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ORIGINAL ARTICLE

Influence of phosphodiesterases and cGMP on cAMP generation and on phosphorylation of phospholamban and troponin I by 5-HT$_4$ receptor activation in porcine left atrium

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Abstract Our objective was to investigate the role of phosphodiesterase (PDE)3 and PDE4 and cGMP in the control of cAMP metabolism and of phosphorylation of troponin I (TnI) and phospholamban (PLB) when 5-HT$_4$ receptors are activated in pig left atrium. Electrically paced porcine left atrial muscles, mounted in organ baths, received stimulators of particulate guanylyl cyclase (pGC) or soluble guanylyl cyclase (sGC) and/or specific PDE inhibitors followed by 5-HT or the 5-HT$_4$ receptor agonist prucalopride. Muscles were freeze-clamped at different moments of exposure to measure phosphorylation of the cAMP/protein kinase A targets TnI and PLB by immunoblotting and cAMP levels by enzyme immunoassay. Corresponding with the functional results, 5-HT only transiently increased cAMP content, but caused a less quickly declining phosphorylation of PLB and did not significantly change TnI phosphorylation. Under combined PDE3 and PDE4 inhibition, the 5-HT-induced increase in cAMP levels and PLB phosphorylation was enhanced and sustained, and TnI phosphorylation was now also increased. Responses to prucalopride per se and the influence thereupon of PDE3 and PDE4 inhibition were similar except that responses were generally smaller. Stimulation of pGC together with PDE4 inhibition increased 5-HT-induced PLB phosphorylation compared to 5-HT alone, consistent with functional responses. sGC stimulation hastened the fade of inotropic responses to 5-HT, while cAMP levels were not altered. PDE3 and PDE4 control the cAMP response to 5-HT$_4$ receptor activation, causing a dampening of downstream signalling. Stimulation of pGC is able to enhance inotropic responses to 5-HT by increasing cAMP levels, while sGC stimulation decreases contraction to 5-HT cAMP independently.

Keywords 5-HT$_4$ receptor · Pig atrium · Phosphodiesterases · cAMP · cGMP

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Introduction

5-HT$_4$ receptors are G$_s$-protein-coupled receptors, which mediate a wide range of effects in various tissues triggered via cAMP generation (Tonini 2005; Kaumann and Levy 2006; Bockaert et al. 2011). In human and porcine atrium, 5-HT$_4$ receptor activation by 5-HT promotes a transient increase in inotropic and a more sustained increase in lusitropic effects (Sanders and Kaumann 1992; De Maeyer et al. 2006). The transient nature of the inotropic response is due to a rapid degradation of the second messenger cAMP by phosphodiesterases (De Maeyer et al. 2006). Phosphodiesterases are a superfamily of cyclic nucleotide (cAMP or cGMP) hydrolysing enzymes, and expression of PDE2, 3, 4 and 5 subtypes has been reported in pig heart (Zimmermann et al. 1994; Jakobsen et al. 2006). 5-HT$_4$ receptor-mediated responses are controlled predominantly by PDE3 in human and PDE3 and PDE4 in porcine heart (Afzal et al. 2008; Galindo-Tovar et al. 2009). The 5-HT$_4$ receptor agonist prucalopride, which was approved for use in Europe in 2009 and in Canada in 2011, for the treatment of laxative-resistant chronic constipation, behaves as a weak partial agonist in human and porcine heart, inducing a very small inotropic response compared to the native agonist

5-HT (Krobert et al. 2005; De Maeyer et al. 2006). We recently showed that these responses are also enhanced by and do not fade under concomitant PDE3 and PDE4 inhibition in pig atrium (Weninger et al. 2012).

Phosphodiesterases, in particular PDE3 and PDE2, can mediate cross-talk between cAMP and cGMP signalling pathways (Zaccolo and Movsesian 2007). PDE3 is specific for cAMP and cGMP, but shows a much lower reaction velocity for cGMP, resulting in a functional inhibition by cGMP, while PDE2 shows dual specificity for both cGMP and cAMP and is stimulated by cGMP (Fischmeister et al. 2006). cGMP production in the cell is catalysed by two enzymes, a particulate guanylyl cyclase (pGC), which is located in the plasma membrane and activated by natriuretic peptides, and a nitric oxide (NO)-activated soluble guanylyl cyclase (sGC) in the cytoplasm. Indeed, cAMP-mediated responses to 5-HT_4 receptor activation are influenced by cGMP elevating agents. Afzal et al. (2011) reported that both stimulation of pGC using C-type natriuretic peptide (CNP) and sGC using the NO-donor Sin-1 increased inotropic responses to 5-HT, presumably through inhibition of PDE3, in failing rat heart. In a previous study, we showed that pGC stimulation prolonged inotropic responses to 5-HT in porcine atrium when PDE4 and PDE2 subtypes were inhibited as well. We proposed that CNP increased cGMP levels, thereby inhibiting PDE3, and this inhibition was enhanced by preventing cGMP degradation by PDE2. Because in pig atrium cAMP is controlled by PDE3 and PDE4 in a redundant way, we also had to inhibit PDE4 to unveil the effect of CNP. In contrast, activation of sGC resulted in a hastened fading of the inotropic response to 5-HT in pig atrium (Weninger et al. 2012).

It has been demonstrated in human heart that activation of β-adrenergic and 5-HT_4 receptors increases phosphorylation of the contractile proteins phospholamban (PLB) on Ser^{16} and troponin I (TnI) on $Ser^{23/24}$, through increased cAMP production and a subsequent activation of protein kinase A (Bartel et al. 1996; Gergs et al. 2009). Therefore, in this study, the role of PDEs and pGC stimulation on 5-HT_4 receptor-mediated responses in pig left atrium was further investigated by measuring the phosphorylation levels of these proteins as well as tissue cAMP content in function of exposure time to 5-HT. The effect of sGC activation on responses to 5-HT was studied by measuring cGMP and cAMP content only.

Methods

Tissue preparation

The study was approved by the Ethical Committee for Animal Experiments from the Faculty of Medicine and Health Sciences at Ghent University. Adolescent male pigs (breed line 36, 10–12 weeks of age, 15–25 kg) were obtained from Rattlerow Seghers N.V. (Lokeren, Belgium) and deeply anaesthetized with an intramuscular injection of 5 ml Zoletil 100 (containing 250 mg zolazepam and 250 mg tiletamine) from Virbac Animal Health (Carros, France). After exsanguination, the heart was rapidly dissected and washed free of blood in Krebs–Henseleit solution (composition in millimolar: glucose 11.1, $CaCl_2$ 2.51, $NaHCO_3$ 25, $MgSO_4$ 1.18, KH_2PO_4 1.18, KCl 4.69, $CaNa_2$-EDTA 0.033 and NaCl 118). The left atrium was removed and placed in fresh aerated buffer solution at room temperature. Left atrial pectinate muscles (width, <2 mm; length, between 4 and 10 mm) were rapidly dissected, attached to tissue holders (equipped with two electrodes designed for contact stimulation) and put into 20 or 8 ml tissue baths filled with aerated Krebs–Henseleit solution preheated to 37 °C. Eight muscle strips were obtained per left atrium. To measure changes in isometric force, Statham UTC2 force transducers (Gould, Cleveland, OH, USA) and DBA 18 digital bridge amplifiers (Anerma, Belgium) were used on a Powerlab data acquisition system by ADInstruments (Spechbach, Germany) and recorded with Chart v5.5.6 software (ADInstruments). Electrical field stimulation was performed using a constant voltage stimulator (Janssen Pharmaceutica, Beerse, Belgium).

Experimental protocols

Isometric force was calibrated in milli-newton (mN). Resting load of left atrial pectinate muscles was set to 19.6 mN. Muscle contractions were stimulated with square-wave pulses (0.5 Hz, 5 ms duration, 4 V). Muscle preparations responding with a contraction below 4.9 mN were excluded. During an equilibration time of 90 min, the buffer in the organ bath was changed every 15 min. After equilibration, (*S*)-(−)-propranolol (0.2 μM) and cocaine (6 μM) were added to avoid β-adrenoceptor-mediated effects evoked by the 5-HT-mediated release of noradrenaline and to inhibit 5-HT re-uptake by the cells, respectively (Kaumann 1990). Tissues were allowed to stabilize for another 20 min before the voltage was reduced to a value at which the generated force was reduced to approximately half (between 2 and 4 V). Once a stable response was achieved, PDE inhibitors, CNP or Sin-1 were added (details on drugs and concentrations are given in the "Results" section). After an incubation period of 30 min, 5-HT or the 5-HT_4 receptor agonist prucalopride were added in a concentration of 1 μM; this concentration was selected on the basis of our previous results (De Maeyer et al. 2006), where the maximal effect was reached at this concentration in the presence of IBMX, for both agonists. Two, 10 or 30 min after 5-HT_4 receptor stimulation, muscles were freeze-clamped in liquid N_2 and stored at −80 °C until further processing. In comparison, muscles were also freeze-clamped in the absence of any

compound (basal), and after incubating PDE inhibitors, CNP or Sin-1 without addition of 5-HT_4 receptor agonist.

Western blot analysis

Frozen tissues were homogenized using a Mikro-dismembrator U (B. Braun Biotech, Melsungen, Germany) and taken up in 10× volume/weight of buffer containing 7.5 mM $NaHCO_3$, 5 % SDS and 1 % phosphatase inhibitor cocktail 1 (Sigma-Aldrich, Bornem, Belgium). This buffer was used to minimize dephosphorylation of proteins (Gergs et al. 2009). After an incubation period of 30 min at room temperature, samples were centrifuged (14,000×g for 10 min at room temperature), and the supernatant was collected. Protein concentration was determined using the method of bicinchoninic acid (Thermo Fisher Scientific, Aalst, Belgium). Twenty micrograms of protein samples were separated on 4–12 % Bis-Tris Gels (Life technologies, Ghent, Belgium) and transferred to nitrocellulose membranes. After blocking of the membrane in 5 % skim milk for 1 h, primary antibody incubation was performed overnight at 4 °C. Total and phosphorylated troponin I was detected using anti-troponin I antibody 1:1,000 and anti-phospho-troponin I $Ser^{23/24}$ antibody 1:1,000 (Cell Signaling Technology, Boston, MA, USA), respectively. Total and phosphorylated phospholamban was detected using anti-phospholamban A1 antibody 1:10,000 and anti-phospholamban phospho-Ser^{16} antibody 1:5,000 (Badrilla, Leeds, UK), respectively. All primary antibodies were detected with an anti-rabbit HRP-linked secondary antibody 1:1,000 (Cell Signaling Technology), except the anti-phospholamban A1 antibody, which was detected using an anti-mouse HRP-linked secondary antibody 1:1,000 (Cell Signaling Technology). Secondary antibodies were incubated for 1 h at room temperature. Immunological signals were detected on Amersham Hyperfilm ECL (GE healthcare, Diegem, Belgium) using Pierce® ECL (Thermo Fisher Scientific) chemiluminescent substrate for total troponin I and both phospholamban antibodies and SuperSignal® WestFemto (Thermo Fisher Scientific) chemiluminescent substrate for the phospho-troponin I $Ser^{23/24}$ antibody. After detection membranes were stripped for 45 min at 50 °C with a buffer containing 2 % SDS, 62 mM Tris pH=6.8 and 0.8 % β-mercaptoethanol, then washed extensively and blocked again for 1 h in 5 % skim milk before incubation with a primary antibody against β-tubulin 1:1,000 (Abcam, Cambridge, UK). Films were scanned (hp scanjet 5590), and signal intensities for total proteins and phosphorylated proteins were analysed using ImageJ software, and the ratio with β-tubulin, used as loading control, was calculated.

cAMP and cGMP content

Frozen tissues were homogenized using a Mikro-dismembrator U (B. Braun Biotech) and taken up in 10× volume/weight ice-cold 6 % (*w*/*v*) trichloroacetic acid (TCA). After centrifugation (2,000×g for 10 min at 4 °C), the supernatants were washed 3× with 5 volumes of water-saturated diethyl ether to extract the TCA from the sample. Briefly, diethyl ether was added, samples were vortexed, ether and aqueous phases were allowed to separate and the top ether layer was carefully removed using a vacuum pump. After that, samples were dried in a warm water bath at 60 °C under a stream of nitrogen, and the dried extract was dissolved in buffer provided with the cAMP and cGMP EIA kits (Cayman Chemical, Tallinn, Estonia). cAMP or cGMP content was detected following the manufacturer's instructions. Absorbance at 405 nm was measured using a plate reader (Amersham Biotrak II, GE healthcare). The pellet was dissolved in 5 % sodium dodecyl sulphate (SDS) in 0.1 N NaOH and used for protein quantification employing the method of bicinchoninic acid (Thermo Fisher Scientific). cAMP and cGMP concentrations were expressed in picomoles per milligram protein.

Data analysis and statistics

Contraction force, maximal contraction velocity $(+dF/dT)_{max}$ and maximal relaxation velocity $(-dF/dT)_{max}$ were analysed based on isometric force measurements (mean of 10–15 contractions) taken just before administration of PDE inhibitors, CNP or Sin-1 (basal), then before addition of 5-HT or prucalopride, and finally before the muscles were freeze-clamped. Time to peak force (TPF) as well as time to 50 % relaxation (TR_{50}; mean of two contractions) was measured at the same time points. Drug-induced increases or decreases in cardiac parameters were expressed in percentage of basal values.

All data are represented as means ± SEM of n=number of pectinate muscles from different animals. Graph Pad Prism V5.03 was used to draw graphs and to calculate the statistics. Data in the presence of compound versus those in basal conditions were compared with unpaired Student's t tests. Differences between 5-HT_4 receptor-mediated responses at a given time of exposure to 5-HT in the absence and presence of compounds were assessed using one-way ANOVA and Bonferroni post-testing when more than two groups were compared. A P value <0.05 was considered significant.

Drugs

EHNA hydrochloride, cilostamide, rolipram, C-type natriuretic peptide (CNP) and amino-3-morpholinyl-1,2,3-oxadiazolium chloride (Sin-1) were purchased at Tocris bioscience (Huissen, The Netherlands). (*S*)-(−)-propranolol hydrochloride, IBMX and serotonin creatinine sulphate salt monohydrate (5-HT) were purchased at Sigma-Aldrich (Bornem, Belgium). Cocaine hydrochloride was from Belgopia (Louvain-La-Neuve, Belgium). Prucalopride succinate was a gift from Shire-Movetis (Turnhout, Belgium). IBMX, cilostamide and

rolipram were dissolved in dimethyl sulphoxide (DMSO). Sin-1 was dissolved in Krebs–Henseleit solution. All other chemicals were dissolved in deionized water. The DMSO concentration in the organ baths did not exceed 0.1 %, which by itself did not modify muscle contractions.

Results

Effect of PDE inhibition on cardiac responses, cAMP generation and PLB and TnI phosphorylation by 5-HT

In concordance with our previous results (see De Maeyer et al. 2006; Weninger et al. 2012), 5-HT (1 μM) caused a transient increase in contraction force in pig left atrial preparations (Fig. 1a). Inhibition of PDEs using the non-selective inhibitor IBMX (20 μM) completely prevented the fade of the inotropic response to 5-HT (Fig. 1b). PDE4 inhibition with rolipram (1 μM) slightly increased and prolonged responses to 5-HT (Fig. 1d). However, in muscles which were pre-treated for 30 min with the specific PDE3 and PDE4 inhibitors cilostamide and rolipram, responses to 5-HT were increased, and the fade of the response was completely abolished (Fig. 1c), similar to responses in the presence of the non-selective PDE inhibitor IBMX (Fig. 1b). The mean inotropic responses are summarized in Fig. 2a, which shows the force increase 30 min after addition of the PDE inhibitors and 2, 10 and 30 min after addition of 5-HT in the absence and presence of PDE inhibition, in percentage of the basal contraction force. The decrease in time to peak force (TPF) as well as time to 50 % relaxation (TR_{50}) in percentage of basal values is given in Fig. 2c, d. Absolute force as well as $(+dF/dt)_{max}$ (maximal contraction rate) and R_2 values [obtained by dividing the maximal contraction rate $(dF/dt)_{max}$ by the maximal relaxation rate $(dF/dt)_{min}$] is listed in Online resource 1. Basal TPF and TR_{50} values were similar between groups. TPF and TR_{50} were both clearly decreased by IBMX as well as by cilostamide plus rolipram (Fig. 2c, d), e.g. TPF from 53.4±3.4 to 47.8±1.5 ms and TR_{50} from 41.8±1.3 to 34.2±1.3 ms (n=5) by 30 min incubation with cilostamide plus rolipram. 5-HT decreased TPF to −11±1 % below basal at 2 min (Fig. 2c). Unlike contraction force, this decrease in TPF did not fade (−9±5 % below basal at 30 min). With pre-incubation of IBMX or cilostamide and rolipram, the decrease in TPF by 5-HT was significantly enhanced (to about −20 % below basal, P<0.05) and was also maintained for 30 min. 5-HT decreased TR_{50} 2 min after addition to the same extent as TPF (about −10 % below basal), but TR_{50} showed a further decrease after 10 min to −22±6 % where it stabilized (Fig. 2d). With PDE inhibition using IBMX or cilostamide and rolipram, the decrease in TR_{50} by 5-HT was significantly enhanced (to −35±3 % and −32±3 % at 2 min, respectively, P<0.05) and remained at that level after 10 and 30 min.

At the same time points where force of contraction was measured, the muscles were freeze-clamped, and cAMP content (not tested 30 min after addition of 5-HT) as well as phosphorylation of PLB at Ser^{16} and TnI at $Ser^{23/24}$ was detected. Total PLB and TnI levels were also measured but were not changed by any compounds tested (representative blots are shown in Fig. 2e, f). The influence of PDE inhibition per se using IBMX, or concomitant cilostamide plus rolipram on basal muscle contractility, cAMP levels and the degree of PLB and TnI phosphorylation were minor; the increases of functional responses and PLB phosphorylation by IBMX as well as the small increase in TnI phosphorylation by concurrent cilostamide and rolipram reached statistical significance. 5-HT significantly increased tissue cAMP content to about 1.5-fold 2 min after addition (P<0.05), while after 10 min, cAMP levels had almost returned to basal (Fig. 2b), which is in accordance to the contractile response (Fig. 2a). In the presence of cilostamide and rolipram, 5-HT increased cAMP levels three- and four-fold, 2 and 10 min after its addition, respectively; this was significantly different from 5-HT alone (P<0.05; Fig. 2b). Phosphorylation of PLB on Ser^{16} was increased four-fold in muscles freeze-clamped 2 min after addition of 5-HT (P<0.05; Fig. 2e). Dephosphorylation appears to be slower than the fade of the inotropic response (Fig. 2a) because 10 and 30 min after adding 5-HT, phosphorylation of PLB was still significantly increased by about four- and two-fold, respectively, compared to basal (P<0.05; Fig. 2e). With concomitant PDE3 and PDE4 inhibition, 5-HT increased phosphorylation of PLB approximately six-fold versus basal, and protein phosphorylation did not decrease over the 30 min tested; this was significant compared to 5-HT alone (P<0.05). The same response was observed for PDE inhibition using the non-selective PDE inhibitor IBMX, which also showed a stable approximately six-fold increase in PLB phosphorylation (Fig. 2e). The change in TnI phosphorylation by 5-HT at 2 min from 0.6 to 1.0 did not reach significance (Fig. 2f). Under PDE inhibition with IBMX or cilostamide plus rolipram, TnI phosphorylation after 5-HT was significantly increased three to four-fold compared to basal (Fig. 2f).

Effect of PDE inhibition on cardiac responses, cAMP generation and PLB and TnI phosphorylation by the $5\text{-}HT_4$ receptor agonist prucalopride

As observed before (see Weninger et al. 2012), contractile responses to prucalopride (1 μM; Fig. 3a) in porcine left atrium were considerably smaller than responses to 5-HT showing a maximal increase of only 17 % above basal compared to 98 % for 5-HT (see Fig. 2a). Interestingly, TPF and TR_{50} showed a maximal decrease in response to prucalopride of −10 % and −18 % below basal, respectively (Fig. 3c, d), which is similar as with 5-HT (compare with Fig. 2c, d). Responses to prucalopride developed slower

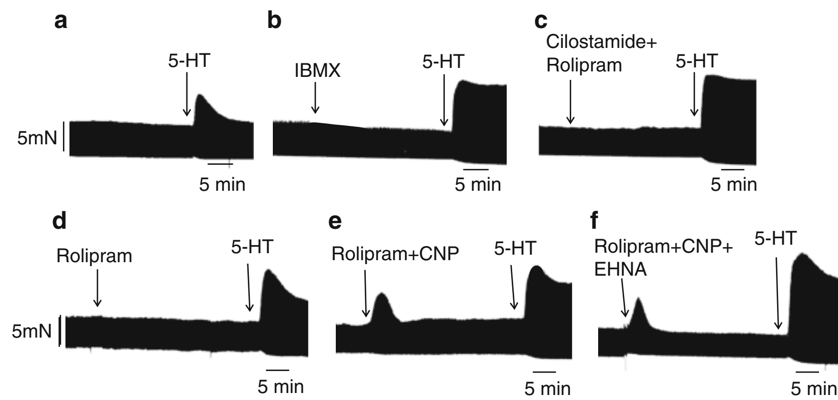


Fig. 1 Representative tracings from electrically paced porcine left atrial pectinate muscles showing the responses to 1 μM 5-HT alone (**a**) or in the presence of PDE inhibition using IBMX (20 μM) (**b**) and concomitant cilostamide (0.3 μM) plus rolipram (1 μM) (**c**). The *lower traces* show representative responses to 5-HT in the presence of rolipram (**d**), rolipram plus C-type natriuretic peptide (CNP, 0.3 μM) (**e**) and rolipram plus CNP plus the PDE2 inhibitor EHNA (10 μM) (**f**). Ten minutes after addition of 5-HT, the traces end because at this time point, the muscles were freeze-clamped for biochemical analysis

than responses to 5-HT, showing maximal force increase 10 min after addition and maximal decrease of TPF as well as TR_{50} only at 30 min. As seen with 5-HT, contraction force faded with time and even decreased below basal after 30 min (Fig. 3a), while TPF continually decreased over the 30 min measured (Fig. 3c), and TR_{50} strongly decreased from 2 to 10 min and then stabilized (Fig. 3d). Pre-incubation of muscles with IBMX (20 μM) or concomitant cilostamide (0.3 μM) and rolipram (1 μM) significantly further increased contraction force in response to prucalopride compared to prucalopride alone; these responses did not fade over the observed period of 30 min (Fig. 3a; Weninger et al. 2012). The decrease in TPF by prucalopride developed faster and was more pronounced under PDE inhibition with IBMX or cilostamide plus rolipram, being stable at approximately −20 % from 2 to 30 min (Fig. 3c). The same was observed for the decrease in TR_{50} (approximately −30 % from 2 to 30 min; Fig. 3d). cAMP content did not significantly increase after addition of prucalopride alone. Only when PDE3 and 4 were inhibited using cilostamide plus rolipram, cAMP content was significantly increased by about 1.5- and 2-fold, 2 and 10 min after addition of prucalopride, respectively ($P<0.05$; Fig. 3b). Prucalopride increased PLB phosphorylation two-fold 10 min after its addition ($P<0.05$; Fig. 3e), but not at 2 and 30 min. Phosphorylation of PLB in response to prucalopride was increased five-fold compared to basal in the presence of IBMX ($P<0.05$) and concomitant cilostamide and rolipram ($P<0.05$), which was significantly higher versus prucalopride alone at some time points. The results for the phosphorylation state of TnI were less clear. A significant increase in TnI phosphorylation was observed 2 and 30 min after addition of prucalopride alone ($P<0.05$; Fig. 3f). With PDE inhibition, phosphorylation of TnI in response to prucalopride increased slowly, reaching a maximal increase (three-fold for IBMX and five-fold for cilostamide and rolipram) 30 min after addition of prucalopride. These increases in TnI phosphorylation at 30 min, however, were not significantly different from responses to prucalopride in the absence of PDE inhibition.

Effect of particulate guanylyl cyclase activation on inotropic responses, cAMP generation and PLB and TnI phosphorylation by 5-HT

Tissue cGMP content was increased about two-fold ($P<0.05$, $n=5$; Fig. 4) in muscles freeze-clamped 2 min after addition of CNP, confirming that CNP indeed stimulates cGMP production. However, 30 min after incubation of CNP, cGMP was not higher than basal cGMP levels (Fig. 4). As reported before (Weninger et al. 2012), CNP alone did not affect inotropic responses to 5-HT (data not shown). PDE4 inhibition using rolipram (1 μM) increased and prolonged the inotropic response mediated by 5-HT, showing a contraction force of 61 % above basal at 10 min, compared to 7 % above basal 10 min after addition of 5-HT in the absence of the PDE inhibitor (Fig. 1d and 5a; Weninger et al. 2012). CNP and rolipram administered together showed a further enhancement of inotropic responses to 5-HT to 113 % above basal at 10 min (Fig. 1e and 5a); this was not further enhanced in the additional presence of 10 μM of the PDE2 inhibitor EHNA (115 %; Fig. 5a). We also noticed that CNP had a small inotropic effect of its own (Fig. 5a), which was enhanced when rolipram was administered together with CNP (Fig. 1e, f and 5a).

Tissue cAMP content was increased 2 min after addition of CNP ($P<0.05$) and after simultaneous addition of CNP and rolipram ($P=0.06$; Fig. 5b). Tissue cAMP content in response to 5-HT was significantly increased by about two-fold compared to basal levels 10 min after administration in muscles receiving rolipram, and concomitant rolipram plus

CNP ($P<0.05$). In the presence of the three compounds rolipram, CNP and EHNA, cAMP content in response to 5-HT was increased three-fold, which was significant versus 5-HT in the absence of these compounds ($P<0.05$; Fig. 5b). PLB phosphorylation essentially mirrored the functional effects depicted in Fig. 5a. Phosphorylation of PLB was increased two-fold ($P<0.05$) after the simultaneous addition of CNP and rolipram (Fig. 5c). PLB phosphorylation was not significantly increased 10 min after addition of 5-HT in this set of experiments, while there was significant phosphorylation in the same condition in the set of experiments depicted in Fig. 2e. In the presence of rolipram, PLB phosphorylation was increased four-fold 10 min after addition of 5-HT ($P<0.05$; Fig. 5c). The combinations of rolipram plus CNP, and rolipram plus CNP plus EHNA, both increased PLB phosphorylation in response to 5-HT about six-fold ($P<0.05$ versus basal; Fig. 5c); however, this was not significant versus 5-HT in the presence of rolipram alone. TnI phosphorylation showed the same tendencies as PLB phosphorylation, but the effects were less pronounced (Fig. 5d). Phosphorylation of TnI was increased 1.5 times 2 min after the simultaneous addition of CNP and rolipram ($P<0.05$; Fig. 5d), while no significant effect was observed with CNP alone. Ten minutes after administration of 5-HT, TnI phosphorylation was not significantly increased, while in the presence of rolipram, rolipram plus CNP and the triple combination rolipram plus CNP plus EHNA, the phosphorylation level of TnI was increased by about two to three-fold versus basal ($P<0.05$; Fig. 5d); however, no significance was observed when compared to the condition with 5-HT alone.

Effect of soluble guanylyl cyclase activation on inotropic responses and cAMP generation by 5-HT

Sin-1 (300 μM) alone significantly decreased muscle contractility to −34 % below basal ($P<0.05$; Fig. 6c). 5-HT could still increase muscle contractility to 56 % above basal 2 min after its addition in the presence of Sin-1 (300 μM), which was not significantly different from responses to 5-HT in the absence of Sin-1. Ten minutes after addition of 5-HT, force increase had decreased to −26 % below basal in the presence of Sin-1, while remaining 3 % above basal in the absence of the drug (Fig. 6c; Weninger et al. 2012). Sin-1 increased tissue cGMP content massively by 30-fold ($P<0.05$; Fig. 4), confirming that production of cGMP is stimulated. Interestingly, also cAMP content was increased by about 1.5-fold after administration of Sin-1 ($P<0.05$; Fig. 6d). 5-HT in the presence of Sin-1 significantly increased tissue cAMP content 2 min after addition ($P<0.05$), while after 10 min, cAMP levels had almost returned to basal; these responses were similar to cAMP levels observed with 5-HT in the absence of Sin-1.

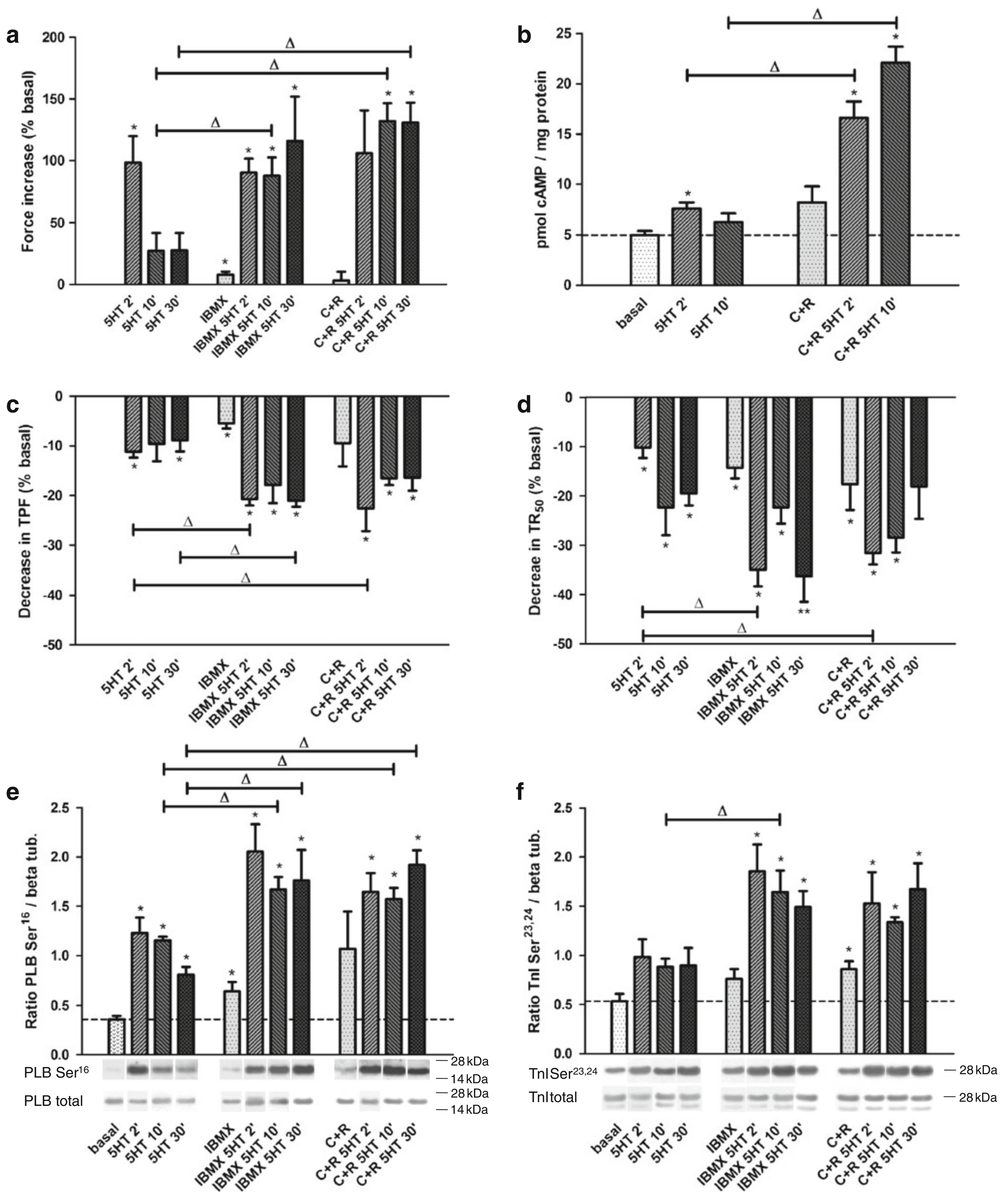
Fig. 2 Effect of 5-HT (1 μM) alone or in the presence of PDE inhibition with IBMX (20 μM) or cilostamide (0.3 μM) plus rolipram (1 μM) on force of contraction (**a**), tissue cAMP content (**b**), time to peak force (TPF) (**c**), time to 50 % relaxation (TR_{50}) (**d**), phosphorylation of phospholamban (**e**) and phosphorylation of troponin I (**f**) of $n=3$ to 5 (**c**, **d**), $n=5$ (**a**, **e**, **f**) and $n=6$ (**b**) electrically paced porcine left atrial pectinate muscles. On the *x*-axis, the different conditions are plotted; basal values were taken before addition of any compounds; IBMX and C+R show values 30 min after addition of IBMX and cilostamide plus rolipram, respectively; *all other bars* depict responses to 5-HT either 2 min (2′), 10 min (10′) or 30 min (30′) after its administration, in the absence (5-HT) or presence of IBMX (IBMX 5-HT) or cilostamide plus rolipram (C+R 5-HT). Force of contraction as well as TPF and TR_{50} was calculated in percentage to basal values (**a**, **c**, **d**), cAMP content is shown as picomole cAMP per milligram protein (**b**) and phosphorylation of phospholamban on Ser^{16} (**e**) and troponin I on $Ser^{23/24}$ (**f**) is expressed relative to the household protein β-tubulin [representative western ?blots are inserted in panels **e** and **f** showing phosphorylated protein (PLB Ser^{16}, TnI $Ser^{23,24}$) and total protein (PLB total, TnI total)]. *$P<0.05$ versus basal, unpaired *t* test; $^{\Delta}P<0.05$ for 5-HT in the presence of IBMX or C+R versus 5-HT in their absence, one-way ANOVA with Bonferroni post-testing

Discussion

The aim of this study was to further investigate the role of PDEs and cGMP in the control of the response to 5-HT_4 receptor stimulation in porcine left atrium by analysing the concentration of cAMP and the degree of phosphorylation of phospholamban (PLB) and troponin I (TnI). Both proteins have a direct role in the establishment of the functional response, and activation of human cardiac 5-HT_4 receptors has been correlated with their phosphorylation (Gergs et al. 2009). TnI is a member of the troponin protein complex of the contractile machinery in the cell, which upon phosphorylation decreases myofibrillar Ca^{2+} sensitivity, resulting in a faster relaxation, thus a lusitropic response (Matsuba et al. 2009). PKA phosphorylation of PLB Ser^{16} causes the protein to release its inhibition on SERCA, the sarcoplasmic reticulum (SR) Ca^{2+} pump, thereby allowing a faster reuptake of Ca^{2+} into the SR. This allows for a faster clearance of Ca^{2+} from the myoplasm during diastole augmenting relaxation and increases Ca^{2+} in the SR to be released during the next systole which increases contraction (MacLennan and Kranias 2003). Another SR protein, the type 2 ryanodine receptor Ca^{2+} channel, is also phosphorylated by PKA, which increases its sensitivity to Ca^{2+}-induced activation enhancing cardiac contraction (Marx et al. 2000).

Influence of PDE3 and PDE4 on the signal transduction pathway of 5-HT_4 receptor stimulation

In human right atrium, the inotropic response to 5-HT (2 μM) was stable 7 min after its addition; at this moment, the cAMP content and the phosphorylation of PLB and TnI were increased 2.8-, 1.7- and 1.5-fold (Gergs et al. 2009). In

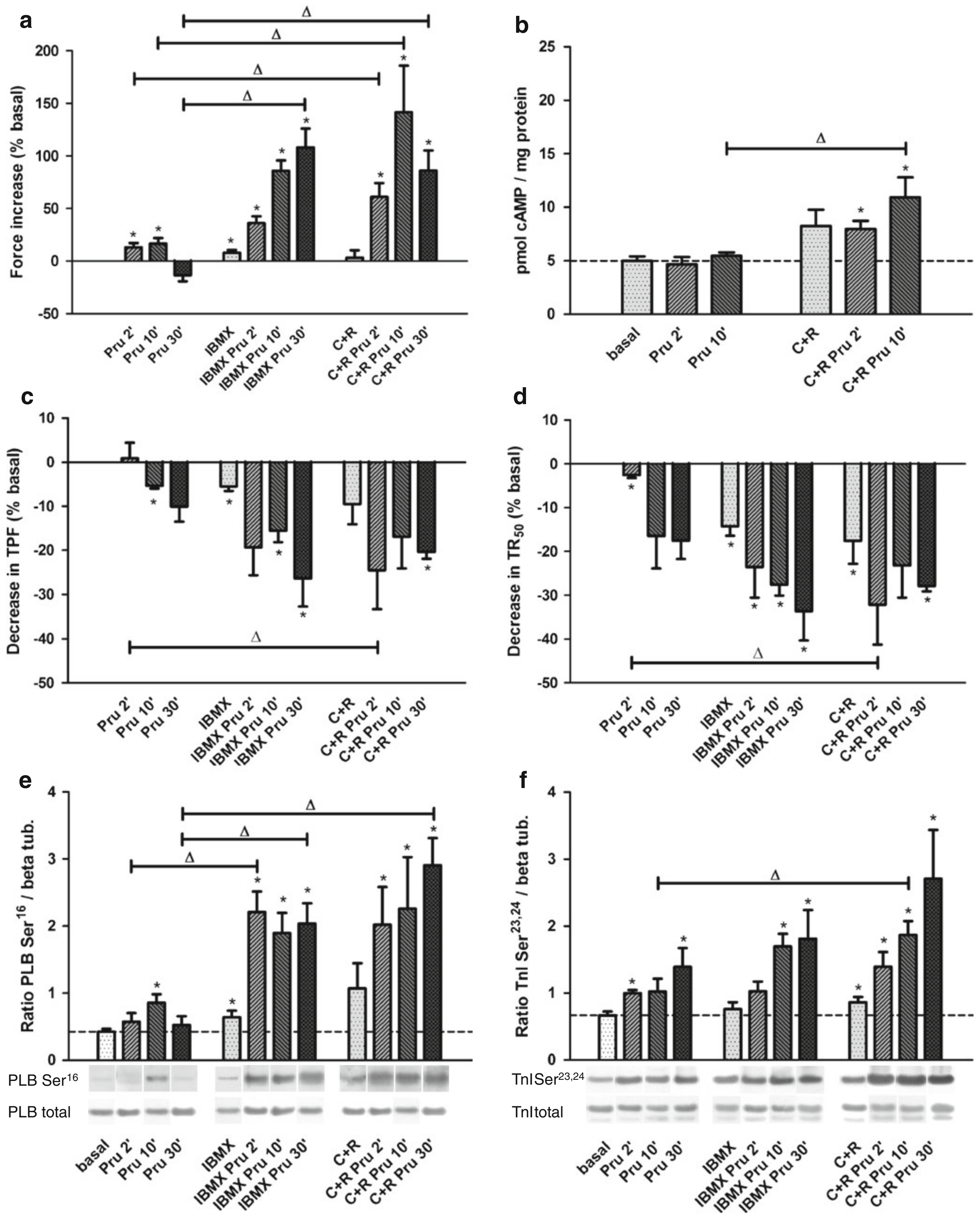


the actual study in porcine left atrium, the inotropic response to 5-HT (1 μM) peaked at 2 min after its addition, and the cAMP content and PLB phosphorylation were significantly increased at this time point, while TnI phosphorylation was not. At 10 and 30 min after administration of 5-HT, the inotropic response had declined to a very moderate level

above basal corresponding with a no longer increased cAMP level. However, the degree of PLB phosphorylation remained increased at 10 and 30 min after addition of 5-HT. There was a clear tendency to increased TnI phosphorylation by 5-HT which was persistent over the 30-min observation period. It was shown before that in isolated rabbit and guinea pig hearts, TnI phosphorylation persists, while PLB phosphorylation reverses within 15 min after β-agonist withdrawal (Garvey et al. 1988; Stemmer et al. 2000). These data are mostly in line with our results, except for the dephosphorylation velocity of PLB. However, caution should be taken when comparing dephosphorylation rates of PLB and TnI, observed after β-agonist withdrawal, to the results here since we studied PLB and TnI phosphorylation in response to continued stimulation with 5-HT. Sustained TnI and PLB phosphorylation could be due to limited access by protein phosphatases (PP) or continued phosphorylation by PKA. PLB and TnI have been reported to be dephosphorylated by PP1 and PP2A which, like PKA, are tightly regulated and compartmentalized by binding to scaffolding proteins (Redden and Dodge-Kafka 2011). Taken together, our results suggest that the inotropic mechanisms initiated by 5-HT fade quickly with the decline in cAMP, while the lusitropic effects are sustained as also reported in De Maeyer et al. (2006), due to maintained phosphorylation of TnI and PLB. The strong further decrease of TR_{50} from 2 to 10 min is also indicative of a maintained lusitropic effect. Prucalopride behaves as a partial 5-HT_4 receptor agonist in porcine left atrium, inducing slower and weaker inotropic responses than 5-HT (Weninger et al. 2012). This was confirmed in the actual study showing a mild but significant inotropic response at 2 and 10 min after addition of prucalopride. Interestingly, prucalopride decreased both TPF and TR_{50} to about the same level as 5-HT, but decreases developed slower. As seen for 5-HT, we observed a strong further decrease in TR_{50} from 2 to 10 min after addition of prucalopride. This suggests that lusitropic responses to prucalopride develop slower but are equally well developed compared to 5-HT. Prucalopride induced increased phosphorylation of TnI and PLB, which was well maintained for TnI corresponding with a sustained lusitropic effect. The cAMP level in response to prucalopride was not increased at any time point, suggesting that whole tissue cAMP analysis cannot detect the small and possibly highly compartmentalized cAMP increase induced by the partial agonist prucalopride.

In the left atrium of adolescent pigs, it was previously shown that single inhibition of PDE3 only slightly increases responses to 5-HT but does not prevent the fade, while single PDE4 inhibition causes a small increase and prolongation (Weninger et al. 2012), the latter also being confirmed in the actual study. However, concomitant PDE3 and PDE4 inhibition completely prevents the fade of the response to 5-HT and induces a clearly more pronounced and sustained response to the 5-HT_4 receptor agonist prucalopride (Galindo-Tovar et al. 2009; Weninger et al. 2012). This was confirmed in the actual study, now also showing that combined PDE3 and PDE4 inhibition largely increased and sustained cAMP levels after addition of 5-HT, corroborating a redundant control by PDE3 and PDE4 of the cAMP response to 5-HT. Combined PDE3 and PDE4 inhibition also led to increased levels of cAMP after addition of prucalopride; although the cAMP levels obtained were clearly lower than with 5-HT, this is sufficient to drive the inotropic response to prucalopride to a comparable degree as with 5-HT. Probably a certain amount of cAMP is sufficient to induce the maximal achievable contractile response within the tissue, as also illustrated by the fact that the cAMP content 2 min after addition of 5-HT is strongly increased in the presence of concurrent cilostamide and rolipram compared to that in their absence, while the increase in PLB and TnI phosphorylation is less pronounced, and the functional responses are almost the same at this time point (Fig. 2a, b). It was found before that pronounced β-adrenergic stimulation or moderate β-adrenergic stimulation combined with PDE inhibition causes excessive levels of cAMP in cardiac myocytes but fails to show equivalent increases in contraction responses (Xiang et al. 2005; De Arcangelis et al. 2008). This discrepancy can be explained by increased protein phosphatase (PP) activity in response to high levels of cAMP, which prevents a hyperphosphorylation of PKA target proteins and consequentially limits contraction responses (De Arcangelis et al. 2008). Phosphorylation of PLB and TnI in response to 5-HT and prucalopride is increased to the same extent under general PDE inhibition with IBMX and under combined PDE3 plus PDE4 inhibition, further illustrating that these two PDE subtypes maintain a tight control on the cAMP response to 5-HT_4 receptor activation. Decreases in TR_{50} as well as TPF in response to 5-HT_4 receptor stimulation are both significantly larger under PDE inhibition using IBMX or concomitant cilostamide and rolipram, indicating an enhanced lusitropic effect. This is supported by the increased and sustained phosphorylation

Fig. 3 Effect of prucalopride (Pru, 1 μM) alone or in the presence of PDE inhibition with IBMX (20 μM) or cilostamide (0.3 μM) plus rolipram (1 μM) on force of contraction (**a**), tissue cAMP content (**b**), TPF (**c**), TR_{50} (**d**), phosphorylation of phospholamban (**e**) and phosphorylation of troponin I (**f**) of n=3 to 5 (**c**, **d**), n=5 (**a**, **e**, **f**) and n=6 (**b**) electrically paced porcine left atrial pectinate muscles. See legend in Fig. 2 for detailed explanation of the different conditions depicted on the x-axis of the graphs and the way of expressing the results. *$P<0.05$ versus basal, unpaired t test; $^{\triangle}P<0.05$ for Pru in the presence of IBMX or C+R versus Pru in their absence, one-way ANOVA with Bonferroni post-testing


a
Force increase (% basal)
Pru 2'
Pru 10'
Pru 30'
IBMX
IBMX Pru 2'
IBMX Pru 10'
IBMX Pru 30'
C+R
C+R Pru 2'
C+R Pru 10'
C+R Pru 30'
b
pmol cAMP / mg protein
basal
Pru 2'
Pru 10'
C+R
C+R Pru 2'
C+R Pru 10'
c
Decrease in TPF (% basal)
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PLB Ser16
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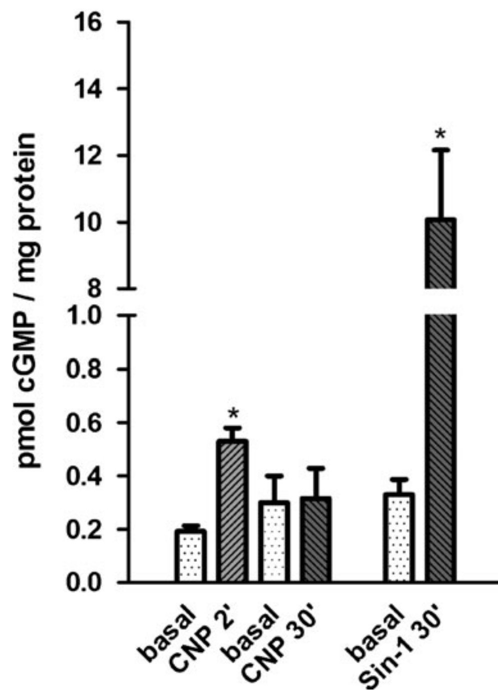

Fig. 4 Effect of particulate guanylyl cyclase stimulation with C-type natriuretic peptide (CNP, 0.3 μM) 2 and 30 min after administration and soluble guanylyl cyclase stimulation using the NO-donor Sin-1 (300 μM) 30 min after administration on tissue cGMP content (in picomole cGMP per milligram protein), in n=4 (CNP 30′) and n=6 (CNP 2′, Sin-1 30′) electrically paced porcine left atrial pectinate muscles. *P<0.05 versus basal cGMP content, unpaired t test

of the regulatory proteins TnI and PLB in these conditions. The further decrease in TR_{50} from 2 to 10 min after 5-HT_4 receptor stimulation (see Fig. 2d and 3d) was not observed in the presence of PDE inhibition. Possibly the maintained inotropic response under PDE inhibition, due to preservation of cAMP levels, prevents a further decrease in relaxation time.

Influence of cGMP generated by sGC and pGC on basal muscle function and on the response to 5-HT

Both C-type natriuretic peptide (0.3 μM) and the NO-donor Sin-1 (300 μM) significantly increased tissue cGMP content by about 2- and 30-fold, respectively (Fig. 4). The influence on basal muscle contractility and on the inotropic response to 5-HT however is different.

CNP transiently increases basal muscle contractility, while Sin-1 continuously decreases it (Figs. 5a and 6b; Weninger et al. 2012). Both effects have been observed before and were reported to be mediated by protein kinase G I (PKG; Layland et al. 2005). Like cAMP signalling, cGMP signalling is highly compartmentalized, and pGC and sGC increase cGMP close to the plasma membrane and in the bulk cytosol, respectively, thereby activating different subsets of PKG (Castro et al. 2006); this might explain the differential effects of CNP and Sin-1 on basal muscle contractility. The transient inotropic response to CNP was augmented in the presence of PDE4 inhibition (Weninger et al. 2012; Fig. 5a). In the literature, both a negative inotropic response and a biphasic response to CNP consisting of an initially positive inotropic and lusitropic response followed by a negative inotropic effect are described (Brusq et al. 1999; Pierkes et al. 2002; Qvigstad et al. 2010). The suggested mechanism is a cGMP/PKG-mediated phosphorylation of phospholamban and troponin I. Indeed, PLB on Ser^{16} and troponin I on $Ser^{23/24}$ are phosphorylated by PKG with rates similar to that of PKA and about 100-fold slower than that of PKA, respectively (Raeymaekers et al. 1988; Matsuba et al. 2009). In accordance to that, in our study, CNP tended to increase phosphorylation of PLB and TnI, reaching significance in the concomitant presence of the PDE4 inhibitor rolipram (Fig. 5c, d). We have no clear-cut explanation for the additional effect of PDE4 inhibition, since PDE4 is strictly cAMP specific and the effect of CNP has always been reported to be mediated by cGMP (Brusq et al. 1999; Layland et al. 2002; Pierkes et al. 2002; Su et al. 2005); to our knowledge, non-specific effects of rolipram not related to PDE inhibition have not been reported. A cross-talk between cGMP and cAMP signalling might be involved possibly through inhibition of PDE3 by CNP-generated cGMP (see below). We indeed observed a small but significant increase in cAMP content in response to CNP. This is in contrast to reports in rodent hearts where CNP increased cGMP but did not change cAMP levels (Brusq et al. 1999; Pierkes et al. 2002). Surprisingly, Sin-1 also slightly increased cAMP levels, which is in contrast to a study in guinea pig myocytes, where 500 μM of the NO-donor did not significantly modify cAMP (Malan et al. 2003).

As observed before (Weninger et al. 2012), CNP and Sin-1 also had a differential effect on the inotropic response to 5-HT, Sin-1 hastening its fade while CNP plus rolipram reduced the fade. An influence of elevated cGMP on cAMP signalling, predominantly mediated by phosphodiesterases 2 and 3, has been reported (Mery et al. 1993; Zaccolo and Movsesian 2007). PDE2 is specific for both cAMP and cGMP, but its cAMP-degrading activity is stimulated by cGMP. In the presence of increased cGMP, PDE2 significantly contributes to cAMP degradation and blunting of β-adrenergic receptor-mediated inotropic effects in mouse and rat cardiomyocytes (Mongillo et al. 2006). PDE3 also has cGMP and cAMP degrading activity, but a much lower V_{max} for cGMP, making it a cGMP-inhibited cAMP-specific phosphodiesterase (Fischmeister et al. 2006). In failing rat heart an enhancing effect of cGMP, generated by activation of pGC with CNP, on β-adrenergic and 5-HT_4 receptor-mediated inotropic responses has been reported, presumably through inhibition of PDE3 (Qvigstad et al. 2010; Afzal et al. 2011). In contrast to that, we showed before that CNP alone does not have an effect on the 5-HT-induced inotropic response

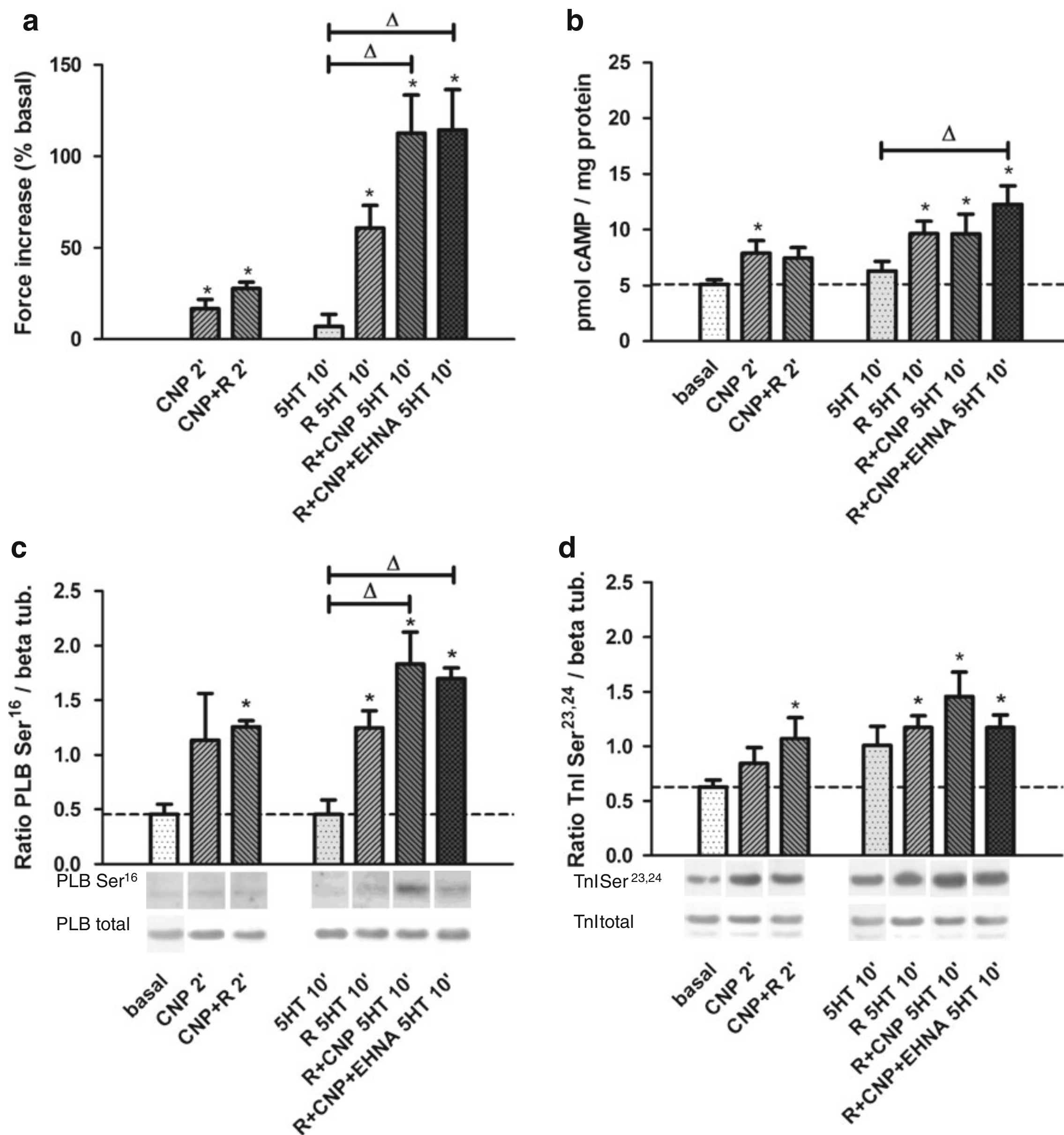

Fig. 5 Effect of particulate guanylyl cyclase stimulation using C-type natriuretic peptide (CNP, 0.3 μM) on basal muscle responses and on responses to 5-HT (1 μM). Measured were force of contraction (**a**), tissue cAMP content (**b**), phosphorylation of phospholamban (**c**) and phosphorylation of troponin I (**d**) of n=11 (**a**), n=6 (**b**) and n=5 (**c**, **d**) electrically paced porcine left atrial pectinate muscles. On the x-axis, the different conditions are plotted; basal values were taken before addition of any compounds; CNP 2′ and CNP+R 2′ show the own effect of CNP and CNP administered together with rolipram (1 μM) after 2 min, respectively. Responses to 5-HT were evaluated at 10 min after its administration in the absence (5-HT) or presence of rolipram (R 5-HT), concomitant rolipram plus CNP (R+CNP 5-HT) and rolipram plus CNP plus EHNA (10 μM; R+CNP+EHNA 5-HT). Functional responses were calculated as force increase in percentage to basal force (**a**), cAMP content is shown as picomole cAMP per milligram protein (**b**) and phosphorylation of phospholamban (**c**) and troponin I (**d**) is expressed relative to the household protein β-tubulin [representative western blots are inserted in panels **c** and **d** showing phosphorylated protein (PLB Ser^{16}, TnI $Ser^{23,24}$) and total protein (PLB total, TnI total)]. *P<0.05 versus basal, unpaired t test; $^{\triangle}P$< 0.05 versus 5-HT 10′, one-way ANOVA with Bonferroni post-testing

in porcine atrium. However, in the presence of PDE4 and PDE2 inhibition, CNP significantly prolonged the inotropic response to 5-HT (Weninger et al. 2012). We proposed that increases in cGMP, mediated by pGC stimulation and further enhanced by inhibition of its breakdown through PDE2, inhibit PDE3. Since PDE3 and PDE4 work in a redundant way in porcine atrium, it was required to inhibit PDE4 as well in order to unravel an inotropic effect. In the actual study, we show that the functional response to 5-HT was significantly larger in the presence of concurrent CNP plus rolipram, compared to 5-HT alone, and tended to be larger than the response to 5-HT in the presence of rolipram. We did not detect an additional effect of

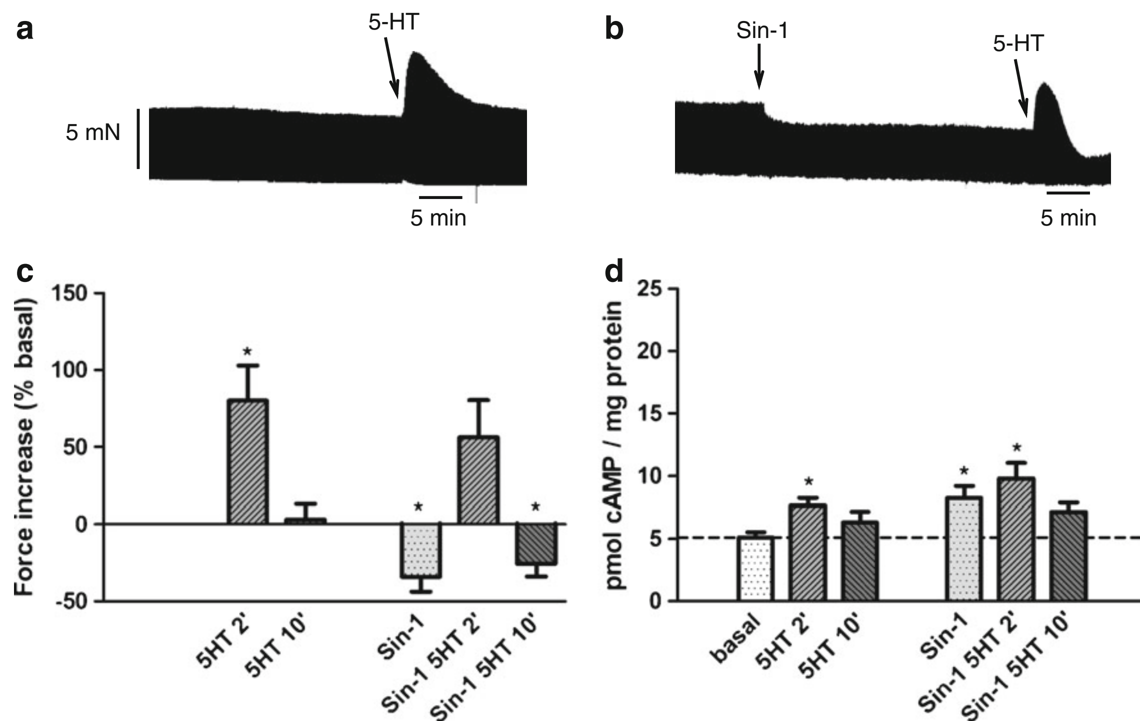

Fig. 6 Effect of soluble guanylyl cyclase stimulation using the NO-donor Sin-1 (300 μM) on responses to 5-HT (1 μM). Displayed are representative traces showing responses to 5-HT alone (**a**) or 5-HT in the presence of Sin-1 (**b**) as well as graphs showing mean force of contraction (**c**) and tissue cAMP content (**d**) of n=6 (**c**, **d**) electrically paced porcine left atrial pectinate muscles. On the x-axis of the graphs, the different conditions are plotted; basal values were taken before addition of any compounds; Sin-1 values were taken 30 min after addition of the NO-donor; the *other bars* show responses to 5-HT 2 min (2′) and 10 min (10′) after its administration in the absence (5-HT) or presence of the NO-donor (Sin-1 5-HT). Functional responses were calculated as force increase or decrease in percentage to basal force (**c**) and cAMP content is shown as picomole cAMP per milligram protein (**d**). *$P<0.05$ versus basal, unpaired t test

PDE2 inhibition with EHNA (Fig. 5a). In line with this, PLB phosphorylation levels in response to 5-HT were also increased to the same extent by both combinations (CNP plus rolipram, CNP plus rolipram plus EHNA); this level of PLB phosphorylation was significantly higher than with 5-HT alone (Fig. 5c). This supports the assumption that cGMP elevated through CNP partially inhibits PDE3, which together with PDE4 inhibition considerably slows down cAMP degradation, mediating the prolonged inotropic response to 5-HT. As for the cAMP levels, a significantly different result compared to 5-HT alone was only obtained in the presence of the triple combination CNP plus rolipram plus EHNA, again suggesting that PDE2 inhibition might be required to fully obtain inhibition of PDE3 with CNP. sGC stimulation using the NO-donor Sin-1 hastened the fade of the response to 5-HT_4 receptor stimulation in porcine atrium in this study and previously (Weninger et al. 2012; Fig. 6c). This is in contrast to a study in rat failing ventricle where Sin-1 increased 5-HT_4 receptor-mediated inotropic responses, presumably by inhibition of PDE3 (Afzal et al. 2011). However, β_1-adrenergic receptor signalling in healthy and failing rat heart was attenuated by Sin-1, the proposed mechanism being a PKG-mediated inhibition of L-type Ca^{2+} channels (Ebihara and Karmazyn 1996; Wang et al. 2009; Afzal et al. 2011). Preliminary experiments with the L-type Ca^{2+} channel blocker verapamil suggest that L-type Ca^{2+} channels are not the main mechanism by which Sin-1 decreases responses to 5-HT in pig left atrium. Changes in cAMP are not involved in the effect of Sin-1 because tissue cAMP content in response to 5-HT was the same in the presence of the NO-donor as in its absence (Fig. 6d). Sin-1 simultaneously generates NO and superoxide anion (O_2^-) which react at an almost diffusion-limited rate to peroxynitrite ($ONOO^-$; Pacher et al. 2007). Peroxynitrite has been shown to depress contractility in perfused rat hearts by lowering the Ca^{2+} sensitivity of the contractile apparatus (Schulz et al. 1997; Brunner and Wolkart 2003). In murine cardiomyocytes, β-AR responses were reduced by a high concentration of Sin-1 (200 μM), which was attributed to a peroxynitrite-mediated increased dephosphorylation of PLB by protein phosphatase 1 (PP1; Kohr et al. 2008a, b). We suggest that the effect of 300 μM Sin-1 in pig left atrium might also be mediated by peroxynitrite as opposed to NO. This is supported by the fact that SNAP (100 μM), an NO-donor which does not release O_2^-, had no effect on basal nor on 5-HT_4 receptor-mediated responses in a previous study performed in pig atrium (Weninger et al. 2012). Our data point towards a compartmentation of signalling maintained by the action of PDE3 and PDE4, where the cAMP pool in the vicinity of the 5-HT_4

receptor is not accessible to cGMP generated by sGC, while it is accessible to cGMP generated by pGC.

Conclusions

We showed that in pig left atrium, PDE3 and PDE4 subtypes are responsible for the fade of the inotropic response to 5-HT_4 receptor activation by controlling the generation of cAMP and the downstream phosphorylation of PLB and TnI. Opposed to inotropic responses, lusitropic responses to 5-HT_4 receptor stimulation do not fade, suggesting that they are less sensitive to PDE regulation. Generation of cGMP by pGC and sGC activation differentially influences basal muscle responses and the inotropic response to 5-HT. Elevated cGMP by pGC leads to increased cAMP, PLB phosphorylation and inotropic responses to 5-HT provided PDE4 is inhibited. In contrast, the negative inotropic responses caused by Sin-1 are cAMP independent; the mechanism remains to be elucidated.

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Conflict of interest Prucalopride belongs to the portfolio of Shire-Movetis NV. J. H. De Maeyer is employed by Shire-Movetis NV. R. A. Lefebvre performs contract studies for Shire-Movetis NV.

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