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RESEARCH PAPER

Selective desensitization of the 5-HT$_4$ receptor-mediated response in pig atrium but not in stomach

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Background and purpose: The time dependency of the effect of 5-HT$_4$ receptor agonists depends on many specific regulatory mechanisms, which vary between tissues. This has important implications with regard to the effects of endogenous 5-HT, as well as to the clinical use of 5-HT$_4$ receptor agonists, and might contribute to tissue selectivity of agonists.
Experimental approach: The progression and desensitization of 5-HT$_4$ receptor-mediated responses were evaluated in an organ bath set-up using two, clinically relevant, porcine *in vitro* models: gastric cholinergic neurotransmission and atrial contractility.
Key results: Exposure of gastric tissue to 5-HT or to the selective 5-HT$_4$ receptor agonists prucalopride and M0003 results in a sustained non-transient effect during exposure; after washout, the response to a subsequent challenge with 5-HT shows no clear desensitization. Incubation of left atrial tissue with 5-HT resulted in a transient response, leading after washout to a marked desensitization of the subsequent response to 5-HT. The selective 5-HT$_4$ receptor agonists prucalopride and M0003 induce only very weak atrial responses whereas they are very effective in desensitizing the atrial response to 5-HT. The observations also suggest that the properties of prucalopride and M0003 to bind to and/or activate the 5-HT$_4$ receptor differ from those of 5-HT. This difference might have contributed to the observed desensitization.
Conclusions and implications: The high potency of prucalopride and M0003 in desensitizing the response to 5-HT together with their low efficacy in the atrium emphasizes the cardiac safety of this class of 5-HT$_4$ receptor agonists.
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Keywords: 5-HT$_4$ receptor; proximal stomach; left atrium; desensitization; tachyphylaxis; prucalopride; M0003

Abbreviations: EFS, electrical field stimulation; GI, gastrointestinal; GRK2, G protein-coupled receptor kinase 2; L-NAME, N^G-nitro-L-arginine-methylester; IBMX, 3-isobutyl-1-methylxanthine; mAChR, muscarinic acetylcholine receptor; PDE, phosphodiesterase; PKA, protein kinase A; PLB, phospholamban; R2, contraction-relaxation coupling parameter equal to (+dF dt^{-1})/($^-$dF dt^{-1}); SR, sarcoplasmatic reticulum; TnI, troponin I

Introduction

5-HT$_4$ receptors are widely expressed in the body and they exert pleiotropic effects after being activated by their endogenous ligand, 5-HT. Selective 5-HT$_4$ receptor agonists therefore have a large therapeutic potential to treat patients suffering from a variety of diseases. Their beneficial effect mainly arises from the interaction with neuronal 5-HT$_4$ receptors, resulting in a facilitation of neurotransmitter release in the brain and the periphery. In the brain, 5-HT$_4$ receptors reside mainly in the limbic system. In animal models, their activation results in increased acetylcholine release, suggesting a role for this receptor in memory and learning (Langlois and Fischmeister, 2003). Also in the enteric nervous system of the gastrointestinal (GI) tract, 5-HT$_4$ receptor agonists are able to strengthen cholinergic neurotransmission. Although the effects of 5-HT$_4$ receptor agonists go beyond the cholinergic system, their impact on the enteric cholinergic axis is considered the predominant mechanism by which 5-HT$_4$ receptor activation stimulates peristaltic reflex activity (Gershon and Tack, 2007). This mechanism has been described in the human GI tract, for neurons innervating the circular muscle layer of the human proximal stomach (Leclere and Lefebvre, 2002a) and both muscle layers in the colon (Prins *et al.*, 2000; Leclere *et al.*, 2005), and underlies the clinical efficacy of 5-HT$_4$ receptor agonists such as cisapride in patients suffering from reflux or gastroparesis (Feldman and Smith, 1987) and prucalopride in patients with chronic constipation (Bouras *et al.*, 1999; Schiller, 2004). The human heart also accommodates a small population of muscular 5-HT$_4$ receptors and 5-HT$_4$ receptor agonists therefore have the potential to affect cardiac contraction and relaxation parameters (Kaumann and Levy, 2006).

To be successful in clinical practice, 5-HT$_4$ receptor agonists should not show tachyphylaxis of their GI effects and should

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thus remain effective during the course of treatment and not respond only to the initial doses. On the other hand, the occurrence of such tachyphylaxis in tissues outside the primary target, for example the heart, could reduce potential side effects of the agonists. Tachyphylaxis, or desensitization, denoting the attenuation of a biological response to sustained or repeated intervention, results from various, often cell-type-specific mechanisms that limit further stimulation of downstream effectors either by the inhibition of downstream signalling or direct receptor inactivation. It is well established that 5-HT$_4$ receptors can desensitize (become inactivated) upon stimulation by their ligands. As shown in recombinant systems, the 5-HT$_4$ receptor is initially uncoupled from G proteins resulting in attenuation of the primary response (the formation of cAMP). This process involves G protein-coupled receptor kinase 2 (GRK2) without any requirement of the kinase activity of GRK2 (Barthet *et al.*, 2005); by binding to the receptor and to the G protein, GRK2 presumably prevents their interaction (Ferguson, 2007). In a second step, receptors can be removed from the cell surface by endocytosis, a process involving GRK2-dependent phosphorylation of the receptor and the recruitment of β-arrestins (Barthet *et al.*, 2005; Ponimaskin *et al.*, 2005). Interestingly, the use of heterologous cells clearly shows the cell-type dependence of 5-HT$_4$ receptor desensitization, which is rapid and strong in CHO cells (Mialet *et al.*, 2003) and weak and slow in COS and HEK293 cells, explained by low levels of GRK2 in these cells (Barthet *et al.*, 2005). GRK levels might also influence the mechanism of receptor desensitization for certain agonists within a given tissue, and thus influence functional selectivity (Kelly *et al.*, 2008). Additionally, the desensitization process is dependent on the 5-HT$_4$ receptor splice variant that is involved (Mialet *et al.*, 2003; Pindon *et al.*, 2004).

These observations underscore the importance of studying the desensitization process in native tissue. Unfortunately, apart from two studies describing a rapid and homologous desensitization of the adenylyl cyclase response in neurons from the mouse colliculi (Ansanay *et al.*, 1992) and the rat oesophagus (Ronde *et al.*, 1995), little information is available on the desensitization of 5-HT$_4$ receptors in their natural environment. It has been suggested, referring to two studies by Kaumann *et al.* (1991) and Kaumann and Sanders (1994), that 5-HT$_4$ receptors in the human atrium desensitize to a lesser extent. However, the studies from which the underlying data were derived were not designed to address this question. The limitations of recombinant expression systems are also emphasized by the observation that, despite desensitization of 5-HT$_4$ receptors in mouse coliculli neurons, a brief application of 5-HT results in a prolonged cAMP-mediated blockade of K^+ channels (Ansanay *et al.*, 1995), ending up in a persistent enhancement of transmitter release. In the left atrium, 5-HT$_4$ receptor-induced responses have the opposite fate. In this tissue, inotropic responses decrease over time despite the continuous presence of the agonist. This phenomenon presumably results from the recruitment of phosphodiesterases (PDEs) that rapidly quench any inotropic response elicited by 5-HT$_4$ receptor agonists (De Maeyer *et al.*, 2006a).

We have now evaluated the progression and desensitization of 5-HT$_4$ receptor-mediated responses in two clinically relevant porcine *in vitro* models: gastric cholinergic neurotransmission and left atrial contractility. The time dependency of the effects of 5-HT and the selective 5-HT$_4$ receptor agonists prucalopride and M0003 (R149402) as well as their capacity to reduce the responsiveness to 5-HT were compared in these assays. Previously, the efficacy of both agonists was compared in both assays with prucalopride showing full agonism in the stomach and partial agonism in the atrium (De Maeyer *et al.*, 2006b). The degree of agonism is indeed tissue-dependent and is related to differences in receptor density or coupling efficiency (De Maeyer *et al.*, 2008). Divergence of the desensitization properties of these agonists from their activation properties (De Maeyer *et al.*, 2006b) might reveal functional selectivity of desensitization (Kelly *et al.*, 2008). Prucalopride and M0003 (R149402; De Maeyer *et al.*, 2006b) are two compounds underdevelopment by Movetis NV currently in preregistration phase and phase 2 respectively. The pig was chosen as an experimental model because, apart from humans, it is one of the very few species available with cardiac 5-HT$_4$ receptors.

Methods

Tissue preparation

All animal procedures and this study were approved by the ethical committee from Johnson & Johnson Pharmaceutical Research & Development, a division of Janssen Pharmaceutica N.V., Beerse, Belgium and by the Ethical Committee for Animal Experiments of the Faculty of Medicine and Health Sciences, Ghent University.

Female pigs (10–11 weeks, 22–27 kg), obtained from local farms, were anaesthetized with an intravenous (50 mg kg^{-1}) sodium pentobarbital (Kela N.V., Hoogstraten, Belgium) injection. After exsanguination, the heart and the entire stomach were dissected and placed in Krebs-Henseleit solution (composition in mmol·L^{-1}: glucose 11.1, $CaCl_2$ 2.51, $NaHCO_3$ 25, $MgSO_4$ 1.18, KH_2PO_4 1.18, KCl 4.69, EDTA 0.033 and NaCl 118). The preparation of muscle strips from the left atrium and the proximal stomach was essentially as described before (De Maeyer *et al.*, 2006a,b). All tissues were used on the day of preparation.

Left atrium Briefly, under continuous aeration, left atrial pectinate muscles (9–12 per left atrium) with a thickness <1 mm and a length varying between 3 and 7 mm were dissected away from the endocardial surface. Tissues were suspended in 20 mL organ baths containing Krebs-Henseleit solution at 37°C and oxygenated with 95% O_2 and 5% CO_2. The preparations were continuously electrically stimulated using two platinum wire electrodes [0.5 Hz, 5 ms, just above threshold voltage, resting length being half of L_{max}, the length at which maximal active tension was developed upon electrical field stimulation (EFS)], except during wash steps. Before starting the experimental protocol, the bath solution was routinely supplemented with propranolol (0.2 μmol·L^{-1}), to avoid β-adrenoreceptor-mediated effects due to released noradrenaline and cocaine (6 μmol·L^{-1}), to inhibit the uptake of 5-HT.

Proximal stomach Gastric muscle strips of approximately 1.5 cm in length were prepared from the ventral side of the proximal stomach in the direction of the longitudinal muscle layer and stripped of the mucosa and most of the circular muscle layer. The longitudinal muscle strips were mounted between two coaxially aligned platinum wire electrodes under a resting tension of 20 mN in organ baths containing 20 mL of continuously aerated, Krebs-Henseleit solution which was kept at 37°C. The bath solution also contained the nitric oxide (NO) synthase inhibitor N^G-nitro-L-arginine-methylester (L-NAME; 0.1 mmol·L^{-1}), to avoid functional antagonism by the release of endogenous NO and 1 μmol·L^{-1} indomethacin to avoid prostaglandin-mediated contractions. Viability of the tissue was checked with carbachol (3 μmol·L^{-1}), administered at 30 min intervals until two similar successive responses to carbachol were obtained (usually three times). After the last washing step organ baths were supplied with 1 μmol·L^{-1} of methysergide, a 5-HT$_1$, 5-HT$_2$, 5-ht$_5$, 5-HT$_6$ and 5-HT$_7$ receptor [nomenclature according to Alexander *et al.* (2008)] antagonist, 0.3 μmol·L^{-1} of the 5-HT$_3$ receptor antagonist granisetron and 6 μmol·L^{-1} cocaine, to reduce the uptake of 5-HT by nerve endings. Thirty minutes later, the gastric muscle strips were electrically stimulated (every 3 min a 10 s pulse train at 4 Hz, 0.5 ms and 20 V). Once successive electrically evoked contractions became reproducible, the applied voltage was adjusted to reduce the contraction force to 50% of the force developed at 20 V and the electrical stimulation was set on hold for 20 min. EFS was then reinstated at this adjusted voltage and from then on, this electrical stimulation pattern was maintained.

Changes in isometric force of the preparations were recorded via Statham UC2 force transducers (Gould, Cleveland, USA) and DBA 18 digital bridge amplifiers (Anerma, Belgium) on a Powerlab data acquisition system and recorded using Chart v5.1.1 software. EFS was performed with a constant voltage stimulator (Janssen Pharmaceutica, Belgium).

Experimental desensitization protocols

The applied protocols followed the previously described methods to study 5-HT$_4$ receptor desensitization in native cells or tissue (Ansanay *et al.*, 1992; Ronde *et al.*, 1995) and are illustrated in the Figures. Responses were monitored during the complete duration of the experiment. In all protocols, parallel preparations were exposed to increasing concentrations of 5-HT$_4$ receptor agonists (desensitization period). The choice of the range of desensitizing concentrations for the atrium was determined in preliminary experiments. For experiments with gastric tissue, this was not possible (as there was no desensitization) and the range of desensitizing concentrations was chosen 1 log unit lower than in the atrium because the agonists are more potent in gastric tissue (De Maeyer *et al.*, 2006b). After removal of the agonists, tissues were challenged with a fixed concentration of 5-HT (test period). A submaximal concentration (as determined from previously obtained non-cumulative concentration-response curves) was chosen as the test concentration, being 1 μmol·L^{-1} in the atrium and 3 nmol·L^{-1} in the stomach (De Maeyer *et al.*, 2006a,b).

Proximal stomach

Protocol A The protocol was initiated by changing the physiological bathing solution. Seven EFS trains (20 min) were then obtained, followed by challenging parallel tissues with different concentrations of 5-HT, prucalopride or M0003. After 1 h (desensitization period), incubation was terminated by three 1 min washes with fresh physiological solution and after recovery of stable response to EFS (seven EFS trains), a test concentration of 3 nmol·L^{-1} 5-HT was administered and left in contact with the tissues for at least 20 min (test period).

Protocol B The protocol was initiated by changing the physiological bath solution, followed by seven EFS trains before the addition of a control concentration of 3 nmol·L^{-1} 5-HT (control period). When the maximal potentiating effect of 5-HT on the EFS-induced contractions was reached (seven EFS trains), the tissues were washed with physiological solution and allowed to recover for 1 h, during which time they were washed every 15 min. Next, different desensitizing concentrations of 5-HT were added to parallel tissues (desensitization period). After 1 h, incubation was terminated by three 1 min washes with fresh physiological solution and after recovery of stable response to EFS (seven EFS trains), a test concentration of 3 nmol·L^{-1} 5-HT was administered and left in contact with the tissues for at least 20 min (test period). A representative tracing of an experiment performed according to protocol B is shown in Figure 1a.

The susceptibility of the responses in the stomach to reversal by the administration of a selective 5-HT$_4$ receptor antagonist was examined by adding GR113808 (0.1 μmol·L^{-1}) 1 h after the administration of the agonists.

Left atrium

Protocols A and B Protocols A and B in this tissue were similar to protocols A and B in the proximal stomach, except that 1 μmol·L^{-1} 5-HT was used as control and test concentration and that the protocol was ended by the administration of a saturating concentration of isoprenaline (0.1 mmol·L^{-1}). The maximal response to the control concentration of 5-HT was reached in 2–3 min. During wash steps, EFS was set on hold. Time for recovery of a stable EFS-induced response after reinstating EFS was between 10 and 15 min. The test concentration was left in contact with the tissues for approximately 6 min. A representative tracing of an experiment performed according to protocol B is shown in Figure 1b.

Protocol C Nine consecutive concentrations of 5-HT, including a solvent concentration, were added as a single concentration to parallel left atrial pectinate muscles and the response was followed for 30 min (desensitization period). Tissues were washed three times by changing the bath solution every minute under non-EFS conditions. EFS was reinstated and once a stable response was obtained (10–15 min), a cumulative concentration-response curve to 5-HT with half log units ascending concentration increments was established (test period). Experiments were terminated by the administration of a saturating concentration of isoprenaline (0.1 mmol·L^{-1}). The same protocol was also performed in the

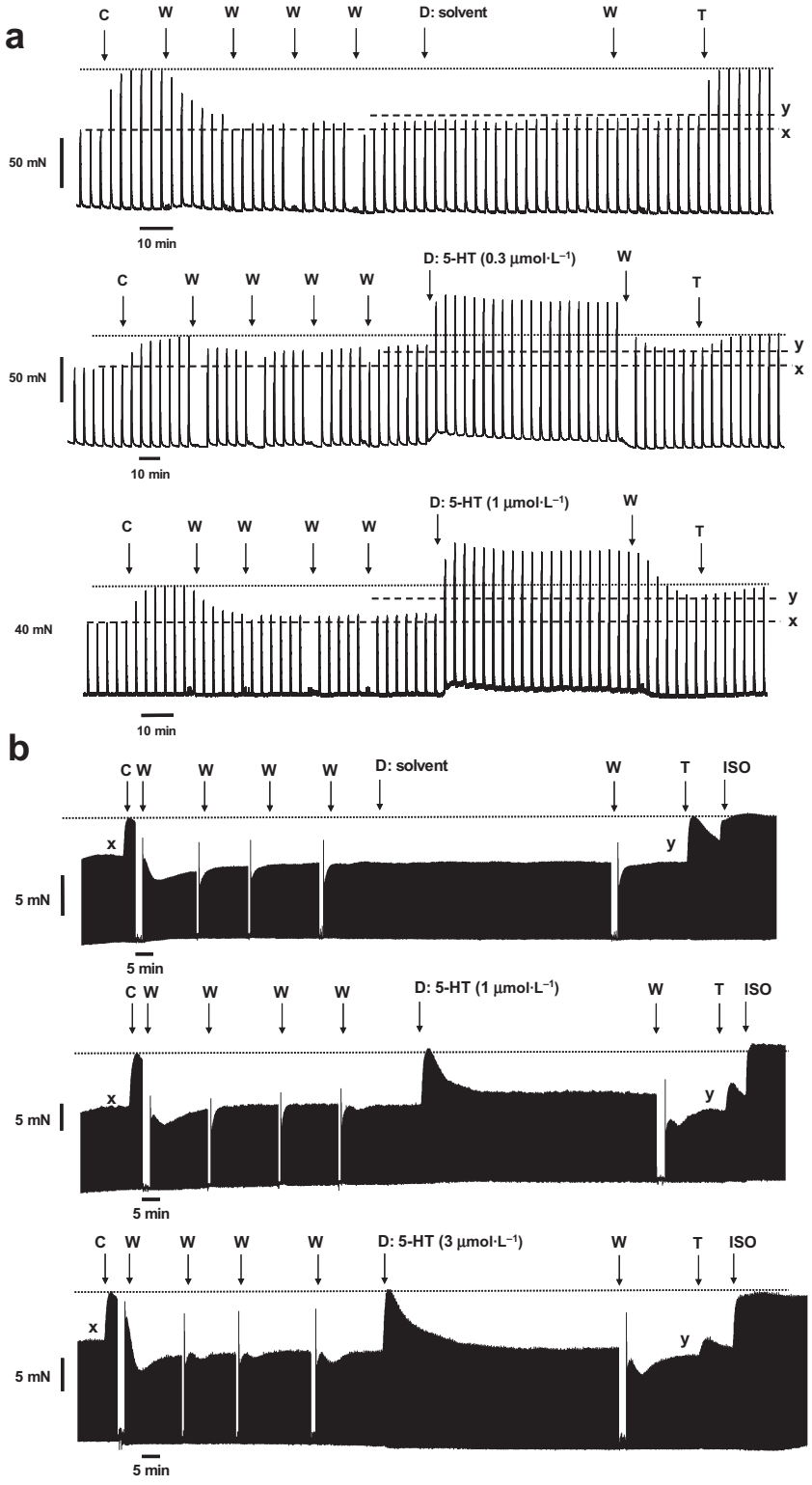

Figure 1 Representative tracings showing experiments with 5-HT according to protocol B with longitudinal muscle strips of the proximal stomach (a) or left atrial pectinate muscles (b). A control (C) concentration of 3 nmol·L^{-1} (a) or 1 μmol·L^{-1} (b) 5-HT (control period) was administered and when the maximal potentiating effect of 5-HT on the EFS-induced contractions was reached, the tissues were washed (W) with physiological solution and allowed to recover for 1 h, during which time they were washed every 15 min. Next, solvent or a desensitizing concentration of 0.3 μmol·L^{-1} or 1 μmol·L^{-1} (a) or 1 μmol·L^{-1} or 3 μmol·L^{-1} (b) 5-HT was added. After 1 h, incubation was terminated by three 1 min washes with fresh physiological solution and after recovery of a stable response to EFS, a test concentration of 3 nmol·L^{-1} (a) or 1 μmol·L^{-1} (b) 5-HT was administered. The atrial experiments were terminated by the administration of 0.1 mmol·L^{-1} isoprenaline (ISO). The increase in the level of the EFS-induced contractions at time point y (before administration of T) versus time point x (before administration of C) in gastric tissue (a) is indicated by dashed lines.

presence of the non-selective PDE inhibitor 3-isobutyl-1-methylxanthine (IBMX, 20 μmol·L^{-1}).

Data analysis

Electrically induced gastric contractions were expressed as means of the contraction amplitude. Atrial contractions were analysed using the peak of positive force derivative, i.e. the contraction velocity (+dF·dt^{-1}), rather that the contraction force because the latter is also dependent on the relaxation phase and a hastening of relaxation might appear as a negative inotropic effect. The average contraction to two EFS trains (stomach) or contraction velocity during 2 min (left atrium) before the addition of agonist was taken as the basal value. Responses to 5-HT, prucalopride and M0003 (i.e. increase in amplitude of the electrically induced gastric contractions, and increase in +dF·dt^{-1} for the electrically induced atrial contractions) were expressed relative to the basal value *per se* for gastric experiments or relative to the increase above the basal value caused by 0.1 mmol·L^{-1} isoprenaline for atrial experiments.

To follow the time course of the drug-induced changes in lusitropy, variations in contraction and relaxation must be considered simultaneously because changes in the contraction phase induce coordinated changes in the relaxation phase and the peak of the negative force derivative, –dF·dt^{-1}, cannot assess the isometric relaxation independently of the contraction phase. We therefore analysed the coefficient R2 = (+dF·dt^{-1})/(–dF·dt^{-1}) which tests the coupling between contraction and relaxation under high load and thus the lusitropic state under high load in a manner that is less dependent on inotropic changes (Hanouz *et al*., 2004). When the muscle contracts isometrically, sarcomeres shorten very little. Because of an increased sensitivity of myofilament for Ca^{2+} in such heavy loading conditions, the relaxation time course is mainly determined by Ca^{2+} dissociation from troponin C rather than by Ca^{2+} sequestration by the sarcoplasmic reticulum (SR) or Ca^{2+} extrusion via Na^+/Ca^{2+} exchange. Thus, R2 indirectly reflects myofilament calcium sensitivity (Mattiazzi *et al*., 1986; Hanouz *et al*., 2004).

Statistical methods

Concentration-response curves were fitted to the Hill equation using GraphPad Prism v4.02. The same software was used for statistical analysis. Unless otherwise stated, data are presented as mean ± SEM, based on results from six different animals. Comparisons between different pre-treatment groups were made using one-way ANOVA with a *post hoc* Dunnett's multiple comparison test to compare different groups against the solvent-treated tissues. Concentration dependency was tested with a *post hoc* test for a trend between mean and column number. Significance is associated with a *P*-value < 0.05. Of note, GraphPad Prism v4.02 never reports a *P*-value < 0.001 for the Dunnett's test for which *P* < 0.01 is the limit. Concentration dependency of desensitization was statistically verified *post hoc* by a test for a trend between mean and column number.

Drugs

The following drugs were used (abbreviations and respective suppliers in parentheses): 3-isobutyl-1-methylxanthine (IBMX) and L-N^G-nitro-arginine methyl ester (L-NAME; Fluka, Switzerland); propranolol HCl (Sigma, Belgium); methysergide maleate (Research Biochemical INS, USA); indomethacin (Merck Belgolabo N.V.); [1-[2-[(methylsulphonyl)amino] ethyl]-4-piperidinyl]methyl 1-methyl-1H-indole-3-carboxylate (GR 113808 ; Tocris Cookson, UK); 5-hydroxytryptamine creatinine sulphate (5-HT; Acros Chimica, Belgium); cocaine HCl, granisetron HCl, isoprenaline HCl, prucalopride HCl, 4-amino-5-chloro-2,2-dimethyl-2,3-dihydro-benzofuran-7-carboxylic acid [3-hydroxy-1-(3-methoxy-propyl)-piperidin-4ylmethyl]-amide (M0003, previously R149402) and carbachol (Johnson & Johnson Research and Development, Beerse, Belgium). All compounds were dissolved and diluted in distilled water, except for GR113808 and indomethacin. GR113808 was freshly dissolved in dimethyl sulphoxide to obtain a stock solution of 10 mmol·L^{-1}; dilutions were made with distilled water; indomethacin was dissolved in 9.1 mL distilled water supplemented with 0.9 mL 2% Na_2CO_3. These solutions were stored at –20°C.

Results

The capacity of 5-HT and the selective 5-HT$_4$ receptor agonists prucalopride and M0003 to reduce the responsiveness to 5-HT was studied in gastric muscle strips and left atrial pectinate muscles. Unless otherwise stated, experiments were performed according to protocol A, as described in the methods section.

Agonist-induced desensitization of the response to 5-HT in gastric tissue

5-HT Activation of 5-HT$_4$ receptors with 5-HT (30 nmol·L^{-1} to 1 μmol·L^{-1}) in porcine gastric muscle strips resulted in an enhancement of EFS-induced muscle contractions. The maximal increase was rapidly reached (6 min or two EFS trains) and was preserved during the 1 h exposure time. Consistent with our previous results, despite the presence of methysergide and granisetron, 5-HT also induced a tonic contraction that was previously shown to be insensitive to GR113808 (De Maeyer *et al*., 2006b). This is in line with a study of Janssen *et al*. (2002) who showed the involvement of an uncharacterized receptor and of the 5-HT$_{2A}$ receptor in the basal contractile response to 5-HT.

Washout of 5-HT returned the EFS-induced response to a level not different from that in control strips, although a tendency to a remaining increase in basal response can be observed in the strips that received 0.3 and 1 μmol·L^{-1} 5-HT (Figure 2a). Re-exposing the tissues to 3 nmol·L^{-1} 5-HT showed an apparent concentration-dependent ($P < 0.001$) loss of efficacy, coinciding with the trend in increased baseline levels. When responses were expressed relative to the initial baseline, that is, before the addition of desensitizing agent, no significant effect on efficacy could be demonstrated (Figure 2c). However, a trend towards a desensitizing response was visible.

To clarify this ambiguity, the desensitizing potential of 5-HT was analysed using protocol B (Figures 1a and 3a), in which each muscle strip first receives a control concentration of 5-HT (3 nmol·L^{-1}) followed by 1 h recovery period with extensive washing, after which the protocol continues as

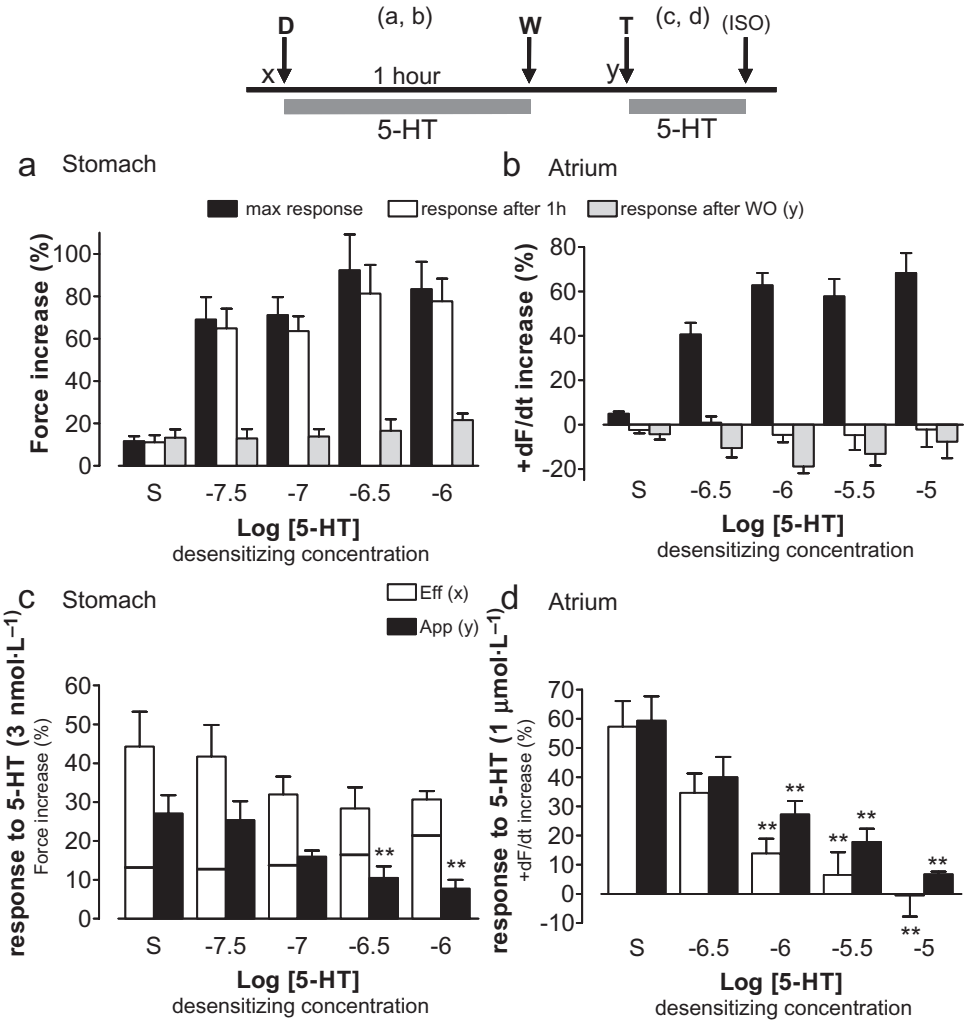

Figure 2 Desensitization of the 5-HT$_4$ receptor-mediated responses induced by 5-HT in longitudinal muscle strips of the proximal stomach (a, c) and left atrial pectinate muscles (b, d). The protocol (protocol A) of the desensitization experiment is illustrated at the top of the figure. Gastric and atrial tissues were incubated with solvent (S) or different concentrations of 5-HT for 1 h (desensitization period from D to W). The maximal effect of this treatment as well the response after 1 h, at the end of the desensitization period, is shown for stomach (a) and atrium (b). All responses are expressed relative to the basal tissue response at time point x. After 1 h, tissues were washed (W) to remove 5-HT; the remaining tissue response at time point y is shown in (a) and (b). The effect of pre-treatment with 5-HT on the response to a subsequent exposure to 5-HT (test period T: 3 nmol·L^{-1} in stomach and 1 µmol·L^{-1} in left atrium) is shown in (c) and (d). Responses were expressed relative to the baseline at time point y, named apparent desensitization (App) as well as to the baseline at time point x, named effective desensitization (Eff). In (c), the level of the baseline at time point y, which is also shown in (a), is marked by a line in the bar showing the response relative to the baseline at time point x. Atrial experiments were terminated by the administration of a supramaximal concentration of isoprenaline (0.1 mmol·L^{-1}) and responses were expressed relative to the effect caused by this β-adrenoreceptor agonist. $^{**}P < 0.01$ versus solvent treated tissues (Dunnett).

described above. In a control strip it was confirmed that after the 1 h recovery period, the response to 3 nmol·L^{-1} 5-HT was not yet affected. Within each tissue, responses to the 5-HT test application were compared with the effect of the control application (Figures 1a and 3a). This experiment confirmed that responses only apparently desensitized ($P < 0.001$) because of the maintained increase in basal response before adding the test concentration. This is also clearly demonstrated by the apparently reduced response to 5-HT in the solvent-treated strips (Figure 3a), resulting from the increase in EFS-induced contractions (drift) during the desensitization period with solvent (Figures 1a and 2a).

In order to maximize comparability with the atrial experiments, we also performed the above-described experiments with 10 µmol·L^{-1} 5-HT as the desensitizing concentration. The experiments with protocol A showed that 10 µmol·L^{-1} 5-HT increased the EFS-induced contractions above the maximum of the previously obtained concentration-response curve (87.1 ± 8.1%; De Maeyer *et al.*, 2006b). Also in strips that only weakly responded to EFS (<2 g), 5-HT induced a robust increase in the EFS-induced response, resulting in a large variation when the responses were expressed as % increase, ranging from 76% to 1019% with a median value of 242% ($n = 11$ tissues from three animals); this response did not fade during the 1 h exposure. The 5-HT$_4$ receptor antagonist GR113808 (even in a concentration of 3 µmol·L^{-1}) was unable to affect this increased response when administered 1 h after the administration of 5-HT. This is in contrast with the results obtained for all 5-HT

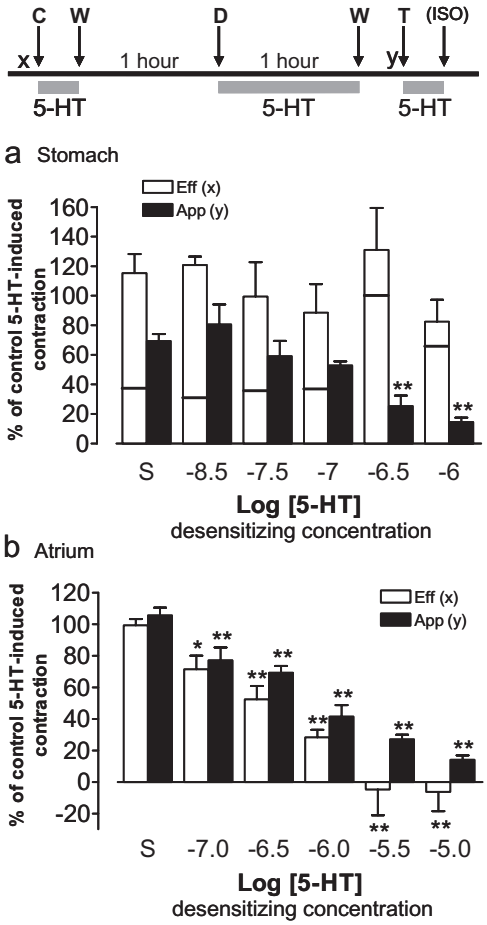

Figure 3 Desensitization of the 5-HT$_4$ receptor-mediated responses induced by 5-HT in longitudinal muscle strips of the proximal stomach (a) and left atrial pectinate muscles (b). The protocol (protocol B) of the desensitization experiment is illustrated at the top of the figure. Gastric and atrial tissues were challenged with 3 nmol·L^{-1} or 1 μmol·L^{-1} 5-HT (control period C to W) respectively followed by 1 h recovery period with extensive washing. Next, tissues were pre-incubated with solvent (S) or different concentrations of 5-HT for 1 h (desensitization period D to W). After washing, the effect to 3 nmol·L^{-1} or 1 μmol·L^{-1} 5-HT (test period T) respectively was assessed. The response was expressed as a percentage of the 5-HT-induced contractions in the same tissue in the absence of any desensitization, that is, during the control period (taken as 100%). Responses were expressed relative to the baseline at time point y, named apparent desensitization (App) as well as to the baseline at time point x, named effective desensitization (Eff). In (a), the level of the baseline at time point y is marked in the effective desensitization bar (also expressed as a percentage of the maximal 5-HT-induced contraction during the control period). Atrial experiments were terminated by the administration of a supramaximal concentration of isoprenaline (0.1 mmol·L^{-1}) and responses were expressed relative to its effect. The maximal contraction induced by 5-HT in the control period (C to W) was 39 ± 2% (contraction force increase) and 56 ± 3% (maximal contraction velocity increase relative to the increase caused by isoprenaline) in gastric and atrial tissue respectively. *$P < 0.05$, **$P < 0.01$ versus solvent treated tissues (Dunnett).

concentrations up to 1 μmol·L^{-1} 5-HT which were all at least partially reversed by 0.1 μmol·L^{-1} GR113808 (previous experiments, results not shown). This result greatly hampers interpretation of the results as well as comparison with the other desensitizing concentrations of 5-HT, because other than 5-HT$_4$ receptors appear to be involved.

Prucalopride and M0003

Prucalopride and M0003 concentration-dependently increased the EFS-induced cholinergic contraction, an effect that was sustained for 1 h and, in contrast to 5-HT, after removal of the agonists by three washes (Figures 4a and 5a). Consequently, 15 min after ending the desensitizing exposure, control tissues displayed smaller contractions than tissues pre-treated with prucalopride or M0003. Application of GR113808 (0.1 μmol·L^{-1}) before washing reversed the effects induced by the agonists (illustrated in Figure 6 for prucalopride). In strips pre-treated with the agonists, application of 3 nmol·L^{-1} 5-HT, 15 min after the wash step, induced only a limited additional increase of the contraction force, decreasing with higher pre-treatment concentrations of the agonist ($P < 0.001$; Figures 4c and 5c). However, when these responses were expressed relative to baseline contractions before the desensitization period, responses were constant (Figures 4c and 5c), or even increased (after 3 nmol·L^{-1} of M0003; Figure 5c). These observations thus indicate that the potentiating effects of prucalopride and M0003 on electrically induced contractions are wash-resistant and that the apparent reduced response to 5-HT is a reflection of this wash resistance. This is only reflected by the apparently reduced response to 5-HT in solvent-treated tissues.

Agonist-induced desensitization of the response to 5-HT in atrial tissue

5-HT As previously described (De Maeyer *et al*., 2006a), 5-HT induced a transient increase in the electrically induced contractile response in atria. After reaching a maximum within 2 min, the atrial contractile response rapidly returned to basal (Figure 2b). When the strips were subsequently exposed to 1 μmol·L^{-1} 5-HT, a loss of efficacy was observed dependent on the preceding desensitizing 5-HT concentration ($P < 0.001$) and irrespective of the chosen baseline value (Figure 2d). The pDC$_{50}$ (negative logarithm of the concentration at which the response was reduced by 50%) was 6.2 ± 0.2. As with the gastric preparations, these results were confirmed in atria by experiments performed according to protocol B (Figures 1b and 3b). Thus, after the 1 h recovery period upon washout of the 5-HT control (C) concentration, the response to 1 μmol·L^{-1} 5-HT was not yet affected (middle tracing of Figure 1b). The pDC$_{50}$ in this protocol was 6.3 ± 0.2.

To elucidate whether the diminishing response to a fixed concentration of 5-HT upon pre-incubation of left atrial trabeculae with 5-HT was due to an effect on potency, efficacy or both, a full concentration-response curve to 5-HT was elicited during the test period (protocol C). Pre-treatment with 1, 3 and 10 μmol·L^{-1} 5-HT caused a reduction of the maximum as well as a rightward shift of the bell-shaped concentration-response curve (Figure 7c,e; Table 1). Similar results were obtained when the experiments were conducted in the presence of 20 μmol·L^{-1} IBMX to assess the desensitizing potential of 5-HT in the absence of any involvement of PDEs: a rightward shift of the (non-bell-shaped) concentration-response curve as well as a reduction of the maximal response after pre-treatment with 1–10 μmol·L^{-1} 5-HT (Figure 7d,f; Table 1). Interestingly, in the presence of IBMX, 5-HT responses showed a concentration-dependent wash-resistance (compare

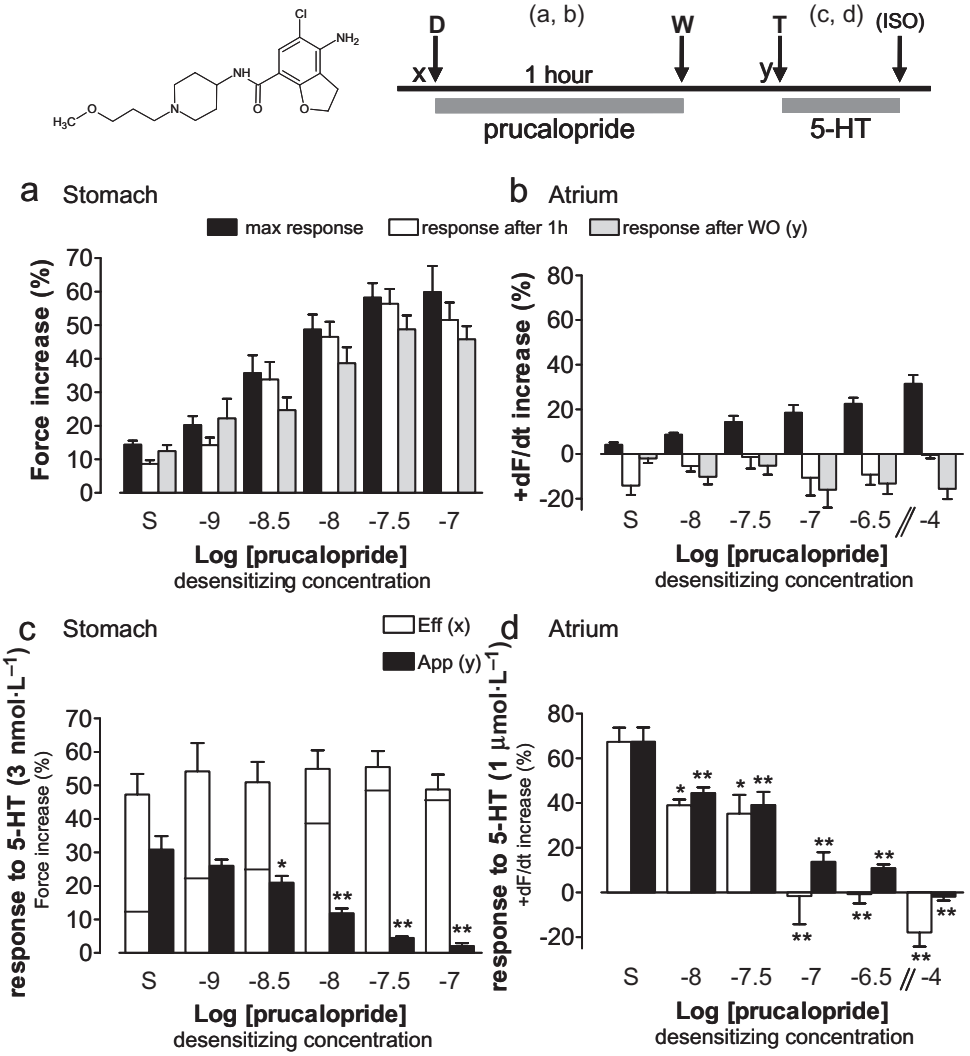

Figure 4 Desensitization of the 5-HT$_4$ receptor-mediated responses induced by prucalopride in longitudinal muscle strips of the proximal stomach (a, c) and left atrial pectinate muscles (b, d). The chemical structure of prucalopride and the protocol of the desensitization experiment are illustrated at the top of the figure. Further explanation is as for Figure 2 except that prucalopride was used instead of 5-HT during the desensitization period. *$P < 0.05$, **$P < 0.01$ versus solvent treated tissues (Dunnett).

the 30 min response in Figure 7b with baseline responses in Figure 7d; $P < 0.05$ for 0.1 µmol·L^{-1} 5-HT and $P < 0.01$ for 1 µmol·L^{-1} 5-HT). Therefore, the effect on the maximal response largely depends on the representation of the data (Figure 7d,f). GR113808 was able to reverse the maintained response to 5-HT (results not shown). In the experiments with IBMX, IBMX by itself increased the inotropic response from 295 ± 18 mN·s^{-1} to 421 ± 27 mN·s^{-1} ($n = 53$, $P < 0.0001$), and responses were measured relative to this increased baseline.

Prucalopride and M0003

Prucalopride exerted positive inotropic effects on the atrial preparations, which were considerably weaker than those induced by 5-HT (Figure 4b). No significant effect on the atrial contraction force was observed with M0003 (Figure 5b). However, despite the weak inotropic response, both compounds had a conditioning influence on the tissue as after their removal by repeatedly changing the bath solution, the response to 1 µmol·L^{-1} 5-HT was diminished, the degree of decrease correlating with the pre-treatment concentration ($P < 0.001$; Figure 4d and 5d). The pDC$_{50}$ for the cross-desensitization of the 5-HT response was 7.5 ± 0.1 and 8.4 ± 0.1 for prucalopride and M0003 respectively.

Lusitropy and variation of R2 ratios

In contrast to the transient positive inotropic response, activation of atrial 5-HT$_4$ receptors by the desensitizing substances (5-HT, prucalopride and M0003) resulted in a sustained lusitropic response (results not shown), that is, a larger effect on the relaxation phase than on the contraction phase of the contraction-relaxation cycle and hence a decrease in R2. After washout, the lusitropic state of the tissues remained elevated, as illustrated by the sustained significant decrease of the contraction-relaxation coupling parameter R2 (Figure 8).

In experiments with IBMX, IBMX itself caused a reduction of R2 from 1.39 ± 0.02 to 1.28 ± 0.02 ($n = 51$, $P < 0.0001$). As

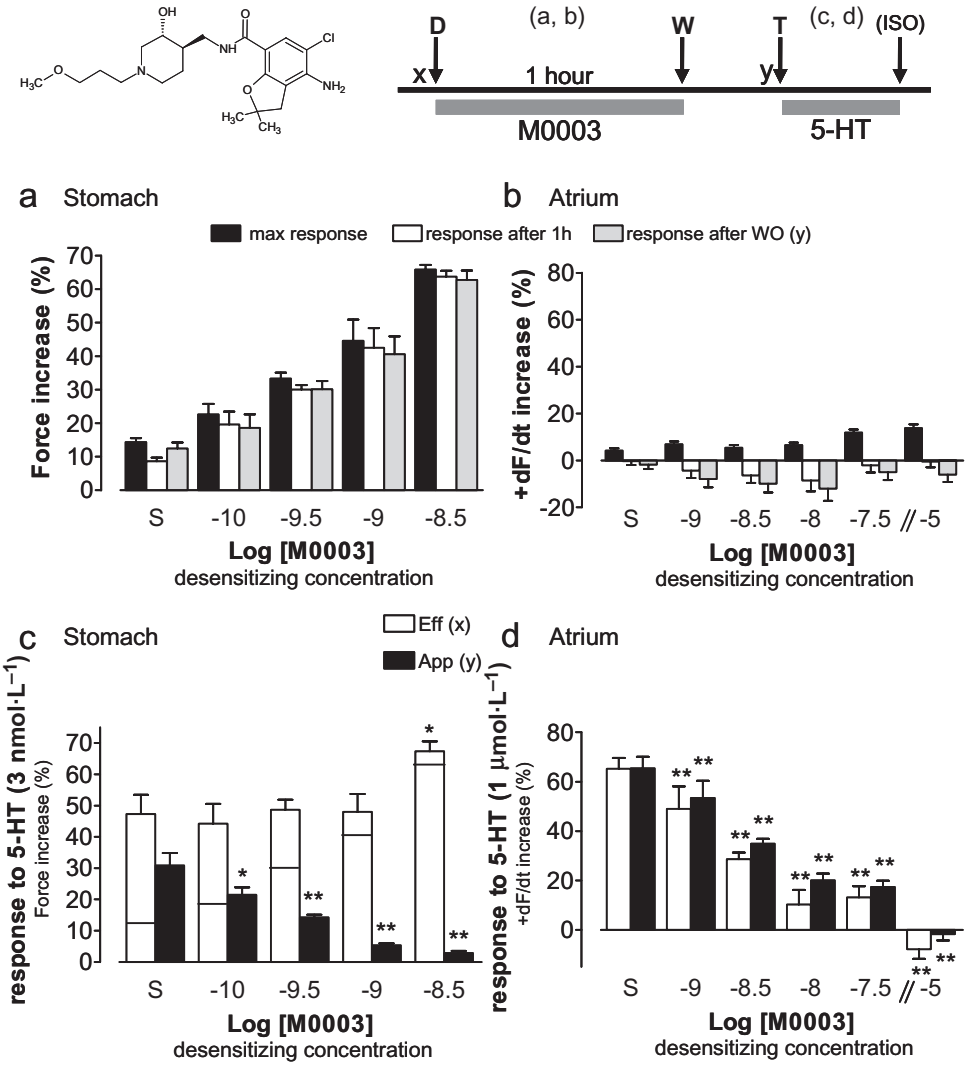

Figure 5 Desensitization of the 5-HT$_4$ receptor-mediated responses induced by M0003 in longitudinal muscle strips of the proximal stomach (a, c) and left atrial pectinate muscles (b, d). The chemical structure of M0003 and the protocol (protocol A) of the desensitization experiment are illustrated at the top of the figure. Further explanation is as for Figure 2 except that M0003 was used in stead of 5-HT during the desensitization period. *$P < 0.05$, **$P < 0.01$ versus solvent treated tissues (Dunnett).

also previously reported (De Maeyer *et al.*, 2006a), under this condition no further decrease of R2 occurred after the addition of 5-HT, or prucalopride. Actually, R2 rather increased upon addition of a high concentration of 5-HT or prucalopride in the presence of IBMX. This was also observed after the addition of isoprenaline at the end of the protocol (results not shown).

Discussion and conclusions

The progression and desensitization of 5-HT$_4$ receptor-mediated responses were evaluated in two clinically relevant *in vitro* models: a porcine GI model of cholinergic neurotransmission (De Maeyer *et al.*, 2006b), and the porcine atrium. Summarized, our results show that exposure of the gastric tissue to 5-HT or selective 5-HT$_4$ receptor agonists results in a sustained, non-transient effect during exposure; after washout this does not result in a clear desensitization of the response to a subsequent challenge with 5-HT. Incubation of left atrial tissue with 5-HT results in a transient response, after washout leading to a potent desensitization of the subsequent response to 5-HT. The selective 5-HT$_4$ receptor agonists prucalopride and M0003 induced only very weak atrial responses but appeared very effective in desensitizing the response to 5-HT. Our observations also suggest that the properties of prucalopride and M0003 to bind and activate the 5-HT$_4$ receptor differ from those of 5-HT, and this might have contributed to the observed desensitization.

Stomach

In the gastric muscle preparations, 5-HT$_4$ receptors are presumably located presynaptically on myenteric cholinergic nerves (De Maeyer *et al.*, 2006b). By facilitating the release of acetylcholine, their activation results in a reinforcement of muscle contraction through activation of M_3 receptors on the smooth muscle cells (Leclere and Lefebvre, 2002b). 5-HT,

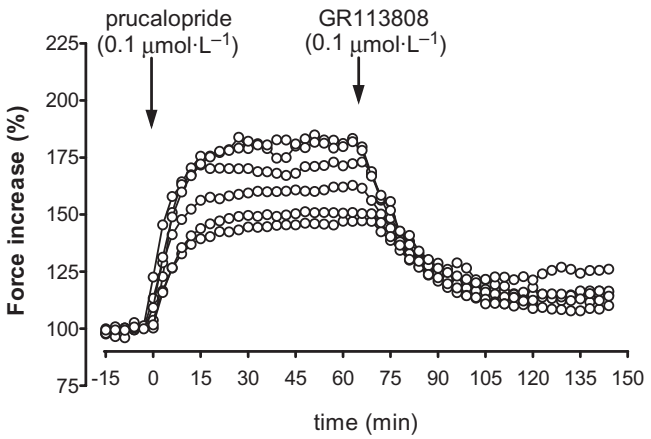

Figure 6 The effect of 0.1 μmol·L^{-1} prucalopride on the EFS-induced contractions of longitudinal muscle strips of the proximal stomach. GR113808 (0.1 μmol·L^{-1}) was added in the presence of prucalopride. Data points represent six individual experiments and are connected by a line, for each tissue. Experiments were performed in the presence of L-NAME (0.1 mmol·L^{-1}) and indomethacin (1 μmol·L^{-1}) and methysergide (1 μmol·L^{-1}). Similar results were obtained with M0003.

prucalopride and M0003 all persistently increased the cholinergic contraction of the gastric muscle strips, confirming our previous observations (De Maeyer *et al.*, 2006b). This suggests either the absence of receptor desensitization or the sustained activation of a mechanism downstream of the receptor. In the latter scenario, the 5-HT$_4$ receptors themselves could well be desensitized while the response initiated by their activation is not. Indeed, in spite of a rapidly desensitizing adenylyl cyclase response to 5-HT$_4$ receptor activation in mouse colliculi neurons (Ansanay *et al.*, 1992), their activation does result in long-term effects. In these cells, a 1 min application and washout of 5-HT causes a 5-HT$_4$ receptor-mediated transient protein kinase A (PKA) response and a long-lasting inhibition of phosphatases resulting in a progressive and long-lasting blockade of K^+ channels (Ansanay *et al.*, 1995). Similarly, the effect of prucalopride appeared to be wash-resistant in rat hippocampal slices (Spencer *et al.*, 2004). In our experiments, the effects mediated by prucalopride and M0003 were also resistant to drug washout, theoretically corresponding with the possibility of sustained facilitation of transmitter release by these two drugs via a mechanism downstream of the receptor. However, two observations cast doubt on the validity of this hypothesis in the gastric preparations. First, the phenomenon is absent for 5-HT as the effects induced by 5-HT were reversed after washout of the drug (regardless of a small trend for 0.3 and 1 μmol·L^{-1} 5-HT). Second, the 5-HT$_4$ receptor antagonist GR113808 was able to nullify the sensitized cholinergic contractions before and after (results not shown) washout of the drugs, suggesting that the receptors are still active. In colliculi neurons, the effect of 5-HT was abolished by pre-incubation with a 5-HT$_4$ receptor antagonist, but its effect was not reversed when the antagonist was applied during washout of the drug (Ansanay *et al.*, 1995). In our experiments, an activated state of the receptor is thus required to sustain the response and no long-term effect on a downstream effector has occurred. There may be a mechanistic analogy with the colon because Cellek *et al.* (2006) have described the non-transient effect of prucalopride on cholinergic contractions and nitrergic relaxations in the human colon, both effects being reversed upon administration of a 5-HT$_4$ receptor antagonist. The observation that application of 5-HT, 15 min after a conditioning application of 5-HT, prucalopride or M0003 had an additional facilitatory effect on the EFS-induced contractions resulting in similar EFS-induced contractions as in solvent-treated strips also argues against the occurrence of desensitization; in other words, the maximal response was always reached, whatever the level of the basal value. It is also possible that because of the high receptor reserve for 5-HT in this preparation (De Maeyer *et al.*, 2006b), 5-HT$_4$ receptor desensitization has only moderate effects on the agonist-induced responses. Similarly, we cannot exclude the possibility that receptors already have been (partially) resensitized, because in colliculi neurons and CHO cells, partial recovery of the 5-HT$_4$ receptor-induced cAMP production was already observed after 30 min (Ansanay *et al.*, 1992; Mialet *et al.*, 2003); because of the high receptor reserve for 5-HT in the stomach, only part of the receptors need to be operational again to achieve the same tissue response.

The experiments with 10 μmol·L^{-1} 5-HT show that at this concentration, a receptor that is resistant to high concentrations of GR113808 is involved in the EFS-induced responses. Because our experiments were performed in the presence of granisetron and methysergide, 10 μmol·L^{-1} 5-HT either surmounts antagonism by these antagonists, or an unknown 5-HT receptor is involved. The tonic contractile response to 5-HT in the same preparation as used in this study has previously been shown to be mediated by 5-HT$_{2A}$ and a receptor that could not be characterized (Janssen *et al.*, 2002).

It appears that prucalopride and M0003 are unique 5-HT$_4$ receptor agonists in that they interact with the receptor both in an antagonist-reversible but wash-resistant manner. Although we cannot exclude the possibility that the compounds partition into biological membranes, resulting in the development of a substantial depot of agonist that exhibits relative resistance to washing, we question this explanation because the hydrophobicity of prucalopride, M0003 and 5-HT is comparable (data on file, Movetis NV). Interestingly, the persistent nature of the interaction of the two benzofurancarboxamides, prucalopride and M0003, shows many similarities to xanomeline, a muscarinic M_1 acetylcholine receptor (mAChR) agonist (Christopoulos *et al.*, 1998; De Lorme *et al.*, 2007): (i) resistance to extensive washout and persistent agonistic effect; (ii) concentration dependency of this effect; and (iii) susceptibility to reversal by the addition of the antagonist atropine. For xanomeline, a model was proposed that incorporated two possible modes of interaction for this ligand with the M_1 receptor (Christopoulos *et al.*, 1998). The first mode involves a reversible, syntopic interaction with the classic binding site on the receptor, shared by other agonists or antagonists. The second mode involves the subsequent development of a persistent attachment with specific receptor regions that allowed xanomeline to continue to activate the M_1 mAChR via the classic binding site but did not allow it to be readily removed from the receptor compartment. In contrast to 5-HT, prucalopride and M0003 have the structural features to interact in a hydrophobic binding pocket on the 5-HT$_4$ receptor (Rivail *et al.*, 2004; De Maeyer *et al.*, 2008).

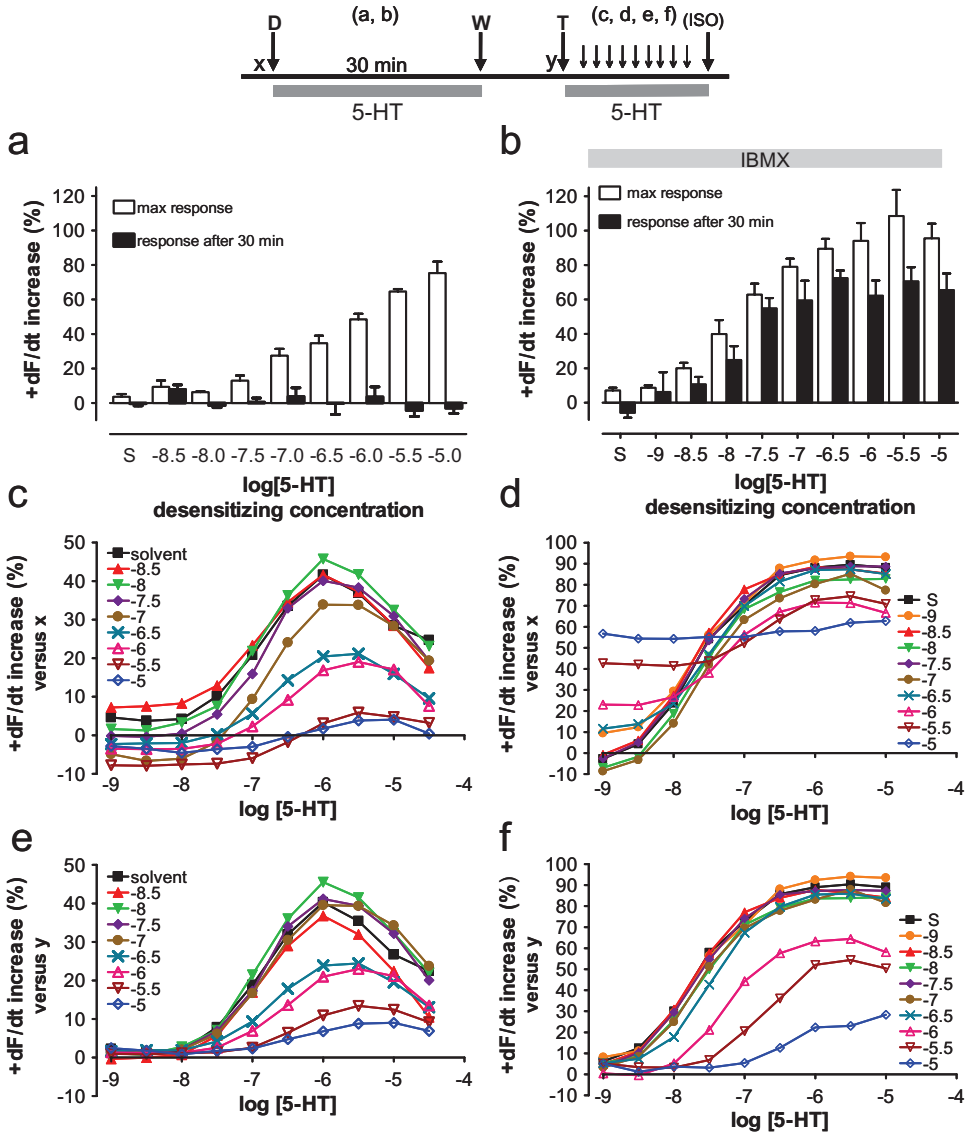

Figure 7 Desensitization of the 5-HT$_4$ receptor-mediated responses induced by 5-HT in left atrial pectinate muscles (c, d, e, f). The protocol (protocol C) of the desensitization experiment is illustrated at the top of the figure. Panels on the right (b, d, f) represent experiments in the presence of IBMX. Atrial tissues were incubated with different concentrations of 5-HT for 30 min (desensitization period from D to W). The time course of the 5-HT-induced effects in the atrium is shown in (a). After 30 min, tissues were washed (W) to remove 5-HT and a concentration-response curve to 5-HT was established. Responses were expressed relative to the baseline at time point x (c, d) or at time point y (e, f). Atrial experiments were terminated by the administration of a supramaximal concentration of isoprenaline (0.1 mmol·L^{-1}) and responses were expressed relative to the effect caused by this β-adrenoreceptor agonist.

This pocket might fulfil the role of the wash-resistant binding site. However, whether prucalopride and M0003 do, indeed, interact with the 5-HT$_4$ receptor in a way similar to that of xanomeline with M$_1$ receptors, needs further investigation.

Atrium

In order to evaluate the long-term off-target effects of 5-HT, prucalopride and M0003, these compounds were also studied in the porcine left atrium. Next to human, the pig is one of the few animals expressing functional 5-HT$_4$ receptors in the heart. In contrast to the response in gastric tissue, the increase in contraction force upon application of 5-HT rapidly faded and was not reproducible after washout. Because we only monitored changes in the EFS-induced contraction-relaxation cycle, we cannot deduce the exact underlying mechanism of this response desensitization. Several mechanisms might be involved, each contributing to the observed diminishing response and these mechanisms will be discussed below.

We previously described that the transient nature of the influence of 5-HT on electrically induced atrial contractions does not result from ligand breakdown but involves the recruitment of PDEs, limiting the magnitude and duration of cAMP signals elicited by a 5-HT$_4$ receptor agonist in the atrial tissue (De Maeyer *et al.*, 2006a). In a recent abstract, using paced left atrium of newborn piglets, Vargas *et al.* (2006) showed that after a 2 min incubation period with 10 μmol·L^{-1} 5-HT, left atrial contraction force was increased but not the

Table 1 Curve parameters for concentration-effect curves of the left atrial inotropic effect of 5-HT upon pre-treatment with different concentrations of 5-HT

Desensitizing 5-HT concentration	*No IBMX*		*IBMX*	
	pEC$_{50}$	*Maximal effect*	*pEC$_{50}$*	*Maximal effect*
Solvent	6.98 ± 0.05	38.8 ± 5.0	7.72 ± 0.13	90.2 ± 4.7
1 nmol·L^{-1}	nd	nd	7.64 ± 0.14	94.4 ± 7.1
3 nmol·L^{-1}	6.95 ± 0.14	34.7 ± 6.1	7.75 ± 0.14	87.2 ± 5.9
10 nmol·L^{-1}	6.98 ± 0.07	44.8 ± 4.0	7.69 ± 0.12	83.7 ± 1.8
30 nmol·L^{-1}	6.92 ± 0.05	41.6 ± 4.3	7.67 ± 0.09	89.5 ± 8.2
0.1 μmol·L^{-1}	6.84 ± 0.08	41.15 ± 5.5	7.59 ± 0.14	86.2 ± 3.8
0.3 μmol·L^{-1}	6.83 ± 0.04	25.5 ± 3.1	7.48 ± 0.04	86.5 ± 5.6
1 μmol·L^{-1}	6.64 ± 0.06*	23.2 ± 2.8*	7.23 ± 0.09*	64.3 ± 3.8*
3 μmol·L^{-1}	6.47 ± 0.08**	13.6 ± 1.4**	6.74 ± 0.09**	56.1 ± 9.8**
10 μmol·L^{-1}	6.54 ± 0.15**	9.90 ± 2.1**	6.55 ± 0.18**	33.7 ± 9**

The data used for fitting were those expressed relative to the baseline at time point y (see Figure 7). Data were obtained using tissues from six animals and are presented as mean ± SEM ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$ versus solvent-treated tissues.
nd: not determined.

(global) left atrial cAMP levels. After 20 min, the inotropic response had faded while cAMP levels were markedly increased. In the presence of PDE3 and PDE4 inhibitors, the inotropic response as well as the tissue cAMP levels were increased after 2 min and still increased after 20 min. This confirms the involvement of PDEs in the fading response and suggests that PDEs contribute to the compartmentation of the 5-HT$_4$ receptor-mediated cAMP signalling pathway in the left atrium by limiting the diffusion of the second messenger. PDE activation might provide a framework for a negative feedback that controls global cAMP homeostasis beneath the membrane. The inhibition of PDEs therefore generates a condition that is very different from the physiological situation, with no control over subsarcolemal cAMP levels (Fischmeister *et al.*, 2006).

Still, it is conceivable that the 5-HT$_4$ receptor-mediated inotropic response is affected not only by PDE recruitment, but also by receptor desensitization (inactivation), by analogy with β_2-adrenoreceptors for which the transient cAMP response in HEK-293 cells could be explained by a GRK-β-arrestin-mediated receptor inactivation as well as a PKA-mediated induction of PDE activity (Rochais *et al.*, 2004; Violin *et al.*, 2008). In recombinant systems, 5-HT$_4$ receptor activation has indeed been shown to be followed by rapid receptor desensitization through a process involving GRK and β-arrestin (Barthet *et al.*, 2005; Ponimaskin *et al.*, 2005). The results obtained in protocol C showed that the pEC$_{50}$ and the maximal response of the concentration-response curve for the inotropic effect of 5-HT were decreased after pre-treatment with 5-HT, also in the presence of the PDE inhibitor IBMX. This also implies that the decreased inotropic response upon repeated administration of 5-HT is not a purely PDE-mediated phenomenon, although inhibition of PDE activity by IBMX might not be complete.

The expression level of 5-HT$_4$ receptors in the atrium is very low, i.e. ~0.3 and ~4 fmol·mg^{-1} protein in newborn piglets and adult man, respectively (Kaumann *et al.*, 1995; 1996), compared with ~ 225 fmol·mg^{-1} protein in human brain (Domenech *et al.*, 1994). Additionally, in contrast to gastric tissue, there is no 5-HT$_4$ receptor reserve for 5-HT in atrial tissue (De Maeyer *et al.*, 2006b). This might explain why, in atria in contrast to gastric tissue, there is a robust desensitization of the response to 5-HT.

We do acknowledge two additional factors that might have added to the observed diminishing response to 5-HT. One factor is the incomplete washout of prucalopride and M0003. As described above, our data obtained with gastric tissue indeed suggest an antagonist-reversible but wash-resistant receptor interaction of these agonists. Both agonists, as well as GR113808, have the structural features to interact in a hydrophobic binding pocket on the 5-HT$_4$ receptor, putatively fulfilling the role of a wash-resistant binding site. In the atrium, the response to these agonists already fades during the exposure period because of PDE recruitment and this could hide the fact that they are still present after the washing step. If the agonists are bound in a wash-resistant way, they could act as an antagonist for subsequently administered 5-HT; antagonism of 5-HT was previously shown for prucalopride at human and porcine atrial 5-HT$_4$ receptors (Krobert *et al.*, 2005). Beyond these considerations, because the agonists are still bound to the receptor after the washing step, PDE recruitment would persist at the time of 5-HT administration. In the experiments with 5-HT itself as desensitizing agent in the presence of IBMX, the 5-HT-induced response was maintained during the desensitizing exposure period and after washout, theoretically suggesting that wash-resistant binding might also occur for 5-HT. We are, however, not aware of any reports on wash-resistant binding of 5-HT to the 5-HT$_4$ receptor.

The second contributing factor can be found in the activation of the lusitropic apparatus. We previously demonstrated that 5-HT$_4$ receptor activation caused a non-transient lusitropic effect which is resistant to inhibition by PDEs (De Maeyer *et al.*, 2006a). We now show that this enhancement of cardiac diastolic muscle relaxation is also resistant to drug washout. The effect of 5-HT$_4$ stimulation in accelerating relaxation is believed to involve a combination of an increased rate of SR Ca^{2+} uptake due to phospholamban (PLB) phosphorylation, and a reduction in myofilament Ca^{2+} sensitivity and an increase in cross-bridge cycling rate due to troponin I (TnI) phosphorylation (Li *et al.*, 2000; Birkeland *et al.*, 2007). Our

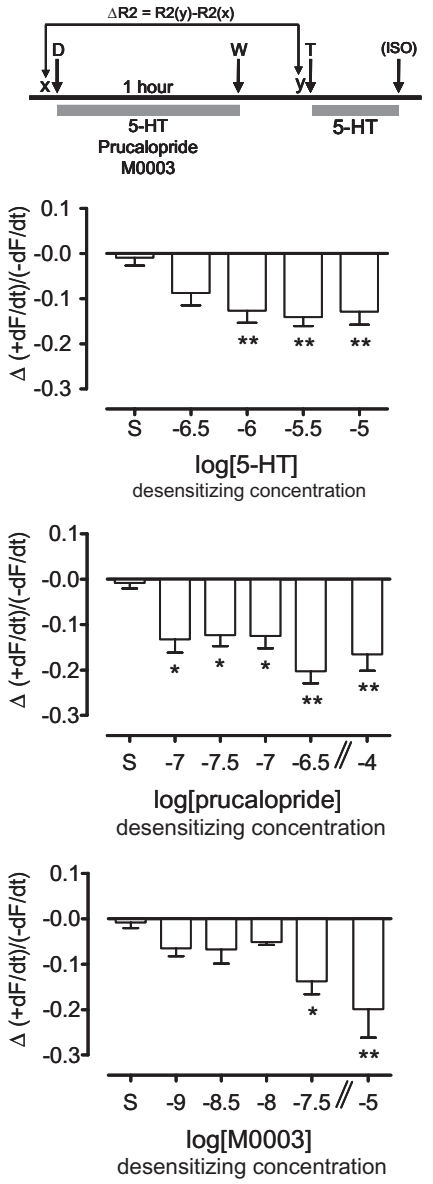

Figure 8 Concentration-dependent decrease of R_2, shown as $\Delta(+dF{\cdot}dt^{-1})/(-dF{\cdot}dt^{-1})$, measured after washout of 5-HT, prucalopride or M0003 following a 1 h incubation with these compounds. On average, the basal value of R_2 was 1.41 ± 0.04 (n = 30), 1.46 ± 0.04 (n = 36) and 1.44 ± 0.03 (n = 36) in the experiments with 5-HT, prucalopride and M0003 respectively. Data were analysed using one-way ANOVA with Dunnett's *post hoc* test (*P < 0.05, **P < 0.01 versus solvent-treated tissues). For all compounds, a significant trend between the effect and desensitizing concentration was found (P < 0.001).

results suggest that neither PDEs nor drug washout interferes with the different intracellular processes involved in the positive lusitropic effect of 5-HT$_4$ receptor stimulation. This can be explained by the slow dephosphorylation rates for PLB and TnI (Garvey *et al.*, 1988). The relative contribution of both proteins to the lusitropic effect is highly dependent on the loading conditions (Li *et al.*, 2000). It has been suggested that under isometric conditions, because of an increased sensitivity of myofilament for Ca^{2+} in such heavy loading conditions, the relaxation time course is mainly determined by Ca^{2+} dissociating from troponin C, and that the lusitropic coefficient R2 indirectly reflects myofilament Ca^{2+} sensitivity (Hanouz *et al.*, 1998). A primary role for TnI is indirectly supported by our results because the lusitropic response was still maintained after fade of the inotropic response. Because phosphorylation of PLB also has an inotropic effect by increasing Ca^{2+} load of the SR (Birkeland *et al.*, 2007), one would not expect fading to basal values. The situation might be different when blocking PDEs, because this disrupts spatially compartmentalized cAMP production (Rochais *et al.*, 2004). The involvement of long-term phosphorylation of PLB in the wash-resistant positive inotropic effect of high concentrations of 5-HT in the presence of IBMX can be ruled out by the ability of GR113808 to reverse this maintained response to 5-HT (preliminary results not shown). The long-term activation of the lusitropic machinery implies that the effect of 5-HT$_4$ receptor activation on the subsequent response to 5-HT was assessed under conditions of reduced Ca^{2+} sensitivity of the myofilaments, which might in part explain the desensitized inotropic responses to 5-HT.

Because the inotropic effects of prucalopride and M0003 were very small, these compounds allowed us to examine further the desensitization properties of left atrial 5-HT$_4$ receptor-mediated responses. For many GPCRs there is a good correlation between agonist efficacy and the ability to induce receptor desensitization (Clark *et al.*, 1999). However, pre-treatment with prucalopride or M0003 very effectively blunted the response to 5-HT, arguing against this generalization. This finding can be interpreted in three ways. First, in colliculi neurons, a good correlation was found between the potencies of several 5-HT$_4$ receptor agonists and their abilities to desensitize the adenylyl cyclase response (Ansanay *et al.*, 1992). According to the authors, the dependence on potency, and not on efficacy, reflects the influence of the agonists' binding affinity or its mean occupancy time of the receptor. Our results fit into this theory because pre-treatment with prucalopride or M0003 very effectively blunted the response to 5-HT with a potency proportional to their 5-HT$_4$ receptor affinity. Second, the correlation with affinity is also in accordance with antagonism by residually bound agonist (see above). Finally, this can also be interpreted in light of 'functional selectivity'. The idea that different agonists are able to produce different response profiles by stabilizing different receptor conformations and activating different signal transduction pathways or mechanisms of desensitization is extending (Kelly *et al.*, 2008). This has clearly been shown for the μ-opioid receptor for which it has been demonstrated that different agonists induce different mechanisms of μ-opioid receptor desensitization (Kelly *et al.*, 2008).

In conclusion, our results show a clear-cut difference between the desensitization of the 5-HT$_4$ receptor-mediated responses in stomach and left atrium. In the stomach, the responses were only apparently desensitized while in the atrium the responses to 5-HT were clearly not reproducible. In addition to the efficacy differences of 5-HT$_4$ receptor agonists such as prucalopride and M0003 in stomach versus atrium (De Maeyer *et al.*, 2006b), the phenomenon might contribute to tissue selectivity of 5-HT$_4$ receptor agonists.

Acknowledgements

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Part of the experimental work was performed at the Department of GI Pharmacology, Johnson & Johnson Pharmaceutical Research and Development, Beerse, Belgium.

Conflicts of interest

Prucalopride and M0003 are in the portfolio of Movetis NV. J. De Maeyer and J. Schuurkes are employees of Movetis.

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