effects of TG and CNP on contractility (Figure 4A, 4Ai and 4Aii) led us to suggest that PLB phosphorylation and thus SERCA2 activity might be an important mechanism for CNP-induced functional responses. To further examine the involvement of SERCA2, we studied the effects of CNP when added to the muscle strips after pre-incubation with TG. TG significantly reduced the maximum NIR to CNP (ΔNIR_{max} = 8.6 ± 2.4%, n = 6, $P < 0.05$; Figure 4B). The LR_{max} to CNP (16.9 ± 1.6%, n = 6) was also significantly reduced in the presence of TG (ΔLR_{max} = –10.8 ± 2.4%, n = 6, $P < 0.05$; Figure 4C). Subsequent addition of Iso induced a reduced but robust LR ($LR_{max(TG\ CNP\ Iso)}$ = 19.5 ± 1.4%, n = 6; Figure 4C). As the CNP-mediated NIR was lower in the presence than in the absence of TG and in the light of increased PLB phosphorylation, increased SERCA2 activity can be assumed to be involved in CNP-mediated NIR. The ability of CNP and Iso to induce a small LR in the presence of TG might indicate incomplete inhibition of SERCA2 activity by TG, but possibly also that other mechanisms are involved in the CNP-elicited LR. To evaluate more directly whether CNP increases SERCA2 activity, we used a standard protocol for evaluating SERCA2 activity in isolated cardiomyocytes. CNP caused a faster decay of the Ca^{2+} transient [rate constant of decay ($1/\tau$) 8.0 ± 0.9 vs. 5.8 ± 0.6, n = 11, $P < 0.005$], in line with an increase in SERCA2 activity and a LR to CNP. Furthermore, after the addition of caffeine (10 mM), which opens ryanodine receptors and evokes a rapid release of SR Ca^{2+} stores, the rate constant of decay ($1/\tau$) was unaltered by CNP (1.08 ± 0.14, n = 8) compared with basal (1.05 ± 0.13, n = 8). The amplitude of the Ca^{2+} transient was also unaltered by CNP in the presence of caffeine (data not shown). Based on the Ca^{2+} extrusion rate with and without caffeine, we calculated the SERCA2 activity (SERCA2 rate constant) in isolated cardiomyocytes to be 53% higher after adding CNP (4.98 ± 0.60 vs. 7.61 ± 0.90, $P < 0.05$; Figure 4D). Overall, we conclude that CNP increases SERCA2 activity in failing cardiomyocytes.

CNP reduces myofilament Ca^{2+} sensitivity in HF muscle strips

To investigate whether CNP decreased myofilament Ca^{2+} sensitivity, we calculated the ratio between the maximal cell contraction (fractional shortening) and maximal Ca^{2+} fluorescence. Unstimulated cells had a contraction/Ca^{2+} ratio of 4.5 ± 0.2, which was reduced to 3.9 ± 0.2 by CNP ($P < 0.05$; Figure 5A). We next assessed whether the Ca^{2+} concentration–contraction response relationship in muscle strips was altered by adding CNP. By gradually increasing extracellular Ca^{2+} with and without CNP present, we observed that the Ca^{2+} concentration–contraction response curve was significantly shifted to higher Ca^{2+} concentrations in the presence of CNP (EC_{50} = 1.01 ± 0.11 mM vs. 0.77 ± 0.12 mM, $P < 0.05$, n = 4; Figure 5B).

The CNP-induced NIR is reduced in 8 week SERCA2-KO mice

As our results so far indicated that increased SERCA2 activity is involved in the CNP-induced NIR and LR, we investigated this in another model where SERCA2 was eliminated. We used an inducible cardio-specific SERCA2-KO mouse model to further explore the involvement of SERCA2 in the functional responses elicited by CNP. To evaluate the functional responses in these mice, we used Iso as a control. In 4 week SERCA2-KO mice, both a positive inotropic response and a LR (37.2 ± 2.4% of basal, n = 4) were obtained to Iso, possibly because of residual SERCA2 still present (Louch *et al.*, 2010). After 7 weeks, this mouse model has previously been shown to have no detectable SERCA2 activity (Louch *et al.*, 2010). Consistent with this, the 8 week SERCA2-KO mice in this study showed no LR to Iso (2.1 ± 2.9%, n = 3) (Figure 6A). However, Iso was still able to cause a positive inotropic response in these mice (Figure 6A). CNP (300 nM) did not cause a NIR in 8 week SERCA2-KO mice (1.4 ± 3.5%, n = 3), whereas in 4 week SERCA2-KO CNP still induced a robust NIR [23.4 ± 7.6%, n = 4, $P < 0.05$ (CNP vs. basal); Figure 6B,C]. These results revealed that in the absence of functional SERCA2, the CNP-induced NIR was abolished.

Discussion and conclusions

This work is the first to demonstrate that CNP produces both a NIR and a LR in a model of HF. These functional effects were attenuated by a PKG blocker, which suggests they were mediated by cGMP and PKG. We found that CNP increased Ca^{2+} transients, increased SERCA2 activity, and increased PLB and TnI phosphorylation. Our data further demonstrated that CNP reduces the sensitivity of myofilaments to Ca^{2+}. Hence, the effects of CNP on contractility are clearly complex. However, our results indicate that it is the concerted effects of PLB and TnI phosphorylation, that represent the main mechanisms responsible for the PKG-mediated functional responses to CNP in failing myocardium.

Both negative and positive inotropic effects of CNP?

The present findings that CNP induces an inotropic response during adrenoceptor blockade may help to explain the discrepancies with regard to the functional effects of CNP in normal myocardium reported in the literature. CNP stimulation of mouse myocardium has been shown to produce positive inotropic responses and increase Ca^{2+} transients (Wollert *et al.*, 2003), whereas in another study in rats it induced a NIR but the Ca^{2+} transients were unaltered (Nir *et al.*, 2001). Further, similar to our results, CNP was shown to induce an initial increase in contractility followed by a slowly developing NIR in mice (Pierkes *et al.*, 2002). In some of our muscle preparations and in cardiomyocytes, we observed a biphasic effect of CNP with an initial transient positive inotropic response followed by a slowly developing NIR. The apparent disagreement between earlier studies, with some showing positive and others NIRs to CNP, could reflect differences between species, tissues (atria vs. ventricles) or experimental conditions. Furthermore, in left ventricular muscle strips, in the presence of β-adrenoceptor stimulation CNP induced a positive inotropic effect due to PDE3 inhibition by cGMP (Qvigstad *et al.*, 2010). This positive inotropic response to CNP could be as a result of small amount of β-adrenoceptor

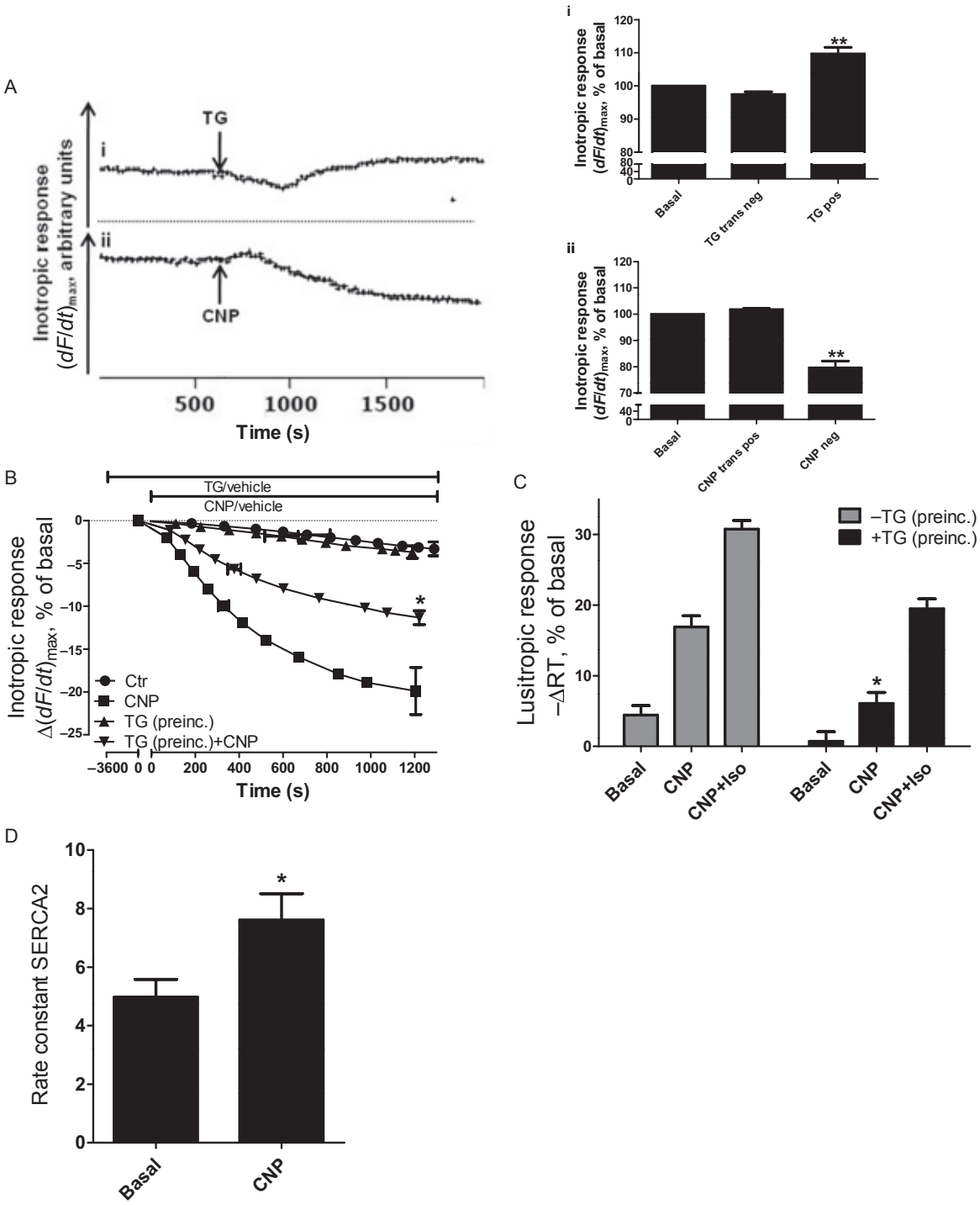

Figure 4

The SERCA inhibitor thapsigargin reduces the functional responses to CNP and CNP increases SERCA2 activity. (A) Original tracings from contracting HF left ventricular muscle strips in the presence of the SERCA-inhibitor TG (1 μM; i) and CNP (100 nM; ii) showing opposite effects of the two. One representative of five to six experiments is shown. (i) The inotropic response to TG showing a biphasic response. Basal, before addition of TG; TG trans neg, transient negative inotropic response phase of TG (~5 min); TG pos, positive inotropic response to TG (~25 min). $n = 6$. (ii) The inotropic response to CNP showing a biphasic response. Basal, before addition of CNP; CNP trans pos, transient positive inotropic response to CNP (~3.5 min); CNP neg, negative inotropic response to CNP (~22 min). (B) The time course of CNP-induced negative inotropic response in the absence and presence of TG (TG preinc.). TG was added to the organ bath 60 min before CNP (added at time zero). $n = 6$, *$P < 0.05$ CNP versus TG (preinc.) + CNP. (C) The lusitropic response to CNP alone and to Iso combined with CNP (Iso was added subsequently to CNP) in the absence [–TG (preinc.)] and presence of TG [+TG (preinc.)]. $n = 6$–8; TG: 1 μM; Iso: 800 μM. *$P < 0.05$ CNP {–TG [preinc] versus CNP [+TG (preinc.)]}. (D) SERCA2 rate constant in isolated cardiomyocytes ($n = 8$).

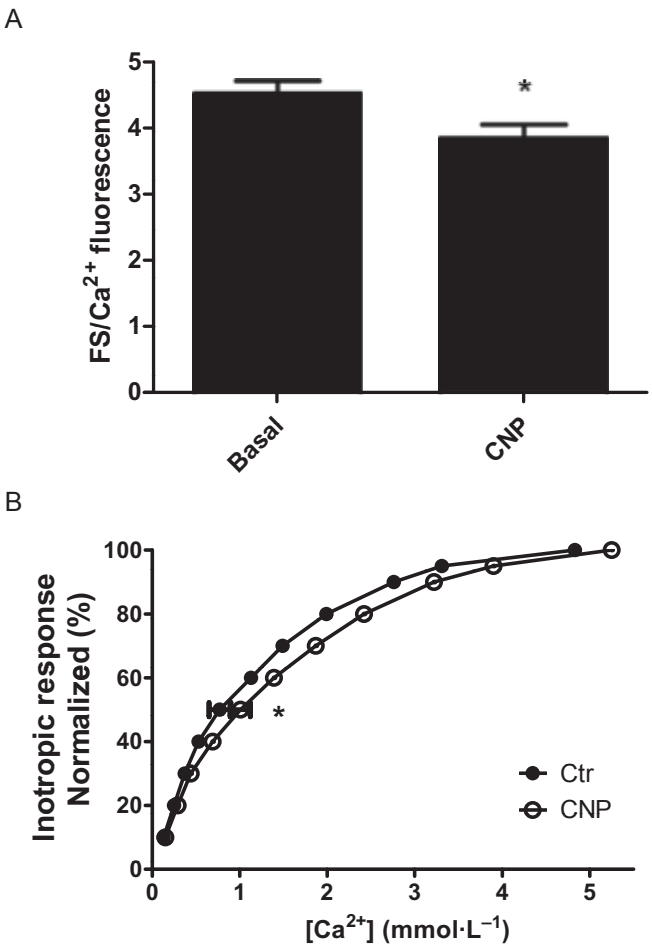

Figure 5

Decreased Ca^{2+} sensitivity by CNP. (A) The ratio between maximal contraction (fractional cell shortening) and maximal amplitude of the Ca^{2+} transient in cardiomyocytes was decreased by CNP (300 nM). (B) CNP shifted the Ca^{2+} concentration–contraction response relationship in muscle strips to higher Ca^{2+} concentrations, indicating reduced myofilament Ca^{2+} sensitivity in the presence of CNP ($n = 4$). FS, fractional shortening; *$P < 0.05$.

stimulation due to traces of endogenous noradrenaline in the preparations. Hence, the presence of a β-adrenoceptor blocker, as in the present study, is clearly needed to avoid this pitfall. Thus, in our study we clarified that NIR is the dominant effect of CNP when the cAMP pathway is not activated.

Mechanism of the functional responses to CNP

The effects of CNP develop relatively slowly and are concentration-dependent. Our results are consistent with a CNP-induced NIR mediated through the PKG pathway as shown earlier in normal hearts (Su *et al.*, 2005; Zhang *et al.*, 2005a). We found that CNP increased the phosphorylation of both PLB Ser^{16} and TnI $Ser^{23/24}$, which corresponds to earlier findings in normal hearts (Brusq *et al.*, 1999; Wollert *et al.*, 2003). However, there are other mechanisms besides PLB and TnI phosphorylation that might contribute to the functional responses to CNP. Increased forward mode Na^{+}–Ca^{2+} exchanger (NCX) currents are a potential mechanism for increasing Ca^{2+} transient decay. In our HF rat model, we previously demonstrated that the NCX contribution to Ca^{2+} removal is about 11%, which is increased compared with the Sham group (Bøkenes *et al.*, 2008). However, our present results in the same rat model do not support a major role for forward-mode NCX in Ca^{2+} removal by CNP as there is no effect of CNP on the Ca^{2+} extrusion in the presence of caffeine. CNP could also reduce L-type Ca^{2+} channel (LTCC) activity. In this case, one would expect to see a functional response to CNP in the presence of maximal Iso stimulation, as earlier studies have shown that the inhibition of LTCC by PKG might still occur when cAMP is increased (Mery *et al.*, 1991). However, we found that in the presence of maximal β-adrenoceptor stimulation by Iso, CNP was unable to induce either a NIR or a LR but these two responses were both obtained in the presence of LTCC activation by BAY-K8644. Iso causes an inotropic response through the activation of the cAMP pathway and the concomitant phosphorylation of different downstream targets such as LTCC, ryanodine receptor/SR release channel (RyR), PLB and TnI. From our results it is assumed that CNP and Iso both act by phosphorylation of PLB Ser^{16} and TnI $Ser^{23/24}$ by PKA, leaving no mechanisms for CNP to act upon in the presence of Iso. This accords with the results from another study where it was found that a cGMP analogue did not reduce the functional effects of Iso, but reduced the BAY-K8644-induced inotropic response (Shah *et al.*, 1991). However, although we cannot rule out the possibility that CNP can also influence LTCC, RyR and NCX, our results support an assumption that PLB and TnI phosphorylation are sufficient to account for the PKG-mediated functional responses to CNP.

The interplay between SERCA2 and TnI in the mechanism of CNP-induced functional responses

In addition to the increased PLB Ser^{16} phosphorylation and SERCA2 activity in the presence of CNP, we also found, we also found increased Ca^{2+} transients and SR Ca^{2+} load. CNP also increases SERCA2 activity in normal hearts (Brusq *et al.*, 1999). Our findings thus support and extend these results. Further, our results suggest TnI $Ser^{23/24}$ phosphorylation contributes to the CNP-mediated NIR, as CNP reduced contractility in isolated cardiomyocytes despite increased Ca^{2+} transients, suggestive of reduced Ca^{2+} sensitivity. In support of this hypothesis, we also observed that the curve describing the relationship between contraction in muscle strips and Ca^{2+} concentration was shifted to higher Ca^{2+} concentrations in the presence of CNP. Based on these results from contracting/relaxing cardiomyocytes with preserved cellular signalling pathways, and the finding of increased phosphorylation of TnI, we suggest that reduced myofilament Ca^{2+} sensitivity also contributes to the CNP-mediated NIR.

The exact mechanism of the effect of CNP on the myofilaments cannot be elucidated from the present indirect measurements of reduced Ca^{2+} sensitivity. In accordance with our observations, PKG activation was found to decrease myofilament Ca^{2+} sensitivity (Pfitzer *et al.*, 1982; Shah *et al.*, 1994). Decreased myofilament Ca^{2+} sensitivity is caused by increased off-rate of Ca^{2+} from troponin C (TnC), which will

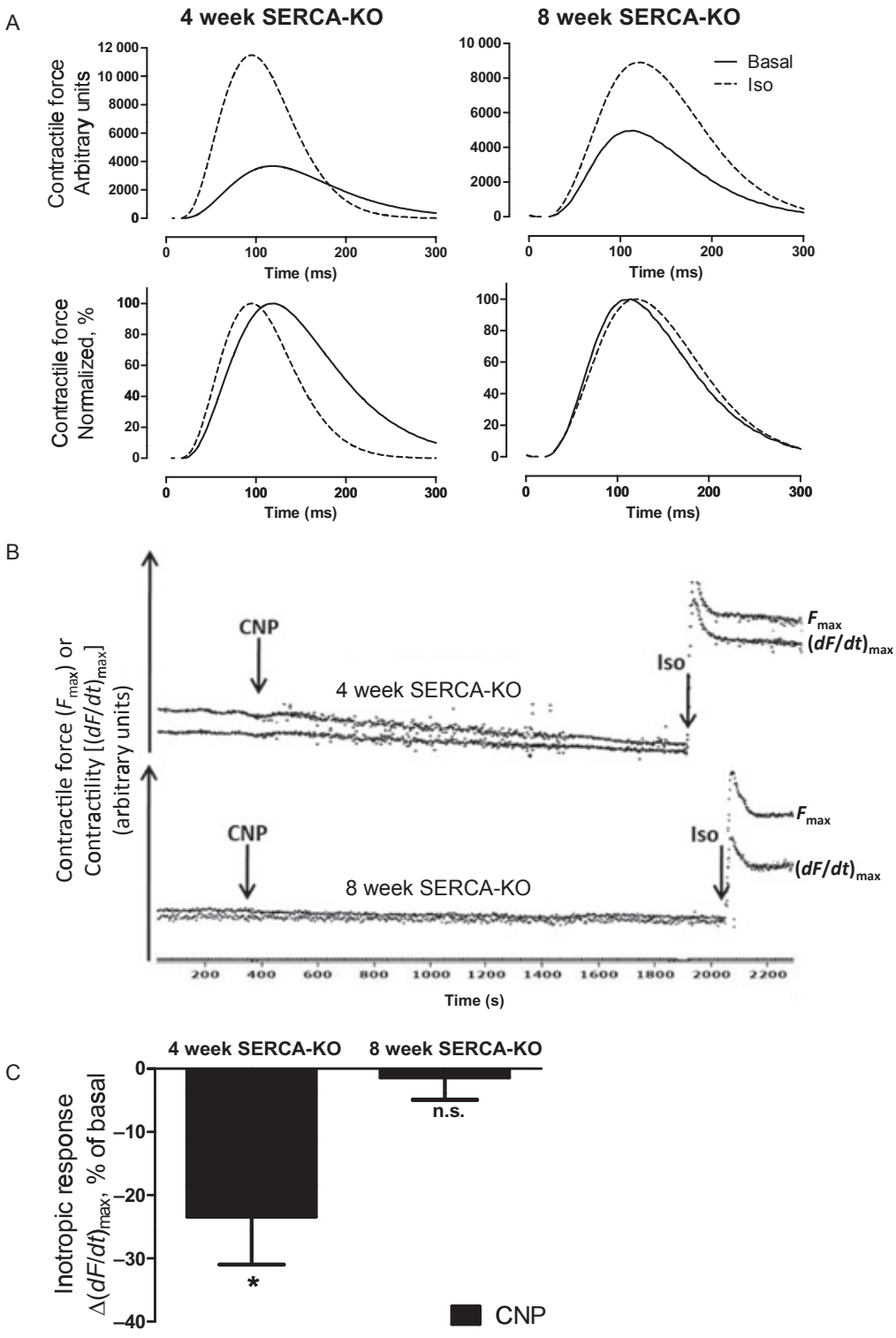

Figure 6

In 8 week SERCA2-KO mice, there are no functional responses to CNP. (A) Illustration of contraction–relaxation cycles (signal averaged across about 25 cycles), arbitrary units (upper part) and normalized (lower part) showing increased contraction (positive inotropic response) and shortening (lusitropic response) of the CRC in the presence of Iso in 4 and 8 week SERCA2-KO. There was a positive inotropic response to Iso with no shortening in 8 week SERCA2-KO indicating an absence of SERCA2 activity. One representative muscle strip from 4 and 8 week SERCA2-KO of three to four experiments. (B) Original tracings from contracting muscle strips in SERCA2-KO mice. CNP (300 nM) elicits a negative inotropic response in 4 week SERCA2-KO mice, whereas in the 8 week SERCA2-KO mice the response is abolished. (C) The inotropic response to CNP (300 nM) in 4 and 8 week SERCA2-KO. $n = 3–4$, $^*P < 0.05$ (CNP in 4 weeks KO vs. basal), n.s., not significant (CNP in 8 weeks KO vs. basal).

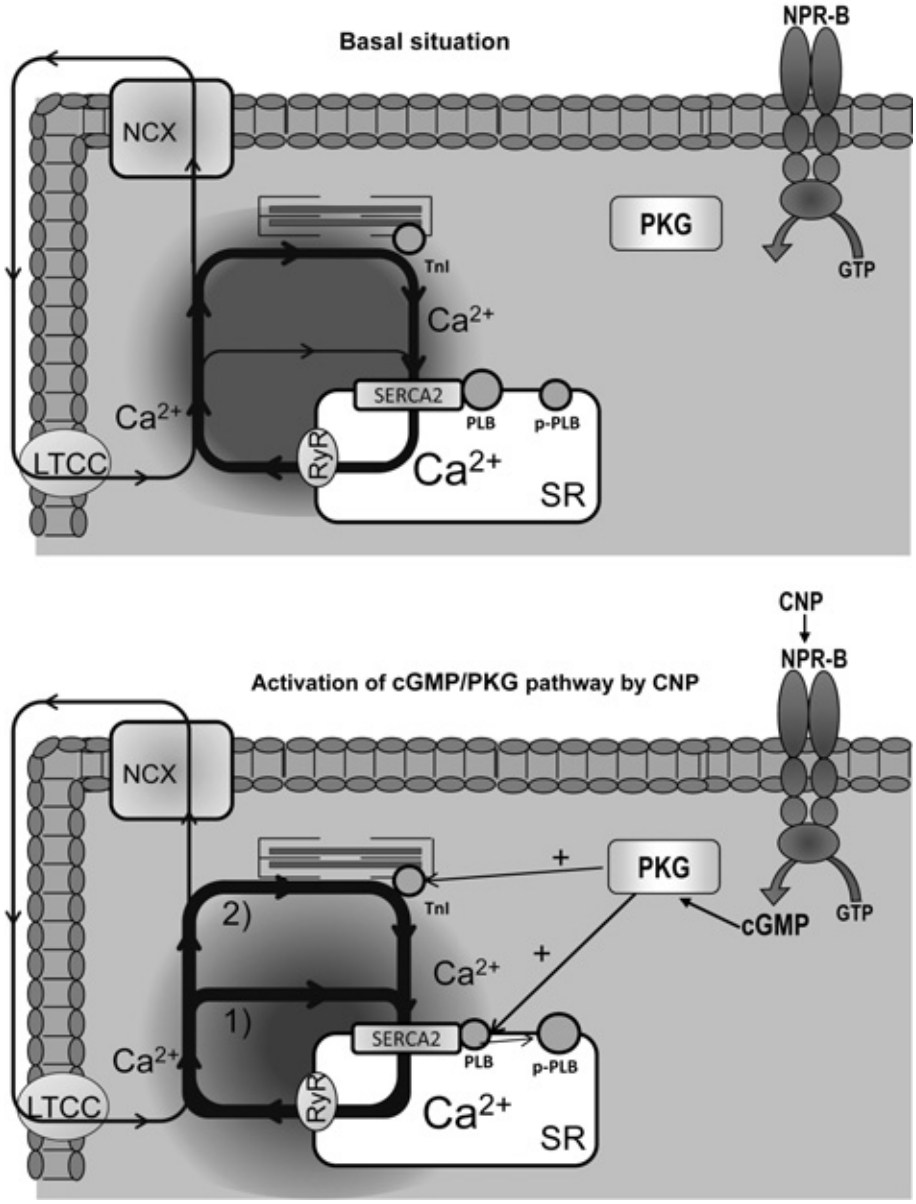

Figure 7

Two plausible models to explain the mechanism of CNP-induced functional responses. CNP activates the cGMP/PKG pathway and increases PLB and TnI phosphorylation followed by: (1) increased SERCA2 activity that causes a higher fraction of the cytosolic Ca^{2+} to be sequestered back into SR without achieving equilibrium with TnC and thus activation of the myofilaments, resulting in a NIR and LR to CNP and/or (2) reduced sensitivity of the myofilaments to Ca^{2+} and increased rate of Ca^{2+} dissociation from TnC caused by CNP-induced TnI $Ser^{23/24}$ phosphorylation, resulting in NIR and LR to CNP.

contribute to faster reversal of the actin–myosin interaction and facilitate the increased Ca^{2+} sequestration induced by increased SERCA2 activity. Other possible negative effects on Ca^{2+} sensitivity in addition to phosphorylation of TnI, such as, for example, the role of cMyBPC, were not investigated in the present study. The observed reduction in contractility in the presence of increased Ca^{2+} transients could theoretically be due to either reduced Ca^{2+} sensitivity in the myofilaments, or less Ca^{2+} binding to TnC. We suggest two plausible models to explain the functional responses to CNP: (i) Increased SERCA2 activity by CNP, causing a higher fraction of the cytosolic Ca^{2+} to be sequestrated back into SR without activating the myofilaments, resulting in a NIR and LR. (ii) Reduced Ca^{2+} sensitivity of the myofilaments to Ca^{2+} and increased rate of Ca^{2+} dissociation from TnC as a result of CNP-induced TnI $Ser^{23/24}$ phosphorylation, leading to a NIR and LR (Figure 7) (Layland *et al*., 2005). A contribution of TnI phosphorylation to the LR is one possible reason for the ability of CNP to elicit a LR, although small, in the presence of the SERCA-inhibitor TG (Figure 4C), and would also be in line with the results of Yasuda *et al*. (2007); they showed, indirectly through mutagenesis studies, that phosphorylation

of TnI is partly responsible for mediating the LR induced by β-adrenoceptor activation of the myocardium, thus demonstrating the ability of decreased myofilament Ca^{2+} sensitivity *per se* to mediate a LR. The increased dissociation rate of Ca^{2+} from TnC after TnI phosphorylation attenuates systolic binding of Ca^{2+} to TnC (Robertson *et al.*, 1982; Chandra *et al.*, 1997). TnI phosphorylation may also induce desensitization to the (changes caused by the) Ca^{2+}–TnC complex (Solaro and Van Eyk, 1996).

The role of the increased SERCA2 activity induced by CNP in the failing myocardium

Increased SERCA2 activity and increased Ca^{2+} transients, as shown in this study, are usually followed by increased cardiac contractility. However, we found that cardiac contractility was reduced by CNP, which potentially could be explained by the concomitant increase in TnI phosphorylation, with the expected shift in the myofilament Ca^{2+}–force relationship. Another possible explanation for the NIR in the presence of increased Ca^{2+} transients is the sequestrating role of increased SERCA2 activity. In the present study, we obtained an apparent opposite inotropic response to the SERCA-inhibitor TG and CNP, indicative of CNP leading to decreased contractility despite increased SERCA2 activity, in contrast to the decreased SERCA2 activity and increased contractility seen after TG administration. However, as CNP induces biphasic responses, the initial increased contractility induced by CNP could be mediated through increased SERCA2 activity and Ca^{2+} transient, while the second, stable phase could be explained by the expected reduction in myofilament Ca^{2+} sensitivity induced by TnI phosphorylation during increased SERCA2 activity. Earlier studies have shown that the presence of TG attenuates the NIR to cGMP mediated through PKG (Zhang *et al.*, 2005b; 2009), which corresponds to the reduction in the CNP-mediated response in the presence of TG obtained here. The role of SERCA2 in CNP-mediated responses was, therefore, investigated further in a mouse model with cardiomyocyte-specific inducible excision of the *SERCA2* gene (Andersson *et al.*, 2009); these mice showed complete loss of SERCA2 activity 7 weeks after SERCA2-KO (Louch *et al.*, 2010). In 8 week SERCA2-KO mice, CNP was unable to produce an inotropic response, whereas in the 4 week SERCA2-KO mice, which still possessed some SERCA2 activity (Louch *et al.*, 2010), CNP induced a NIR. Our results demonstrate that the presence of SERCA2-mediated Ca^{2+} extrusion is necessary for inducing the CNP-mediated NIR, as shown by the lack of NIR to CNP in 8 week SERCA2-KO mice. Furthermore, CNP is also unable to produce a NIR in PLB-KO mice, which have persistant high SERCA2 activity (Zhang *et al.*, 2007). Taken together, these findings suggest that TnI phosphorylation alone cannot cause the functional responses seen with CNP. Thus, CNP-induced PLB and TnI phosphorylation may work in concert. However, this needs further investigation.

Concluding remarks

Our results indicate that the presence of SERCA2 activity is necessary for the CNP-induced functional responses. However, the relative role of increased SERCA2 activity and TnI phosphorylation needs further investigation. Knockout models will not fully answer these questions as the concerted action of SERCA2 and TnI is probably involved in the functional responses to CNP. Experimental models where CNP could separately phosphorylate either PLB or TnI without changing their basal phosphorylation state, respectively, are needed to elucidate the precise role of each. However, such experimental models are not available at present.

In conclusion, we present, for the first time, evidence that CNP mediates functional responses in failing myocardium. Our main findings were (i) CNP concentration-dependently induced a NIR and LR, which were both higher in HF than in Sham-operated hearts; (ii) the functional responses were consistent with a cGMP–PKG-mediated effect of CNP; (iii) CNP increased the Ca^{2+} transient and Ca^{2+} decay, corresponding to PLB phosphorylation and associated increased SERCA2 activity; (iv) the myofilament Ca^{2+} sensitivity was decreased by CNP; and (v) CNP-mediated NIR was not present in experimental conditions in which SERCA2 activity was abolished. These results combined with the increase in TnI phosphorylation indicate a complex response with CNP lowering cardiac contractility even though it also increases Ca^{2+} transients.

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Conflicts of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1 Representative echocardiographic tracing of a heart failure (HF) rat heart 6 weeks after myocardial infarction compared with a Sham-operated rat heart. IVSd, inter-ventricular septum diameter; LA, left atrial diameter; LVDd, left ventricular diameter in diastole; PWd, posterior wall diameter.

Figure S2 Biphasic response to CNP in cell contraction. The first phase was positive in all cells examined, while the second, stable phase was negative in all cells compared with basal contractility. *$P < 0.05$.

Figure S3 The maximal inotropic and lusitropic response to CNP as % of basal in the presence of IMBX and Iso ($n = 6$); CNP: 300 nM; IBMX: 100 μM; Iso: 1 μM (no timolol present in this experiment).

Table S1 Animal characteristics, echocardiographic and haemodynamic data of HF and Sham hearts.