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RESEARCH PAPER

Differential regulation of β_2-adrenoceptor-mediated inotropic and lusitropic response by PDE3 and PDE4 in failing and non-failing rat cardiac ventricle

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BACKGROUND AND PURPOSE
β-Adrenoceptors play a major role in regulating myocardial function through cAMP-dependent pathways. Different phosphodiesterases (PDEs) regulate intracellular cAMP-pools and thereby contribute to the compartmentalization of cAMP-dependent effects. We explored the involvement of PDEs in limiting the β_2 adrenoceptor-mediated positive inotropic (PIR) and lusitropic (LR) responses in sham-operated (Sham) and failing rat hearts.

EXPERIMENTAL APPROACH
Extensive myocardial infarctions were induced by coronary artery ligation in Wistar rats. Rats developing heart failure were studied 6 weeks after surgery. Contractility was measured in left ventricular strips from failing and Sham hearts. cAMP was quantified by RIA.

KEY RESULTS
In ventricular strips, stimulation of β_2-adrenoceptors with (-)-adrenaline (300 nM CGP20712A present) exerted a small PIR and LR. In Sham hearts, β_2-adrenoceptor-mediated as well as β_1-adrenoceptor-mediated PIR and LR were increased by selective inhibition of either PDE3 (1 μM cilostamide) or PDE4 (10 μM rolipram). In failing rat hearts, PDE3 inhibition enhanced PIR and LR to both β_1- and β_2-adrenoceptor stimulation while PDE4 inhibition had no effect on these responses despite a significant increase in cAMP levels. Combined PDE3/4 inhibition further enhanced the PIR and LR of β_2- and β_1-adrenoceptor activation both in Sham and failing hearts, compared with PDE3 inhibition alone. PDE4 enzyme activity was reduced in failing hearts.

CONCLUSIONS AND IMPLICATIONS
Both PDE3 and PDE4 attenuated β_2- and β_1-adrenoceptor-mediated contractile responses in Sham hearts. In failing hearts, these responses are attenuated solely by PDE3 and thus even selective PDE3 inhibitors may provide a profound enhancement of β-adrenoceptor-mediated responses in heart failure.

Abbreviations
EHNA, erythro-9-(2-hydroxy-3-nonyl) adenine; HF, heart failure; LR, lusitropic response; PIR, positive inotropic response; RT, relaxation time

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Introduction

The stimulation of β-adrenoceptors is one of the most powerful mechanisms to increase myocardial contractility (Brodde and Michel, 1999). The adrenoceptor subtypes, β_1 and β_2, coexist in mammalian hearts although the former usually dominates (Brodde and Michel, 1999; receptor nomenclature follows Alexander *et al.*, 2009). The activated β-adrenoceptors couple to G_s proteins, elevating cytosolic cAMP. Cyclic AMP activates protein kinase A which promotes phosphorylation of L-type Ca^{2+}-channels, ryanodine receptors, phospholamban and troponin I, responsible for regulation of intracellular Ca^{2+} and other components of the excitation–contraction coupling process (Brodde and Michel, 1999). Despite the fact that both β_1- and β_2-adrenoceptors couple to G_s and increase cAMP production, several differences in β_1- and β_2-adrenoceptor-mediated effects are observed. In rodents, the stimulation of β_1-adrenoceptors evokes a cAMP signal propagating throughout the cardiomyocyte and elicits a maximal positive inotropic response (PIR), whereas the β_2 adrenoceptor-mediated cAMP signal appears more compartmentalized and elicits a smaller PIR (Nikolaev *et al.*, 2006; Xiao *et al.* 2006). Suggested explanations for the compartmentation in rodents include localization of β_2-adrenoceptors inside caveolae in cardiomyocytes or coupling of β_2-adrenoceptors to G_i-dependent pathways, acting as negative regulators of contractility (Jo *et al.*, 2002; Xiang *et al.*, 2002; Pavoine and Defer, 2005). The stimulation of β_1-adrenoceptors hastens the relaxation (lusitropic response, LR) along with increasing the contraction (PIR) while β_2-adrenoceptor stimulation has been found to evoke a PIR with no LR in cardiac tissue from rodents, cat and sheep (Xiao *et al.*, 1999), but not human (Kaumann *et al.*, 1999; Molenaar *et al.*, 2000; 2007) and canine hearts (Xiao *et al.*, 1999). In accordance with the suggestion that G_i is able to compartmentalize downstream β_2-adrenoceptor signalling, inhibition of G_i by *Pertussis* toxin disclosed an LR to β_2-adrenoceptor stimulation along with increased phosphorylation of phospholamban (Kuschel *et al.*, 1999).

Phosphodiesterases (PDEs), the only enzymes hydrolyzing cAMP, play important roles in limiting elevation of cAMP and thus the downstream effects of cAMP (Fischmeister *et al.*, 2006). The β_1- and β_2-adrenoceptor-activated cAMP signals are differentially regulated by various PDE isoforms in cardiomyocytes (Nikolaev *et al.*, 2006; Rochais *et al.*, 2006; Galindo-Tovar and Kaumann, 2008). In non-failing myocardium, PDE3 and PDE4 provide about 90% of total cAMP-PDE activity, and the β-adrenoceptor-mediated increase in cAMP is mainly limited by PDE4 (Mongillo *et al.*, 2004). However, it is not known whether this corresponds to compartments of cAMP that are relevant for contractile effects. There is evidence of reduced PDE3 and PDE4 activities or expression in heart failure (Movsesian *et al.*, 1991; Lehnart *et al.*, 2005). However, another study reported enhanced activity and expression of PDE3 and PDE4 during heart failure (Takahashi *et al.*, 2002). β_2-Adrenoceptor-mediated signalling is known to be altered during heart failure (Brodde and Michel, 1999). However, whether the suppression of β_2 adrenoceptor-mediated signalling and contractile effects by various PDE isoforms is changed in heart failure, is not known.

Accordingly, the aims of this study were to characterize β_2 adrenoceptor-mediated PIR and LR in failing and non-failing ventricular myocardium and to determine and compare the role of different PDE isoforms in suppression or compartmentalization of β_2-adrenoceptor-mediated signalling in comparison with the corresponding characteristics of β_1-adrenoceptor-mediated contractile responses. We show that the stimulation of myocardial β_2-adrenoceptors mediated a PIR as well as a LR, both of which were suppressed in similar ways by PDE3 and PDE4. Dual PDE3/4 inhibition augmented the β_2 adrenoceptor-mediated responses to similar levels as β_1-adrenoceptor-mediated responses as recently reported (Christ *et al.*, 2009). In failing myocardium, PDE3 exerted a clear limitation of the β_2- and β_1-adrenoceptor-mediated functional responses, despite a hardly detectable increase in total cAMP levels after inhibition. In contrast, there was a lacking primary role of PDE4 in suppressing the β_2- and β_1-adrenoceptor-mediated functional responses, despite a considerable increase in cAMP after inhibition of this isoform. The PDE4 activity was reduced in failing myocardium.

Methods

Animal model

Animal care and experimental procedures were according to the Norwegian Animal Welfare Act, which conforms to the *European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes* (Council of Europe, 1985) and were approved by the Norwegian National Animal Research Committee. As described, an extensive myocardial infarction was induced in 320 g male Wistar rats by coronary artery ligation for 6 weeks. Left-ventricular end diastolic (LVEDP) pressures were measured by catheterization as

described earlier (Sjaastad *et al.*, 2003) and were included in the study if LVEDP was 15 mm Hg or higher after 6 weeks; these animals were considered to be in heart failure (HF) (Sjaastad *et al.*, 2000). Sham-operated animals (Sham) underwent identical surgical procedures without coronary artery ligation.

Isolated ventricular strips

Left ventricular strips were prepared, field-stimulated at 1 Hz in organ baths, and the contraction-relaxation cycles (CRCs) were recorded and analyzed as described previously (Sjaastad *et al.*, 2003) with respect to maximal developed force (F_{max}, mN) and maximal development of force ($(dF/dt)_{max}$). The $(dF/dt)_{max}$ was used as an index of contractility as it is traditionally recognized to be a better measure of the intensity of contraction, that is, contractile active state, than F_{max} (Sonnenblick, 1967), particularly during action of inotropic agents causing asymmetrical changes in CRC, that is, a PIR accompanied by an LR, as for β-adrenoceptors (Aass *et al.*, 1983; Skomedal and Osnes, 1983). Moreover, F_{max} tends to underestimate the PIR compared with $(dF/dt)_{max}$ in the presence of an accompanying LR, due to an early initiation of relaxation (Aass *et al.*, 1983; Skomedal and Osnes, 1983). PIR to agonists were expressed by increases in $(dF/dt)_{max}$ in percentage of control levels. LRs were expressed as decrease of relaxation time (time to 80% relaxation–time to peak force; RT), as a percentage of RT before the addition of the agonist (–ΔRT %). Blockers of α_1-adrenoreceptors (prazosin 1 μM), muscarinic cholinergic receptors (atropine 1 μM) and the β_2-adrenoceptor-selective antagonist ICI118551 (1-[2,3-dihydro-7-methyl-1*H*-inden-4-yl]oxy-3-[(1-methylethyl)amino]-2-butanol) or the β_1-adreno ceptor-selective antagonist CGP20712A (2-hydroxy-5-[2-[[2-hydroxy-3-[4-[1-methyl-4-(trifluorometyl)-1H-imidazol-2-yl]phenoxy]propyl]amino]ethoxy]-benzamide) (as specified next) were added 90 min before the agonists. These drugs did not influence the basal contraction–relaxation cycle characteristics (data not shown). (-)-Noradrenaline in the presence of 50 nM ICI118551 and (-)-adrenaline in the presence of 300 nM CGP20712A were used to selectively stimulate β_1- and β_2-adrenoreceptors respectively. PDE inhibitors were added, as specified, 45 min before the agonists. (-)-Noradrenaline or (-)-adrenaline was added to the organ bath cumulatively to obtain concentration–response relationships. Concentration–response curves were constructed by estimating centiles (EC_{10}–EC_{100}) for the receptor selective effects for each experiment and calculating the corresponding means and the horizontal positioning expressed as –log EC_{50} (Sjaastad *et al.*, 2003).

cAMP content in ventricular strips

Ventricular strips were prepared, mounted in organ baths and equilibrated for 90 min with antagonists and 45 min with PDE inhibitors as described above. The strips were then freeze-clamped following 2-min stimulation with a sub-maximal concentrations of agonist; with 1 μM (-)-noradrenaline (50 nM ICI118551 present) or 1 μM (-)-adrenaline (300 nM CGP20712A present). Frozen strips were homogenized at 4°C in 1 mL of 5% trichloroacetic acid by a Retsch MM301 mechanical mill (Retsch GmbH & Co., Haan, Germany) and cAMP content was determined by radioimmunoassay (Skomedal *et al.*, 1980).

A 1 μM of (-)-adrenaline was chosen as an appropriate concentration in the experiments where cAMP was assayed as it did not evoke any PIR in the concomitant presence of ICI118551 and CGP20712A (data not shown), ensuring selective activation of β_2-adrenoceptors. Additionally, 1 μM (-)-adrenaline elicits a β_2 adrenoceptor-mediated PIR significant enough to evaluate the effect of intervention with different PDE inhibitors as presented in Results.

PDE activity assay

Ventricular strips (described previously) were equilibrated for 90 min in organ baths and freeze-clamped. The left ventricle tissue samples were homogenized in ice cold 20 mM Tris-HCl (pH 7.4 at room temperature), 1 mM EDTA, 1 mM dithiothreitol, 1 mM benzamidine, 0.2 mM PMSF, 0.1 mM Na_3VO_4, 20 μg/mL leupeptin and 10 μg/mL trypsin inhibitor. PDE activity was assayed using a modification of a two-step procedure (Marchmont and Houslay, 1980). Briefly, 50 μL of 2 μM mixture of [^{3}H]cAMP and unlabelled cAMP in 20 mM Tris-HCl (pH 7.4 at room temperature), 10 mM $MgCl_2$ assay buffer was mixed with muscle homogenates to a final concentration of 1 μM cAMP. To determine the fraction of activities of PDE3s and PDE4s, PDE activities were measured with and without the selective inhibitors cilostamide (Cil; 1 μM) or rolipram (Rol; 10 μM) respectively. Reaction mixtures were incubated at 30°C for 20 min, with frequent agitation. Cyclic AMP hydrolysis was stopped by boiling the tubes for 3 min and then placing them on ice for 10 min. *Crotalus atrox* venom (25 μL; 1 μg/μL) was added and the samples incubated at 30°C for a further 10 min to convert 5'AMP extensively to adenosine. Dowex 1X8-400 resin, pH 3, stored as a 50:50 Dowex/water mixture, was prepared immediately prior to the assay by the addition of one volume of 96% ethanol to two volumes of the

Dowex/water mixture to create a slurry. Dowex slurry (400 µL) was added to each reaction mixture, placed on ice and mixed frequently for 15 min. Samples were centrifuged at 13 000×g_{av} at 4°C for 3 min. Supernatant fraction (150 µL) from each tube was taken for scintillation counting in a beta counter. [^{14}C]-AMP was processed in parallel tubes and recovery of 5′AMP was estimated and used for correction.

Statistics

All results are expressed as mean ± SEM unless otherwise indicated and statistical significance assessed with unpaired or paired Student's *t*-tests as appropriate. When appropriate, Bonferroni corrections were made. $P < 0.05$ was regarded as statistically significant.

Materials

(-)-Adrenaline (+)-bitartrate salt (-)-noradrenaline bitartrate salt (hydrate), prazosin hydrochloride, atropine sulphate, *Crotalus atrox* venom and Dowex 1X8-400 resin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rol, Cil, CGP20712A, ICI118551 and EHNA hydrochloride were from Tocris Bioscience (Bristol, UK). Isoflurane (Forene) was from Abbot Scandinavia (Solna, Sweden). [^{3}H]cAMP and [^{14}C]-AMP were purchased from GE HealthCare (Buckinghamshire, UK).

Results

All rats in the HF group had large anterolateral infarcts and signs of HF including tachypnea and pulmonary congestion. The characteristics and haemodynamic data are shown in Table 1.

Selection of PDE inhibitor concentrations

We wanted to use optimal concentrations of the PDE inhibitors Cil and Rol, allowing both selective and maximal or nearly maximal inhibition of PDE3 and PDE4 respectively. Thus, we determined the effect of increasing concentrations of Cil and Rol in Sham ventricular strips preincubated with 0.1 µM (-)-noradrenaline (Figure 1A and B) and 3 µM (-)-adrenaline (Figure 1C and D) respectively. Cil concentration dependently elicited PIR with a plateau from about 2 µM (–log M = 5.7) to at least 5 µM (–log M = 5.3), indicating maximal PDE3 inhibition, sensitizing the β_1-adrenoceptor-mediated PIR (Figure 1B). Cil (50 µM) (–log M = 4.3) further increased the PIR, revealing non-selective PDE inhibition only at concentrations higher than at least 5 µM. The strips were then challenged with 10 µM Rol which further increased the PIR, indicating a dual PDE regulation of β_1 adrenoceptor-mediated PIR (presented in more details and discussed next), which was reversed by timolol (Figure 1A). Based on these observations, 1 µM was chosen as an appropriate concentration of Cil eliciting a clearly selective and nearly maximal PDE3 inhibition. Increasing concentrations of Cil in the absence of agonist did not elicit any inotropic response (Figure 1B).

In Sham ventricular strips preincubated with 0.1 µM (-)-noradrenaline, 1 µM Rol increased the PIR which was not further enhanced by 10 µM Rol or even by concentrations up to 100 µM Rol (data not shown). In Sham ventricular strips preincubated with 3 µM (-)-adrenaline in the presence of 300 nM CGP20712A, 1 µM Rol significantly increased the PIR compared with (-)-adrenaline (Figure 1D). In three of the six strips, 10 µM Rol further enhanced the PIR suggesting that 1 µM Rol in some muscle strips, but not all, is sufficient to inhibit PDE4 to an extent that maximally amplifies functional effects of β_2-adrenoceptor stimulation (Figure 1C). The PIR to Rol was reversed by 50 nM ICI118551, indicating selective β_2-adrenoceptor activation. Due to this variability, 10 µM Rol was chosen as an optimal concentration to inhibit PDE4 in order to ensure a maximal but still selective PDE4 inhibition. Increasing concentrations of Rol in the absence of agonist did not elicit any inotropic response (Figure 1D).

Table 1
Animal characteristics

	Sham rats (n = 64)	HF rats (n = 56)
Body weight (g)	399 ± 5	378 ± 3
Heart weight (g)	1.32 ± 0.03	2.49±0.05*
Heart weight /body weight (g/kg)	3.3 ± 0.1	6.6 ± 0.1*
LVEDP (mmHg)	2.1 ± 0.2	24.0 ± 0.6*
LVSP (mmHg)	124 ± 3	102 ± 2*
Lung weight (g)	1.65 ± 0.04	3.81 ± 0.17*

*$P < 0.05$.
Data represent mean ± SEM.
HF, heart failure; LVEDP, left ventricular end-diastolic pressure; LVSP, left ventricular systolic pressure.

In tissues from Sham rats, β_2-adrenoceptor-mediated PIR and LR are suppressed by PDE3 and PDE4

Selective β_2-adrenoceptor stimulation (by (-)-adrenaline in the presence of CGP20712A) elicited a maximal positive PIR of 11.1 ± 1.8% (n = 11,

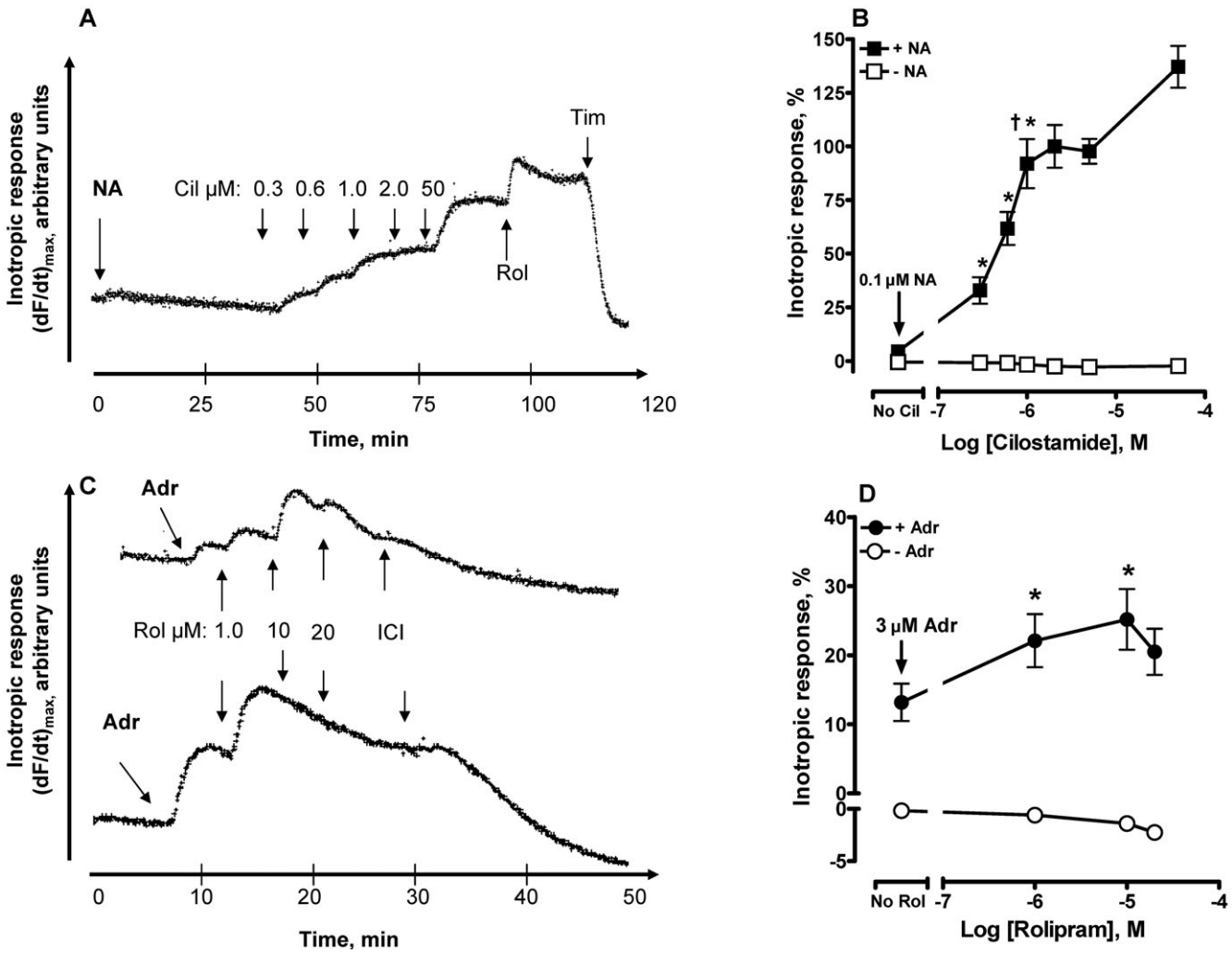

Figure 1

(A) Representative tracing of concentration–response relationship of cilostamide (Cil) in the presence of NA. The arrows indicate addition of NA, of various concentrations of Cil in µM, of 1 µM timolol (Tim) and of rolipram (Rol; 10 µM). (B) Concentration-dependent inotropic response to Cil in Sham ventricular strips in absence of (-)-noradrenaline but presence of 1 µM Tim (- NA, $n = 6$) and strips preincubated with 0.1 µM (-)-noradrenaline (+NA, $n = 4$) in the presence of 50 nM ICI118551. Abscissa: Logarithmic concentrations of Cil. Ordinate: Inotropic response (percent increase in $(dF/dt)_{max}$) above steady state contractility after NA. Vertical bars represent SEM for inotropic response. (C) Representative tracings of two independent experiments illustrating inotropic response to different concentrations of Rol in the presence of adrenaline (Adr), illustrating the variability in sensitivity to Rol between different hearts. The arrows indicate addition of Adr, various concentrations of Rol in µM and of 50 nM ICI118551 (ICI). (D) Concentration-dependent inotropic response to Rol in Sham ventricular strips in absence of (-)-Adr but presence of 1 µM Tim (-Adr, $n = 5$) and strips preincubated with 3 µM (-)-adrenaline (+Adr, $n = 6$) in the presence of 300 nM CGP20712A. Abscissae: Logarithmic concentrations of Rol. Ordinate: inotropic response (increase in $(dF/dt)_{max}$) in percent above steady state contractility after adrenaline. Vertical bars represent SEM for inotropic response. *$P < 0.05$ versus noradrenaline or Adr; †$P < 0.05$ versus –6.5 log M (0.3 µM) Cil.

$P < 0.05$), with a –log EC_{50} value of 5.58 ± 0.09 and reduced the RT by 4.5 ± 1.4% ($n = 11$, $P < 0.05$). The concentration–response curves for (-)-adrenaline in the presence of CGP20712A were biphasic (Figure 2E), where the first phase is the selective β_2 adrenoceptor-mediated component while the second rather incomplete phase is β_1 adrenoceptor-mediated. This was further verified by the lacking first phase in the presence of both CGP20712A and ICI118551 (data not shown). Table 2 lists the number of experiments (n) in each group, basal ($F_{max}B$, mN/mm^2), maximal contractile ($F_{max}M$, mN/mm^2) force to (-)-adrenaline and –log EC_{50} values in terms of F_{max} for each intervention with PDE inhibitors.

The β_2-adrenoceptor-mediated PIR and LR were insensitive to PDE2 inhibition by 10 µM EHNA (Figure 2A and B). In the presence of 1 µM Cil or 10 µM Rol, the maximal β_2 adrenoceptor-mediated PIR was enhanced compared with control. There was no increase in the potency of (-)-adrenaline in the presence of Rol. However, in the presence of Cil,

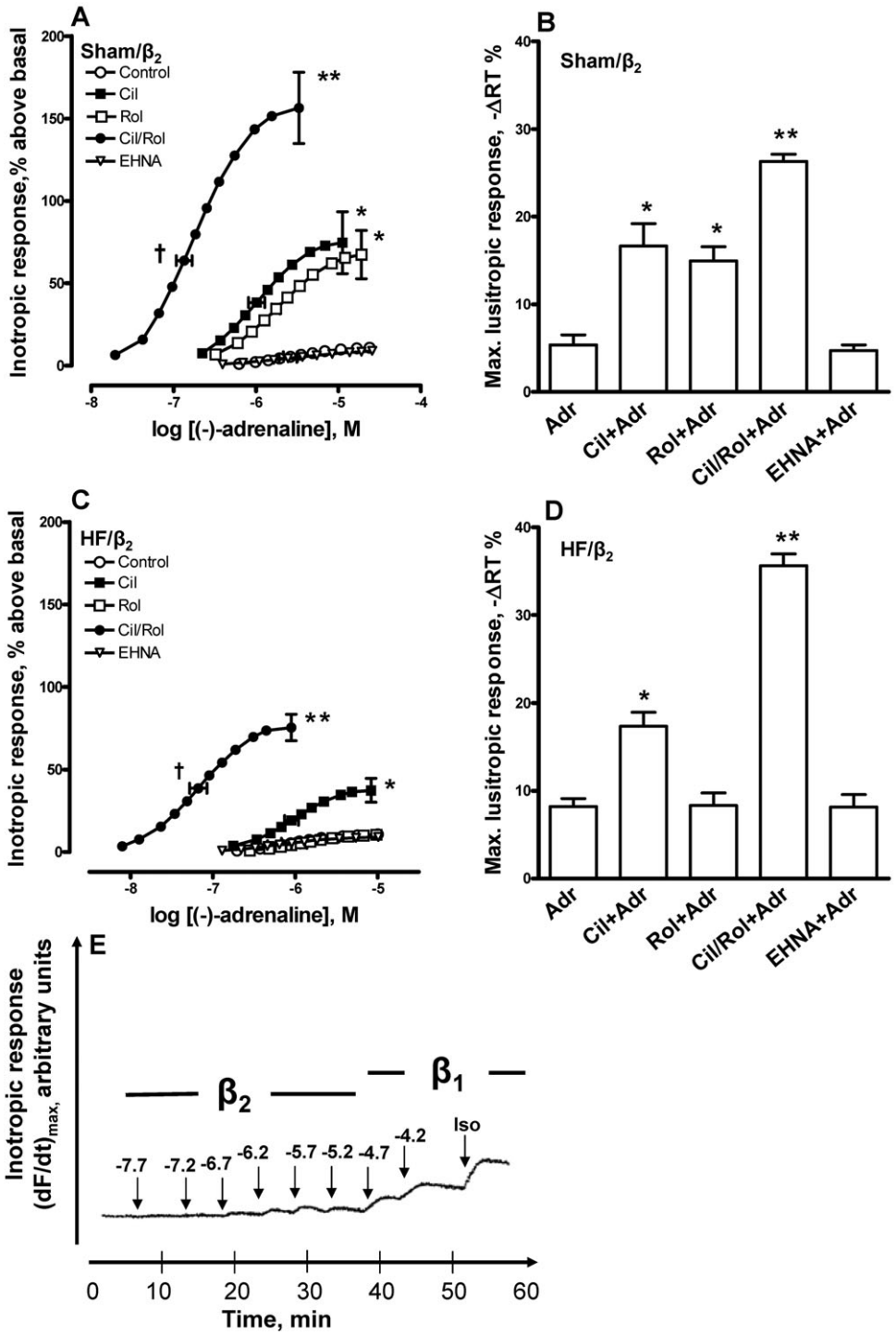

Figure 2

Concentration–response curves of inotropic response (A,C) and maximal lusitropic response (B,D) to selective β_2-adrenoceptor stimulation in the absence and presence of PDE inhibitors [1 μM cilostamide (Cil), 10 μM rolipram (Rol), the combination of cilostamide and rolipram, 10 μM EHNA; n = 6–11] in Sham (A,B) and heart failure (HF) (C,D) strips. A,C: Abscissae: Logarithm of (-)-adrenaline (Adr) concentration (300 nM CGP20712A present). Horizontal bars: SEM of –log EC_{50}. Ordinates: Inotropic response [increase in $(dF/dt)_{max}$ in percent above basal]. (B,D) Ordinates: Maximal lusitropic response as change in relaxation time (–ΔRT) in %. Vertical error bars: SEM. (E) Representative trace of cumulative concentration–response curve for (-)-adrenaline in the presence of 300 nM CGP20712A in an HF ventricular strip. The arrows indicate the addition of agonist at various concentrations indicated as logarithmic values. A biphasic relationship was obtained, where the first phase is consistent with a β_2-adrenoceptor-mediated component and the second rather incomplete phase consistent with a β_1-adrenoceptor-mediated component as the first phase is prevented by 50 nM ICI118551 (not shown). Experiments were terminated with addition of 10 μM isoprenaline (Iso). *P < 0.05 versus control, for maximal response; **P < 0.05 versus Cil, for maximal response; †P < 0.05 versus Cil, for log EC_{50}.

Table 2

Contractile force (mN/mm^2) and the sensitivity to agonist in Sham and HF ventricular strips in the absence and presence of PDE inhibitors

		Control	Cil	Rol	Cil/Rol	EHNA
β_2-Sham	n	11	11	11	11	6
	F_{max}B (mN/mm^2)	8.4 ± 0.9	8.1 ± 1.1	8.0 ± 0.9	9.0 ± 1.3	8.1 ± 1.4
	F_{max}M (mN/mm^2)	9.2 ± 1.0	13.2 ± 1.8*	13.6 ± 1.5*	23.0 ± 3.3*	8.8 ± 1.6
	–log EC_{50}	5.61 ± 0.09	5.95 ± 0.11*	5.78 ± 0.06#	6.70 ± 0.09**	5.64 ± 0.11
β_2-HF	n	11	11	11	11	6
	F_{max}B (mN/mm^2)	9.3 ± 0.9	9.7 ± 0.9	9.2 ± 1.0	10.0 ± 1.2	9.3 ± 1.3
	F_{max}M (mN/mm^2)	10.0 ± 1.0	12.7 ± 1.2*	10.0 ± 1.1	15.5 ± 1.8†	10.0 ± 1.4
	–log EC_{50}	6.00 ± 0.11	6.05 ± 0.10#	5.91 ± 0.09#	7.25 ± 0.10**	6.09 ± 0.12
β_1-Sham	n	8	7	11	6	7
	F_{max}B (mN/mm^2)	8.4 ± 0.9	8.2 ± 0.9	8.0 ± 1.0	8.2 ± 1.0	9.1 ± 1.4
	F_{max}M (mN/mm^2)	23.4 ± 2.5	23.0 ± 2.5	20.4 ± 2.6	20.8 ± 2.5	23.0 ± 2.3
	–log EC_{50}	5.95 ± 0.09	6.49 ± 0.09*	6.47 ± 0.10*	7.74 ± 0.09**	5.99 ± 0.13
β_1-HF	n	9	8	9	6	7
	F_{max}B (mN/mm^2)	9.7 ± 0.9	9.4 ± 1.0	9.4 ± 1.3	9.8 ± 1.1	9.3 ± 1.4
	F_{max}M (mN/mm^2)	14.0 ± 1.3†	12.8 ± 1.4	13.1 ± 1.8	14.3 ± 1.6†	12.9 ± 2.0
	–log EC_{50}	6.90 ± 0.11	7.30 ± 0.12*	6.94 ± 0.13	8.28 ± 0.80**	6.76 ± 0.11

*$P < 0.05$ versus Control.
**$P < 0.05$ versus Cil.
†$P < 0.05$ versus Sham.
#Non-significant versus Control.
Number of experiments 'n', basal contractile force F_{max}B (mN/mm^2), maximal contractile force during stimulation of the receptors F_{max}M (mN/mm^2) and the sensitivity to the agonist in terms of contractile force (–log EC_{50}) in the presence of different PDE inhibitors.
Cil, cilostamide; HF, heart failure; Rol, rolipram.

Table 3

Effect of concomitant addition of cilostamide and rolipram on basal contractility

	Sham $(dF/dt)_{max}$	–ΔRT	HF $(dF/dt)_{max}$	–ΔRT
ICI118551	7.6 ± 1.0% (n = 6)	16.9 ± 3.5% (n = 6)	5.5 ± 2.1% (n = 6)	9.8 ± 2.4% (n = 6)
CGP20712A	Non-significant (n = 11)	6.7 ± 0.9% (n = 11)	3.9 ± 1.6% (n = 11)	8.8 ± 0.9% (n = 11)

The effect of concomitant application of 1 μM cilostamide and 10 μM rolipram on the basal contractility or on the time course of the contraction-relaxation cycle in Sham or HF strips in the presence of 50 nM ICI118551 or 300 nM CGP20712A, all $P < 0.05$ versus basal. The application of EHNA (10 μM), cilostamide (1 μM) or rolipram (10 μM) alone had no effect on the basal contractility or on the time course of the contraction-relaxation cycle in Sham or HF strips.
HF, heart failure.

the potency of (-)-adrenaline was increased by 0.34 log units, consistent with a recent report (Christ *et al.*, 2009). The presence of Cil or Rol also increased the maximal β_2-adrenoceptor-mediated LR. In the presence of Cil and Rol together, both the maximal PIR and the sensitivity to β_2-adrenoceptor stimulation were further increased compared with Cil alone. The effects of concomitant addition of Cil and Rol on basal contractility are given in Table 3. In the presence of both Cil and Rol, the LR was also increased. The maximal PIR as well as LR to β_2-adrenoceptor stimulation during combined PDE3 and PDE4 inhibition is quantitatively similar to the β_1-adrenoceptor-mediated PIR and LR (see next, Figure 4). These data indicated that the β_2-adrenoceptor-mediated PIR and LR were suppressed by both PDE3 and PDE4.

For comparison, similar experiments were performed with selective β_1-adrenoceptor stimulation (by (-)-noradrenaline in the presence of ICI118551)

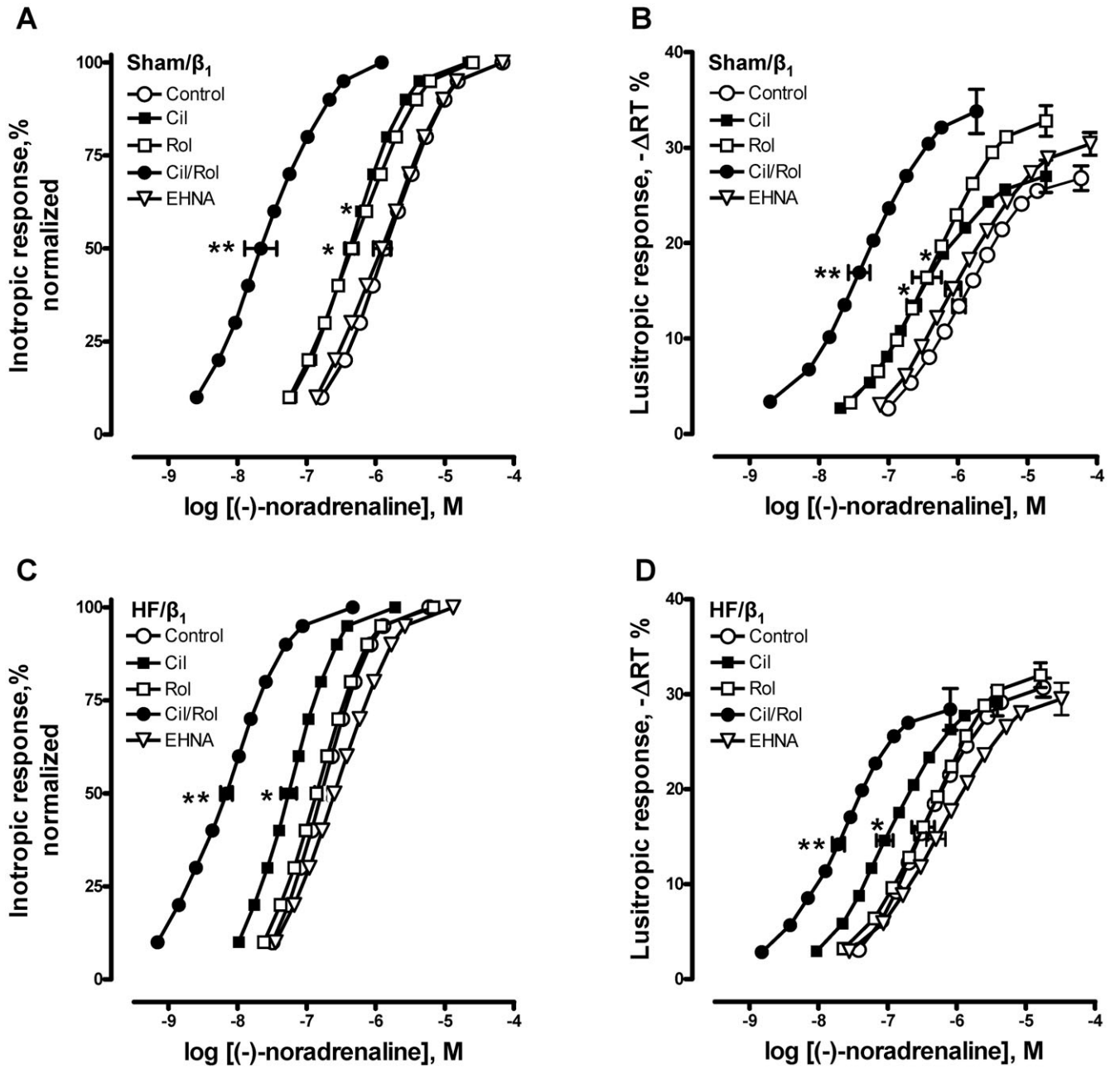

Figure 3

Concentration-response curves of selective β_1-adrenoceptor stimulation in the absence and presence of PDE inhibitors [1 μM cilostamide (Cil), 10 μM rolipram (Rol), the combination of Cil and Rol, 10 μM EHNA; $n = 6–9$] in Sham (A,B) and heart failure (HF) (C,D) strips. Abscissae: Logarithm of (-)-noradrenaline concentration (50 nM ICI118551 present). Horizontal bars: SEM of –log EC_{50}. Ordinate (A,C): Inotropic response [increase in $(dF/dt)_{max}$] in percent of maximum in each experiment. Ordinate (B,D): Lusitropic response as change in relaxation time (-RT) in %. Vertical bars: SEM of lusitropic response. *$P < 0.05$ versus control, for log EC_{50}; **$P < 0.05$ versus Cil, for log EC_{50}.

(Figure 3A and B). PDE3 or PDE4 inhibition did not increase the maximal β_1-adrenoceptor-mediated PIR, but increased the potency of (-)-noradrenaline. The maximal β_1 adrenoceptor-mediated LR was also unchanged during separate PDE3 and PDE4 inhibition. While PDE3 inhibition significantly increased the potency of (-)-noradrenaline, PDE4 inhibition only nominally increased the potency ($P = 0.09$). Combined PDE3 and PDE4 inhibition further increased the potency of (-)-noradrenaline for both the PIR and the LR. In summary, in Sham tissues, the functional responses mediated by both β_2- and β_1-adrenoceptors are regulated by both PDE3 and PDE4.

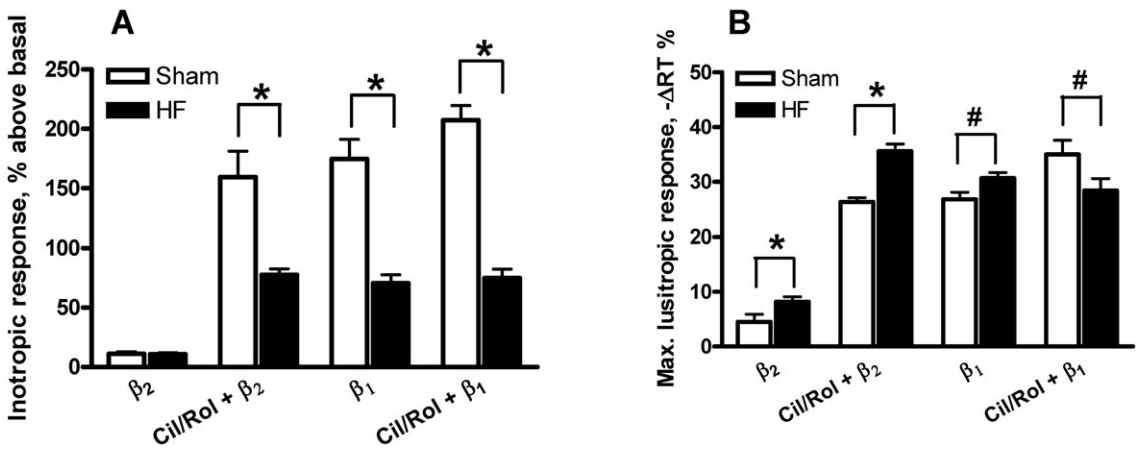

Figure 4

Maximal inotropic responses as increase in $(dF/dt)_{max}$ in percent above basal (A), and maximal lusitropic response as change in relaxation time (–ΔRT) in % (B), upon selective stimulation of β_2-adrenoceptors and β_1-adrenoceptors, respectively, in Sham and heart failure (HF) strips in the absence and presence of dual PDE3 and PDE4 inhibition (cilostamide + rolipram; Cil/Rol). Mean ± SEM. *$P < 0.05$; #Non-significant.

In tissues from HF rats, β_2-adrenoceptor-mediated PIR and LR are primarily suppressed by PDE3

Similar experiments were conducted in HF strips (Figure 2C and D). In the absence of PDE inhibitors, the maximal β_2-adrenoceptor-mediated PIR and LR in HF were not different from the responses in Sham. Table 2 lists the number of experiments (n) in each group, basal (F_{max}B, mN/mm^2), maximal (F_{max}M, mN/mm^2) contractile force to (-)-noradrenaline and –log EC_{50} values in terms of F_{max} for each intervention with PDE inhibitors.

In HF strips, PDE2 inhibition by 10 μM EHNA had no effect on the β_2-adrenoceptor-mediated PIR and LR. Cil enhanced both the maximal β_2 adrenoceptor-mediated PIR and LR compared with control. In contrast to Sham hearts, Rol had no effect on the β_2-adrenoceptor-mediated PIR or LR. However, when both PDE3 and PDE4 were inhibited, there was a further significant increase in the maximal PIR as well as increase in the sensitivity to (-)-adrenaline compared with the presence of Cil alone. Similarly, the LR was also increased compared with Cil alone. Thus, PDE3 inhibition unmasked a secondary role of PDE4 in suppressing β_2-adrenoceptor-mediated contractile responses in HF.

During dual PDE inhibition, the β_2-adrenoceptor-mediated PIR in HF was considerably lower than in Sham, as also seen for β_1-adrenoceptor-mediated PIR. However, the maximal β_2-adrenoceptor-mediated LR during dual PDE inhibition was modestly increased in HF compared with Sham, indicating that the β_2-adrenoceptor-mediated PIR in HF is potentially downregulated in accordance with β_1-adrenoceptor-mediated PIR (Figure 4) while the LR is preserved.

For comparison, we studied how the PDEs were regulating β_1-adrenoceptor-mediated PIR and LR in HF and specifically if the effect of PDE4 inhibition was lacking (Figure 3C and D), as for β_2-adrenoceptor-mediated responses. Consistent with the β_2-adrenoceptor-mediated effects in HF, PDE4 inhibition did not increase the sensitivity to β_1-adrenoceptor-mediated PIR or LR. In the presence of PDE3 inhibition, the potency of (-)-noradrenaline was increased regarding both PIR and LR with similar maximal response. Combined PDE3 and PDE4 inhibition further increased the potency of (-)-noradrenaline but not the maximal response. The maximal PIR mediated by selective β_1-adrenoceptor stimulation was decreased in HF compared with Sham (Figure 4A), suggesting a down-regulation in heart failure. The β_1-adrenoceptor-mediated maximal LR, however, was preserved in HF and Sham (Figure 4B), as also seen for the β_2-adrenoceptor-mediated LR.

Decreased total PDE activity and PDE4 activity in HF rats

We measured and compared total cardiac cAMP phosphodiesterase activities in HF and Sham rats and used Cil and Rol to address the fractions of the total activity that were due to PDE3 (Cil-sensitive) and PDE4 (Rol-sensitive). In both HF and Sham rats, PDE3 and PDE4 provided about 90% of total cAMP-PDE activity in the left ventricle, with the activity of PDE4 being approximately twice that of PDE3. The total cAMP PDE activity was decreased by 22 ± 4% ($P < 0.05$, Figure 5) in HF compared with Sham, mainly represented by a decrease in PDE4 activity (18 ± 5%, $P < 0.05$, Figure 5). This reduction in PDE4 activity might hypothetically occur in a

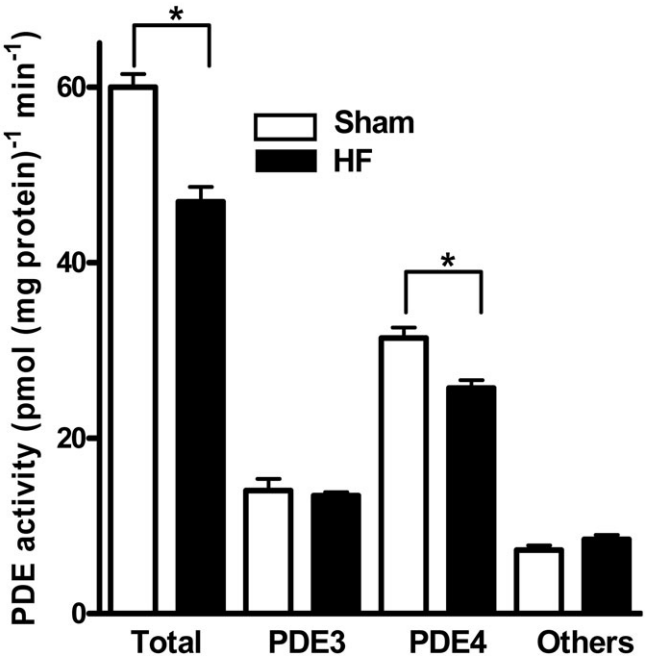

Figure 5

cAMP PDE activity in left ventricle homogenate from Sham and heart failure (HF) hearts. PDE3 and PDE4 activity was determined as the fraction of total PDE activity inhibited by cilostamide (1 μM) and rolipram (10 μM) respectively. Ordinate: PDE activity as cAMP degraded (pmol mg/protein/min). Mean ± SEM. (Sham: $n = 4$, HF: $n = 5$); *$P < 0.05$.

critically localized compartment, and thus might be associated with a reduced contribution of PDE4 in limiting the β_2- and β_1-adrenoceptor-evoked contractile response only, but not necessarily, affecting the overall cAMP response in HF.

Differential PDE modulation of total cAMP and PIR to β-adrenoceptor stimulation

We further investigated how PDE inhibition influenced total cAMP generated during β_2-adrenoceptor stimulation in Sham and HF strips (Figure 6) and how the enhancement of cAMP increase corresponded with the PIRs (Figure 7). Strips stimulated with agonist in the absence or presence of PDE inhibitors were compared with parallel strips not stimulated with agonist. Strips used for PIR measurement were snap-frozen for later measurement of total cAMP.

PDE3 inhibition amplified the β_2-adrenoceptor-mediated PIR without enhancing the cAMP increase in both Sham and HF. Even though PDE3 inhibition by Cil markedly increased the β_2-adrenoceptor-mediated PIR, it did not change the modest β_2-adrenoceptor-mediated increase in cAMP levels in Sham or HF strips (Figures 6A, B and 7A, B). Thus, PDE3 inhibition amplified the β_2 adrenoceptor-mediated PIR without enhancing the total cAMP increase.

PDE4 inhibition enhanced the β_2-adrenoceptor-mediated cAMP increase and PIR in Sham, but failed to increase PIR in HF. In Sham, PDE4 inhibition by Rol enhanced both the β_2-adrenoceptor-mediated increase in cAMP levels (borderline significance) (Figure 6A) and the PIR (to levels similar to those of PDE3 inhibition) (Figure 7A). However, PDE4 inhibition, while enhancing the β_2-adrenoceptor-mediated cAMP increase as in Sham (Figure 6B), did not increase the β_2-adrenoceptor-mediated PIR in HF, thus disclosing a mismatch between the increase in cAMP and the PIR (Figures 6B and 7B).

During concomitant PDE3 and PDE4 inhibitions, β_2-adrenoceptor stimulation substantially increased the cAMP levels (Figure 6A and B) and evoked a PIR of 86 ± 10% and 51 ± 15%, in Sham and HF respectively.

PDE4 inhibition enhanced the β_1-adrenoceptor-mediated cAMP increase in both Sham and HF, but failed to sensitize the PIR in HF. For comparison, we determined how selective PDE inhibition influenced the effect of β_1-adrenoceptor stimulation on total cAMP levels in ventricular strips and related it to the effect on the PIR. As the same concentration of (-)-noradrenaline (1 μM) elicits a maximal PIR, both without and with PDE inhibitors, the increase in total cAMP levels was plotted against the sensitivity of the strips (–log EC_{50}) to (-)-noradrenaline.

In Sham, while PDE4 inhibition (Rol) both enhanced the β_1-adrenoceptor-mediated increase in cAMP levels (Figures 6C and 7C) and potentiated the β_1-adrenoceptor-mediated PIR (Figure 7C), PDE3 inhibition (Cil) sensitized the PIR (Figure 7C) without enhancing the cAMP increase (Figures 6C and 7C). However, in HF only PDE3 inhibition potentiated the β_1-adrenoceptor-mediated PIR (Figure 7D) despite a substantial enhancement of β_1-adrenoceptor-mediated increase in cAMP during PDE4 inhibition alone and PDE3 inhibition alone (Figures 6D and 7D). Thus, the amplified cAMP increase during PDE3 inhibition matched the potentiation of the β_1-adrenoceptor-mediated PIR, whereas during PDE4 inhibition the markedly enhanced cAMP increase without an effect on the β_1-adrenoceptor-mediated PIR again disclosed a mismatch between the two responses, as also seen with β_2-adrenoceptor stimulation in HF.

Thus, these results suggest that PDE3 predominantly regulates apparently confined pools of cAMP,

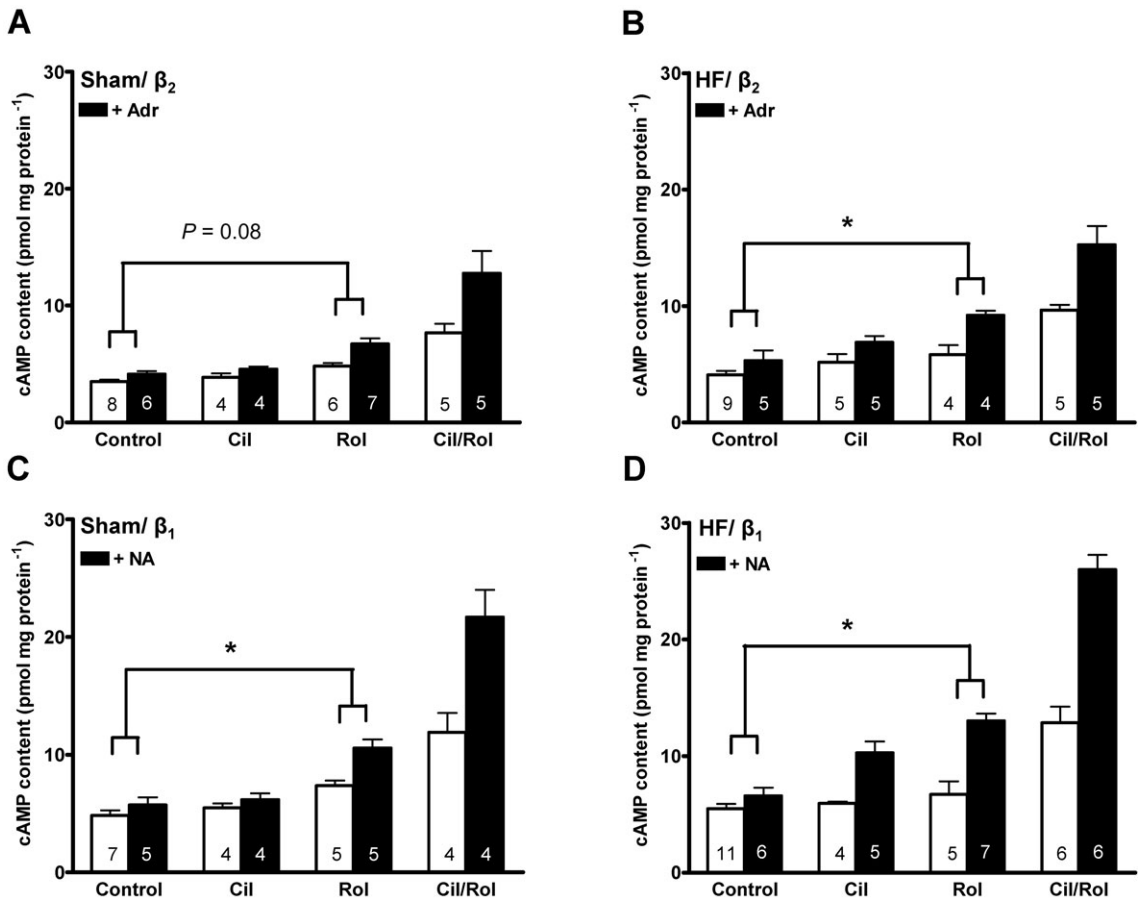

Figure 6

cAMP levels in Sham (A, C) and heart failure (HF) (B, D) strips without (open bars) and 2 min after selective stimulation of β_2-adrenoceptors (with adrenaline, +Adr) and β_1-adrenoceptors (with noradrenaline, +NA) in the absence and presence of PDE inhibitors [1 μM cilostamide (Cil), 10 μM rolipram (Rol), and the combination of Cil and Rol]. Numbers of experiments are shown in the bars. Mean ± SEM.*$P < 0.05$ for increase in cAMP levels in each group.

relevant to contractile response, whereas PDE4 regulates more global cAMP pools that are not necessarily related to contractile responses.

Discussion

The study shows that the stimulation of cardiac β_2-adrenoceptors mediates a PIR as well as an LR which are suppressed by PDEs in Sham and failing hearts. While both PDE3 and PDE4 regulate the PIR and LR in Sham hearts, only PDE3 is the primary regulator of both these responses in HF. This may be explained by a possible elimination of a localized PDE4 pool comprising only a small part of the total PDE4 activity. Combined PDE3 and PDE4 inhibition increases the maximal responses to β_2-adrenoceptor stimulation to become similar to those of β_1-adrenoceptor stimulation. The receptor-mediated increases in total cAMP levels in the presence of selective PDE inhibition did not exhibit a uniform correlation with alterations in contractility, reflecting compartmentalization of distinct functional pools of cAMP.

Suppression of functional responses by PDEs

In non-failing myocardium, the β_2-adrenoceptor-mediated PIR was small compared with the PIR mediated by β_1-adrenoceptor stimulation. There was, however, a substantial increase in the PIR to β_2-adrenoceptor stimulation during either PDE3 or PDE4 inhibition, demonstrating that both PDE isoenzymes are modulating the PIR. The increase in β_2-adrenoceptor-mediated PIR during PDE3 inhibition was recently also reported by Christ *et al.* (2009). When both PDE3 and PDE4 were simultaneously inhibited, the β_2-adrenoceptor-mediated PIR further increased to a maximum comparable with the maximal β_1-adrenoceptor-mediated PIR, as also reported by Christ *et al.* (2009). As PDE3 or

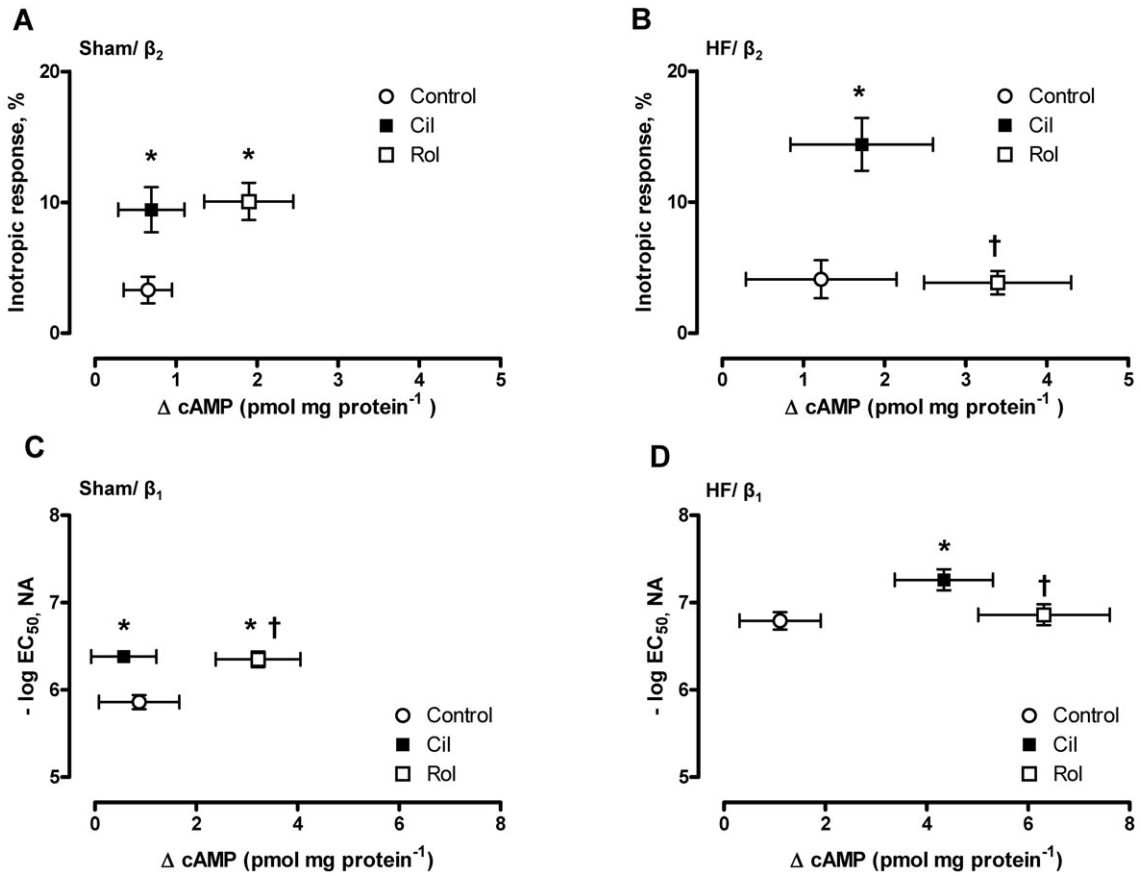

Figure 7

Increase in cAMP levels upon selective stimulation of β_2-adrenoceptors and β_1-adrenoceptors versus functional effects. Abscissae: Increase in cAMP levels (ΔcAMP pmol mg/protein) in strips compared to their respective controls, in the absence and presence of PDE inhibitors [cilostamide (Cil); rolipram (Rol)], upon selective stimulation of β_2-adrenoceptors in Sham (A) and heart failure (HF) (B) and of β_1-adrenoceptors (with noradrenaline, NA) in Sham (C) and HF (D). Horizontal bars: SEM of Δ cAMP. (A,B) Ordinate: Inotropic effect as increase in $(dF/dt)_{max}$ in % above basal 2 min after stimulation with agonist in the same strips. (C,D) Ordinate: –log EC_{50} the agonist concentration (in M; potency) in concentration–response curve experiments. Vertical bars: SEM for inotropic response or –log EC_{50}. *$P < 0.05$ versus controls, for inotropic response or –log EC_{50}; † $P < 0.05$ versus controls, for ΔcAMP.

PDE4 inhibition alone only increased the efficacy with a modest or no increase in the potency of the agonist, the stimulation of β_2-adrenoceptors alone only partially activates the downstream signalling. This is in contrast to β_1-adrenoceptor stimulation which can fully activate the downstream signalling as either PDE3 or PDE4 inhibition only increased the potency of the agonist to elicit the PIR with no change in efficacy. During combined PDE3 and PDE4 inhibition, however, stimulation of β_2-adrenoceptors revealed an approximately full activation of the cAMP/PKA signalling systems as there were increases in both efficacy and potency of the agonist. Thus, PDE3 and PDE4 play prominent roles in the functional limitation of the cardiac β_2-adrenoceptor signals. In the present study, the β_1-adrenoceptor-mediated PIR was also found to be regulated by both PDE3 and PDE4, which is different from the findings by others that only PDE4 played such a role in non-failing mouse and rat right ventricle (Vargas *et al.* 2006; Galindo-Tovar and Kaumann, 2008) and left ventricle (Christ *et al.*, 2009). Interestingly, another study reported an increase in the inotropic potency of isoprenaline in non-failing rat right ventricle during PDE4 inhibition but not during PDE3 inhibition by 10 μM milrinone (Katano and Endoh, 1992), a concentration which inhibits approximately 97% of PDE3 (Shakur *et al.*, 2002). In contrast, 1 μM Cil which inhibits approximately 96% of PDE3 (Shakur *et al.*, 2002), increased the inotropic sensitivity of (-)-noradrenaline in the present study. However, PDE3 inhibition by 10 μM milrinone increased the inotropic potency of isoprenaline in rabbit hearts (Katano and Endoh, 1992) and sensitized both β_1- and β_2-adrenoceptor-mediated PIR in human atrial

myocardium (Carceles *et al.*, 2007). The sensitization of β_1- and β_2-adrenoceptor-mediated PIR in human atrium was also reported during PDE3 inhibition by Cil but not during PDE4 inhibition by Rol (Christ *et al.*, 2006). However, 300 nM Cil sensitized the β_2-adrenoceptor-mediated PIR to a greater extent compared with the β_1-adrenoceptor-mediated PIR. These differences may reflect possible different effects of PDE3 inhibitors in different species or be related to different experimental conditions (discussed next). In human atrium, 300 nM Cil increased the sensitivity of β_1-adrenoceptor-mediated PIR by approximately 0.5 log units (Christ *et al.*, 2006) whereas in the present study, 1 μM Cil sensitized the β_1-adrenoceptor-mediated PIR to a similar extent and 300 nM sensitized to a lesser extent (Figure 1B), suggesting distinct sensitivities of human and rat PDE3 to Cil. PDE2 was reported to blunt the PIR to β_1-AR stimulation (Mongillo *et al.*, 2006). However, our data do not support a role of PDE2 in the regulation of β_1- or β_2-adrenoceptor-mediated PIR.

We demonstrated that the stimulation of β_2-adrenoceptors mediated a small LR in rat ventricles, which is limited by PDEs as inhibition of PDE3 or PDE4 enhanced the LR. Combined PDE3 and PDE4 inhibition further augmented the LR substantially to a level similar to the β_1-adrenoceptor-mediated LR. Thus, the β_2-adrenoceptor-coupled signalling systems mediating LR is functionally limited by PDEs. Previous reports have claimed that cardiac β_2-adrenoceptor-mediated LR was absent in most mammalian species such as rats and mice (Xiao *et al.*, 1999). Methodological limitations might explain the discrepancy, as the β_2-adrenoceptor-mediated LR in the absence of PDE inhibition is small and needs accurate measurement techniques to be detected. Our method was based on computerized averaged time courses of 20–30 normalized contraction–relaxation cycles. It is also reported that the LR to β_2-adrenoceptors is compartmentalized by G_i-dependent signalling (Kuschel *et al.* 1999; Jo *et al.*, 2002). This may contribute to limit LR in addition to the PDEs. However, these studies, performed mostly on rodents, are not in line with the similar ventricular lusitropic results from human failing hearts (Kaumann *et al.*, 1999) and non-failing hearts (Molenaar *et al.*, 2000), showing robust lusitropic effects and potencies through activation of both β_2- and β_1-adrenoceptors, reflecting possible differences in β_2-adrenoceptor-mediated LR between rodent and human hearts.

A recent study reported on effects of PDE3 and PDE4 inhibition on β_1- and β_2-adrenoceptor-mediated responses in non-failing rat hearts (Christ *et al.*, 2009). Despite a large number of similarities, there are some discrepancies between their and our results which are discussed next. In accordance with our results, β_2-adrenoceptor-mediated responses were regulated by both PDE3 and PDE4 in healthy rat left and right ventricle (Christ *et al.*, 2009). However, the authors detected only an enhancement of β_2-adrenoceptor-mediated LR, but not of PIR upon PDE4 inhibition. Moreover, they did not detect any effect of PDE3 inhibition on β_1-adrenoceptor-mediated PIR. A reason for this discrepancy between their and our results might be that the lower concentrations of PDE inhibitors (0.3 μM Cil and 1 μM Rol) did not reveal effects large enough to be detected by the methods used. We found that although both 1 μM and 10 μM Rol enhances β_2-adrenoceptor-mediated PIR (reversible by ICI118551) in Sham ventricular strips, the observed variation between the hearts indicated that 10 μM Rol was apparently a better choice. We also found that Cil sensitised the β_1 adrenoceptor-mediated responses as Cil elicited PIR in a concentration-dependent manner in the presence of 0.1 μM (-)-noradrenaline and this PIR was reversed by timolol (Figure 1A). Figure 1B indicates that 300 nM (–log M = 6.5) Cil also inhibits PDE3 sufficiently to sensitize the β_1 adrenoceptor-mediated responses but to a lesser extent than 1 μM. Moreover, a plateau for maximal and selective PDE3 inhibition, which is of functional relevance, is reached around 2 μM (–log M = 5.7) to 5 μM (–log M = 5.3) Cil. The biphasic effect of Cil on PIR is most likely caused by PDE4 inhibition revealing non-selectivity at higher concentrations such as 50 μM (–log M = 4.3).

β_1- and β_2-adrenoceptor responsiveness in HF

The receptor density of β_1-adrenoceptors and their functional effects are known to be down-regulated in HF (Kompa *et al.*, 1999). Accordingly, we also show that the responsiveness to β_1-adrenoceptors is reduced in HF compared with Sham (Figure 4A). However, there is an increase in the sensitivity of β_1-adrenoceptor-mediated PIR in HF compared with Sham (Figures 3C and 7D) which is in accordance with our previous reports using both (-)-noradrenaline and isoprenaline as β-adrenoceptor agonists (Sjaastad *et al.*, 2003; Qvigstad *et al.*, 2005). A possible explanation for this leftward shift of the concentration–response curve might be the reduced activity of PDE4 in HF compared with Sham which is consistent with the observation that PDE4 inhibition does not further sensitize the β_1-adrenoceptor-mediated PIR in HF. It was reported that the neuronal transporter for noradrenaline is down-regulated in failing hearts (Backs *et al.*, 2001). However, downregulation of the transporter may

at best contribute to this sensitization of β_1-adrenoceptor-mediated PIR in HF only for (-)-noradrenaline, as isoprenaline is not a substrate for the neuronal transporter. Moreover, the increased sensitivity of β_1-adrenoceptor-mediated PIR by (-)-noradrenaline in HF compared with Sham is also observed during inhibition of neuronal transporter in nerve terminals by cocaine (E. Qvigstad *et al.*, unpubl. data). Interestingly and unexpectedly, the sensitivity of β_2-adrenoceptor-mediated PIR in HF compared with Sham appears to be enhanced although to a lesser extent than PIR to β_1-adrenoceptors (Table 2). Mechanisms similar to those discussed for β_1-adrenoceptors might be responsible for these findings but an influence of reduced neuronal transporters in HF should at least be smaller because adrenaline (Adr) is a poor substrate for this uptake mechanism.

Although the PIRs obtained by β_2-adrenoceptor stimulation in the absence of PDE inhibition were similar in failing and non-failing hearts, the maximal PIR evoked by β_2-adrenoceptor stimulation during combined PDE3 and PDE4 inhibition was attenuated in failing hearts, comparable with that of β_1-adrenoceptor stimulation. It has been reported that the density of β_2-adrenoceptors is preserved or increased in rat, rabbit and human failing hearts (Bristow *et al.*, 1986; Kompa *et al.*, 1999; Desantiago *et al*. 2008), and their functional effects are reported to be enhanced (Altschuld *et al.*, 1995; Desantiago *et al*. 2008). In accordance with the down-regulated β_1-adrenoceptor-mediated PIR, the attenuated responsiveness to β_2-adrenoceptor stimulation during combined PDE3 and PDE4 inhibition in failing hearts compared with Sham hearts might indicate a down-regulation of potential β_2-adrenoceptor signalling in failing rat myocardium in accordance with findings from some earlier studies on human heart failure (Brodde *et al.*, 1995). Interestingly, following the same logic, it could be expected that the β_2-adrenoceptor-mediated PIR would be lower in HF compared with Sham under normal conditions, that is, in the absence of PDE inhibition, but according to our results it is in fact similar in both Sham and HF as mentioned previously. This apparent discrepancy might at least partly be explained by the reduced activity of PDE4 in HF compared with Sham as any further PDE4 inhibition does not increase the β_2-adrenoceptor-mediated PIR in HF.

In contrast to the maximal PIRs, the LRs to both β_1- and β_2-adrenoceptor stimulation were not attenuated in heart failure. The β_1-adrenoceptor-mediated maximal LR might seem to be slightly increased in HF compared with Sham whereas during combined PDE3 and PDE4 inhibition it might appear to be somewhat decreased in HF compared with Sham. However, these apparent changes were not statistically significant (Figure 4B). Thus, as a whole the β_1-adrenoceptor-mediated maximal LR was preserved in HF compared with Sham. This corresponds essentially to findings in our previous reports (Sjaastad *et al.*, 2003; Qvigstad *et al.*, 2005) that the LR to non-selective β-adrenoceptor stimulation by isoprenaline was unchanged in post-infarction HF compared with Sham. The β_2-adrenoceptor-mediated LR was significantly increased in HF compared with Sham, both in the absence and in the presence of Cil/Rol. These findings were novel and unexpected. Thus, the regulatory processes related to β-adrenoceptor-mediated PIR and LR, respectively, are obviously differentially adapted to HF. As discussed previously (Sjaastad *et al.*, 2003), this may reflect 'differential changes of phosphorylation of regulatory proteins and/or differential consequences of such changes' in relation to PIR and LR respectively. The details, however, remain to be investigated.

Differential effects of PDE3 and PDE4 indicate various cAMP compartments

PDE4 plays a secondary role in regulating β_2- and β_1-adrenoceptor-mediated PIR and LR in failing hearts as its role was only visible when the primary regulator PDE3 was inhibited. This is in accordance with our previous finding that 5-HT_4 receptor-mediated PIR in post-infarction rats is regulated by PDE4 only during PDE3 inhibition (Afzal *et al.*, 2008). In non-failing hearts, the 5-HT_4 receptor-mediated PIR is only visible when PDE3 and PDE4 are inhibited simultaneously (Afzal *et al.*, 2008), which might be related to the moderate reduction in failing myocardium as discussed next. PDE4 inhibition increases the elevation of cAMP considerably upon β_2- and β_1-adrenoceptor activation in both Sham and HF. Despite the fact that the increases in cAMP elevation in HF are at least as large in Sham, PDE4 inhibition has no effect on the PIR in HF compared with Sham. In contrast to PDE4 inhibition, during PDE3 inhibition the increases in cAMP elevation upon activation of β-adrenoceptors are not detected except for β_1-adrenoceptor activation in HF where cAMP levels were significantly increased compared with control (Figure 7D). However, PDE3 inhibition significantly amplifies the β-adrenoceptor-mediated PIR under all conditions. These mismatches between cAMP levels and functional responses most likely reflect compartmentation of cAMP, with functionally relevant pools regulating contractility. Similar observations concerning small compartmentalized cAMP pools regulated by PDE3 were also reported by Christ *et al.*

(2009), who showed that only PDE3 inhibition, and not PDE4 inhibition, uncovered a small but significant increase in $I_{Ca\text{-}L}$ during activation of β_2-adrenoceptors which correlated with inotropic potentiation of the effects of (-)-adrenaline. Recent studies have shown that β-adrenoceptor-mediated increase in cAMP is mostly regulated by PDE4 and to a lesser extent by PDE3 when measured as increase in cAMP by fluorescence resonance energy transfer (FRET)-based sensors but without contractility as an end-effect (Mongillo *et al*. 2004; Leroy *et al*., 2008). Our data on cAMP levels measured by RIA are in line with these studies as PDE4 inhibition markedly increased the cAMP elevation indicating a more global cAMP increase while PDE3 inhibition only modestly increased cAMP. The latter is most likely to reflect confined cAMP pools responsible for the contractile effects. The further increases in the cAMP elevation and their end-effects during concomitant PDE3 and PDE4 inhibition illustrate a potential dual PDE-regulation of β_1- and β_2-adrenoceptor-mediated signalling. Although FRET-based techniques are increasingly used to illustrate compartmentalization of cAMP-mediated signalling, the utility of such techniques depends on the intracellular localization of the cAMP sensor. Because there is so far no known cAMP sensor which reflects the inotropic or lusitropic effects, studies like the present one would be better able to reflect the functional compartmentalization of the cAMP signal.

The reduced PDE4 activity and functional role in failing myocardium are most likely to reflect a specific PDE4 subtype located in a compartment regulating the contractility in non-failing myocardium. The reported association between reduced PDE4D activity in the ryanodine-receptor complex and heart failure (Lehnart *et al*., 2005) should be considered in this context. The subtype of PDE4 that is affected and contributes to the reduced activity remains to be identified. Our data can also be interpreted as some redistribution of a particular subpopulation of PDE4 in failing cardiac tissue. Our data show a preserved activity of PDE3 in contrast to PDE4, which is in contrast to some studies indicating a reduced expression or activity of PDE3 in failing canine, dog, mouse and human hearts (Smith *et al*., 1997; 1998; Ding *et al*., 2005). The differences observed might be attributable to different species or aetiology of heart failure.

Involvement of β_2-adrenoceptors in HF

The selective down-regulation of β_1-adrenoceptors and the preservation of β_2-adrenoceptors are believed to increase the relative importance of the latter in both human and rodent heart failure (Pavoine and Defer, 2005). Cardiac β_2-adrenoceptors in rodents are capable of coupling to both G_s and G_i (Xiao *et al*., 2006). The β_2-adrenoceptor–G_i signalling, which is reported to be increased in failing rodent hearts (Pavoine and Defer, 2005), is proposed to be cardio-protective as it reduces apoptosis and hypertrophy, and improves contractility in rodents (Xiao *et al*., 2006). Beneficial effects of selective β_2-adrenoceptor stimulation during β_1-adrenoceptor blockade have been proposed as a therapeutic approach in treatment of HF based on experimental studies (Ahmet *et al*., 2008). On the other hand, some recent studies have failed to reproduce the proposed coupling of β_2-ARs to G_i in rodents (Christ *et al*., 2009; LaFlemme and Becker, 1998). Moreover, in human myocardium the functional responses mediated by β_2-adrenoceptors are consistent with coupling of β_2-adrenoceptors to G_s and not G_i (Kaumann *et al*., 1999; Molenaar *et al*., 2000; 2007). Additionally, it is also reported that Adr preferentially activates G_s rather than G_i through activation of human β_2 -adrenoceptors as approximately 1000 times higher concentrations of Adr are required to activate G_i compared with G_s through human β_2-adrenoceptors, and noradrenaline does not activate G_i at all (Heubach *et al*., 2004). Nevertheless, the activation of G_s-dependent mechanisms elevating cAMP is injurious to hearts as they increase energy expenditure and cause cardiac tissue damage (Lohse *et al*., 2003). The activation of β_2-adrenoceptors is arrhythmogenic in failing canine and human hearts (Martin *et al*. 1998; Desantiago *et al*., 2008). During PDE inhibition, there is apparently an increase of G_s signalling from the β_2-adrenoceptors relative to G_i signalling. Our data show that PDE3 becomes the key PDE regulating contractile responses to β-adrenoceptor stimulation in rat HF, thus resembling human hearts (Movsesian *et al*., 1991). Moreover, a recent study reported that in human atrium only PDE3 inhibition, and not PDE4 inhibition, enhances both β_1- and β_2-adrenoceptor-mediated PIR (Christ *et al*., 2006), which is consistent with our data in failing myocardium of rats. However, a substantial additional contribution of PDE4 was unmasked by concomitant inhibition of PDE3 in failing rat hearts. This additional contribution of PDE4 resembles that in non-failing hearts, as also supported by others (Christ *et al*., 2009). PDE activity is suggested to be reduced in HF (Movsesian *et al*. 1991; Smith *et al*., 1997; Lehnart *et al*., 2005), and the treatment of human HF with PDE3 inhibitors has shown deleterious effects (Packer *et al*., 1991; Cuffe *et al*., 2002; Felker *et al*. 2003). Our results indicate that during PDE3 inhibition, β_2-adrenoceptors play a considerable role

in regulating the G_s-mediated contractility in failing hearts, thus contributing to the injurious effect of β-adrenoceptor stimulation in the myocardium.

Conclusion

The stimulation of myocardial β_2-adrenoceptors mediated a PIR as well as an LR, both of which were suppressed by PDE3 and PDE4. In non-failing myocardium, PDE3 and PDE4 suppressed the β_2-adrenoceptor-mediated functional responses to a similar extent. In failing myocardium, only PDE3 primarily limited the responses. However, PDE3 inhibition unmasked a secondary role of PDE4. Dual PDE3/4 inhibition augmented the β_2-adrenoceptor-mediated responses to equal the β_1-adrenoceptor-mediated functional responses. PDE3 inhibition revealed functionally important cAMP pools, an increase of which could scarcely be detected by cAMP measurements, at least for β_2-adrenoceptor stimulation. One such pool might be located close to the L-type calcium channel domain (Christ *et al.*, 2009). PDE4 inhibition in failing myocardium unmasked an additional increase of cAMP that was not associated with increased functional responses to β_2- or β_1-adrenoceptor stimulation. Thus, PDE3 and PDE4 to some extent regulated separate cAMP pools. During PDE inhibition, and especially in HF, the myocardial β_2-adrenoceptors play a significant functional role.

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Conflicts of interest

None.

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