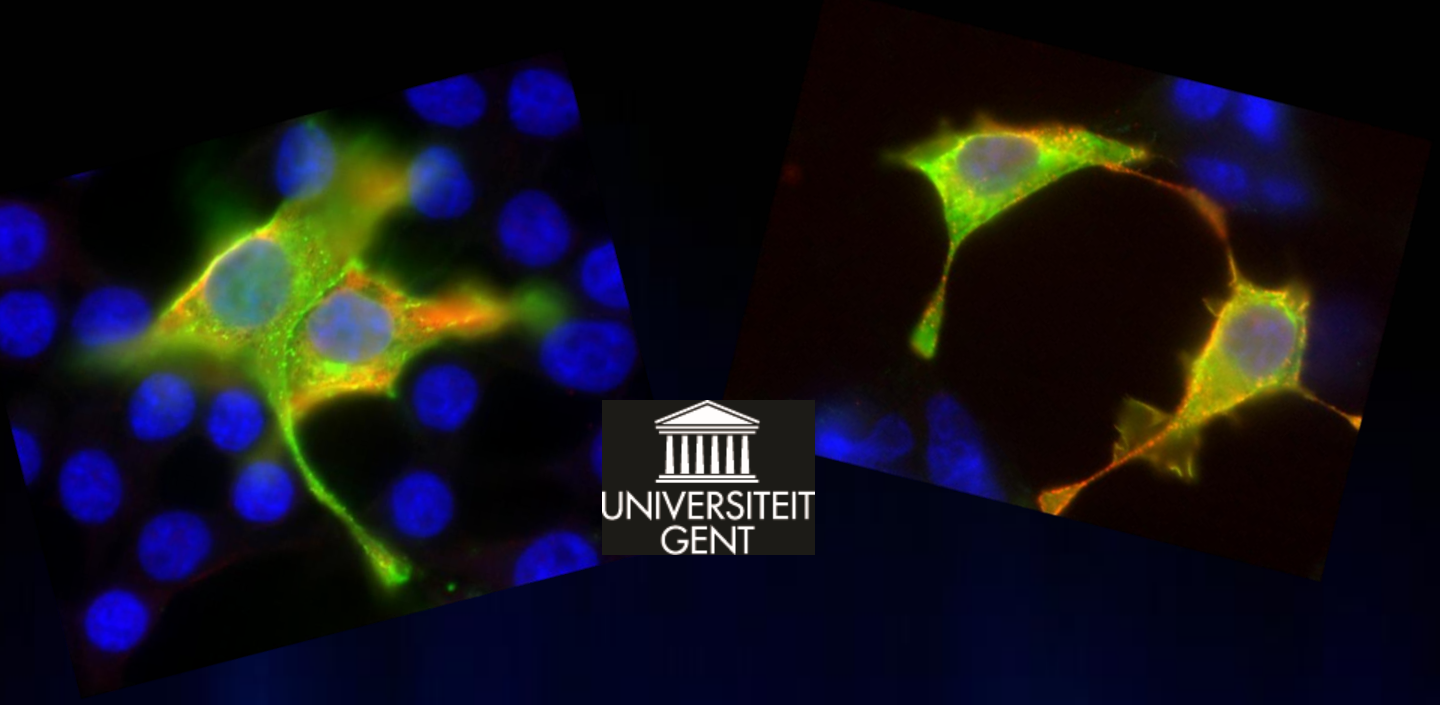

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# Cardiac 5-$HT_4$ receptor signalling:

## Regulation by phosphodiesterases and cross-talk with cGMP signalling pathways.

**Sabine Weninger**

Promotor Prof. Dr. R.A. Lefebvre
Faculty of Medicine and Health Sciences
Heymans Institute of Pharmacology
2014

Thesis submitted as partial fulfilment of the requirements for the degree of Doctor in Medical Sciences

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## *Table of Contents*

*List of abbreviations:*

5-HT: 5-hydroxytryptamine

AC: adenylyl cyclase

ADP: adenosine diphosphate

AKAP: A-kinase anchoring protein

ANP: A-type or atrial natriuretic peptide

ATP: adenosine triphosphate

BNP: B-type or brain natriuretic peptide

CNP: C-type natriuretic peptide

DMSO: dimethylsulfoxide

EC: enterochromaffin

ERK: extracellular signal-regulated protein kinase

GDP: guanosine diphosphate

GI: gastro-intestinal

GTP: guanosine triphosphate

GPCR: G protein-coupled receptor

GRK: G protein-coupled receptor kinase

HEK: human embryonic kidney cell line

hERG: human Ether-a-go-go Related Gene potassium channel

IBMX: isobutylmethylxanthine

MβC: methyl-beta-cyclodextrin

NO: nitric oxide

PDE: phosphodiesterase

pGC: particulate guanylyl cyclase

PKA: protein kinase A

PKG: protein kinase G

PLB: phospholamban

PTX: pertussis toxin

RyR2: ryanodine receptor 2 $Ca^{2+}$ channel

sGC: soluble guanylyl cyclase

SERCA: sarco(endo)plasmic reticulum $Ca^{2+}$-ATPase

SERT: serotonin transporter

SR: sarcoplasmic reticulum

TnI: troponin I

# Chapter I

## *Literature survey*

# *I Literature survey*

In this thesis the regulation of the 5-$HT_4$ receptor signalling pathway in the heart was investigated. 5-$HT_4$ receptor stimulation leads to an increase of contractility in the atrium of pig and human. Before focusing on 5-$HT_4$ receptors and the effect their stimulation has on cardiac contractility, it is important to understand the transduction of an electrical current into mechanical force in the cardiomyocyte and the control mechanisms involved in the physiological regulation of heart function. Therefore, the process of excitation-contraction coupling and the regulation of cardiac contractility via β-adrenoceptor stimulation will first be explained.

## I.1 Excitation-contraction (E-C) coupling in the heart

This section will discuss how an electrical signal causes an excitation in cardiomyocytes and induces a contraction in heart muscle; a process called excitation-contraction coupling. **Figure I.1** gives an overview of the cellular structures and protein complexes important in E-C coupling.

The crucial ion for cardiomyocyte contraction is calcium. Upon depolarization of the membrane during a cardiac action potential L-type $Ca^{2+}$ channels located in the transverse tubules (T tubules) open (see **Figure I.1**), allowing extracellular $Ca^{2+}$ to enter the cell (this current is called $I_{Ca,L}$). This local increase in $Ca^{2+}$ concentration stimulates a release of $Ca^{2+}$ from the sarcoplasmic reticulum (SR) via type 2 ryanodine receptor (RyR2) $Ca^{2+}$ channels, in a process called $Ca^{2+}$-induced $Ca^{2+}$ release (CICR). Efficient CICR is ensured by juxtaposition of sarcolemma T-tubules located at the sarcomeric z-line and cisternae of the SR in a structure called dyad. $Ca^{2+}$ entry via L-type $Ca^{2+}$ channels and RyR2s causes a global increase in $Ca^{2+}$ concentration in the cytoplasm which then activates the myofilaments (Bers 2002; Diviani et al. 2011; Harvey and Calaghan 2011).

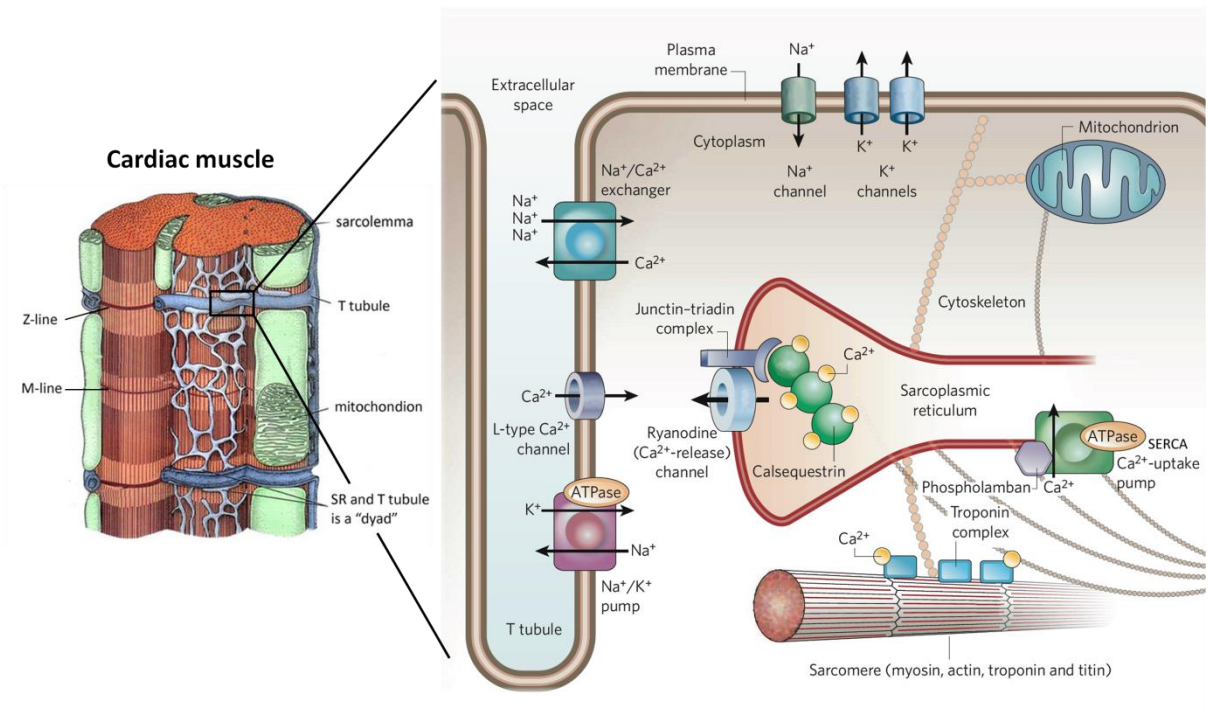


**Figure I.1** Excitation-contraction coupling in cardiac myocytes. *Left*: Cardiac muscle with T-tubules. *Right*: Protein complexes and organelles involved in E-C coupling. Adapted from http://quizlet.com/ and Knollmann and Roden (2008)

Muscle contraction is the result of a sliding of thick filaments (consisting mainly of myosin) against thin filaments (actin filaments). At relaxed state the binding sites for myosin on the actin filament are blocked by the troponin complex. The troponin complex consists of troponin C ($Ca^{2+}$ sensor), troponin I (inhibitory subunit) and troponin T (connects troponin complex to tropomyosin). $Ca^{2+}$ binding to troponin C causes a conformational change in the troponin complex, which exposes the binding sites for myosin on actin; thus allowing a cross-bridge formation of the myofilaments and causing contraction (Ruegg 1998). Contractions are ended when $Ca^{2+}$ channels close and cytosolic $Ca^{2+}$ is removed. In human ~70% of cytosolic $Ca^{2+}$ is transported back to the SR by the sarco(endo)plasmic reticulum $Ca^{2+}$-ATPase (SERCA), ~25% is pumped into the extracellular space by the $Na^{+}/Ca^{2+}$ exchanger and the remaining percent is removed by the sarcolemmal $Ca^{2+}$-ATPase and mitochondrial $Ca^{2+}$ uniporter (generally referred to as the slow 'systems') (Bers 2002). SERCA activity is regulated by sympathetic stimulation via the small pentameric protein phospholamban (PLB) (MacLennan and Kranias 2003).

## I.2 Regulation of cardiac contractility by sympathetic stimulation

### I.2.1 General

Heart contractility is regulated by sympathetic stimulation via activation of β-adrenergic receptors (e.g. during the fight-or-flight response). Three subtypes of β-adrenergic receptors exist ($\beta_1$, $\beta_2$ and $\beta_3$), and the $\beta_1$- and $\beta_2$-adrenoceptor subtypes are important in the regulation of myocardial contraction (Woo and Xiao 2012). The $\beta_1$ subtype is expressed more abundantly (70-80 %) compared to $\beta_2$ subtype (remaining 20-30 %) (Xiao et al. 2006; El-Armouche and Eschenhagen 2009). While expression of $\beta_3$-adrenergic receptor protein has been demonstrated, existence of functional $\beta_3$ receptors in the heart is controversial (Christ et al. 2011; Michel et al. 2011). $\beta_1$-adrenoceptor stimulation strongly increases contractility in atrium and ventricle and heart rate in atrium of man. $\beta_2$-adrenoceptor stimulation can also fully increase heart rate and contractility in atrium, however the inotropic effect in ventricle is smaller compared to $\beta_1$-adrenergic stimulation (Kaumann et al. 1989; Motomura et al. 1990; El-Armouche and Eschenhagen 2009). Three different effects on heart function can be distinguished in response to β-adrenergic stimulation. They are: increased inotropy, lusitropy and chronotropy. Inotropy refers to increased force of myocardial contraction, lusitropy to increased rate of relaxation (see **Figure I.2**) and chronotropy refers to changes in heart rate (El-Armouche and Eschenhagen 2009). Inotropic and chronotropic effects are measured as changes in tension (in millinewton or milligram) and beats per minute respectively. Lusitropic responses, often measured as the time to 50 % or 75 % relaxation, depend to a certain extent on inotropic changes, making them difficult to assess independently (see Figure VI.1).

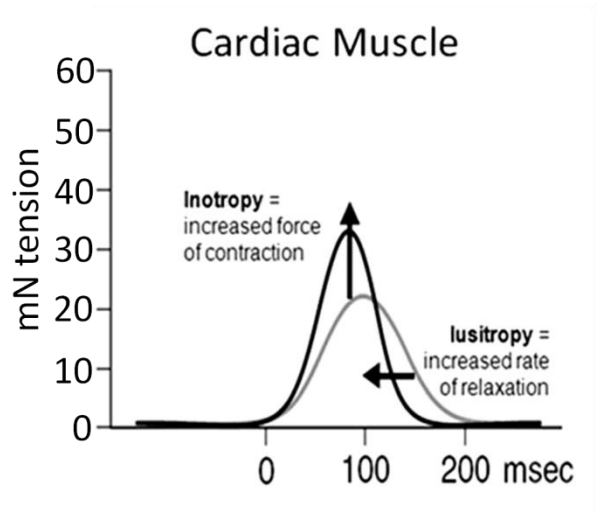


**Figure I.2** Inotropy and lusitropy in cardiac muscle. mN = millinewton; msec = milliseconds; from Kenakin (2012)

Stimulation of the G protein-coupled β-adrenergic receptors causes an increase in the second messenger cAMP and subsequent protein kinase A (PKA) phosphorylation of several proteins involved in E-C coupling. PKA phosphorylation of L-type $Ca^{2+}$ channels and RyR2s increases their sensitivity to $Ca^{2+}$ which leads to increased $Ca^{2+}$ currents through these channels and enhances contraction (Hove-Madsen et al. 1996; Marx et al. 2000; Eschenhagen 2010). Phosphorylation of PLB at $Ser^{16}$ releases its inhibition on SERCA, resulting in a faster clearing of $Ca^{2+}$ from the myoplasm which enhances relaxation. Furthermore the enhanced up-take of $Ca^{2+}$ into the SR increases the amount of $Ca^{2+}$ which can be released during the next systole and increases contraction (Bartel et al. 1996; MacLennan and Kranias 2003). PKA phosphorylates the myofibrillar proteins troponin I (TnI) at two adjacent serines ($Ser^{22,23}$), and myosin-binding protein C (MyBPC) at $Ser^{282}$. Phosphorylated TnI and MyBPC decrease $Ca^{2+}$ affinity of troponin C allowing for a faster relaxation (Bartel et al. 1996; Ruegg 1998; El-Armouche and Eschenhagen 2009; Matsuba et al. 2009).

The effects of increased cAMP by β-adrenergic stimulation are counteracted by parasympathetic nerves, releasing acetylcholine (ACh). ACh activates muscarinic M2 receptors on cardiomyocytes which signal through $G_i$ proteins and inhibit cAMP production (Fleming et al. 1987).

### I.2.2 Signalling pathway of G protein-coupled receptors (GPCRs) with focus on β-adrenergic receptors

β-adrenergic receptors, like 5-HT receptors (with the exception of 5-$HT_3$ receptors), are seven-transmembrane spanning receptors which couple to guanine nucleotide binding proteins (G proteins) (**Figure I.3**). G proteins exist as heterotrimers of $G_\alpha$, $G_\beta$ and $G_\gamma$ subunits. In their inactive form GDP is bound to the $G_\alpha$ subunit. Agonist-bound receptors induce the release of GDP from $G_\alpha$, which successively binds GTP, present in a much higher concentration in the cell than GDP. GTP-bound $G_\alpha$ dissociates from $G_{\beta\gamma}$ and both are now able to interact with down-stream effectors. Due to its intrinsic GTPase activity $G_\alpha$ hydrolyzes GTP to GDP, causing a reassociation with $G_{\beta\gamma}$ and returning the protein to its inactive state (Hamm 1998; McCudden et al. 2005; Beazely and Watts 2006). $G_\alpha$ subunit proteins fall into 4 major classes ($G_{\alpha s}$, $G_{\alpha i/0}$, $G_{\alpha q}$ and $G_{\alpha 12/13}$), with stimulatory $G_{\alpha s}$ (activates adenylyl cyclase) and inhibitory $G_{\alpha i/o}$ (inhibits adenylyl cyclase) being the best known members. But also the other $G_\alpha$ subunit classes as well as $G_{\beta\gamma}$ bind to cellular targets which only recently were begun to unravel (McCudden et al. 2005).

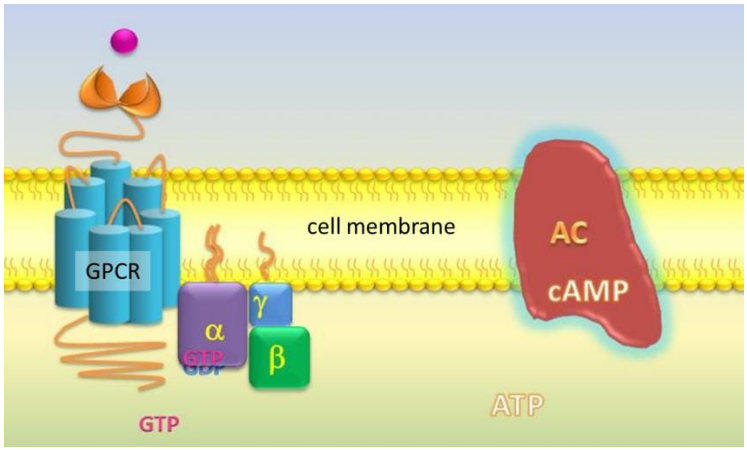


**Figure I.3** G protein-coupled receptor (GPCR) signaling. GPCR with tightly associated G protein, consisting of $G_{\alpha}$, $G_{\beta}$ and $G_{\gamma}$ subunits. Active $G_{\alpha s}$ and $G_{\alpha i}$ activate and inhibit adenylyl cyclase (AC), respectively. From http://www.picscience.net/animations/gProtein.php

$\beta_1$-adrenergic receptors predominantly couple to $G_{\alpha s}$. $\beta_2$-adrenergic receptors couple to both $G_{\alpha s}$ and $G_{\alpha i}$ in rodent and feline heart (Xiao et al. 1999), whereas $G_{\alpha i}$ coupling in human heart is controversial (Kilts et al. 2000; Molenaar et al. 2007). $G_{\alpha s}$ activates while $G_{\alpha i}$ inhibits adenylyl cyclase (AC), an enzyme which converts ATP into cAMP. In mammals 9 isoforms of particulate/membrane-bound AC (AC1 – AC9) and one soluble AC (sAC) were characterized, with AC5 and AC6 being the main isoforms in the heart (Timofeyev et al. 2013). AC5 and AC6 co-localize with L-type $Ca^{2+}$ channels and other GPCR signalling components in sarcolemma and T-tubules in cardiac myocytes (Head et al. 2005; Nichols et al. 2010). Furthermore AC5 and AC6 activity is negatively regulated by sub-micromolar $Ca^{2+}$ concentrations, providing a negative feedback mechanism for increased cAMP signalling which leads to a PKA-mediated phosphorylation and activation of $Ca^{2+}$ channels (Yu et al. 1993; Hanoune and Defer 2001; Mou et al. 2009).

GPCR-mediated activation of adenylyl cyclase increases cellular cAMP levels and subsequently activates protein kinase A (PKA). Inactive PKA exists as a tetramer of two catalytic subunits and two regulatory subunits. cAMP binding to the regulatory subunits of PKA leads to the dissociation of the holoenzyme and the release of active catalytic subunits (Dodge-Kafka et al. 2008). The catalytic subunits are serine/threonine kinases which phosphorylate and activate many target proteins involved in excitation-contraction coupling (phospholamban, troponin I, ryanodine receptor $Ca^{2+}$ channel, L-type $Ca^{2+}$ channel etc.) and thereby elicit a functional response in the heart. cAMP also has PKA-independent targets in the heart, such as Epac

(exchange protein directly activated by cAMP) and cyclic nucleotide-gated ion channels such as the hyperpolarization-activated cyclic nucleotide-gated (HCN) cation channel which regulates heart rate (Mika et al. 2012). Epac is a guanine-nucleotide-exchange factor which activates the small GTP-ases Rap1 and Rap2. Epac has been proposed to be involved in cellular processes such as cardiac gap junction formation (Somekawa et al. 2005) and has been shown to increase the frequency of RyR2 triggered $Ca^{2+}$ sparks (Pereira et al. 2007).

### I.2.3 Regulation of cAMP signalling (focus on β-adrenergic regulation of heart function)

Many GPCRs stimulate cAMP production, but the functional responses can be quite different. For example β-adrenergic stimulation in perfused rat hearts and cardiomyocytes leads to increased cAMP levels and enhances contractility. In contrast stimulation of prostaglandin E series receptors with prostaglandin $E_1$ ($PGE_1$) or $E_2$ ($PGE_2$) also enhances cAMP production but has no effect on basal contractility. (Hayes et al. 1979; Buxton and Brunton 1983; Steinberg and Brunton 2001; Liu et al. 2012). Liu et al. (2012) recently reported that stimulation with the endogenous ligand $PGE_2$ even seems able to dampen β-adrenergic receptor mediated contractile responses, while $PGE_1$ stimulation does not affect β-adrenergic signalling (Hayes et al. 1979). These differences in response to the same second messenger can be explained by a compartmentation of signalling within the cell. But how is a compartmentation of signalling maintained with such a small and highly diffusible messenger like cAMP? The next pages aim to answer this question.

#### I.2.3.1 Phosphodiesterases (PDEs)

PDEs play an important role in localizing the cAMP signal generated by stimulation of GPCRs. Gradients of cAMP are generated within the cell by the dynamic interplay between cAMP production by AC and degradation by PDEs, which act as localized ‘sinks’. Additionally diffusion of cyclic nucleotides is restricted by specialized membrane structures (Fischmeister et al. 2006; Houslay et al. 2007).

PDEs are a super-family of cyclic nucleotide degrading enzymes and represent the only means of cAMP degradation in the cell. They catalyse the cleaving of the 3’,5’-cyclic phosphate moiety of cAMP and/or cGMP to generate the corresponding 5’-nucleotide, thereby abolishing their second messenger activity (Fischmeister et al. 2006). The cGMP level in cardiomyocytes can be

increased by stimulation of soluble guanylyl cyclase (sGC) by NO and of particulate guanylyl cyclase (pGC) by natriuretic peptides. At least 11 PDE families were identified to date of which five (PDE1 to PDE5) were also detected in human heart (Fischmeister et al. 2006). PDE1, has dual specificity for both cAMP and cGMP. PDE2 also hydrolyses both cAMP and cGMP and its cAMP degrading activity is stimulated by cGMP. Even though PDE2 has a low affinity for cAMP, compared to other PDEs, it was shown to contribute to the regulation of L-type $Ca^{2+}$ channel activity in human atrium when cGMP levels are increased (Vandecasteele et al. 2001). PDE3 is specific for cAMP and cGMP, but shows a much lower reaction velocity for cGMP, resulting in a functional inhibition by cGMP (Fischmeister et al. 2006). PDE4 hydrolyses cAMP with high affinity and PDE5 is specific for cGMP. In cardiac myocytes PDE2, 3, 4 and 5 were found to contribute to the compartmentation of cAMP and cGMP pathways (see **Figure I.4**) (Fischmeister et al. 2006). All of these PDEs are also present in porcine heart, with the exception of PDE1 (Zimmermann et al. 1994; Jakobsen et al. 2006). PDE3, and the "long" PDE4 isoforms (see **Figure I.5**) can be activated by PKA phosphorylation (Sette and Conti 1996; Ekholm et al. 1997; Shakur et al. 2000; MacKenzie et al. 2002), providing a negative feedback mechanism to cAMP signalling.

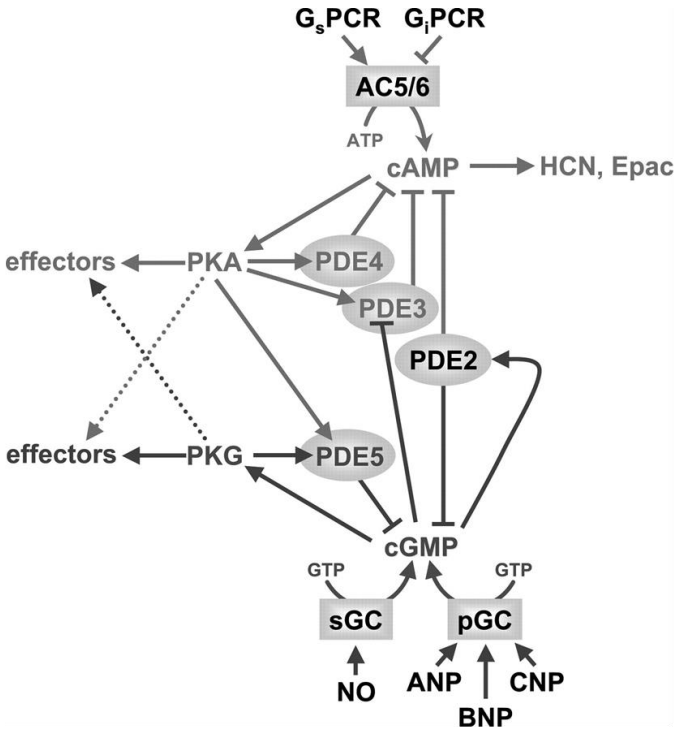


**Figure I.4** cAMP and cGMP signalling effectors in porcine cardiac myocytes. cGMP, produced by sGC and pGC and degraded by PDE2 and PDE5, activates PKG. cAMP, generated by AC5/6 and degraded by PDE2, PDE3 and PDE4, activates PKA. From Fischmeister et al. (2006)

Within these families different isoforms, generated as products of different genes or by alternative splicing, exist. In the heart PDE1C, PDE2A, PDE3A-94, PDE3A-118, PDE3A-136, PDE3B, PDE4B, PDE4D3, PDE4D5, PDE4D8, PDE4D9 and PDE5A have been described (Hambleton et al. 2005; Fischmeister et al. 2006). PDEs themselves localize to distinct regions in the cell. In cardiomyocytes PDE2 has been found at the plasma membrane and particularly in cell-to-cell junction but also associated with the sarcomeric Z-line (Mongillo et al. 2006). PDE3 was also found in both cytosolic and membrane fractions of cardiac myocytes. Most membranous PDE3 activity was found to be associated with the SR in canine and human heart (Kauffman et al. 1986; Lugnier et al. 1993; Shakur et al. 2000; Fischmeister et al. 2006). In mammalian cells at least 16 different isoforms of PDE4 encoded by four genes (PDE4A – PDE4D) have been described, which are characterized by an unique N-terminal region (see **Figure I.5**). This N-terminal domain mediates the specific localization of PDE4 isoforms to signalling complexes (interacting with A-kinase anchoring proteins, β-arrestins etc.; see also section I.2.3.3) and membranes within the cell (Conti et al. 2003; Houslay and Adams 2003; Mongillo et al. 2004; Houslay et al. 2007). In rat cardiomyocytes most cAMP degrading PDE activity is provided by PDE3 and PDE4, together ~90 % (Mongillo et al. 2004).

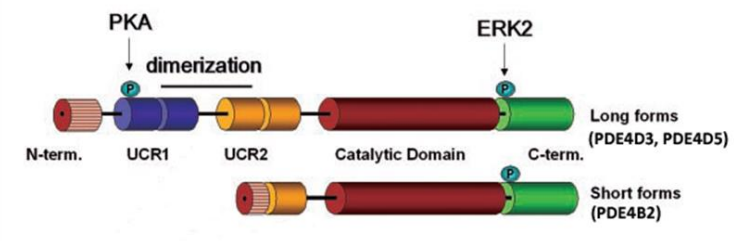


**Figure I.5** Organization of domains of PDE4 short and long forms. At least 16 PDE isoforms from four genes are expressed in mammalian cells. They can be divided into short and long forms. Only long forms contain upstream conserved region 1 (UCR1) which holds the PKA phosphorylation site. From Conti et al. 2003

In human atrium and failing ventricle $\beta_1$- and $\beta_2$-adrenergic responses are potentiated by inhibition of PDE3, but not PDE4 (Christ et al. 2006; Kaumann et al. 2007; Molenaar et al. 2013). Conversely in the atrium and ventricle of newborn pigs and adult rats PDE4 controls the inotropic response to $\beta_1$-adrenoceptor stimulation, with the response to $\beta_2$-adrenergic

stimulation being more tightly controlled by both PDE3 and PDE4 (Vargas et al. 2006; Christ et al. 2009; Galindo-Tovar et al. 2010). Another study reported that $\beta_1$-adrenergic receptor-mediated responses are regulated by both PDE3 and PDE4 in rat ventricle (Afzal et al. 2011a). In failing rat heart PDE4 activity is reduced and PDE3 is the dominant phosphodiesterase regulating $\beta_1$- and $\beta_2$-adrenergic receptor-mediated responses (Afzal et al. 2011a), while in failing dog and mouse heart PDE3 activity was shown to be reduced, but the effects on β-adrenergic signalling were not measured (Smith et al. 1998; Ding et al. 2005).

### I.2.3.2 Cross-talk between cAMP and cGMP signalling

It has been established that there is cross-talk between cAMP and cGMP signalling events, mediated by the cGMP-activated PDE2 and cGMP-inhibited PDE3 (see **Figure I.4**) (Zaccolo and Movsesian 2007). In human cardiac myocytes small elevations in cGMP, increase cAMP levels and stimulate $I_{Ca}$ through inhibition of PDE3, while higher concentrations of cGMP counteract this effect by activation of PDE2 (Kirstein et al. 1995; Vandecasteele et al. 2001). As already mentioned, cGMP is produced by two types of guanylyl cyclase, the nitric oxide (NO) activated sGC and the membrane-bound pGC. In cardiomyocytes, cGMP primarily activates protein kinase G I (PKGI), which phosphorylates proteins such as the L-type $Ca^{2+}$ channel causing its inhibition and TnI which decreases myofilament $Ca^{2+}$ sensitivity; cGMP also activates cGMP-gated cation channels (Layland et al. 2005; Takimoto 2012). Thus cGMP generally exerts negative inotropic and positive lusitropic effects and attenuates β-adrenergic signalling (Fischmeister et al. 2005; Takimoto 2012).

NO, the activator of sGC, is produced from L-arginine by the NO synthase (NOS) enzyme group, consisting of 3 isozymes: neuronal NOS (nNOS or NOS1), inducible NOS (iNOS or NOS2) and endothelial NOS (eNOS or NOS3); all three isoforms are expressed in cardiac myocytes (Hammond and Balligand 2011; Takimoto 2012). eNOS and nNOS are both constitutively expressed in healthy heart and activated by $Ca^{2+}$/calmodulin, while iNOS expression is induced by inflammatory mediators and works $Ca^{2+}$ independently (Umar and van der Laarse 2010; Takimoto 2012). NO is an important modulator of normal and pathological heart function, having effects on inotropy, lusitropy and chronotropy (Massion et al. 2003). NO has been shown to also signal via sGC-independent pathways; mainly by S-nitrosylation of signalling proteins (Stamler et al. 2001; Ziolo 2008). However, in the presence of superoxide ($O_2^{\cdot}$) NO reacts to peroxynitrite ($ONOO^-$), which has been shown to influence heart contractility (Kohr et al. 2012).

Natriuretic peptides (NPs) are a family of peptide hormones, produced in the heart, the brain and the vasculature. They include A-type NP (ANP) and B-type NP (BNP), which are expressed primarily in the atria and ventricles of the heart, and C-type NP (CNP), which is secreted by endothelial cells. ANP and BNP are used as biomarkers for the detection of cardiac pathologies, since they are increased in the development of heart failure (Hammond and Balligand 2011; Takimoto 2012). The receptors for NP are pGCs; GC-A (receptor for ANP and BNP) and GC-B (receptor for CNP) are the important forms in the cardiovascular system (Takimoto 2012).

Cellular cGMP, similar to cAMP, is strictly regulated and compartmentalized by the dynamic interplay between generation and degradation. In rat cardiomyocytes activation of pGC raises cGMP beneath the membrane while activation of sGC leads to increased cGMP levels in the soluble fraction and barely affects subsarcolemmal cGMP levels (Castro et al. 2006). Furthermore cGMP in the soluble fraction is degraded by both PDE2 and PDE5 subtypes, while cGMP in the particulate fraction is under the exclusive control of PDE2 (Castro et al. 2006). Compelling evidence for a strict compartmentalization of cGMP signalling comes from studies showing that increases in cGMP by sGC and pGC have different functional outcomes in cardiac myocytes. The functional outcome of stimulation of sGC using NO-donors depends on the concentration of NO; often showing a positive inotropic and lusitropic response at low concentrations and a negative inotropic and positive lusitropic response at high concentrations (Vila-Petroff et al. 1999; Muller-Strahl et al. 2000; Layland et al. 2002; Massion et al. 2003). Stimulation of pGC by natriuretic peptides increases chronotropy and lusitropy but diverse and species-dependent effects were observed on inotropy. Positive inotropic effects, negative inotropic effects or a biphasic effect with an initial increase followed by a decrease in inotropy were observed for natriuretic peptides in dog, rat and mouse heart respectively (Hirose et al. 1998; Brusq et al. 1999; Pierkes et al. 2002; Qvigstad et al. 2010).

cGMP generated by both sGC and pGC is able to modulate β-adrenergic signalling, either directly via activation of PKGI or via modulation of cAMP signalling through activation of PDE2 and inhibition of PDE3. In healthy and failing rat heart $\beta_1$-adrenergic signalling was attenuated by sGC stimulation, the proposed mechanism being a PKGI-mediated inhibition of L-type $Ca^{2+}$ channels (Ebihara and Karmazyn 1996; Wang et al. 2009; Afzal et al. 2011b). Other authors also observed a decrease in β-adrenoceptor-mediated inotropy when cGMP levels are increased through an NO/sGC dependent pathway, but attributed this to enhanced cAMP degradation by stimulation of PDE2 (Mongillo et al. 2006). In contrast pGC stimulation by natriuretic peptides

increased $\beta_1$-adrenergic receptor stimulated inotropy in failing rat heart, presumably via a cGMP-mediated inhibition of PDE3 (Qvigstad et al. 2010; Afzal et al. 2011b).

### I.2.3.3 A-kinase Anchoring Proteins (AKAPs)

In cardiac myocytes signalling molecules such as PKA and PDEs are targeted to distinct subcellular locations by scaffolding proteins. An important group of scaffolding proteins are AKAPs, a family of over 50 functionally related proteins, which are defined by their ability to bind the regulatory subunit of PKA (Dodge-Kafka et al. 2008). AKAPs assemble multi-enzyme signalling complexes, thereby bringing PKA into close proximity with its substrates and regulatory proteins such as PDEs, to ensure the specificity and tight regulation of signalling events. St-Ht31, a small peptide inhibitor of PKA-AKAP interaction, helped to reveal the importance of AKAPs in heart physiology. Adenoviral expression of Ht31 in rat heart or in cultured cardiomyocytes altered contractile responses to β-adrenergic stimulation and decreased PKA dependent phosphorylation of several proteins involved in excitation-contraction coupling (Fink et al. 2001; McConnell et al. 2009).

Many different AKAPs were shown to be expressed in cardiomyocytes (Diviani et al. 2011). The nomenclature of AKAPs is quite confusing and the names of several AKAPs differ between species. The following AKAPs, indicated by their gene names with protein names given in parenthesis, are involved in $Ca^{2+}$ handling in the heart: **AKAP5** (AKAP79 in human, AKAP150 in rodents), AKAP6 (**mAKAP**), AKAP7 (**AKAP**15/**18**) and AKAP12 (AKAP250, **gravin**) (see **Figure I.6**).

**AKAP5** forms a large signalling complex at the plasma membrane of cardiomyocytes containing PKA, AC5/6, protein phosphatase 2B, $\beta_1$- and $\beta_2$-adrenergic receptors and L-type $Ca^{2+}$ channels that is tightly associated with caveolin-3 (a protein typically present in caveolae membrane structures; see section I.2.3.4) (Fraser et al. 2000; Nichols et al. 2010). It was postulated that this complex generates a microdomain of cAMP that is juxtaposed to the RyR2 containing regions of the SR which is essential for adrenergic stimulation of whole-cell $Ca^{2+}$ transients (Nichols et al. 2010). **AKAP18δ** (large splice variant of the AKAP7 gene) forms a complex with PKA, phospholamban and SERCA2 in cardiac myocytes. This complex regulates PKA phosphorylation of PLN in response to adrenergic stimulation and thereby facilitates enhanced SERCA2-mediated uptake of $Ca^{2+}$ into the SR (Lygren et al. 2007). **AKAP18α** anchors PKA to L-type $Ca^{2+}$ channels and is important for the regulation of channel current under β-adrenergic stimulation (Hulme et

al. 2003). **mAKAP** forms a signalling complex on the perinuclear membrane including PDE4D3, a subset of nuclear envelope associated ryanodine receptor2 and protein phosphatase 2A. This complex implements a negative feedback loop between PKA and PDE4D3 and regulates extracellular signal-regulated kinase (ERK) signalling to the nucleus, involved in the induction of cardiac hypertrophy (Kapiloff et al. 2001; Dodge-Kafka et al. 2005; Dodge-Kafka and Kapiloff 2006). A similar complex was suggested to exist in the sarcoplasmic reticulum which could enhance ryanodine receptor-mediated $Ca^{2+}$ release, however whether mAKAP is present in the SR is a matter of discussion (Marx et al. 2000; Dodge et al. 2001; Diviani et al. 2011).

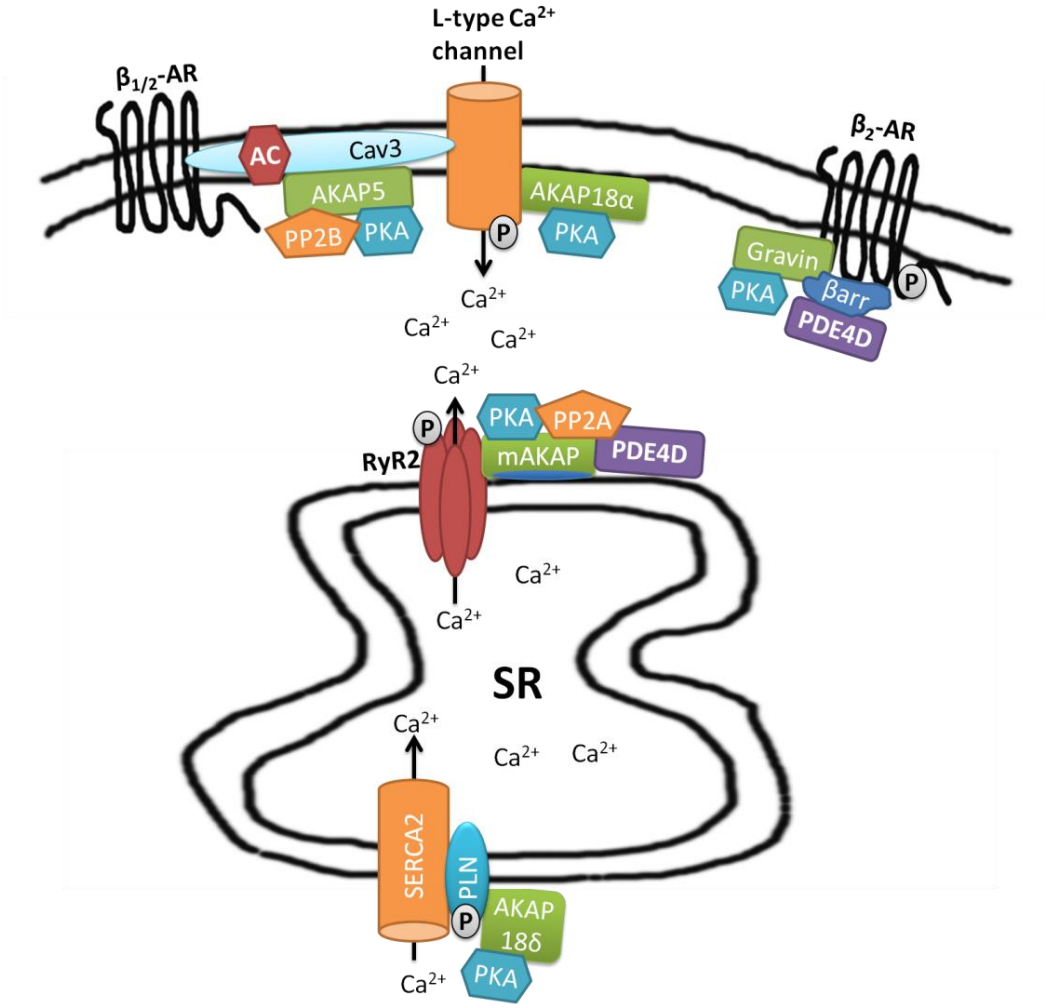


**Figure I.6** AKAPs (A-kinase anchoring proteins), important in $Ca^{2+}$ handling in the heart. AKAP5 builds a complex with $\beta_1$- and $\beta_2$-adrenergic receptors (AR), including caveolin3 (cav3) and protein phosphatase 2B (PP2B). AKAP18α targets PKA to L-type $Ca^{2+}$ channels. Gravin forms a complex including β-arrestin2 (βarr), PDE4D and the $\beta_2$-AR. mAKAP builds a complex with ryanodine receptor2 (RyR) and PDE4D and may enhance RyR mediated $Ca^{2+}$ release. AKAP18δ targets PKA to PLN and facilitates the re-uptake of $Ca^{2+}$ into the sarcoplasmic reticulum (SR). Figure based on Diviani et al. (2011)

**Gravin** targets the $\beta_2$-adrenergic receptor and forms a complex including PKA, PDE4D and β-arrestin which is believed to be involved in signalling and desensitization of the receptor (Lin et al. 2000; Fan et al. 2001; Tao et al. 2003; Willoughby et al. 2006).

#### I.2.3.4 Caveolae

Caveolae are cholesterol enriched invaginations in the plasma membrane that contain the protein caveolin. Several authors (Okamoto et al. 1998; Gratton et al. 2004; Calaghan and White 2006) suggested a role of these structures in the compartmentation of cAMP signalling. Three main isoforms of caveolin were found: Cav1, Cav2 and Cav3. All of them are expressed in adult ventricular myocytes, but Cav3 was reported to play the primary role in the formation of caveolae in these cells (Harvey and Calaghan 2011; Harvey and Hell 2013). The cAMP signal generated by stimulation of $\beta_1$-adrenergic receptors is global, stimulating L-type $Ca^{2+}$ channels in the sarcolemma but also increasing the phosphorylation of SR proteins such as RyR and PLN. While the cAMP signal generated by $\beta_2$-adrenergic receptor stimulation in rat cardiomyocytes is localized to the sarcolemma, where it stimulates L-type $Ca^{2+}$ channels, it does not affect proteins in the SR. Cardiac $\beta_2$-adrenoceptors were shown to almost exclusively localize in caveolae as well as many downstream and regulatory components of this pathway including PKA, AC5/6, $G_s$, $G_i$ and L-type $Ca^{2+}$ channels (Steinberg 2004; Head et al. 2005). It was proposed that caveolae limit $\beta_2$-adrenergic receptor signalling (Macdougall et al. 2012), which is supported by the results of Calaghan et al. (2008) who showed that the disruption of caveolae changes the $\beta_2$-adrenoceptor-induced response from one confined at the sarcolemma to a global cAMP signal. A subset of $\beta_1$-adrenergic receptors is also localized in caveolae and their disruption increases the sensitivity of the functional response to receptor stimulation (Agarwal et al. 2011; Harvey and Calaghan 2011).

## I.3 What is 5-HT? General information and research history

The history of research on serotonin or 5-hydroxytryptamine dates back to the 1930s, when Erspamer and Vialli found that a substance in isolated enterochromaffin (EC) cells of the gut, named enteramine, caused contractions in rat uterus (Erspamer and Vialli 1937; Whitaker-Azmitia 1999; Hannon and Hoyer 2008). In 1948, Rapport et al. isolated a substance in serum involved in contracting blood vessels and named it serotonin (Rapport et al. 1948). In 1952, after

the structure of serotonin was found to be 5-hydroxytryptamine (Rapport 1949), Erspamer and Asero realized that enteramine and serotonin were in fact the same substance (Erspamer and Asero 1952). By now, it is clear that serotonin or 5-hydroxytryptamine (5-HT) is a messenger, which is involved in many functions in various organs including the brain, the gastro-intestinal (GI) tract and the cardiovascular system. It is synthetized, starting from (L)-tryptophan, in two steps by the enzymes tryptophan hydroxylase (TpH) and L-amino acid decarboxylase (AAD) (see **Figure I.7**). TpH is the rate limiting step of this pathway. The enzyme exists in two isoforms: *TpH1* is expressed in the periphery, while *TpH2* is found exclusively in the brain (Walther et al. 2003).

5-HT is widely spread in nature, being present in plants and animals. In mammals more than 95 % of 5-HT in the body is located in the gut, where it is synthesized by EC cells in the GI mucosa and to a small extent in serotonergic neurons of the myenteric plexus. EC cells produce high enough amounts of 5-HT for it to overflow into the lumen of the GI tract and into the blood. Overflowing 5-HT is quickly taken up and concentrated by platelets, which represent virtually the only source of 5-HT in the cardiovascular system (Gershon and Tack 2007; Gershon 2013). 5-HT is inactivated by transporter-mediated uptake into cells. The main molecule responsible for 5-HT uptake is the serotonin reuptake transporter (SERT); however in case SERT is inhibited, backup transporters such as the dopamine transporter and organic cation transporters can partly compensate for deficient SERT function (Gershon and Tack 2007). Within cells, 5-HT is degraded by monoamine oxidase (MAO) and aldehyde dehydrogenase to 5-hydroxyindole acetic acid (Monassier et al. 2010; Watts et al. 2012).

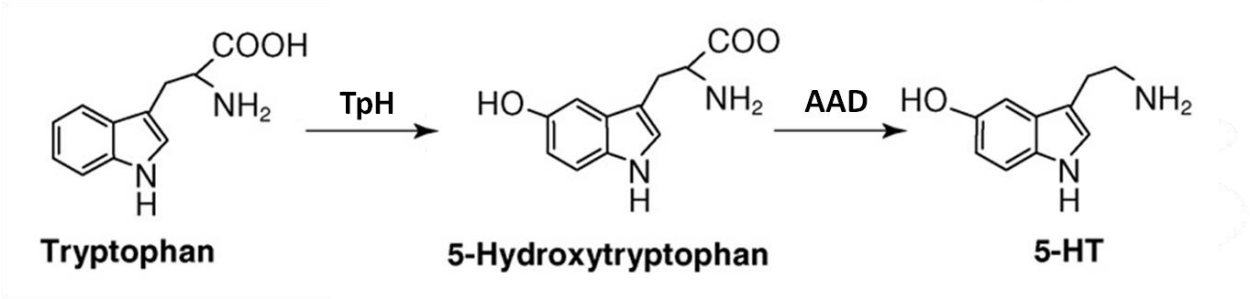


**Figure I.7** Biosynthesis of 5-HT; adapted from Sanger (2008)

Effects of 5-HT are mediated by at least 14 different receptor subtypes which fall into seven receptor classes (named $5\text{-HT}_1$ to $5\text{-HT}_7$). They all belong to the family of the seven

transmembrane-spanning G protein-coupled receptors (GPCRs), except for the 5-$HT_3$ receptor, which is a ligand gated ion channel (De Maeyer et al. 2008b; Monassier et al. 2010). They are called seven-transmembrane spanning because these receptors contain a conserved structure of seven transmembrane α-helices, with an extracellular amino-terminus and an intracellular carboxyl-terminus (see **Figure I.8**). The diversity of 5-HT receptors is thought to be a result of the long evolutionary history of this signalling system, which predates the separation of vertebrates and invertebrates, some 600 million years ago (Hoyer et al. 2002).

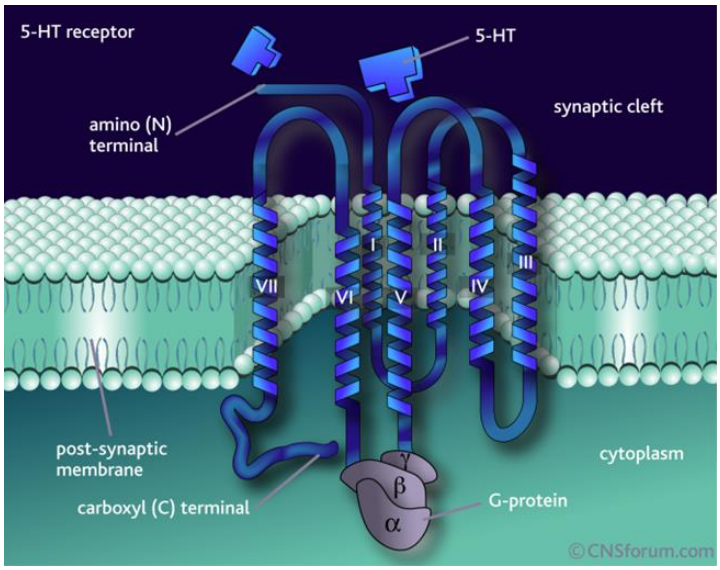


**Figure I.8** Structure of a G protein-coupled 5-HT receptor containing seven transmembrane α-helices, with an extracellular amino-terminus and an intracellular carboxyl-terminus. From Lundbeck Institute – CNS forum (http://www.cnsforum.com/imagebank/item/5HT_struc_level2/default.aspx)

The distinction between 5-HT receptor classes began with the work of Gaddum and Picarelli in 1957, who found that in guinea pig ileum part of the contractile effect of 5-HT could be blocked by **m**orphine and part by **d**ibenzyline (phenoxybenzamine). They suggested two kinds of tryptamine receptors are responsible for mediating the effects to 5-HT and named them M and D, respectively (Gaddum and Picarelli 1957; Hannon and Hoyer 2008). Further classification advanced in the late seventies when Peroutka and Snyder distinguished 5-$HT_1$ and 5-$HT_2$ receptors in rat brain by differential binding of radioligands (Peroutka and Snyder 1979; Hoyer et al. 2002). The aforementioned D receptor proved to be pharmacologically similar to the 5-$HT_2$ binding site, while the M receptor was distinct from both 5-$HT_1$ and 5-$HT_2$ binding sites and in

1986 was classified as 5-$HT_3$ by Bradley et al. (Bradley et al. 1986; Hoyer et al. 2002). In the following years further progress was made and new receptor subtypes were added within the 5-$HT_1$ and 5-$HT_2$ receptor classes. The existence of the 5-$HT_4$ receptor was suggested in the late eighties in mouse embryo colliculi neurons (Dumuis et al. 1988; Clarke et al. 1989). Soon thereafter putative 5-$HT_4$ receptors were found in the heart and the GI tract (Eglen et al. 1990; Kaumann et al. 1990; Villalon et al. 1990). With the start of the era of molecular biology many new and suspected 5-HT receptors were cloned, greatly advancing our understanding in the field (Hoyer et al. 2002; Hannon and Hoyer 2008) and culminating in the classification of receptors into seven groups (Hoyer et al. 1994).

In the periphery 5-HT receptors in the GI tract received most attention, because of the fact that agonists for several receptor classes have prokinetic effects (see I.4 for description of Agonists and antagonists) (Dumuis et al. 1989). Clinical advances began in 1964 with metoclopramide, which was shown to stimulate gastric motility and inhibit emesis (Sanger 2009). Several GI prokinetic drugs moved to the clinic before their pharmacology was fully understood. After the discovery of the 5-$HT_4$ receptor in 1988, Dumuis et al. discovered that benzamide derivates, such as cisapride, zacopride and metoclopramide, with GI prokinetic effect, actually acted as agonists on the newly found receptor (Dumuis et al. 1989). In 1989 cisapride (Prepulsid$^{TM}$) was marketed worldwide for the treatment of gastroparesis, gastro-oesophageal reflux disease and severe dyspepsia (De Maeyer et al. 2008b; Sanger 2009). After 10 years use in the clinic rare side-effects of cisapride including cardiac dysrhythmias based on QT interval prolongation emerged, leading to its withdrawal in 2000. These side effects are actually unrelated to activation of 5-HT receptors, but arise from the drugs activity at the human Ether-a-go-go Related Gene (hERG) potassium channel (De Maeyer et al. 2008b; Sanger 2009). Many of these prokinetic benzamides, including cisapride, are unselective and also show activity at several other receptors; the rank order of affinity for cisapride is 5-$HT_{2A}$ > 5-$HT_4$ = $\alpha_1$ > hERG > 5-$HT_3$ (De Maeyer et al. 2008b). The story of the cardiac side effects of cisapride brought to attention that there are indeed 5-$HT_4$ receptors present on human cardiac muscle, that need to be considered when developing 5-$HT_4$ receptor agonists for the treatment of motility disorders of the GI tract. More recently developed 5-$HT_4$ receptor agonists such as prucalopride, which was marketed in 2009 for the treatment of laxative-resistant chronic constipation, are highly specific and devoid of off-target effects. In vitro prucalopride behaves as a weak partial agonist on cardiac 5-$HT_4$ receptors (De Maeyer et al. 2006b). Clinically, no cardiovascular safety concerns were reported for patients using prucalopride (Camilleri et al. 2010; Tack et al. 2012).

## I.4 Agonists and antagonists

An agonist is a substance that binds to a receptor and gives the same response as the natural ligand for this receptor. Agonists are defined by their potency and efficacy towards a receptor. Potency refers to the amount of agonist needed to obtain a certain response, often indicated as $EC_{50}$ (agonist concentration needed to induce a half-maximal response). Efficacy refers to the maximal response ($E_{max}$) induced by a saturating concentration of agonist. A full agonist can maximally activate the receptor, while a partial agonist has lower efficacy and is not able to induce a maximal response even when occupying all receptors. An antagonist does not stimulate the receptor itself but blocks the action of agonists. When a receptor is constitutively active, it can shift between an active and an inactive state. An agonist then increases the proportion of receptors in the active state, while an inverse agonist silences the constitutive activity and increases the proportion of inactive receptors. An antagonist does not influence the degree of constitutive activity, but blocks the effect of both agonists and inverse agonists (see also section I.6.1).

Pharmacologically the 5-$HT_4$ receptor is defined by selective agonists such as BIMU8, ML10302 and RS67506 and selective antagonists such as GR113808, SB204070 and RS100235 (Hegde and Eglen 1996; Alexander et al. 2011). 5-$HT_4$ receptor agonists and antagonists belong to very different chemical classes: 5-HT and structurally related indoles (e.g. tegaserod), substituted benzamides (e.g. metoclopramide, cisapride, renzapride), substituted benzimidazolones (e.g. BIMU1, BIMU8), napthalimides (RS66331, RS56532), aryl ketones (RS67333, RS67506) and dihydrobenzofuran-carboxamides (e.g. prucalopride, R149402, R199715) (Hoyer et al. 1994; Eglen et al. 1995; Hegde and Eglen 1996; De Maeyer et al. 2006b). The potency and efficacy of 5-$HT_4$ receptor agonists is tissue specific, depending on the receptor density and splice variant composition in a given tissue. For example prucalopride acts as a full agonist in the stomach but is only a partial agonist in the left atrium of pigs (De Maeyer et al. 2006a).

## I.5 5-HT and its receptors in the cardiovascular system

5-HT receptors have been found in every major organ of the cardiovascular system and the effects of 5-HT are diverse. In vessels 5-HT can mediate both vasoconstriction and vasorelaxation. It is interesting to note that even though virtually all of the 5-HT in the blood is released by platelets (see section I.3), a full serotonergic system exists in peripheral arteries,

which are able to synthesize, take up, metabolize and release 5-HT (Beazely and Watts 2006; Watts 2009). In isolated left atrium 5-HT mediates both an inotropic and lusitropic response and in right atrium additionally an increase in heart rate is observed (Kaumann 1990; Medhurst and Kaumann 1993). Effects by 5-HT in the cardiovascular system are mediated by different receptor subtypes. This section will give an overview over the receptors found in the cardiovascular system (**Figure I.9**).

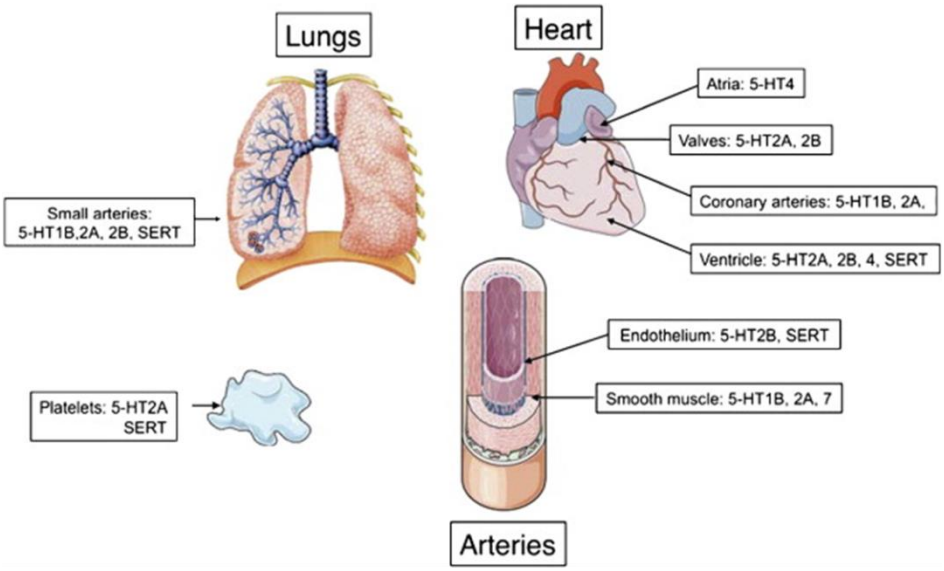


**Figure I.9** Distribution of 5-HT receptors and SERT in the rodent cardiovascular system. From Monassier et al. (2010)

### I.5.1 5-$HT_1$ receptors

Five receptors belong to this class (5-$HT_{1A}$, 5-$HT_{1B}$, 5-$HT_{1D}$, 5-$ht_{1e}$, 5-$HT_{1F}$), all coupling to $G_i/G_0$ and negatively regulating AC function (Kaumann and Levy 2006). 5-$HT_{1A}$ receptors are widely spread in the central nervous system, but were not reported to be present in the cardiovascular system. 5-$HT_{1B}$ receptors are abundantly expressed on endothelial cells and smooth muscle cells of several vessels including coronary and pulmonary arteries (Tepper et al. 2002; Kaumann and Levy 2006). 5-$HT_{1B}$ and $_{1D}$ receptors have been implicated in the pathophysiology of migraine. 5-$HT_{1B/1D}$ receptor agonists (triptans) have anti-migraine effects, promoting 5-$HT_{1B}$ receptor-mediated vasoconstriction of painfully dilated intracranial arteries while effects mediated by the 5-$HT_{1D}$ receptor are believed to be neuronal (Tepper et al. 2002; Kaumann and Levy 2006).

Indeed 5-$HT_{1D}$ receptors are mostly located in the central nervous system (CNS) and messenger RNA for the receptor was not found in human and rat endothelial and smooth muscle cells of blood vessels (Ullmer et al. 1995). No cardiovascular expression of the 5-$ht_{1e}$ receptor has been reported (Kaumann and Levy 2006). According to the International Union of Pharmacology (IUPHAR) nomenclature this receptor is depicted in lower case letters, since it is not fully characterized and no functional relevance was reported yet (Vanhoutte et al. 1996). 5-$HT_{1F}$ receptor agonists are also in development for the treatment of migraine. However, 5-$HT_{1F}$ receptor-mediated effects are neuronal and no vasoconstrictive activity of 5-$HT_{1F}$ receptor agonists was observed in cerebral and peripheral arteries (Neeb et al. 2010).

### I.5.2 5-$HT_2$ receptors

Three subtypes belong to this class, namely the 5-$HT_{2A}$ (corresponding to the originally described D receptor by Gaddum and Picarelli), 5-$HT_{2B}$ (first indicated as 5-$HT_{2F}$ for its high expression in fundus) and 5-$HT_{2C}$ (originally called 5-$HT_{1C}$) receptors. They all couple to $G_q$ or $G_{11}$ and primarily stimulate phospholipase C and elevate cytosolic [$Ca^{2+}$]. Although, members of this class contain introns at conserved positions in their coding sequence (Gerhardt and van Heerikhuizen 1997; Kaumann and Levy 2006), no functional splice variants have been detected as yet (Kaumann and Levy 2006). The 5-$HT_{2A}$ receptor is widely expressed in the CNS, in platelets and in vascular and uterine smooth muscle. Correspondingly 5-$HT_{2A}$ receptors have been implicated in a variety of physiological functions including platelet aggregation, vascular smooth muscle contraction and thrombus formation (Nagatomo et al. 2004). Isolated arteries (many were studied including coronary, pulmonary and mesenteric vessels) respond to 5-HT with constriction; this response is predominantly mediated by 5-$HT_{2A}$ and 5-$HT_{1B}$ receptors (Kaumann and Levy 2006; Watts et al. 2012). The 5-$HT_{2A}$ receptor might also contribute to systemic blood pressure regulation. The 5-$HT_{2A}$ receptor antagonist ketanserin was used clinically for the treatment of hypertension. However, the mechanism of action for ketanserin is controversial, as it also shows affinity for $\alpha_1$-adrenergic receptors and histamine $H_1$ receptors (Nagatomo et al. 2004; Monassier et al. 2010). Less is known about the action of 5-$HT_{2B}$ receptors. Originally they were found to mediate smooth muscle contraction in fundus of rats (Gerhardt and van Heerikhuizen 1997). Expression of 5-$HT_{2B}$ receptors in the cardiovascular system was detected in pulmonary artery endothelium, heart valves and ventricular myocytes (Fitzgerald et al. 2000; Nebigil et al. 2000; Kaumann and Levy 2006). The receptor received attention for its involvement in pulmonary hypertension and

heart valve disease caused by drugs like fenfluramines (appetite suppressants), which were subsequently removed from the market (Blanpain et al. 2003; Hutcheson et al. 2011). Fenfluramines cause release of 5-HT from platelets; furthermore their main metabolite norfenfluramine is a direct agonist at 5-$HT_{2B}$ and 5-$HT_{2C}$ receptors. Stimulation of 5-$HT_{2B}$ receptors on heart valve leaflets by these drugs via 5-HT and norfenfluramine leads to excessive mitosis and secretion of extracellular matrix components and ultimately causes stiffening and thickening of the valves (Elangbam 2010; Hutcheson et al. 2011). One particular problem with drugs such as anti-psychotics and anorexigens targeting 5-$HT_{2A}$ or 5-$HT_{2C}$ receptors in the brain is that they display activity at all three 5-$HT_2$ receptor subtypes. This lack of specificity is due to a high sequence homology and structural similarity between the three subtypes of 5-$HT_2$ receptors and can cause off-target effects on 5-$HT_{2B}$ receptors (Roth et al. 1998; Barnes and Sharp 1999; Hutcheson et al. 2011). To avoid such side effects, in silico models are now developed for new drugs which predict their toxicity on valves based on 5-$HT_{2B}$ receptor binding (Kim et al. 2011; Reid et al. 2013). There is also strong support for an important role of 5-$HT_2$ receptors in normal heart development, because genetic deletion of the receptor leads to incomplete cardiac development in mice (Nebigil et al. 2000; Hutcheson et al. 2011). Additionally 5-$HT_{2B}$ together with 5-$HT_7$ receptors were shown to mediate 5-HT-induced relaxation of pulmonary and coronary arteries of rat (Watts et al. 2012). The 5-$HT_{2C}$ receptor has been associated with motor behaviour and appetite modulation (Gerhardt and van Heerikhuizen 1997) but to our knowledge has not been found in the cardiovascular system.

### I.5.3 The 5-$HT_3$ receptor

Unlike the other 5-HT receptors, the 5-$HT_3$ receptor is a ligand gated ion channel similar to neuronal nicotinic acetylcholine receptors, and inhibitory neurotransmitter receptors for GABA and glycine (Kaumann and Levy 2006). The functional 5-$HT_3$ receptor consists of five subunits that are organized as a homopentamer or heteropentamer around the ion conducting pore. The first subunit identified was 5-$HT_3$A, which is the only subunit which can form functional homopentamers. All other subunits identified to date (5-$HT_3$B-E) are only functional as heteropentamers with 5-$HT_3$A (Lummis 2012). 5-$HT_3$ receptors are found in the central and peripheral nervous system where they mediate fast depolarization. 5-$HT_3$ antagonists are used in the clinic for the treatment of nausea and emesis in cancer patients (Lummis 2012). In the heart 5-$HT_3$ receptors were shown to mediate reflex bradycardia and hypotension in response

to intrapericardial 5-HT (the von Bezold-Jarisch reflex) (Pascual et al. 2003; Kaumann and Levy 2006).

### I.5.4 The 5-$HT_4$ receptor

This receptor is discussed in detail in section I.6.

### I.5.5 5-$ht_5$ receptors

The 5-$ht_5$ receptors are among the least studied of the 5-HT receptor family. Two receptors belong to this class, namely the 5-$ht_{5a}$ and 5-$ht_{5b}$ receptor. Both 5-$ht_{5a}$ and 5-$ht_{5b}$ are expressed in mouse and rat while in human only the 5-$ht_{5a}$ receptor is functional; the 5-$ht_{5b}$ receptor is present as a pseudogene but early stop codons interrupt expression of a functional protein (Thomas 2006). 5-$ht_{5a}$ receptors were shown to couple to $G_i/G_o$ and inhibit forskolin (AC activator) induced AC activity in HEK293 cells (Nelson 2004). These receptors are expressed in several brain areas but to date no evidence of 5-$ht_5$ receptor expression in the cardiovascular system exists.

### I.5.6 The 5-$HT_6$ receptor

5-$HT_6$ receptors were discovered quite late (in the early 1990's). They signal via $G_s$ to activate AC and mediate excitatory neurotransmission. 5-$HT_6$ receptors are expressed in various regions of the brain but there is no evidence of expression in the cardiovascular system so far (Woolley et al. 2004).

### I.5.7 The 5-$HT_7$ receptor

Three 5-$HT_7$ receptor splice variants were detected in both human (5-$HT_{7(a)}$, 5-$HT_{7(b)}$, 5-$HT_{7(d)}$) and rat (5-$HT_{7(a)}$, 5-$HT_{7(b)}$, 5-$HT_{7(c)}$, 5-$HT_{7(e)}$); all activate AC via coupling to $G_s$ (Vanhoenacker et al. 2000; Gellynck et al. 2013). 5-$HT_7$ receptors are mostly expressed in the brain, but peripheral expression has been detected in various blood vessels, including human coronary artery and

human vascular smooth muscle (Vanhoenacker et al. 2000; Kaumann and Levy 2006). It has been shown that 5-$HT_7$ receptor activation causes vasodilation in cranial blood vessels of rats, which could partly explain the beneficial effects of migraine prophylactic 5-HT receptor antagonists with high affinity for 5-$HT_7$ receptors (Vanhoenacker et al. 2000; Terron and Martinez-Garcia 2007). In organ bath experiments nearly every blood vessel responds to 5-HT with vasoconstriction, predominantly mediated by 5-$HT_{2A}$ and 5-$HT_{1B}$ receptors (Kaumann and Levy 2006; Watts et al. 2012). However, in some vessels also relaxant effects are observed, which were reported to be mediated by 5-$HT_{2B}$ and 5-$HT_7$ receptors (Watts et al. 2012).

## I.6 5-$HT_4$ receptors

5-$HT_4$ receptors preferentially couple to $G_s$, activating AC and promoting cAMP formation. 5-$HT_4$ receptors are wide-spread in the brain and in the periphery. In the brain 5-$HT_4$ receptors have been linked to functions such as memory and cognition and impaired signalling of the receptor might be implicated in pathologies like Alzheimer's disease, feeding disorders (e.g. anorexia nervosa) and depression (Bockaert et al. 2011). In the periphery effects of 5-HT mediated by these receptors have been described in the GI tract, the heart, the bladder and adrenal gland. In the GI tract 5-HT stimulates excitatory motor neurons in the myenteric plexus, facilitating the release of the contractile neurotransmitter acetylcholine and subsequently increasing contractility (De Maeyer et al. 2008b). Several 5-$HT_4$ receptor agonists have been used in the clinic for the treatment of motility disorders of the GI tract (Tonini 2005; De Maeyer et al. 2008b). 5-$HT_4$ receptors are expressed on cardiomyocytes in human and porcine atrium and to a lower extent in ventricle. Stimulation of atrial 5-$HT_4$ receptors leads to positive inotropic and lusitropic responses in left atrium (Kaumann 1990; Sanders and Kaumann 1992) and additionally increases chronotropy in right atrium of pig and man (Villalon et al. 1990; Medhurst and Kaumann 1993).

### I.6.1 Protein and gene structure

As mentioned earlier 5-$HT_4$ receptors belong to the family of seven-transmembrane spanning G protein-coupled receptors. 5-$HT_4$ receptors were shown to behave as dimers or oligomers and full G protein activation by the receptor dimer requires the stimulation of both receptor

protomers (Berthouze et al. 2005; Pellissier et al. 2009; Pellissier et al. 2011). Berthouze et al. (2007) found that the transmembrane 4 subunit (TM4) is important for receptor dimerization, involving covalent and hydrophobic interactions. 5-$HT_4$ receptors C-terminally contain several sites for post-translational modifications and undergo agonist dependent cysteine palmitoylation and serine phosphorylation (Ponimaskin et al. 2001; Ponimaskin et al. 2005). Palmitoylation of the 5-$HT_{4(a)}$ receptor affects the phosphorylation of the receptor by G protein-coupled receptor kinases (GRKs), which in turn regulates desensitization and β-arrestin dependent internalization (also see section I.6.2) (Ponimaskin et al. 2001; Ponimaskin et al. 2005).

In the classic model GPCRs are in an inactive R state in the cell membrane. Agonist binding (for definition see section I.4) shifts the receptor conformation towards the G-protein activating R* state. However, recent studies on recombinant 5-$HT_4$ receptors in model systems and on the purified protein showed that 5-$HT_4$ receptors exist in at least three states: a $R_g$ (ground) state, which is a state of complete inactivation, stabilized by inverse agonists; an R state which can with low efficacy activate G proteins and is responsible for the constitutive activity of the receptor and an R* state which is supported by full agonists (Bender et al. 2000; Joubert et al. 2002; Baneres et al. 2005). Indeed some 5-$HT_4$ receptor splice variants (especially the short C-terminal isoforms) show a relatively high constitutive activity (Mialet et al. 2000b; Bockaert et al. 2004; Chang et al. 2007). Evidence suggests that receptor palmitoylation is important for the regulation of constitutive activity (Ponimaskin et al. 2002).

The 5-$HT_4$ receptor gene is very large (700 kb) and complex. It comprises 38 exons (see **Figure I.10**) and in most species multiple splice variants also called 'isoforms' are formed. Isoforms are made by a post-transcriptional process called alternative splicing. The pre-RNA is modified; introns are removed and combinations of alternative exons are joined to generate different products (Coupar et al. 2007). 5-$HT_4$ receptor splice variants are potentially interesting for the development of new drugs; tissue specific isoforms could be targeted to increase the specificity and safety of the drug. In human at least 11 5-$HT_4$ receptor splice variants are expressed. The divergence of the splice variants starts after amino acid $Leu^{358}$, giving rise to intracellular COOH-terminal splice variants, with the exception of h5-$HT_{4(hb)}$, which has an internal insertion in the second extracellular loop and was only observed in combination with the 5-$HT_{4(b)}$ C-terminal tail (Kaumann and Levy 2006). The most common 5-$HT_4$ receptor splice variants are 5-$HT_{4(a)}$ and 5-$HT_{4(b)}$, which were found in the heart, the brain, the kidney and the GI tract (Coupar et al. 2007).

5-$HT_4$ receptor splice variants found in human atrium are a, b, c, e, g, i and n and in human ventricle a, b, g and i. (Blondel et al. 1998; Mialet et al. 2000a; Bach et al. 2001; Vilaro et al. 2002; Brattelid et al. 2004a; Coupar et al. 2007). Some splice variants show pronounced tissue specificity, for example the 5-$HT_{4(d)}$ isoform has only been found in human small intestine and colon (Coupar et al. 2007). Furthermore the splice variant expression was found to be different in pig compared to man, indicating species specificity (De Maeyer et al. 2008a).

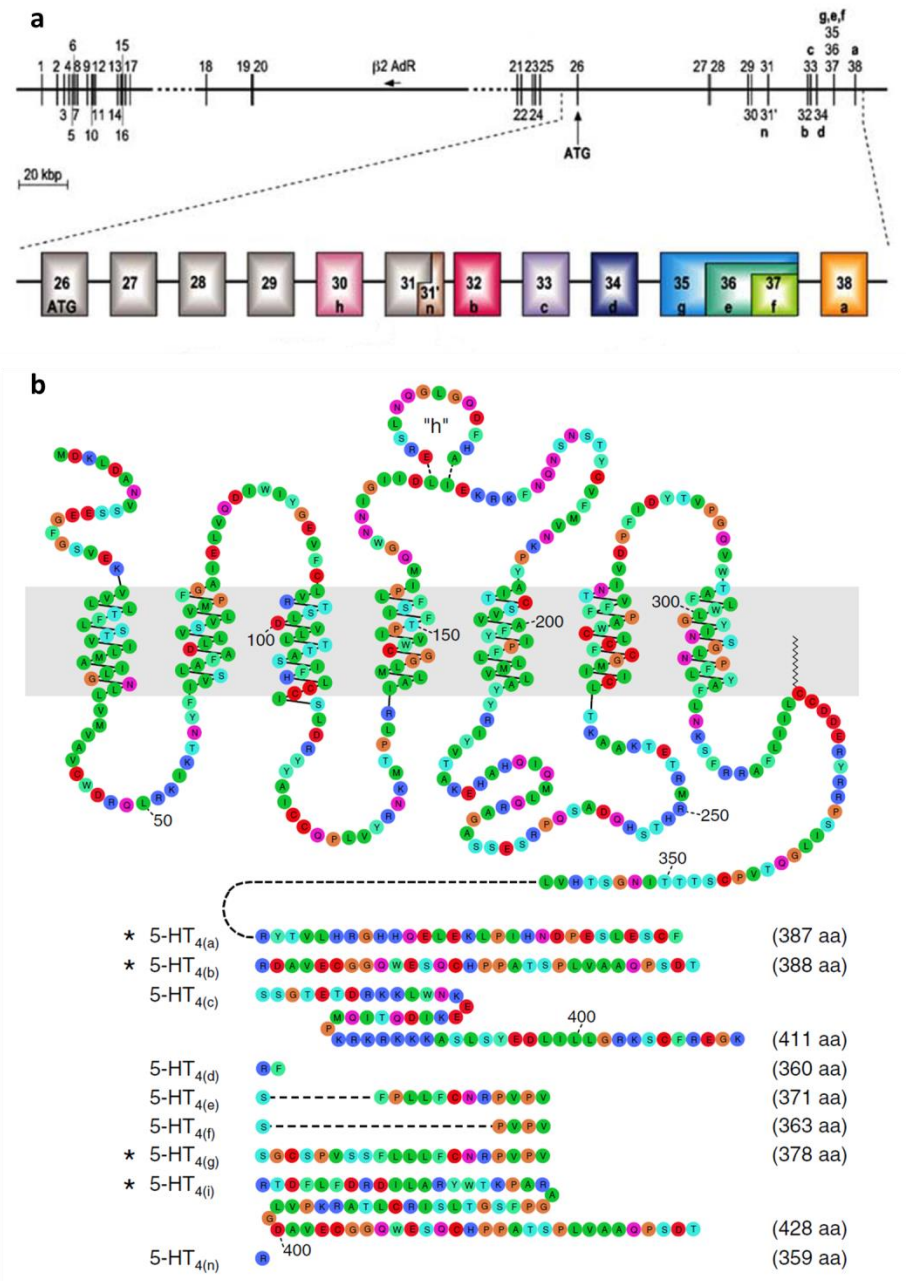


**Figure I.10** 5-$HT_4$ receptor gene structure and splice variants, not including the 5-$HT_4$ "short" c splice variant. **a**) Organization of the human 5-$HT_4$ receptor gene; **b**) Human 5-$HT_4$ receptor splice variants. From Bockaert et al. 2004 and Kaumann and Levy, 2006

### I.6.2 Coupling to G proteins and desensitization

The observed tissue and species specific differences in 5-$HT_4$ receptor splice variant expression gain importance in light of evidence suggesting that splice variants have different properties, with regard to agonist potency, coupling to G proteins and desensitization (Bockaert et al. 2004; Coupar et al. 2007; Irving et al. 2010). A study in human embryonic kidney (HEK) cells has shown that the 5-$HT_4$ receptor agonist 5-methoxytryptamine is more potent at 5-$HT_{4(a)}$ compared to 5-$HT_{4(b)}$ receptors. Additionally pertussis toxin (an inhibitor of $G_i$ proteins) increased cAMP levels in response to 5-$HT_{4(b)}$ receptor stimulation, suggesting a coupling of 5-$HT_{4(b)}$ not only to $G_s$ but also to $G_i$; this was not observed for the 5-$HT_{4(a)}$ receptor (Pindon et al. 2002). Further evidence for this dual coupling of the 5-$HT_{4(b)}$ receptor comes from a study overexpressing the receptor in rat cardiomyocytes, where pertussis toxin potentiated the effect of 5-HT on L-type $Ca^{2+}$ currents (Castro et al. 2005).

Receptor desensitization is a multistep process leading to a decreased functional response in the continued presence of the receptor agonist. Four main steps can be distinguished, which are: 1) uncoupling of the receptor from G proteins which attenuates the primary response (eg. generation of cAMP), 2) endocytosis/internalization of the receptor (removal of the receptor from the cell membrane) followed by 3) resensitization or 4) degradation of the receptor (Barthet et al. 2005). Uncoupling was found to be mainly dependent on GPCR kinase 2 (GRK2), but without requiring the kinase activity of GRK2 (Barthet et al. 2005; Ponimaskin et al. 2005). In contrast endocytosis requires phosphorylation of the receptor by GRK2 followed by recruitment of β-arrestins (Barthet et al. 2005; Ponimaskin et al. 2005). 5-$HT_4$ receptor desensitization was found to be rapid in mouse colliculus neurons but rather slow in most cell systems such as COS-7 and HEK293 cells, possibly because these cells express low levels of endogenous GRK2 (Barthet et al. 2005). Furthermore De Maeyer et al. (2009) found a marked desensitization of 5-$HT_4$ receptor-mediated responses in pig atrium but not in pig stomach. These differences may result from different expression levels of GRK2 and β-arrestins and may also reflect differences in the composition of 5-$HT_4$ receptor splice variants (Barthet et al. 2005; Ponimaskin et al. 2005). Indeed splice variant dependent differences in internalization were described. Pindon et al. (2004) showed in HEK293 cells that the 5-$HT_{4(a)}$ receptor is not internalized after agonist treatment, while the 5-$HT_{4(b)}$ receptor underwent a time-dependent internalization after agonist treatment; this difference is not due to the $G_i$ coupling of the 5-$HT_{4(b)}$ receptor, since it also

occurred in the presence of pertussis toxin (PTX). However, another study did observe the same internalization pattern for 5-$HT_{4(a)}$, $_{(b)}$ and $_{(e)}$ splice variants (Barthet et al. 2005).

### I.6.3 5-$HT_4$ receptor signalling pathway and regulation

5-$HT_4$ receptors predominantly couple to $G_s$, thereby increasing cAMP and activating PKA (Kaumann et al. 1990; Kaumann et al. 1991; Sanders and Kaumann 1992). $G_{\alpha s}$ mediated increases in cAMP concentration in turn activate PKA. PKA phosphorylates a wide range of proteins, which differ according to the type of cell, leading to different functional outcomes. It has been shown that 5-$HT_4$ receptor stimulation inhibits voltage-activated $K^+$ current in colliculi neurons (Fagni et al. 1992; Ansanay et al. 1995) and modulates GABAergic signalling in neurons of the prefrontal cortex (Cai et al. 2002) via activation of PKA. Direct stimulation of 5-$HT_4$ receptors on colon smooth muscle leads to a PKA-dependent relaxation (McLean et al. 1995; McLean and Coupar 1996). 5-$HT_4$ receptor stimulation in heart muscle increases contractility through increased PKA mediated phosphorylation of the contractile proteins phospholamban and troponin I (Gergs et al. 2009). Furthermore PKA-independent effects of 5-$HT_4$ stimulation were described. In HEK293 cells stimulation of overexpressed 5-$HT_4$ receptors produced a transient activation of the ERK1/2 pathway, an important pathway involved in learning and memory (Norum et al. 2003; Barthet et al. 2007). This activation of ERK1/2 was shown to be independent of $G_s$/cAMP/PKA, but might require Src tyrosine kinase, which associates with the 5-$HT_4$ receptor (Barthet et al. 2007). The GTPase Ras, acting down-stream of Src tyrosine kinase, was shown to also be involved in the 5-$HT_4$ receptor mediated stimulation of the ERK1/2 pathway (Norum et al. 2003). In line with this 5-$HT_4$ receptor-mediated activation of the ERK pathway is inhibited by GPCR kinase 5 (GRK5) mediated phosphorylation of β-arrestin1, which in turn prevents an activation of Src tyrosine kinase (Barthet et al. 2009).

#### I.6.3.1 Regulation by PDEs

As the only means of cAMP degradation, PDEs play an important role in the regulation of predominantly $G_s$ coupled 5-$HT_4$ receptor-mediated signalling. 5-$HT_4$ receptor stimulation causes an inotropic response in porcine atrium which fades very quickly with time. This fade can be entirely prevented by inhibition of both PDE3 and PDE4 (Galindo-Tovar et al. 2009), while in

human atrium and failing rat ventricle 5-$HT_4$ receptor-mediated responses are regulated solely by PDE3 (Afzal et al. 2008; Galindo-Tovar et al. 2009) (also see section I.7 5-$HT_4$ receptors in the heart). Regulatory effects of PDEs on 5-$HT_4$ receptor-mediated responses have also been observed in other tissues. In pig stomach the facilitating effect of 5-$HT_4$ receptor stimulation on cholinergic neurotransmission is regulated by PDE4 (Priem et al. 2012). In human colon the relaxant effect of 5-$HT_4$ receptor stimulation was shown to be enhanced in the presence of PDE4 inhibition (McLean and Coupar 1996).

#### I.6.3.2 Influence of elevated cGMP

Influence of cGMP signalling, mediated by PDE2 and PDE3, on cAMP-mediated signal transduction pathways, has been suggested (Zaccolo and Movsesian 2007). Indeed the cAMP-mediated response to 5-$HT_4$ receptor activation was shown to be influenced by cGMP elevating agents. Afzal et al. (2011b) 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 (Afzal et al. 2011b). Furthermore, endogenous NO signalling, via NOS and sGC, was shown to augment the 5-$HT_4$ receptor mediated inotropic response in failing rat heart; this was also proposed to be mediated through an inhibition of PDE3 (Afzal et al. 2011b).

#### I.6.3.3 Involvement of AKAPs and caveolae

AKAPs are important in the regulation of β-adrenergic signalling in the heart. However, to our knowledge no studies exist on whether AKAPs are involved in 5-$HT_4$ receptor-mediated signalling.

Caveolae were reported to play an important role in the signalling of many GPCR, including β-adrenergic receptors (as discussed in detail) but also $\alpha_1$-adrenoceptors, muscarinic receptors and prostaglandin receptors (Harvey and Calaghan 2011). However, no reports about an involvement of caveolae in 5-$HT_4$ receptor signalling exist to date.

## I.7 5-HT$_4$ receptors in the heart

The receptor type by which 5-HT mediates inotropic, lusitropic and chronotropic effects in the heart is species-dependent. For example 5-HT increases heart rate through activation of 5-HT$_{2A}$ receptors in the rat, 5-HT$_4$ receptors in the pig and human, and 5-HT$_7$ receptors in the cat (Watts et al. 2012). Therefore the lab animal options to study responses to 5-HT in the heart are limited. Next to humans only pigs and monkeys were shown to express functional 5-HT$_4$ receptors in healthy heart (Kaumann 1990). The 5-HT$_4$ receptor was shown to be the only functional receptor for 5-HT in pig and human heart (Lorrain et al. 1992; Medhurst and Kaumann 1993; Parker et al. 1995). In the rat functional 5-HT$_4$ receptors are present in late foetal development and are reactivated in heart failure (Qvigstad et al. 2005; Brattelid et al. 2012). In human failing heart an increase of 5-HT$_4$ receptor mRNA was detected, suggesting an up-regulation of 5-HT$_4$ receptor-mediated signalling in heart failure (Brattelid et al. 2004b).

Functional 5-HT$_4$ receptors were first detected in human and porcine atrium, but in these early studies no evidence for their presence in the ventricle was obtained (Lorrain et al. 1992; Sanders and Kaumann 1992; Schoemaker et al. 1992). 5-HT was shown to mediate an increase in contraction force as well as increased relaxation in left atrium of pig and human (Sanders and Kaumann 1992; Parker et al. 1995). Additionally in right atrium an increase in heart rate in response to 5-HT was observed (Kaumann 1990; Kaumann et al. 1991; Medhurst and Kaumann 1993). The mechanism by which 5-HT increases inotropy and lusitropy via 5-HT$_4$ receptors is similar to β-adrenergic receptors. 5-HT raises cAMP production and PKA activity in human atrium resulting in increased phosphorylation of the contractile proteins PLB and TnI (Kaumann et al. 1990; Gergs et al. 2009). Additionally it has been shown that 5-HT$_4$ receptor activation in human atrial myocytes and failing rat ventricular myocytes PKA-dependently increases L-type $Ca^{2+}$ current and SR $Ca^{2+}$ content (Ouadid et al. 1992; Birkeland et al. 2007). The chronotropic response to 5-HT$_4$ receptor stimulation can be explained by the direct effect of cAMP on hyperpolarization-activated cyclic nucleotide-gated (HCN) cation channels which causes an increase in inward pacemaker current $I_f$ in sinoatrial myocytes (DiFrancesco and Tortora 1991; Pino et al. 1998; Ulens and Tytgat 2001; Lonardo et al. 2005). The activity of 5-HT on these channels as well as on L-type $Ca^{2+}$ channels was suggested to contribute to the development of experimental arrhythmias observed in porcine and human right atrium after 5-HT$_4$ receptor stimulation, presumably by $Ca^{2+}$ overload; this was facilitated by chronic β-adrenergic receptor blockade (Villalon et al. 1990; Kaumann and Sanders 1994; Pino et al. 1998; Workman and

Rankin 1998; Pau et al. 2003). Later mRNA for 5-$HT_{4(a)}$ and 5-$HT_{4(b)}$ receptors was detected in human atrium and ventricle (Bach et al. 2001), indicating an existence of the receptor also in the ventricle. Finally functional 5-$HT_4$ receptors were uncovered in pig and human ventricle by phosphodiesterase inhibition (Brattelid et al. 2004b). In concordance with the smaller functional effects in ventricle, 5-$HT_4$ receptor expression is lower in human ventricle compared to atrium (Blondel et al. 1997; Gergs et al. 2009). Important age related differences in 5-$HT_4$ receptor-mediated responses were observed. In the atrium and ventricle of newborn piglets 5-HT was less potent and efficacious in inducing an inotropic response in the presence of IBMX than in young (~3 months old) pigs (Brattelid et al. 2004b; De Maeyer et al. 2006b). It was suggested that this might be due to an increase in 5-$HT_4$ receptor expression during development (De Maeyer et al. 2006b). Compared to β-adrenergic receptors the expression level of 5-$HT_4$ receptors is very low in the atrium of newborn piglets (174 and 22 times lower than $\beta_1$- and $\beta_2$-adrenergic receptors respectively), while in adult human atrium the expression level of 5-$HT_4$ receptors is only 10 and 5 times lower than that of $\beta_1$- and $\beta_2$-adrenergic receptors respectively (Kaumann et al. 1995; Kaumann et al. 1996). Another possibility is that the coupling of 5-$HT_4$ receptors to their effectors might become more effective during development (De Maeyer et al. 2006b).

PDEs clearly play an important role in 5-$HT_4$ receptor signalling in the heart. In the left atrium of human and pig, PDEs cause a quick fading of the inotropic response to 5-HT (De Maeyer et al. 2006b; Galindo-Tovar et al. 2009) and in the ventricle they completely prevent a functional response to 5-HT (Brattelid et al. 2004b). Galindo-Tovar et al. (2009) found that the PDE subtypes controlling 5-$HT_4$ receptor-mediated responses are PDE3 and PDE4 together in porcine heart and PDE3 in human heart, with another mechanism (possibly receptor desensitization) also involved. In human failing heart responses to 5-HT are also predominantly controlled by PDE3, however in this condition an additional role of PDE4 emerged; a similar control by PDEs was found in failing rat heart (Afzal et al. 2008). Interestingly a change in the control of 5-$HT_4$ receptor-mediated responses was observed during development. In the atrium of newborn piglets, PDE4 was the predominant PDE subtype controlling the inotropic response to 5-HT, while in the ventricle it was PDE3. During development this control changed and both PDE3 and PDE4 controlled inotropic responses to 5-HT in the atrium and ventricle of adolescent pigs (Galindo-Tovar et al. 2009). Unlike inotropic responses, lusitropic responses in porcine left and right atrium are less sensitive to regulation by PDEs, as they do not fade (De Maeyer et al. 2006b). As mentioned earlier 5-$HT_4$ receptor activation increases phosphorylation and activation

of the primarily relaxant proteins PLB and TnI. Both proteins have slow dephosphorylation kinetics, which might explain the prolonged lusitropic response to 5-$HT_4$ receptor stimulation (Garvey et al. 1988). PDEs were shown to play an important role in the control of basal sinoatrial beating rate, nevertheless chronotropic responses to 5-HT are not affected by PDE-inhibition (De Maeyer et al. 2006b; Vinogradova et al. 2008; Galindo-Tovar et al. 2009). This suggests that in sinoatrial cells the cAMP compartment mediating responses to 5-HT is protected from the action of PDEs, while PDEs are active in a distinct compartment where they regulate basal heart rate.

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# Chapter II

## *Aims*

# *II Aims*

Soon after the discovery of the 5-$HT_4$ receptor in colliculus neurons in 1988 (Dumuis et al. 1988), the receptor was also found in the atrium and in the GI tract (Eglen et al. 1990; Kaumann et al. 1990). 5-$HT_4$ receptors in the GI tract received a lot of attention because agonists acting on this receptor have prokinetic effects (Dumuis et al. 1989). The 5-$HT_4$ receptor agonist cisapride (Prepulsid®), marketed for the treatment of several GI motility disorders, was removed from the market in 2000 due to some rare cardiac side effects. Although these side effects arose from an unspecific effect of the drug and were unrelated to activation of cardiac 5-$HT_4$ receptors, this brought more attention to the 5-$HT_4$ receptor in the heart. New, more selective, 5-$HT_4$ receptor agonists were developed for the treatment of GI disorders. One of them being prucalopride (Resolor®), which was marketed in 2009 for the treatment of laxative-resistant chronic constipation. Prucalopride was shown to fully stimulate 5-$HT_4$ receptors in the gut, but gives only a submaximal response in the heart, increasing the safety profile of the drug (De Maeyer et al. 2009). However, in order to completely exclude cardiac side effects it is important to fully understand 5-$HT_4$ receptor signalling in the heart.

Yet the role of 5-$HT_4$ receptors in heart physiology is still not known very well. β-adrenergic receptors were studied extensively for their importance in sympathetic stimulation of heart function (Motomura et al. 1990; Brodde et al. 2001). Like β-adrenergic receptors, 5-$HT_4$ receptors mediate positive inotropic and lusitropic responses in left and right atrium (Kaumann et al. 1990; Sanders and Kaumann 1992). Additionally chronotropic responses were observed in the right atrium (Villalon et al. 1990; Medhurst and Kaumann 1993). Both β-adrenergic receptors and 5-$HT_4$ receptors are $G_s$ coupled receptors which signal via the production of the second messenger cAMP and subsequent activation of PKA. However, compared to $\beta_1$-receptor-mediated responses, 5-$HT_4$ receptor-mediated inotropic responses are rather weak and short lasting. This might reflect the lower expression level of 5-$HT_4$ receptors (Kaumann et al. 1995; Kaumann et al. 1996) but mostly is due to a strict regulation of cardiac 5-$HT_4$ receptor signalling by phosphodiesterases (PDEs), which by breaking down cAMP diminish inotropic responses in the atrium and prevent a response in the ventricle (Brattelid et al. 2004; De Maeyer et al. 2006; Galindo-Tovar et al. 2009). PDEs are a superfamily of at least 11 subtypes, and PDE3 and PDE4 subtypes were found to control 5-$HT_4$ receptor signalling in the heart (Fischmeister et al. 2006; Afzal et al. 2008; Galindo-Tovar et al. 2009; Weninger et al. 2012). However, PDEs might not be the only regulatory mechanism for 5-$HT_4$ receptor signalling. Elevations in cGMP can influence

cAMP signalling via the cGMP-activated PDE2 and cGMP-inhibited PDE3 (Tsai and Kass 2009). Furthermore A-kinase anchoring proteins (AKAPs), which scaffold signaling molecules such as PKA and PDEs to distinct subcellular locations, were found to be important in β-adrenergic signaling (Dessauer 2009). Their possible role in 5-$HT_4$ receptor signaling was not yet investigated.

Therefore the aim of this thesis was to further unravel 5-$HT_4$ receptor signalling in the heart, investigating the role of PDE subtypes, the possible interaction between cAMP and cGMP signalling events and the possible role of AKAPs in compartmentalized 5-$HT_4$ receptor signalling.

Specifically the aims were as follows:

- In a first project (**chapter III**) the role of PDE subtypes in 5-$HT_4$ receptor signalling was further investigated by studying the influence of PDE2, 3, 4 and 5 subtype inhibitors on the inotropic response to 5-HT and prucalopride in pig atrium. The influence of PDE inhibition once the inotropic response had completely faded was also studied. Secondly, the influence of cGMP on 5-$HT_4$ receptor signalling was investigated by elevating cellular cGMP through activation of particulate guanylyl cyclase (pGC) and soluble guanylyl cyclase (sGC) and through inhibition of PDE2 and PDE5, which both hydrolyse cGMP. Thirdly, the possible role of caveolae and $G_i$ proteins in containing the response to 5-HT was investigated.
- In a second project (**chapter IV**) in pig atrium the role of PDEs and the influence of elevated cGMP by activation of pGC and sGC on 5-$HT_4$ receptor signalling was further evaluated by taking a closer look at the 5-$HT_4$ receptor signalling pathway. This was done by measuring tissue cAMP and cGMP content as well as the phosphorylation levels of the contractile proteins phospholamban and troponin I, down-stream targets of PKA which showed increased phosphorylation after activation of β-adrenergic and 5-$HT_4$ receptors in human heart (Bartel et al. 1996; Gergs et al. 2009).
- In the final project (**chapter V**) we aimed to detect proteins interacting with the 5-$HT_4$ receptor by immunoprecipitation. For that the tagged 5-$HT_{4(b)}$ receptor splice variant was overexpressed in a human cell line (HEK293). Investigated as potential interaction partners of the 5-$HT_4$ receptor were PDEs, as they limit signalling through the receptor and are therefore likely to co-localize with it, and AKAPs, known to co-localize with the $\beta_2$-adrenergic receptor and playing an important role in receptor signalling.

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# Chapter III

## *Study of the regulation of the inotropic response to 5-$HT_4$ receptor activation via phosphodiesterases and its cross-talk with C-type natriuretic peptide in porcine left atrium*

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# *III Study of the regulation of the inotropic response to 5-HT$_4$ receptor activation via phosphodiesterases and its cross-talk with C-type natriuretic peptide in porcine left atrium*

## III.1 Abstract

**Purpose**. We studied how 5-HT$_4$ receptor-mediated inotropic responses are regulated at the level of cAMP in porcine left atrium. We used selective PDE inhibitors, to assess which PDE-subtypes are responsible for the fade with time of inotropic responses to 5-HT$_4$ receptor activation with 5-HT and the 5-HT$_4$ receptor agonists prucalopride and RS67333. A possible cross-talk via PDEs between cGMP and 5-HT$_4$ receptor-induced cAMP signalling was evaluated. Furthermore we tested the possible influence of caveolae and $G_i$ proteins on responses to 5-HT.

**Methods**. Electrically paced left atrial pectinate muscles from young male pigs (15-25 kg) were studied *in vitro*.

**Results**. Simultaneous inhibition of PDE3- plus PDE4-subtypes was necessary to increase the amplitude and completely prevent the fade of the inotropic response to 5-HT and prucalopride. In this condition an inotropic response to RS67333 was uncovered as well. When responses to 5-HT or prucalopride had faded one hour after addition, the nonspecific PDE-inhibitor IBMX still fully recovered inotropic responses. Stimulation of particulate guanylyl cyclase, together with PDE2 and PDE4 inhibition, delayed the fade of the response to 5-HT, while stimulation of soluble guanylyl cyclase independently of PDEs accelerated the fade of the response to 5-HT.

**Conclusions**. Both PDE3- and PDE4-subtypes are responsible for the suppression and the fade of the inotropic response to 5-HT, prucalopride and RS67333. Signalling through the 5-HT$_4$ receptor remains fully active for at least 90 min with PDEs continuously regulating the response. cGMP levels, elevated by activation of particulate guanylyl cyclase under PDE2 inhibition, can indirectly enhance 5-HT$_4$ receptor-mediated signalling, at least when also PDE4 is inhibited, presumably through inhibition of PDE3. Elevation of cGMP generated by soluble guanylyl cyclase attenuates responses to 5-HT independently of PDEs.

**Keywords**. Porcine atrium, 5-$HT_4$ receptor, PDE, cAMP/cGMP signalling, prucalopride, serotonin

**Abbreviations**. CNP, C-type natriuretic peptide; IBMX, isobutylmethylxanthine; MβC, methyl-beta-cyclodextrin; pGC, particulate guanylyl cyclase; PDE, phosphodiesterase; PKA, protein kinase A; PTX, pertussis toxin; sGC, soluble guanylyl cyclase

## III.2 Introduction

Serotonin exerts its action through a number of 5-HT receptors, of which only the 5-$HT_4$ receptor subtype is functionally present in human atrium (Blondel et al. 1997). The pig is one of the few species to possess atrial 5-$HT_4$ receptors and porcine atrium is considered a good model for human atrial 5-$HT_4$ receptors (Kaumann et al. 1990; Schoemaker et al. 1992; Medhurst and Kaumann 1993). Activation of G protein-coupled 5-$HT_4$ receptors starts a complex signalling cascade involving cAMP-mediated activation of protein kinase A (PKA) that ultimately leads to positive inotropic and lusitropic responses in left and right atrium (Kaumann et al. 1990; Sanders and Kaumann 1992). In addition activation of sinoatrial 5-$HT_4$ receptors also promotes chronotropic effects (Villalon et al. 1990; Medhurst and Kaumann 1993).

In a given tissue, several agents can stimulate the cAMP pathway via different receptors but the functional responses can be quite different (Steinberg and Brunton 2001). This can be explained by a compartmentation of the signal (Lissandron and Zaccolo 2006). Phosphodiesterases (PDEs), which represent the sole mechanism of cAMP degradation in cells, undoubtedly play an important role in limiting cAMP diffusion and therefore keeping the signal localized (Fischmeister et al. 2006). PDEs in porcine heart can be grouped into at least 4 families: PDE2 and PDE3, with dual specificity for both cAMP and cGMP; PDE4 which is selective for cAMP; and PDE5, which selectively hydrolyses cGMP (Zimmermann et al. 1994; Jakobsen et al. 2006). The importance of PDEs in cardiac 5-$HT_4$ receptor signalling is shown by the fact that the fade of the inotropic response to 5-$HT_4$ receptor activation by 5-HT (in contrast to $\beta_1$-receptor mediated responses induced by isoprenaline) in porcine atrium can be prevented by non-selective PDE inhibition (De Maeyer et al. 2006). Galindo-Tovar et al. (2009) found that the fade of the inotropic response to 5-HT in porcine atrium is prevented by jointly blocking PDE3 and PDE4 isoforms, while in human atrium PDE3, but not PDE4, is partly responsible for fading of the response with another mechanism (possibly receptor desensitization) also adding to this fade. We have shown before in porcine atrium that the inotropic response to the selective 5-$HT_4$ receptor agonist prucalopride is very weak compared to that to 5-HT, but in the presence of the non-selective PDE inhibitor isobutylmethylxanthine (IBMX), a clear-cut sustained response was unmasked (De Maeyer et al. 2006); the PDE subtypes involved were not yet investigated.

PDEs were also reported to play an important role in the cross-talk between cGMP and cAMP signalling pathways (Zaccolo and Movsesian 2007). cGMP synthesis is catalyzed by two types of guanylyl cyclase, the nitric oxide (NO) activated soluble guanylyl cyclase (sGC) and the

membrane-bound particulate guanylyl cyclase (pGC), which is activated by natriuretic peptides. Elevations of cGMP can influence cAMP signalling particularly via the cGMP-activated PDE2 and cGMP-inhibited PDE3 (Tsai and Kass 2009). It was shown in human and frog cardiac myocytes that small elevations in cGMP, evoked by an NO donor or an intracellular application of cGMP, modulate cAMP levels and stimulate $I_{Ca}$ through inhibition of PDE3, while higher concentrations of cGMP counteract this effect by activation of PDE2 (Mery et al. 1993; Vandecasteele et al. 2001). Furthermore Afzal et al. (2011) demonstrated that activation of pGC and sGC increased the 5-$HT_4$ receptor mediated inotropic response in failing rat ventricle, presumably through inhibition of PDE3.

Several authors (Okamoto et al. 1998; Gratton et al. 2004; Calaghan and White 2006) suggested a role of caveolae in compartmentation of the cAMP signal. Caveolae are cholesterol enriched membrane structures that contain the protein caveolin. Cardiac $\beta_2$-adrenoceptors localize in caveolae as well as many downstream components of this pathway (Steinberg 2004; Head et al. 2005). Calaghan et al. (2008) showed that the disruption of caveolae changes the $\beta_2$-adrenoceptor induced response from one confined at the sarcolemma to a global cAMP signal. For some receptors (a prominent example being the $\beta_2$-adrenoceptor), G protein switching from $G_s$ to $G_i$ also represents a mechanism of limiting the amount of cAMP signal generated (Baillie et al. 2003), because $G_i$ inhibits adenylate cyclase.

In this study we sought to further analyse the control of 5-$HT_4$ receptor signalling in porcine atrium. First, the role of PDE subtypes was further investigated by studying the influence of PDE2, 3, 4 and 5 inhibitors on the inotropic response to 5-HT but also to the selective 5-$HT_4$ receptor agonists prucalopride and RS67333. The influence of PDE inhibition once the inotropic response had completely faded was also studied. Secondly, the influence of cGMP on cAMP mediated 5-$HT_4$ receptor signalling was investigated by elevating cellular cGMP through activation of pGC and sGC and through inhibition of PDE2 and PDE5, which both hydrolyse cGMP. Thirdly, the possible role of caveolae and $G_i$ proteins in containing the response to 5-HT was investigated by use of methyl-β-cyclodextrin and pertussis toxin, respectively.

## III.3 Methods

### III.3.1 Tissue preparation

The study was approved by the Ethical Committee for Animal Experiments from the Faculty of Medicine and Health Sciences at Ghent University. Young 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 Zoletil 100 from Virbac Animal Health (Carros, France). After exsanguination the heart was rapidly dissected and washed free of blood in Krebs-Henseleit solution (composition in mM: 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 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, Spechbach, Germany). Electrical field stimulation was performed using a constant voltage stimulator (Janssen Pharmaceutica, Belgium).

### III.3.2 Experimental protocols

Isometric force was calibrated in gram (g). Resting load of left atrial pectinate muscles was set to 2 g. Muscle contractions were stimulated with square-wave pulses (0.5 Hz, 5 ms duration, 4 V). During an equilibration time of 90 min the buffer in the organ bath was changed every 15 min. After equilibration 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, compounds were added (details on drugs, concentrations and incubation times are given in the Results section). After the incubation period, 5-HT or the 5-$HT_4$ receptor agonists prucalopride and RS67333 were added in a concentration of 1 µM, unless otherwise indicated;

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 5HT and prucalopride and for RS67333 was based on concentration-response curves established in the actual study (see Results). The effect of pertussis toxin was studied on responses to 5-HT and on carbachol as control. Responses were recorded for 60 min. After that time the 5-$HT_4$ receptor antagonist GR113808 (0.1 µM) was added. After another 30 min a saturating concentration of isoprenaline (100 µM) was given, still in the presence of all previously added compounds. At the end of the experiment the $CaCl_2$ concentration was elevated to 7 mM.

Deviations from this protocol were made for the assessment of the activation status of the 5-$HT_4$ receptor with time, where the protocol was as follows: 60 min after addition of 5-HT or prucalopride, IBMX was administered followed after 30 min by GR113808, and vice versa (GR113808 at 60 min and IBMX 30 min later).

### III.3.3 Data analysis

Isometric force measurements (mean of 10-14 contractions) were taken successively during the protocol (before addition of PDE-inhibitors, before 5-$HT_4$ receptor activation, at multiple time points after 5-$HT_4$ receptor activation) to evaluate the evolution of the drug-induced changes in inotropic force in function of time. Values for isoprenaline and $CaCl_2$ were taken at the maximum response to these agents. The maximal first derivative ($(dF/dt)_{max}$) of isometric force recordings was used as an indicator for contractility. All values were expressed relative to the isoprenaline-mediated increase above the initial value (right before adding the 5-$HT_4$ receptor agonist). Muscle responses to carbachol were expressed relative to the $CaCl_2$-mediated increase above the initial value, since carbachol significantly reduced responses to isoprenaline ($P<0.05$, n=6, data not shown). Muscle preparations which showed a basal contraction below 0.4 g or an isoprenaline-induced contraction below 1 g were excluded. By dividing $(+dF/dt)_{max}$ (maximal contraction rate) by $(-dF/dt)_{max}$ (maximal relaxation rate) the coefficient $R_2$ was calculated, which is the contraction-relaxation coupling under high load and indirectly reflects myofilament $Ca^{2+}$ sensitivity. $R_2$ assesses lusitropic effects, independent of inotropic changes (Mattiazzi et al. 1986; Hanouz et al. 2004). Changes of $R_2$ in the course of time are expressed percentually, relative to the basal value (before addition of PDE inhibitors).

### III.3.4 Statistics

All data are represented as means ± s.e.m. of n=number of pectinate muscles from different animals. Differences between means of compound-treated and control strips for every second time point taken (change in force as a % of change in force by isoprenaline), were assessed using unpaired, two-tailed student's t-test or One-way ANOVA (Bonferroni post-testing) when more than two groups were compared. Paired, two-tailed student's t-test or Repeated Measures One-way ANOVA (Bonferroni post-testing) was performed for comparisons of $(dF/dt)_{max}$ at different time points within the same muscle strips (when comparing effects of compounds/agonists to basal force and when comparing effects of IBMX/GR113808 to contraction force before addition of these compounds in Figure III.8). Graph Pad Prism V5.03 was used to draw graphs and to calculate the statistics. A P value <0.05 was considered significant.

### III.3.5 Drugs

GR113808, EHNA hydrochloride, cilostamide, rolipram, salbutamol hemisulfate, (RS)-atenolol, C-type natriuretic peptide (CNP), (*S*)-Nitroso-*N*-acetylpenicillamine (SNAP), Amino-3-morpholinyl-1,2,3-oxadiazolium chloride (Sin-1), pertussis toxin (PTX), 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) and RS67333 hydrochloride were purchased at Tocris bioscience (Huissen, The Netherlands). (S)-(-)-propranolol hydrochloride, IBMX, isoprenaline hydrochloride, serotonin creatinine sulfate salt monohydrate (5-HT), zaprinast, cholesterol-water soluble, carbachol and filipinIII were purchased at Sigma-Aldrich (Bornem, Belgium). Methyl-beta-cyclodextrin was purchased from Cyclolab (Budapest, Hungary). Prucalopride succinate was a gift from Shire-Movetis (Turnhout, Belgium). IBMX, GR113808, cilostamide, rolipram, zaprinast, filipinIII and ODQ were dissolved in dimethylsulphoxide (DMSO). Methyl-beta-cyclodextrin, SNAP and Sin-1 were 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.

## III.4 Results

### III.4.1 Effects of the 5-HT$_4$ receptor agonist RS67333 on muscle contractility (unpublished results)

Single concentrations of RS67333 did not induce any inotropic response till 0.1 µM. At 1 and 10 µM, a small inotropic response was obtained that faded during the 60 min incubation period; for 10 µM this decreased response was clearly lower than the basal contraction force (-28 ± 7 %, n=5). When the 5-HT$_4$ receptor antagonist GR113808 (0.1 µM) was added 1h after RS67333, it did not significantly decrease responses further (**Figure III.1a**).

When added cumulatively, with a contact time of 10 min at each concentration, no positive inotropic response was obtained; at 10 µM, contractions even decreased below the basal EFS-induced contractile force (-8 ± 6 %, n=6, ns). However, when the cumulative concentration-response curve was constructed in the presence of the non-selective PDE inhibitor IBMX (20 µM), a clear concentration-dependent positive inotropic response was obtained with a maximal force increase of 33 ± 5 %, n=6, at 1 µM RS67333 (**Figure III.1b**).

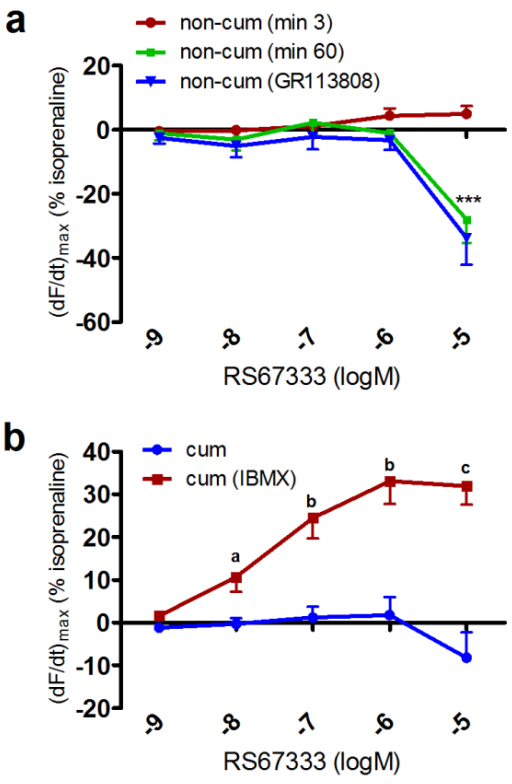


**Figure III.1** Concentration-dependent increase in contractile force by addition of RS67333 in left atrial pectinate muscles. Maximal contraction responses (non-cum (min 3)) as well as force 60 min after addition of RS67333 (non-cum (min 60)) and minimal contraction force after addition of 0.1 µM GR113808 (non-cum GR113808), were plotted against the concentration of RS67333 (1 nM to 10 µM) added non-cumulatively (**a**). Contraction force in response to RS67333 added in a cumulative manner without (cum) and with (cum (IBMX)) pre-incubation of IBMX (20 µM, 30 min) was taken 9 min after addition of each concentration of RS67333 (**b**). Data points were expressed as mean percentage ± s.e.m. (n=5 atria in **a** and 6 atria in **b**) of the contraction caused by isoprenaline. *** P<0.001 non-cum (min 60) versus non-cum (min 3), Repeated measures ANOVA (**a**); a (P<0.05), b (P<0.01), c (P<0.001) cum (IBMX) compared to cum, unpaired t-test (**b**).

### III.4.2 Influence of pre-incubation with selective PDE inhibitors on the responses to 5-HT, prucalopride and RS67333 (unpublished results for RS67333)

***5-HT*** The selective PDE-inhibitors EHNA (PDE2, 10 µM), cilostamide (PDE3, 0.3 µM), rolipram (PDE4, 1 µM) and zaprinast (PDE5, 10 µM) were tested versus 1 µM 5-HT by pre-incubating them for 30 min alone or in combination. IBMX (20 µM) was studied in comparison. The selective PDE inhibitors EHNA and zaprinast did not influence basal contraction force and did not cause a significant change in the response to 5-HT (n=6, results not shown). The PDE3 inhibitor cilostamide, marginally increased muscle contractions in response to 5-HT (significant only at min 4 to 6, n=6) (**Figure III.2a**). There was a pronounced variability of inotropic responses to 5-HT in muscles pre-treated with rolipram. In a first series of experiments rolipram had no significant effect on responses to 5-HT (n=6, data not shown). However, in subsequent series, rolipram pre-treated muscles showed an enhanced inotropic effect to 5-HT reaching significance in the series depicted in **Figures III.5d, 6a** and **6b**. Next, combinations of PDE inhibitors were tested. The combination of EHNA plus cilostamide did not have an effect on basal contraction force nor did it influence responses to 5-HT (n=6; data not shown). The combination of EHNA plus rolipram also did not influence basal muscle contractility and did not show a difference to responses to 5-HT in the presence of rolipram, which itself also had no significant effect (n=6, data not shown). Similarly the combination of zaprinast plus rolipram did not influence basal muscle contractility and did not show a difference to responses to 5-HT in the presence of rolipram, which in this series had a significant effect of its own (n=8, data not shown).

The combination of cilostamide plus rolipram did not influence basal contraction force but it largely increased inotropic responses and completely prevented the fade of the response to 5-HT. 4 min after addition of 5-HT, contraction force reached 93 ± 3 % and remained stable (98 ± 5 % vs. -1 ± 4 % for 5-HT control after 60 min, n=6, $P<0.001$). Muscle responses to 5-HT under PDE inhibition by cilostamide plus rolipram were better maintained compared to those in the presence of IBMX ($P<0.01$ at min 60, n=6, **Figure III.2b**). The addition of 0.1 µM GR113808 60 min after addition of 5-HT decreased the contraction force to -13 ±3 %, -20 ± 4 % and 6 ± 9 % in muscles receiving 5-HT only, 5-HT in the presence of IBMX and 5-HT in the presence of cilostamide plus rolipram, respectively.

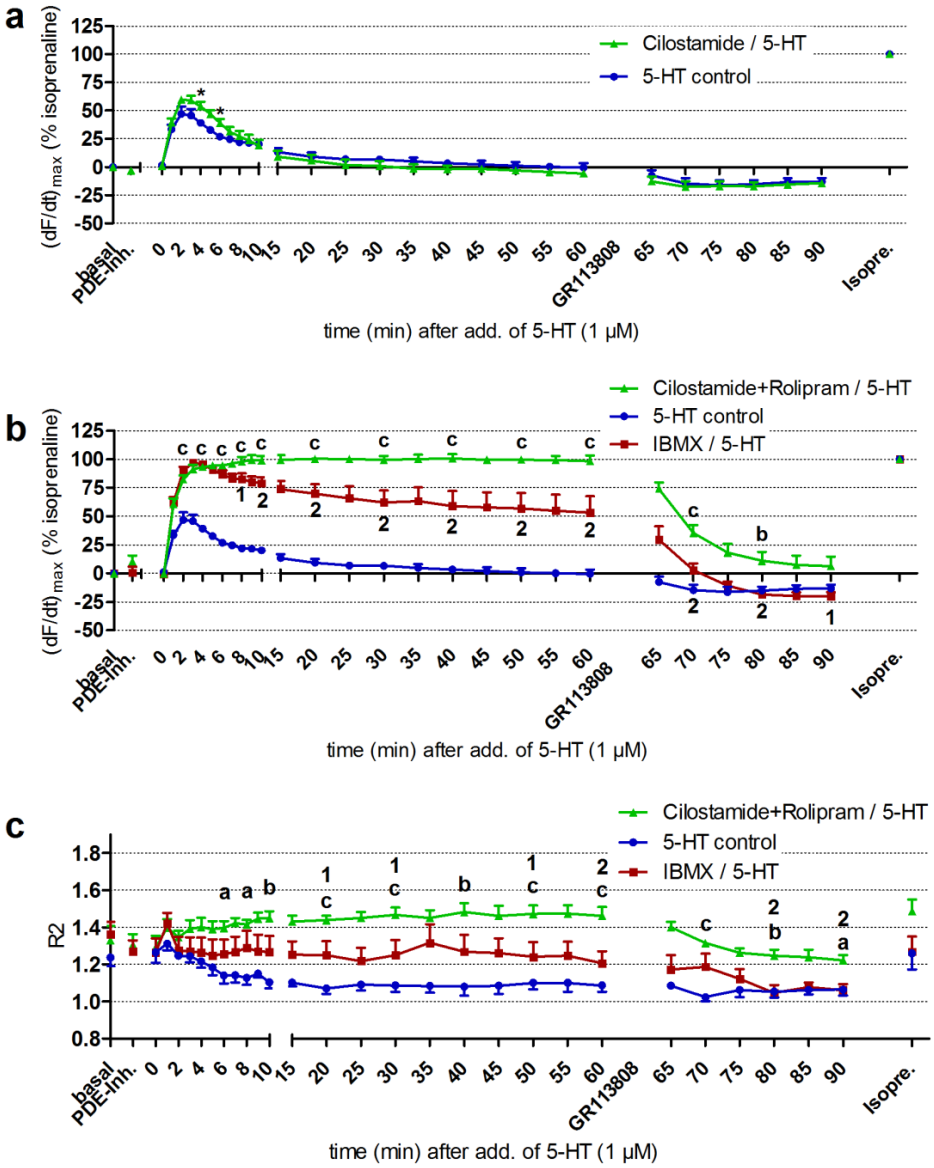


**Figure III.2** Time line of the effects of PDE inhibition with 0.3 µM cilostamide (**a**) or 0.3 µM cilostamide plus 1 µM rolipram (**b,c**) on responses to 1 µM 5-HT. After 60 min GR113808 (0.1 µM) was added and contraction force was followed for another 30 min. Each time point represents averaged contraction force ± s.e.m. of n=6 atrial strips as a % of the response generated by 100 µM isoprenaline (Isopre.) (**a**) (**b**) or the $R_2$ value calculated at the same time (**c**). Basal values were taken just before addition of the PDE inhibitors and presented as 0 % force increase. The contraction force at the end of the 30 min incubation period with the PDE inhibitors just before adding 5-HT is shown as PDE-Inh.; this value was then normalized to 0 % force increase (time point 0) and taken as the new basal value to measure the responses to 5-HT. Responses of muscles which only received 5-HT (5-HT control) or were incubated with IBMX for 30 min before addition of 5-HT (IBMX / 5-HT) are shown in comparison. * ($P<0.05$) Cilostamide / 5-HT compared to 5-HT control; a ($P<0.05$), b ($P<0.01$), c ($P<0.001$) Cilostamide+Rolipram / 5-HT compared to 5-HT control; 1 ($P<0.05$), 2 ($P<0.01$) Cilostamide+Rolipram / 5-HT compared to IBMX / 5-HT, t-test (**a**) and One-way ANOVA (**b**) (**c**)

A small increase in $R_2$ was observed after addition of 5-HT, which faded within 10 min and stabilized below the basal value (-11 ± 2 % at min 10). In the presence of IBMX, $R_2$ showed a temporary small increase by 5-HT and was then stable for 60 min (**Figure III.2c**). Addition of GR113808 decreased the $R_2$ of muscle pre-treated with IBMX from -11 ± 5 % to -21 ± 4 %. In the presence of cilostamide and rolipram, a significant increase in $R_2$ was observed after addition of 5-HT, which did not fade (+12 ± 6 % vs. -12 ± 2 % for 5-HT control after 30 min). GR113808 decreased the $R_2$ in muscle pre-treated with cilostamide plus rolipram (from +11 ± 6 % to -7 ± 5 %) (**Figure III.2c**).

The triple combination EHNA plus cilostamide plus rolipram did not increase inotropic responses further than the combination cilostamide plus rolipram (n=6) (data not shown).

***Prucalopride*** Addition of prucalopride (1 μM) only weakly increased contraction force, reaching a maximum of 17 ± 3 % at min 4 (Figure III.3a). This response, like the response to 5-HT, faded with time and settled at -4 ± 8 % below basal, 60 min after addition of prucalopride. In the presence of cilostamide plus rolipram responses to prucalopride increased significantly (89 ± 14 % vs. -4 ± 9 % for control at min 60, n=6, P<0.001; **Figure III.3a**) but built slower than responses to 5-HT (see **Figure III.2a**) and then remained stable till min 60. Addition of GR113808 1 h after adding prucalopride decreased contraction force in control and IBMX pre-treated muscles to the same level below basal (-14 ± 6 % and -17 ± 3 %, respectively) while in cilostamide plus rolipram pre-treated muscles contraction force remained above basal 30 min after incubation with GR113808 (26 ± 12 %, n=6; **Figure III.3a**).

After a short increase, $R_2$ values declined below basal after addition of prucalopride (-5 ± 2 % at min 10). In the presence of IBMX, prucalopride induced a short increase of $R_2$ followed by a slight decrease. In the presence of cilostamide plus rolipram, a significant increase of $R_2$ was observed after addition of prucalopride (+13 ± 9 % vs. -11 ± 2 % of prucalopride control at min 30) (**Figure III.3b**). Addition of GR113808 one hour after prucalopride did not change $R_2$ values of prucalopride control, but decreased the $R_2$ of muscles pre-treated with IBMX (from -7 ± 6 % to -15 ± 6 %) and cilostamide plus rolipram (from +10 ± 8 % to -6 ± 4 %), respectively (**Figure III.3b**).

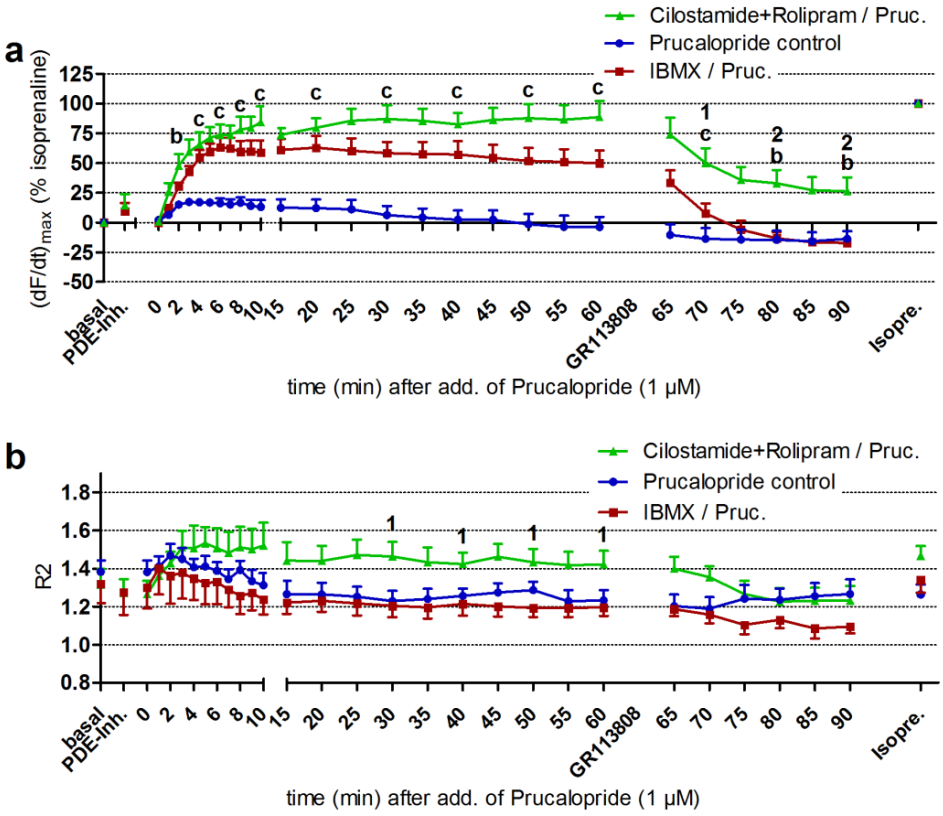


**Figure III.3** Time line of the effects of PDE inhibition with 0.3 µM cilostamide plus 1 µM rolipram on responses to 1 µM prucalopride. After 60 min GR113808 (0.1 µM) was added and contraction force was followed for another 30 min. Each time point represents averaged contraction force ± s.e.m. of n=6 atrial strips as a % of the response generated by 100 µM isoprenaline (Isopre.) (**a**) or the $R_2$ value calculated at the same time (**b**). For description of basal and PDE-Inh. see legend Figure III.2. Responses of muscles which only received 5-HT (5-HT control) or were incubated with IBMX for 30 min before addition of 5-HT (IBMX / 5-HT) are shown in comparison. a (P<0.05), b (P<0.01), c (P<0.001) Cilostamide+Rolipram / Pruc. compared to Prucalopride control; 1 (P<0.05), 2 (P<0.01) Cilostamide+Rolipram / Pruc. compared to IBMX / Pruc., One-way ANOVA

***RS67333*** Under PDE inhibition with cilostamide plus rolipram, RS67333 (1 µM) significantly increased contraction force compared to control (50 ± 5 % vs. 4 ± 6 % at min 4, n=6, P<0.001). As seen with prucalopride responses built slowly after which they remained stable. Responses of IBMX pre-treated muscles built up quicker and decreased slightly throughout the 60 min measured. Therefore contraction force by RS67333 at min 60 was significantly higher when PDEs were blocked with cilostamide plus rolipram than when they were blocked using IBMX (80 ± 5 % vs. 38 ± 7 %, P<0.001, n=6) (**Figure III.4a**). When GR113808 was added 1 h after receptor activation, contraction force in control and IBMX pre-treated muscles decreased to -7 ± 3 % and 15 ± 5 %, respectively. In muscles receiving cilostamide plus rolipram however, the decrease in

contraction force was very limited and the contraction force remained at 72 ± 7 % (n=6, P<0.001 vs. control and IBMX treated muscles) (**Figure III.4a**).

$R_2$ values did not change much after addition of 1 µM RS67333 in control and IBMX pre-treated muscles. Cilostamide plus rolipram pre-treated muscles showed slightly increased $R_2$ values after addition of RS67333 (+8 ± 6 % vs. 4 ± 3 % of RS67333 control at min 30) but this effect was not significant versus control (**Figure III.4b**).

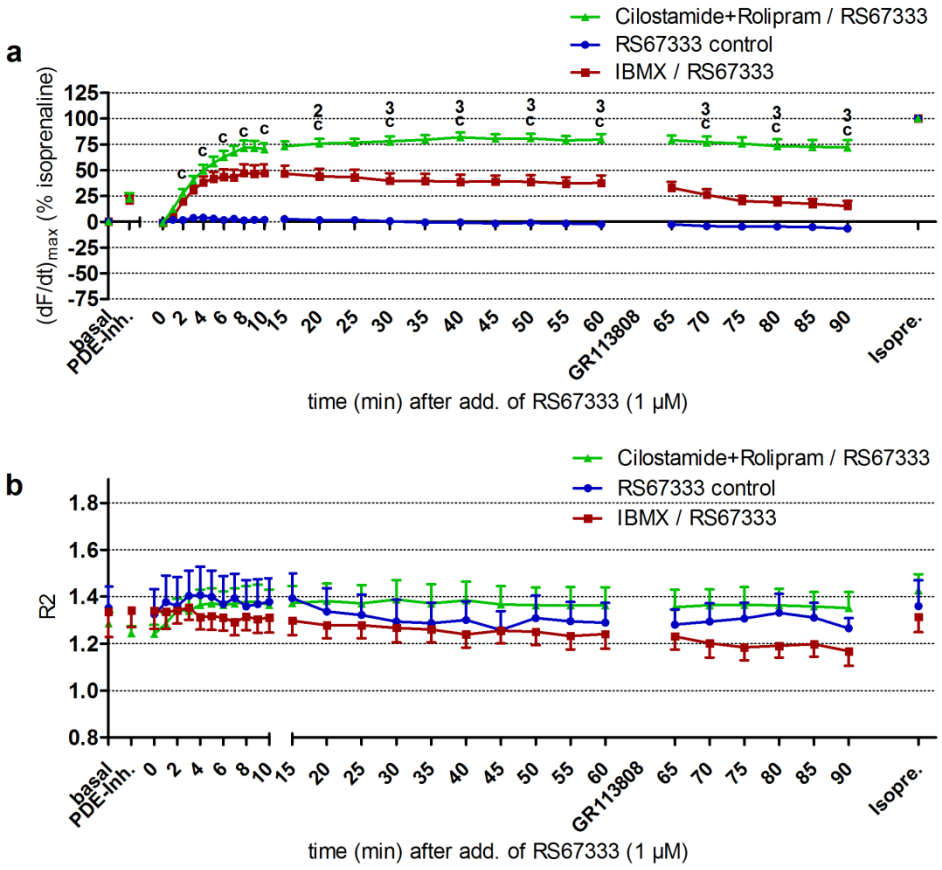


**Figure III.4** Time curve of the effects of PDE inhibition with 0.3 µM cilostamide plus 1 µM rolipram on responses to 1 µM RS67333. After 60 min GR113808 (0.1 µM) was added and contraction force was followed for another 30 min. Each time point represents averaged contraction force ± s.e.m. of n=6 atria in % of the response generated by 0.1 mM isoprenaline (Isopre.) (**a**) or the $R_2$ value calculated at the same time (**b**). For description of basal and PDE-Inh. see legend figure III.2. Responses of muscles which only received 5-HT (5-HT control) or were incubated with IBMX for 30 min before addition of 5-HT (IBMX/5-HT) are shown in comparison. c (P<0.001) Cilostamide+Rolipram/RS67333 compared to RS67333 control; 2 (P<0.01), 3 (P<0.001) Cilostamide+Rolipram¬/RS67333 compared to IBMX/RS67333, One-way ANOVA.

### III.4.3 Influence of cGMP elevating agents on responses to 5-HT

Since cGMP has been shown to influence cAMP-mediated cardiac responses, we investigated whether cGMP elevating agents have an influence on responses to 5-HT. In detail, we activated pGC via C-type natriuretic peptide (CNP, 0.3 µM). sGC activity was increased by using the NO-donors SNAP (100 µM) and Sin-1 (300 µM). These drugs, which could inhibit PDE3 through elevations in cGMP levels, were also tested together with rolipram, since a clear and maintained effect on muscle responses to 5-HT was observed only when both PDE3 and PDE4 were inhibited (see **Figure III.2a**). cGMP hydrolysis is controlled by phosphodiesterases 2 and 5 (Zhang and Kass 2011). Therefore we tested the cGMP elevating drugs in combination with PDE2 and 5 inhibitors to maximally increase cGMP levels.

CNP tended to increase muscle contractions (P=0.08 at min 2). When CNP was given within 1 min after rolipram muscle contractility increased to 13 ± 5 % above basal 2 min after administration (n=12, P<0.01, **Figure III.5a**), which faded quickly and stabilized slightly above basal. CNP, incubated for 30 min, did not have an effect on responses to 5-HT (**Figure III.5c**). Under combination of pGC stimulation by CNP plus PDE4 inhibition with rolipram, the response to 5-HT, although not significant, seemed to be better maintained than in the presence of rolipram alone (**Figure III.5c**). Cumulative concentration-response curves to 5-HT (1 nM to 10 µM) were similar for control muscles and in the presence of rolipram and rolipram plus CNP ($IC_{50}$ as well as $E_{max}$), except for a significant effect between rolipram pre-treated and control muscles at 10 µM 5-HT, when responses to control muscles had already started to fade (n=6, P<0.05; **Figure III.5b**). To further increase cGMP levels we tested the effect of CNP plus rolipram in combination with the PDE2 inhibitor EHNA, which was found to control the cGMP pool generated by pGC in rat cardiac myocytes (Castro et al. 2006). The combination of rolipram plus EHNA plus CNP increased the inotropic response to 5-HT, reaching a maximum of 84 ± 3 % at min 3, which was not significantly different from the response to 5-HT of muscles receiving only rolipram (81 ± 9 % at min 3). However, the fade of the inotropic response was slowed down, giving responses of 68 ± 5 % at min 10 and 47 ± 9 % at min 60; this was significant from min 20 onwards compared to muscles receiving 5-HT in the presence of rolipram (P<0.05, **Figure III.5d**).

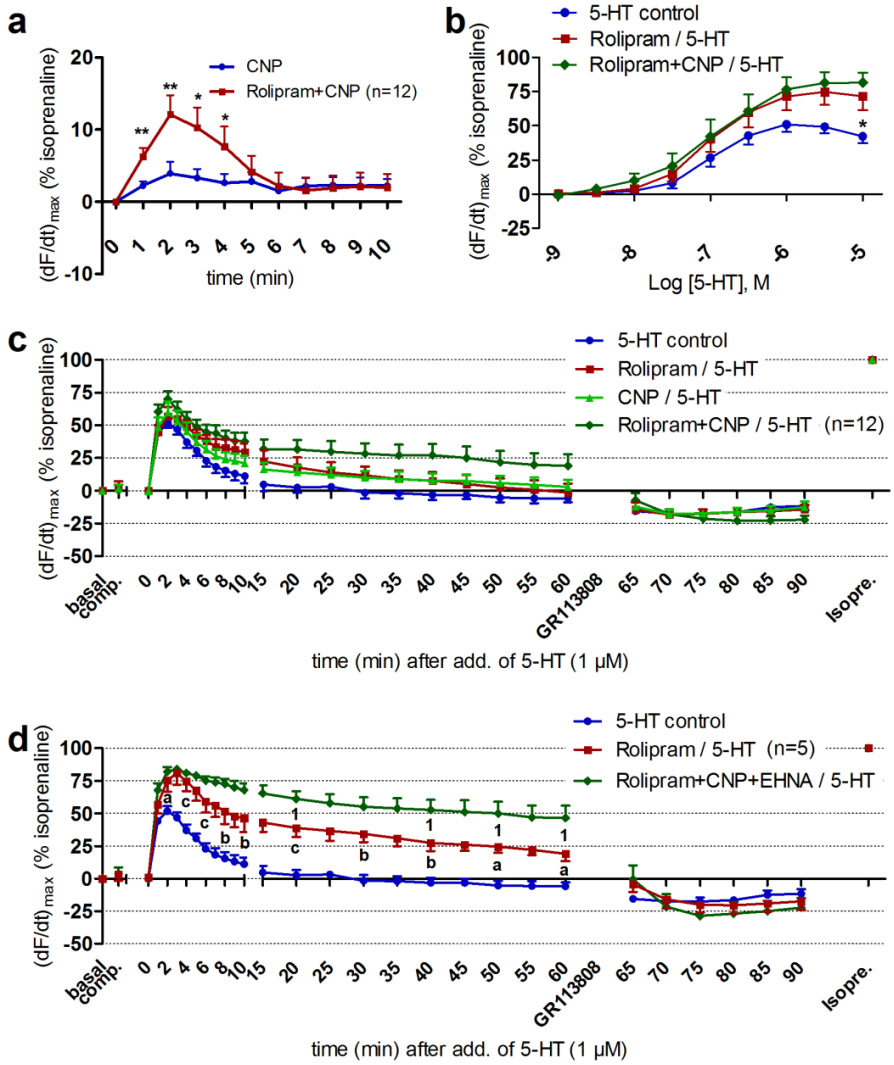


**Figure III.5** Effect of C-type natriuretic peptide, alone or in combination with rolipram on contraction force, expressed as mean ± s.e.m. of the contraction to isoprenaline. * (P<0.05), ** (P<0.01) Rolipram+CNP compared to basal, paired t-test (**a**). Cumulative concentration-response curves to 5-HT (1 nM to 10 µM) in the absence (5-HT control) and presence of rolipram (Rolipram / 5-HT) and rolipram plus CNP (Rolipram+CNP / 5-HT) expressed as mean percentage ± s.e.m. of the contraction to isoprenaline. * (P<0.05) Rolipram / 5-HT compared to 5-HT control (**b**). Time line of the response to 5-HT in muscles receiving CNP, and rolipram plus CNP (**c**) or the triple combination rolipram plus CNP plus EHNA (**d**). The averaged experimental data points were expressed as mean percentage ± s.e.m. of the contraction to isoprenaline (100 µM, Isopre.) of n=6 atrial strips, unless indicated otherwise. Basal values were taken just before addition of any compounds and presented as 0 % force increase. The contraction force at the end of the 30 min incubation period with the compounds just before adding 5-HT is shown as comp.; this value was then normalized to 0 % force increase (time point 0) and taken as the new basal value to measure responses to 5-HT. Responses of muscles which only received 5-HT (5-HT control) or were incubated with rolipram for 30 min before addition of 5-HT (Rolipram / 5-HT) are shown in comparison; a (P<0.05), b (P<0.01), c (P<0.001) Rolipram / 5-HT compared to 5-HT control; 1 (P<0.05) Rolipram+CNP+EHNA / 5-HT compared to Rolipram / 5-HT, One-way ANOVA

Stimulation of sGC by addition of the NO-donor SNAP did not have an effect on basal muscle contractility or on responses to 5-HT (**Figure III.6a**). When given in combination with rolipram, we observed a prolonged inotropic response to 5-HT, which was not significantly different to the one obtained by rolipram alone (n=5, data not shown). To further increase cGMP levels, SNAP was tested in combination with the PDE5 inhibitor zaprinast, because Castro et al. (2006) showed that the cGMP pool generated by sGC is controlled mainly by PDE5 in rat cardiac myocytes (n=4, **Figure III.6a**). However this combination did not influence the response to 5-HT. In the presence of the triple combination of rolipram plus zaprinast plus SNAP (n=5, data not shown), the response to 5-HT did not differ from that in the presence of rolipram. Finally, rolipram plus SNAP was tested in combination with the PDE2 inhibitor EHNA as well, since PDE2 has cGMP and cAMP degrading activity and might therefore lower cGMP levels. But this combination also did not significantly alter responses to 5-HT in the presence of rolipram (n=3, data not shown). Muscle responses to 5-HT in the presence of another NO-donor Sin-1 showed a similar maximal response compared to 5-HT control (43 ± 3 % and 54 ± 15 % respectively at min 2, n=4). However, the fade of the response was much quicker in muscles receiving Sin-1 compared to control, becoming significant at min 6 after addition of 5-HT (-11 ± 3 % vs. 19 ± 4 % respectively, n=4, $P<0.05$; **Figure III.6b**). After contraction force was maximally decreased at min 8, muscle responses to 5-HT in the presence of Sin-1 recovered slowly, stabilizing at a value slightly below basal (-6 ± 10 %, n=4). This effect of Sin-1 on responses to 5-HT was not influenced by rolipram (**Figure III.6b**), nor by any combination of PDE inhibitors tested (rolipram plus zaprinast, rolipram plus EHNA, rolipram plus zaprinast plus EHNA; data not shown). The sGC inhibitor ODQ was added 10 min before Sin-1 to test if the effect of the NO-donor on responses to 5-HT is mediated by activation of sGC. The inotropic response to 5-HT was better maintained in muscles receiving 100 µM ODQ compared to control muscles receiving 0.1 % DMSO (the solvent for ODQ) ($P<0.05$ from min 5 to 7, n=4 and 3, respectively; data not shown), giving responses of 2 ± 2 % and -7 ± 2 % at 6 min after addition of 5-HT, respectively; lower concentrations of ODQ were not effective.

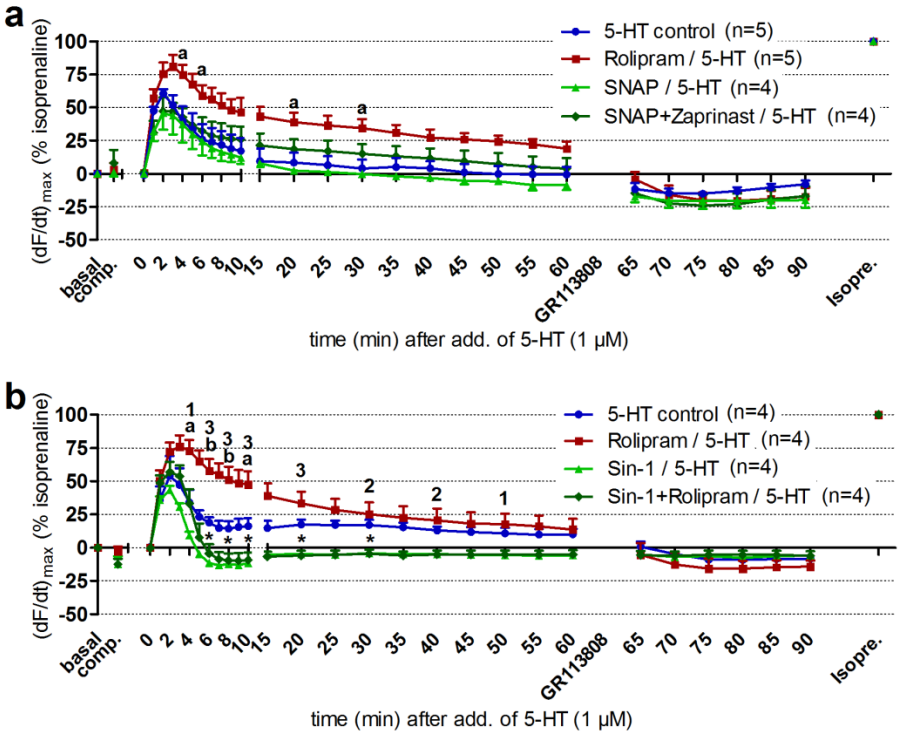


**Figure III.6** Time line of the effects of the NO-donors SNAP (**a**) and Sin-1 (**b**) alone or in combination with PDE inhibitors on responses to 5-HT. The averaged experimental data points were expressed as mean percentage ± s.e.m. of the contraction to isoprenaline (100 µM; Isopre.). Number of atrial strips is indicated in parenthesis. For description of basal and comp. see legend Figure III.5. Responses of muscles which only received 5-HT (5-HT control) or were incubated with rolipram for 30 min before addition of 5-HT (Rolipram / 5-HT) are shown in comparison; * (P<0.05) Sin-1 / 5-HT compared to 5-HT control; a (P<0.05), b (P<0.01) Rolipram / 5-HT compared to 5-HT control; 1 (P<0.05), 2 (P<0.01), 3 (P<0.001) Sin-1+Rolipram / 5-HT compared to Rolipram / 5-HT, One-way ANOVA

### III.4.4 Influence of IBMX, when added 60 min after 5-HT or prucalopride

60 min after addition of 5-HT or prucalopride, inotropic responses have completely faded to 3 ± 7 % and 0 ± 1 %, respectively. Addition of IBMX (20 µM) to the organ bath at this time point fully recovered these responses, increasing contraction force significantly to 66 ± 10 % (n=6, P<0.001) and 62 ± 6 % (n=6, P<0.001) for 5-HT and prucalopride stimulation, respectively. When the 5-$HT_4$ receptor antagonist GR113808 (0.1 µM) was given another 30 min later, inotropic responses declined again significantly and settled at -2 ± 9 % (n=6, P<0.001) and 12 ± 5 % (n=6, P<0.001) for 5-HT and prucalopride stimulation, respectively (**Figure III.7a** and **8a,b**).

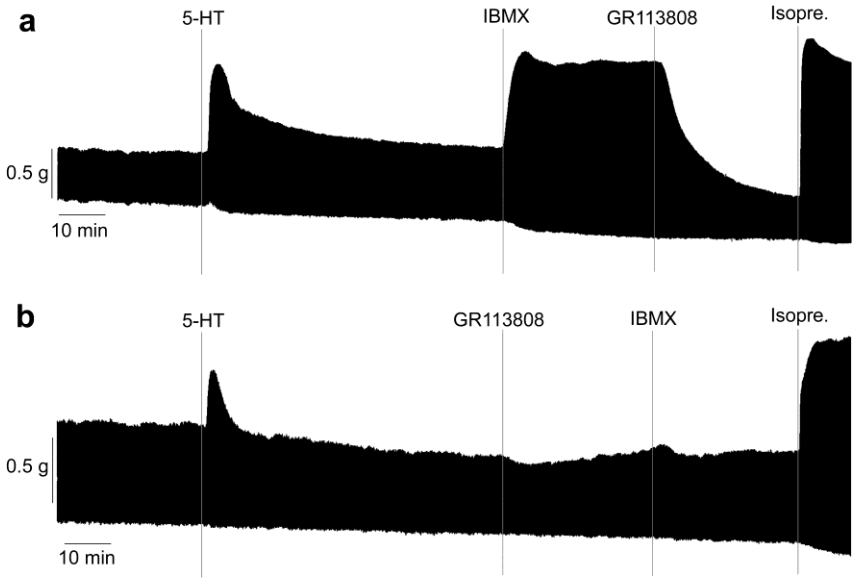


**Figure III.7** Representative tracings showing the responses to 5-HT (1 µM), IBMX (20 µM) and GR113808 (0.1 µM) in porcine left atrial pectinate muscles. In the upper tracing (**a**) IBMX was given 60 min after 5-HT and GR113808 was added 30 min later. In the lower tracing (**b**) GR113808 was added 60 min after 5-HT and IBMX was added 30 min later, still in the presence of previously administered substances. At the end of every experiment isoprenaline (100 µM) was added.

When GR113808 was added one hour after addition of 5-HT or prucalopride (the inotropic response to the agonists had almost completely faded), force of muscle contraction declined a bit further and settled slightly below basal at -2 ± 5 % and -1 ± 4 %, respectively. Addition of IBMX (20 µM) to 5-HT and prucalopride exposed muscles, in the presence of GR113808 (0.1 µM) again significantly increased force to 12 ± 6 % (n=6, P<0.05) and 42 ± 5 % (n=6, P<0.001), respectively (**Figure III.7b** and **8c,d**).

### III.4.5 Influence of caveolae and $G_i$ proteins on contractile responses to 5-HT (unpublished results)

Methyl-beta-cyclodextrin (MβC), which removes cholesterol from cell membranes and thereby destroys the cholesterol-dense caveolae, added in a concentration of 10 mM was deleterious to muscle function. Pre-incubation of 3 mM MβC for 60 min did not destroy muscle function and increased the basal contraction force of muscle strips by 22 ± 4 % (P<0.001, n=6, data not shown). Addition of 5-HT in the presence of MβC (3 mM) showed a decreased response in amplitude (43 ± 7 % vs. 64 ± 7 % of 5-HT control at min 2, n=6), reaching significance at 20 min

after addition of the agonist. 60 min after addition of 5-HT the inotropic response had faded below basal (-35 ± 7 %) in MβC pre-treated muscles which was significantly different from control strips (4 ± 8 %, P<0.01, n=6, data not shown). When MβC was washed-out and cholesterol content was re-constituted with cholesterol-supplemented MβC (water-soluble cholesterol, 1 mg/ml), responses to 5-HT did not return to normal as expected, but were still reduced (38 ± 7 % vs. 58 ± 5 % of 5-HT control at min 2, n=3). Interestingly control muscles treated with 3 mM MβC, showed normal inotropic responses to 5-HT after a wash-out of the substance (68 ± 5 % at min 2, n=3; data not shown). This suggests that the lowered responsiveness to 5-HT in muscles receiving MβC is probably unspecific and is not caused by the destruction of caveolae.

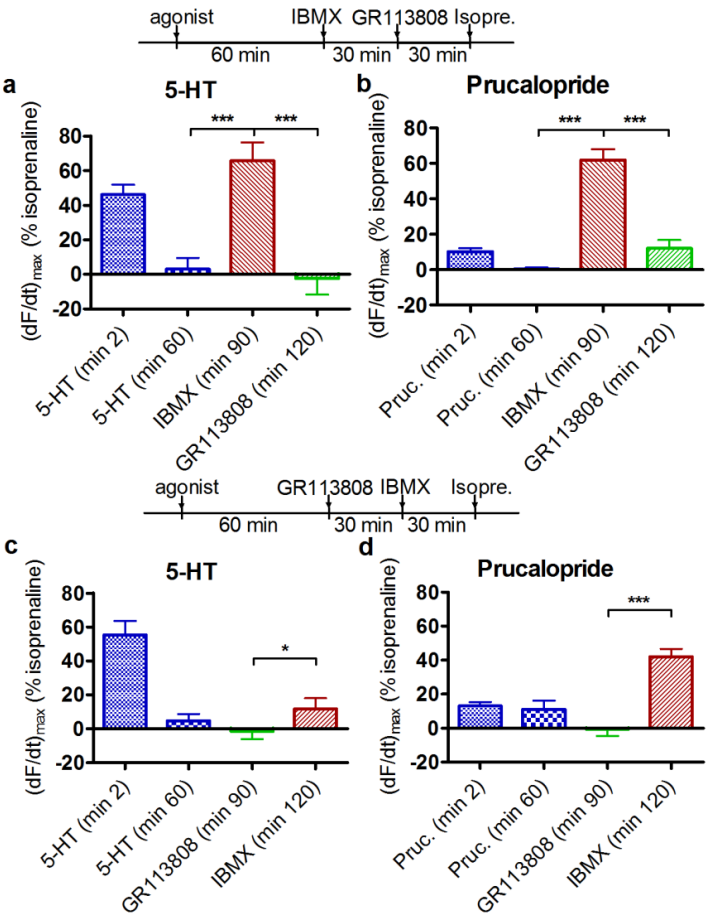


**Figure III.8** Contractile responses to 1 µM 5-HT (panels **a**, **c**) and 1 µM prucalopride (panels **b**, **d**) when 20 µM IBMX and 0.1 µM GR113808 were added at 60 and 90 min after the addition of agonist (**a**, **b**) or vice versa (**c**, **d**). Mean responses of n=6 atrial strips are shown at 2, 60, 90 and 120 min after addition of the agonist according to the time line of the respective protocols shown above the panels. Panels **a**, **b**: *** P<0.001 IBMX (min 90) compared to 5-HT (min 60) and Pruc. (min 60), respectively; GR113808 (min 120) compared to IBMX (min 90); Panels **c**, **d**: * P<0.05, *** P<0.001 IMBX (min 120) compared to GR113808 (min 90); Repeated measured ANOVA

In comparison, the influence of MβC was also tested on the response to the $\beta_2$ agonist salbutamol (50 µM) in the presence of the $\beta_1$ antagonist atenolol (1 µM, 30 min). No difference was observed, in the inotropic response to salbutamol between muscles pre-treated for 60 min

with 3 mM MβC and controls (67 ± 8 % and 67 ± 7 % at min 2, respectively; n=6). The effect of filipinIII (2 µg/ml), an inhibitor of the caveolae pathway, was also tested on responses to 5-HT. FilipinIII, like MβC, increased basal muscle contractility to 19 ± 10 %. Contrary to MβC, filipinIII slightly increased inotropic responses to 5-HT (84 ± 11 % vs. 57 ± 10 % of 5-HT control at min 2, n=6, data not shown), an effect which did not reach significance. This provides further evidence that the effect of MβC on responses to 5-HT is unspecific.

Incubation of muscles with pertussis toxin (1.5 $\mu g ml^{-1}$) for 3 h did not influence the inotropic responses to addition of 0.1 µM (n=3, data not shown) or 1 µM 5-HT (n=3, **Figure III.9a**). As a positive control we tested the effect of PTX on responses to carbachol, a muscarinic receptor agonist. Carbachol (0.3 µM) reduced muscle contractility to -21 ± 5 % below basal 1 min after addition. This response was short lasting and contractility recovered fully by min 15 (**Figure III.9b**). Incubation of muscles with PTX reduced the carbachol effect to -6 ± 3 % below basal at min 1 (ns, n=5), but could not completely abolish the reduction in contractile force by carbachol (**Figure III.9b**).

## III.5 Discussion

The aim of this study was to analyse control mechanisms of 5-$HT_4$ receptor signalling at the level of cAMP in porcine left atrium, paying special attention to the action of PDEs and the cross-talk by cGMP with cAMP signalling and exploring a possible role of caveolae and $G_i$ proteins. Furthermore we looked at the time course of 5-$HT_4$ receptor signalling and investigated whether it was still active 60 min after stimulation. Experiments were performed with 5-HT as agonist but the role of PDEs and the time course was also studied for the selective 5-$HT_4$ receptor agonist prucalopride (Resolor®, Shire-Movetis, Turnhout, Belgium), that has been granted in 2009 the European market authorisation for the symptomatic treatment of chronic constipation in women in whom laxatives fail to provide adequate relief. RS67333, another selective agonist with high affinity to 5-$HT_4$ receptors (Eglen et al. 1995) that is commercially available was studied in comparison. De Maeyer et al. (2006) already tested the inotropic effects of prucalopride on pig left atrium. Having only a very small effect in the absence of IBMX, prucalopride was equally potent as but less effective than 5-HT in the presence of IBMX. The aromatic ketone RS67333 acted as a partial agonist in *in vivo* studies investigating the heart rate of anaesthetized micropigs (Eglen et al. 1995). In the actual study RS67333 also showed the characteristics of a very weak partial agonist; in the presence of IBMX, RS67333 was less

effective than 5-HT and prucalopride. The rank order of efficacy by the observed maximal effect ($E_{max}$) upon cumulative administration in the presence of IBMX is 5-HT ($E_{max}$: 89 ± 5 %) > prucalopride ($E_{max}$: 64 ± 7 %) > RS67333 ($E_{max}$: 33 ± 5 %). (data for 5-HT and prucalopride from De Maeyer et al., 2006; Table 2).

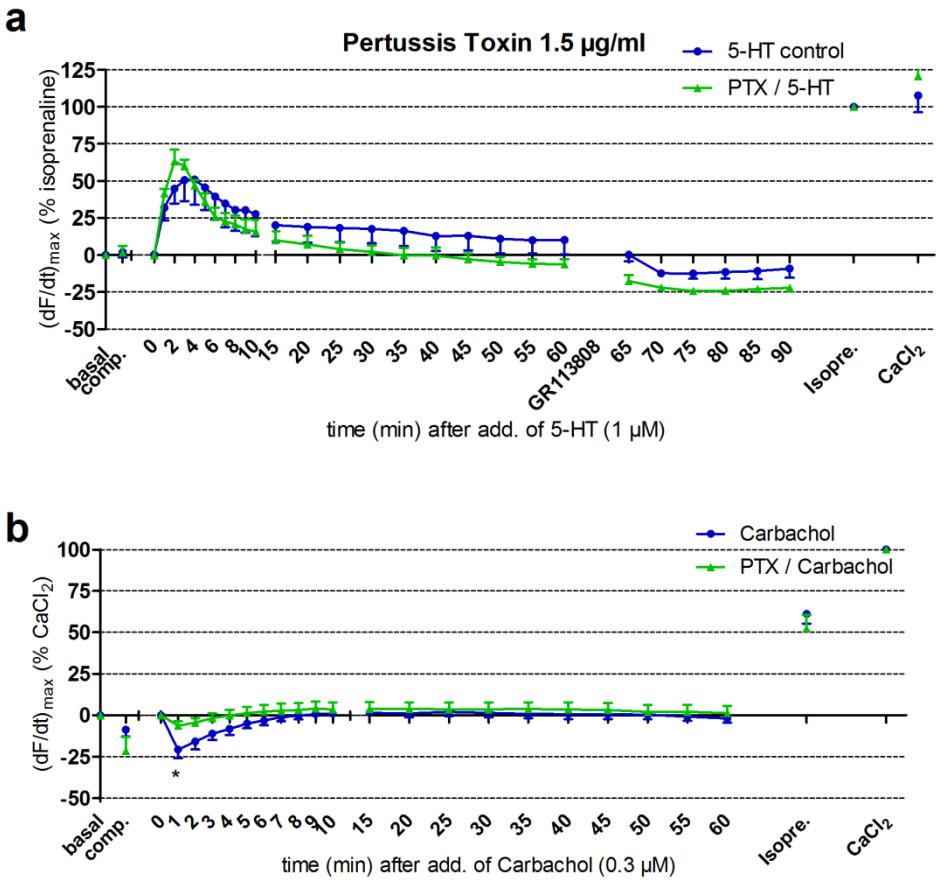


**Figure III.9** Time line of the effects of pertussis toxin (PTX) on responses to 5-HT (1 µM) (**a**) and carbachol (0.3 µM) (**b**). The averaged experimental data points were expressed as mean percentage ± s.e.m. (n=3 atrial strips in **a** and 6 atrial strips in **b**) of the contraction to isoprenaline (0.1 mM; Isopre.) (**a**) or $CaCl_2$ (7 mM) (**b**) and connected by a line. For description of basal and comp. see legend Figure III.5. Responses of muscles which only received 5-HT (5-HT control) (**a**) or carbachol (**b**) are shown in comparison; * P<0.05 Carbachol vs. PTX / Carbachol; Student's t-test

### III.5.1 Maintenance of the PDE-mediated control of the inotropic response to 5-$HT_4$ receptor agonists; no evidence for a role of $G_i$ proteins or caveolae

From experiments with pre-treatment with PDE-inhibitors (De Maeyer et al. 2006; Galindo-Tovar et al. 2009), it is clear that the inotropic response to 5-$HT_4$ receptor agonists in pig left atrium is controlled by PDEs. We now show that IBMX, added when the responses to 5-HT or

prucalopride had already completely faded one hour after their addition, increased the contraction force to the same extent as when studying the response to 5-HT or prucalopride after pre-treatment with IBMX (**Figure III.8a,b**). Signalling through the 5-$HT_4$ receptor is thus still fully active at this moment and PDEs continue to control the inotropic response. Addition of the 5-$HT_4$ receptor antagonist GR113808 30 min after IBMX (that is 90 min after addition of 5-HT or prucalopride) was able to reduce contraction force to a level close to basal, also indicating that the 5-$HT_4$ receptor is still available for binding of GR113808 and that 5-$HT_4$ receptor activation is on-going at this moment.

In muscles treated with 5-HT and then GR113808 at 60 min, IBMX added at the 90$^{th}$ min could still induce a moderate but significant increase in contraction force (**Figure III.8c**), despite the presence of the 5-$HT_4$ receptor antagonist in the organ bath. The same concentration of GR113808 was able to fully reduce contraction force when added after IBMX (**Figure III.8a**). When prucalopride was used to stimulate the 5-$HT_4$ receptor, IBMX added after GR113808 also significantly increased contraction force to an even higher level compared to 5-HT receiving muscles (**Figure III.8d**). In the case of prucalopride, addition of GR113808 in the presence of IBMX did not fully reduce contraction force (**Figure III.8b**). However there is still a marked difference in the prucalopride-induced inotropic response in the presence of GR113808 given after IBMX (**Figure III.8b**) than when GR113808 is added before IBMX (**Figure III.8d**), even though at this time point the same drugs have been administered (with only the order of administration being different). We have no clear-cut explanation for this. The differences in contractile force in the presence of both IBMX and GR113808 between 5-$HT_4$ receptor stimulation with prucalopride and 5-HT can be explained by the higher affinity of prucalopride at 5-$HT_4$ receptors than 5-HT. $K_i$ values (obtained by displacement of [$^3$H]GR113808) for 5-$HT_4$ receptor a, b, g and i splice variants are lower for prucalopride compared to 5-HT (Krobert et al. 2005). Therefore 0.1 µM GR113808 might effectively displace 5-HT from the 5-$HT_4$ receptor, while the concentration might not completely displace prucalopride.

The clear effect of IBMX added 60 min after 5-HT illustrates that, if 5-$HT_4$ receptor desensitization or a switch in G protein specificity from stimulatory ($G_s$) to inhibitory ($G_i$) proteins occurs after addition of 5-HT, this is minimal. Receptor desensitization leads to an uncoupling of the receptor from G proteins, which reduces cAMP signalling; IBMX should then only increase contraction force minimally when added 60 min after the agonist. The same holds true for a switch of $G_s$ to $G_i$ coupling, where cAMP signalling is reduced as well. Still a dual

coupling to $G_s$ and $G_i$ proteins has been reported for the h5-$HT_{4(b)}$ receptor splice variant when stably transfected into human embryonic kidney 293 cells (Pindon et al. 2002) and rat cardiomyocytes (Castro et al. 2005). Therefore we deemed it interesting to investigate, whether the 5-$HT_4$ receptor in pig atrium couples to both $G_s$ and $G_i$ by looking at responses to 0.1 and 1 µM 5-HT in the presence of pertussis toxin (PTX), an inhibitor of $G_i$ signalling. We tested these two concentrations of 5-HT because Lonardo et al. (2005) found that the pacemaker current ($I_f$) in human atrial myocytes to 0.1 µM but not 1 µM 5-HT is potentiated in PTX pre-treated cells. As a positive control we tested the effect of pertussis toxin on muscle responses to carbachol, a cholinergic receptor agonist. In cardiac muscle the predominant cholinergic receptor subtype is the $M_2$ receptor (Dhein et al. 2001). In human atrial cells $M_2$ receptor activation leads to a decrease in cAMP levels and a reduction in L-type $Ca^{2+}$ current (Vandecasteele et al. 1998), which is attributed to the inhibition of adenylate cyclase (AC) by a PTX-sensitive G protein (Wang and Lipsius 1995; Harvey and Belevych 2003). Indeed, carbachol was shown to reduce contraction force in murine atrium, an effect which was reversed by PTX pre-treatment (Neumann et al. 2003; Hara et al. 2009). In pig atrium carbachol mediated a short-lasting decrease in contraction force, after which contractility returned back to basal. This negative inotropic effect was clearly reduced in PTX-treated muscles, but was not completely abolished. Tissue penetration of pertussis toxin in porcine atrial muscle strips might not be complete, leading only to a partial inhibition of $G_i$ proteins.

The tight and maintained control of the 5-HT-induced response by PDEs does not fully exclude that still other mechanisms might contribute. Caveolar membrane structures are described to be involved in compartmentation of the signalling components of G-protein-coupled receptors (Rybin et al. 2000; Head et al. 2005). We disrupted caveolae by addition of methyl-beta-cyclodextrin (MβC), which depletes the cell membrane of cholesterol and especially targets caveolae as they are highly enriched in cholesterol (Rothberg et al. 1992). In porcine heart MβC increased basal muscle contractility, while responses to 5-HT were decreased. This is in line with a study in coronary artery myocytes, where the 5-HT mediated increase in $Ca^{2+}$ transients was greatly reduced in the presence of MβC (Prendergast et al. 2010). In comparison, we also tested the effect of MβC on responses to salbutamol (a $\beta_2$ receptor agonist) in the presence of atenolol (a $\beta_1$ antagonist), because in adult rat ventricular myocytes MβC was reported to increase responses to $\beta_2$ stimulation (cell shortening and $Ca^{2+}$ transients) (Calaghan and White 2006). In pig left atrium MβC had no effect on responses to salbutamol. To exclude unspecific effects of MβC, we first treated muscles with MβC and then, after a wash-out step, we reconstituted

cholesterol content by adding cholesterol-supplemented MβC (water-soluble cholesterol). We expected this to restore caveolae (Christian et al. 1997; Buxton and Vittori 2005) and bring muscle responses to 5-HT back to normal. However, muscles re-constituted with cholesterol-supplemented MβC showed decreased responses to 5-HT, while muscles which had also received MβC followed by a wash-out step but were not reconstituted with cholesterol, showed normal responses to 5-HT. These results strongly indicate that the decreased response to 5-HT in the presence of MβC is not due to the destruction of caveolae but an unspecific effect of MβC. Because of its hydrophobic pocket MβC is expected to interact with hydrophobic protein domains and can therefore cause a number of unspecific effects. MβC was shown to interact with proteins such as ubiquitin, chymotrypsin inhibitor 2, insulin and a number of other proteins and even remove proteins from the cell surface (Zidovetzki and Levitan 2007). However, to our knowledge no reports exists about possible cholesterol-independent effects on cardiac myocytes. The observed effect of MβC being unspecific is further supported by additional experiments with filipinIII, an inhibitor of the caveolae pathway (McGookey et al. 1983). FilipinIII also increased basal muscle contractility but did not have a significant influence on responses to 5-HT. In the literature conflicting effects of MβC on basal cardiac cell shortening and $Ca^{2+}$ transients have been reported. In one study on adult rat ventricular myocytes the disruption of caveolae decreased cell shortening and $Ca^{2+}$ transients (Calaghan and White 2006), while in another study it did not have an effect on basal cell shortening and $Ca^{2+}$ transients (Agarwal et al. 2011). To our knowledge MβC has not been tested in heart whole muscle strips. But a study on guinea pig myometrium strips reported an increase in tension after application of MβC (Buxton and Vittori 2005), which is in line with our results.

### III.5.2 PDE isoforms involved in controlling the inotropic response to 5-$HT_4$ receptor agonists

Inhibition of PDE2 or PDE5 did not have an influence on responses to 5-HT and inhibition of PDE3 showed only a marginal effect. There was a big variability in responses to 5-HT of muscles under PDE4 inhibition. In a first set of experiments we could not detect any effect of PDE4 inhibition on responses to 5-HT. However, in subsequent studies rolipram treated muscles showed a moderately prolonged inotropic response to 5-HT. Galindo-Tovar et al. (2009) reported that the inotropic response to 5-HT (10 μM) in left atria of adolescent pigs tended to be prolonged when PDE3 was inhibited, while PDE4 inhibition did not have such an effect. We

do not have an explanation for these differences in both studies, but would not consider these to be important differences, as the variability was quite large in both studies. More importantly we could fully confirm the results of Galindo-Tovar et al. (2009), that PDE3 and PDE4 inhibitors jointly prevent the fade of the inotropic response to 5-$HT_4$ receptor activation with 5-HT (**Figure III.2b**) and showed that the same applies for the response to the 5-$HT_4$ receptor agonists prucalopride (**Figure III.3a**) and RS67333 (**Figure III.4a**). The other tested combinations of PDE inhibitors did not have any effect on the response to 5-HT (cilostamide plus EHNA) or did not enhance responses to 5-HT more than rolipram alone (rolipram plus EHNA, rolipram plus zaprinast). This suggests that PDE3 and PDE4 act in a redundant way, where one PDE can replace the function of the other. Galindo-Tovar et al. (2009) proposed that the compartmentalized increase of cAMP, with single inhibition of either PDE3 or PDE4, could lead to an increased PKA-mediated phosphorylation and activation of the other, thereby maintaining the control of the inotropic response to 5-HT.

Some interesting differences were observed, when comparing the responses to 5-HT, prucalopride and RS67333 in the presence of IBMX and in the presence of cilostamide plus rolipram, respectively. While the maximal amplitude of the inotropic response to 5-HT and prucalopride was the same when PDEs were inhibited with IBMX compared to cilostamide plus rolipram, the response seemed better maintained in the presence of cilostamide plus rolipram. The inotropic responses to RS67333 were significantly higher in the presence of cilostamide and rolipram compared to IBMX and responses were also significantly better maintained in that condition (**Figure III.4a**). We suggest that specific inhibition of PDE3 and PDE4 with cilostamide plus rolipram is more effective in blocking the activities of these PDEs than nonselective inhibition with IBMX. Indeed cilostamide and rolipram have much lower $IC_{50}$ values for PDE3 and PDE4, respectively than IBMX. Especially for agonists with low efficiency as RS67333, it is important that cAMP degradation is completely blocked; only then high enough levels of cAMP can accumulate to achieve maximal stimulation.

Under PDE inhibition with cilostamide plus rolipram, responses to 5-HT built up fastest, while responses to prucalopride and to RS67333 took longer to fully develop. It is interesting that in this condition of PDE3 and PDE4 inhibition, 0.1 µM of the 5-$HT_4$ receptor antagonist GR113808 given one hour after addition of agonist, decreased inotropic responses faster and more effectively in 5-HT-stimulated muscles, while it had less effect in prucalopride- and almost no effect in RS67333-stimulated muscles. The slower building up of the inotropic response to the

partial agonists prucalopride and especially RS67333 can be explained by the lower level of cAMP generation or a slower tissue penetration; the lowered responsiveness to GR113808 (under PDE3 and 4 inhibition), however, is puzzling. Maybe regulatory mechanisms such as de-phosphorylation of PKA target proteins by phosphatases are more active in muscles receiving 5-HT, leading to a more efficient decrease of the inotropic response once stimulation is terminated. The binding affinity of prucalopride is slightly higher than that of 5-HT for all 5-$HT_4$ receptor splice variants (Krobert et al. 2005). RS67333 was reported to have a higher potency than 5-HT for 5-$HT_4$ receptors in rat oesophagus (Eglen et al. 1995). Therefore we conclude that the used concentration of 100 nM GR113808 was sufficient to effectively displace 1 µM 5-HT from 5-$HT_4$ receptors. However it only led to a reduction but not to a complete abolishment of receptor occupancy by 1 µM prucalopride and 1 µM RS67333 due to the higher affinity of these substances for the 5-$HT_4$ receptor.

### III.5.3 Interaction of cGMP and cAMP pathways

Cross-talk between cGMP and cAMP signalling pathways, mediated mainly by PDE2 and PDE3 has been reported (Zaccolo and Movsesian 2007). PDE2 is a dual specificity enzyme, which hydrolyses both cGMP and cAMP. cGMP allosterically stimulates cAMP hydrolysis of PDE2 via binding to regulatory GAF domains (Zaccolo and Movsesian 2007). PDE3 can also hydrolyse both cAMP and cGMP, but has a lower $V_{max}$ for cGMP, which makes it a cGMP-inhibited cAMP PDE (Degerman et al. 1997). Taking binding affinities into account Zaccolo and Movsesian (2007) predicted that small elevations in cGMP would selectively inhibit PDE3, while higher cGMP concentrations would also stimulate PDE2. Indeed it was shown that cGMP-mediated inhibition of PDE3 leads to increases in cAMP and stimulation of $I_{Ca}$ in human atrial myocytes (Kirstein et al. 1995; Vandecasteele et al. 2001). In order to evaluate if cGMP has an influence on 5-$HT_4$ receptor signalling we stimulated pGC using C-type natriuretic peptide and sGC using the NO-donors SNAP and Sin-1. Furthermore we inhibited specific PDEs to eliminate the break-down of cGMP and further raise the cGMP concentration in the cell.

We were particularly interested in the effect of CNP, because it has been demonstrated that $\beta_1$-adrenoceptor and 5-$HT_4$ receptor signalling in failing rat heart is enhanced by CNP, presumably through inhibition of PDE3 (Qvigstad et al. 2010; Afzal et al. 2011). However, in pig atrium, CNP alone did not influence responses to 5-HT. Therefore, we tested CNP in combination with PDE

inhibitors. Since in porcine heart 5-$HT_4$ receptor signalling is controlled by both PDE3 and PDE4 subtypes (see **Figure III.2**), we tested cGMP elevating agents in combination with rolipram. To further increase cGMP levels, CNP was used in combination with a PDE2 inhibitor, because the cGMP pool generated by pGC was found to be under the exclusive control of PDE2 in rat cardiac myocytes (Castro et al. 2006). The combination of rolipram plus CNP did not significantly increase inotropic responses to 5-HT. The triple combination of rolipram plus EHNA plus CNP, also did not increase inotropic responses to 5-HT further than rolipram alone, but the fade of the response was significantly reduced (**Figure III.5d**). These results can be explained by (A) the cGMP levels produced by pGC stimulation are further enhanced by inhibition of their break-down by PDE2 with EHNA, resulting in the inhibition of PDE3, which together with PDE4 inhibition by rolipram leads to the better maintained inotropic response or (B) pGC stimulation generates sufficiently high levels of cGMP to inhibit PDE3, but those higher cGMP levels also stimulate the cAMP degrading activity of PDE2, which in this activated state could significantly contribute to cAMP degradation, thereby maintaining the PDE-mediated control of the response to 5-HT in the presence of rolipram; an increased inotropic effect to 5-HT will then also only be observed when PDE2 is inhibited as well.

CNP per se showed a very small positive inotropic effect on basal muscle contractility, which was augmented when given in combination with rolipram (**Figure III.5a**). In cardiac myocytes, transient positive inotropic effects of CNP followed by negative inotropic effects have been reported (Pierkes et al. 2002; Wollert et al. 2003). It was proposed that the positive inotropic effects are mediated by a cGMP/protein kinase G (PKG) pathway that leads to phosphorylation of phospholamban (Pierkes et al. 2002; Wollert et al. 2003). Effects by CNP were to our knowledge always reported to be mediated by cGMP (Brusq et al. 1999; Pierkes et al. 2002; Wollert et al. 2003; Layland et al. 2005; Su et al. 2005), therefore it is puzzling that inhibition of PDE4 significantly increased the inotropic response mediated by CNP. It is unlikely that an increase in cGMP by CNP will transiently inhibit PDE3 causing the inotropic effect, as an inhibition of PDE3 and PDE4 does not show a clear influence on basal muscle contractility.

Influence of cGMP generated by sGC on responses to 5-HT was tested using the NO-donors SNAP and Sin-1. SNAP alone and in combination with PDE inhibitors did not show a significant effect on responses to 5-HT. In contrast to that the NO-donor Sin-1 showed a clear acceleration of the fade of the inotropic response to 5-HT. This effect of Sin-1 was not influenced by any of the PDE inhibitors tested or combinations of them. The differential effect of the two NO-donors

might be due to the different concentrations used (SNAP 100 µM; Sin-1 300 µM), with 100 µM SNAP not releasing enough NO to produce an effect. The effect of Sin-1 on responses to 5-HT could be partly prevented by the sGC inhibitor ODQ (100 µM), verifying that the NO-donor works through stimulation of sGC. The fact that ODQ only partly prevented the effect of Sin-1, could be due to the high concentration of the NO-donor used, because the binding of ODQ to sGC is competitive with NO (Schrammel et al. 1996). Our results with Sin-1 are in contrast with the results of Afzal et al. (2011), who reported an increase in the 5-$HT_4$ receptor-elicited inotropic response in failing rat ventricle by this compound presumably through inhibition of PDE3. However a suppression of $\beta_1$ adrenergic responses by NO-donors has been observed in healthy and failing rat heart (Ebihara and Karmazyn 1996; Afzal et al. 2011). The proposed mechanism for this is a PKG-mediated inhibition of L-type $Ca^{2+}$ currents, with the functional consequence of suppressing inotropic responses to $\beta_1$ adrenergic stimulation (Mery et al. 1993; Fischmeister et al. 2005). We propose that the accelerated fade of the inotropic response to 5-HT in the presence of Sin-1 is mediated by a similar mechanism involving PKG. There is ample evidence that cGMP as well as cAMP signalling in the cell are strictly compartmentalized (Fischmeister et al. 2005; Fischmeister et al. 2006; Stangherlin et al. 2011). Our results support this view, as stimulation of pGC (under PDE2 and PDE4 inhibition) prolonged inotropic responses to 5-HT, while stimulation of sGC with Sin-1 showed quite the opposite effect by accelerating the fade of the response to 5-HT.

### III.5.4 Influence of PDE inhibition on the lusitropic responses to 5-$HT_4$ receptor agonists

To assess the lusitropic responses to 5-$HT_4$ receptor agonism in pig left atrium, $R_2$ values were calculated for the three agonists under control conditions, in the presence of IBMX and under PDE3 plus PDE4 inhibition. In control conditions, 5-HT and prucalopride induced an initial short lasting very moderate increase in $R_2$ which then progressively decreased reaching a stable value below basal from 10 min after agonist addition on. The initial increase in $R_2$ probably correlates with the inotropic response after addition of agonist, but the following decrease of $R_2$ below basal suggests that 5-HT and prucalopride have lusitropic effects, that are less sensitive to PDE regulation than the positive inotropic response (De Maeyer et al. 2006). GR113808, added 60 min after agonist addition, did not change the decreased $R_2$ values while further reducing contraction force that had stabilized slightly below basal at 60 min. This suggests that the lusitropic response is reversed more slowly than the inotropic response and that therefore the

latter is preferentially antagonized by GR113808. The maintained lusitropic but rapidly fading inotropic responses to 5-$HT_4$ receptor activation may be due to a differential rate of dephosphorylation of the different PKA targets. Indeed different rates of dephosphorylation have been shown for several PKA target proteins after β adrenoceptor stimulation in rabbit heart (Garvey et al. 1988).

Upon pre-treatment with efficient PDE inhibitors, the lusitropic response to 5-HT and prucalopride is masked, as $R_2$ did not change (in the presence of IBMX) or even increased (in the presence of cilostamide plus rolipram) after addition of 5-HT and prucalopride. This reflects the maintenance of the inotropic response under PDE inhibition, which is most pronounced under selective PDE3 and PDE4 inhibition. GR113808 added 60 min after 5-HT or prucalopride under PDE inhibition was now able to reduce $R_2$, corresponding with the reduction of the still present positive inotropic response to 5-HT and prucalopride at 60 min under PDE inhibition.

RS67333 (1 µM) did not induce a change in contractile force nor did it reduce $R_2$ suggesting that receptor activation and signal transduction was too moderate to induce any effect. Under the most efficient PDE inhibition (cilostamide plus rolipram), a clear-cut and sustained positive inotropic response was obtained, explaining the tendency to an increased $R_2$. GR113808 added 60 min after RS67333 did not reduce $R_2$, in contrast to the observations for 5-HT and prucalopride, corresponding to the very weak effect of GR113808 on the inotropic response.

The main conclusions of this study in pig left atrium are: (A) PDE3 and PDE4 isoforms are responsible for the fade of the inotropic response to 5-HT (already assessed by Galindo-Tovar et al. 2009) and prucalopride and completely prevent a response to RS67333. (B) PDE mediated degradation of cAMP upon 5-$HT_4$ receptor activation is maintained, as inotropic responses can be fully recovered by adding the nonspecific PDE inhibitor IBMX one hour after addition of the 5-$HT_4$ receptor agonists. (C) Elevation of cGMP levels via pGC together with inhibition of PDE2 and PDE4, leads to a delayed fade of the response to 5-HT, while elevation of cGMP levels via sGC accelerates the fade of the response to 5-HT independently of PDEs suggesting that there is a compartmentalized interaction between cGMP and cAMP signalling pathways.

## III.6 Acknowledgements

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## III.7 Conflict of interest

Prucalopride belongs to the portfolio of Shire-Movetis NV. J.H. De Maeyer is employed by Shire-Movetis NV. RA Lefebvre receives funding from Shire-Movetis in the framework of a governmentally funded research collaboration, unrelated to the current study.

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# Chapter IV

## *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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# *IV 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*

## IV.1 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 signaling. 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

## IV.2 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 particulate guanylyl cyclase 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 soluble guanylyl cyclase activation on responses to 5-HT was studied by measuring cGMP and cAMP content only.

## IV.3 Methods

### IV.3.1 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 mM: 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 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).

### IV.3.2 Experimental protocols

Isometric force was calibrated in gram (g). Resting load of left atrial pectinate muscles was set to 2 g. Muscle contractions were stimulated with square-wave pulses (0.5 Hz, 5 ms duration, 4 V). Muscle preparations responding with a contraction below 0.5 g 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.

### IV.3.3 Western Blot Analysis

Frozen tissues were homogenized using a Mikro-dismembrator U (B. Braun Biotech, Melsungen, Germany) and taken up in 10x 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). 20 µg 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 over-night 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, 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.

### IV.3.4 cAMP and cGMP content

Frozen tissues were homogenized using a Mikro-dismembrator U (B. Braun Biotech) and taken up in 10x 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 3x 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 sulfate (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 pmol/mg protein.

### IV.3.5 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 2 contractions) were measured at the same time points. Drug-induced increases or decreases in cardiac parameters were expressed in percentage of basal values. The exception were experiments with verapamil, where $(+dF/dT)_{max}$ values were expressed relative to the response generated by 100 µM isoprenaline, given 60 min after 5-HT.

All data are represented as means ± s.e.m. 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.

### IV.3.6 Drugs

EHNA hydrochloride, cilostamide, rolipram, C-type natriuretic peptide (CNP), amino-3-morpholinyl-1,2,3-oxadiazolium chloride (Sin-1) and verapamil hydrochloride were purchased at Tocris bioscience (Huissen, The Netherlands). (S)-(-)-propranolol hydrochloride, IBMX, serotonin creatinine sulfate 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 dimethylsulphoxide (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.

## IV.4 Results

### IV.4.1 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 and Weninger et al. 2012), 5-HT (1 µM) caused a transient increase in contraction force in pig left atrial preparations (**Figure IV.1a**). Inhibition of PDEs using the non-selective inhibitor IBMX (20 µM) completely prevented the fade of the inotropic response to 5-HT (**Figure IV.1b**). PDE4 inhibition with rolipram (1 µM) slightly increased and prolonged responses to 5-HT (**Figure IV.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 (**Figure IV.1c**), similar to responses in the presence of the non-selective PDE inhibitor IBMX (**Figure IV.1b**).

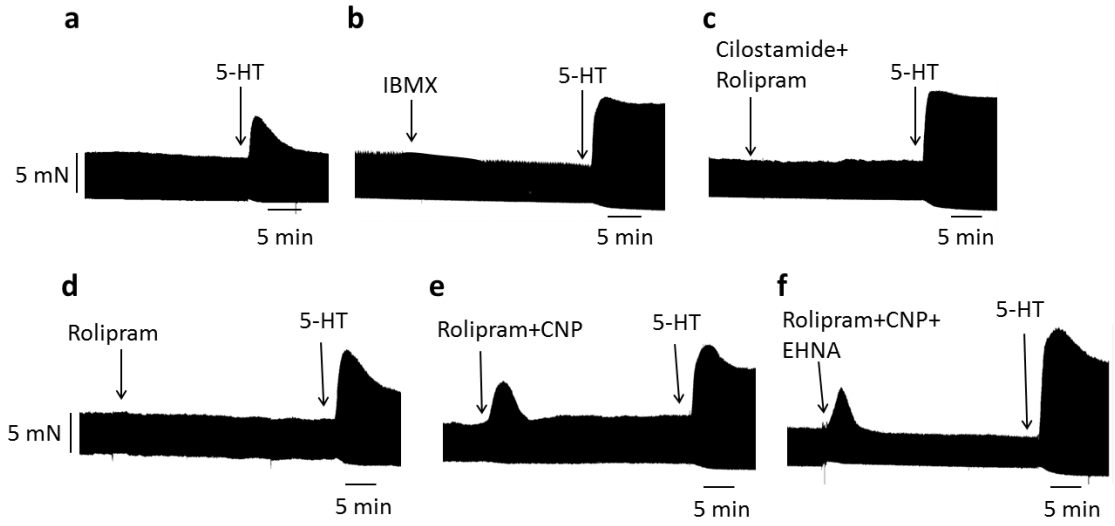


**Figure IV.1** Representative tracings from electrically paced porcine left atrial pectinate muscles showing the responses to 1 µM 5-HT alone (**a**) or in the presence of PDE-inhibition using IBMX (20 µM) (**b**) and concomitant cilostamide (0.3 µM) plus rolipram (1 µM) (**c**). The lower traces show 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

The mean inotropic responses are summarized in **Figure IV.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 are given in **Figure IV.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 $(dF/dt)_{min}$) are listed in **Table IV.1**.

**Table IV.1** Effect of PDE inhibition on 5-HT$_4$ receptor mediated cardiac responses

| | absolute force (mN) | | | $(+dF/dt)_{max}$ | | | $R_2$ | | |
|---|---|---|---|---|---|---|---|---|---|
| | basal | clamp. | Δ | basal | clamp. | Δ | basal | clamp. | Δ |
| **5-HT 2'** | 5.9 ± 0.8 | 11.8 ± 2.1 | 5.9 ± 1.6 | 18.2 ± 2.0 | 36.0 ± 6.0 | 17.8 ± 4.5 | 1.27 ± 0.08 | 1.38 ± 0.09 | 0.11 ± 0.06 |
| **5HT 10'** | 8.1 ± 1.6 | 10.1 ± 2.2 | 2.1 ± 1.1 | 21.0 ± 4.5 | 27.4 ± 6.7 | 6.4 ± 2.7 | 1.32 ± 0.16 | 1.21 ± 0.08 | -0.12 ± 0.11 |
| **5HT 30'** | 6.9 ± 1.3 | 8.5 ± 1.7 | 1.7 ± 0.7 | 18.3 ± 3.3 | 23.6 ± 3.8 | 5.2 ± 1.5 | 1.14 ± 0.03 | 1.14 ± 0.02 | 0.00 ± 0.03 |
| **Pru 2'** | 6.2 ± 0.3 | 7.0 ± 0.5 | 0.8 ± 0.3 | 16.8 ± 0.6 | 18.5 ± 1.1 | 1.7 ± 0.7 | 1.37 ± 0.02 | 1.4 ± 0.02 | 0.03 ± 0.02 |
| **Pru 10'** | 5.5 ± 0.3 | 6.4 ± 0.6 | 1.0 ± 0.3 | 14.7 ± 1.0 | 17.6 ± 1.0 | 2.9 ± 1.1 | 1.26 ± 0.06 | 1.28 ± 0.13 | 0.02 ± 0.10 |
| **Pru 30'** | 10.1 ± 1.9 | 8.5 ± 1.4 | -1.6 ± 0.7 | 21.6 ± 3.0 | 20.7 ± 2.8 | -0.9 ± 1.1 | 1.36 ± 0.17 | 1.22 ± 0.09 | -0.13 ± 0.13 |
| **IBMX 30'** | 10.0 ± 2.7 | 10.7 ± 2.9 | 0.8 ± 0.3 | 28.6 ± 6.4 | 31.6 ± 7.6 | 3.0 ± 1.3 | 1.43 ± 0.05 | 1.27 ± 0.07 | -0.15 ± 0.05 |
| **IBMX 5HT 2'** | 12.1 ± 3.0 | 21.0 ± 4.5 | 8.9 ± 1.7 | 34.9 ± 6.9 | 66.7 ± 12.8 | 31.8 ± 6.3 | 1.23 ± 0.03 | 1.13 ± 0.03 | -0.09 ± 0.04 |
| **IBMX 5HT 10'** | 8.2 ± 1.9 | 14.1 ± 1.9 | 5.9 ± 0.4 | 23.2 ± 5.0 | 41.5 ± 5.3 | 18.3 ± 1.1 | 1.28 ± 0.05 | 1.23 ± 0.05 | -0.05 ± 0.05 |
| **IBMX 5HT 30'** | 7.5 ± 2.1 | 14.9 ± 2.7 | 7.4 ± 1.7 | 23.0 ± 4.6 | 48.5 ± 8.1 | 25.5 ± 5.3 | 1.25 ± 0.04 | 1.23 ± 0.11 | -0.02 ± 0.12 |
| **IBMX Pru 2'** | 8.9 ± 3.0 | 11.9 ± 3.9 | 3.0 ± 1.0 | 25.1 ± 7.0 | 35.2 ± 10.1 | 10.1 ± 3.1 | 1.27 ± 0.05 | 1.38 ± 0.05 | 0.11 ± 0.04 |
| **IBMX Pru 10'** | 11.9 ± 1.3 | 21.5 ± 1.8 | 9.6 ± 1.3 | 31.1 ± 2.2 | 57.2 ± 4.0 | 26.1 ± 3.4 | 1.22 ± 0.05 | 1.33 ± 0.10 | 0.11 ± 0.10 |
| **IBMX Pru 30'** | 5.5 ± 0.9 | 13.2 ± 2.2 | 7.7 ± 1.4 | 14.7 ± 1.0 | 39.2 ± 5.9 | 24.5 ± 5.1 | 1.16 ± 0.07 | 1.28 ± 0.09 | 0.12 ± 0.15 |
| **C+R 30'** | 6.1 ± 1.1 | 6.5 ± 1.6 | 0.4 ± 0.6 | 18.3 ± 2.9 | 20.9 ± 3.9 | 2.6 ± 1.7 | 1.25 ± 0.08 | 1.24 ± 0.03 | -0.01 ± 0.07 |
| **C+R 5HT 2'** | 6.9 ± 1.0 | 17.3 ± 2.0 | 10.4± 2.9 | 20.2 ± 1.6 | 54.6 ± 6.9 | 34.4 ± 8.0 | 1.16 ± 0.05 | 1.13 ± 0.04 | -0.03 ± 0.08 |
| **C+R 5HT 10'** | 8.0 ± 1.4 | 16.8 ± 2.2 | 8.8 ± 1.0 | 23.0 ± 3.4 | 50.1 ± 5.8 | 27.1 ± 2.6 | 1.23 ± 0.05 | 1.21 ± 0.03 | -0.02 ± 0.03 |
| **C+R 5HT 30'** | 8.8 ± 1.7 | 19.0 ± 3.1 | 10.2 ± 1.8 | 25.1 ± 4.3 | 55.5 ± 8.4 | 30.3 ± 5.2 | 1.23 ± 0.07 | 1.24 ± 0.07 | 0.01 ± 0.11 |
| **C+R Pru 2'** | 4.7± 1.3 | 8.9 ± 2.0 | 4.2 ± 1.1 | 15.3 ± 2.9 | 27.5 ± 5.4 | 12.2 ± 3.2 | 1.17 ± 0.04 | 1.39 ± 0.12 | 0.22 ± 0.11 |
| **C+R Pru 10'** | 7.9 ± 1.3 | 17.4 ± 2.3 | 9.5 ± 2.5 | 22.5 ± 3.1 | 48.6 ± 3.9 | 26.0 ± 4.5 | 1.13 ± 0.03 | 1.42 ± 0.10 | 0.29 ± 0.12 |
| **C+R Pru 30'** | 6.4 ± 1.0 | 11.4 ± 0.8 | 5.0 ± 0.7 | 19.7 ± 2.7 | 38.2 ± 2.8 | 18.5 ± 2.0 | 1.17 ± 0.03 | 1.29 ± 0.08 | 0.12 ± 0.07 |

*basal* value before addition of IBMX and C+R for "IBMX 30" and "C+R 30", respectively; for all other condition the value right before addition of the agonist was used as basal; *clamp.* value after addition of all drugs, right before clamping of the muscle; *Δ* difference between clamp. and basal values; mean ± s.e.m. of n=5 left atrial pectinate muscles

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 (**Figure IV.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 (**Figure IV.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 (**Figure IV.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 **Figure IV.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 (**Figure IV.2b**) which is in accordance to the contractile response (**Figure IV.2a**). In the presence of cilostamide and rolipram, 5-HT increased cAMP levels 3- and 4-fold, 2 and 10 min after its addition, respectively; this was significantly different from 5-HT alone (P <0.05; **Figure IV.2b**). Phosphorylation of PLB on $Ser^{16}$ was increased 4-fold in muscles freeze-clamped 2 min after addition of 5-HT (P <0.05; **Figure IV.2e**). De-phosphorylation appears to be slower than the fade of the inotropic response (**Figure IV.2a**), because 10 min and 30 min after adding 5-HT, phosphorylation of PLB was still significantly increased by about 4- and 2-fold respectively compared to basal (P <0.05; **Figure IV.2e**). With concomitant PDE3 and PDE4 inhibition, 5-HT increased phosphorylation of PLB approximately 6-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 6-fold increase in PLB phosphorylation (**Figure IV.2e**). The change in TnI phosphorylation by 5-HT at 2 min from 0.6 to 1.0 did not reach significance (**Figure IV.2f**). Under PDE inhibition with IBMX or cilostamide plus rolipram, TnI phosphorylation after 5-HT was significantly increased 3-4-fold compared to basal (**Figure IV.2f**).

### IV.4.2 Effect of PDE inhibition on cardiac responses, cAMP generation and PLB and TnI phosphorylation by the 5-HT$_4$ receptor agonist prucalopride

As observed before (see Weninger et al. 2012) contractile responses to prucalopride (1 µM; **Figure IV.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 **Figure IV.2a**). Interestingly TPF and $TR_{50}$ showed a maximal decrease in response to prucalopride of -10 and -18 % below basal, respectively (**Figure IV.3c,d**), which is similar as with 5-HT (compare with **Figure IV.2c,d**). Responses to prucalopride developed slower 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 (**Figure IV.3a**), while TPF continually decreased over the 30 min measured (**Figure IV.3c**) and $TR_{50}$ strongly decreased from 2 to 10 min and then stabilized (**Figure IV.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 (**Figure IV.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 (**Figure IV.3c**). The same was observed for the decrease in $TR_{50}$ (approximately -30 % from 2 to 30 min; **Figure IV.3d**). cAMP content did not significantly increase after addition of prucalopride alone. Only when PDE 3 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 ($P < 0.05$; **Figure IV.3b**). Prucalopride increased PLB phosphorylation 2-fold 10 min after its addition ($P < 0.05$; **Figure IV.3e**), but not at 2 and 30 min. Phosphorylation of PLB in response to prucalopride was increased 5-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 ($P < 0.05$; **Figure IV.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.

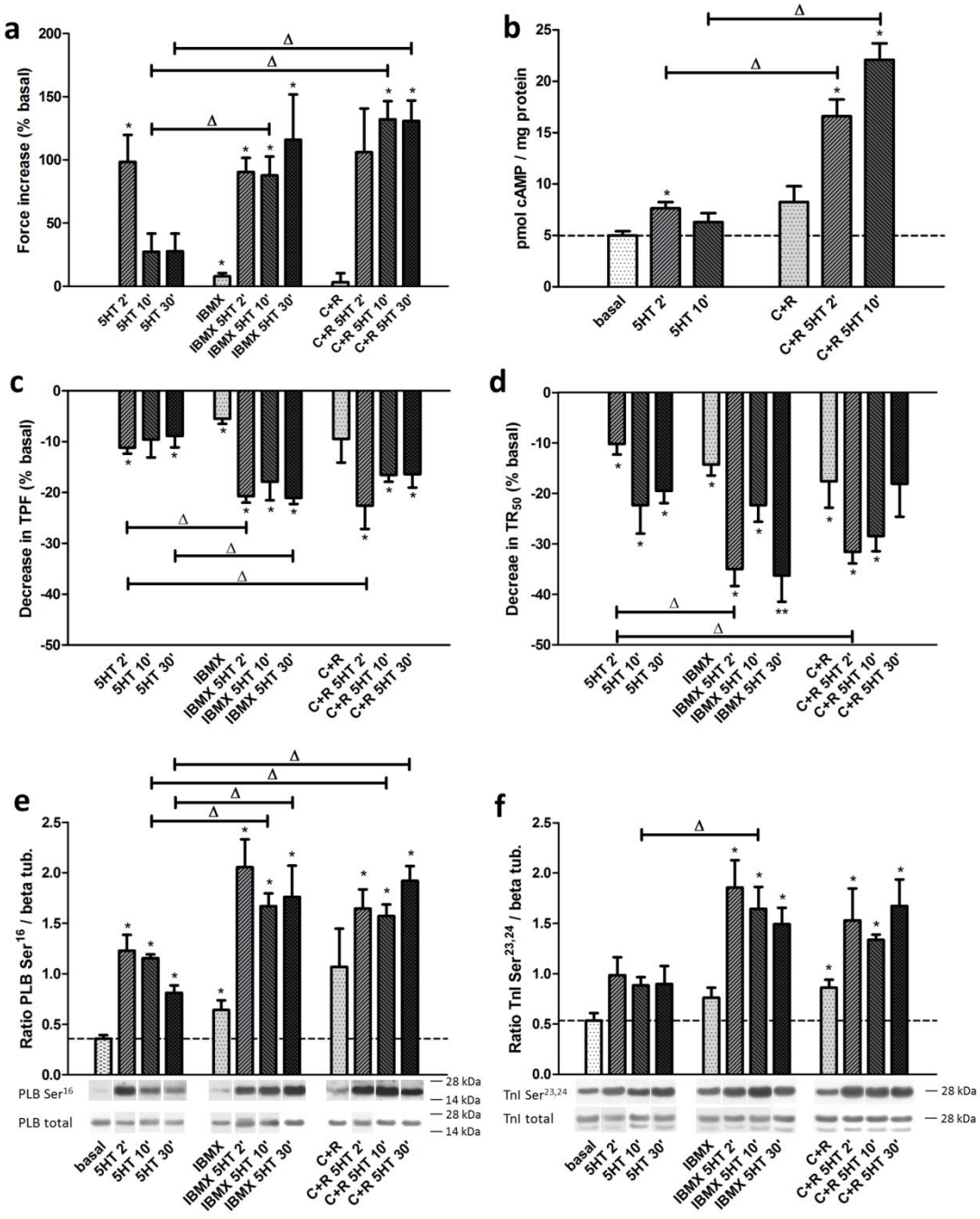


**Figure IV.2** Effect of 5-HT (1µM) alone or in the presence of PDE inhibition with IBMX (20 µM) or cilostamide (0.3 µM) plus rolipram (1 µM) on force of contraction (**a**), tissue cAMP content (**b**), time to peak force (TPF) (**c**), time to 50 % relaxation ($TR_{50}$) (**d**), phosphorylation of 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 (5HT) or presence of IBMX (IBMX 5HT) or

cilostamide plus rolipram (C+R 5-HT). Force of contraction as well as TPF and $TR_{50}$ were calculated in percentage to basal values (**a**,**c**,**d**), cAMP content is shown as pmol cAMP per mg 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; Δ *P*<0.05 for 5HT in the presence of IBMX or C+R versus 5HT in their absence, one-way ANOVA with Bonferroni post-testing

### IV.4.3 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 2-fold (P <0.05, n=5, **Figure IV.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 (**Figure IV.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 (**Figure IV.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 (**Figure IV.1e** and **5a**); this was not further enhanced in the additional presence of 10 μM of the PDE2-inhibitor EHNA (115 %, **Figure IV.5a**). We also noticed that CNP had a small inotropic effect of its own (**Figure IV.5a**), which was enhanced when rolipram was administered together with CNP (**Figure IV.1e,f** and **Figure IV.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) (**Figure IV.5b**). Tissue cAMP content in response to 5-HT was significantly increased by about 2-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 3-fold, which was significant versus 5-HT in the absence of these compounds (P <0.05; **Figure IV.5b**).

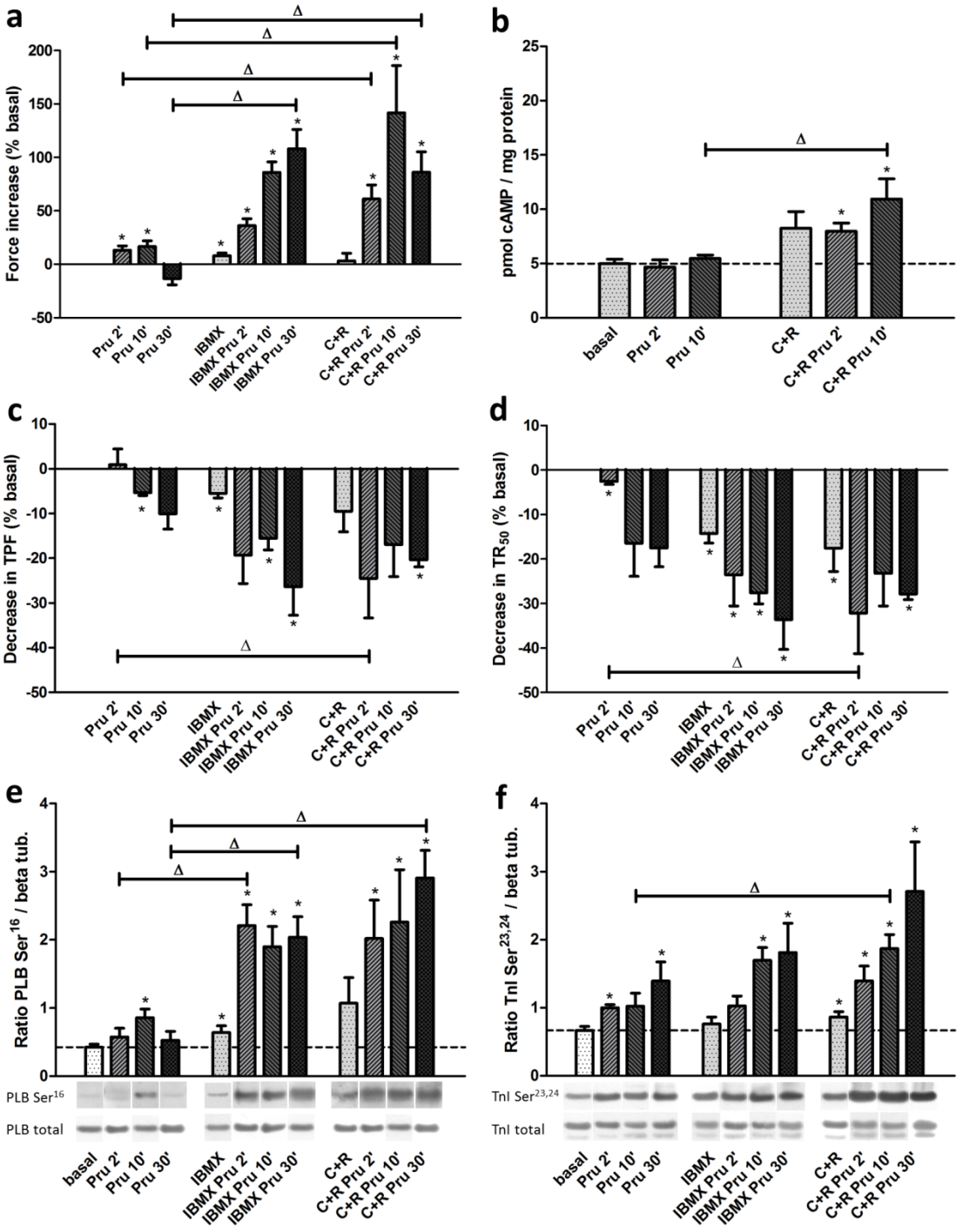


**Figure IV.3** Effect of prucalopride (Pru, 1µM) alone or in the presence of PDE inhibition with IBMX (20 µM) or cilostamide (0.3 µM) plus rolipram (1 µM) on force of contraction (**a**), tissue cAMP content (**b**), TPF (**c**), $TR_{50}$ (**d**), phosphorylation of phospholamban (**e**) and phosphorylation 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 Figure IV.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; Δ *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

PLB phosphorylation essentially mirrored the functional effects depicted in **Figure IV.5a**. Phosphorylation of PLB was increased 2-fold (P <0.05) after the simultaneous addition of CNP and rolipram (**Figure IV.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 **Figure IV.2e**. In the presence of rolipram, PLB phosphorylation was increased 4-fold 10 min after addition of 5-HT (P <0.05; **Figure IV.5c**). The combinations of rolipram plus CNP, and rolipram plus CNP plus EHNA, both increased PLB phosphorylation in response to 5-HT about 6-fold (P <0.05 versus basal; **Figure IV.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 (**Figure IV.5d**). Phosphorylation of TnI was increased 1.5-times 2 min after the simultaneous addition of CNP and rolipram (P <0.05; **Figure IV.5d**), while no significant effect was observed with CNP alone. 10 min 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 2-3-fold versus basal (P <0.05; **Figure IV.5d**); however, no significance was observed when compared to the condition with 5-HT alone.

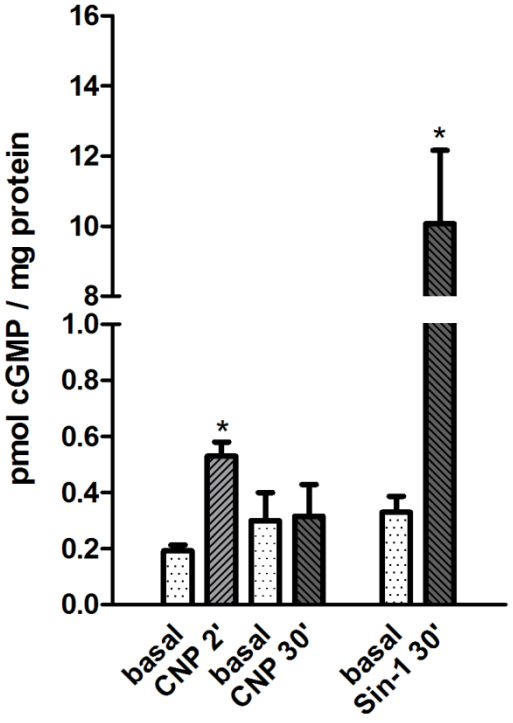


**Figure IV.4** Effect of particulate guanylyl cyclase stimulation with C-type natriuretic peptide (CNP, 0.3 µM) 2 and 30 min after administration and soluble guanylyl cyclase stimulation using the NO-donor Sin-1 (300 µM) 30 min after administration on tissue cGMP content (in pmol cGMP per mg protein), 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

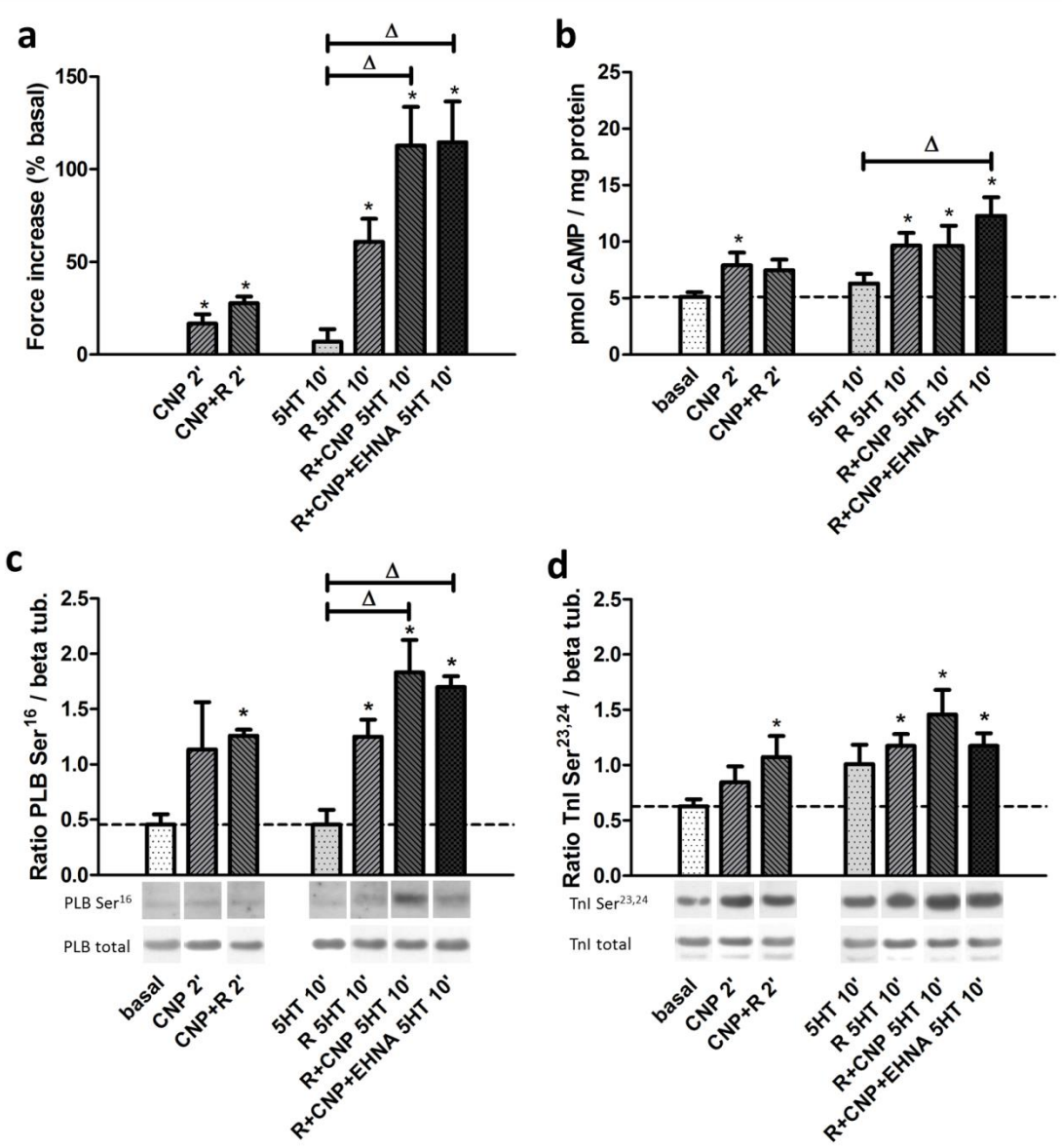


**Figure IV.5** Effect of particulate guanylyl cyclase stimulation using C-type natriuretic peptide (CNP, 0.3 µM) on basal muscle responses and on responses to 5-HT (1 µM). Measured were force of contraction (**a**), tissue cAMP content (**b**), phosphorylation of phospholamban (**c**) and phosphorylation 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 (5HT) or presence of rolipram (R 5HT), concomitant rolipram plus CNP (R+CNP 5HT) and rolipram plus CNP plus EHNA (10 µM) (R+CNP+EHNA 5HT). Functional responses were calculated as force increase in percentage to basal force (**a**), cAMP content is shown as pmol cAMP per mg 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; Δ *P*<0.05 versus 5HT 10', one-way ANOVA with Bonferroni post-testing

### IV.4.4 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 ($P < 0.05$; **Figure IV.6b,c**). 5-HT could still increase muscle contractility to 56 % above basal 2 min after its addition in the presence of Sin-1 (300 µM; **Figure IV.6b,c**), which was not significantly different from responses to 5-HT in the absence of Sin-1 (**Figure IV.6a,c**). 10 min 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 (**Figure IV.6c**; Weninger et al. 2012). Sin-1 increased tissue cGMP content massively by 30-fold ($P < 0.05$; **Figure IV.4**), confirming that production of cGMP is stimulated. Interestingly also cAMP content was increased by about 1.5-fold after administration of Sin-1 ($P < 0.05$; **Figure IV.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. To test whether the effect of Sin-1 is due to a cGMP/protein kinase G I (PKGI) mediated inhibition of L-type $Ca^{2+}$ channels, experiments were performed using the L-type $Ca^{2+}$ channel inhibitor verapamil (**Figure IV.7**; unpublished results). From a preliminary experiment studying the concentration-dependency of the effect of verapamil per se on contraction force, 30 and 100 nM verapamil were selected. 30 nM verapamil decreased basal contraction force to -12 ± 2 % and 100 nM verapamil decreased contraction force to -23 ± 9 %. Both concentrations of verapamil decreased responses to 5-HT similarly, giving contractile responses of 41 ± 12 % and 39 ± 9 % 2 min after addition of 5-HT; compared to 60 ± 14 % in control muscles receiving only 5-HT; this did not reach significance, however. An effect of verapamil was only observed in the first 8-9 min after addition of 5-HT, while after 10 min all responses (in muscles receiving verapamil and control muscles) had faded to ~10 %. Sin-1 also tended to decrease immediate responses to 5-HT giving a contraction force of 36 ± 2 %, 2 min after addition of 5-HT. But contrary to verapamil, Sin-1 clearly accelerated the fade of the response to 5-HT showing responses below basal already 4 min after addition of 5-HT and decreasing contraction force to a minimal value of -10 ± 5 % at min 6. After that responses recovered slowly and stabilized at a value close to basal (also see Weninger et al. 2012). Verapamil 30 nM and 100 nM in combination with Sin-1 seemed to decrease immediate responses to 5-HT somewhat further than verapamil or Sin-1 alone, giving values of 31 ± 13 % and 22 ± 12 % respectively, 2 min after addition of 5-HT (**Figure IV.7**). From min 4 onwards muscles exposed to verapamil 30 nM + Sin-1 / 5-HT and verapamil 100 nM + Sin-1 / 5-HT

showed basically the same response as muscles only receiving Sin-1 / 5-HT, with the accelerated fade of the response followed by a slow recovery (**Figure IV.7**).

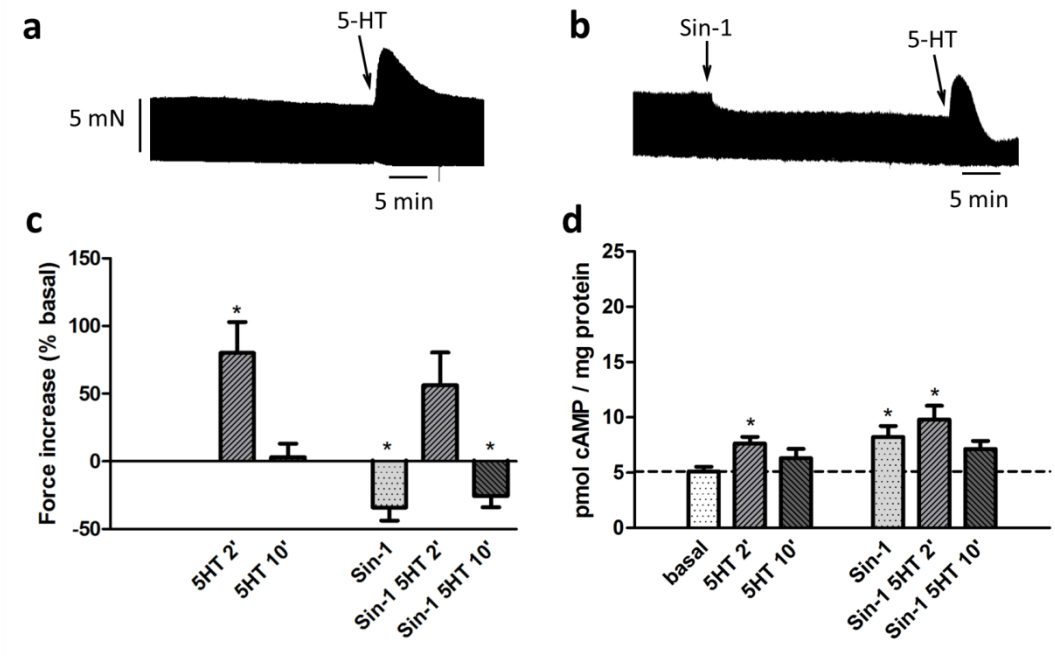


**Figure IV.6** Effect of soluble guanylyl cyclase stimulation using the NO-donor Sin-1 (300 µM) on responses to 5-HT (1 µM). Displayed are representative traces showing responses to 5-HT alone (**a**) or 5-HT in the presence of Sin-1 (**b**) as well as graphs showing mean force of contraction (**c**) 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 (5HT) 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 pmol cAMP per mg protein (**d**). * *P*<0.05 versus basal, unpaired *t* test

## IV.5 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 re-uptake 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).

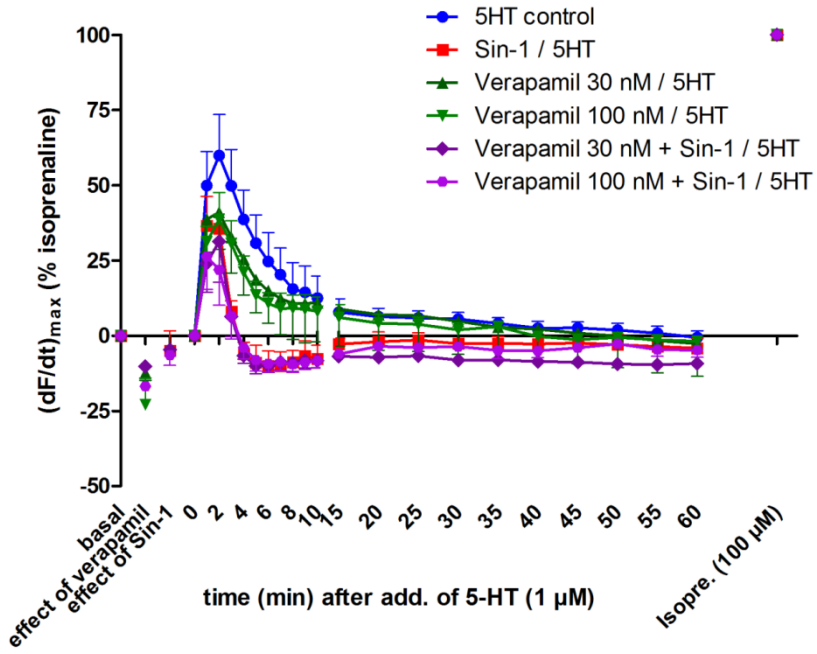


**Figure IV.7** Time line of the effects of Sin-1 (300 µM) in the absence or presence of verapamil (30 nM or 100 nM) on responses to 1 µM 5-HT; Sin-1 was added 40 min after verapamil. Each time point represents averaged contraction force ± s.e.m. of n=4 atrial strips as a % of the response generated by isoprenaline (Isopre. 100 µM). Basal values were taken just before addition of verapamil or Sin-1 and presented as 0 % force increase. The contraction force at the end of the 40 min incubation period with verapamil is shown as "effect of verapamil". The contraction force at the end of the 20 min incubation period with Sin-1 is calculated in reference to the basal value before its own addition and is shown as "effect of Sin-1". Values taken right before addition of 5-HT were normalized to 0 % force increase (time point 0) and taken as the new basal value to measure the responses to 5-HT. Responses of muscles which only received 5-HT (5HT control) are shown in comparison.

### IV.5.1 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 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 (**Figure IV.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 are 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 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 **Figure IV.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.

### IV.5.2 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 (**Figure IV.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 (**Figure IV.5a** and **6b**; Weninger et al. 2012). Both effects have been observed before and were reported to be mediated by PKGI (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-inhibiton (Weninger et al. 2012; **Figure IV.5a**). In the literature positive and negative inotropic responses as wells as a biphasic response to CNP consisting of an initially positive inotropic and lusitropic response followed by a negative inotropic effect are described (Hirose et al. 1998; 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 (**Figure IV.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 PDEs 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 induced inotropic response 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 break-down 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 PDE2-inhibition with EHNA (**Figure IV.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 (**Figure IV.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. Soluble guanylyl cyclase 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 (**Figure IV.6c**, Weninger et al. 2012). 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, $\beta_1$-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. The immediate response to 5-HT, 2 min after addition, is decreased further in the combined presence of verapamil and Sin-1 compared to Sin-1 or verapamil alone, indicating action through different mechanisms. Moreover verapamil 30 or 100 nM alone decreased responses to 5-HT but did not show an acceleration of the fade of the response like Sin-1, also suggesting different mechanisms. 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 (**Figure IV.6c, d**). 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 de-phosphorylation of PLB by protein phosphatase 1 (PP1) (Kohr et al. 2008b; Kohr et al. 2008a). 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.

## IV.6 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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## IV.8 Conflict of Interest

Prucalopride belongs to the portfolio of Shire-Movetis NV. JH De Maeyer is employed by Shire-Movetis NV. RA Lefebvre performs contract studies for Shire-Movetis NV.

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# Chapter V

# *Interaction of PDE4Ds with the 5-$HT_{4(b)}$ receptor in HEK293 cells.*

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# *V Interaction of PDE4Ds with the 5-HT$_{4(b)}$ receptor in HEK293 cells.*

## V.1 Abstract

**Purpose.** Phosphodiesterase (PDE) 3 and PDE4, which degrade cAMP, are important regulators of 5-HT$_4$ receptor signalling in cardiac tissue. Therefore we investigated whether they interact with the 5-HT$_{4(b)}$ receptor on protein level. Furthermore it was studied whether A-kinase anchoring proteins (AKAPs), scaffolding proteins which contribute to the spacial-temporal control of cAMP signalling, are involved in the regulation of 5-HT$_{4(b)}$ receptor signalling.

**Methods.** Signalling of the overexpressed HA-tagged 5-HT$_{4(b)}$ receptor in HEK293 cells and the regulation by PDEs was investigated by measuring protein kinase A (PKA) activity in the absence and presence of PDE3- and PDE4-inhibitors. An interaction of overexpressed PDE3B as well as PDE4D3 and PDE4D5 splice variants with the 5-HT$_{4(b)}$ receptor was examined by co-immunoprecipitation (co-IP) and for PDE4D3 also by PDE activity measurements in immunoprecipitates of the receptor. Furthermore, a possible involvement of AKAPs in 5-HT$_{4(b)}$ receptor signalling was studied employing the St-Ht31 inhibitor peptide, which disrupts the interaction of AKAPs with PKA, and by investigating the interaction of endogenous AKAP79 and gravin with the 5-HT$_{4(b)}$ receptor by co-IP.

**Results.** PDE4 inhibition strongly increased PKA activity in 5-HT$_{4(b)}$ receptor overexpressing HEK293 cells while PDE3 inhibition had no effect. Furthermore, both overexpressed PDE4D3 and PDE4D5 splice variants co-immunoprecipitated with the 5-HT$_{4(b)}$ receptor; no change was observed upon receptor stimulation. PDE3B as well as AKAP79 and gravin were not found in IPs of the 5-HT$_{4(b)}$ receptor and the St-Ht31 peptide had no influence on 5-HT$_{4(b)}$ receptor stimulated PKA activity.

**Conclusion**. 5-HT$_{4(b)}$ receptor signalling to PKA in HEK293 cells is regulated predominantly by PDE4. In line with that PDE4D3 and PDE4D5 splice variants, but not PDE3B, interact with the 5-HT$_{4(b)}$ receptor on protein level. No evidence for an involvement of AKAPs in 5-HT$_{4(b)}$ receptor signalling was obtained.

**Keywords.** 5-HT$_4$ receptor signalling, PDE4D3, PDE4D5, PDE3B, AKAP79, gravin

## V.2 Introduction

The 5-$HT_4$ receptor is a $G_s$ protein-coupled receptor signalling through adenylyl cyclase, generation of cAMP and activation of protein kinase A (PKA). It is widely distributed in the brain and periphery. In human at least 11 5-$HT_4$ receptor splice variants are expressed with important tissue specific differences (Bockaert et al. 2004; Coupar et al. 2007; Irving et al. 2010). 5-$HT_{4(a)}$ and 5-$HT_{4(b)}$ splice variants are most commonly expressed and were detected in the heart, the brain, the kidney and the gastrointestinal tract (Coupar et al. 2007).

Phosphodiesterases (PDEs), which degrade the second messenger cAMP, were shown to play a paramount role in regulating 5-$HT_4$ receptor signalling in various tissues including the heart, the stomach and the colon (McLean and Coupar 1996; De Maeyer et al. 2006; Afzal et al. 2008; Galindo-Tovar et al. 2009; Priem et al. 2012; Weninger et al. 2012; Weninger et al. 2013), yet it was not tested if they interact on a molecular basis with the 5-$HT_4$ receptor. At least 11 PDE families exist, including 20 genes and many more isoforms (Mika et al. 2012). PDE3 and PDE4 are the most important cAMP-specific PDEs in the heart (Afzal et al. 2008; Galindo-Tovar et al. 2009) and together provide nearly all cAMP-PDE activity in HEK293 cells (Lynch et al. 2005). Two PDE3 genes were detected (PDE3A and PDE3B), and further diversity stems from PDE3A splice variants (Shakur et al. 2000; Wechsler et al. 2002). Conversely four PDE4 genes (PDE4A - PDE4D) generate more than 20 different PDE4 isoforms by alternative splicing coupled to the use of different promoters, allowing tissue- and development-specific expression. Each PDE4 isoform has a unique N-terminal domain responsible for its specific localization to signalling complexes and membrane structures within the cell (Conti et al. 2003; Houslay et al. 2007). PDEs are often found in complex with A-kinase anchoring proteins (AKAPs), a family of scaffolding enzymes which tether PKA and other enzymes involved in the regulation of cAMP signalling to defined locations in the cell. AKAPs were shown to play an important role in the signalling of some G protein-coupled receptors, a prime example being the $\beta_2$-adrenergic receptor (Dessauer 2009).

PDEs and AKAPs were both shown to build signalling complexes with β-adrenergic receptors, a classic model of $G_s$ protein-coupled receptors acting via cAMP and PKA. Upon agonist stimulation PDE4D3 and PDE4D5 isoforms are recruited via β-arrestin-2 to the $\beta_2$-adrenergic receptor both in HEK293 cells and cardiomyocytes (Perry et al. 2002; Baillie et al. 2003; Lynch et al. 2005). In contrast, the interaction between PDE4Ds and the $\beta_1$-adrenergic receptor was found to be independent of β-arrestin (Richter et al. 2008). Also two AKAPs, AKAP79 and gravin were found to immunoprecipitate with the $\beta_2$-adrenergic receptor, and AKAP79 tethers the pool

of PKA responsible for phosphorylation of the receptor upon stimulation (Fraser et al. 2000; Lin et al. 2000; Fan et al. 2001; Tao et al. 2003; Lynch et al. 2005). As little is known about the interaction of these proteins with the 5-$HT_4$ receptor, this was investigated in the present study in HEK293 cells overexpressing the 5-$HT_{4(b)}$ receptor.

## V.3 Materials and methods

### V.3.1 Plasmids and antibodies

The HA-tagged 5-$HT_{4(b)}$ receptor cloned into pcDNA3.1+ (Life Technologies, Carlsbad, California) was purchased from the Missouri S&T cDNA Resource Center. VSV-tagged PDE4D3 and PDE4D5 in pcDNA3 were described before (Bolger et al. 1997; Baillie et al. 2007). GFP-tagged PDE3B in pEGFP-C1 (Clonetech Laboratories, Mountain View, California) was a kind gift from Prof. Maurice (Queen's University, Kingston, Ontario, Canada).

Primary antibodies used in this study were mouse monoclonal anti-HA (1/2,000, clone 16B12; Covance Inc., Princeton, New Jersey), rat anti-HA (1/2,000; Roche, Basel, Switzerland), rabbit anti-PDE4D (1/250; Abcam, Cambridge, UK), rabbit anti-VSV-G (1/5,000; Sigma-Aldrich, St. Louis, Missouri), mouse monoclonal anti-Flag M2 HRP (1/1,000; Sigma-Aldrich), rabbit anti-PDE3B (1/200; Santa Cruz Biotechnology, Inc., Santa Cruz, California), mouse monoclonal anti-AKAP79 (1/2,000; BD Biosciences, Franklin Lakes, New Jersey) and rabbit anti-AKAP250 (gravin; 1/1,000; Thermo Fisher Scientific, Waltham, Massachusetts). IRDye® 800CW and IRDye® 680LT secondary antibodies (1/15,000; LI-COR Biosciences, Lincoln, Nebraska) were used for visualization of primary antibodies. Alexa-Fluor 488- or 594-coupled goat anti-mouse antibodies (1/500; Life Technologies) were used to visualize the primary anti-HA antibody in immunofluorescence.

### V.3.2 Cell culture and transfection

HEK293T cells and HEK293A cells were cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10 % (v/v) fetal bovine serum with L-glutamine (2 mM), penicillin (100 U $ml^{-1}$) and streptomycin (0.1 mg $ml^{-1}$), in a controlled environment (37°C, 98% humidity, 5% $CO_2$). For transfection, cells were grown until 50% confluency.

HEK293A cells stably expressing the HA-tagged 5-$HT_{4(b)}$R (293A/5-$HT_{4(b)}$R) were generated using the calcium phosphate transfection method (using 25 µg DNA per 75 $cm^2$ flask) with addition of 0.52 mg chloroquine (Sigma-Aldrich). Stable transfectants were selected with geneticin (G418, 100 mg $ml^{-1}$, Life Technologies) over a period of 3 weeks. 5-$HT_{4(b)}$R expression at the plasma membrane was confirmed by immunofluorescence (Figure V.1b).

HEK293T cells were transiently transfected with plasmids encoding HA-5-$HT_{4(b)}$R as well as PDE4D3, PDE4D5 or PDE3B using the calcium phosphate transfection method (using 10 µg DNA per 10 cm dish) as described previously (Skieterska et al. 2013). In HEK293A cells the calcium phosphate method did not yield a high transfection efficiency. Therefore 293A/5-$HT_{4(b)}$R cells as well as parental HEK293A cells were transiently transfected with PDE4D3 or PDE4D5 using the poly(ethylenimine) transfection method (adding 10 µg DNA per 10 cm dish), as described previously (Van Craenenbroeck et al. 2011). Cells were grown for at least 36 h and starved for more than 12 h in serum-free DMEM before addition of drugs. Drug concentrations and incubation times are given in the results section. After stimulation cells were washed twice with ice-cold phosphate-buffered saline and harvested.

### V.3.3 Immunofluorescence

HEK293T and 293A/5-$HT_{4(b)}$R cells were grown on poly-D-lysine-coated glass coverslips in 6-well plates. HEK293T cells were transiently transfected with the HA-tagged 5-$HT_{4(b)}$ receptor. At about 50 % confluency cells were serum-starved overnight before incubation with a mouse anti-HA antibody (1/1,000) for 1 h at 4°C. After carefully washing the cells and adding fresh serum-free DMEM, cells were transferred to 37°C where 5-HT (10 µM) was added to the medium of selected cells for 2, 10 or 30 min. Then cells were fixed in 10 mM sodium phosphate buffer at pH 7.4 containing 3.7 % formaldehyde and 150 mM sodium chloride, washed extensively and permeabilized in blocking buffer (3 % skim milk, 1 mM $CaCl_2$, 50 mM Tris-HCl pH 7.5) containing 0.1 % Triton X-100. Secondary antibody incubation with Alexa-Fluor 488- or 594-conjugated goat anti-mouse antibody was performed for 30 min at room temperature. Cells were washed and cover slips were mounted onto slides using Mowiol mounting medium. Fluorescent images were taken on a Zeiss Axiovert 200 M microscope with a 63x oil immersion lens. The excitation and emission filters for Alexa-Fluor 594 were $\lambda_{ex}$=590 nm and $\lambda_{em}$=617 nm and for Alexa-Fluor 488 $\lambda_{ex}$=495 nm and $\lambda_{em}$=519 nm.

### V.3.4 Co-immunoprecipitation (co-IP) assay and Western blot analysis

HEK293T cells as well as HEK293A parental and 293A/5-$HT_{4(b)}$R cells were grown in 10 cm dishes and transfected, stimulated as described in the results section and harvested. The co-IP and Western blot assays were performed as described previously (Spooren et al. 2010). Briefly, cells were lysed in 400 µl of RIPA buffer (150 mM NaCl, 5 mg/ml sodium deoxycholate, 50 mM Tris/HCl pH 7.5, 1 % Nonidet P-40, 0.1 % sodium dodecyl sulfate) supplemented with freshly added protease inhibitors (2.5 µg/ml aprotinin, 1 mM pefablock, 10 µg/ml leupeptin, 10 mM β-glycerophospate) for 1 h at 4°C with rotation, after which the lysates were cleared by centrifugation (8,000 g for 10 min at 4°C). 40 µl of the cleared lysate was denatured in SDS loading buffer (62 mM Tris⁄HCl, pH 6.8, 4 % sodium dodecyl sulfate, 20 % glycerol, 0.01 bromophenol blue plus 20 mM dithiothreitol) and analysed by Western blot for detection of protein expression. To the remaining lysates 2 µg of monoclonal mouse anti-HA (Clone 16B12, Covance) antibody was added and incubated for 4 h at 4°C with rotation before addition of 20 µl washed protein A UltraLink® resin (Thermo Fisher Scientific) followed by another incubation overnight at 4°C with continuous rotation. After extensive washing with RIPA buffer, SDS-loading buffer was added to the resin followed by denaturation at 95 °C for 5 min or 37°C for 10 min for detection of PDEs and AKAPs, respectively. Proteins were separated on a 10 % SDS/ polyacrylamide gel and transferred to a nitrocellulose membrane. Primary antibody incubation was performed overnight at 4°C with rotation or for 2 h at room temperature with rotation. Secondary antibodies were incubated for 1 h at room temperature with rotation in the dark. For the anti-Flag HRP antibody, bands were visualized with Amersham ECL detection reagent (GE healthcare, Little Chalfont, United Kingdom) and detected with a Digital Science Image Station (Kodak, Rochester, NY). IRDye® 800CW and IRDye® 680LT (LI-COR Biosciences) secondary antibodies were used to visualize all other primary antibodies and detected using an Odyssey® infrared imaging system (LI-COR Biosciences) and Odyssey software version 3.0.21 (LI-COR Biosciences).

### V.3.5 PKA activity assay

HEK293T cells as well as HEK293A parental and 293A/5-$HT_{4(b)}$R cells, grown in 6-well plates, were transfected and stimulated as indicated in the Results section. Immediately after harvesting, cells were lysed by adding 100 µl lysis buffer (0.2 M $K_2HPO_4$, 0.2 M $KH_2PO_4$, 2 %

Triton X-100) per well. The plate was then placed at -20°C for more than one hour before the lysate was collected and debris removed by centrifugation at 14,000 g for 5 min at 4°C. 10 µl of the supernatant was used to analyse PKA activity using the PepTag® non-radioactive cAMP-dependent protein kinase assay (Promega, Fitchburg, Wisconsin) according to the manufacturers' instructions, without addition of extra cAMP. Shortly the dye-tagged PKA substrate peptide (L-R-R-A-S-L-G; PepTag® A1 Peptide) was incubated in the provided buffer (20 mM Tris/HCl pH 7.4, 10 mM $MgCl_2$, 1 mM ATP) with the cell lysate and kept for 30 min at room temperature. During this time the PepTag® A1 peptide is phosphorylated by the PKA in the cell lysate, adding a negative charge. The phosphorylation reaction was stopped by heating the sample to 95°C for 10 min. Phosphorylated and non-phosphorylated peptide bands were then separated on a 0.8 % agarose gel. Bands were quantified using Image J software (version 1.47v) and the ratio between non-phosphorylated and phosphorylated peptide bands was calculated.

### V.3.6 PDE activity assay

PDE activity was measured directly in cell lysates and in immunoprecipitates (IP) of the 5-$HT_4$ receptor. 293A/5-$HT_{4(b)}$R as well as parental HEK293A cells were grown in 10 cm dishes, transfected with PDE4D3, stimulated as described in the Results section and harvested. For preparation of lysates, cells were suspended in 400 µl 20 mM Tris/HCl pH 7.4, freeze-thawed and lysed by passing through a 25 gauge needle. IP samples were prepared by lysing cells in 400 µl buffer containing 50 mM NaCl, 50 mM NaF, 25 mM HEPES, 5 mM EDTA, 30 mM sodium pyrophosphate, 10% glycerol, 1% Triton X-100 at pH 7.5, supplemented with protease inhibitor cocktail (Roche). The IP of the 5-$HT_{4(b)}$ receptor was performed as described above. Lysates and IP samples were snap frozen and kept at -70°C until further analysis. PDE activity was assayed using a modification of the Thompson and Appleman procedure (Thompson and Appleman 1971) as described previously (Lynch et al. 2005). Measurements of lysates were performed in the presence of the selective PDE3- and PDE4-inhibitors cilostamide (1 µM) and rolipram (10 µM) respectively, to determine the fraction of activity corresponding to each PDE family. The cAMP substrate solution containing a 2 µM mixture of [$^3$H]cAMP (Amersham Biosciences) and unlabelled cAMP in 20 mM Tris/HCL, 10 mM $MgCl_2$ at pH 7.4 was added to all samples to a final concentration of 1 µM cAMP, followed by an incubation period of 20 min at 30°C with frequent agitation. To stop cAMP hydrolysis samples were placed in a 100 °C hot water bath for 1 min followed by an incubation on ice for 10 min. 1 mg/ml snake venom (from *Crotalus atrox*, Sigma)

was added and incubated for 10 min at 30°C. Dowex resin (1X8-400, Sigma) was prepared by supplementing a Dowex/water mixture with absolute alcohol (ratio 2:1). This Dowex slurry was added to the samples, vortexed and incubated on ice for 10 min. After centrifugation (13,000 g, 3 min, 4°C), to pellet the dowex resin, the supernatant was transferred to a scintillation vial containing 1 ml of Optiscint scintillator (PerkinElmer, Waltham, Massachusetts) and cAMP hydrolysis was measured on a Wallac 1409 liquid scintillation counter (PerkinElmer).

PKA and PDE activities are represented as means ± s.e.m. of n=number of different experiments. GraphPad Prism V5.03 was used to draw graphs and to calculate the statistics (using unpaired Student's *t* test). A *P* value <0.05 was considered significant.

### V.3.7 Drugs

GR113808, GR125487, cilostamide and rolipram were purchased at Tocris Bioscience (Bristol, UK). Isobutylmethylxanthine (IBMX), isoprenaline hydrochloride and serotonin creatinine sulfate salt monohydrate (5-HT) were obtained from Sigma-Aldrich. The InCELLect™ AKAP St-Ht31 inhibitor peptide was purchased from Promega. IBMX, GR113808, cilostamide and rolipram were dissolved in dimethylsulphoxide (DMSO). GR125487, 5-HT and isoprenaline were dissolved in deionized water.

## V.4 Results

### V.4.1 Localization and signalling of the overexpressed 5-$HT_{4(b)}$ receptor

Membrane localization of the overexpressed 5-$HT_{4(b)}$ receptor was confirmed by immunofluorescence in both HEK293T cells transiently transfected with the receptor and in cells stably expressing the receptor (293A/5-$HT_{4(b)}$R cells) (**Figure V.1a,b**). In line with literature data (Pindon et al. 2004; Barthet et al. 2005), a time-dependent internalization of the 5-$HT_{4(b)}$ receptor was observed upon stimulation with 10 µM 5-HT. No internalization was visible after 2 min of stimulation but after 10 and 30 min dots appeared inside of HEK293T cells transiently transfected with the receptor, indicating internalization (**Figure V.1a**). In 293A/5-$HT_{4(b)}$R cells the receptor was also internalized after exposure to 5-HT for 10 min (**Figure V.1b**); other time points were not tested in this cell line.

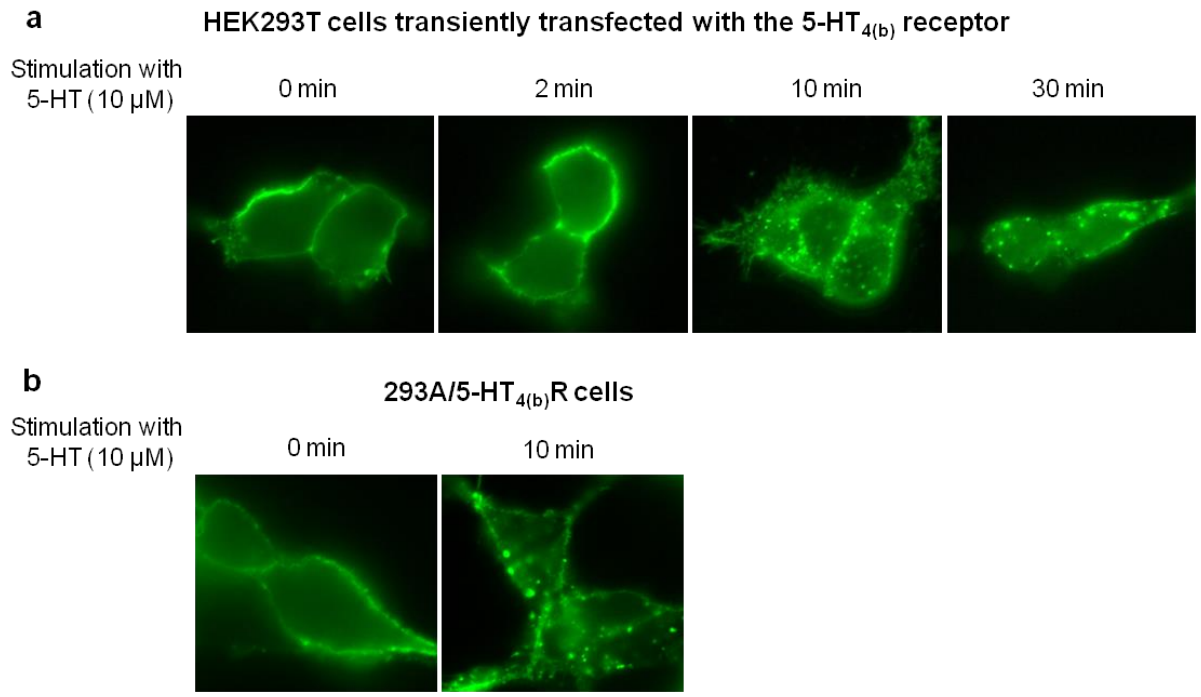


**Figure V.1** Membrane localization and stimulation-dependent internalization of the 5-HT$_{4(b)}$ receptor in HEK293T cells transiently transfected with the HA-tagged receptor (**a**) and in 293A/5-HT$_{4(b)}$R cells stably expressing the receptor (**b**). Living cells were incubated with anti-HA antibody before addition of 10 µM 5-HT to the culture medium. Cells were then fixed and incubated with Alexa-Fluor 594 conjugated secondary antibody. Fluorescence microscopy visualized localization of 5-HT$_{4(b)}$ receptors before (0 min) or after stimulation with 5-HT (time indicated above the image).

cAMP signalling of the overexpressed 5-HT$_{4(b)}$ receptor was analyzed by measuring PKA activity. Parental HEK293A cells did not show an increase in PKA activity in response to 5-HT (10 µM for 5 min; **Figure V.2b**). This concentration and incubation time of 5-HT were selected based on previous studies in HEK293 cells overexpressing the 5-HT$_4$ receptor (Barthet et al. 2007; Barthet et al. 2009). Transient overexpression of the 5-HT$_{4(b)}$ receptor nominally increased basal PKA activity 1.6-fold in HEK293T cells, compared to vector transfected cells (**Figure V.2a**); no further increase was observed after stimulation (10 µM 5-HT for 5 min) of the receptor. In 293A/5-HT$_{4(b)}$R cells PKA activity was increased 1.3-fold compared to parental cells not expressing the receptor ($P<0.001$; **Figure V.2b**). Stimulation of the receptor with 10 µM 5-HT significantly further increased PKA activity in these cells to about 1.7-fold of the activity in parental cells ($P<0.01$; **Figure V.2b**). The constitutive activity of the 5-HT$_{4(b)}$ receptor observed in transiently transfected cells and both the constitutive activity and the additional effect of the agonist 5-HT observed in 293A/5-HT$_{4(b)}$R cells were specific for the 5-HT$_4$ receptor, since they were blocked by

pre-incubation of cells for 30 min with the 5-$HT_4$ receptor antagonist GR113808 (10 µM) or GR125487 (10 µM) (**Figure V.2a,b**).

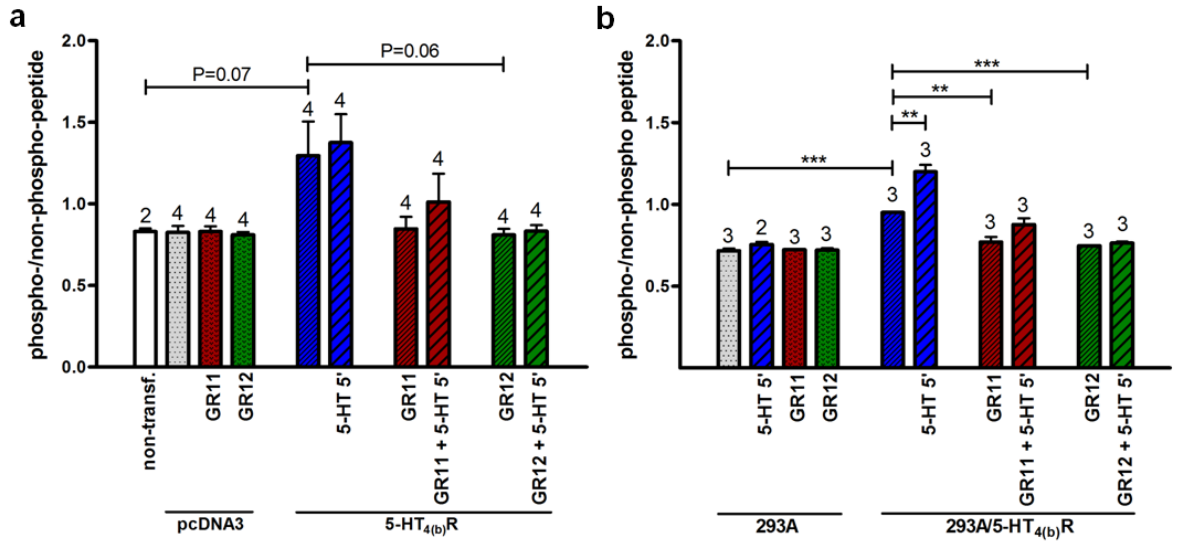


**Figure V.2** PKA activity in response to 5-$HT_{4(b)}$ receptor signalling in HEK293T cells transiently transfected with the 5-$HT_{4(b)}$ receptor (**a**) and in 293A/5-$HT_{4(b)}$R cells (**b**). Controls were non-transfected and pcDNA3 (empty vector) transfected HEK293T cells (**a**) or parental HEK293A cells (**b**). Experiments were performed without or with pre-incubation of the 5-$HT_4$ receptor antagonists GR113808 (10 µM; GR11) or GR125487 (10 µM; GR12) for 30 min. 5-$HT_{4(b)}$ receptor stimulation with 10 µM 5-HT for 5 min is indicated (5-HT 5'). PKA activity is expressed as the ratio of phosphorylated to non-phosphorylated PepTag® A1 peptide. Values are mean ± s.e.m. of n=2-4 independent experiments as indicated above the bars. ** P<0.01; *** P<0.001 for indicated comparisons; unpaired t-test

### V.4.2 Regulation of 5-$HT_{4(b)}$ receptor signalling by PDEs

PKA activity assay data from 293A/5-$HT_{4(b)}$R cells showed less variability compared to data from transiently transfected cells (see **Figure V.2a,b**), presumably because with transient transfection 5-$HT_{4(b)}$ receptor density varied between experiments. Therefore the role of PDEs in 5-$HT_{4(b)}$ receptor signalling was studied in the 293A/5-$HT_{4(b)}$R cell line by measuring PDE and PKA activity. As determined by PDE4- and PDE3-inhibition with rolipram (10 µM) or cilostamide (1 µM), respectively, about 63 % of cAMP-degrading PDE activity is provided by PDE4 in HEK293A cells with the remaining activity being provided by PDE3 **(Figure V.3a**). Even though total PDE activity was increased ~1.7 fold in 293A/5-$HT_{4(b)}$R cells compared to HEK293A parental cells (P<0.05), the ratio between PDE3- and PDE4-activities was similar (**Figure V.3a,b**). As expected overexpression of PDE4D3 strongly increased total PDE activity in 293A/5-$HT_{4(b)}$R and in HEK293A parental cells (P<0.05 and P<0.001 vs. vector transfected 293A/5-$HT_{4(b)}$R and non-

transfected HEK293A cells, respectively). Addition of 5-HT (10 µM) for 10 min had no effect on PDE activity in 293A/5-HT$_{4(b)}$R cells per se, while in 293A/5-HT$_{4(b)}$R cells overexpressing PDE4D3 a tendency towards increased PDE activity was observed with stimulation; this was not significant (**Figure V.3b**).

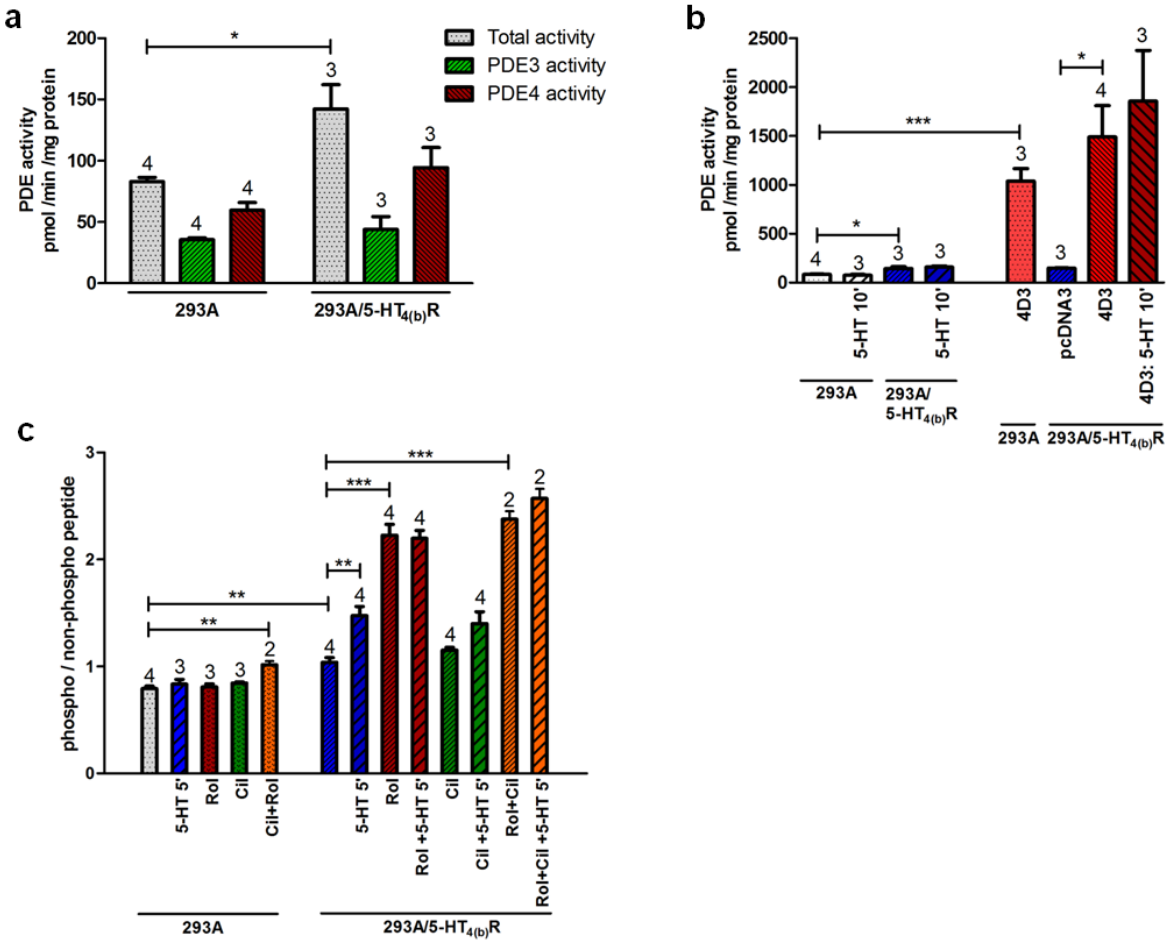


**Figure V.3** PDE activities in HEK293A parental and 293A/5-HT$_{4(b)}$R cells (**a,b**) and effect of PDE inhibition on 5-HT$_{4(b)}$ receptor mediated PKA activity (**c**). Total PDE activity as well as PDE3 and PDE4 activity are compared between HEK293A parental and 293A/5-HT$_{4(b)}$R cells. PDE4 activity is defined as the proportion inhibited by 10 µM rolipram and PDE3 activity is defined as the proportion inhibited by 1 µM cilostamide (**a**). Effect of stimulation with 10 µM 5-HT for 10 min (5-HT 10') on total PDE activity in HEK293A parental and in 293A/5-HT$_{4(b)}$R cells without and with transient overexpression of empty vector (+pcDNA3) or PDE4D3 (+4D3) (**b**). PDE activities are shown as pmol cAMP hydrolysed /min /mg protein (**a, b**). Effect of PDE inhibition on PKA activity in HEK293A parental and 293A/5-HT$_{4(b)}$R cells (**c**). Experiments were performed without and with pre-incubation of rolipram (10 µM; Rol), cilostamide (3 µM; Cil) or both together (Cil+Rol) for 30 min followed by receptor stimulation with 10 µM 5-HT for 5 min in indicated samples (5-HT 5'). PKA activity is expressed as the ratio of phosphorylated to non-phosphorylated PepTag® A1 peptide (**c**). Values are mean ± s.e.m. of n=2-4 independent experiments as indicated above the bars. * P<0.05, ** P<0.01, *** P<0.001 for indicated comparisons; unpaired t-test

PKA activity in parental HEK293A cells was not affected by the PDE3 inhibitor cilostamide nor the PDE4 inhibitor rolipram alone when incubated for 30 min, while the combination of cilostamide and rolipram significantly increased PKA activity by about 1.3 fold in these cells ($P<0.01$; **Figure V.3c**). Cilostamide alone had no effect on PKA activity in 293A/5-$HT_{4(b)}$R cells. In contrast rolipram increased constitutive signalling of the 5-$HT_{4(b)}$ receptor 2.1-fold ($P<0.001$; **Figure V.3c**). In this condition no further increase in PKA activity was observed with receptor stimulation (10 µM 5-HT for 5 min). Incubation of 293A/5-$HT_{4(b)}$R cells for 30 min with both cilostamide and rolipram tended to further increase PKA activity compared to rolipram alone. It is possible that a further increase in PKA phosphorylation was prevented by limited PepTag® A1 substrate peptide, which was almost maximally phosphorylated in this condition.

These results show that more than 60 % of PDE activity is provided by PDE4 in parental HEK293A cells with the remaining activity being provided by PDE3. In 293A/5-$HT_{4(b)}$R cells total PDE activity is increased, while stimulation with 5-HT has no significant further effect on PDE activity. Furthermore, $5HT_{4(b)}$ receptor mediated PKA activity is controlled almost exclusively by PDE4, with a possible role of PDE3 in the presence of PDE4 inhibition.

### V.4.3 Association of PDEs with the 5-$HT_{4(b)}$ receptor

Although convincing evidence was obtained that PDE3 and PDE4 are expressed in HEK293 cells and are involved in 5-$HT_{4(b)}$ receptor signalling from the PKA and PDE activity assays (see **Figure V.3**), endogenous PDE4 or PDE3 protein was not detected in HEK293A and HEK293T cells with the antibodies tested (results not shown). Therefore, PDE4D3, PDE4D5 and PDE3B were overexpressed in order to test the interaction with the 5-$HT_{4(b)}$ receptor.

Overexpressed VSV-tagged PDE4D3 and PDE4D5 splice variants, producing protein bands in transfected cells at ~95 kDa and ~105 kDa respectively, co-immunoprecipitated with the transiently expressed 5-$HT_{4(b)}$ receptor in HEK293T cells; this interaction did not change upon stimulation of the receptor (10 µM 5-HT for 10 min) (**Figure V.4a**). Incubation times with 5-HT of 2, 10 and 30 min were tested and gave similar results (data not shown). In the 293A/5-$HT_{4(b)}$R cell line, overexpressed PDE4D3 co-immunoprecipitated with the 5-$HT_{4(b)}$ receptor; no change in interaction was observed with stimulation of the receptor (10 µM 5-HT for 10 min) (**Figure V.4b**). PDE4D5 was not detected in the immunoprecipitate (results not shown).

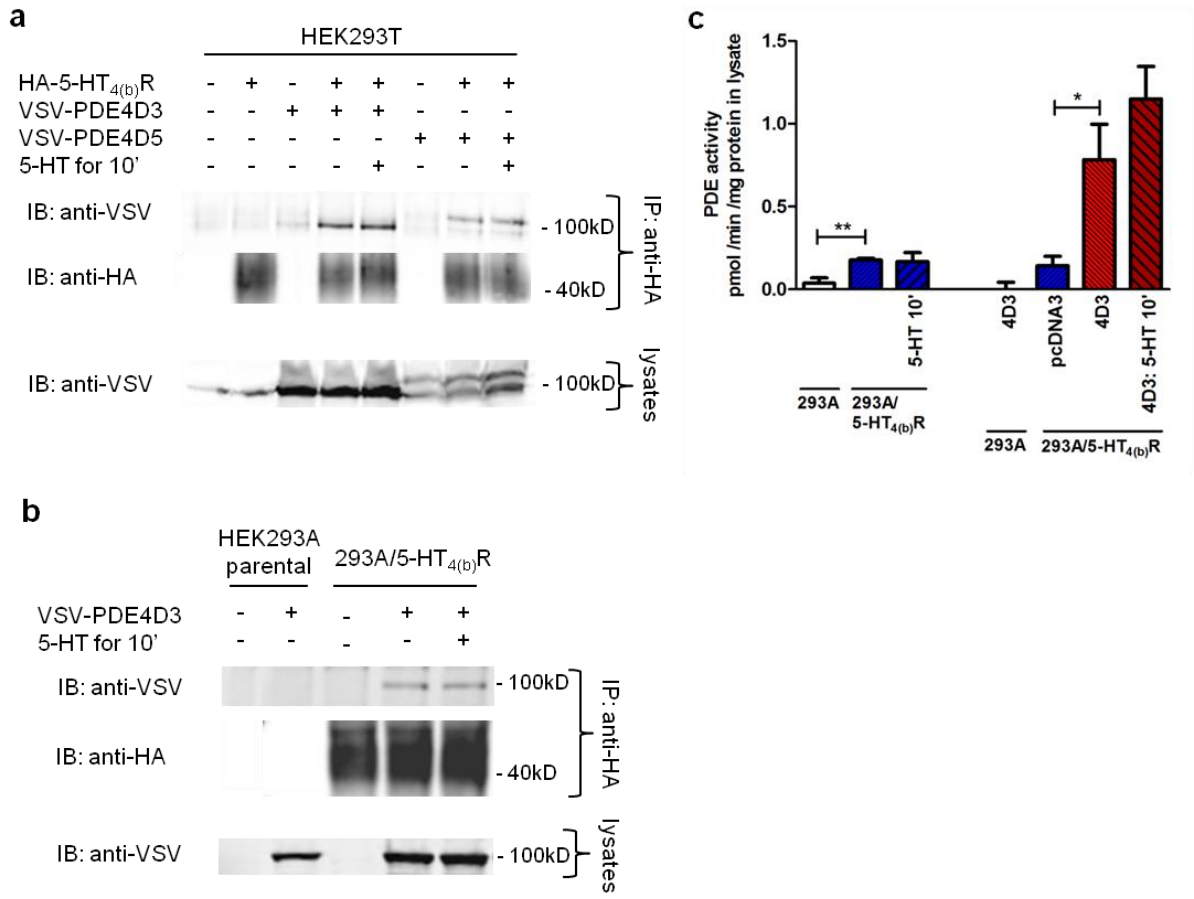


**Figure V.4** Interaction of overexpressed PDE4D3 and PDE4D5 with the 5-HT$_{4(b)}$ receptor: representative immunoblots (**a**)(**b**) and PDE activity (**c**) are shown. HEK293T cells were transiently transfected with empty vector, HA-5-HT$_{4(b)}$R, VSV-PDE4D3, VSV-PDE4D5 or combinations of these (**a**). HEK293A parental cells (first two lanes) and 293A/5-HT$_{4(b)}$R cells (three last lanes) were transiently transfected with empty vector or VSV-PDE4D3 (**b**). Indicated cells were stimulated for 10 min with 10 µM 5-HT. Cell lysates were either used directly for detection of PDE4Ds (lysates) or the 5-HT$_{4(b)}$ receptor was immunoprecipitated using an anti-HA antibody (IP: anti-HA) before separation of proteins by SDS-PAGE and detection by immunoblotting of the 5-HT$_{4(b)}$R (IB: anti-HA) and co-immunoprecipitated PDE4Ds (IB: anti-VSV). Representative immunoblots from three similar experiments are shown (**a, b**). HEK293A parental cells (293A) and 293A/5-HT$_{4(b)}$R cells were used directly or were transiently transfected with empty vector (pcDNA3) or VSV-PDE4D3 (4D3). Stimulation with 10 µM 5-HT was performed for 10 min in indicated samples (5-HT 10'). PDE activity was measured in 5-HT$_{4(b)}$ receptor immune complexes and shown as pmol cAMP hydrolysed /min /mg protein in cell lysates. Data shown are mean ± s.e.m. of n=3 independent experiments. * P<0.05, ** P<0.01 for indicated comparisons; unpaired t-test (**c**)

To confirm the results obtained by co-IP, PDE activity was measured in immunoprecipitates (IPs) of the 5-HT$_{4(b)}$ receptor obtained from 293A/5-HT$_{4(b)}$R cells. Low endogenous PDE activity was detected in association with the 5-HT$_{4(b)}$ receptor (P<0.01 vs. parental HEK293A cells; **Figure V.4c**). In 293A/5-HT$_{4(b)}$R cells overexpressing PDE4D3, a clear ~5.5 fold increase in PDE activity

was observed in IPs of the 5-$HT_{4(b)}$ receptor ($P<0.05$ vs. vector-transfected 293A-5-$HT_{4(b)}$ cells; **Figure V.4c**), supporting an interaction of this PDE4D splice variant with the 5-$HT_{4(b)}$ receptor. In cells stimulated with 10 µM 5-HT for 10 min a trend towards increased PDE activity was observed; this did not reach significance however.

In lysates of GFP-PDE3B transfected cells a Western blot band was detected, corresponding with the overexpressed protein of 130 kDa for PDE3B plus 27 kDa for GFP (**Figure V.5**). Overexpressed PDE3B did not co-immunoprecipitate with the 5-$HT_{4(b)}$ receptor in transiently transfected cells (**Figure V.5**); this was not tested in 293A/5-$HT_{4(b)}$R cells, also in view of the mild effect of cilostamide on 5-$HT_{4(b)}$ receptor stimulated PKA activity (see **Figure V.3c**).

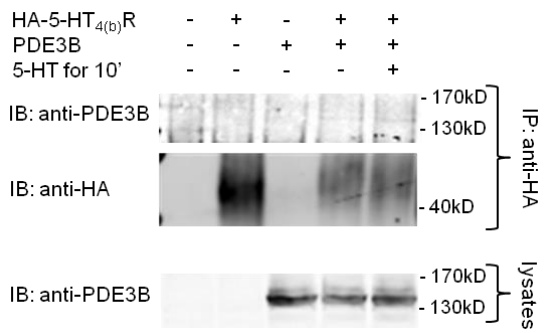


**Figure V.5** Study of the interaction of overexpressed PDE3B with the 5-$HT_{4(b)}$ receptor. HEK293T cells were transiently transfected with empty vector, HA-5-$HT_{4(b)}$R, GFP-PDE3B or combinations of these. Indicated cells were stimulated for 10 min with 10 µM 5-HT. Cell lysates were used directly or 5-$HT_{4(b)}$ receptor immune complexes were isolated (IP: anti-HA) and the 5-$HT_{4(b)}$R (IB: anti-HA) and co-immunoprecipitated PDE3B (IB: anti-PDE3B) detected by immunoblotting. Representative immunoblots from three similar experiments are shown.

### V.4.4 Role of AKAPs in 5-$HT_{4(b)}$ receptor signalling

A possible involvement of AKAPs in 5-$HT_{4(b)}$ receptor signalling was investigated using the InCELLect™ AKAP St-Ht31 inhibitor peptide, the stearated, cell-permeable, form of the Ht31 peptide. Ht31 is derived from human thyroid AKAP and binds the regulatory II (RII) subunit of PKA with high affinity thereby preventing the binding of endogenous AKAPs (Carr et al. 1992; Fink et al. 2001). Incubation of cells with St-Ht31 did not affect PKA activity in untransfected cells, in cells transiently transfected with the 5-$HT_{4(b)}$ receptor (**Figure V.6a**) nor in the 293A/5-$HT_{4(b)}$R cell line (**Figure V.6b**).

Despite the negative results of the St-Ht31 inhibitor peptide in the PKA activity assay, an interaction on protein level of the AKAPs 79 and gravin with the 5-$HT_{4(b)}$ receptor was investigated. Both AKAP79 and gravin were detected endogenously in HEK293 cells. The anti-

AKAP79 antibody detected a protein band slightly above the 70 kDa mark, corresponding with the size of human AKAP79 of 79 KDa (**Figure V.6c**). The anti-gravin antibody produced a double-band clearly above the last marker band of 191 kDa, corresponding with gravin (AKAP250), a large protein of 250 kDa (**Figure V.6d**). Neither AKAP79 nor gravin co-immunoprecipitated with the transiently transfected 5-HT$_{4(b)}$ receptor (**Figure V.6c,d**).

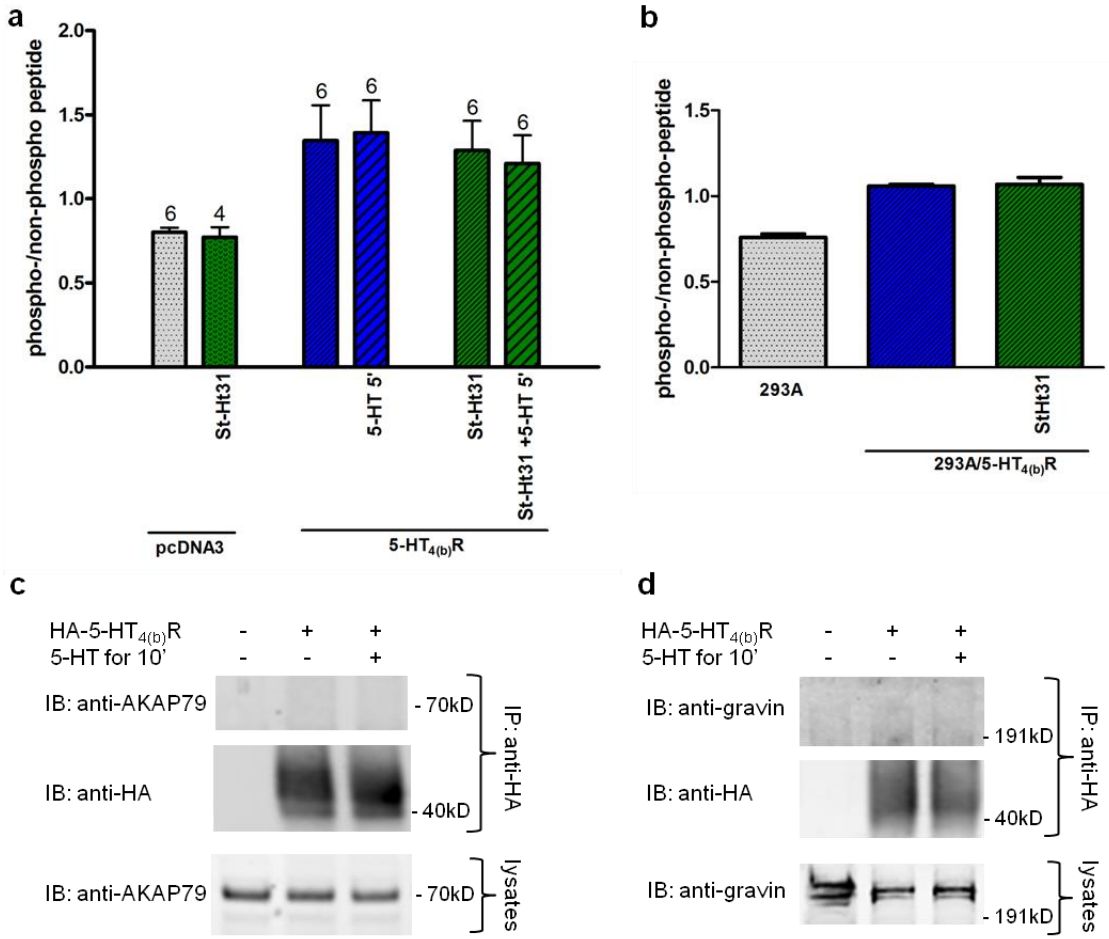


**Figure V.6** Influence of StHt31 inhibitor peptide on 5-HT$_{4(b)}$ receptor mediated PKA activity in cells transiently transfected with the 5-HT$_{4(b)}$ receptor (**a**) or in 293A/5-HT$_{4(b)}$R cells (**b**) and co-immunoprecipitation of AKAP79 and AKAP250 with the 5-HT$_{4(b)}$ receptor (**c,d**). HEK293T cells, transiently transfected with empty vector (pcDNA3) or the 5-HT$_{4(b)}$R (**a**) and 293A/5-HT$_{4(b)}$R cells (**b**) were treated with St-Ht31 inhibitory peptide (St-Ht31; 50 µM) for 30 min. Receptor stimulation with 10 µM 5-HT for 5 min is indicated (5-HT 5') (**a**). PKA activity is expressed as the ratio of phosphorylated to non-phosphorylated PepTag® A1 peptide. Values are mean ± s.e.m. of n=2-6 independent experiments as indicated above the bars (**a**) and n=2 (**b**). HEK293T cells were transiently transfected with empty vector or HA-5-HT$_{4(b)}$R and stimulated for 10 min with 10 µM 5-HT as indicated. Cell lysates were used directly or 5-HT$_{4(b)}$ receptor immune complexes were isolated (IP: anti-HA) and the 5-HT$_{4(b)}$R (IB: anti-HA) and co-immunoprecipitated endogenous AKAP79 (IB: anti-AKAP79) (**c**) or AKAP250 (IB: anti-AKAP250) (**d**) detected by immunoblotting. Representative immunoblots from three similar experiments are shown.

### V.4.5 Interaction of β-arrestin-2 with the 5-HT$_{4(b)}$ receptor

We observed an internalization of the 5-HT$_{4(b)}$ receptor 10 min after stimulation with 5-HT both in HEK293T cells transiently transfected with the receptor and in 293A/5-HT$_{4(b)}$R cells (see **Figure V.1a,b**).

Uncoupling and internalization of G protein-coupled receptors is often mediated by β-arrestins (DeFea 2011), scaffolding proteins which were also shown to associate with the 5-HT$_4$ receptor (Barthet et al. 2009). In line with this, an interaction between overexpressed β-arrestin-2 and the 5-HT$_{4(b)}$ receptor in transiently transfected HEK293T cells was detected. However, no increase in β-arrestin-2, associated with the 5-HT$_{4(b)}$ receptor was observed after stimulation for 10 min with 5-HT (**Figure V.7**).

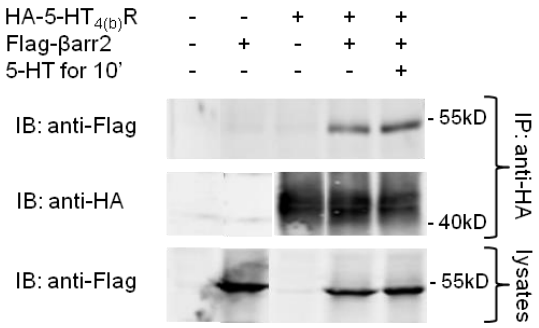


**Figure V.7** Interaction of overexpressed β-arrestin-2 with the 5-HT$_{4(b)}$ receptor. HEK293T cells were transiently transfected with empty vector, HA-5-HT$_{4(b)}$R, Flag-βarr2 or combinations of these. Indicated cells were stimulated for 10 min with 10 µM 5-HT. Cell lysates were used directly or 5-HT$_{4(b)}$ receptor immune complexes were isolated (IP: anti-HA) and the 5-HT$_{4(b)}$R (IB: anti-HA) and co-immunoprecipitated β-arrestin-2 (IB: anti-Flag) detected by immunoblotting. Representative immunoblots from three similar experiments are shown.

## V.5 Discussion

The aim of this study was to investigate whether PDE3 and PDE4, which were shown to regulate 5-HT$_4$ receptor signalling in many tissues, interact with the 5-HT$_{4(b)}$ receptor at protein level. Furthermore an involvement of AKAPs in 5-HT$_4$ receptor signalling was examined.

In parental HEK293 cells 10 µM 5-HT did not induce an increase in PKA activity, suggesting that no $G_s$ protein-coupled receptors for 5-HT, including the 5-HT$_4$ receptor, are expressed and functional in these cells. In contrast the β-adrenergic receptor agonist isoprenaline induced a strong increase in PKA activity in HEK293 cells (data not shown). The effect of exogenously expressed 5-HT$_{4(b)}$ receptors on PKA activity was tested in both transiently and stably transfected cells. We observed a strong constitutive activity of the overexpressed 5-HT$_{4(b)}$

receptor, which was higher in transiently transfected cells compared to 293A/5-HT$_{4(b)}$R cells stably expressing the receptor. Furthermore, In 293A/5-HT$_{4(b)}$R cells but not in cells transiently transfected with the 5-HT$_{4(b)}$ receptor, a significant further increase in PKA activity was observed after stimulation of the receptor with 5-HT. We suggest that these differences are due to a lower expression level of the receptor in the 293A/5-HT$_{4(b)}$R cell line. It was observed before that the 5-HT$_{4(a)}$ receptor has a high constitutive activity, much larger than the $\beta_2$-adrenergic receptor tested in comparison, which increased dramatically with increased receptor expression (Claeysen et al. 1999; Bockaert et al. 2004). Indeed, we did not observe a significant constitutive activity of the $\beta_2$-adrenergic receptor overexpressed in HEK293 cells (data not shown). We conclude that in the 293A/5-HT$_{4(b)}$R cell line the receptor is functional as its signalling was increased by 5-HT, while in the transiently transfected cells the very high constitutive activity of the 5-HT$_{4(b)}$ receptor might have prevented an even further increase in PKA activity upon stimulation, possibly because the system was already saturated. Immunofluorescence experiments revealed an internalization of the 5-HT$_{4(b)}$ receptor in response to stimulation with 5-HT in HEK293T cells transiently overexpressing the receptor and in 293A/5-HT$_{4(b)}$R cells, suggesting that the receptor is available for binding to 5-HT. This is in accordance with literature data showing a time-dependent internalization of the 5-HT$_{4(b)}$ receptor (Pindon et al. 2004; Barthet et al. 2005).

We found that in both HEK293A parental and 293A/5-HT$_{4(b)}$R cells, more than 60 % of endogenous cAMP-PDE activity is provided by PDE4 and the remaining part by PDE3. This is in line with data in the literature, which show that PDE4 provides the majority of PDE activity in HEK293 cells, with the remaining activity being provided by PDE3 (Lynch et al. 2005). Interestingly in 293A/5-HT$_{4(b)}$R cells total PDE activity was increased compared to HEK293A parental cells. Possibly the high constitutive activity of the 5-HT$_{4(b)}$ receptor led to a PKA-mediated activation of PDE activity. Indeed, PDE3 and most PDE4 splice variants can be activated by PKA phosphorylation (Sette and Conti 1996; Ekholm et al. 1997; Shakur et al. 2000; MacKenzie et al. 2002). Receptor stimulation with 10 µM 5-HT had no effect on PDE activity in 293A/5-HT$_{4(b)}$R cells, even though we observed a further increase in PKA activity in this condition. 5-HT$_{4(b)}$ receptor induced cAMP signalling is controlled predominantly by PDE4, with a possible role of PDE3 in the presence of PDE4 inhibition, as determined by PKA activity measurements in 293A/5-HT$_{4(b)}$R cells (see **Figure V.3c**). From the PDE4 family, HEK293 cells were shown to express PDE4A, PDE4B and PDE4D isoforms, with PDE4D3 and PDE4D5 providing the majority of activity (Lynch et al. 2005). From the two known PDE3 genes (PDE3A and

PDE3B), PDE3B was found endogenously in HEK293 cells (Wilson et al. 2011). However, we did not detect endogenous PDE3 and PDE4 in HEK293 cells by Western blot, probably because the binding of the antibodies was too weak to detect low levels of endogenous protein. Therefore PDE4D3, PDE4D5 and PDE3B were overexpressed to test for a possible interaction with the 5-$HT_{4(b)}$ receptor.

PDE activity was strongly increased in cells transiently transfected with PDE4D3, confirming the functionality of the overexpressed protein. Co-immunoprecipitation data revealed an interaction of the PDE4D3 splice variant with the 5-$HT_{4(b)}$ receptor; the signal was less strong in 293A/5-$HT_{4(b)}$R cells compared to cells transiently transfected with the receptor. Furthermore an interaction of PDE4D5 with the 5-$HT_{4(b)}$ receptor was found in transiently transfected cells but not in 293A/5-$HT_{4(b)}$R cells. The differences observed between transiently transfected cells compared to 293A/5-$HT_{4(b)}$R cells are probably due to the lower expression level of the 5-$HT_{4(b)}$ receptor in the latter (as discussed above). PDE4D5 might only associate with the 5-$HT_{4(b)}$ receptor when both proteins are present in a high concentration, as is the case in transiently transfected cells. Stimulation of 5-$HT_{4(b)}$ receptor signalling with 10 µM 5-HT did not influence its interaction with PDE4D3 and PDE4D5, suggesting a constitutive association. This might be due to the high constitutive activity of the 5-$HT_{4(b)}$ receptor. In contrast PDE4D3 and PDE4D5 were reported to barely interact with the $\beta_2$-adrenergic receptor in basal condition; but upon stimulation they are recruited to the $\beta_2$-adrenergic receptor in a β-arrestin-2 dependent manner in HEK293 cells and cardiomyocytes (Perry et al. 2002; Baillie et al. 2003). Interestingly PDE4D8, PDE4D9 and PDE4D5 splice variants were shown to interact with the $\beta_1$-adrenergic receptor independent of β-arrestin-2, and PDE4D8 dissociates from the receptor upon stimulation (Richter et al. 2008; De Arcangelis et al. 2009). It remains to be determined whether the interaction of PDE4Ds with the 5-$HT_{4(b)}$ receptor requires β-arrestins. β-arrestin-2 was shown to have a binding site for the common catalytic subunit of PDE4Ds and additional sites conferring specific interaction with PDE4D5 (Baillie et al. 2007). Furthermore, we detected an interaction of overexpressed β-arrestin-2 with the 5-$HT_{4(b)}$ receptor, which was, similar to the interaction with PDE4Ds, not influenced by receptor stimulation. This is partly in line with results from Barthet et al. (2009) who showed that endogenous β-arrestin-2 interacts with the 5-$HT_4$ receptor per se and is further recruited to the receptor upon stimulation in HEK293 cells and neurons, being important for receptor desensitization.

To test if activity of PDEs in complex with the 5-$HT_{4(b)}$ receptor increases upon receptor stimulation, PDE activity was tested in immunoprecipitates of the 5-$HT_{4(b)}$ receptor from 293A/5-$HT_{4(b)}$R cells. The low endogenous PDE activity detected did not change upon receptor activation. In cells overexpressing PDE4D3, PDE activity in immunoprecipitates of the 5-$HT_{4(b)}$ receptor was increased, confirming the interaction of PDE4D3 with the 5-$HT_{4(b)}$ receptor. A trend towards a further increase in PDE activity in response to stimulation with 5-HT was observed in lysates and in 5-$HT_{4(b)}$ receptor immunoprecipitates from 293A/5-$HT_{4(b)}$R cells overexpressing PDE4D3. This provides evidence that PDE activity might be increased upon stimulation of the receptor, probably by phosphorylation by PKA. Such a PKA-mediated activation of PDE4 activity was suggested to cause the fade of the cAMP signal in response to prostaglandin E1 treatment in HEK293 cells (Rich et al. 2007).

PDE3s were found both in cytosolic and membrane fractions in cardiomyocytes and were predominantly membrane-associated in HEK293 cells and adipocytes (Shakur et al. 2000; Matthiesen and Nielsen 2011). Membranous PDE3 activity was shown to be mainly associated with sarcoplasmic reticulum in human and dog heart (Kauffman et al. 1986; Lugnier et al. 1993; Fischmeister et al. 2006). Furthermore transfected PDE3B associated mostly with endoplasmic reticulum in Cos-7 cells and adipocytes (Shakur et al. 2000). Therefore it is not surprising that we did not find an interaction of PDE3B with the 5-$HT_{4(b)}$ receptor in HEK293 cells transiently overexpressing both proteins. To our knowledge a direct association of PDE3 with G protein-coupled receptors was never reported.

AKAPs scaffold signalling enzymes into clusters at specific subcellular locations and by doing so they support the formation of cAMP microdomains which ensure specificity of cAMP signalling (Dodge-Kafka et al. 2008). The $\beta_1$-adrenergic receptor was found in a complex including AKAP79, and both AKAP79 and gravin were shown to associate with the $\beta_2$-adrenergic receptor in HEK293 cells (Tao et al. 2003; Lynch et al. 2005). Therefore, we deemed it interesting to explore a possible influence of AKAPs on 5-$HT_4$ receptor signalling. However, the St-Ht31 peptide, shown to disturb the binding of AKAPs to PKA in HEK293 cells and cardiomyocytes (Carr et al. 1992; Fink et al. 2001; Lynch et al. 2005), had no influence on 5-$HT_{4(b)}$ receptor mediated PKA activity. Furthermore, no association of AKAP79 and gravin was found with the 5-$HT_{4(b)}$ receptor. Thus 5-$HT_{4(b)}$ receptor signalling in HEK293 cells might be regulated differently than β-adrenergic signalling, with AKAPs not playing a prominent role. The control by PDEs of 5-$HT_{4(b)}$ receptor

signalling can be achieved independently of AKAPs by association of PDE4s with the receptor, either directly, via β-arrestin-2 or by another mechanism.

In conclusion our data show that 5-$HT_{4(b)}$ receptor signalling in HEK293 cells is controlled by PDE4, with a possible role of PDE3 in the presence of PDE4 inhibition. PDE4D3 associates with the 5-$HT_{4(b)}$ receptor both in cells transiently expressing the receptor and in stably transfected cells. Receptor stimulation does not change its interaction with PDE4D3 but it might lead to an increase in the activity of the PDE. An interaction of PDE4D5 with the 5-$HT_{4(b)}$ receptor was only found in cells transiently expressing the receptor and might therefore only occur when both proteins are present at a high concentration.

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# Chapter VI

## *General discussion and conclusion*

# *VI General discussion and conclusion*

5-$HT_4$ receptors are present in human and porcine atrium where they signal via cAMP to activate protein kinase A (PKA), ultimately leading to positive inotropic, lusitropic and chronotropic responses (Kaumann 1990; Kaumann et al. 1990; Villalon et al. 1990; Kaumann and Sanders 1994). The bulk of research in the periphery however focused on 5-$HT_4$ receptors in the GI tract, located on excitatory cholinergic neurons. Stimulation of these receptors facilitates release of the neurotransmitter acetylcholine, promoting GI motility (Gershon and Tack 2007; Gershon 2013). Several 5-$HT_4$ receptor agonists were developed for the treatment of GI motility disorders. However, early 5-$HT_4$ receptor agonists were often unspecific and therefore had problems with side-effects; an example being cisapride which was withdrawn from the market in 2000 for showing rare cardiac side-effects (De Maeyer et al. 2008; Sanger 2008). The more recently developed 5-$HT_4$ receptor agonist prucalopride, which was marketed in 2009 under the brand name Resolor® for the treatment of laxative-resistant chronic constipation, is more specific and was also tested for its effect on cardiac 5-$HT_4$ receptors (Krobert et al. 2005; De Maeyer et al. 2006b; Camilleri et al. 2010). Prucalopride behaves as a full agonist in the stomach and colon while it acts as a partial agonist on cardiac 5-$HT_4$ receptors, increasing its safety profile (De Maeyer et al. 2006a; Priem and Lefebvre 2011). Nevertheless, in order to exclude cardiac side-effects that might arise from the combination of 5-$HT_4$ receptor agonists with drugs acting on down-stream effectors of the pathway it is important to fully understand 5-$HT_4$ receptor signalling in the heart.

Our laboratory and others previously found that phosphodiesterases (PDEs), an enzyme group which degrade the cyclic nucleotides cAMP and cGMP, are of utmost importance in cardiac 5-$HT_4$ receptor signalling, inducing a fade of the inotropic response to 5-HT in the atrium and completely blocking the inotropic response in the ventricle (Brattelid et al. 2004; De Maeyer et al. 2006b). PDEs fall into at least 11 subfamilies and PDE1 to PDE5 subtypes were found in human and PDE2 to PDE5 in porcine heart (Zimmermann et al. 1994; Fischmeister et al. 2006; Jakobsen et al. 2006). Especially PDE2 and PDE3 can mediate a cross-talk between cAMP and cGMP mediated signalling events, because their cAMP degrading activities are stimulated and inhibited by cGMP, respectively (Fischmeister et al. 2006; Zaccolo and Movsesian 2007). 5-$HT_4$ receptor signalling could also be influenced by other proteins such as $G_i$ proteins which inhibit the production of cAMP and A-kinase anchoring proteins (AKAPs) which scaffold PKA activity to

specific locations, thereby ensuring specific and effective signalling (Dessauer 2009; Mauban et al. 2009).

Therefore our goals were to evaluate the role of PDE subtypes in cardiac 5-$HT_4$ receptor signalling to 5-HT and prucalopride and to test whether increased cGMP signalling can affect responses to 5-HT. This was done by recording functional responses to 5-$HT_4$ receptor stimulation in pig left atrial muscle strips in an organ bath setup, by evaluating tissue cAMP and cGMP content as well as measuring the phosphorylation of the contractile proteins phospholamban (PLB) and troponinI (TnI). PLB negatively regulates activity of the sarcoplasmic reticulum $Ca^{2+}$ pump (SERCA); this inhibition is released upon PLB phosphorylation by PKA, causing a faster re-uptake of $Ca^{2+}$ into the SR during diastole and a faster relaxation. Furthermore more $Ca^{2+}$ can be released from the SR during systole increasing contractility (MacLennan and Kranias 2003). TnI phosphorylation by PKA leads to a decrease in $Ca^{2+}$ sensitivity of the myofilaments, also resulting in a faster relaxation (Matsuba et al. 2009). We used the pig as a model system because it is one of the few species which expresses functional 5-$HT_4$ receptors in the heart and is suggested to be a good experimental model for studying human atrial 5-$HT_4$ receptors (Kaumann 1990). Finally we investigated the interaction between the 5-$HT_4$ receptor and PDEs at the protein level by immunoprecipitation in a human cell line overexpressing the 5-$HT_{4(b)}$ receptor; in this paradigm a possible interaction between the 5-$HT_4$ receptor and AKAPs was also studied.

## VI.1 Cardiac response to stimulation of 5-$HT_4$ receptors with 5-HT and prucalopride

In concordance with previous studies (De Maeyer et al. 2006b; Galindo-Tovar et al. 2009), 5-HT induced a short lasting inotropic response and a maintained lusitropic response in pig left atrium. The fade of the inotropic response is reflected by tissue cAMP content.

The lusitropic response to 5-$HT_4$ receptor stimulation is difficult to measure because it is masked to some extent by the inotropic response. Lusitropic responses are often described by the time to peak force (TPF) and time to 50 % relaxation ($TR_{50}$) of the muscle contraction (**Figure VI.1a**) (Mattiazzi et al. 1986; Molenaar et al. 2007; Afzal et al. 2011a). However, the onset of relaxation as measured by TPF and the relaxation velocity as measured by $TR_{50}$ are not independent of the contraction force. Therefore, when contraction force is maximally increased in pig left atrium

two min after addition of 5-HT, the full extent of the lusitropic effect cannot be assessed; as suggested by only a minimal decrease in $TR_{50}$ (**Figure VI.1b**). After 10 min, when the inotropic effect mostly faded the lusitropic effect becomes apparent; this is reflected by the further decrease in $TR_{50}$ (**Figure VI.1c**). Another method to measure lusitropic responses is by calculating the coefficient $R_2$, which is $(+dF/dt)_{max}$ (maximal contraction rate) divided by $(-dF/dt)_{max}$ (maximal relaxation rate). The $R_2$ shows contraction-relaxation coupling under high load and was described as assessing lusitropic effects, independent of inotropic changes (Mattiazzi et al. 1986; Hanouz et al. 2004), with decreasing $R_2$ illustrating lusitropy. However, two minutes after addition of 5-HT the $R_2$ was slightly increased and only decreased with the fading of the inotropic response, going below the basal value after about five minutes. This immediate increase in $R_2$ indicates a masking of the lusitropic effect by the inotropic response and only when the inotropic response fades the lusitropic response becomes apparent with the $R_2$ value going below basal values.

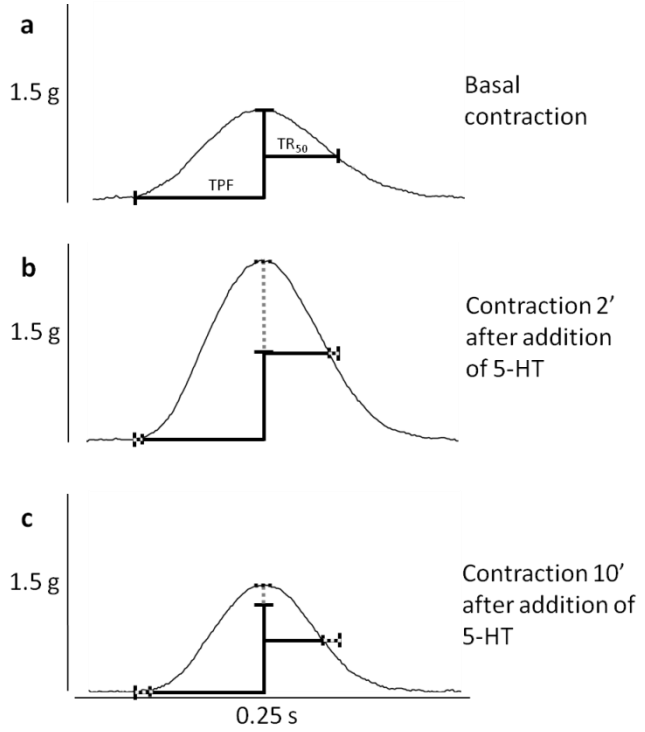


**Figure VI.1** Traces of a single contraction of a pig left atrial muscle strip. The lusitropic parameters time to peak force (TPF) and time to 50 % relaxation ($TR_{50}$) are indicated. Contraction in the following conditions are depicted: before addition of 5-HT (**a**), 2 min after addition of 5-HT (**b**) and 10 min after addition of 5-HT (**c**). Filled lines represent TPF, contraction force and $TR_{50}$ at basal condition and dashed lines indicate changes of these parameters after stimulation with 5-HT.

In line with the maintained lusitropic response the increase in phosphorylation of the mainly lusitropic proteins PLB and TnI to 5-HT faded slower and did not fade, respectively. The prolonged increase in PLB and TnI phosphorylation is probably due to a slow de-phosphorylation caused by limited access by phosphatases and not a result of continued phosphorylation by PKA,

since cAMP levels in response to 5-HT declined quickly notwithstanding ongoing 5-$HT_4$ receptor activation. The main enzymes responsible for de-phosphorylation of both PLB and TnI are protein phosphatase 1 (PP1) and PP2A (MacDougall et al. 1991; Sulakhe and Vo 1995). Murine models overexpressing PP1 or PP2A show significantly reduced phosphorylation of PLB and TnI and depressed cardiac function, highlighting the importance of these enzymes (Carr et al. 2002; Gergs et al. 2004). PP1 and PP2A activity is tightly controlled by their regulatory subunits; furthermore they are tethered to signalling complexes by AKAPs, culminating in a stringent spatiotemporal control (Redden and Dodge-Kafka 2011). It would have been interesting to also study PKA phosphorylation of L-type $Ca^{2+}$ channels and ryanodine receptor $Ca^{2+}$ channels which primarily mediate the inotropic response (Marx et al. 2000; Harvey and Hell 2013). Unfortunately, the phosphorylation sites as well as the mechanism of action of PKA phosphorylation are still not fully unravelled/understood for both of these proteins (Xiao et al. 2006; Huke and Bers 2008; Capes et al. 2011; Ullrich et al. 2012; Harvey and Hell 2013), making them unsuitable for determining PKA/protein phosphatase activity in response to 5-$HT_4$ receptor activation.

Prucalopride acts as a partial agonist on cardiac 5-$HT_4$ receptors and induced a slower developing and weaker inotropic response. Prucalopride did not produce a measurable increase in tissue cAMP, probably because the amount of cAMP produced in response to prucalopride is strongly compartmentalized and too small to be detected by whole tissue cAMP content analysis. In contrast to the inotropic response, the lusitropic response to prucalopride almost reached the same maximum as seen with 5-HT but developed slower. This is in line with the phosphorylation of the lusitropic protein TnI by prucalopride, which does not fade with time, but in contrast to a previous study where prucalopride was less efficacious in producing a lusitropic effect compared to 5-HT (De Maeyer et al. 2006b).

## VI.2 PDEs regulate 5-$HT_4$ receptor signalling in the heart

Galindo-Tovar et al. (2009) showed that the inotropic response to 5-HT in pig atrium is controlled by both PDE3 and PDE4 together. We could confirm this result and additionally show that the inotropic response to the 5-$HT_4$ receptor agonists prucalopride and RS67333 is similarly controlled by PDE3 and PDE4 subtypes. PDE3- or PDE4-inhibition alone had no effect or only a slight effect on the functional response to 5-HT receptor stimulation respectively, while

inhibition of both PDE subtypes together strongly increased and prevented the fade of the inotropic response to 5-HT and prucalopride. In this condition a non-fading inotropic response to RS67333 was also unravelled. It was suggested that PDE3 and PDE4 act in a redundant way, where one PDE can replace the function of the other (Galindo-Tovar et al. 2009). In concordance with the functional response, inhibition of PDE3- and PDE4-subtypes together led to a further and persistent increase in cAMP content in response to 5-HT. In the presence of PDE3- and PDE4-inhibition prucalopride also induced an increase in cAMP content, which however developed slower than the cAMP response to 5-HT and was considerably lower. These lower levels of cAMP were sufficient to obtain comparable levels of PLB- and TnI-phosphorylation and yielded the same inotropic response. We propose that a certain amount of cAMP is sufficient to maximally stimulate the contractile apparatus. Additionally control mechanisms, e.g. increased protein phosphatase activity, may prevent hyper-phosphorylation of proteins to excessive cAMP. Indeed, De Arcangelis et al. (2008) found that stimulation of cells with a high concentration of the β-adrenergic receptor agonist isoprenaline led to increased activity of PP2A, counteracting excessive protein phosphorylation by PKA.

We found that the maintained lusitropic response to 5-HT and prucalopride is enhanced in the presence of combined PDE3- and PDE4-inhibition as indicated by a further decrease in TPF and $TR_{50}$. This is supported by the strong further increase in phosphorylation of PLB and TnI observed in this condition. $R_2$ values in response to 5-HT and prucalopride in the presence of PDE-inhibition did not decrease, but instead remained stable or even increased. Probably the maintained inotropic response completely masks the lusitropic effect as measured by the $R_2$ value.

Given the importance of PDEs in cardiac 5-$HT_4$ receptor signalling, we assessed whether PDE3 and PDE4 subtypes also interact with the receptor on protein level. This was done by overexpressing the HA-tagged 5-$HT_{4(b)}$ receptor in HEK293 cells and determining co-immunoprecipitation of overexpressed PDE4D3, PDE4D5 and PDE3B splice variants. In contrast to porcine atrium where 5-$HT_4$ receptor signalling is controlled jointly by PDE3 and PDE4, in HEK293 cells 5-$HT_{4(b)}$ receptor mediated PKA activity is controlled predominantly by PDE4. This is in concordance to overall PDE activity in HEK293 cells, which is provided mostly by PDE4 and to a smaller extent by PDE3 (this work; Lynch et al. 2005). Endogenous PDE4 was not detected by Western blot but overexpressed PDE4D3 and PDE4D5, which provide the majority of PDE4 activity in this cell line (Lynch et al. 2005), associated with the 5-$HT_{4(b)}$ receptor. No change was

observed upon stimulation of the receptor with 5-HT, indicating a constitutive interaction. However, receptor stimulation might still enhance the control of PDEs on 5-$HT_4$ receptor signalling by stimulating PDE activity. Indeed, PDE3 and the majority of PDE4 isoforms have been shown to be phosphorylated and activated by PKA, providing a negative feedback mechanism to cAMP signalling (Sette and Conti 1996; Ekholm et al. 1997; Shakur et al. 2000; MacKenzie et al. 2002). This is supported by PDE activity measurements showing that PDE4D3 activity in whole cell lysates and in immunoprecipitates of the 5-$HT_{4(b)}$ receptor tended to be increased after stimulation of the receptor. In contrast to PDE4Ds, overexpressed PDE3B did not interact with the 5-$HT_{4(b)}$ receptor in HEK293 cells. This is not surprising since PDE3 was shown to be mainly localized at the sarcoplasmic/endoplasmic reticulum in human and dog heart and Cos-7 cells, with little or no association with the plasma membrane (Kauffman et al. 1986; Lugnier et al. 1993; Shakur et al. 2000).

In this context we also investigated a possible influence of the AKAPs 79 and gravin on 5-$HT_4$ receptor signalling. AKAPs are scaffolding enzymes which tether PKA and other enzymes involved in the regulation of cAMP signalling to defined locations in the cell. The $\beta_2$-adrenergic receptor was found in a complex including PDE4D, AKAP79 and the AKAP gravin in HEK293 cells (Lin et al. 2000; Fan et al. 2001; Tao et al. 2003; Willoughby et al. 2006). The St-Ht31 peptide, shown to disrupt the interaction between AKAPs and PKA (Fink et al. 2001; Lynch et al. 2005), did not influence 5-$HT_{4(b)}$ receptor signalling in HEK293 cells overexpressing the receptor. Furthermore endogenous AKAP79 and gravin did not co-immunoprecipitate with the 5-$HT_{4(b)}$ receptor. However, cell studies often do not fully reflect receptor signalling in native tissue. Thus an involvement of AKAPs in 5-$HT_4$ receptor signalling could be further studied in primary cardiomyocytes, which maintain many aspects of native tissue while having the same advantages as cell systems.

## VI.3 5-$HT_4$ receptor desensitisation and G-protein specificity

It has been reported that the 5-$HT_{4(b)}$ receptor splice variant couples to both $G_s$ (stimulating adenylyl cylase) and $G_i$ (inhibiting adenylyl cylase) proteins in HEK293 cells and rat cardiomyocytes (Pindon et al. 2002; Castro et al. 2005). Furthermore, Lonardo et al. (2005) found evidence for a $G_i$ coupling of the 5-$HT_4$ receptor in human right atrial myocytes. Therefore we deemed it interesting to investigate whether a switching in G protein specificity occurs for 5-

$HT_4$ receptors in pig left atrium. However, we could not detect an effect of pertussis toxin (PTX), an inhibitor of $G_i$ proteins, on responses to 5-$HT_4$ receptor stimulation. In contrast the negative inotropic response to muscarinic receptor stimulation with carbachol was clearly reduced, but not completely abolished, in muscle strips pre-incubated with PTX. This demonstrates that PTX works in our experimental setup but tissue infiltration might not be optimal. This possibly poor tissue penetration of PTX makes a straightforward conclusion on $G_i$ protein contributing to 5-$HT_4$ receptor signalling in pig left atrium difficult.

In order to further exclude a switch to $G_i$ signalling and to investigate 5-$HT_4$ receptor desensitization we explored whether cAMP signalling in response to 5-HT remains active despite the fade with time of the inotropic response. This was done by adding the non-selective PDE inhibitor IBMX 60 min after 5-HT to pig left atrial muscle strips. While IBMX had almost no effect on basal muscle contractility, when added 60 min after 5-$HT_4$ receptor stimulation, IBMX fully restored the inotropic response to 5-HT and prucalopride. These results show that cAMP signalling in response to 5-$HT_4$ receptor activation is still on-going at this moment and that PDEs continue to control the inotropic response. Therefore we consider a switching to $G_i$ signalling or receptor desensitization unlikely, since both would result in decreased cAMP signalling. This is in contrast to the functional study by De Maeyer et al. (2009), who observed 5-$HT_4$ receptor desensitization in pig atrium. In their approach they stimulated the 5-$HT_4$ receptor with 5-HT and after a wash-out of the drug, added 5-HT again, obtaining a lower inotropic response this time. It is possible that this lowered response to the second administration of 5-HT is due to stimulated PDE activity after the first contact with the agonist, which will result in an even tighter control of the cAMP signal and a lower response to a second stimulation of the 5-$HT_4$ receptor. Indeed receptor stimulation tended to increase PDE4D3 activity in HEK293 cells (see above).

In contrast to our results in pig left atrium, we observed a time-dependent internalization of the 5-$HT_{4(b)}$ receptor splice variant in HEK293 cells overexpressing the receptor, indicating desensitization. These results are in line with other studies in HEK293 cells, which also found a desensitization/internalization of the 5-$HT_4$ receptor (Pindon et al. 2004; Barthet et al. 2005). While Pindon et al. (2004) only observed an internalization of the 5-$HT_{4(b)}$, but not the 5-$HT_{4(a)}$ receptor splice variant, Barthet et al. (2005) observed a similar internalization for $5HT_{4(a)}$, $_{(b)}$ and $_{(e)}$ splice variants. These differences in receptor behaviour demonstrate that results from

overexpression studies in cell systems must be interpreted with caution and highlight the importance of organ bath studies using native tissue.

## VI.4 Stimulation of sGC and pGC have differential effects on the inotropic response to 5-HT in pig heart

We found differential effects of stimulation of sGC and pGC on basal muscle contractility and on responses to 5-HT in pig left atrium. Responses to increased cGMP, as a result of sGC or pGC stimulation, can be mediated by cGMP-dependent protein kinase I (PKGI) or via stimulation/inhibition of PDEs (Takimoto 2012). cGMP activates PKGI, which negatively modulates inotropy and accelerates relaxation of cardiomyocytes by phosphorylating proteins such as the L-type $Ca^{2+}$ channel, phospholamban and troponin I. PKGI phosphorylation of the L-type $Ca^{2+}$ channel has the opposite effect of phosphorylation by PKA, inhibiting the channel's activity and resulting in decreased $Ca^{2+}$ entry and contractility. PLB and TnI are phosphorylated at the same sites by PKGI and PKA, resulting in increased lusitropy (Yang et al. 2007; Francis et al. 2010). Increased cGMP can indirectly modify muscle responses via changes in cAMP levels mediated by PDE2 and PDE3 (Zaccolo and Movsesian 2007). PDE2 is specific for both cAMP and cGMP and its cAMP degrading activity is stimulated by cGMP. PDE3 can also break-down both cAMP and cGMP, but has a lower $V_{max}$ for cGMP, resulting in a functional inhibition of cAMP degradation by cGMP (Fischmeister et al. 2006).

### VI.4.1 Stimulation of pGC by CNP augments the inotropic response to 5-HT in the presence of PDE4-inhibition

CNP per se produced a small and short-lasting inotropic response in pig left atrium. Both positive and negative inotropic effects as well as a biphasic effect with a short positive inotropic followed by a negative inotropic effect of CNP were observed before in mouse and rat heart and were reported to be mediated by compartmentalized PKGI activity (Hirose et al. 1998; Pierkes et al. 2002; Wollert et al. 2003). In the presence of PDE4-inhibition the inotropic effect of CNP on basal muscle contractility was enlarged; we do not have an explanation for this. The inotropic response to 5-$HT_4$ receptor activation was not influenced by CNP alone in pig left atrium. However, in the combined presence of CNP plus the PDE4-inhibitor rolipram the fade of the

inotropic response to 5-HT was slowed down, corresponding with a higher degree of phosphorylation of phospholamban and a tendency to increased phosphorylation of troponin I. These results support the theory that increased cGMP by pGC stimulation with CNP inhibits PDE3 but since the increase in cAMP by 5-HT is jointly controlled by PDE3 and PDE4 in porcine atrium, PDE4 needs to be inhibited as well to uncover an increase in cAMP levels and a contractile response. This is in line with the results of Afzal et al. (2011b) in rat failing left ventricle, who observed an increased inotropic response to 5-HT in the presence of CNP, and an even further increase with the combination of CNP plus rolipram; they attributed these effects to the cGMP-mediated inhibition of PDE3.

PDE2 might limit the increase in cAMP levels in response to CNP, either by breaking down cGMP thereby preventing full inhibition of PDE3, or by degrading cAMP since in the presence of high cGMP PDE2 activity would be increased. Indeed 10 min after addition of 5-HT cAMP levels were higher in the combined presence of CNP plus rolipram plus the PDE2-inhibitor EHNA compared to the absence of EHNA. However, in the combined presence of CNP plus rolipram, EHNA did not further increase PLB and TnI phosphorylation in response to 5-HT and its effect on functional responses was minimal producing a slightly better maintained response from 20 min onwards after addition of 5-HT. Therefore, we believe that PDE2 has the potential to limit increases in cAMP in the presence of high cGMP levels, but this has no or only minimal influence on the functional outcome in pig left atrium.

### VI.4.2 sGC stimulation with an NO-donor reduces the inotropic response to 5-HT

Sin-1 (300 µM) produced a very pronounced (~30-fold) increase in tissue cGMP content and a slight increase in cAMP, 30 min after administration. In the literature a ~3-fold and ~10-fold increase in cGMP content in response to Sin-1 (300 and 200 µM), was found in failing rat left ventricle and rat left ventricular myocytes respectively (Stojanovic et al. 2001; Afzal et al. 2011b). However, in contrast to pGC stimulation using CNP, sGC stimulation using the NO-donor Sin-1 (300 µM) decreased basal muscle contractility and hastened the fade of the inotropic response to 5-HT in porcine left atrium. This is in line with results found for β-adrenergic signalling in failing rat ventricle, whereby Sin-1 attenuated the inotropic effect (Ebihara and Karmazyn 1996; Afzal et al. 2011b), but in contrast to the increased inotropic response to 5-HT

in the presence of the NO-donor in the same tissue; this was attributed to cGMP-mediated inhibition of PDE3 (Afzal et al. 2011b).

From our experiments we can exclude that the effect of Sin-1 on responses to 5-HT is obtained via a modulation of cAMP levels, since tissue cAMP content in response to 5-HT was the same in the presence and absence of Sin-1. Furthermore PDE-inhibition could not overcome the dampening of 5-$HT_4$ receptor signalling in the presence of the NO-donor. The Sin-1 mediated increase in cGMP could lead to a PKGI mediated inhibition of L-type $Ca^{2+}$ channels (Levi et al. 1989; Mery et al. 1991; Sumii and Sperelakis 1995; Wegener et al. 2002). Such a mechanism was proposed by several authors for the reduction of β-adrenergic receptor signalling by increased NO/cGMP (Ebihara and Karmazyn 1996; Wang et al. 2009; Afzal et al. 2011b). However, from preliminary experiments with the L-type $Ca^{2+}$ channel blocker verapamil, we conclude that an inhibition of these channels is not the main mode of action of Sin-1 in pig left atrium. Alternatively Sin-1 can act independently of cGMP, by the production of peroxynitrite (Stamler et al. 2001; Ziolo 2008; Kohr et al. 2012). Sin-1 simultaneously produces NO and superoxide anion ($O_2^{\cdot}$), which react at a near diffusion-limited rate to peroxynitrite ($ONOO^-$)(Pacher et al. 2007). Low amounts of peroxynitrite are produced endogenously in the heart under physiological conditions and are increased in pathological conditions such as heart failure (Zhang et al. 2007; Kohr et al. 2012). While low physiological concentrations of peroxynitrite have been shown to increase basal and β-adrenergic receptor mediated contractility, high concentrations of peroxynitrite, as expected to be produced by 300 µM Sin-1, have the opposite effect; reducing basal and β-adrenergic receptor mediated cardiac contractility (Paolocci et al. 2000; Kohr et al. 2008a; Kohr et al. 2008b). The negative inotropic action of high levels of peroxynitrite was suggested to be due to stimulated PP2A activity leading to dephosphorylation of PLB and RyR2 $Ca^{2+}$ channels, mitochondrial dysfunction and myofilament protein cleavage (Stojanovic et al. 2001; Kohr et al. 2008b; Kohr et al. 2009; Kohr et al. 2012). To test whether Sin-1 in our experiments works via generation of peroxynitrite, it would have been interesting to test 5-$HT_4$ receptor-mediated PLB phosphorylation in the presence of Sin-1. Peroxynitrite as opposed to NO being responsible for the effect of Sin-1 on 5-$HT_4$ receptor mediated signalling is supported by experiments we performed with S-Nitroso-N-Acetyl-D,L-Penicillamine (SNAP; 100 µM). SNAP, an NO donor that does not produce $O_2^{\cdot}$, did not influence basal muscle activity nor did it affect responses to 5-HT. Still, cGMP content after administration of SNAP was not measured in these experiments so that we have no absolute proof of sGC stimulation by SNAP.

## VI.5 Conclusion

In conclusion, the results of this thesis have shown that the inotropic response to stimulation of 5-$HT_4$ receptors in pig left atrium is strongly controlled by PDE3 and PDE4 in a redundant way. In overexpression studies in HEK293 cells it was confirmed that 5-$HT_4$ receptors can indeed form a complex with PDE4. In pig left atrium, CNP in the presence of PDE4-inhibition prolonged the inotropic response to 5-HT, presumably by a cGMP-mediated inhibition of PDE3. In contrast, sGC stimulation using the NO-donor Sin-1, hastened the fade of the inotropic response to 5-HT, by a cAMP-independent mechanism.

As cardiac β-adrenergic signalling was well known for its inotropic effects, β-blockers were originally contraindicated for the treatment of heart failure. However, long-lasting enhanced cAMP signalling has been associated with heart deterioration in heart failure, explaining why β-blockers are now part of the standard treatment scheme for heart failure (Lohse et al. 2003). 5-$HT_4$ receptor stimulation mediates a short lasting increase in cAMP signalling. However, in heart failure patients cardiac 5-$HT_4$ receptor mRNA levels were increased (Brattelid et al. 2004), which might result in enhanced 5-$HT_4$ receptor signalling. Furthermore, Kaumann and Sanders (1994) found that occurrence of arrhythmias in response to 5-$HT_4$ receptor stimulation was increased in atria from patients on chronic β-blocker therapy, providing evidence that chronic blockade of β-adrenergic signalling might lead to an upregulation of 5-$HT_4$ receptor signalling. Therefore it would be important to further investigate in our pig model whether the inotropic response to 5-HT is increased in failing heart and to explore a possible influence of β-blocker treatment on 5-$HT_4$ receptor signalling.

We showed that CNP is able to increase 5-$HT_4$ receptor-mediated responses in the presence of PDE4-inhibition in pig atrium. In human heart the effect of CNP might be observable without the need for PDE4-inhibition, because responses to 5-$HT_4$ receptor stimulation are mainly regulated by PDE3 (Galindo-Tovar et al. 2009). Moreover, in chronic heart failure CNP plasma levels are significantly increased; this is related to the severity of the disease (Del Ry et al. 2005). Considering that the response to 5-$HT_4$ receptor stimulation might be more pronounced in the failing heart which could be further enhanced by the high level of plasma CNP, normal plasma levels of serotonin, reported were concentrations of 2 to 10 nmol/L (Alvarez et al. 1999; Kereveur et al. 2000; Lee et al. 2000), might have a detrimental effect in heart failure patients.

In line with the above, 5-$HT_4$ receptor antagonists were already tested for possible beneficial effects in failing rat and human heart. In rats with induced heart failure, treatment with the 5-$HT_4$ receptor antagonist SB207266 was found to improve to some extent *in vivo* and *ex vivo* heart function (Birkeland et al. 2007). However, in a human study the significant but slight improvement of cardiac output observed upon treatment with the 5-$HT_4$ receptor antagonist piboserod was not accompanied by a significant improvement of other efficacy parameters; as the study was limited to one dose of piboserod tested over a limited amount of time, clinical relevance in man remains uncertain and would need further investigation (Kjekshus et al. 2009).

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# Chapter VII

## *Summary*

# *VII Summary*

Serotonin (5-hydroxytryptamine; 5-HT) is a messenger, involved in many functions in various organs such as the brain, the gastro-intestinal (GI) tract and the cardiovascular system. Most serotonin in the body is produced by enterochromaffin cells (EC) in the GI mucosa. Overflowing serotonin into the blood is avidly taken up by platelets which serve as the main source of 5-HT in the cardiovascular system. 5-HT acts through 5-HT receptors, a family of seven receptor classes, named 5-$HT_1$ to 5-$HT_7$. All 5-HT receptors, except for the 5-$HT_3$ receptor, belong to the superfamily of seven-transmembrane spanning, G protein-coupled receptors. In the GI tract the 5-$HT_4$ receptor gained attention because agonists acting on this receptor have prokinetic effects. In 2009 the 5-$HT_4$ receptor agonist prucalopride (Resolor®) was marketed for the treatment of laxative-resistant chronic constipation. The 5-$HT_4$ receptor is also expressed in human and pig heart and activation of 5-$HT_4$ receptors in pig atrium results in a short lasting increase in contraction force (an inotropic response) and a better maintained enhanced relaxation (a lusitropic response). 5-$HT_4$ receptors are $G_s$ protein-coupled receptors which signal via adenylyl cyclase and cAMP to activate protein kinase A (PKA). PKA in turn phosphorylates key proteins involved in excitation-contraction coupling in cardiomyocytes. Among them are L-type $Ca^{2+}$ channels, ryanodine receptor $Ca^{2+}$ channels, phospholamban (PLB) and troponin I (Tn I). L-type $Ca^{2+}$ channels open upon electrical excitation of cardiomyocytes, allowing $Ca^{2+}$ to enter the cells. This local increase in $Ca^{2+}$ leads to an opening of ryanodine receptor $Ca^{2+}$ channels in the sarcoplasmic reticulum (SR) membrane. The following release of $Ca^{2+}$ from the SR causes a further global rise in cytoplasmic calcium which then activates the myofilaments resulting in a contractile response. PKA phosphorylation of L-type $Ca^{2+}$ channels and ryanodine receptor $Ca^{2+}$ channels increases their opening probability, leading to enhanced $Ca^{2+}$ cycling and an increased contraction force. PLB phosphorylation releases the protein's inhibition on the SR $Ca^{2+}$ pump, causing faster re-uptake of $Ca^{2+}$ into the SR during diastole and faster relaxation. PKA phosphorylation of TnI reduces the $Ca^{2+}$ affinity of the myofilaments also resulting in enhanced relaxation. Cardiac 5-$HT_4$ receptor signalling is strongly controlled by phosphodiesterases (PDEs), a family of cAMP and cGMP degrading enzymes, which cause the fade of the inotropic response to 5-HT in the atrium and completely prevent a response to 5-HT in the ventricle of pig and man. Porcine heart contains PDEs 2 to 5. PDE3 and PDE4 are cAMP-specific, while PDE2 has dual specificity for both cAMP and cGMP and PDE5 degrades only cGMP. PDEs can also mediate a cross-talk between cGMP and cAMP signalling pathways. Elevated cGMP can have an influence

on cAMP signalling via activation of the cAMP degrading function of PDE2 and via a functional inhibition of PDE3.

The **aim** of this thesis was to further investigate the regulation of cardiac 5-$HT_4$ receptor signalling. This was done in two functional studies in isolated pig atrium and in one study in HEK293 cells overexpressing 5-$HT_4$ receptors. The pig was used as a model system, since it is one of the rare species, next to humans, to express 5-$HT_4$ receptors in healthy heart. In detail it was investigated which PDE subtypes are important for the regulation of 5-$HT_4$ receptor signalling. Furthermore a possible influence of increased cGMP on cAMP mediated responses to 5-HT was examined. Finally the association of PDE3 and PDE4 with the 5-$HT_4$ receptor was investigated on protein level in HEK293 cells overexpressing the receptor. In this context an involvement of A-kinase anchoring proteins (AKAPs) in 5-$HT_4$ receptor signalling was also studied. AKAPs are scaffolding proteins which tether PKA and other important regulators of cAMP signalling to specific subcellular locations and thereby ensure specificity of signalling.

In the first study (**chapter III**) the influence of PDE subtypes and of elevated cGMP levels on 5-$HT_4$ receptor signalling in porcine atrium was investigated. cGMP is produced by the cytosolic soluble guanylyl cyclase (sGC), which is activated by nitric oxide (NO) and by the membrane-bound particulate guanylyl cyclase (pGC), which is activated by natriuretic peptides. In this functional study contractile responses of electrically paced pig left atrial muscle strips to 5-HT and to the 5-$HT_4$ receptor agonists prucalopride and RS67333 were measured in the absence and presence of PDE2, 3, 4 and 5 inhibitors, and of the NO-donor Sin-1 and C-type natriuretic peptide (CNP) stimulating the production of cGMP via sGC and pGC, respectively. Prucalopride behaved as a partial agonist in pig atrium, inducing a smaller inotropic response than 5-HT which also faded with time. This fade of the inotropic response to 5-HT and prucalopride could not be prevented by any of the subtype-specific inhibitors tested alone. However, in the combined presence of the PDE3- and PDE4-inhibitors cilostamide and rolipram, respectively, contractile responses to 5-HT and prucalopride were increased and the fade was abolished. In this condition an inotropic response to RS67333 was uncovered. This suggests that inotropic responses to 5-$HT_4$ receptor activation in pig atrium are controlled by both PDE3 and PDE4 in a redundant way, where one PDE can take over the function of the other. sGC stimulation using Sin-1 decreased the amplitude and hastened the fade of the inotropic response to 5-HT; the mechanism remains to be determined. In contrast, stimulation of pGC using C-type natriuretic peptide (CNP) per se had no influence on contractile responses to 5-HT. However, in the

presence of rolipram, CNP tended to prolong the inotropic response to 5-HT, corresponding with increased phosphorylation of phospholamban and a tendency to increased troponin I phosphorylation. CNP exerts its action presumably via the production of cGMP leading to a partial inhibition of PDE3. To obtain the functional effect on 5-HT, PDE4 needs to be inhibited as well because of the redundant control of both PDE-subtypes on 5-$HT_4$ receptor signalling.

The next study (**chapter IV**) aimed to gain a deeper understanding of the control by PDE3 and PDE4 and the effect of sGC and pGC stimulation on 5-$HT_4$ receptor signalling in pig left atrium. As in the previous study pig left atrial muscle strips were incubated with PDE-inhibitors and/or cGMP elevating agents before 5-$HT_4$ receptor stimulation with 5-HT or prucalopride. After 2, 10 and 30 min muscles were freeze-clamped and tissue cAMP and cGMP levels as well as phosphorylation of the lusitropic proteins PLB and TnI was determined. Corresponding with the inotropic response, 5-HT transiently increased cAMP levels, while prucalopride failed to increase tissue cAMP. 5-HT and prucalopride increased and tended to increase phosphorylation of PLB and TnI, respectively. This increase in PLB and TnI phosphorylation faded very slowly and did not fade, respectively, corresponding with the non-fading lusitropic response. As expected, in the presence of PDE3- and PDE4-inhibition, 5-HT caused a more pronounced and stable increase in cAMP and prucalopride increased tissue cAMP now as well. Also lusitropic responses to 5-HT and prucalopride were increased in the presence of PDE3- and PDE4-inhibition and the phosphorylation of PLB and TnI was significantly further increased in this condition. These data confirm the importance of PDE3 and PDE4 in controlling inotropic and lusitropic responses to 5-$HT_4$ receptor stimulation. Both pGC and sGC stimulation by CNP and Sin-1, respectively caused an increase in tissue cGMP. Consistent with the inotropic response, the 5-$HT_4$ receptor-mediated phosphorylation of PLB tended to be larger in the presence of CNP plus rolipram, compared to rolipram alone. In this study no further effect of PDE2-inhbition on functional responses and on PLB and TnI phosphorylation was detected. These data support the theory that cGMP generated by pGC inhibits PDE3, which in the presence of PDE4 inhibition enhances 5-$HT_4$ receptor signalling. Sin-1, which hastened the fade of the inotropic response to 5-HT, had no effect on the cAMP response to 5-HT, suggesting a cAMP-independent mechanism of action.

In the last study (**chapter V**) it was investigated whether PDE3 and PDE4 also interact with the 5-$HT_4$ receptor on protein level. Furthermore, a possible involvement of AKAPs in 5-$HT_4$ receptor signalling was studied. This was done in HEK293 cells overexpressing the HA-tagged 5-$HT_{4(b)}$ receptor splice variant. The influence of PDEs as well as an involvement of AKAPs on 5-$HT_{4(b)}$

receptor signalling was studied by measuring PKA activity. Furthermore the association of proteins with the 5-$HT_{4(b)}$ receptor was determined by co-immunoprecipitation. 5-$HT_{4(b)}$ receptor signalling in HEK293 cells was found to be regulated predominantly by PDE4, with a possible role of PDE3 in the presence of PDE4-inhibition. Additionally overexpressed PDE4D3 and PDE4D5, the splice variants providing the majority of PDE4 activity in HEK293 cells, were found to interact with the 5-$HT_{4(b)}$ receptor; this interaction did not change upon receptor stimulation. However, PDE activity measurements provided evidence that PDE activity might be increased upon stimulation with 5-HT. Overexpressed PDE3B did not interact with the 5-$HT_{4(b)}$ receptor. A possible involvement of AKAPs in 5-$HT_{4(b)}$ receptor signalling was tested employing the St-Ht31 peptide, which disrupts the binding of AKAPs to PKA. 5-$HT_{4(b)}$ receptor-stimulated PKA activity was not affected by the presence of St-Ht31. Furthermore the AKAPs 79 and gravin, did not co-immunoprecipitate with the 5-$HT_{4(b)}$ receptor, suggesting that AKAPs do not play an important role in 5-$HT_{4(b)}$ receptor signalling.

In conclusion the results of this thesis have shown that PDE3 and PDE4 redundantly regulate 5-$HT_4$ receptor signalling in porcine left atrium. Furthermore overexpressed PDE4D splice variants associated with the overexpressed 5-$HT_{4(b)}$ receptor on protein level in HEK293 cells, providing evidence that PDE4s might be in a complex with the 5-$HT_4$ receptor in cardiac cells. cGMP generated by stimulation of pGC increased the inotropic response to 5-HT in pig left atrium, when PDE4 was inhibited as well. The mechanism is probably an inhibition of PDE3 by elevated cGMP. In contrast, sGC stimulation using the NO-donor Sin-1 caused a hastening of the fade of the inotropic response to 5-HT in pig left atrium, which is independent of an influence on cAMP.

# Chapter VIII

## *Samenvatting*

# *VIII Samenvatting*

Serotonine (5-hydroxytryptamine; 5-HT) is een boodschappermolecule, betrokken in talrijke functies in diverse organen zoals de hersenen, de gastro-intestinale (GI) tractus en het cardiovasculaire stelsel. Het grootste deel van de hoeveelheid 5-HT in het lichaam wordt geproduceerd door enterochromaffiene cellen (EC) in het GI slijmvlies. Wanneer 5-HT overloopt naar het bloed, wordt het gretig opgenomen door bloedplaatjes, welke de belangrijkste bron vormen van 5-HT in het cardiovasculaire systeem. 5-HT is werkzaam via 5-HT receptoren, een familie van 7 receptorklassen aangeduid als 5-$HT_1$ tot 5-$HT_7$. Alle 5-HT receptoren, uitgezonderd de 5-$HT_3$ receptor, behoren tot de superfamilie van zeven-transmembranaire, aan G proteïne gekoppelde receptoren. In de GI tractus trok de 5-$HT_4$ receptor meest aandacht omdat agonisten van deze receptor prokinetische effecten hebben. In 2009 werd de 5-$HT_4$ receptor agonist prucalopride (Resolor®) op de markt gebracht voor de behandeling van chronische constipatie resistent aan laxatieven. De 5-$HT_4$ receptor wordt ook tot expressie gebracht in het hart van mens en varken; activatie van 5-$HT_4$ receptoren in het atrium van het varken veroorzaakt een kortdurende toename in contractiekracht (een inotroop antwoord) en een beter volgehouden toename in relaxatie (een lusitroop effect). 5-$HT_4$ receptoren zijn gekoppeld aan $G_s$ proteïne en geven hun signaal door via adenylaatcyclase, vorming van cAMP en activatie van proteïne kinase A (PKA). PKA fosforyleert op zijn beurt een aantal sleutelproteïnen in de koppeling van excitatie-contractie in cardiomyocyten, zoals L-type $Ca^{2+}$ kanalen, $Ca^{2+}$ kanalen gekoppeld aan de ryanodine receptor, fosfolamban (PLB) en troponine I (TnI). L-type $Ca^{2+}$ kanalen openen bij elektrische excitatie van de cardiomyocyten, wat toelaat dat $Ca^{2+}$ in de cel binnenkomt. Deze lokale toename van $Ca^{2+}$ leidt tot opening van $Ca^{2+}$ kanalen gekoppeld aan de ryanodine receptor in de membraan van het sarcoplasmatisch reticulum (SR). De hierdoor uitgelokte vrijstelling van $Ca^{2+}$ uit het SR veroorzaakt een verdere globale stijging van cytoplasmatisch $Ca^{2+}$, wat de myofilamenten activeert leidend tot een contractiel antwoord. Fosforylatie door PKA van L-type $Ca^{2+}$ kanalen en $Ca^{2+}$ kanalen gekoppeld aan de ryanodine receptor verhoogt hun openingsprobabiliteit, wat leidt tot verhoogd circuleren van $Ca^{2+}$ en toegenomen contractiekracht. Fosforylatie van PLB heft zijn inhiberend effect op de $Ca^{2+}$ pomp van het SR op, wat een snellere heropname van $Ca^{2+}$ in het SR veroorzaakt gedurende de diastole en een snellere relaxatie. Fosforylatie van TnI vermindert de affiniteit van de myofilamenten voor $Ca^{2+}$ wat eveneens leidt tot een snellere relaxatie. De signaaltransductie van de cardiale 5-$HT_4$ receptoren wordt sterk gecontroleerd door fosfodiësterasen (PDEs), een

familie van enzymes welke cAMP en cGMP afbreken. Zij veroorzaken het verval van het inotroop antwoord op 5-HT in het atrium en voorkomen een antwoord op 5-HT in het ventrikel van varken en mens. Het varkenshart bevat PDEs 2 tot 5. PDE3 en PDE4 zijn cAMP-specifiek, terwijl PDE2 specificiteit heeft voor zowel cAMP als cGMP en PDE5 enkel cGMP afbreekt. PDEs kunnen ook de interactie tussen cGMP- en cAMP-gemedieerde signalisatiewegen regelen. Een toename in cGMP-gehalte kan een invloed hebben op de signalisatie via cAMP door activatie van de afbraak van cAMP door PDE2 en via een functionele inhibitie van PDE3.

Het **doel** van dit proefschrift was om de regulatie van de signaaltransductie der cardiale 5-$HT_4$ receptoren verder te onderzoeken. Dit werd uitgevoerd in twee functionele studies met geïsoleerd atriumweefsel van het varken en in één studie met HEK293 cellen, die 5-$HT_4$ receptoren tot overexpressie brengen. Het varken werd als model gebruikt omdat het een van de weinige species is die zoals de mens 5-$HT_4$ receptoren tot expressie brengt in gezond hartweefsel. Er werd onderzocht welke PDE subtypes belangrijk zijn voor de regulatie van de signaaltransductie van de 5-$HT_4$ receptoren. Verder werd een mogelijke invloed van een stijging in cGMP op de door cAMP gemedieerde effecten van 5-HT onderzocht. Tenslotte werd de associatie van PDE3 en PDE4 met de 5-$HT_4$ receptor onderzocht op proteïneniveau in HEK293 cellen, die de receptor tot overexpressie brengen. In dit verband werd ook de mogelijke betrokkenheid van A-kinase verankerende proteïnen (AKAPs) op de signalisatie door 5-HT bestudeerd. AKAPs zijn schragende proteïnen, welke PKA en andere belangrijke regulatoren van de door cAMP geïnitieerde signalisatie fixeren op specifieke subcellulaire plaatsen en hierdoor specifieke signalisatie verzekeren.

In de eerste studie (**hoofdstuk III**) werd de invloed van de PDE subtypes en van verhoogde spiegels van cGMP op de signaaltransductie van 5-$HT_4$ receptoren onderzocht in het atrium van het varken. cGMP wordt geproduceerd door cytosolair oplosbaar guanylaatcyclase (sGC), dat geactiveerd wordt door stikstofmonoxide (NO), en door membraan-gebonden guanylaatcyclase (pGC), dat geactiveerd wordt door natriuretische peptiden. In deze functionele studie werden de contractiele antwoorden van elektrisch aangestuurde spierstrips van het linker atrium van het varken, uitgelokt door 5-HT en door de 5-$HT_4$ receptor agonisten prucalopride en RS67333, gemeten in af- en aanwezigheid van inhibitoren van PDE2, 3, 4 en 5, van de NO-donor Sin-1 en van C-type natriuretisch peptide (CNP); deze laatste stimuleren de productie van cGMP via respectievelijk sGC en pGC. Prucalopride gedroeg zich als een partiële agonist in het atrium van het varken en lokte een kleiner inotroop antwoord uit dan 5-HT, dat eveneens terugviel in

functie van de tijd. Dit verval van het inotroop antwoord op 5-HT en prucalopride kon niet voorkomen worden door één van de subtype-specifieke PDE-inhibitoren. In de gezamelijke aanwezigheid van de PDE3- en PDE4-inhibitoren cilostamide en rolipram, verhoogde het contractiel antwoord op 5-HT en prucalopride en werd het verval van het antwoord opgeheven. In deze experimentele conditie werd ook een antwoord op RS67333 ontsluierd. Dit suggereert dat inotrope antwoorden in reactie op activatie van 5-$HT_4$ receptoren in het atrium van het varken gecontroleerd worden door zowel PDE3 als PDE4 op een manier dat het ene PDE de functie van het andere kan overnemen. Stimulatie van sGC met Sin-1 verminderde de amplitude en versnelde het verval van het inotroop antwoord op 5-HT; het mechanisme hiervan moet nog opgehelderd worden. Stimulatie van pGC met CNP had op zich geen invloed op de contractiele antwoorden op 5-HT. Maar in de gezamelijke aanwezigheid van rolipram en de PDE2-inhibitor EHNA, verlengde CNP het inotroop antwoord op 5-HT significant, waarschijnlijk door partiële inhibitie van PDE3 onder invloed van het door CNP gegenereerde cGMP. Om de invloed van CNP versus 5-HT functioneel te kunnen observeren, moet ook PDE4 geïnhibeerd worden vermits dit de taak van PDE3 kan overnemen; inhibitie van PDE2 versterkte het effect wellicht omdat PDE2, dat een cGMP-geactiveerd PDE enzyme is, significant bijdraagt aan de degradatie van cAMP in aanwezigheid van een verhoogd gehalte aan cGMP.

De volgende studie (**hoofdstuk IV**) had tot doel om de kennis over de controlerende rol van PDE3 en PDE4, en over het effect van stimulatie van sGC en pGC op de signaaltransductie van 5-$HT_4$ receptoren in het linker atrium van het varken te verdiepen. Zoals in de vorige studie werden spierstrips van het linker atrium van het varken geïncubeerd met PDE-inhibitoren en/of producten die het gehalte aan cGMP verhogen, vooraleer de 5-$HT_4$ receptoren te stimuleren met 5-HT of prucalopride. Na 2, 10 en 30 minuten stimulatie werden de spierstrips bevroren. De weefselspiegels aan cAMP en cGMP en de fosforylatiegraad van de lusitrope proteïnen PLB en TnI werden bepaald. Correlerend met het functionele inotrope antwoord, verhoogde 5-HT transiënt de spiegels van cAMP, terwijl prucalopride niet in staat was om het weefselgehalte aan cAMP te verhogen. 5-HT en prucalopride verhoogden de fosforylatie van PLB en toonden een tendens om de fosforylatie van TnI te verhogen. De toename in fosforylatie van PLB verviel slechts traag en deze van TnI verviel helemaal niet, wat overeenkwam met het niet vervallende lusitrope antwoord. Zoals te verwachten veroorzaakte 5-HT in de gezamelijke aanwezigheid van PDE3- en PDE4-inhibitie een meer uitgesproken en stabiele toename in cAMP, en in deze omstandigheden verhoogde ook prucalopride het weefselgehalte aan cAMP. Ook de lusitrope antwoorden op 5-HT en prucalopride namen toe onder gezamelijke PDE3- en PDE4-inhibitie en

de fosforylatie van PLB en TnI werd significant verder opgedreven. Deze resultaten bevestigen het belang van PDE3 en PDE4 in de controle van de inotrope en lusitrope antwoorden, uitgelokt door stimulatie van 5-$HT_4$ receptoren in het atrium. Zowel stimulatie van pGC met CNP als van sGC met Sin-1 veroorzaakten een toename in weefselgehalte aan cGMP. Correlerend met het inotrope antwoord, vertoonde de fosforylatie van PLB door stimulatie van de 5-$HT_4$ receptoren de tendens om te verhogen in de aanwezigheid van CNP plus rolipram, in vergelijking met de graad van fosforylatie in aanwezigheid van rolipram alleen. In deze studie werd geen bijkomend effect van PDE2-inhibitie op de functionele antwoorden of op de fosforylatie van PLB en TnI gedetecteerd. Deze gegevens ondersteunen de stelling dat cGMP, gegenereerd door pGC, PDE3 inhibeert, wat in de aanwezigheid van PDE4-inhibitie de signalisatie van 5-$HT_4$ receptoren versterkt. Sin-1, dat het verval van het inotroop antwoord op 5-HT versnelde, had geen effect op het cAMP-gehalte uitgelokt door 5-HT, wat een cAMP-onafhankelijk mechanisme suggereert.

In de laatste studie (**Hoofdstuk V**) werd onderzocht of PDE3 en PDE4 ook interageren met de 5-$HT_4$ receptor op proteïneniveau. Verder werd een mogelijke rol van AKAPs in de signalisatie van 5-$HT_4$ receptoren bestudeerd. Dit werd gedaan in HEK293 cellen, waarin de HA-gemerkte 5-$HT_{4(b)}$ receptor tot overexpressie werd gebracht. De invloed van PDEs zowel als de mogelijke betrokkenheid van AKAPs op de signalisatie van de 5-$HT_{4(b)}$ receptor werd bestudeerd door het meten van de PKA-activiteit. Verder werd de associatie van proteïnen met de 5-$HT_{4(b)}$ receptor bepaald door co-immunoprecipitatie. De signalisatie van de 5-$HT_{4(b)}$ receptor in HEK293 cellen bleek predominant geregeld door PDE4, met een mogelijke rol van PDE3 in aanwezigheid van PDE4-inhibitie. Verder werd geconstateerd dat tot overexpressie gebracht PDE4D3 en PDE4D5, de varianten die het merendeel van de PDE4-activiteit in HEK293 cellen verzekeren, interageerden met de 5-$HT_{4(b)}$ receptor; de graad van deze interactie veranderde niet bij stimulatie van de receptor. Maar analyse van de PDE-activiteit toonde dat deze leek te verhogen bij stimulatie met 5-HT. Tot overexpressie gebracht PDE3B interageerde niet met de 5-$HT_{4(b)}$ receptor. De mogelijke betrokkenheid van AKAPs in de signalisatie van 5-$HT_{4(b)}$ receptoren werd getest met behulp van St-Ht31 peptide, dat de binding van AKAPs aan PKA onderbreekt. De PKA-activiteit, verhoogd door stimulatie van de 5-$HT_{4(b)}$ receptor, werd evenwel niet beïnvloed door St-Ht31 peptide. Verder co-immunoprecipiteerden AKAPs 79 en gravin niet met de 5-$HT_{4(b)}$ receptor, wat suggereert dat AKAPs geen belangrijke rol spelen in de signalisatie van de 5-$HT_{4(b)}$ receptor.

Tot besluit kan gesteld worden dat de resultaten van dit proefschrift tonen dat PDE3 en PDE4 de signalisatie van 5-$HT_4$ receptoren in het linker atrium van het varken controleren, waarbij ze elkaars functie kunnen overnemen. Verder associeerden tot overexpressie gebrachte PDE4 varianten met de tot overexpressie gebrachte 5-$HT_{4(b)}$ receptor op proteïneniveau in HEK293 cellen, wat suggereert dat PDE4 zich in een complex met de 5-$HT_4$ receptor zou kunnen bevinden in cardiale cellen. cGMP, gegenereerd door stimulatie van pGC verhoogde het inotroop antwoord op 5-HT in het linker atrium van het varken, wanneer ook PDE4 geïnhibeerd was. Het mechanisme berust waarschijnlijk op de inhibitie van PDE3 door het verhoogde gehalte aan cGMP. Stimulatie van sGC met de NO-donor Sin-1 daarentegen versnelde het verval van het inotrope antwoord op 5-HT in het linker atrium van het varken, wat onafhankelijk is van een wijziging in cAMP.

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TAJEL, IT'S PERFECTLY OK FOR YOU TO GO ON VACATION.
I MEAN, YOU DID TAKE WORK WITH YOU, RIGHT?
EXCUSE ME?
YOU SPENT THE WHOLE TIME THINKING AND OBSESSING ABOUT YOUR RESEARCH PROJECT, I ASSUME?
UH...
IN ACADEMIA, "VACATIONS" JUST MEAN YOU'RE DOING WORK SOMEWHERE ELSE.
I DON'T THINK WE'RE USING THE SAME DICTIONARY.
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