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RESEARCH PAPER

5-HT$_4$-elicited positive inotropic response is mediated by cAMP and regulated by PDE3 in failing rat and human cardiac ventricles

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Background and purpose: The left ventricle in failing hearts becomes sensitive to 5-HT parallelled by appearance of functional G_s-coupled 5-HT$_4$ receptors. Here, we have explored the regulatory functions of phosphodiesterases in the 5-HT$_4$ receptor-mediated functional effects in ventricular muscle from failing rat and human heart.
Experimental approach: Extensive myocardial infarctions were induced by coronary artery ligation in Wistar rats. Contractility was measured in left ventricular papillary muscles of rat, 6 weeks after surgery and in left ventricular trabeculae from explanted human hearts. cAMP was quantified by RIA.
Key results: In papillary muscles from postinfarction rat hearts, 5-HT$_4$ stimulation exerted positive inotropic and lusitropic effects and increased cAMP. The inotropic effect was increased by non-selective PDE inhibition (IBMX, 10 μM) and selective inhibition of PDE3 (cilostamide, 1 μM), but not of PDE2 (EHNA, 10 μM) or PDE4 (rolipram, 10 μM). Combined PDE3 and PDE4 inhibition enhanced inotropic responses beyond the effect of PDE3 inhibition alone, increased the sensitivity to 5-HT, and also revealed an inotropic response in control (sham-operated) rat ventricle. Lusitropic effects were increased only during combined PDE inhibition. In failing human ventricle, the 5-HT$_4$ receptor-mediated positive inotropic response was regulated by PDEs in a manner similar to that in postinfarction rat hearts.
Conclusions and implications: 5-HT$_4$ receptor-mediated positive inotropic responses in failing rat ventricle were cAMP-dependent. PDE3 was the main PDE regulating this response and involvement of PDE4 was disclosed by concomitant inhibition of PDE3 in both postinfarction rat and failing human hearts. 5-HT, PDE3 and PDE4 may have pathophysiological functions in heart failure.
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Keywords: phosphodiesterase; cAMP; heart failure; serotonin; 5-HT$_4$ receptor; 5-HT

Abbreviations: EHNA, erythro-9-(2-hydroxy-3-nonyl) adenine; IBMX, 3-isobutyl-1-methyl-xanthine; PDE, phosphodiesterase

Introduction

Functional 5-HT$_4$ receptors and increased mRNA levels were recently discovered in chronic failing human and rat ventricle (Brattelid *et al.*, 2004; Qvigstad *et al.*, 2005a). In acute failing rat ventricle, increased expression of 5-HT$_{2A}$ receptors was also detected, as well as contributions of both 5-HT$_{2A}$ and 5-HT$_4$ receptors to the inotropic response to 5-HT (Qvigstad *et al.*, 2005b). Rat ventricular 5-HT$_4$ mRNA and 5-HT$_4$-mediated inotropic effects are increased with increasing severity of heart failure regardless of the aetiology (Brattelid *et al.*, 2007). Chronic stimulation of 5-HT$_4$ receptors during heart failure might be hazardous as treatment with a 5-HT$_4$ receptor antagonist yielded potentially beneficial effects in rats with heart failure (Birkeland *et al.*, 2007a). Collectively, these reports underline a possible role of 5-HT$_4$ receptors in the pathophysiology of heart failure.

Phosphodiesterases (PDEs) degrade cAMP and modulate cAMP-dependent signalling. PDE inhibition potentiates cAMP-dependent inotropic responses in line with classical criteria for involvement of cAMP in any given intracellular signalling cascade (Robison *et al.*, 1971). PDEs provide barriers to cAMP diffusion, giving rise to specific compartments

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or microdomains where PKA-mediated effects are modulated (Beavo and Brunton, 2002; Fischmeister *et al*., 2006). In cardiomyocytes, four PDE isoenzymes have been identified, PDE1, PDE2, PDE3 and PDE4. PDE1 and PDE2 can hydrolyse both cAMP and cGMP. PDE3 has a 10-fold higher capacity to hydrolyse cAMP than cGMP (Lugnier, 2006). PDE4 is strictly cAMP-selective (Lugnier, 2006). Several reports have shown the importance of PDEs in the pathophysiology of heart failure (Smith *et al*., 1997; Takahashi *et al*., 2002; Ding *et al*., 2005; Lehnart *et al*., 2005).

Cardiac β_1- and β_2-adrenoceptors mediate their effects through G_s/AC/cAMP/PKA-dependent pathways but their effects may be differentially sensitive to different PDEs (Christ *et al*., 2006). Recent studies have focused on the involvement of PDEs in the regulation of cAMP levels during the stimulation of β-adrenoceptors in different cardiac models (Jurevicius *et al*., 2003; Mongillo *et al*., 2004, 2006; Christ *et al*., 2006; Rochais *et al*., 2006; Vargas *et al*., 2006; Galindo-Tovar and Kaumann, 2008). The function of PDEs in the regulation of cAMP and inotropic response following 5-HT_4 receptor stimulation in failing hearts is however relatively unknown. In the presence of the non-selective PDE inhibitor 3-isobutyl-1-methyl-xanthine (IBMX), 5-HT_4 receptor-mediated inotropic responses to 5-HT were uncovered in porcine and failing human ventricle (Brattelid *et al*., 2004), and fading of 5-HT_4 receptor-mediated responses was abolished in porcine atrium (De Maeyer *et al*., 2006).

In failing rat ventricle, the 5-HT_4 receptor-mediated inotropic response was of similar magnitude with striking qualitative similarities to the response elicited by β-adrenoceptors, especially regarding time course and accompanying lusitropic effect (Qvigstad *et al*., 2005a). Stimulation of 5-HT_4 receptors has also been shown to increase the L-type Ca^{2+} current and the phosphorylation of phospholamban and troponin I in failing rat cardiomyocytes (Birkeland *et al*., 2007b). However, the increase of cAMP levels following 5-HT_4 receptor stimulation was considerably lower than following β-adrenoceptor stimulation by isoprenaline, despite the quantitatively and qualitatively similar inotropic responses (Qvigstad *et al*., 2005a), possibly reflecting differential compartmentation of cAMP-mediated signalling following stimulation of 5-HT_4 or β-adrenoceptors. In this study, we examined the function of different PDEs in regulating the inotropic and lusitropic responses to 5-HT_4 receptor stimulation in postinfarction rat as well as failing human myocardium. We find that PDE3 is the main PDE isoenzyme regulating 5-HT_4-mediated functional responses in both postinfarction rat and failing human hearts and a regulatory function of PDE4 is uncovered by concomitant inhibition of PDE3.

Materials and methods

Animal model

Animal care was according to the Norwegian Animal Welfare Act, which conforms with the *European Convention for the protection of Vertebrate animals used for Experimental and other Scientific Purposes* (Council of Europe no. 123, Strasbourg 1985). Two animals per cage in a temperature-regulated room on a 12-h/12-h day/night cycle were given access to food and water *ad libitum*. As described earlier (Sjaastad *et al*., 2000), an extensive myocardial infarction was induced in anaesthetized (68% N_2O/29% O_2/1.5–2.5% isoflurane) ~320 g male Wistar rats by proximal ligation of the left coronary artery (MI rats). Six weeks later, the rats were again anaesthetized and intubated, and left ventricular pressures were measured as described previously (Sjaastad *et al*., 2003). Sham-operated animals (Sham) underwent identical surgical procedure without coronary artery ligation. The chest was opened, heart and lungs were excised before the hearts were retrogradely perfused. The left ventricles were cut open, and the posterior papillary muscle was excised. Then the left ventricle was pinned to a plate, and the endocardial surface was digitally photographed and infarct size (% of inner surface) was traced on screen (KS 100, Kontron Electronics, Germany). Animals were included in the study if infarct size was larger than 30% of the left ventricular inner surface.

Isolated papillary muscles

Posterior left ventricular papillary muscles were prepared, mounted in organ baths (31 °C) with a physiological salt solution with a Ca^{2+} concentration of 1.8 mM, equilibrated and field-stimulated at 1 Hz (Sjaastad *et al*., 2003). Lidocaine (10 μM) and ascorbic acid (100 μM) were also present in the baths. Contraction–relaxation cycles were recorded and analysed as described previously (Skomedal *et al*., 1997; Sjaastad *et al*., 2003) with respect to maximal developed force (F_{max}, mN), maximal development of force ($(dF/dt)_{max}$), TPF (time to peak force), time to 80% relaxation (TR80) and relaxation time (RT = TR80-TPF). Inotropic responses were expressed as increase of F_{max} and $(dF/dt)_{max}$ in percentage of control levels. Lusitropic effects were expressed as decrease of RT. Blockers of α_1-adrenoreceptors (prazosin 1 μM), β-adrenoceptors (timolol 1 μM), muscarinic cholinergic receptors (atropine 1 μM) and 5-HT_{2A} receptors (ketanserin 0.1 μM) were added 90 min before the agonists. These drugs did not influence the basal contraction–relaxation cycle characteristics or electrical stimulation threshold (data not shown). PDE inhibitors (IBMX (4.6 μM and 10 μM), erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA; 10 μM), rolipram (10 μM), cilostamide (1 μM) were added, as specified, 45 min before the agonists. 5-HT was added to the organ bath cumulatively to obtain a concentration–response relationship. Concentration–response curves were constructed by estimating centiles (EC_{10}–EC_{100}) and calculating the corresponding means and the horizontal positioning expressed as $-\log EC_{50}$ (Sjaastad *et al*., 2003).

cAMP content in papillary muscles

Papillary muscles were prepared, mounted and equilibrated for 90 min with antagonists as described above and then freeze-clamped following 1-min stimulation with 5-HT (10 μM). Frozen papillary muscles (~10 mg) were homogenized at 4 °C in 1 ml of 5% trichloroacetic acid by a Retsch MM301 mechanical mill and cAMP content was determined by radioimmunoassay (Skomedal *et al*., 1980).

Human trabeculae

Human left ventricular trabeculae were obtained from five explanted hearts from patients with heart failure undergoing heart transplantation at Rikshospitalet University Hospital, Oslo (Ethics approval #S01025). Characteristics of the five patients are shown in Table 1. Trabeculae from the patients were first placed into relaxing solution containing (mM): NaCl (118.3), KCl (3.0), $CaCl_2$ (0.2), $MgSO_4$ (4.0), KH_2PO_4 (2.4), $NaHCO_3$ (24.9), glucose (10.0) and mannitol (2.2) and kept in this solution until the trabeculae were mounted in organ baths to contract (1 Hz, 37 °C) in the same solution except that Ca^{2+} was 2.5 mM and Mg^{2+} 1.2 mM as described (Skomedal *et al*., 1997). F_{max} and $(dF/dt)_{max}$ were recorded as described (Skomedal *et al*., 1997).

Statistics

All results are expressed as mean ± s.e.mean 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

5-HT hydrochloride, (−)isoprenaline hydrochloride, timolol maleate, prazosine hydrochloride, atropine sulphate, 3-isobutyl-1-methylxanthine, lidocaine (2-diethylamino-N-[2,6-dimethylphenyl]-acetamide) hydrochloride and L-ascorbic acid were purchased from Sigma-Aldrich (St Louis, MO, USA). Rolipram, cilostamide, EHNA hydrochloride and ketanserin tartrate were from Tocris Cookson Inc. (Bristol, UK). Isoflurane (1-chloro-2,2,2-trifluoroethyl difluoromethyl ether; Forene) was from Abbot Scandinavia (Solna, Sweden). The drug and receptor nomenclature used here is in accordance with the *BJP*'s Guide to Receptors and Channels (Alexander *et al*., 2008).

Results

Characteristics of MI rats

All rats had large anterolateral infarcts that comprised more than 30% of the inner myocardial surface (MI rats). We have previously demonstrated in hearts with infarct size >30% a 5-HT_4 receptor-mediated inotropic effect of the same magnitude as observed in heart failure (Qvigstad *et al*., 2005a). For characteristics and haemodynamic data, see Table 2.

Characteristics of the mechanical responses to 5-HT$_4$ receptor stimulation in MI rat hearts

5-HT elicited in concentration–response experiments, a maximal positive inotropic response of 16.1 ± 2.3% above control and revealed a $-\log EC_{50}$ value of 7.44 ± 0.05 in MI rats ($n=9$). The lusitropic effect exerted by 5-HT stimulation caused a reduction in RT of 19.4 ± 2.4 ms compared with control (Figure 1, Table 3). In comparison, β-adrenoceptor stimulation by isoprenaline exerted a maximal positive inotropic response of 36.5 ± 4.8% ($n=6$) above control and displayed a $-\log EC_{50}$ value of 8.47 ± 0.07. RT was reduced by 31 ± 3.8 ms (Figure 1). The observed faster relaxation along with the inotropic effect of both 5-HT_4 receptor and β-adrenoceptor stimulation was compatible with a cAMP-dependent signalling pathway.

Effects of phosphodiesterase inhibition on 5-HT$_4$-mediated inotropic and lusitropic responses in MI rat hearts

To determine the function of different PDEs in 5-HT_4 receptor signalling, the papillary muscles were challenged with 5-HT in the presence of both non-selective and selective PDE inhibition (PDE2, PDE3 and PDE4).

Non-selective PDE inhibition. High concentrations of non-selective PDE inhibitors such as IBMX are reported to increase the force of contraction alone without an agonist present (Shahid and Nicholson, 1990). To find a concentration of IBMX that only minimally increases basal force of contraction, to avoid artificial influence on estimation of the inotropic responses mediated by 5-HT_4 receptor and

Table 2 Animal and papillary muscle characteristics

	MI rats (n = 119)
Body wt, g	377 ± 2.4
Heart wt, g	2.2 ± 0.05
Heart wt/body wt, g kg^{-1}	5.8 ± 0.1
LVEDP, mm Hg	14.4 ± 1.8
LVSP, mm Hg	103 ± 2.0
Lung wt, g	2.4 ± 0.1
Basal F_{max}, mN	4.9 ± 0.3
CSA, mm^2	0.74 ± 0.01

Abbreviations: CSA, cross-sectional area of the contracting papillary muscles; F_{max}, maximal developed force; LVEDP, left ventricular end-diastolic pressure; LVSP, left ventricular systolic pressure; MI rats: rats with myocardial infarction larger than 30% of inner surface area. Mean ± s.e.mean.

Table 1 Details of the patients

Patient no.	*Aetiology*	*Age (years)*	*Sex*	*CI (l min^{-1} m^{-2})*	*Drug treatment*
1	CAD	58	Male	2.0	Aa, Ac, ACEI, Alda, Crv, D, Dig, Esp
2	CAD	64	Male	2.0	Ac, ACEI, Alda, Crv, D, Dig, Esp, FA, Fe, Lip
3	Cardiomyopathy	63	Male	1.6	ACEI, AD, Alda, D, Dig, Esp
4	CAD	64	Male	1.9	Ac, ACEI, Alda, Ap, AT1, Crv, D, Dig, In, Mg, N
5	CAD	63	Male	1.9	Aa, Ac, AD, Ap, Crv, D, Dig, K, Lip

Abbreviations: Aa, antiarrhythmic; Ac, anticoagulant; ACEI, ACE inhibitor; AD, antidepressants; Alda, aldosterone antagonist; Ap, allopurinol; AT1, AT receptor antagonist; CAD, coronary artery disease; CI, cardiac index; Crv, carvedilol (α- and β-adrenoceptor antagonist); Dig, digitoxin; D, diuretic; Esp, esomeprazole; FA, folic acid supplement; Fe, iron supplement; Lip, lipid-lowering drug; K, potassium supplement; Mg, magnesium supplement; N, nitrate.

β-adrenoceptor stimulation, we determined the concentration–response relationship of IBMX itself on papillary muscles. IBMX elicited a maximal positive inotropic response of $54.3 \pm 7.1\%$ with a $-\log EC_{50}$ value of 4.56 ± 0.09 ($n=7$) (Figure 2). The RT was decreased by 34.0 ± 2.0 ms ($n=7$). Subsequent addition of isoprenaline did not cause a further inotropic or lusitropic effect suggesting that IBMX at a maximal concentration was sufficient to fully activate the cAMP–PKA pathway (data not shown). The concentrations 4.6 and 10 μM IBMX were chosen for the interaction studies with 5-HT as they have minimal effect on basal contractility; 10 μM IBMX increased the $(dF/dt)_{max}$ by only $5.0 \pm 2.4\%$ ($n=5$).

The maximal inotropic response to 5-HT was increased concentration-dependently in the presence of 4.6 μM IBMX ($n=5$) and 10 μM IBMX ($n=5$) compared with control ($P<0.05$; Figure 3a, Table 3). IBMX also increased the maximal lusitropic effect of 5-HT (Table 3). However, the sensitivity to 5-HT was unaffected by IBMX.

In comparison, 4.6 μM IBMX did not increase the maximal positive inotropic effect of isoprenaline, but increased its potency compared with control ($-\log EC_{50}$: 9.10 ± 0.08, $n=6$ vs 8.47 ± 0.07, $n=6$, $P<0.05$; Figure 3b).

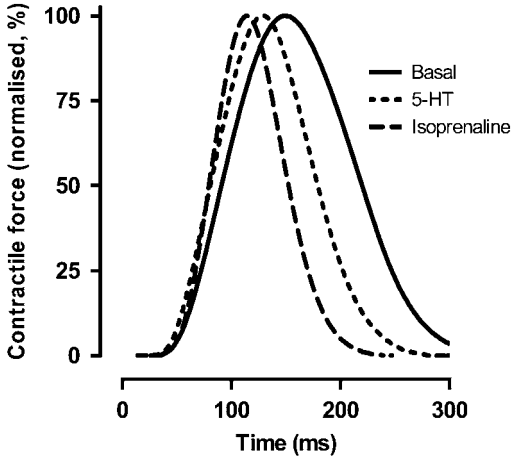

Figure 1 Representative averaged contraction–relaxation cycles in a papillary muscle before the addition of an agonist (basal) and at maximal steady-state inotropic response to 10 μM 5-HT (5-HT) or 10 μM isoprenaline, normalized to maximal force (100%). Abscissa: time in milliseconds after the electrical pulse triggering contraction. Ordinate: contractile force, normalized to maximum as 100%.

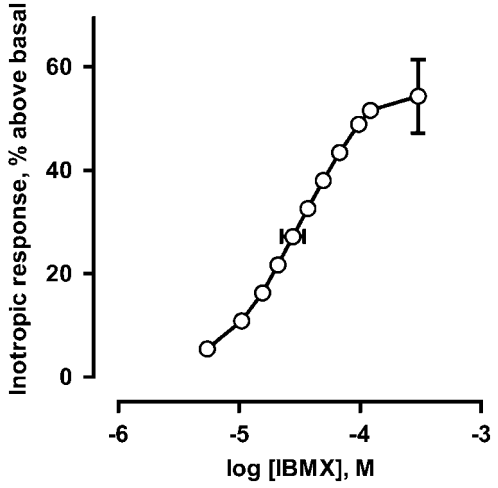

Figure 2 Concentration–response curve of IBMX. IBMX elicited a maximal positive inotropic response of $54.3 \pm 7.1\%$ above basal with a $-\log EC_{50}$ value of 4.56 ± 0.09 ($n=7$). Ordinate: inotropic effect expressed as increase in $(dF/dt)_{max}$ in percentage above basal. Vertical bars represent ± s.e.mean of inotropic response. Abscissa: log concentration of IBMX. Horisontal bars represent ± s.e.mean of $-\log EC_{50}$. $(dF/dt)_{max}$, maximal development of force.

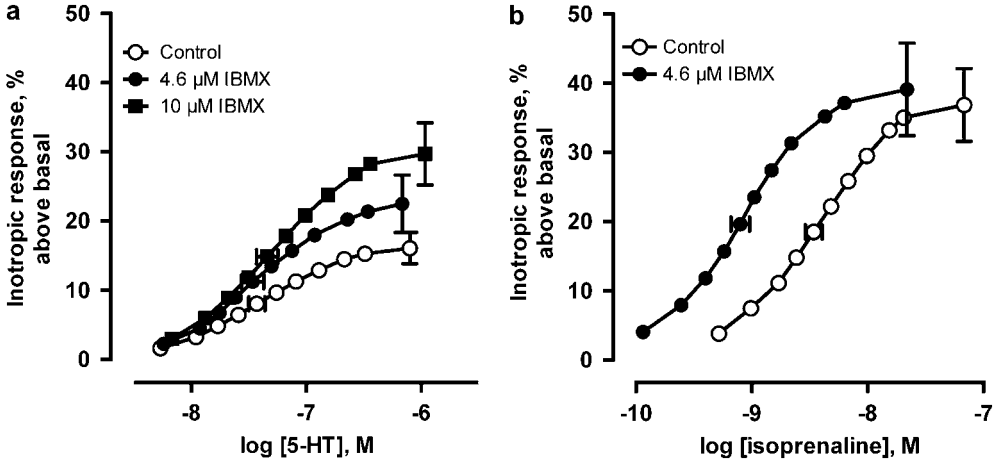

Figure 3 (**a**) Concentration–response curves of inotropic responses to 5-HT_4 receptor stimulation in the absence ($n=9$) and presence of IBMX (4.6 μM ($n=5$) or 10 μM ($n=5$)) in papillary muscles from MI rat ventricles. IBMX was added 45 min before 5-HT. (**b**) Concentration–response curves of inotropic responses to β-adrenoceptor stimulation by isoprenaline in the absence ($n=6$) and presence of 4.6 μM IBMX ($n=6$) in papillary muscles from MI rat ventricles. IBMX was added 45 min before isoprenaline. Ordinate: inotropic effect expressed as increase in $(dF/dt)_{max}$ in percentage above basal. Vertical bars represent ± s.e.mean of inotropic response. Abscissa: log concentration of agonist. Horisontal bars represent ± s.e.mean of $-\log EC_{50}$. $(dF/dt)_{max}$, maximal development of force. MI rats, rats with myocardial infarction larger than 30% of inner surface area.

Table 3 Maximal inotropic and lusitropic responses and sensitivity to 5-HT during selective and non-selective PDE inhibition in MI rat hearts

PDE inhibitor	*Maximal inotropic response to 5-HT (dF/dt_{max}, percentage above basal)*	*Sensitivity: -logEC_{50} of inotropic response to 5-HT*	*Maximal lusitropic response to 5-HT -ΔRT (ms)*
None	16.1 ± 2.3	7.44 ± 0.05	19.4 ± 2.4
4.6 μM IBMX	22.5 ± 4.2	7.47 ± 0.1	23.5 ± 0.9
10 μM IBMX	29.7 ± 4.5*	7.34 ± 0.09	26.6 ± 4.4*
10 μM EHNA	18.9 ± 2.1	7.33 ± 0.14	15.2 ± 2.7
1 μM cilostamide	49.0 ± 5.6*	7.32 ± 0.08	21.7 ± 2.5
10 μM rolipram	19.5 ± 3.2	6.48 ± 0.3	19.6 ± 6.8
Cilostamide/EHNA	38.7 ± 6.7	7.37 ± 0.05	20.6 ± 2.0
Cilostamide/rolipram	65.7 ± 5.9	7.74 ± 0.12**	31.8 ± 1.3*

Abbreviations: PDE, phosphodiesterase; $(dF/dt)_{max,}$ maximal development of force; MI rats: rats with myocardial infarction larger than 30% of inner surface area.
*$P<0.05$ vs no PDE inhibition.
**$P<0.05$ vs 1 μM cilostamide.

Selective PDE inhibition. We determined the influence of selective inhibition of PDE2, PDE3 and PDE4 on the 5-HT_4-mediated inotropic and lusitropic responses. The values of maximal inotropic effects ($(dF/dt)_{max}$, percentage above basal) and maximal lusitropic effects (shortening of RT in the contraction–relaxation cycles) and sensitivities to 5-HT ($-\log EC_{50}$) are given in Table 3.

PDE2 inhibition. In the presence of 10 μM EHNA, there was no significant change in the maximal inotropic response, the maximal lusitropic response or the sensitivity to 5-HT ($n=5$; Figure 4, Table 3). This indicates that PDE2 is not directly involved in the regulation of 5-HT_4-mediated functional responses. EHNA (10 μM) by itself did not affect the basal force or time course of contraction.

PDE3 inhibition. Cilostamide (1 μM) significantly increased the inotropic response to 5-HT ($n=7$) compared with control ($P<0.05$; Figures 4 and 5, Table 3), without changing the sensitivity. This demonstrates the key role of PDE3 in the regulation of the 5-HT_4-mediated inotropic response. Cilostamide did not significantly increase the lusitropic effect of 5-HT_4 receptor stimulation. Cilostamide (1 μM) alone had no effect on the basal force or time course of contraction.

PDE4 inhibition. In the presence of rolipram (10 μM), there was no significant increase in the maximal positive inotropic response, the maximal lusitropic effect, or in the sensitivity to 5-HT ($n=6$) compared with control (Figure 4, Table 3). These data suggest that PDE4 is apparently not involved in the regulation of the 5-HT_4-mediated inotropic response. Rolipram (10 μM) alone had no effect on the mechanical characteristics of the papillary muscles.

Combined PDE2 and PDE3 inhibition. We then determined the effect of PDE2 inhibition in the presence of PDE3 inhibition on the responses to 5-HT. There was no further change in the maximal inotropic response, lusitropic response or the sensitivity to 5-HT in the presence of both cilostamide (1 μM) and EHNA (10 μM) ($n=5$) compared with cilostamide alone (Figure 4, Table 3), indicating that PDE2 does not have a function in the regulation of the 5-HT_4-mediated inotropic response even when PDE3 is inhibited.

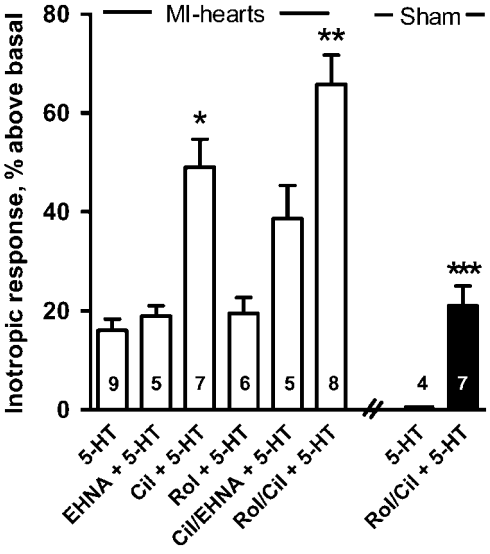

Figure 4 The maximal inotropic responses to 5-HT_4 receptor stimulation, in MI rat or Sham ventricle, in the absence of PDE inhibitor or in the presence of 10 μM EHNA, 1 μM cilostamide (Cil), 10 μM rolipram (Rol), the combination of 10 μM EHNA/1 μM Cil (Cil/EHNA) and the combination of 10 μM Rol/1 μM Cil (Rol/Cil), as indicated. PDE inhibitors were added 45 min before 5-HT. Number of experiments in each group is given in the bars. Ordinate: inotropic response expressed as increase in $(dF/dt)_{max}$ in percentage above basal. Error bars represent the s.e.mean of the inotropic responses. *$P<0.05$ vs 5-HT in MI hearts. **$P=0.06$ vs Cil + 5-HT. ***$P<0.05$ vs 5-HT in Sham ventricle. $(dF/dt)_{max,}$ maximal development of force; MI-hearts, hearts with myocardial infarction larger than 30% of inner surface area; PDE, phosphodiesterase.

Combined PDE3 and PDE4 inhibition. The effect of combined inhibition of PDE3 and PDE4 on the inotropic response to 5-HT was explored, as these two subtypes together constitute the major part of the total PDE activity in the myocardium. When the papillary muscles were challenged with 5-HT in the presence of 10 μM rolipram and 1 μM cilostamide ($n=8$), the inotropic response was nominally further enhanced, but without reaching statistical significance, compared with PDE3-inhibition alone (Figures 4 and 5, Table 3). There was also an increase in the sensitivity to 5-HT ($P<0.05$). In addition, there was an increase in the lusitropic effect compared with the response in the presence of PDE3 inhibition alone ($P<0.05$). The decrease in RT during 5-HT

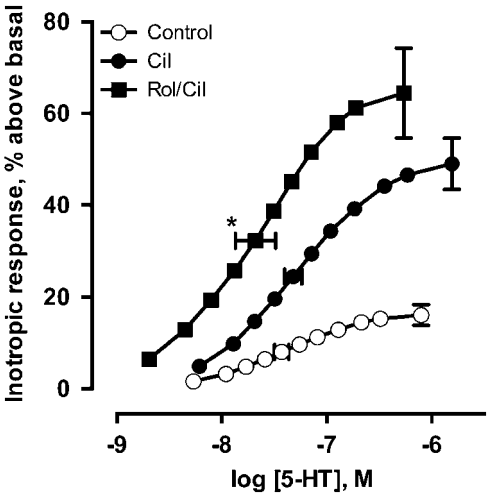

Figure 5 Concentration–response curves of inotropic responses to 5-HT_4 receptor stimulation in the absence and presence of 1 μM cilostamide (Cil) and 10 μM rolipram + 1 μM Cil (Rol/Cil) in papillary muscles from MI rat ventricles. PDE inhibitors were added 45 min before 5-HT. Ordinate: inotropic effect expressed as increase in $(dF/dt)_{max}$ in percentage above basal. Verticals bars represent ± s.e.mean of inotropic response. Abscissa: log concentration of 5-HT (5-HT). Horizontal bars represent ± s.e.mean of $-\log EC_{50}$. *$P<0.05$, $-\log EC_{50}$ Rol/Cil ($n=8$) vs Cil ($n=7$). $(dF/dt)_{max}$, maximal development of force; MI rat, rat with myocardial infarction larger than 30% of inner surface area; PDE, phosphodiesterase.

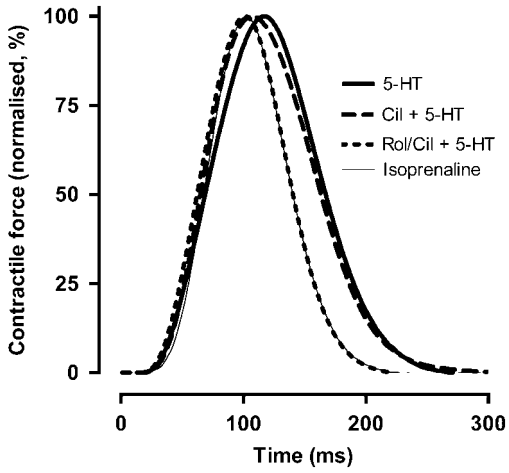

Figure 6 Representative averaged contraction–relaxation cycles in papillary muscles at maximal steady-state inotropic responses to 10 μM 5-HT alone (5-HT), in the presence of 1 μM cilostamide (Cil + 5-HT), in the presence of 10 μM rolipram + 1 μM cilostamide (Rol/Cil + 5-HT) or 10 μM isoprenaline (partly colocalized with the curve for Rol/Cil + 5-HT), normalized to maximal force (100%). PDE inhibitors were added 45 min before 5-HT. Abscissa: time in milliseconds after the electrical pulse triggering contraction. Ordinate: contractile force, normalized to 100%. PDE, phosphodiesterase.

stimulation in the presence of combined PDE3 and PDE4 inhibition was of a similar size as the lusitropic effect exerted by isoprenaline (31.8 ± 1.3 vs 31.0 ± 3.8 ms, respectively; Figure 6, Table 3). When the papillary muscles were simultaneously incubated with rolipram and cilostamide, the basal $(dF/dt)_{max}$ was increased by 9.0 ± 2.9% ($P<0.05$ compared with basal).

Effects of phosphodiesterase inhibition on 5-HT_4 receptor stimulation in ventricular muscle from sham-operated rats (Sham ventricles)

We have previously demonstrated a complete lack of mechanical response to 5-HT in Sham ventricle in contrast to a marked 5-HT_4 receptor-evoked responsiveness and increased expression of 5-$HT_{4(b)}$ mRNA levels in heart failure (Qvigstad *et al*., 2005a). Thus, we wanted to investigate whether PDE inhibition could unmask a 5-HT_4-mediated positive inotropic response in non-failing, Sham ventricles. Papillary muscles that were challenged with 5-HT in the absence or presence of 10 μM rolipram or 1 μM cilostamide alone, exhibited no inotropic response (data not shown). However, in the simultaneous presence of both rolipram (10 μM) and cilostamide (1 μM), 5-HT_4 receptor stimulation elicited a maximal positive inotropic response of 21.0 ± 4.0% with a $-\log EC_{50}$ value of 6.88 ± 0.08 ($n=7$; Figure 4).

Effects of receptor stimulation on cAMP levels in the absence and presence of PDE inhibitors in MI rat hearts

5-HT (10 μM) increased tissue levels of cAMP in papillary muscles (Figure 7) by 29 ± 7% compared with unstimulated

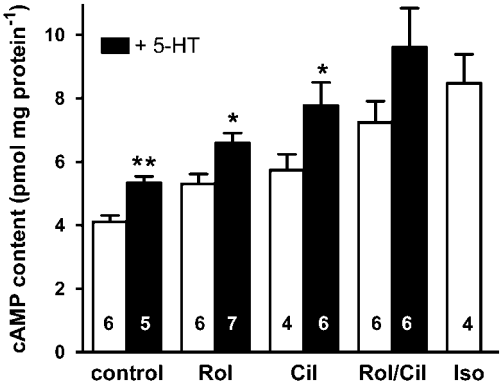

Figure 7 cAMP levels in the papillary muscles without and after stimulation with 5-HT (5-HT, 10 μM) and isoprenaline (Iso, 10 μM) in the absence and presence of PDE inhibitors. Number of experiments in each group is given in the bars. Stimulation of the 5-HT_4 receptors significantly increases the cAMP levels in papillary muscles pre-incubated without or with rolipram (10 μM) or cilostamide (1 μM) (*$P<0.05$, **$P<0.01$). PDE inhibitors were added 45 min before 5-HT. cAMP levels measured upon 5-HT stimulation in the presence of rolipram and cilostamide were not significantly different from those measured after stimulation with isoprenaline. PDE, phosphodiesterase.

muscles (($n=5$), $P<0.01$). In the presence of rolipram, 5-HT (10 μM) increased tissue levels of cAMP by 25 ± 8% compared with unstimulated muscles preincubated with rolipram (($n=7$), $P<0.05$). Thus, the increase of cAMP during stimulation by 5-HT was similar in the absence and presence of PDE4 inhibition (1.2 ± 0.3 vs 1.3 ± 0.4 pmol mg protein^{-1}, respectively). In papillary muscles preincubated with cilostamide, 5-HT (10 μM) increased the cAMP levels by 37 ± 15% (($n=6$), $P<0.05$). Only a tendency towards higher cAMP increase during stimulation by 5-HT was observed in the

presence of PDE3 inhibition compared with the absence of PDE inhibition or with the presence of PDE4 inhibition (non-significant for both), although the inotropic effect was markedly increased. The various levels and changes in cAMP levels in the absence and presence of PDE inhibitors were not entirely in accordance with the corresponding changes of functional responses.

In the simultaneous presence of both rolipram and cilostamide, cAMP levels in the papillary muscles were nominally increased by 33 ± 19% without reaching significance when challenged with 10 μM 5-HT ($n=6$, $P=0.06$). Isoprenaline (10 μM) increased the tissue levels of cAMP by 107% compared with unstimulated muscles ($n=6$, $P<0.01$). The cAMP levels upon isoprenaline stimulation were similar to those obtained when the muscles were stimulated with 5-HT in the presence of both PDE4 and PDE3 inhibition (non-significant). These results are shown in Figure 7.

Effect of selective PDE inhibition on 5-HT$_4$-mediated inotropic response in failing human ventricular tissue

In trabeculae from failing human left ventricles, 10 μM 5-HT elicited a small but significant positive inotropic response above basal values ($n=8$; $P<0.05$; Figure 8). The inotropic response to 5-HT (10 μM) in the presence of rolipram (10 μM) was similar ($P<0.05$, $n=6$). In the presence of 1 μM cilostamide, the positive inotropic response to 5-HT was further increased ($n=3$), although with borderline significance (after Bonferroni correction), compared with the inotropic response in the presence of cilostamide alone. These data indicate that PDE3 is the main regulatory PDE isoenzyme of the 5-HT$_4$-mediated inotropic response in the failing human left ventricle, and that a role for PDE4 is revealed upon PDE3 inhibition.

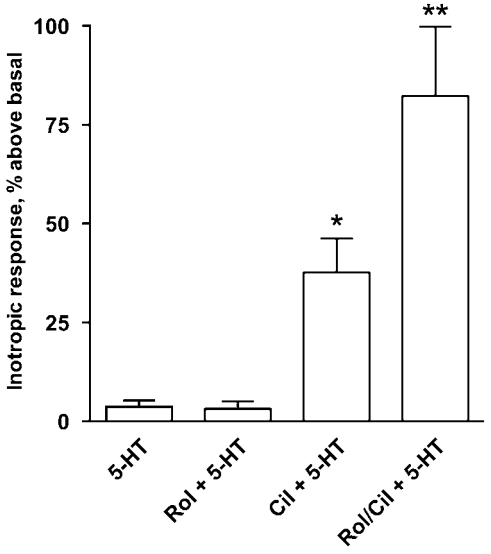

Figure 8 The maximal inotropic responses expressed as increase in $(dF/dt)_{max}$, percentage above basal (ordinate) generated in the trabeculae from failing human ventricles upon stimulation with 5-HT (single-bolus experiments; 10 μM 5-HT). Experiments were conducted in the absence of PDE inhibitors and after pretreatment with and in the presence of 10 μM rolipram (Rol), 1 μM cilostamide (Cil), or the combination of 10 μM Rol and 1 μM Cil (Rol/Cil), respectively. PDE inhibitors were added 45 min before 5-HT. Error bars represent the s.e.mean of the inotropic responses. *$P<0.05$, Cil + 5-HT ($n=4$) vs 5-HT ($n=6$). **$P=0.06$, Rol/Cil + 5-HT ($n=3$) vs Cil + 5-HT ($n=4$). Bonferroni correction was used, as the Cil + 5-HT group was compared also with 5-HT alone. $(dF/d)t_{max}$, maximal development of force; PDE, phosphodiesterase.

Discussion

Our findings demonstrate that PDE3 is the main PDE isoenzyme regulating the 5-HT$_4$-mediated positive inotropic response to 5-HT in postinfarction rat ventricle. Involvement of PDE4 is visible only when PDE3 is inhibited. On the other hand, PDE3 and PDE4 can substitute for each other in regulating the lusitropic response to 5-HT. In failing human hearts, the inotropic response to 5-HT$_4$ receptor stimulation was regulated in a manner similar to that in postinfarction failing rat ventricles. The influence of PDE inhibitors on the levels and increases of total cAMP during 5-HT stimulation only partially reflects the functional responses. Thus, the functional effects of 5-HT seem to be related to discrete compartments of cAMP.

Participation of PDEs and thus involvement of cAMP in the signalling cascade of 5-HT$_4$-mediated inotropic response in postinfarction rat ventricle was initially demonstrated by non-selective PDE inhibition by IBMX, in line with previous observations in porcine and failing human ventricle (Brattelid *et al*., 2004) and porcine and human atrium (Kaumann and Levy, 2006a, b). Further investigation using isoenzyme-selective PDE inhibitors showed that PDE3 inhibition amplified the inotropic response markedly. Thus, PDE3 was the primary isoenzyme regulating the inotropic response to 5-HT$_4$ receptor stimulation, and a contribution from PDE4 was revealed only when PDE3 was inhibited. It is known that different PDEs are localized differentially inside the cells (Lugnier, 2006). A specific intracellular localization of PDE3 somewhat closer to the inotropic signalling pathway than PDE4, would explain why the effect of PDE4 inhibition was unmasked by PDE3 inhibition. Reported differences in the kinetics between these PDE isoenzymes may also contribute to their differential regulatory functions (Lugnier, 2006). Our data do not support a role of PDE2 in regulating 5-HT$_4$-mediated inotropic responses.

The 5-HT$_4$-mediated inotropic response was previously reported to be absent in Sham ventricles despite the detectable 5-HT$_4$ mRNA (Qvigstad *et al*., 2005a). We now report that combined PDE3 and PDE4 inhibition unmasks functional 5-HT$_4$ receptors eliciting an inotropic response in Sham ventricles. Although the 5-HT$_4$ response obtained in Sham ventricles, in the presence of combined PDE3 and PDE4 inhibition, was considerably lower than that observed in MI or failing ventricle, a possible modification in the PDE activity during heart failure may partly account for the observed difference between MI/failing and Sham ventricles, but this requires further investigation. As the ventricular 5-HT$_4$ receptor mRNA levels are much lower in Sham than in MI and failing ventricle, increased 5-HT$_4$ receptor levels

probably explain partly the increased responsiveness to 5-HT in heart failure.

The 5-HT_4 receptor-mediated lusitropic effect is unaffected in the presence of separate PDE3 or PDE4 inhibition. Interestingly, the lusitropic response is enhanced when both PDE3 and PDE4 are inhibited simultaneously. This is demonstrated in our experiments conducted in the presence of non-selective PDE inhibition and in the presence of both rolipram and cilostamide. These results show that PDE3 and PDE4 can substitute for each other in regulating the lusitropic effect and might indicate that the functional compartment responsible for the lusitropic response is confined equally by PDE3 and PDE4 in contrast to the compartment responsible for the inotropic response where PDE3 is the main regulatory PDE. However, the influence of different PDE isoenzymes on the various proteins responsible for the lusitropic response upon 5-HT_4 receptor stimulation is still unknown and requires further investigation.

The increase in total cAMP levels upon 5-HT_4 receptor stimulation only to some extent supported the functional data concerning PDE3 as the main regulator of this response. The total cAMP elevation in the presence of PDE3 inhibition was slightly larger than the elevations without and with PDE4 inhibition, which is somewhat at variance with the marked difference in their influences on the functional responses. The increases in total cAMP levels upon 5-HT_4 receptor stimulation were similar in the presence and absence of PDE4 inhibition, in accordance with the lack of influence on the inotropic and lusitropic responses. The increase in total cAMP levels upon stimulation of 5-HT_4 receptors during combined PDE3 and PDE4 inhibition was of similar magnitude as that in the presence of PDE3 inhibition alone, illustrating a mismatch between the total cAMP levels and the functional responses. Thus, PDE4 did not seem to have a significant regulatory function in the total cAMP increase elicited by 5-HT_4 receptor stimulation either in the absence or in the presence of PDE3 inhibition. In the absence of receptor stimulation, however, the total cAMP levels were increased by PDE3 and PDE4 inhibitors alone and in combination without eliciting corresponding inotropic effects.

As it is known that PDEs have a major function in compartmentation of cAMP-dependent signalling (Rich *et al*., 2001; Zaccolo and Pozzan, 2002), and the different isoforms of PDEs have their specific subcellular localization (Okruhlicova *et al*., 1996; Lugnier *et al*., 1999), the regulatory functions of the various PDE isoenzymes may be different based on the measurements of total cAMP levels and on specific end effects such as increase of contractile force. The regulatory function of PDEs may also vary between different receptor subtypes and agonists, between atrium and ventricle, between normal and failing myocardium, between neonatal and adult myocardium, between myocardium from left and right ventricle and between species. Most of our results are based on the functional effects of PDE inhibition where the readout is 5-HT_4 receptor-mediated increase in mechanical activity of postinfarction adult ventricular myocardium. These aspects may account for some of the differences between our results and those found by others measuring cAMP during β-adrenoceptor stimulation in the absence or presence of PDE inhibition in neonatal and adult ventricular myocytes (Mongillo *et al*., 2004; Rochais *et al*., 2006). Mongillo *et al*. (2004) reported that β-adrenoceptor-evoked effects, measured as increase in cAMP using fluorescence resonance energy transfer-based techniques where the readout was cAMP-dependent PKA activation, are regulated mainly by PDE4 in cardiomyocytes, whereas PDE3 has a minor function. Another study (Rochais *et al*., 2006) reported that β_1-adrenoceptor-evoked effects (i.e., increase in submembrane cAMP levels measured as activation of cyclic nucleotide-gated channels in adult rat ventricular cardiomyocytes) are mainly regulated by PDE4, whereas β_2-adrenoceptor-evoked effects are regulated by both PDE3 and PDE4. Other studies showed that β_2-adrenoceptor-evoked effects, measured as recruitment of β-arrestins and contractility, are regulated by different isotypes of PDE4 in different models (Baillie *et al*., 2003; Bolger *et al*., 2003; Xiang *et al*., 2005). Altogether, the mentioned studies underscore the role of PDE4 in degrading cAMP produced as a result of β-adrenoceptor stimulation by catecholamines. The total cAMP, however, might not necessarily be directly related to discrete functional effects. Even considering this limitation, the myocardial 5-HT_4 receptors may at least partly couple to other signalling compartments than the β-adrenoceptors in cardiomyocytes. Two recent studies indicate differential effects of PDE3 and PDE4 on β_1- and β_2-adrenoceptor-mediated effects in human atrium (Christ *et al*., 2006) as well as mouse heart (Galindo-Tovar and Kaumann, 2008). The studies show that, in human atrium, PDE3 inhibition is more efficient in potentiating β_2-adrenoceptor-mediated effects than effects through β_1-adrenoceptors. In mice, β_1-adrenoceptor-mediated inotropic effects are mainly regulated by PDE4, whereas β_2-adrenoceptor-mediated inotropic effects are regulated by both PDE3 and PDE4. Another study has reported differential regulation of positive inotropic effects mediated through the high- and low-affinity site of the β_1-adrenoceptor in healthy rat right ventricle, where effects through the high affinity site, stimulated by noradrenaline, are regulated by PDE4, whereas effects through the low-affinity site are regulated by both PDE3 and PDE4 (Vargas *et al*., 2006).

On the basis of the present results, we can assume that even maximal 5-HT_4 receptor activation in the postinfarction rat heart only partially activates the downstream cAMP signalling cascade, as IBMX increased the efficacy (maximal inotropic response) without changing the potency ($-\log EC_{50}$) of 5-HT_4-mediated inotropic effects. In contrast, the concentration–response curves of isoprenaline, which presumably fully activates the cAMP/PKA downstream signalling through β-adrenoceptors, revealed increased potency, whereas the maximal inotropic effect remained unchanged during PDE inhibition (Schumann *et al*., 1974). Our data suggest that combined PDE3/PDE4 inhibition enables 5-HT_4 receptor stimulation to fully, or nearly fully, activate the downstream signalling system in postinfarction hearts, as there was a significant increase in sensitivity compared with control. In addition, in that situation, the lusitropic effect of 5-HT was quantitatively similar to that following the stimulation of β-adrenoceptors and to the

maximal lusitropic effect of IBMX, indicating full activation of the cAMP/PKA pathway. Furthermore, the total cAMP levels measured upon 5-HT_4 receptor stimulation during combined PDE3 and PDE4 inhibition are of similar magnitude as after stimulation of β-adrenoceptors, illustrating the same phenomenon. Interestingly, our findings in failing human ventricular tissue exhibit a similar pattern of PDE regulation of the 5-HT_4 receptor-mediated inotropic effect as in the postinfarction rat hearts, demonstrating a primary function of PDE3 and a secondary function of PDE4.

It has been demonstrated that the levels of circulating 5-HT are increased during coronary heart disease and heart failure (Sole *et al*., 1979; Chandra *et al*., 1994). Abnormal activation of cAMP-dependent signalling mechanisms in heart is known to cause cardiac tissue damage including necrosis and fibrosis (Iwase *et al*., 1996; Antos *et al*., 2001). The stimulation of β-adrenoceptors is known to be particularly deleterious to the heart as they elevate cAMP that activates processes leading to high energy consumption and possibly other injurious mechanisms (Lohse *et al*., 2003). Similarly, it is reasonable to assume that 5-HT_4 receptor stimulation and its inotropic effect may increase the energy expenditure of the heart in the same manner and promote other pathological processes hazardous to failing hearts. PDE3 is known to account for most of the PDE activity in human hearts (Movsesian *et al*., 1991). It has also been suggested that during dilated cardiomyopathy and heart failure, the gene expression and the activity of PDE3 is reduced (Movsesian *et al*., 1991; Smith *et al*., 1997). The relatively selective PDE3 inhibitor milrinone has been used as a therapeutic agent in treatment of heart failure, but the trials showed deleterious effects of milrinone (Packer *et al*., 1991; Cuffe *et al*., 2002; Felker *et al*., 2003). It has also been proposed that the reduced PDE activity, especially in combination with the activation of G_s-coupled receptors, can cause arrhythmia and promote heart failure because of hyperphosphorylated, and 'leaky' ryanodine receptors because of their tight coupling to PDE4D3 (Lehnart *et al*., 2005). The question whether this is also the case during 5-HT_4 receptor stimulation remains to be explored.

In conclusion, this study demonstrates a primary regulatory function of PDE3 and a secondary function of PDE4 in regulating the cAMP-dependent inotropic effects of 5-HT_4 receptor stimulation in postinfarction rat ventricle and failing human ventricle. In contrast, PDE3 and PDE4 seem to have an equal regulatory function in the lusitropic response to 5-HT_4 receptor stimulation.

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Conflict of interest

FOL is the inventor of a published patent application (WO03097065) covering the potential use of 5-HT_4 antagonists for treatment of heart failure. The patent is owned by the Norwegian biotech company Bio-Medisinsk Innovasjon AS and the contribution of several of the authors (IS, TS, FOL, JBO and EQ) is acknowledged through contractual agreements.

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