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RESEARCH PAPER

Cardiovascular responses to retigabine in conscious rats – under normotensive and hypertensive conditions

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BACKGROUND AND PURPOSE

Retigabine is a recently approved antiepileptic agent which activates $K_v7.2$–7.5 potassium channels. It is emerging that these channels have an important role in vascular regulation, but the vascular effects of retigabine in the conscious state are unknown. Hence, in the present study we assessed the regional haemodynamic responses to retigabine in conscious rats.

EXPERIMENTAL APPROACH

Male Sprague Dawley rats were chronically instrumented with pulsed Doppler flow probes to measure regional haemodynamic responses to retigabine under control conditions and during acute hypertension induced by infusion of angiotensin II and arginine vasopressin. Further experiments were performed, using the β-adrenoceptor antagonists CGP 20712A, ICI 118551 and propranolol, to elucidate the roles of β-adrenoceptors in the responses to retigabine *in vivo* and *in vitro*.

KEY RESULTS

Under normotensive conditions, retigabine induced dose-dependent hypotension and hindquarters vasodilatation, with small, transient renal and mesenteric vasodilatations. In the acutely hypertensive state, the renal and mesenteric, but not hindquarters, vasodilatations were enhanced. The response of the hindquarters vascular bed to retigabine was mediated, in part, by β_2-adrenoceptors. However, *in vitro* experiments confirmed that retigabine did not act as a β-adrenoceptor agonist.

CONCLUSIONS AND IMPLICATIONS

We demonstrated that retigabine causes regionally specific vasodilatations, which are different under normotensive and hypertensive conditions, and are, in part, mediated by β_2-adrenoceptors in some vascular beds but not in others. These results broadly support previous findings and further indicate that K_v7 channels are a potential therapeutic target for the treatment of vascular diseases associated with inappropriate vasoconstriction.

Abbreviations

AII, angiotensin II; AVP, arginine vasopressin; CGP 20712A, 1-[2-((3-carbamoyl-4-hydroxy)phenoxy)ethylamino]-3-[4-(1-methyl-4-trifluoromethyl-2-imidazolyl)phenoxy]-2-propanol dihydrochloride; ICI 118551, (±)-erythro-(S*,S*)-1-[2,3-(dihydro-7-methyl-1H-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol hydrochloride

Introduction

Voltage-dependent potassium (K^+) channels have a well-established role in the determination and stabilization of the resting membrane potential and electrical excitability in many cell types, including neurons, cardiomyocytes and vascular smooth muscle cells (Iannotti *et al.*, 2010; Jepps *et al.*, 2011). The *KCNQ1-5* gene encodes the K_v7 family of K^+ chan-

nels, which comprises the K_v7.1-7.5 subtypes, each with distinct localization and physiological function (for review, see Brown and Passmore, 2009). Impaired activation of K_v7 channels is associated with a number of pathophysiological conditions, including hypertension, neuropathic pain and epilepsy (Goto *et al.*, 2001; Su *et al.*, 2011).

Modulators of K^+ channels, including linopirdine and retigabine (ezogabine), have recently been developed to antagonize and activate these channels, respectively. Linopirdine was developed as a cognition enhancer for treatment of neurodegenerative conditions, such as Alzheimer's disease; however, serious cholinergic hyperstimulation and pro-epileptic side effects have limited its clinical use (Miceli *et al.*, 2008). In 2011, retigabine, a selective K_v7 channel activator, received European approval for use as an adjunctive treatment of partial-onset epileptic seizures in adults (European Medicines Agency, 2011; Stafstrom *et al.*, 2011; Martyn-St James *et al.*, 2012). Retigabine is now considered a first-in-class novel antiepileptic agent with proven efficacy for seizure control and as an anticonvulsant (Ferron *et al.*, 2002; Su *et al.*, 2011; Brickel *et al.*, 2012; Ciliberto *et al.*, 2012). The pharmacological actions of retigabine include positive allosteric modulation of K_v7.2–7.5, but not K_v7.1, channels (Main *et al.*, 2000; Rundfeldt and Netzer, 2000; Wickenden *et al.*, 2000), culminating in increased K^+ conductance, a shift in voltage-dependent channel activation to more hyperpolarized potentials, and a reduction in cell excitability (Main *et al.*, 2000; Rundfeldt and Netzer, 2000; Wickenden *et al.*, 2000; Otto *et al.*, 2002; Gunthorpe *et al.*, 2012).

As recently reviewed by Jepps *et al.* (2013), there is increased recognition of the importance of K_v7 channels in controlling smooth muscle activity. Thus, in addition to the well-described antiepileptic effects of retigabine, K_v7 channel modulators also affect a variety of smooth muscle functions including cardiovascular regulation. Indeed, adverse dose-limiting cardiovascular effects such as symptomatic hypotension, prolongation of the QTc interval and arrhythmias have been reported in healthy volunteers (Ferron *et al.*, 2002), and in patient populations (EMA, 2011; reviewed by Jepps *et al.*, 2013). Recent functional studies have shown that K_v7 channel activators have vasorelaxant effects in rat precontracted, isolated blood vessels (Chadha *et al.*, 2012a), and that K_v7 channels are involved in β-adrenoceptor-mediated vasorelaxation responses (Chadha *et al.*, 2012b). Additionally, there is evidence to suggest that the vasoconstrictor effect of arginine vasopressin (AVP) may be mediated, in part, by inhibition of K_v7 channels (Brueggemann *et al.*, 2007; Mackie *et al.*, 2008). Collectively, these findings suggest that K_v7 channel activators have the potential to be used as antihypertensive agents (Ng *et al.*, 2011), although K_v7 channel function has been found to be impaired in several models of hypertension (Morecroft *et al.*, 2009; Jepps *et al.*, 2011; Chadha *et al.*, 2012b).

The recent review by Jepps *et al.* (2013) provided evidence to support the contention that 'Kv7 channels are vital determinants of resting vascular tone' but to date, there have been very few *in vivo* studies in which the cardiovascular effects of K_v7 channel activation have been investigated and, to our knowledge, none in which the regional haemodynamic effects have been monitored in conscious animals. Therefore, the main aim of the present study was to determine the regional haemodynamic effects of retigabine, administered i.v., in normotensive and hypertensive, conscious rats. The results of the initial studies showed that retigabine induced vasodilator effects that had different time-courses in the various vascular beds investigated. Specifically, in the hindquarters the vasodilatation in response to retigabine was considerably more prolonged than that in the renal and mesenteric vascular beds. Since previous experiments in this experimental model have shown that long-lasting hindquarters vasodilatation is a hallmark of β-adrenoceptor-mediated stimulation (e.g. Gardiner *et al.*, 2010), additional studies were performed to determine whether the activation of β-adrenoceptors is involved in any of the vasodilator effects of retigabine. Hence, we performed additional *in vitro* experiments to address the possibility that the vasodilator responses to retigabine mediated by β-adrenoceptors are as a result of a direct effect of this drug on these receptors. We demonstrated that retigabine causes regionally specific vasodilatations, which are different under normotensive and hypertensive conditions, and are, in part, mediated by β_2-adrenoceptors in some vascular beds but not in others.

Methods

In vivo *studies*

All procedures were approved by the University of Nottingham Ethical Review Committee and were carried out under Home Office Project and Personal Licence authority. Every effort was made to ensure that animals experienced minimal discomfort. Twenty-seven rats were used for this study, and results are recorded in accordance with the ARRIVE guidelines for reporting experiments involving animals (McGrath *et al.*, 2010).

Animals and surgical preparation

Male, Sprague Dawley rats (Charles River, Margate, UK), weighing 300–350 g, were group-housed in individually ventilated cages in a temperature-controlled (21–23°C) environment with a 12 h light-dark cycle (lights on at 0600 h), and free access to food (18% Protein Rodent Diet; Teklad Global, Bicester, UK) and water for at least 7 days after arrival from the supplier before any surgical intervention. Daily welfare checks were carried out.

Surgery was performed in two stages under general anaesthesia (fentanyl and medetomidine, 300 $\mu g \cdot kg^{-1}$ i.p. of each, supplemented as required), with reversal of anaesthesia and postoperative analgesia provided by atipamezole (1 $mg \cdot kg^{-1}$ s.c.) and buprenorphine (0.02 $mg \cdot kg^{-1}$ s.c.). Depth of anaesthesia was assessed by monitoring paw pinch withdrawal. At the first stage, miniature pulsed Doppler flow probes were sutured around the superior mesenteric artery, the left renal artery and the distal abdominal aorta (to monitor hindquarters flow). The wires from the probes were tunnelled s.c. and secured at the back of the neck. Anaesthesia was then reversed and a postoperative analgesic administered (see above), and the animals were placed in individual cages on heat mats to recover. At the end of the day, animals were returned to the holding room, and then re-housed in pairs the following morning. The second stage of surgery took place at least 10 days after probe implantation, following a

satisfactory welfare check from the Named Veterinary Surgeon. Under anaesthesia (as above), catheters were implanted into the distal abdominal aorta, via the caudal artery, for monitoring mean arterial blood pressure and heart rate, and the right jugular vein (three i.v. catheters for concurrent administration of substances). At this stage, the wires from the probes were soldered into a miniature plug (Microtech, Boothwyn, PA, USA) attached to a custom-designed harness worn by the rat. The wires and catheters emerged from the rat at the same point, and were fed through a protective spring and attached to a counter-balanced pivot system. The arterial catheter was connected to a fluid-filled swivel for overnight infusion of saline containing heparin (15 U·mL^{-1}, 0.4 mL·h^{-1}) to maintain patency. Post-surgery, animals were singly housed in experimental cages.

Cardiovascular recordings began 24 h after catheterization, when animals were fully conscious and freely moving, with free access to water and food. Drugs were administered i.v., through the previously implanted catheters, to minimize disruption to the animals during the experiments and allow measurement of the effects immediately after dosing.

Cardiovascular recordings

Continuous recordings of cardiovascular variables [including heart rate, BP, renal, mesenteric and hindquarters Doppler shifts (flows)] were made using a customized, computer-based system [Instrument Development Engineering Evaluation (IDEEQ), Maastricht Instruments Bv, Maastricht, The Netherlands] connected to a transducer amplifier (model 13-4615-50; Gould, Eastlake, OH, USA) and a Doppler flowmeter (Crystal Biotech, Holliston, MA, USA) VF-1 mainframe (pulse repetition frequency 125 kHz) fitted with high-velocity (HVPD-20) modules]. Raw data were sampled by IDEEQ every 2 ms, averaged and stored to disc every cardiac cycle. Changes in vascular conductance were calculated from the changes in BP and Doppler shift.

Experimental protocol

Experiment 1: Cardiovascular responses to retigabine under normotensive and hypertensive conditions. The aim of this experiment was to measure the cardiovascular responses to K_v7 channel activation by retigabine, under normotensive conditions and during acute hypertension, induced by the combined administration of angiotensin II (AII) plus AVP, as described previously (Ho and Gardiner, 2009).

Two groups of animals (n = 8 and n = 7) were used for this experiment, which ran over 3 days. On the first day, animals received an infusion (0.4 mL·h^{-1}) of saline (Group 1; n = 8), or AII-AVP (0.1 μg·kg^{-1}·h^{-1} and 0.01 μg·kg^{-1}·h^{-1}, respectively; Group 2; n = 7). At least 90 min later, three bolus doses (0.1 mL) of retigabine (1 mg·kg^{-1}; 3 mg·kg^{-1}; 5 mg·kg^{-1}) or vehicle (saline) were administered to each rat over 5 s, at least 1 h apart. These doses of retigabine have been used previously in conscious rats (Streng *et al.*, 2004). At least 1 h later, recordings were stopped and animals were re-attached to the fluid-filled swivels. On the second day, no drugs were administered and the arterial catheters were routinely flushed with heparinized saline (30 U·mL^{-1}) to maintain patency. On the final day, animals were again infused with saline (Group 1) or AII-AVP (Group 2), as above, and those which were given retigabine on day 1 were given vehicle and vice versa. Animals were allocated to each group in a randomized order, and this design allowed each animal to act as its own control.

Due to transient behavioural effects triggered by 5 mg·kg^{-1} retigabine in some animals (3 out of 15), 3 mg·kg^{-1} was chosen as the dose for further experiments.

Experiment 2: Responses to retigabine in the presence of β-adrenoceptor antagonism. The aim of this experiment was to elucidate the role of β-adrenoceptors in the responses to retigabine. Two groups of animals were used, and the experiments ran contemporaneously.

The effects of the non-selective β-adrenoceptor antagonist, propranolol. One group of animals (n = 6) received a primed infusion (0.1 mL, 0.4 mL·h^{-1}) of propranolol (1 mg·kg^{-1} bolus, 0.5 mg kg^{-1} h^{-1} infusion) or saline on the first day. At least 90 min later, a bolus dose (0.1 mL) of retigabine (3 mg·kg^{-1}) was administered over 5 s. At least 1 h later, recordings were stopped and animals were re-attached to the fluid-filled swivels. On the second day, no drugs were administered and the arterial catheters were routinely flushed with heparinized saline (30 U·mL^{-1}) to maintain patency. On the final day, animals which were given propranolol on day 1 were given saline and vice versa. Due to a technical failure, the retigabine/saline data from one animal was not included in the analysis.

The effects of selective β_1- or β_2-adrenoceptor antagonism. One group of animals (n = 6) was used for this experiment, which lasted 3 days. On the first day, animals received a bolus dose (0.1 mL) of retigabine (3 mg·kg^{-1}) at least 90 min after the start of a primed infusion of the β_1-adrenoceptor antagonist, CGP 20712A, or the β_2-adrenoceptor antagonist, ICI 118551 (both at 200 μg·kg^{-1} bolus, 100 μg·kg^{-1}·h^{-1} infusion). No drugs were administered on the second day. On the final day, animals which were given CGP 20712A on day 1 were given ICI 118551, and vice versa, as described above. Previously, we have shown that these doses are selective for the appropriate receptor (Baker *et al.*, 2011), and do not affect cardiovascular responses 24 h post-administration (Fretwell and Woolard, unpubl. data), thus we were confident that drugs given on the first day would not affect subsequent treatments.

Experiment 3: In vitro *studies.* These data were kindly provided by Richard Proudman and Jillian Baker. In order to determine whether retigabine was directly interacting with β-adrenoceptors, the ability of retigabine to interact with β_1- and β_2-adrenoceptors stably expressed in CHO cell lines was examined using both a [^{3}H]-CGP 12177 whole cell binding assay and a functional CRE-SPAP (secreted placental alkaline phosphate) production assay, as described previously (Baker, 2005, and Baker, 2010, respectively). Although these CHO cell lines stably express the human β_1- and β_2-adrenoceptors, ligand affinity and efficacy has previously proved to be highly predictive of that seen in the *in vivo* rat model (Baker *et al.*, 2011).

In vivo *data analysis*

Data were analysed offline using IDEEQ software (University of Maastricht, The Netherlands). For all experiments, time-

averaged data are shown as changes from baseline [HR (beats·min^{-1}); BP (mmHg); vascular conductances (%)]. Statistical comparisons between effects of different doses of retigabine, or between effects of retigabine under different conditions, were performed on the integrated changes over specified time periods. As the data were not all normally distributed, a non-parametric, repeated-measures analysis of variance (Friedman's test; Theodorsson-Norheim, 1987) was used for within-group comparisons, and Mann–Whitney or Kruskal–Wallis for between-group comparisons, as appropriate. Vascular conductances were calculated from the BP and Doppler shift (flow) data. $P < 0.05$ was taken as significant.

Each animal represented one experimental unit.

Materials

Retigabine dihydrochloride was from Sequoia Research Products (Pangbourne, UK). AII, AVP and CGP 20712A were from Tocris Biosciences (via R&D Systems Europe Ltd., Abingdon, UK). Propranolol [(*RS*)-1-[(1-methylethyl)amino]-3-(1-naphthalenyloxy)-2-propanol hydrochloride], ICI 118551 and atropine methyl nitrate were from Sigma-Aldrich (Dorset, UK).

Stock solutions of AII and AVP were made up in sterile distilled water. All drugs were dissolved in sterile saline for *in vivo* administration. Bolus doses were given in 0.1 mL and infusions were at 0.4 mL·h^{-1}.

Drugs and molecular target nomenclature conform to the British Journal of Pharmacology's *Guide to Receptors and Channels* (Alexander *et al.*, 2011).

Table 1

Resting cardiovascular variables obtained before the administration of the first dose of retigabine in animals pretreated with either saline ($n = 8$) or AII plus AVP ($n = 7$)

Pretreatment	Saline ($n = 8$)	AII + AVP ($n = 7$)
HR (beats·min^{-1})	324 ± 8	332 ± 14
MAP (mmHg)	101 ± 4	141 ± 6*
RVC (U)	75 ± 11	45 ± 7*
MVC (U)	54 ± 15	39 ± 7*
HVC (U)	44 ± 5	36 ± 5*

Units for VC are (kHz·mmHg^{-1})10^3.
Values are mean ± SEM. *$P < 0.05$ versus saline group (Mann–Whitney test) (Experiment 1).
AII, angiotensin II; AVP, arginine vasopressin; H, hindquarters; HR, heart rate; M, mesenteric; MAP, mean arterial pressure; R, renal; U, units; VC, vascular conductance.

Results

Experiment 1: Cardiovascular responses to retigabine under normotensive and hypertensive conditions

Baseline cardiovascular variables before the administration of retigabine are shown in Table 1, and integrated (area under/over curves, 0–30 min) cardiovascular responses to retigabine are given in Table 2.

Table 2

Integrated (0–30 min) changes in cardiovascular variables following administration of retigabine in animals pretreated with saline ($n = 8$) or AII plus AVP ($n = 7$)

	Retigabine		
	1 mg·kg^{-1}	3 mg·kg^{-1}	5 mg·kg^{-1}
Saline ($n = 8$)			
HR (AOC, beats)	+211 ± 112	+549 ± 202	+1108 ± 510
HR (AUC, beats)	−84 ± 38	−207 ± 92	−159 ± 89
MAP (mmHg min)	−70 ± 12	−216 ± 62	−383 ± 119[1]
RVC (% min)	+105 ± 41	+138 ± 53	+260 ± 104
MVC (% min)	+218 ± 79	+145 ± 62	+219 ± 87
HVC (% min)	+216 ± 53	+801 ± 278[1]	+1614 ± 318[1,3]
AII-AVP ($n = 7$)			
HR (AOC, beats)	+256 ± 132	+617 ± 143	+1197 ± 438
HR (AUC, beats)	−535 ± 220*	−177 ± 80	−168 ± 124
MAP (mmHg min)	−104 ± 47	−335 ± 81	−807 ± 144*
RVC (% min)	+33 ± 15	+312 ± 110	+1137 ± 210*
MVC (% min)	+191 ± 95	+408 ± 117*	+1009 ± 181*
HVC (% min)	+168 ± 77	+758 ± 104	+1548 ± 384

Values are mean ± SEM. [1]$P < 0.05$ vs. 1 mg·kg^{-1}, [3]$P < 0.05$ vs. 3 mg·kg^{-1} (Friedman's test). *$P < 0.05$ vs. saline group (Mann–Whitney test).
For other abbreviations see Table 1 (Experiment 1).
AOC, area over the curve; AUC, area under the curve.

Normotensive conditions. Administration of vehicle (saline) in the presence of saline had no cardiovascular effects (see Figure 1).

The low dose of retigabine (1 mg·kg^{-1}) produced no significant haemodynamic effects other than a slight hindquarters vasodilatation ($P < 0.05$ vs. baseline at 1 min). At 3 mg·kg^{-1}, retigabine caused a transient fall in BP ($P < 0.05$ vs. baseline at 20–60 s), accompanied by small, short-lived renal and mesenteric vasodilatations ($P < 0.05$ vs. baseline at 20 s) and a more prolonged hindquarters vasodilatation ($P < 0.05$ vs. baseline at 10 s–10 min). The highest dose of retigabine (5 mg·kg^{-1}) caused a fall in BP ($P < 0.05$ vs. baseline at 10 s–20 min), with transient renal and mesenteric vasodilatations ($P < 0.05$ vs. baseline at 10–60 s and 10–30 s, respectively), and a prolonged hindquarters vasodilatation ($P < 0.05$ vs. baseline at 10 s–30 min). The integrated (0–30 min) hypotensive and hindquarters vasodilator effects of retigabine were dose-dependent (Table 2).

Heart rate responses to retigabine were very variable, particularly at the lower doses, with some animals showing predominantly tachycardia and others showing bradycardia, hence integrated responses are given for both areas under and over the curves (Table 2).

Hypertensive conditions. The pressor effect of AII-AVP infusion was accompanied by reduced renal, mesenteric and hindquarters vascular conductances ($P < 0.05$ vs. saline group; Table 1). In the presence of AII-AVP (Figure 2), at the low dose (1 mg·kg^{-1}), retigabine caused a slight fall in blood pressure ($P < 0.05$ vs. baseline at 10–20 s), with small renal and mesenteric vasodilatations ($P < 0.05$ vs. baseline at 10 s and 10–20 s, respectively). There was no significant change in hindquarters vascular conductance. At 3 mg·kg^{-1}, retigabine caused a more prolonged fall in BP ($P < 0.05$ vs. baseline at 10 s–30 min), accompanied by transient renal, mesenteric and hindquarters vasodilatations ($P < 0.05$ vs. baseline at 20–60 s, 20–30 s and 20 s–10 min, respectively). At the highest dose (5 mg·kg^{-1}), retigabine caused a fall in BP ($P < 0.05$ vs. baseline at 10 s–30 min) and prolonged vasodilatations in all three vascular beds [$P < 0.05$ vs. baseline at (renal) 10 s–30 min, (mesenteric) 10 s–10 min and (hindquarters) 10 s–30 min]. As before, the heart rate responses to retigabine were very variable.

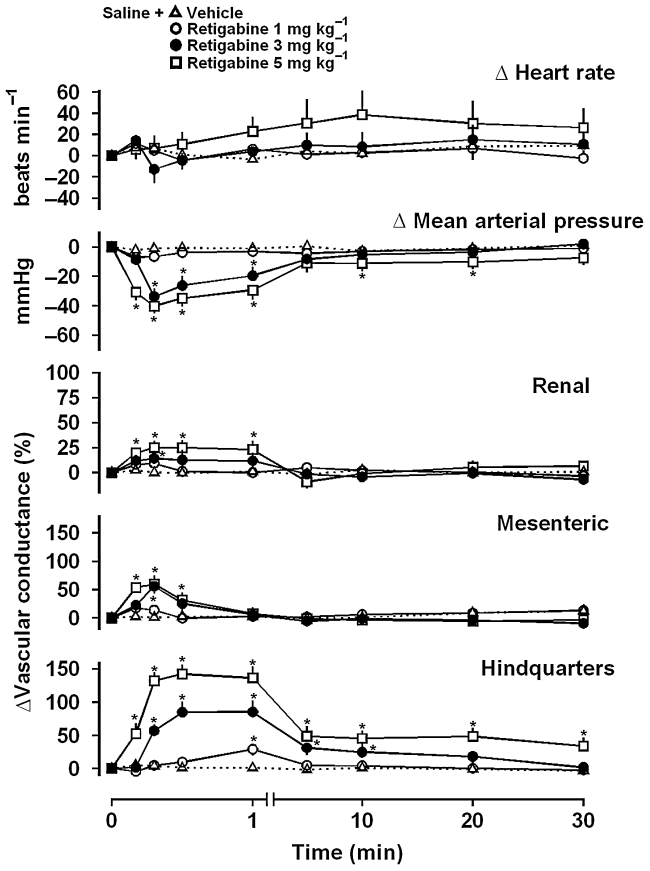

Figure 1

Cardiovascular responses to retigabine (1 mg·kg^{-1}, 3 mg·kg^{-1} or 5 mg·kg^{-1}, $n = 8$) and vehicle (saline, $n = 8$) following pretreatment with saline. Data points are mean and vertical bars represent SEM. *$P < 0.05$ versus baseline (Friedman's test). Note that a non-linear time scale is used for the initial 1 min to illustrate the rapid and transient haemodynamic effects of retigabine (Experiment 1).

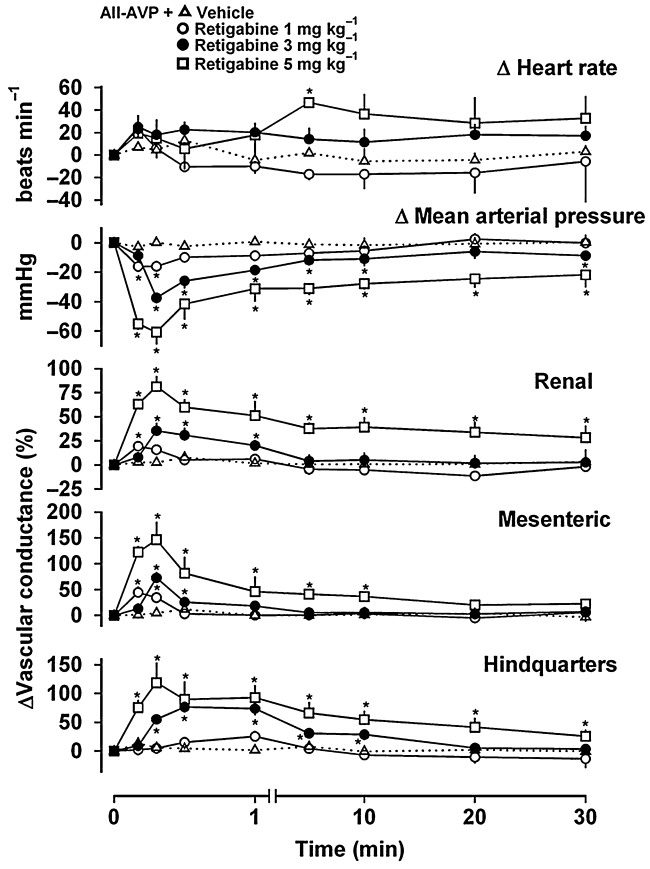

Figure 2

Haemodynamic effects of retigabine (1 mg·kg^{-1}, 3 mg·kg^{-1} and 5 mg·kg^{-1}, $n = 7$) and vehicle (saline, $n = 7$) in conscious, AII-AVP-treated rats. Data points are mean and vertical bars represent SEM. *$P < 0.05$ versus baseline (Friedman's test). Between-group differences are given in the text. AII-AVP, angiotensin II and arginine vasopressin (Experiment 1).

Comparison between normotensive and hypertensive conditions. Under hypertensive conditions, the hypotensive and renal and mesenteric vasodilator effects of the highest dose of retigabine (5 mg·kg^{-1}) were significantly enhanced ($P < 0.05$ vs. saline group). Renal and mesenteric vasodilator effects of 3 mg·kg^{-1} retigabine also tended to be enhanced in the hypertensive condition, but the effect was only significant ($P < 0.05$) for the mesenteric vasodilatation ($P = 0.08$ for renal vasodilator response vs. saline).

Experiment 2: Responses to retigabine in the presence of β-adrenoceptor antagonism

There were no statistically significant differences between resting cardiovascular variables following administration of saline, propranolol, CGP 20712A or ICI 118551 (Table 3); integrated (0–30 min) cardiovascular responses to retigabine are given in Table 4.

As above (see Experiment 1), in the presence of saline, retigabine (3 mg·kg^{-1}) caused a variable heart rate response, and a reduction in BP accompanied by transient renal and mesenteric vasodilatations and a more prolonged hindquarters vasodilatation (Figure 3A).

In the presence of propranolol, retigabine (3 mg·kg^{-1}) caused hypotension and some vasodilatation in all three vascular beds (Figure 3A). Compared to responses seen in the saline-treated animals, the integrated (0–30 min) hypotensive and mesenteric vasodilator effects of retigabine were enhanced by propranolol, whereas the hindquarters vasodilatation was markedly reduced (Table 4). In propranolol-treated animals, there was a consistent bradycardic response to retigabine (Figure 3A, Table 4).

During infusion of the β_1-adrenoceptor antagonist CGP 20712A, retigabine (3 mg·kg^{-1}) caused similar BP and regional haemodynamic changes to those seen in the presence of saline (Figure 3B, Table 4), although under these conditions, as with propranolol, the heart rate response to retigabine was a consistent bradycardia (Figure 3B, Table 4).

In the presence of the β_2-adrenoceptor antagonist ICI 118551, the heart rate, BP and renal and mesenteric vascular responses to retigabine (3 mg·kg^{-1}) were not significantly different from those in the presence of saline, but the hindquarters vasodilatation was significantly attenuated [$P < 0.05$ integrated (0–30 min) responses vs. saline group; Figure 3B, Table 4].

Experiment 3: In vitro *studies*

The β_1-adrenoceptor selective ligand CGP 20712A and the β_2-selective ligand, ICI 118551, inhibited the specific binding of [^{3}H]-CGP 12177 as expected. However, retigabine did not inhibit the specific binding of this ligand at either the β_1- or β_2-adrenoceptor at concentrations up to 1 mM (Figure 4A, Table 5).

CRE-SPAP production

Retigabine (1 μM, 10 μM and 100 μM) did not cause any rightward shift of the cimaterol concentration-response curve

Table 3

Resting cardiovascular variables obtained before the administration of retigabine (3 mg·kg^{-1}) or vehicle

Pretreatment	Saline ($n = 5$)	Propranolol ($n = 6$)	CGP 20712A ($n = 6$)	ICI 118551 ($n = 6$)
HR (beats·min^{-1})	340 ± 9	322 ± 6	326 ± 8	353 ± 15
MAP (mmHg)	100 ± 2	107 ± 2	100 ± 2	110 ± 3
RVC (U)	84 ± 4	82 ± 7	83 ± 5	84 ± 10
MVC (U)	68 ± 6	66 ± 5	95 ± 13	86 ± 10
HVC (U)	50 ± 8	45 ± 3	44 ± 6	40 ± 6

Units (U) for VC are (kHz·mmHg^{-1})10^3. Values are mean ± SEM. For abbreviations see Table 1 (Experiment 2).

Table 4

Integrated (0–30 min) changes in cardiovascular variables following administration of retigabine (3 mg·kg^{-1})

Pretreatment	Saline ($n = 5$)	Propranolol ($n = 6$)	CGP 20712A ($n = 6$)	ICI 118551 ($n = 6$)
HR (AOC, beats)	+340 ± 219	+0.2 ± 0.2*	+0.5 ± 0.3*	+57 ± 38
HR (AUC, beats)	–409 ± 211	–392 ± 56	–461 ± 76	–1055 ± 800
MAP (mmHg min)	–205 ± 87	–508 ± 39*	–391 ± 97	–405 ± 179
RVC (% min)	+220 ± 140	+387 ± 98	+266 ± 103	+344 ± 166
MVC (% min)	+77 ± 39	+551 ± 117*	+288 ± 155	+76 ± 25
HVC (% min)	+726 ± 85	+378 ± 72*	+877 ± 142	+350 ± 146*

Values are mean ± SEM. *$P < 0.05$ versus saline group (Kruskal–Wallis). For other abbreviations see Table 1 (Experiment 2).
AOC, area over the curve; AUC, area under the curve.

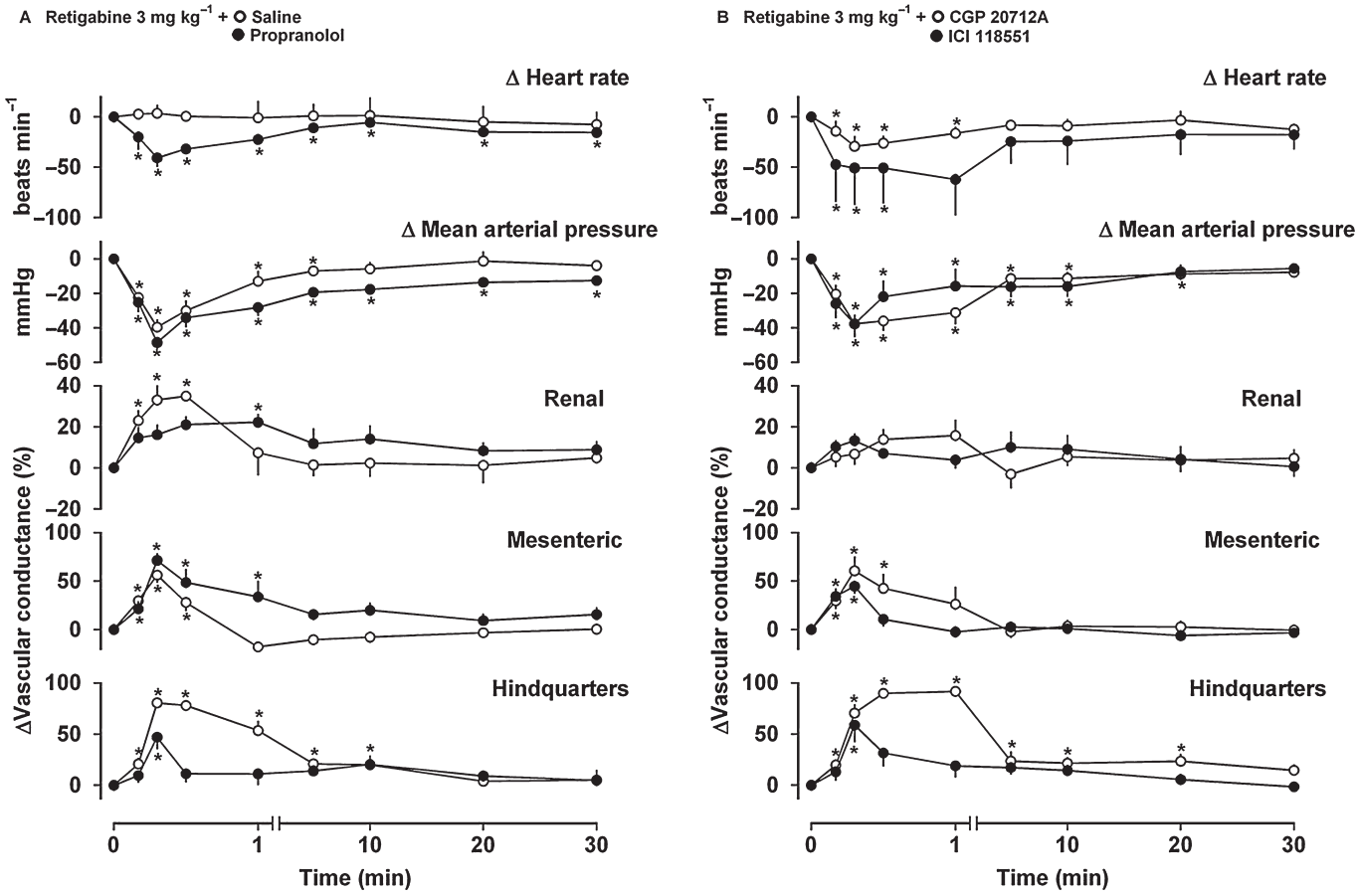

Figure 3

Haemodynamic effects of retigabine (3 mg·kg^{-1}) in conscious rats treated with saline (n = 5, Figure 3A), propranolol (n = 6, Figure 3A), CGP 20712A (n = 6, Figure 3B) or ICI 118551 (n = 6, Figure 3B). Data points are mean and vertical bars represent SEM. *P < 0.05 versus baseline (Friedman's test). Between-group differences are given in the text (Experiment 2).

Table 5

Log K_D values obtained from [^{3}H]-CGP 12177 whole-cell binding studies in CHO cells stably expressing either the human β_1-adrenoceptor or the human β_2-adrenoceptor

	Log K_D					
	CGP 20712A	n	ICI 118551	n	Retigabine	n
β_1	–8.98 ± 0.08	4	–6.76 ± 0.03	4	No binding	4
β_2	–5.88 ± 0.02	4	–9.38 ± 0.04	4	No binding	4

Values are mean ± SEM for n separate experiments (Experiment 3).

obtained at either subtype of β-adrenoceptor (expressed in CHO cells). Also, at concentrations up to 100 μM retigabine had no stimulating or inverse agonist effects on the responses to cimaterol (Figure 4B). Figure 4B shows a representative experiment in CHO cells expressing β_1-receptors. Similar results were obtained in CHO cells expressing β_2-receptors with retigabine concentrations up to 1 mM.

Discussion

The present study demonstrates, for the first time, the regional haemodynamic actions of retigabine in conscious rats. The main findings were that, under normotensive conditions, retigabine caused dose-dependent hypotension and hindquarters vasodilatation, together with transient renal

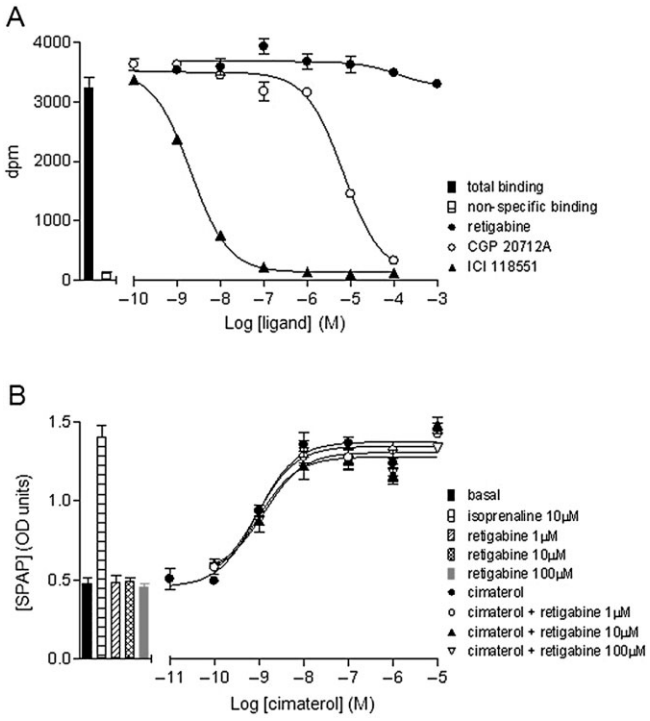

Figure 4

(A) Inhibition of [^{3}H]-CGP12177 specific binding to β_2–adrenoceptors expressed in CHO cells by retigabine, CGP 20712A and ICI 118551. Non-specific binding was determined by 10 μM propranolol and the concentration of [^{3}H]-CGP 12177 in this experiment was 0.631 nM. This single experiment is representative of four separate experiments. (B) CRE-SPAP production in response to cimaterol in β_1–adrenoceptors expressed in CHO cells in the absence and presence of retigabine. Bars represent basal CRE-SPAP production in response to 10 μM isoprenaline and in response to 1 μM, 10 μM or 100 μM retigabine alone. Data points are mean ± SEM of triplicate determinations. This single experiment is representative of four separate experiments. Data were provided by Dr Jillian Baker. Cell lines, methods and analysis as previously reported (Baker, 2005; Baker, 2010; Baker *et al.*, 2011) (Experiment 3).

and mesenteric vasodilatations. In rats made acutely hypertensive by an infusion of AII and AVP, the renal and mesenteric, but not the hindquarters vasodilator effects of higher doses of retigabine were enhanced. Furthermore, we obtained evidence indicating that β-adrenoceptors have a role in the hindquarters vasodilator effect of retigabine (β_2) and in the heart rate response (β_1). The data presented herein support the notion that K_v7 channels have a regulatory role in the maintenance of vascular tone, and suggest that retigabine, an activator of $K_v7.2$–7.5 channels, may have both direct and indirect effects in the vasculature.

K_v7 channels can be functionally expressed in renal and mesenteric arteries *in vitro* (Jepps *et al.*, 2011; Chadha *et al.*, 2012a), and evidence has been obtained suggesting that retigabine-induced vasorelaxation is mediated by an endothelium-independent mechanism (Joshi *et al.*, 2006; Yeung *et al.*, 2007). It is possible, therefore, that the renal and mesenteric vasodilatations are mediated via a direct action of retigabine on K_v7 channels in the vascular smooth muscle. However, the hindquarters responses are more complex and may involve the indirect activation of other intracellular pathways.

The current *in vivo* findings of the vasodilator effects of retigabine in normotensive, conscious rats are broadly consistent with the results from other studies on the role of K_v7 channels in the vasculature. Functional K_v7 channels have been identified in pulmonary arteries (Joshi *et al.*, 2006), murine aorta, carotid, femoral and mesenteric arteries (Yeung *et al.*, 2007), human visceral adipose and mesenteric arteries (Ng *et al.*, 2011), and on baroreceptors (Wladyka *et al.*, 2008) and sympathetic nerves (Brown and Adams, 1980). Activation of K_v7 channels *in vitro*, causes membrane hyperpolarization and smooth muscle relaxation, while inhibition of K_v7 channels leads to smooth muscle contraction (Yeung and Greenwood, 2005; Joshi *et al.*, 2006; 2009; Yeung *et al.*, 2007; Mackie *et al.*, 2008; Ng *et al.*, 2011). Moreover, in anaesthetized rats, it has been reported that activation of $K_v7.2$–7.5 channels with flupirtine, a structural analogue of retigabine, induces a reduction in mesenteric vascular resistance and BP (Mackie *et al.*, 2008).

Recently, it was shown that K_v7 channel function and expression is disrupted in isolated vessels from two different rodent models of hypertension (Jepps *et al.*, 2011). Accordingly, in spontaneously hypertensive rats (SHRs) and in AII-induced hypertensive mice, K_v7 channel activity was impaired, and the vasorelaxant effect of retigabine was diminished. The authors suggested that K_v7 channel-mediated responses were disrupted as a consequence of the development of hypertension, and this may contribute to the maintenance of an elevated BP (Jepps *et al.*, 2011). Others have shown that the vasoactive peptide, AII, inhibits K_v7-mediated currents in sympathetic neurons and CHO cells (Zaika *et al.*, 2006). Furthermore, it has been demonstrated that K_v7-mediated currents are blocked by AVP through a PKC-dependent pathway (Brueggemann *et al.*, 2007), in both mesenteric artery smooth muscle cells and in rat isolated mesenteric arteries (Mackie *et al.*, 2008). These findings suggest that the vasoconstrictor action of AVP is mediated, at least in part, via inhibition of K_v7 channels. Both AII and AVP are thought to have important roles in the pathogenesis of hypertension. Hence, in the present study, we used AII-AVP to induce acute hypertension, and then measured the responses to retigabine under these conditions of increased vascular tone and BP. From the findings reported above, we predicted that the vasodilator effects of retigabine would be reduced under these hypertensive conditions, due to the disruptive effects of AII and AVP on K_v7 channel function. However, contrary to our predictions, the renal and mesenteric vasodilator effects of retigabine were actually enhanced during AII-AVP infusion, suggesting that the scope for K_v7 channel activation is increased under hypertensive conditions.

In previous *in vivo* experiments, it was found that AII causes vasoconstriction in the renal and mesenteric vascular beds, and AVP causes vasoconstriction in the mesenteric and, to a lesser extent, hindquarters vascular beds (Gardiner *et al.*, 1988). Consistent with this, in the present study it was found that AII-AVP infusion caused a greater reduction in vascular conductance in the renal and mesenteric vascular beds compared with the hindquarters vasculature (Table 1). Therefore,

it is possible that the enhanced vasodilator actions of retigabine during AII-AVP infusion were a reflection of the enhanced vascular tone, although it is notable that no enhancement in the vasodilator response to retigabine was observed in the hindquarters, given that AII-AVP also produced some degree of vasoconstriction in that vascular bed. While our results with AII-AVP do not provide any further mechanistic insight into the action of retigabine, they do show that under these acute hypertensive conditions, its vasodilator effect is not impaired.

It was clear from the initial studies that the time course, and possibly mechanism, of the vasodilator effect of retigabine in the hindquarters was quite different from that in the renal and mesenteric vascular beds. Since other experiments in this model have shown that prolonged hindquarters vasodilatation is mediated by β-adrenoceptors (Gardiner *et al.*, 2010), we determined whether β-adrenoceptors are involved in the cardiovascular actions of retigabine by measuring the haemodynamic effects of retigabine during treatment with non-selective and selective β-adrenoceptor antagonists. In the presence of the β_2-adrenoceptor antagonists (either propranolol or ICI 118551), the retigabine-induced increase in hindquarters vascular conductance was markedly attenuated, whereas in the presence of the selective β_1-adrenoceptor antagonist (CGP 20712A) this effect of retigabine was unchanged. Interestingly, the β-adrenoceptor antagonists did not inhibit either the renal or mesenteric vascular effects of retigabine. It has been shown previously that the hindquarters vascular bed expresses a particularly high concentration of β_2-adrenoceptors, and that β-adrenoceptor activation causes a marked hyperaemic hindquarters vasodilatation (Gardiner *et al.*, 1991; 1992). Our results suggest that the observed retigabine-induced hindquarters vasodilatation is, at least in part, mediated by activation of β_2-adrenoceptors. One explanation would be that retigabine acts as a β-adrenoceptor agonist. To address this possibility, we examined whether retigabine (i) elicits a response from functional β_1- and β_2-adrenoceptors, or (ii) binds to human β_1- or β_2-adrenoceptors, expressed in CHO cells. We have recently validated this *in vitro* binding assay as a predictor of ligand affinity and efficacy *in vivo* (Baker *et al.*, 2011). However, our *in vitro* data clearly showed that retigabine has no β-adrenoceptor agonist (or, indeed, antagonist) properties. Another possibility is that retigabine indirectly causes β-adrenoceptor-mediated vasodilatation via sympathetic activation. However, if this was due to a generalized sympathetic activation in the vasculature, then we would have seen a degree of vasoconstriction in the renal and mesenteric vascular beds due to the α-adrenoceptor-mediated effects of noradrenaline (Bennett *et al.*, 2004), but this was not the case. Another hypothesis is that retigabine increases adrenomedullary adrenaline release, which then induces the hindquarters vasodilatation, although we know of no evidence to support this proposal.

The heart rate responses to retigabine deserve some comment. Under most conditions in the present experiments, the heart rate responses to retigabine were extremely variable, but in the presence of either propranolol or CGP 20712A, there was a clear-cut bradycardic effect of retigabine. These findings suggest that β-adrenoceptor-mediated tachycardia contributes to some extent to the heart rate response to retigabine in the intact animal, consistent with the suggestion of sympathoadrenal activation (see above). Recently, K_v7 channels were shown to be functional regulators of adrenergically controlled cardiomyocyte contraction rate (Zaika *et al.*, 2011), further supporting the notion that K_v7 channels interact with the sympathetic nervous system to regulate heart rate. From the results of our experiments we cannot identify the mechanism for this bradycardic action of retigabine, but bradycardia in response to K_v7 channel activation has been reported in other animal models, including conscious, telemetred dogs (EMA, 2011) and anaesthetized rats (Mackie *et al.*, 2008).

In conclusion, we have identified regional vascular effects of retigabine that change under hypertensive conditions and that are, in part, due to β-adrenoceptor activation. Although the exact mechanisms by which retigabine contributes to β-adrenoceptor-mediated vasodilatations in the hindquarters vascular bed are not clear at this stage.

Most studies on K_v7 channels have focused on the neuronal effects of channel modulation, and it is only more recently that interest has developed in their involvement in cardiovascular effects. To date, Phase III clinical trials of retigabine (Brodie *et al.*, 2010; French *et al.*, 2011) have not reported any clinically relevant cardiovascular effects. However, it remains to be seen whether it has adverse effects during prolonged treatment or in patients with pre-existing cardiovascular conditions, such as hypertension. Altered K_v7 channel activity has been implicated in the pathogenesis of primary and secondary hypertension in animal models, where a strong role for these channels in the development of hypertension is emerging (Jepps *et al.*, 2011; Chadha *et al.*, 2012a). In a very recent study, it was shown that the impaired anticontractile effects of periadventitial adipose tissue in skeletal muscle vessels from SHRs can be restored by administration of K_v7 channel openers such as retigabine (Zavaritskaya *et al.*, 2013). These findings further support the proposal that K_v7 channels are a potential therapeutic target for the treatment of vascular diseases associated with inappropriate vasoconstriction. Zavaritskaya *et al.* (2013) also showed that retigabine induced similar *ex vivo* vasorelaxant effects in the gracilis artery, and *in vivo* hypotensive responses in SHRs compared to normotensive control rats, which is perhaps surprising given the results of background studies by Jepps *et al.* (2011), where the vasorelaxant effects of various K_v7 channel openers were found to be reduced in isolated vessels from SHRs. However, in the latter study (Jepps *et al.*, 2011), it was notable that retigabine-induced vasorelaxations were less affected than those to two other structurally different K_v7 channel openers, which had differing subunit specificities.

To the best of our knowledge, the data from the present study are the first to show the regional haemodynamic effects of K_v7 channel activation during acute hypertension *in vivo*. We believe that this body of work provides an important insight into the complex functions of K_v7 channel activation in the cardiovascular system, highlighting direct and indirect vascular control mechanisms and a significant interaction with the sympathoadrenal system. Future work is required to assess the impact of such mechanisms in pathophysiological conditions, and following prolonged treatment with retigabine.

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Conflict of interest

None.

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