Canadian Journal of Physiology and Pharmacology
Revue canadienne de physiologie et pharmacologie

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Journal:	<i>Canadian Journal of Physiology and Pharmacology</i>
Manuscript ID	cjpp-2017-0191.R2
Manuscript Type:	Article
Date Submitted by the Author:	14-Jul-2017
Complete List of Authors:	García-Pedraza, José; Cinvestav-Coapa, Pharmacobiology; Laboratory of Pharmacology. Department of Physiology and Pharmacology, Faculty of Pharmacy. University of Salamanca. Biomedical Research Institute of Salamanca (IBSAL). Hernández-Abreu, Oswaldo; Cinvestav-Coapa, Pharmacobiology García, Mónica; Laboratory of Pharmacology. Department of Physiology and Pharmacology, Faculty of Pharmacy. University of Salamanca. Biomedical Research Institute of Salamanca (IBSAL). Morán, Asunción; Laboratory of Pharmacology. Department of Physiology and Pharmacology, Faculty of Pharmacy. University of Salamanca. Biomedical Research Institute of Salamanca (IBSAL). Villalón, Carlos; Cinvestav-Coapa, Pharmacobiology
Is the invited manuscript for consideration in a Special Issue?:	N/A
Keyword:	5-HT _{1F} receptor, 5-HT ₂ receptor, Cardiac sympatho-inhibition, Sarpogrelate, Tachycardia

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Chronic 5-HT₂ receptor blockade unmasks the role of 5-HT_{1F} receptors in the inhibition of the rat cardioaccelerator sympathetic outflow

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Abstract

Serotonin (5-hydroxytryptamine; 5-HT) inhibits the rat cardioaccelerator sympathetic outflow by 5-HT_{1B/1D/5} receptors. Since chronic blockade of sympatho-excitatory 5-HT₂ receptors is beneficial in several cardiovascular pathologies, this study investigated whether sarpogrelate (a 5-HT₂ receptor antagonist) alters the pharmacological profile of the above sympatho-inhibition. Rats were pretreated during 2 weeks with sarpogrelate in drinking water (30 mg/kg.day; sarpogrelate-treated group) or equivalent volumes of drinking water (control group). Animals were pithed and prepared for spinal stimulation (C₇-T₁) of the cardioaccelerator sympathetic outflow or for intravenous (i.v.) bolus injections of noradrenaline. Both procedures produced tachycardic responses remaining unaltered after saline. I.v. continuous infusions of 5-HT induced a cardiac sympatho-inhibition that was mimicked by the 5-HT receptor agonists 5-carboxamidotryptamine (5-CT; 5-HT_{1/5A}), CP 93,129 (5-HT_{1B}) or PNU 142633 (5-HT_{1D}), but not by indorenate (5-HT_{1A}) in both groups; whereas LY344864 (5-HT_{1F}) mimicked 5-HT only in sarpogrelate-treated rats. In sarpogrelate-treated animals, i.v. GR 127935 (310 µg/kg; 5-HT_{1B/1D/1F} receptor antagonist) attenuated 5-CT- and abolished LY344864-induced sympatho-inhibition; while GR 127935 plus SB 699551 (1 mg/kg; 5-HT_{5A} receptor antagonist) abolished 5-CT-induced inhibition. These results confirm the cardiac sympatho-inhibitory role of 5-HT_{1B}, 5-HT_{1D} and 5-HT_{5A} receptors in both groups; nevertheless, sarpogrelate treatment specifically unmasked a cardiac sympatho-inhibition mediated by 5-HT_{1F} receptors.

Key words: 5-HT_{1F} receptor; 5-HT₂ receptor; cardiac sympatho-inhibition; sarpogrelate; tachycardia.

Introduction

The cardiovascular responses produced by serotonin (5-hydroxytryptamine; 5-HT) involve complex interactions with different receptors at several levels. These include the central nervous system; autonomic ganglia and postganglionic nerve endings; dorsal root ganglia and primary sensory nerves; as well as the heart and blood vessels (Kaumann and Levy 2006; Villalón and Centurión 2007; González-Hernández *et al.* 2011; Watts *et al.* 2012). Depending on the species and the experimental conditions, these responses are mainly mediated by 5-HT₁, 5-HT₂, 5-HT₃, 5-HT₄, putative 5-HT_{5A}/5-HT_{5B} and 5-HT₇ receptors, as well as by a tyramine-like action or unidentified mechanisms (Kaumann and Levy 2006; Villalón and Centurión 2007; Watts *et al.* 2012). Curiously, the 5-HT₆ receptor is not involved (neither centrally nor peripherally) in cardiovascular regulation (Ramage and Villalón 2008; Watts *et al.* 2012).

Regarding the capability of 5-HT to interfere with sympathetic transmission, *i.v.* continuous infusions of this monoamine have been shown to inhibit the cardioaccelerator sympathetic outflow by prejunctional mechanisms in pithed rats (Villalón *et al.* 1999). Subsequent studies using selective 5-HT receptor agonists/antagonists and other compounds (Sánchez-López *et al.* 2003, 2004) have shown that 5-HT-induced cardiac sympatho-inhibition: (i) does not involve serotonin 5-HT_{1A/1E/1F}, 5-HT_{2A/2B/2C}, 5-HT₃, 5-HT₄, 5-HT₆, 5-HT₇ receptors; $\alpha_{1/2}$ -adrenoceptors; or prostaglandins synthesis; and (ii) is mainly mediated by serotonin 5-HT_{1B}, 5-HT_{1D} and methiothepin-sensitive 5-HT_{5A}/5-HT_{5B} receptors. Consistent with these findings, 5-HT₁ (including all subtypes) and 5-HT_{5A} receptors are coupled to heterotrimeric G_{i/o} proteins (Hannon and Hoyer 2008), a signal transduction system usually associated with inhibition of neurotransmitter release (Boehm and Kubista 2002; Villalón and Centurión 2007).

Interestingly, not only are 5-HT₂ receptors associated with vasopressor effects, but also with tachycardic and sympatho-excitatory effects (Ramage and Villalón 2008; Watts *et al.* 2012).

Accordingly, 5-HT₂ receptor blockade can interfere with serotonergic mechanisms at the cardiovascular level and may represent a therapeutic strategy in the treatment of some cardiovascular pathologies in which the sympathetic innervation is altered. For example, the 5-HT₂ receptor antagonist sarpogrelate (Rashid et al. 2003) is therapeutically beneficial in several cardiac disorders, including heart failure and myocardial infarction (Brasil et al. 2002; Doggrell 2004; Saini et al. 2004). Moreover, our group has previously shown that chronic oral treatment with sarpogrelate (30 mg/kg.day for 2 weeks) modified the pharmacological profile of the prejunctional 5-HT receptors that inhibit the rat vasopressor sympathetic outflow (García-Pedraza et al. 2013, 2014). On this basis, the present study has investigated whether chronic oral treatment with sarpogrelate alters the pharmacological profile of the 5-HT receptors that inhibit the rat cardioaccelerator sympathetic outflow. For this purpose, we used most of the pharmacological tools previously reported by our group (Sánchez-López et al. 2003, 2004), except that methiothepin was replaced with SB 699551, a more selective 5-HT_{5A} receptor antagonist (Table 1). This pharmacological approach allowed us to confirm more selectively the role of 5-HT_{5A} receptors mediating cardiac sympatho-inhibition, as 5-HT_{5B} receptors do not seem to have a physiological correlate (Hannon and Hoyer 2008).

Material and methods

Animals

A total of 140 male Wistar normotensive rats (250-300 g) were housed at a 12/12-h light/dark cycle (light beginning at 07:00 hours) at a constant temperature (22±2°C) and humidity (50%), with food and water *ad libitum*. It must be emphasized that all the animal procedures and protocols of the present investigation were reviewed and approved by our Institutional Ethics Committee on the use animals in scientific experiments (CICUAL-Cinvestav; protocol number

507-12) and followed the regulations established by the Mexican Official Norm (NOM-062-ZOO-1999), in accordance with: (i) the *Guide to the Care and Use of Experimental Animals* of the Canadian Council on Animal Care (1993; also available at www.ccac.ca); and (ii) the ARRIVE guidelines for reporting experiments involving animals (McGrath et al. 2010).

General methods

The 140 rats were initially divided into two main sets, namely, set 1 (n=130) and set 2 (n=10) (see Fig. 1). In set 1: (i) 90 rats were orally pretreated during 2 weeks with sarpogrelate dissolved in drinking water (30 mg/kg.day), as previously reported (García-Pedraza et al. 2013, 2014); and (ii) 40 rats were orally pretreated during 2 weeks with equivalent volumes of drinking water (vehicle) to serve as the age-matched control (non-treated) group. In set 2, the 10 rats were orally pretreated with sarpogrelate, as described above. Subsequently, all rats were anaesthetized with sodium pentobarbital (60 mg/kg, i.p.), and its adequacy was judged by corporal relaxation, a negative tail flick test and absence of ocular reflexes. After cannulation of the trachea, the animals were pithed by inserting a stainless steel rod through the orbit and foramen magnum into the vertebral foramen (Gillespie et al. 1970; Villalón et al. 1999). Then, the rats were ventilated with room air by an Ugo Basile pump (56 strokes/min; stroke volume: 20 ml/kg) as previously established (Kleinman and Radford 1964). After cervical bilateral vagotomy, catheters were placed in: (i) the left and right femoral veins, for the infusions of agonists and i.v. bolus injections of antagonists, respectively; and (ii) the left carotid artery, connected to a Grass pressure transducer (P23XL, Grass Instrument Co., Quincy, MA, USA), for the recording of blood pressure. Heart rate was measured with a tachograph (7P4F, Grass Instrument Co.) triggered from the blood pressure signal. A model 7D Grass polygraph (Grass Instrument Co.) recorded both blood pressure and heart rate simultaneously. Then, the responses to i.v. continuous infusions of the 5-HT receptor agonists were investigated on the tachycardic responses induced

by: (i) stimulation of the cardioaccelerator sympathetic outflow (set 1; $n=130$) in non-treated ($n=40$) and sarpogrelate-treated ($n=90$) rats; or (ii) i.v. bolus injections of exogenous noradrenaline (set 2; $n=10$) in sarpogrelate-treated rats (see Fig. 1).

The resulting stimulus-response curves (S-R curves) and dose-response curves (D-R curves) were completed in about 30 min, with no changes in the baseline values of heart rate or diastolic blood pressure. The sympathetic tachycardic stimuli (0.031-3.1 Hz) and the i.v. doses of exogenous noradrenaline (0.031-3.1 $\mu\text{g/kg}$) were given using a sequential schedule in 0.5 log unit increments, at 3-5 min intervals (see below). Each response was elicited under unaltered values of resting heart rate. The body temperature of each pithed rat was maintained at 37°C by a lamp and monitored with a rectal thermometer.

Experimental protocols

After a stable haemodynamic condition for at least 30 min, baseline values of heart rate and diastolic blood pressure (a more accurate indicator of peripheral vascular resistance) were determined. Then, the two sets of rats underwent the following experimental protocols (Fig. 1).

Protocol I. Electrical stimulation of the cardioaccelerator sympathetic outflow: In the first set of rats ($n=130$), the pithing rod was replaced by an electrode enamelled except for 1 cm length 7 cm from the tip, so that the uncovered segment was situated at C₇-T₁ of the spinal cord. This procedure allowed selective preganglionic stimulation of the cardioaccelerator sympathetic outflow (Gillespie et al. 1970; Villalón et al. 1999). Before electrical stimulation, the animals received gallamine (25 mg/kg, i.v.) to avoid electrically-induced muscular twitching. Since the cardiac sympatho-inhibitory responses to several agonists in pithed rats are particularly more pronounced at lower stimulation frequencies, all the animals were pretreated with 50 $\mu\text{g/kg}$ (i.v.) of desipramine (a noradrenaline-reuptake inhibitor) 10 min before each S-R curve. This dose schedule of desipramine enhanced the tachycardic responses to sympathetic stimulation at low

stimulation frequencies (without wearing off during the experiment) when compared to those in animals without desipramine (Villalón *et al.* 1999). Then, the cardioaccelerator sympathetic outflow was stimulated by applying trains of 10 s, consisting of monophasic rectangular pulses of 2 ms duration and 50 V, at increasing frequencies of stimulation (0.031, 0.1, 0.31, 1 and 3.1 Hz). When heart rate had returned to baseline levels, the next frequency was applied; this procedure was systematically performed until the S-R curve was completed. Subsequently, this set of animals was divided into two clusters (n=40 and 90, respectively; see Fig. 1).

The first cluster (non-treated; n=40), used for confirming previous results (Sánchez-López *et al.* 2003, 2004), was divided into 8 groups (n=5 each). These groups received i.v. continuous infusions of: (i) saline (control; 0.02 ml/min), (ii) 5-HT (3.1 µg/kg.min), (iii) 5-HT (10 µg/kg.min), (iv) 5-CT (5-HT₁/5-HT_{5A} receptor agonist; 0.1 µg/kg.min), (v) indorenate (5-HT_{1A} receptor agonist; 31 µg/kg.min), (vi) CP 93,129 (5-HT_{1B} receptor agonist; 31 µg/kg.min), (vii) PNU 142633 (5-HT_{1D} receptor agonist; 31 µg/kg.min), and (viii) LY344864 (5-HT_{1F} receptor agonist; 31 µg/kg.min) (see Table 1 for their affinities). Ten min after starting each infusion, a S-R curve was elicited again during the infusion of the corresponding compound.

The second cluster (sarpogrelate-treated; n=90) was divided into four groups (n=40, 20, 10 and 20; see Fig. 1). The first group (n=40), subdivided into 8 subgroups (n=5 each), received i.v. infusions of saline or the 5-HT receptor agonists exactly as described above for the first cluster (see Fig. 1). Ten min after starting each infusion, a S-R curve was elicited again as described above. The second group (n=20), subdivided into 4 subgroups (n=5 each), received i.v. injections of: (i) saline (1 ml/kg), (ii) GR 127935 (5-HT_{1B/1D/1F} receptor antagonist; 310 µg/kg), (iii) SB 699551 (5-HT_{5A} receptor antagonist; 1 mg/kg), and (iv) the combination of GR 127935 (310 µg/kg) plus SB 699551 (1 mg/kg) (see Table 1 for their affinities). Ten min later, a S-R curve was elicited again to analyse the effects of these compounds *per se* on the sympathetic

tachycardic responses. The third group (n=10), subdivided into 2 subgroups (n=5 each), received i.v. bolus injections of: (i) saline (1 ml/kg), and (ii) GR 127935 (310 µg/kg). Ten min later, both subgroups received an i.v. continuous infusion of LY344864. After 10 min, a S-R curve was elicited again as previously described. The fourth group (n=20), subdivided into 4 subgroups (n=5 each), received i.v. bolus injections of: (i) saline (1 ml/kg), (ii) GR 127935 (310 µg/kg), (iii) SB 699551 (1 mg/kg), and (iv) the combination of GR 127935 (310 µg/kg) plus SB 699551 (1 mg/kg). Ten min later, all subgroups received an i.v. continuous infusion of 5-CT (0.1 µg/kg.min). After 10 min, a S-R curve was elicited again as described above.

Protocol II. Intravenous bolus injections of exogenous noradrenaline: The second set of rats (sarpogrelate-treated; n=10) was prepared as described above, but the pithing rod was left and the administration of both gallamine and desipramine was omitted. Then, the rats received i.v. bolus injections of noradrenaline (0.031, 0.1, 0.31, 1 and 3.1 µg/kg) in order to induce dose-dependent tachycardic responses (*i.e.* a D-R curve) as previously reported (Villalón et al. 1999). Subsequently, this set, divided into two groups (n=5 each), received i.v. continuous infusions of: (i) saline (0.02 ml/min); and (ii) LY344864 (31 µg/kg.min). Ten min later, a D-R curve was elicited again during the infusion of the above compounds.

Other procedures applying to protocols I and II: The doses of the 5-HT receptor agonists and saline were continuously infused at a rate 0.02 ml/min by a WPI model sp100i pump (World Precision Instruments Inc., Sarasota, FL, USA). The intervals between the different stimulation frequencies or noradrenaline doses depended on the duration of the tachycardic responses (5-10 min), as we waited until heart rate had returned to baseline values.

Data presentation and statistical evaluation

All data in the text and figures are presented as the mean $\pm$ SEM. The peak changes in heart rate by sympathetic stimulation or exogenous noradrenaline in the saline- and 5-HT agonists-infused

rats were determined. The difference in the values of heart rate and diastolic blood pressure within one (sub)group of rats before and during the infusions of saline or 5-HT receptor agonists were evaluated with paired Student's t-test. Moreover, the difference between the changes in heart rate within one subgroup of animals was evaluated with the Student-Newman-Keuls test, once a two-way repeated measures ANOVA (randomized block design) had revealed that the samples represented different populations (Steel and Torrie 1980). Additionally, the difference between measured haemodynamic variables in sarpogrelate-treated and non-treated (control) animals was evaluated using a one-way ANOVA, followed by the Student-Newman-Keuls *post hoc* test. Statistical significance was accepted at $P < 0.05$ (two-tailed).

Drugs

Apart from the anaesthetic (sodium pentobarbital), the drugs used in this study (obtained from the sources indicated) were as follows: sarpogrelate hydrochloride (ABBLIS Chemical LLC, Houston, TX, USA); 5-HT creatinine sulphate, gallamine triethiodide, SB 699551 (N-[2-(dimethylamino)ethyl]-N-[[4'-[(2-phenylethyl)amino]methyl][1,1'-biphenyl]-4-yl]methyl]-cyclopentanepropanamide dihydrochloride), desipramine hydrochloride and noradrenaline hydrochloride (Sigma Chemical Co., St. Louis, MO, USA); 5-carboxamidotryptamine (5-CT) maleate and GR 127935 (N-[4-methoxy-3-(4-methyl-1-piperazinyl)phenyl]-2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl)-1,1'-biphenyl]-4-carboxamide hydrochloride monohydrate) (GlaxoSmithKline, Stevenage, Hertfordshire, UK); indorenate (5-methoxytryptamine- β -methylcarboxylate hydrochloride; gift from Prof. Enrique Hong, Cinvestav-IPN, Mexico City, Mexico); CP 93,129 (3-(1,2,5,6-tetrahydropyrid-4-yl)pyrrolo[3,2-b]pyrid-5-one) (Pfizer Inc., Groton, CT, USA); LY344864 (N-[3-(dimethylamino)-2,3,4,9-tetrahydro-1H-carbazol-6-yl]-4-fluorobenzamide) (Eli Lilly & Co., Indianapolis, IN, USA); PNU 142633 [(S)-(-)-3,4-dihydro-1-[2-[4-aminocarbonyl]phenyl]-1-piperazinyl]ethyl]-N-methyl-1H-2-benzopyran-6-carboxamide]

(gift from Dr. R.B. McCall, Pharmacia & Upjohn, Kalamazoo, MI, USA). All compounds were dissolved in saline. This vehicle had no effect on the baseline values of heart rate or diastolic blood pressure (not shown). The doses mentioned in the text refer to the salts of substances, except in the case of the 5-HT receptor agonists, where they refer to the free base.

Results

Systemic haemodynamic variables

The baseline values of heart rate and diastolic blood pressure in the non-treated rats ($n=40$) were 249 ± 7 beats/min and 50 ± 2 mmHg, respectively; whereas in the sarpogrelate-treated rats ($n=100$) these values were 248 ± 9 beats/min and 54 ± 2 mmHg, respectively. These values did not differ ($P>0.05$) between the non-treated and sarpogrelate-treated rats. After the first injection of desipramine, both variables transiently increased ($P<0.05$) to: 259 ± 4 beats/min and 58 ± 1 mmHg in non-treated rats, and 263 ± 6 beats/min and 63 ± 1 mmHg in sarpogrelate-treated rats. These values returned to baseline levels after 10 min. The subsequent desipramine treatments did not modify further ($P>0.05$) these variables. Moreover, in the different subgroups of rats pretreated with desipramine, the baseline values of heart rate and diastolic blood pressure were not significantly modified by the i.v. continuous infusions of saline and of the 5-HT receptor agonists, or by the injections of saline and of the 5-HT receptor antagonists ($P>0.05$; not shown).

Initial effects produced by either electrical stimulation of the cardioaccelerator sympathetic outflow or i.v. noradrenaline on heart rate and diastolic blood pressure

The onset of the tachycardic responses by spinal stimulation (0.031-3.1 Hz) or i.v. injections of noradrenaline (0.031-3.1 $\mu\text{g/kg}$) was immediate and resulted in frequency-dependent or dose-dependent increases in heart rate (see below). In both cases, these responses appeared about 10 s after starting each treatment and reached a maximum around 40 s after the treatment had ended.

Furthermore, the electrically-induced tachycardic responses are due to selective sympathetic cardiostimulation since only negligible blood pressure changes were seen, as reported in other studies (Gillespie *et al.* 1970; Göthert *et al.* 1986). Exogenous noradrenaline (unlike electrical cardio-stimulation) also produced dose-dependent increases in diastolic blood pressure (not shown), as previously described (Villalón *et al.* 1999; Sánchez-López *et al.* 2003, 2004); these responses were not evaluated further. In all cases, the increases in heart rate produced by sympathetic stimulation and exogenous noradrenaline were significant ($P < 0.05$) when compared with their corresponding baseline values.

Effect of i.v. continuous infusions of saline, 5-HT and related agonists on the tachycardic noradrenergic responses in non-treated and sarpogrelate-treated rats

As previously reported by our group (Villalón *et al.* 1999; Sánchez-López *et al.* 2003, 2004), in non-treated rats (data not shown) the continuous infusions of: (i) 5-HT, 5-CT, CP 93,129 and PNU 142633 inhibited the sympathetic tachycardic responses; and (ii) saline, indorenate and LY344864 were inactive. On this basis, the above infusion doses of compounds were chosen for further analysis in sarpogrelate-treated animals. Indeed, as compared with non-treated rats, Fig. 2 shows some similarities in the results obtained in sarpogrelate-treated rats.

For example, the continuous infusions of 5-HT (B and C), 5-CT (D), CP 93,129 (F) and PNU 142633 (G) also inhibited ($P < 0.05$) the sympathetically induced tachycardic responses, while those of saline (A) and indorenate (E) were inactive (Fig. 2). Moreover, in contrast with non-treated rats: (i) the sympatho-inhibition produced by 5-CT (0.1 $\mu\text{g/kg.min}$) was quantitatively more pronounced at lower stimulation frequencies (*i.e.* at 0.031, 0.1 and 0.31 Hz; see Table 2); and (ii) LY344864 (31 $\mu\text{g/kg.min}$) did significantly inhibit the sympathetically induced tachycardic responses at all stimulation frequencies (see Fig. 2H and Fig. 3). Considering the latter finding, Fig. 4 further shows that neither saline (0.02 ml/min; A) nor LY344864

(31 $\mu\text{g/kg.min}$; B) affected ($P>0.05$) the tachycardic responses to exogenous noradrenaline in sarpogrelate-treated rats, as similarly reported by our group in non-treated rats for the same infusion doses of saline, 5-HT, 5-CT, CP 93,129, PNU 142633 and LY344864 (Villalón *et al.* 1999; Sánchez-López *et al.* 2003, 2004).

Effect of GR 127935 and/or SB 699551 on the cardiac sympatho-inhibition produced by LY344864 and 5-CT in sarpogrelate-treated rats

Fig. 5 shows the effect of i.v. injections ($n=5$ each) of: (i) GR 127935 (310 $\mu\text{g/kg}$) on the cardiac sympatho-inhibition to either LY344864 (31 $\mu\text{g/kg.min}$; A) or 5-CT (0.1 $\mu\text{g/kg.min}$; B); and (ii) SB 699551 (1 mg/kg ; C) or GR 127935 plus SB 699551 (D) on the sympatho-inhibition to 5-CT (0.1 $\mu\text{g/kg.min}$). Interestingly, the sympatho-inhibition to LY344864 (Fig. 2H), remaining unaffected after 1 ml/kg saline (not shown), was abolished after 310 $\mu\text{g/kg}$ GR 127935 (Fig. 5A), which completely blocks rat 5-HT_{1F} receptors (González-Hernández *et al.* 2011). Moreover, the sympatho-inhibition to 5-CT (Fig. 2D), remaining unchanged after 1 ml/kg saline (not shown), was: (i) partly blocked after 310 $\mu\text{g/kg}$ GR 127935 (remaining unaffected at 0.31 and 3.1 Hz; Fig. 5B); (ii) apparently unaffected after 1 mg/kg of SB 699551 (Fig. 5C); and (iii) abolished after the combination of GR 127935 plus SB 699551 (Fig. 5D). The above i.v. doses of saline or antagonists were devoid of any effect *per se* ($P>0.05$) on the sympathetically-induced tachycardic responses or on baseline heart rate and diastolic blood pressure (data not shown).

Discussion

General

Our present results confirm the cardiac sympatho-inhibitory role of 5-HT_{1B}, 5-HT_{1D} and 5-HT_{5A} receptors in both non-treated and sarpogrelate-treated rats. Indeed, the use of SB 699551 as a selective 5-HT_{5A} receptor antagonist specifically reinforces the role of this receptor; accordingly,

from now on we can discard the role of “putative 5-HT_{5A}/5-HT_{5B} receptors” mediating cardiac sympatho-inhibition, as previously implied by our group (Sánchez-López *et al.* 2003). Moreover, the most important and novel finding is that, contrasting with non-treated rats, sarpogrelate treatment: (i) potentiated 5-CT-induced sympatho-inhibition (Fig. 2D and Table 2); and (ii) unmasked the role of cardiac sympatho-inhibitory 5-HT_{1F} receptors activated by LY344864 (Fig. 2H and Fig. 3). Table 3 summarizes the most important results of our work. Admittedly, the neurotransmitter release resulting from stimulation of the cardioaccelerator sympathetic outflow was estimated indirectly by measuring the evoked tachycardic responses, as previously described (Villalón *et al.* 1999). Under these conditions, the responses to LY344864 in sarpogrelate-treated rats were considered sympatho-inhibitory as this 5-HT_{1F} receptor agonist (Table 1) inhibited the tachycardic responses to sympathetic stimulation (Fig. 2H) without affecting those to exogenous noradrenaline (Fig. 4B). Indeed, our group (Villalón *et al.* 1999; Sánchez-López *et al.* 2003, 2004) has already shown in sarpogrelate-free pithed rats that, unlike LY344864 and indorenate, 5-HT, 5-CT, CP 93,129 and PNU 142633 inhibited the tachycardic responses to sympathetic stimulation without affecting those to exogenous noradrenaline. This is why we did not analyse again the effects of these agonists on the tachycardic responses to noradrenaline. Furthermore, the effects of all compounds analysed here do not involve central mechanisms as pithed rats were used (see General methods section).

Systemic haemodynamic changes

In view that sarpogrelate potentiated 5-CT-induced sympatho-inhibition (Table 2) and unmasked a sympatho-inhibition by LY344864 (Fig. 2H and Fig. 3), it could be argued that these effects are due to changes in the baseline values of heart rate and diastolic blood pressure. However, this possibility can be excluded as: (i) these values in sarpogrelate-treated rats were not significantly different from those in non-treated rats; and (ii) no remarkable differences were found in the

magnitude of the sympathetically-induced tachycardic responses when comparing non-treated rats and sarpogrelate-treated rats in the different subgroups of animals, despite the potential role of biological variability. These findings are in agreement with previous results in sarpogrelate-treated pithed rats (García-Pedraza *et al.* 2013, 2014).

Moreover, the fact that heart rate and diastolic blood pressure transiently increased after desipramine in both animal groups implies an inhibition of noradrenaline-reuptake mechanisms, as reported earlier (Villalón *et al.* 1999). Accordingly, our results showing the potentiation by desipramine of the sympathetically-induced tachycardic responses (compare Docherty 1988; Villalón *et al.* 1999) are of particular significance within the context of our study, as the sympatho-inhibition produced by several monoamines including 5-HT (and many other agonists) is more pronounced at lower stimulation frequencies (see Langer 1980; Göthert *et al.* 1990).

Furthermore, the tachycardic responses (by sympathetic stimulation or exogenous noradrenaline) in non-treated (not shown) and sarpogrelate-treated (Fig. 2A and 4A) rats were reproducible, as they remained unchanged during i.v. continuous infusions of saline, and after an i.v. injection of saline (not shown), as previously reported (Sánchez-López *et al.* 2003, 2004). Accordingly: (i) the proposed sympatho-inhibition by the above 5-HT receptor agonists is not due to tachyphylaxis of the tachycardic responses; and (ii) no time-dependent fluctuations happened during the course of our experimental protocols. On the other hand, since the doses of the antagonists GR 127935, SB 699551 and the combination of both compounds did not affect *per se* the sympathetically-induced tachycardic responses or baseline heart rate/diastolic blood pressure, these were considered appropriate for identifying (via a direct interaction) the 5-HT receptors involved in 5-CT- and LY344864-induced sympatho-inhibition (see below).

The role of cardiac sympatho-inhibitory 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1F} and 5-HT_{5A} receptors

The role of G_{i/o}-coupled 5-HT_{1B}, 5-HT_{1D} and 5-HT_{5A} receptors stems from the capability of the agonists (see Table 1) CP 93,129 (5-HT_{1B}), PNU 142633 (5-HT_{1D}) and 5-CT (5-HT₁/5-HT_{5A}), but not of indorenate (5-HT_{1A}), to mimic 5-HT-induced cardiac sympatho-inhibition. Indeed, these effects were observed in both non-treated (not shown) and sarpogrelate-treated (Fig. 2) rats, as previously reported by Sánchez-López *et al.* (2003, 2004) in sarpogrelate-free rats. These authors also showed that the antagonists: (i) SB224289 (5-HT_{1B}; 300 µg/kg) and BRL15572 (5-HT_{1D}; 300 µg/kg) abolished the sympatho-inhibition to CP 93,129 and PNU 142633, respectively; (ii) methiothepin (non-selective 5-HT_{1B/1D} and putative 5-HT_{5A}/5-HT_{5B}; 300 µg/kg) abolished the sympatho-inhibition to 5-HT and 5-CT, as previously reported by Docherty (1988), although at that time he suggested the involvement of “5-HT₁ receptors”; and (iii) GR 127935 (5-HT_{1B/1D/1F}; 300 µg/kg) abolished the sympatho-inhibition to 5-CT, but not that to 5-HT. Hence, we concluded that methiothepin was blocking sympatho-inhibitory 5-HT_{1B/1D} and “putative 5-HT_{5A}/5-HT_{5B} receptors”. For this reason, the present study replaced methiothepin (300 µg/kg) with SB 699551 (1 mg/kg), a more selective 5-HT_{5A} receptor antagonist (Table 1).

Surprisingly, the fact that LY344864 (31 µg/kg.min) inhibited the sympathetic tachycardic responses in sarpogrelate-treated rats (Fig. 2H), but failed to do so in non-treated rats (not shown), as previously reported in doses up to 100 µg/kg.min (Sánchez-López *et al.* 2003), suggests the role of sympatho-inhibitory 5-HT_{1F} receptors. This suggestion is reinforced by our findings showing that LY344864-induced sympatho-inhibition was abolished by a supramaximal dose of 310 µg/kg GR 127935 (Fig. 5A), which completely blocks prejunctional 5-HT_{1F} receptors stimulated by LY344864 in pithed rats (González-Hernández *et al.* 2011). It is noteworthy that 100 µg/kg GR 127935: (i) is high enough to completely block cardiac sympatho-inhibitory 5-HT_{1B/1D} receptors in pithed rats (Sánchez-López *et al.* 2004), and (ii) fails to block

prejunctional 5-HT_{1F} receptors stimulated by LY344864 in pithed rats (González-Hernández et al. 2011). This is why the dose of 100 µg/kg GR 127935 was not investigated here (see Table 3 for a comparative analysis of the receptors involved in untreated and sarpogrelate-treated rats).

Moreover, it is notable that 310 µg/kg GR 127935 partly blocked the potentiated sympatho-inhibition to 5-CT in sarpogrelate-treated rats (Fig. 5B), while it abolished this 5-CT response in sarpogrelate-free rats (Sánchez-López et al. 2003). As 5-CT displays very low affinity for 5-HT_{1F} and 5-HT_{1E} receptors (Table 1), we concluded that 5-CT interacts with 5-HT_{1B/1D} receptors in sarpogrelate-free rats, despite displaying a very high affinity for 5-HT_{5A} receptors (Table 1). Hence, since GR 127935 displays very low affinity for 5-HT_{5A} receptors (Table 1), it is possible that (in addition to 5-HT_{1B/1D} receptors) a secondary activation of 5-HT_{5A} receptors by 5-CT was unmasked in sarpogrelate-treated rats (see below), and this may have overshadowed the blockade produced by GR 127935 (Fig. 5B). This possibility is indeed strengthened by our findings showing that the sympatho-inhibition to 5-CT, remaining apparently unaffected after 1 mg/kg SB 699551 (5-HT_{5A} receptor antagonist; see Table 1 and Fig. 5C), was abolished after GR 127935 plus SB 699551 (Fig. 5D). It must be stressed that the doses used of GR 127935 and SB 699551 completely blocked, respectively, 5-HT_{1F} receptors (González-Hernández et al. 2011) and 5-HT_{5A} receptors (Sánchez-Maldonado et al. 2015) in Wistar rats.

Possible mechanisms that may unmask cardiac sympatho-inhibitory responses in sarpogrelate-treated animals, therapeutic relevance and perspectives

The binding affinity (pK_i) of sarpogrelate for the 5-HT_{2A} (8.52), 5-HT_{2B} (6.57) and 5-HT_{2C} (7.43) receptor subtypes (Rashid et al. 2003), together with the role of pharmacokinetics during sarpogrelate oral treatment, make it difficult to discern the extent to which the chronic blockade of each of these receptors would facilitate/unmask cardiac sympatho-inhibitory responses. Moreover, we cannot exclude the potential role of undefined pleiotropic effects or even a change

in the expression of prejunctional 5-HT receptors during sarpogrelate treatment. Admittedly, our sarpogrelate-treatment represents a complex *in vivo* pharmacological scenario that falls beyond any simplistic explanation for our findings. With this notion in mind, it is clear that, in addition to unmasking sympatho-inhibitory responses by 5-HT_{1F} receptors (this study), sarpogrelate attenuates cardiac dysfunction, infarct size and electrocardiographic changes due to myocardial infarction in rats (Brasil et al. 2002). These findings imply that 5-HT and/or activation of 5-HT₂ receptors, which results in many effects including vasoconstriction, tachycardia and sympatho-excitation (Ramage and Villalón 2008; Watts et al. 2012), may participate in the deleterious effects of ischemic heart injury and of other cardiovascular pathologies (Kaumann and Levy 2006; Christ et al. 2014). Thus, it is reasonable to suggest that 5-HT₂ receptor blockade by sarpogrelate may unmask sympatho-inhibitory responses (including those mediated by 5-HT_{1F} receptors) that contribute to heart rate modulation and, thus, to sarpogrelate's therapeutic benefits.

Considering all of the above, many studies have established the presence of abnormal sympathetic signalling in the diabetic heart, namely, an initial sympathetic hyperactivity and elevated noradrenaline spillover (increasing heart rate), eventually culminating in sympathetic attenuation and reduced noradrenaline release (Thackeray et al. 2012). Accordingly, based on our findings, sarpogrelate could also offer potential therapeutic value in the treatment of the acute cardiac sympathetic activation in diabetes. Admittedly, further experiments that fall beyond the scope of the present study will be required to determine whether treatment with sarpogrelate (or with other 5-HT₂ receptor antagonists) alters the expression of cardiac sympatho-inhibitory G_{i/o}-coupled 5-HT receptors in healthy animals and in experimental models of cardiac damage, such as hypertension and diabetes.

Conclusions

As summarized in Table 3, our results support the cardiac sympatho-inhibitory role of 5-HT_{1B}, 5-HT_{1D} and 5-HT_{5A} receptors in both non-treated and sarpogrelate-treated rats. Moreover, sarpogrelate treatment specifically: (i) potentiated 5-CT-induced sympatho-inhibition mediated by 5-HT_{1B/1D} and, to a lesser extent, by 5-HT_{5A} receptors; and (ii) unmasked the role of 5-HT_{1F} receptors mediating cardiac sympatho-inhibition. These findings provide new evidence that may help explain (at least in part) why chronic 5-HT₂ receptor blockade with sarpogrelate is therapeutically beneficial in the treatment of several cardiac disorders.

Acknowledgements

The authors thank Consejo Nacional de Ciencia y Tecnología (CONACyT; grant No. 219707; Mexico City) and Junta de Castilla y León (projects SA147U13 and BIO/SA62/14; Spain) for financial support. J Á García-Pedraza was supported by Junta de Castilla y León and European Social Fund, budgeting application no. 322C1400.

Conflicts of interest

All authors state no conflict of interest whatsoever.

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Figure captions

Fig. 1. Experimental protocols of the study. Number of pithed rats used in the two main sets as well as their divisions for further treatments ($n=5$ each, with no exception). The cardioaccelerator responses were obtained by either electrical sympathetic stimulation (set 1, stimulus-response curves [S-R curves]; $n=130$) or i.v. bolus injections of exogenous noradrenaline (set 2, dose-response curves [D-R curves]; $n=10$). GR: GR 127935; SB: SB 699551.

Fig. 2. Effect of 5-HT receptor agonists on the sympathetic tachycardic responses in sarpogrelate-treated pithed rats. Tachycardic responses produced by sympathetic stimulation before (control) and during i.v. continuous infusions of: saline (A); 5-HT (B and C); 5-CT (D); indorenate (E); CP 93,129 (F); PNU 142633 (G); or LY344864 (H) ($n=5$ each). $*P<0.05$ vs control. Δ Heart rate stands for “increase in heart rate”.

Fig. 3. Differential effects of LY344864 on the sympathetically-induced tachycardic responses in non-treated rats and in sarpogrelate-treated rats. Original experimental recordings illustrating the increases in heart rate (beats/min) produced by electrical stimulation (Hz) of the cardioaccelerator sympathetic outflow in non-treated rats (A) and in sarpogrelate-treated rats (B) before the infusion (left panel) and during the infusion of LY344864 (right panel). Diastolic blood pressure remained unchanged throughout the experiment (not shown). The second S-R curve was elicited 10 min after the infusion of LY344864 had been commenced.

Fig. 4. Effect of LY344864 on the noradrenaline-induced tachycardic responses in sarpogrelate-treated pithed rats. Tachycardic responses to i.v. bolus injections of noradrenaline before (control responses) and during i.v. continuous infusions of saline (A) or LY344864 (B) ($n=5$ each). Note that non-significant effects ($P>0.05$) were produced by saline or LY344864. Δ Heart rate stands for “increase in heart rate”.

Fig. 5. Effect of LY344864 or 5-CT in the presence of 5-HT receptor antagonists on the electrically-induced tachycardic responses in sarpogrelate-treated pithed rats. Effect of i.v. injections of: (i) GR 127935 on the sympatho-inhibition to LY344864 (A) or 5-CT (B) (n=5 each); and (ii) SB 699551 (C) or GR 127935 plus SB 699551 (D) on the sympatho-inhibition to 5-CT (n=5 each). * $P < 0.05$ vs control. Δ Heart rate stands for “increase in heart rate”.

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Table 1. Receptor binding affinities (pK_i) of the different drugs considered in this study.

Drug	Receptor						
	5-HT _{1A}	5-HT _{1B}	5-HT _{1D}	5-HT _{1E} [†]	5-HT _{1F}	5-HT _{5A}	5-HT _{5B} [†]
5-CT	10.3 ¹	8.9 ²	9.2 ³	5.1 ⁴	5.8 ⁴	9.5 ⁵	7.4 ⁵
CP 93,129	5.5 ⁶	8.1 ²	5.7 ⁶	N.D.	N.D.	N.D.	N.D.
GR 127935	6.9 ⁷	8.5 ⁷	8.9 ⁷	6.2 ⁷	7.3 ⁷	5.2 ⁸	N.D.
5-HT	9.2 ¹	8.6 ²	8.4 ³	8.0 ⁴	8.0 ⁴	8.1 ⁵	6.6 ⁵
Indorenate	7.8 ⁹	5.4 ⁹	6.7 ⁹	N.D.	N.D.	N.D.	N.D.
LY344864	6.3 ¹⁰	6.3 ¹⁰	6.2 ¹⁰	5.8 ¹⁰	8.2 ¹⁰	N.D.	N.D.
PNU 142633	N.D.	4.8 ¹¹	8.3 ¹¹	N.D.	N.D.	N.D.	N.D.
SB 699551	6.3 ¹²	6.5 ¹²	6.4 ¹²	<5.0 ¹²	<5.0 ¹²	8.2 ¹²	N.D.

[†], 5-HT_{1E} and 5-HT_{5B} receptors retain their lower case appellation because confirmation of a true physiological role for these receptors is still lacking (Hannon and Hoyer 2008).

Data taken from the following references: ¹(Newman-Tancredi et al. 1997); ²(Beer et al. 1998); ³(Weinshank et al. 1992); ⁴(Adham et al. 1993); ⁵(Hoyer et al. 1994); ⁶(Macor et al. 1990); ⁷(Pauwels et al. 1996); ⁸(Skingle et al. 1996); ⁹(Hoyer D, personal communication); ¹⁰(Phebus et al. 1997); ¹¹(Pregenzer et al. 1999); and ¹²[Corbett et al. 2005].

N.D. stands for “not determined”.

Table 2. Comparative effects of 5-CT (0.1 µg/kg.min) on the sympathetically-induced tachycardic responses in non-treated and sarpogrelate-treated rats.

	Frequency (Hz)				
	0.031	0.1	0.31	1	3.1
	<i>Percentage of inhibition</i>				
Non-treated	59.7	37.7	31.0	26.0	33.2
Sarpogrelate-treated	67.8*	63.8*	58.5*	30.7	37.9

5-CT-induced cardiac sympatho-inhibition (%) in non-treated (control) rats and in sarpogrelate-treated (30 mg/kg.day during 2 weeks) rats. *P< 0.05 *versus* the non-treated (control) group.

Table 3. Summary of the results obtained in this study.

Rats	<i>5-HT receptors mediating cardiac sympatho-inhibition</i>				
	5-HT_{1A}	5-HT_{1B}	5-HT_{1D}	5-HT_{1F}	5-HT_{5A}
<i>Non-treated (control)</i>	—	✓	✓	—	✓
<i>Sarpogrelate-treated</i>	—	✓	✓	✓	✓

Pharmacological profile of the 5-HT receptors mediating cardiac sympatho-inhibition in non-treated (control) and sarpogrelate-treated rats.

Fig. 1. Experimental protocols of the study. Number of pithed rats used in the two main sets as well as their divisions for further treatments ($n=5$ each, with no exception). The cardioaccelerator responses were obtained by either electrical sympathetic stimulation (set 1, stimulus-response curves [S-R curves]; $n=130$) or i.v. bolus injections of exogenous noradrenaline (set 2, dose-response curves [D-R curves]; $n=10$). GR: GR 127935; SB: SB 699551.

254x190mm (300 x 300 DPI)

Fig. 2. Effect of 5-HT receptor agonists on the sympathetic tachycardic responses in sarpgrelate-treated pithed rats. Tachycardic responses produced by sympathetic stimulation before (control) and during i.v. continuous infusions of: saline (A); 5-HT (B and C); 5-CT (D); indorenate (E); CP 93,129 (F); PNU 142633 (G); or LY344864 (H) (n=5 each). * $P < 0.05$ vs control. Δ Heart rate stands for "increase in heart rate".

254x190mm (300 x 300 DPI)

Fig. 3. Differential effects of LY344864 on the sympathetically-induced tachycardic responses in non-treated rats and in sarpogrelate-treated rats. Original experimental recordings illustrating the increases in heart rate (beats/min) produced by electrical stimulation (Hz) of the cardioaccelerator sympathetic outflow in non-treated rats (A) and in sarpogrelate-treated rats (B) before the infusion (left panel) and during the infusion of LY344864 (right panel). Diastolic blood pressure remained unchanged throughout the experiment (not shown). The second S-R curve was elicited 10 min after the infusion of LY344864 had been commenced.

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Fig. 4. Effect of LY344864 on the noradrenaline-induced tachycardic responses in sarpgogrelate-treated pithed rats. Tachycardic responses to i.v. bolus injections of noradrenaline before (control responses) and during i.v. continuous infusions of saline (A) or LY344864 (B) (n=5 each). Note that non-significant effects ($P>0.05$) were produced by saline or LY344864. Δ Heart rate stands for "increase in heart rate".

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Fig. 5. Effect of LY344864 or 5-CT in the presence of 5-HT receptor antagonists on the electrically-induced tachycardic responses in sarpogrelate-treated pithed rats. Effect of i.v. injections of: (i) GR 127935 on the sympatho-inhibition to LY344864 (A) or 5-CT (B) ($n=5$ each); and (ii) SB 699551 (C) or GR 127935 plus SB 699551 (D) on the sympatho-inhibition to 5 CT ($n=5$ each). * $P<0.05$ vs control. Δ Heart rate stands for "increase in heart rate".

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