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Potential Mechanisms Involved in Palmitoylethanolamide-Induced Vasodepressor Effects in Rats

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Abstract

Palmitoylethanolamide is an endogenous lipid that exerts complex vascular effects, enhances the effects of endocannabinoids and induces a direct hypotension, but the mechanisms involved have been poorly explored. Hence, this study investigated in Wistar pithed rats the role of CB_1, CB_2, TRPV1 and GPR55 receptors in the inhibition by palmitoylethanolamide of the vasopressor responses produced by sympathetic stimulation or exogenous noradrenaline. Frequency- and dose-dependent vasopressor responses were analysed before and during intravenous (i.v.) continuous infusions of palmitoylethanolamide in animals receiving i.v. bolus of the antagonists NIDA41020 (CB_1), AM630 (CB_2), capsazepine (TRPV1), and/or cannabidiol (GPR55). Palmitoylethanolamide (0.1–3.1 µg/kg/min) dose-dependently inhibited the sympathetically induced and noradrenaline-induced vasopressor responses. Both inhibitions were: (i) partially blocked by 100 µg/kg NIDA41020, 100 µg/kg capsazepine, or 31 µg/kg cannabidiol; (ii) unaffected by 310 µg/kg AM630; and (iii) abolished by the combination NIDA41020 + capsazepine + cannabidiol (100, 100, and 31 µg/kg, respectively). The resting blood pressure was decreased by palmitoylethanolamide (effect prevented by NIDA41020, capsazepine or cannabidiol, but not by AM630). These results suggest that: (i) palmitoylethanolamide inhibits the vasopressor responses to sympathetic stimulation and exogenous noradrenaline and that it induces hypotension; and (ii) all these effects are mediated by prejunctional and vascular CB_1, TRPV1 and probably GPR55, but not by CB_2, receptors.

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Introduction

Endogenous cannabinoids and related mediators (i.e., endocannabinoids and endocannabinoid-like compounds) play a role in the modulation of a wide variety of physiological functions with potential therapeutic use [1–7]. In cardiovascular homeostasis, they exert actions on systemic blood pressure and local blood flow by mechanisms partially elucidated, including: (i) autonomic and

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sensory neural modulation [8, 9]; (ii) stimulation of endothelial nitric oxide [10]; and (iii) control of smooth muscle cell activity [11]. Modulation of vascular tone by endocannabinoids involves activation of vascular and prejunctional G-protein-coupled receptors (CB$_1$, CB$_2$ and GPR55), capsaicin receptors (TRPV1) and nuclear receptors (PPARα and PPARγ) [8, 9, 12]. The modulation of vascular tone by cannabinoids is of such importance that they induce prolonged hypotension in the nanomolar range doses [13].

Palmitoylethanolamide is an N-acylethanolamine (a fatty acid amide structurally related to the endocannabinoid anandamide) that stands out for its capability to mimic/potentiate some of the vascular effects induced by anandamide, including vasodilatation [14]. Its effects in the cardiovascular system have been mainly related to an "entourage" action. This "entourage" action may involve, among others, an increase in: (i) the levels of other N-acylethanolamines (and its actions) because palmitoylethanolamide has more affinity for the main enzyme involved in endocannabinoids degradation (i.e., fatty acid amide hydrolase); and (ii) the affinity of other endocannabinoids for their targets [15–17].

Interestingly, palmitoylethanolamide: (i) is present in human plasma at 14 pmol/mL [18], and values in the picomolar range are also observed in rodents [19]; (ii) is detected in several tissues where endocannabinoids exert regulatory effects [15–17]; and (iii) displays high affinity for (Table 1) and can activate [18] the cannabinoid/lysophospholipid receptor GPR55. As GPR55 activation results in vasodilatation [8, 10], palmitoylethanolamide might be involved in the vascular physiological actions of activation of GPR55. Nevertheless, the pharmacological nature of the systemic vascular mechanisms by which palmitoylethanolamide induces hypotension and vasorelaxation remains unknown. On this basis, the present study in pithed rats was designed in an attempt to analyse the effects produced by intravenous (i.v.) continuous infusions of palmitoylethanolamide on: (i) the vasopressor responses induced by selective preganglionic sympathetic stimulation or exogenous noradrenaline, and (ii) resting diastolic blood pressure (an indicator of total peripheral vascular resistance; TPVR). Furthermore, the potential role of CB$_1$, CB$_2$, TRPV1, and GPR55 receptors in the above palmitoylethanolamide effects on diastolic blood pressure was investigated by using the corresponding selective antagonists for these receptors (Table 1). Within this context, it is noteworthy that systolic blood pressure is generated during heart contraction, and thus it better reflects the

Table 1. Binding affinity constants (pK_i) of several ligands for CB$_1$, CB$_2$, TRPV1 and GPR55 receptors, except for [α], [β] and [γ], which stand for pEC$_{50}$, PK_B and pIC$_{50}$, respectively, determined in transfected cells

Compound	CB$_1$	CB$_2$	TRPV1	GPR55
Agonist				
Palmitoylethanolamide	<4.5[α, a]	<5.7[α, a]	ND	8.4[α, a]
Antagonists				
NIDA42020	~8.4[b]	6.0[β, c]	ND	ND
AM630	~5.2[d]	~7.5[d]	ND	ND
Capsazepine	ND	ND	~5.8[e] (r)	ND
Cannabidiol	~5.3[f]	~5.4[f]	ND	6.3[γ, a]

(r), interaction of anandamide with vanilloid receptors in CHO cells transfected with rVR1 (TRPV1) receptors; ND, not determined; ~, approximately.

Data taken from: [a] Ryberg et al. [46]; [b] Katoch-Rouse et al. [47]; [c] Donohue et al. [48]; [d] Ross et al. [49]; [e] Ross et al. [50]; [f] Thomas et al. [51].

phenomena in conductance blood vessels [19]. In contrast, diastolic blood pressure (which is generated during left ventricular relaxation) is directly proportional to TPVR [19]; in other words, an increment in TPVR results in a higher diastolic blood pressure [20].

On the other hand, it is important to highlight that in pithed rats the central nervous system has been destroyed. Consequently, the autonomic nervous control of visceral actions is absent, with only the local and hormonal systems remaining (e.g., the renin-angiotensin-aldosterone system; RAAS).

Methods

Animals
Healthy male normotensive Wistar rats (250–300 g) were maintained under a 12/12-h light-dark cycle (with light beginning at 07:00 h) and kept in a special room at 22 ± 2 °C and 50% humidity, with food and water ad libitum in their home cages.

General Methods
Experiments were carried out in 120 rats. After anaesthesia with diethyl ether and cannulation of the trachea, the rats were pithed by inserting a stainless-steel rod as previously reported [21, 22]. Immediately afterwards, the animals were artificially ventilated with room air using a model 7,025 Ugo Basile pump (56 strokes/min; stroke volume: 20 mL/kg), as established by Kleinman and Radford [23]. After bilateral cervical vagotomy, catheters were placed in the left and right femoral and jugular veins for: (i) the infusion of the agonists by a WPI model sp100i pump

(World Precision Instruments Inc., Sarasota, FL, USA); and (ii) bolus injections of the antagonists or vehicles. The left carotid artery was connected to a Grass pressure transducer (P23 XL) for the recording of blood pressure. Heart rate was measured with a tachograph (7P4, Grass Instrument Co., Quincy, MA, USA) triggered from the blood pressure signal. Both blood pressure and heart rate were recorded simultaneously by a model 7 Grass polygraph (Grass Instrument Co., Quincy, MA, USA).

Then, the 120 rats were divided into two main sets, so that the effects produced by i.v. continuous infusions of dimethyl sulfoxide 0.5% (DMSO; vehicle) or palmitoylethanolamide could be investigated on the vasopressor responses induced by either: (i) selective preganglionic (T_7–T_9) stimulation of the vasopressor sympathetic outflow (set 1; $n = 80$), or (ii) i.v. bolus injections of exogenous noradrenaline (set 2; $n = 40$).

The vasopressor stimulus-response curves (S-R curves) and dose-response curves (D-R curves) elicited by sympathetic stimulation and exogenous noradrenaline, respectively, were completed in about 30 min. Moreover, the vasopressor sympathetic stimuli and the noradrenaline injections were given using a sequential schedule, in 0.5 log unit increments at 3- to 5-min intervals. The body temperature of each pithed rat was maintained at 37 °C by a lamp and monitored with a rectal thermometer.

Experimental Protocols

After the animals had been in a stable haemodynamic condition for at least 20 min, baseline values of diastolic blood pressure (an indicator of systemic vascular tone) and heart rate were determined. Subsequently, the following experimental protocols were applied.

Electrical Stimulation of the Vasopressor Sympathetic Outflow

In the first set of rats ($n = 80$), the pithing rod was replaced by an electrode enamelled except for a 1-cm length 9 cm from the tip, so that the uncovered segment was situated at T_7–T_9 in the spinal cord to allow selective preganglionic stimulation of the thoracic sympathetic nerves supplying the systemic vasculature (i.e., the vasopressor sympathetic outflow); an indifferent electrode was placed dorsally [22, 24, 25]. Prior to electrical stimulation, all animals were pretreated with: (i) gallamine (25 mg/kg, i.v.) to avoid electrically induced muscular twitching; and (ii) after 10 min, desipramine (50 µg/kg, i.v.) before each S-R curve to induce higher vasopressor responses at low stimulation frequencies, as previously established [25, 22]. Under these conditions, the vasopressor responses to lower stimulation frequencies are greater than those elicited without desipramine [22, 25–27]. Ten minutes later, baseline values of diastolic blood pressure and heart rate were determined again.

The preganglionic vasopressor sympathetic outflow was then stimulated to elicit vasopressor responses by applying 10-s trains (2 ms monophasic rectangular pulses, 50 V) at increasing frequencies (0.03, 0.1, 0.3, 1 and 3 Hz) using a sequential schedule, as previously described [22, 25–27]. When diastolic blood pressure had returned to baseline levels, the next frequency was applied. This procedure was performed systematically until the S-R curve had been completed (about 30 min). At this point, this set of animals was divided into three treatment groups ($n = 15$, 30 and 35).

The first group ($n = 15$) was subdivided into three subgroups ($n = 5$ each) that received an i.v. continuous infusion of: (i) DMSO 0.5% (vehicle of palmitoylethanolamide, two times; 0.02 mL/min); (ii) palmitoylethanolamide (0.1 and 0.31 µg/kg/min), and (iii) palmitoylethanolamide (1 and 3.1 µg/kg/min). Ten minutes later, an S-R curve was elicited again during the above infusions to analyse their effects on the sympathetically induced vasopressor responses. The intervals between the different infusions ranged between 10 and 15 min, as in each case we waited until the diastolic blood pressure returned to baseline values.

The second group ($n = 30$) was subdivided into six subgroups ($n = 5$ each) that received an i.v. bolus injection of: (i) DMSO 2% (vehicle of antagonists); (ii) NIDA41020 (100 µg/kg); (iii) AM630 (310 µg/kg); (iv) capsazepine (100 µg/kg); (v) cannabidiol (31 µg/kg); and (vi) the combination of 100 µg/kg NIDA41020 + 100 µg/kg capsazepine + 31 µg/kg cannabidiol. Twenty minutes later, an S-R curve was elicited again, as described above, to analyse their effects per se on the sympathetically induced vasopressor responses.

The third group ($n = 35$), subdivided into seven subgroups ($n = 5$ each), received an i.v. bolus injection of: (i) 1 mL/kg DMSO 2%; (ii) 100 µg/kg NIDA41020; (iii) 310 µg/kg AM630; (iv) 31 µg/kg capsazepine; (v) 100 µg/kg capsazepine; (vi) 31 µg/kg cannabidiol; and (vii) the combination of 100 µg/kg NIDA41020 + 100 µg/kg capsazepine + 31 µg/kg cannabidiol.

Ten minutes later, all subgroups received an i.v. continuous infusion of palmitoylethanolamide (3.1 µg/kg/min). After 10 min, an S-R curve was constructed again during the infusion of palmitoylethanolamide as described above, to analyse the effects of the aforementioned compounds on palmitoylethanolamide-induced inhibition of the electrically induced vasopressor responses.

Administration of Exogenous Noradrenaline

The second set of rats ($n = 40$) was prepared as described above, but the pithing rod was left in situ and not replaced by the stimulating electrode throughout the experiment (as electrical stimulation was not used). After determining baseline values of diastolic blood pressure and heart rate, vasopressor responses were elicited by administering i.v. bolus injections of exogenous noradrenaline at increasing doses (0.03, 0.1, 0.3, 1 and 3 µg/kg) as previously described [22, 25–27]. When diastolic blood pressure had returned to baseline levels, the next dose was applied. This procedure was performed until the D-R curve had been completed (about 30 min). Subsequently, the animals were divided into two groups ($n = 10$ and 30, respectively).

The first group ($n = 10$) was subdivided into two subgroups ($n = 5$ each) that, respectively, received i.v. continuous infusions of: (i) DMSO 0.5% (two times; 0.02 mL/min), and (ii) palmitoylethanolamide (1 and 3.1 µg/kg/min). Ten minutes after starting these infusions, a D-R curve for noradrenaline was elicited again.

The second group ($n = 30$) was subdivided into six subgroups ($n = 5$ each) that received an i.v. bolus injection of: (i) 1 mL/kg DMSO 2%; (ii) 100 µg/kg NIDA41020; (iii) 310 µg/kg AM630; (iv) 100 µg/kg capsazepine; (v) 31 µg/kg cannabidiol; and (vi) the combination of 100 µg/kg NIDA41020 + 100 µg/kg capsazepine + 31 µg/kg cannabidiol.

Ten minutes later, all subgroups received an i.v. continuous infusion of palmitoylethanolamide (3.1 µg/kg/min). After 10 min, a D-R curve for noradrenaline was elicited again to analyse the effects of the aforementioned compounds on palmitoylethanolamide-induced inhibition of the vasopressor responses induced by noradrenaline.

Table 2. Effects of the different i.v. continuous infusions of compounds (*n* = 5 each) on the basal values of diastolic blood pressure determined 10 min after a stable condition with no treatment (basal) or 10 min after each corresponding infusion had commenced

Treatment	Diastolic blood pressure, mm Hg		
	basal	after 1st dose treatment	after 2nd dose treatment
DMSO 0.5% (0.02 mL/min; given twice)	49±8	53±5	51±2
Palmitoylethanolamide (0.1 and 0.31 µg/kg/min)	59±6	54±5	51±6
Palmitoylethanolamide (1 and 3.1 µg/kg/min)	54±7	48±6[a]	42±5[a]

All values are expressed as the mean ± SEM. [a] $p < 0.05$ versus the corresponding basal value. DMSO, dimethyl sulfoxide.

Table 3. Effect of the different i.v. bolus injections of compounds (i.e., before starting the infusion of palmitoylethanolamide) on the hypotension induced by 3.1 µg/kg/min palmitoylethanolamide

Pretreatment	Diastolic blood pressure, mm Hg	
	basal	palmitoylethanolamide
DMSO 0.5%, 1 mL/kg	60±5	41±4[a]
DMSO 2%, 1 mL/kg	60±3	43±2[a]
NIDA41020, 100 µg/kg	50±7	46±9
AM630, 310 µg/kg	50±4	40±4[a]
Capsazepine, 31 µg/kg	57±9	49±9
Capsazepine, 100 µg/kg	51±6	46±7
Cannabidiol, 31 µg/kg	52±11	44±9
Combination[b]	64±9	63±3

All values are expressed as the mean ± SEM. The basal values shown in this table correspond to those obtained 10 min after administering the above compounds. [a] $p < 0.05$ versus the corresponding basal value. [b] Combination of 100 µg/kg NIDA41020 + 100 µg/kg capsazepine + 31 µg/kg cannabidiol.

Drugs

Apart from the anaesthetic (diethyl ether), the compounds used in this study (obtained from the sources indicated) were: gallamine triethiodide, desipramine hydrochloride, (−)-noradrenaline bitartrate, palmitoylethanolamide, capsazepine and NIDA41020 (1-[2,4-dichlorophenyl]-5-[4-methoxyphenyl]-4-methyl-N-[1-piperidinyl]-1H-pyrazole-3-carboxamide; Sigma Chemical Co., St Louis, MO, USA); AM630 (6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl(4-methoxyphenyl) methanone; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), and cannabidiol (TOCRIS, Avonmouth, UK).

The compounds were dissolved as follows: (i) palmitoylethanolamide in DMSO 0.5%; (ii) gallamine, desipramine, and noradrenaline in physiological saline, and (iii) NIDA41020, AM630, capsazepine and cannabidiol in DMSO 2%. The resulting solutions were gauged with physiological saline. In the case of noradrenaline, ascorbic acid 1% was used to prevent oxidation. These vehicles had no effect on baseline diastolic blood pressure or heart rate (not shown). Fresh solutions were prepared for each experiment. The doses mentioned in the text refer to the free base of substances, except in the case of gallamine and desipramine, where they refer to their corresponding salts.

Data Presentation and Statistical Analysis

All data in the text and figures are presented as means ± SEM. It must be emphasised that the data and statistical analysis used in the present study comply with recent recommendations on experimental design and analysis in pharmacology, including that data subjected to statistical analysis should have a minimum of *n* = 5 independent samples/individuals per group [28]. The peak changes in diastolic blood pressure by electrical stimulation or exogenous noradrenaline in saline- and agonist-infused animals were determined. The difference between the changes in diastolic blood pressure within one subgroup of animals was evaluated by the Student-Newman-Keuls post hoc test, once a two-way repeated measures analysis of variance (randomised block design) had revealed that the samples represented different populations [29]. Statistical significance was accepted at $p < 0.05$.

Results

Systemic Haemodynamic Variables

The baseline values of diastolic blood pressure and heart rate in the 120 rats were 54 ± 7 mm Hg and 296 ± 13 beats/min, respectively. The i.v. administration of desipramine (50 µg/kg) produced a transient increase of both variables (not shown), but baseline values recovered after 5 min ($p > 0.05$), as previously reported [22, 25–27]. The latter baseline values in desipramine-pretreated rats were not significantly modified during the continuous infusions of: (1) DMSO 0.5% (control, two times, 0.02 mL/min; Table 2), and (2) palmitoylethanolamide (0.1–0.3 µg/kg/min; Table 2). In contrast, the baseline values of diastolic blood pressure were significantly decreased by the continuous infusions of palmitoylethanolamide (1–3.1 µg/kg/min; Table 2). Furthermore, the diastolic blood pressure values (in the absence of agonists) were not modified ($p > 0.05$) after i.v. bolus injections of: (i) 31 and 100 µg/kg NIDA41020; (ii) 310 µg/kg AM630; (iii) 31–100 µg/kg capsazepine; (iv) 31 µg/kg cannabidiol; or (v) the combination of 100 µg/kg NIDA41020 + 100 µg/kg capsazepine + 31 µg/kg cannabidiol (data not shown).
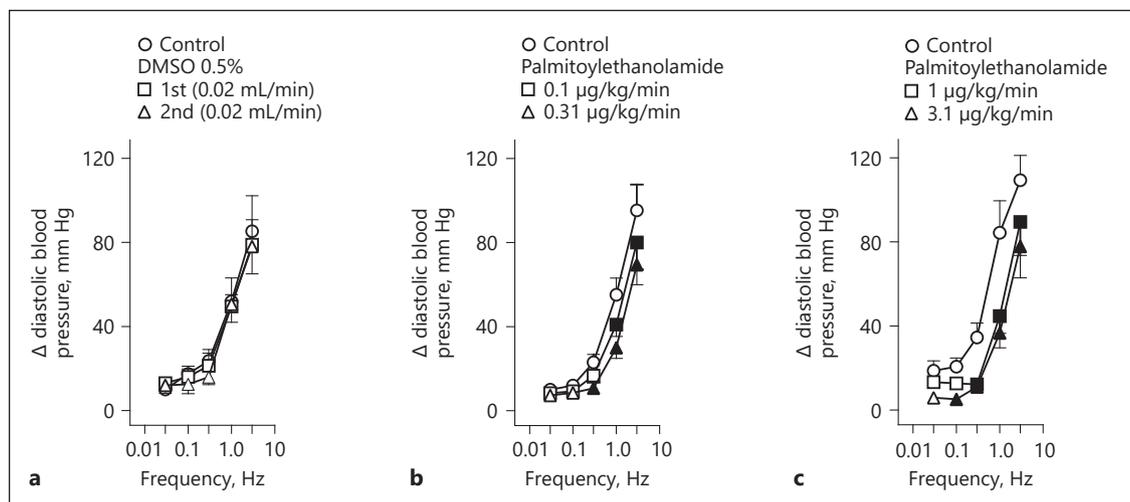
Fig. 1. Effect of an i.v. infusion ($n = 5$ for each figure) of: DMSO 0.5% (0.02 mL/min; twice; **a**) and palmitoyl-ethanolamide (0.1–3.1 µg/kg/min; **b, c**) in the presence of desipramine (50 µg/kg, i.v.) on the vasopressor responses induced by electrical stimulation of the preganglionic (T_7–T_9) sympathetic outflow in pithed rats. Empty symbols depict either control responses (○) or non-significant ($p > 0.05$) responses (△, □) versus the control. Solid symbols (▲, ■) represent significantly different responses ($p < 0.05$) versus the control.

Effect of the Antagonists on the Hypotensive Responses Produced by Palmitoylethanolamide

As shown in Table 3, the hypotensive responses induced by 3.1 µg/kg/min palmitoylethanolamide: (i) remained unaltered after DMSO (0.5 and 2%) or 310 µg/kg AM630, and (ii) were abolished by 100 µg/kg of NIDA41020, 31 or 100 µg/kg of capsazepine, 31 µg/kg of cannabidiol or the combination 100 µg/kg of NIDA41020 + 100 µg/kg of capsazepine + 31 µg/kg of cannabidiol.

Initial Effects Produced by Electrical Sympathetic Stimulation or Exogenous Noradrenaline on Diastolic Blood Pressure and Heart Rate

The increases in diastolic blood pressure induced by electrical stimulation of the sympathetic vasopressor outflow (0.03–3 Hz) or by exogenous noradrenaline (0.03–3 µg/kg) were immediate, frequency-dependent (sympathetic stimulation), or dose-dependent (exogenous noradrenaline; see control responses in Fig. 1–5). As previously described [29, 30], i.v. bolus injections of noradrenaline also produced dose-dependent and transient increases in heart rate (not shown). The vasopressor responses to either electrical stimulation or noradrenaline were significant when compared with their corresponding baseline values (not shown). The electrically induced vasopressor responses are induced by selective sympathetic stimulation, as only negligible changes in heart rate

were observed, as previously reported by our group [5, 29, 31].

Effect of i.v. Continuous Infusions of Vehicle or Palmitoylethanolamide on the Vasopressor Responses Induced by Either Sympathetic Stimulation or Exogenous Noradrenaline

Figure 1 shows the vasopressor responses induced by electrical stimulation before (control) and during the i.v. continuous infusion of DMSO 0.5% (0.02 mL/min; 2 times) and palmitoylethanolamide (0.1, 0.3, 1, and 3.1 µg/kg/min). In animals infused with DMSO 0.5%, the electrically induced vasopressor responses remained essentially unchanged ($p > 0.05$) when repeating two subsequent S-R curves (Fig. 1a). In contrast, the infusions of palmitoylethanolamide (0.1–3.1 µg/kg/min) produced a significant inhibition of the electrically induced vasopressor responses (Fig. 1b, c).

Moreover, Figure 2 shows the vasopressor responses induced by i.v. injections of noradrenaline before (control) and during i.v. continuous infusions of DMSO or palmitoylethanolamide. In animals infused with DMSO 0.5% (0.02 mL/min), the vasopressor responses to noradrenaline remained unchanged when repeating two subsequent D-R curves (Fig. 2a). In contrast, the infusions of 1 and 3.1 µg/kg/min palmitoylethanolamide produced a dose-dependent inhibition of the vasopressor re-

Fig. 2. Effect of an i.v. infusion (*n* = 5 for each figure) of: DMSO 0.5% (0.02 mL/min, twice; **a**) and palmitoylethanolamide (1 and 3.1 µg/kg/min; **b**) on the vasopressor responses induced by exogenous noradrenaline in pithed rats. Empty symbols depict either control responses (○) or nonsignificant (*p* > 0.05) responses (△, □) versus the control. Solid symbols (▲, ■) represent significantly different responses (*p* < 0.05) versus the control. * represents a significantly different response (*p* < 0.05) between palmitoylethanolamide 1 µg/kg/min versus 3.1 µg/kg/min.
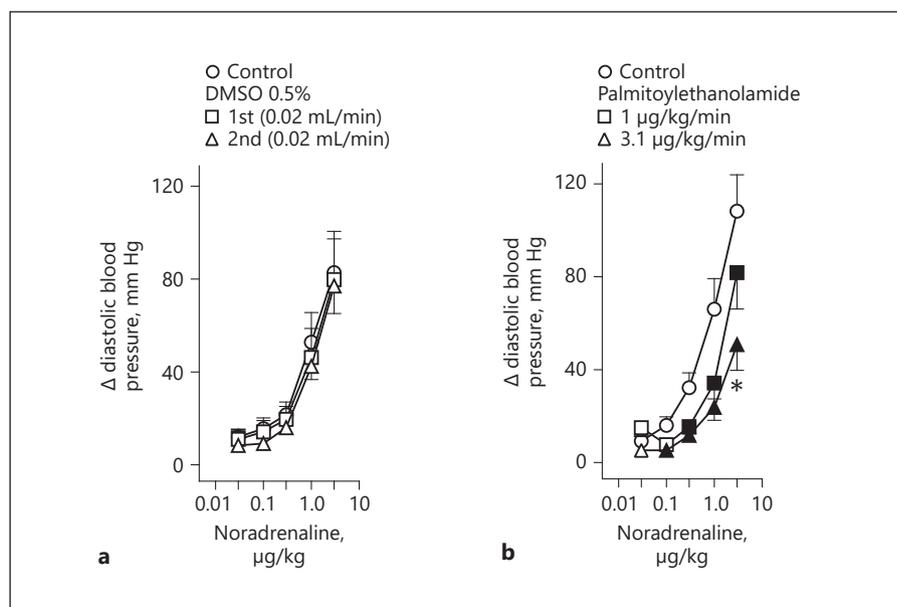
sponses. Indeed, 3.1 µg/kg/min palmitoylethanolamide produced the highest inhibition of the vasopressor responses induced by either electrical stimulation (Fig. 1c) or i.v. noradrenaline (Fig. 2b). For this reason, 3.1 µg/kg/min of palmitoylethanolamide was chosen for further pharmacological analysis (see below).

Effect per se of Vehicles or Antagonists on the Vasopressor Responses by Electrical Stimulation

Figure 3 shows that the electrically induced vasopressor responses remained unchanged (as compared with the control S-R curves; *p* > 0.05) after an i.v. bolus injection of: (i) 1 mL/kg DMSO 2% (Fig. 3a); (ii) 100 µg/kg NIDA41020 (Fig. 3b); (iii) 310 µg/kg AM630 (Fig. 3c); (iv) 31 µg/kg cannabidiol (Fig. 3d); (v) 100 µg/kg capsazepine (Fig. 3e); and (vi) the combination 100 µg/kg NIDA41020 + 100 µg/kg capsazepine + 31 µg/kg cannabidiol (Fig. 3f).

Effect of Vehicles or Antagonists on Palmitoylethanolamide-Induced Inhibition of the Vasopressor Responses to Sympathetic Electrical Stimulation

Figure 4 shows that the sympathoinhibition produced by 3.1 µg/kg/min palmitoylethanolamide was: (i) unaltered after 1 mL/kg of DMSO 0.5% (Fig. 4a) or 310 µg/kg AM630 (Fig. 4c); (ii) partially blocked by 100 µg/kg of NIDA42020 (Fig. 4b), 31 and 100 µg/kg of capsazepine (Fig. 4d, e) or 31 µg/kg of cannabidiol (Fig. 4f); and (iii) abolished by the

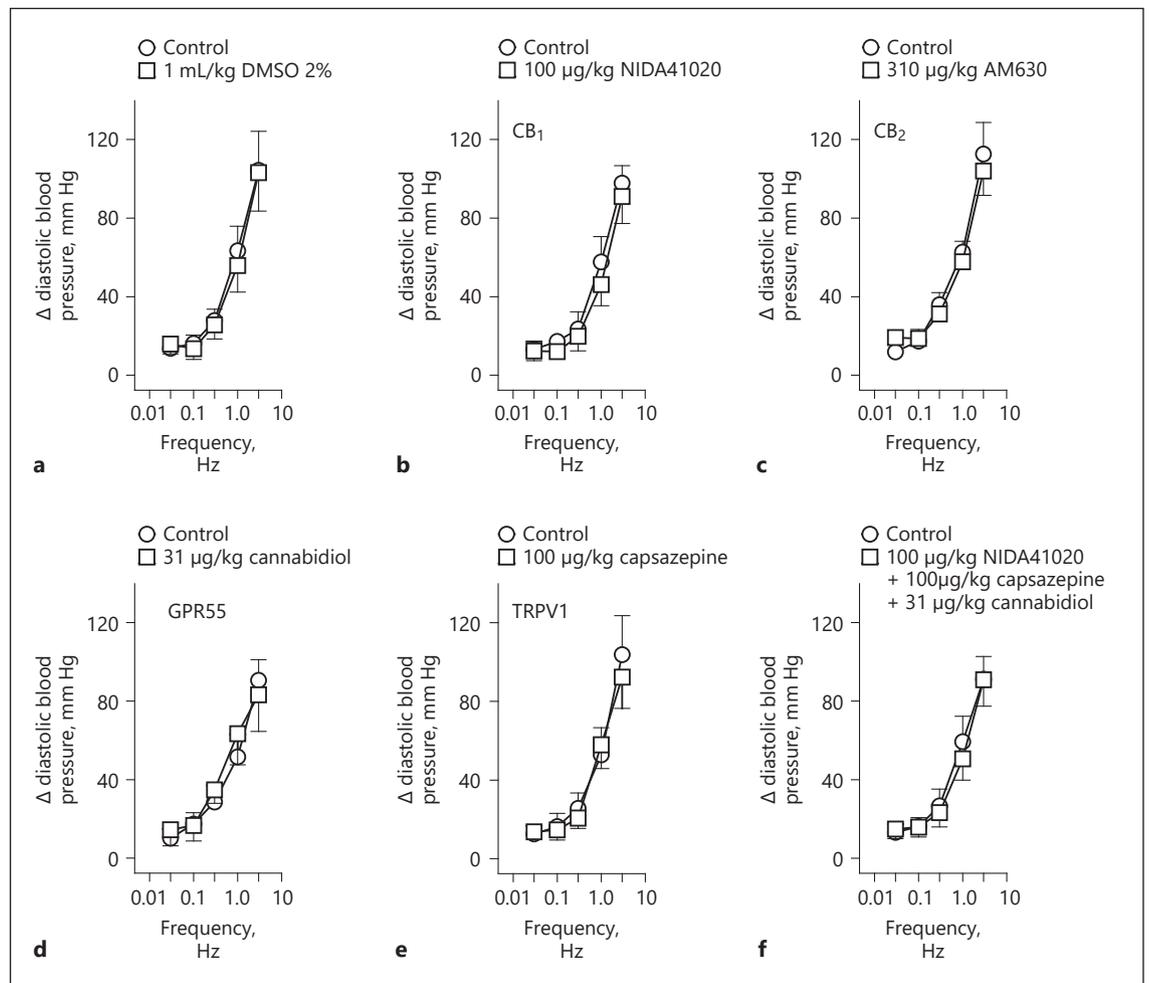combination 100 µg/kg NIDA42020 + 100 µg/kg capsazepine + 31 µg/kg cannabidiol (Fig. 4g).

Effect of Vehicles or Antagonists on Palmitoylethanolamide-Induced Inhibition of the Vasopressor Responses to Exogenous Noradrenaline

As illustrated in Figure 5, the inhibition of the vasopressor responses to noradrenaline by 3.1 µg/kg/min palmitoylethanolamide was: (i) unaltered after 1 mL/kg DMSO 0.5% (Fig. 5a) or 310 µg/kg AM630 (Fig. 5c); (ii) partially blocked by 100 µg/kg NIDA42020 (Fig. 5b), 100 µg/kg capsazepine (Fig. 5d), or 31 µg/kg cannabidiol (Fig. 5e); and (iii) abolished by the combination 100 µg/kg NIDA42020 + 100 µg/kg capsazepine + 31 µg/kg cannabidiol (Fig. 5f).

Discussion

General

Palmitoylethanolamide has emerged as an interesting molecule with several potential therapeutic uses (e.g., in the treatment of neuropathic pain [32]). At the cardiovascular level, palmitoylethanolamide is known to boost the effects of classic endocannabinoids such as anandamide, but its potential direct role in the vascular system remains unclear [14]. Indeed, anandamide and analogues exert complex triphasic effects on blood pressure [33], which

Fig. 3. Effect per se of i.v. bolus injections ($n = 5$ for each figure) of: 1 mL/kg DMSO 2% (**a**); 100 μg/kg NIDA41020 (**b**); 310 μg/kg AM630 (**c**); 100 μg/kg capsazepine (**d**); 31 μg/kg cannabidiol (**e**), and the combination 100 μg/kg NIDA41020 + 100 μg/kg capsazepine + 31 μg/kg cannabidiol (**f**) on the electrically induced vasopressor responses. Empty symbols depict either control responses (○) or non-significant ($p > 0.05$) responses (□) versus the control. Note that no compound produced significant effects ($p > 0.05$ in all comparisons).

basically consist of: (i) a quick transitory decrease in diastolic blood pressure; (ii) a rapid increase in diastolic blood pressure; and (iii) a long-lasting hypotension. Accordingly, this study has made an attempt to identify the pharmacological profile of the palmitoylethanolamide sensitive-receptors mediating inhibition of the vasopressor responses induced by sympathetic stimulation or exogenous noradrenaline. Apart from the implications discussed below and recognising the complex pharmacological properties of the majority of the compounds used (Table 1), the present study shows that, in addition to the hypotension induced by palmitoylethanolamide, this compound inhibits the vasopressor responses induced by

electrical sympathetic stimulation and exogenous noradrenaline. In all cases, the receptors involved resemble the pharmacological profile of CB_1, $TRPV_1$ and GPR55 receptors, but not of CB_2 receptors. Together, these findings shed further light on the potential role of palmitoylethanolamide for modulating per se the systemic vascular tone (i.e., the vasopressor responses to sympathetic stimulation and exogenous noradrenaline) and not only as an "entourage" compound.

It is important to note that all antagonists (used at doses high enough to completely block their respective receptors in pithed rats [8, 9]) failed to modify per se the baseline diastolic blood pressure and heart rate. There-
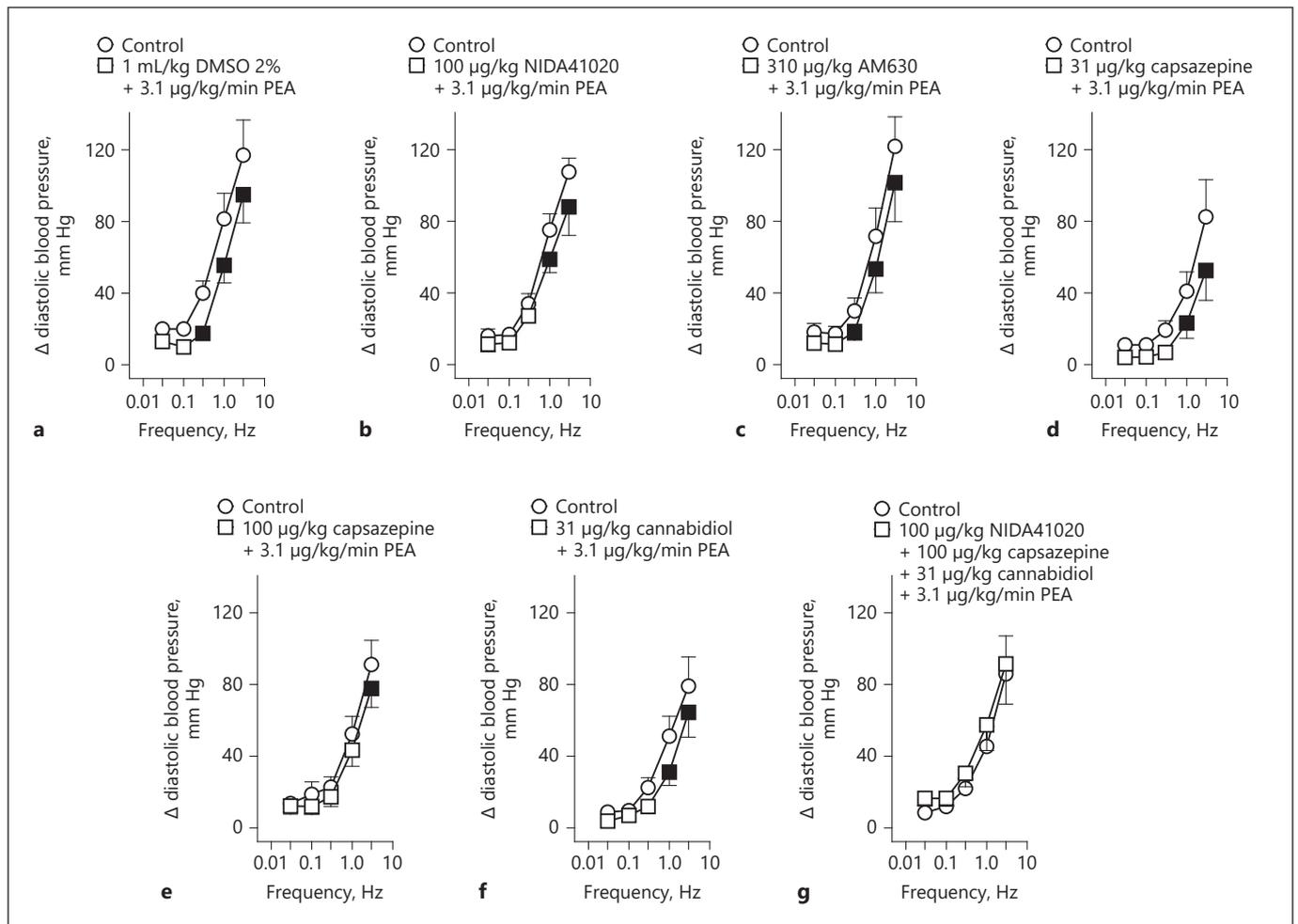
Fig. 4. Effect of i.v. bolus injections of: DMSO 2% (1 mL/kg each; **a**); 100 μg/kg NIDA41020 (**b**); 310 μg/kg AM630 (**c**); 31 and 100 μg/kg capsazepine (**d**, **e**); 31 μg/kg cannabidiol (**f**), and the combination 100 μg/kg NIDA41020 + 100 μg/kg capsazepine + 31 μg/kg cannabidiol (**g**) on palmitoylethanolamide (PEA)-induced inhibition of the vasopressor responses induced by electrical stimulation of the preganglionic (T_7–T_9) sympathetic outflow in pithed rats. Empty symbols depict either control responses (○) or non-significant ($p > 0.05$) responses (□) versus the control. Solid symbols (■) represent significantly different responses ($p < 0.05$) versus the control.

fore, any effect of a given antagonist on the vasopressor sympathoinhibition can be attributed to a direct interaction of the antagonist with its respective receptors on the vasopressor sympathetic nerves and not to physiological antagonism.

Changes on Systemic Haemodynamic Variables Produced by the Different Treatments

A remarkable finding of our study was the capability of 1 and 3.1 μg/kg/min palmitoylethanolamide to produce hypotension in pithed rats (which typically have a low baseline diastolic blood pressure as the central nervous system is not operative; Table 2), and that this re-

sponse was blocked by pretreatment with NIDA41020, capsazepine or cannabidiol, but not by AM630 (Table 3). The simplest interpretation of these findings would be that palmitoylethanolamide-induced hypotension involves the activation of CB_1, TRPV1 and GPR55, but not CB_2, receptors. Nevertheless, palmitoylethanolamide is capable of inducing vasorelaxation by the activation of receptor-dependent and independent mechanisms on vascular smooth muscle [14, 31].

Admittedly, one limitation of the present study is that our results do not allow us to discriminate between direct versus indirect actions induced by palmitoylethanolamide. In this respect, one possibility that this study did
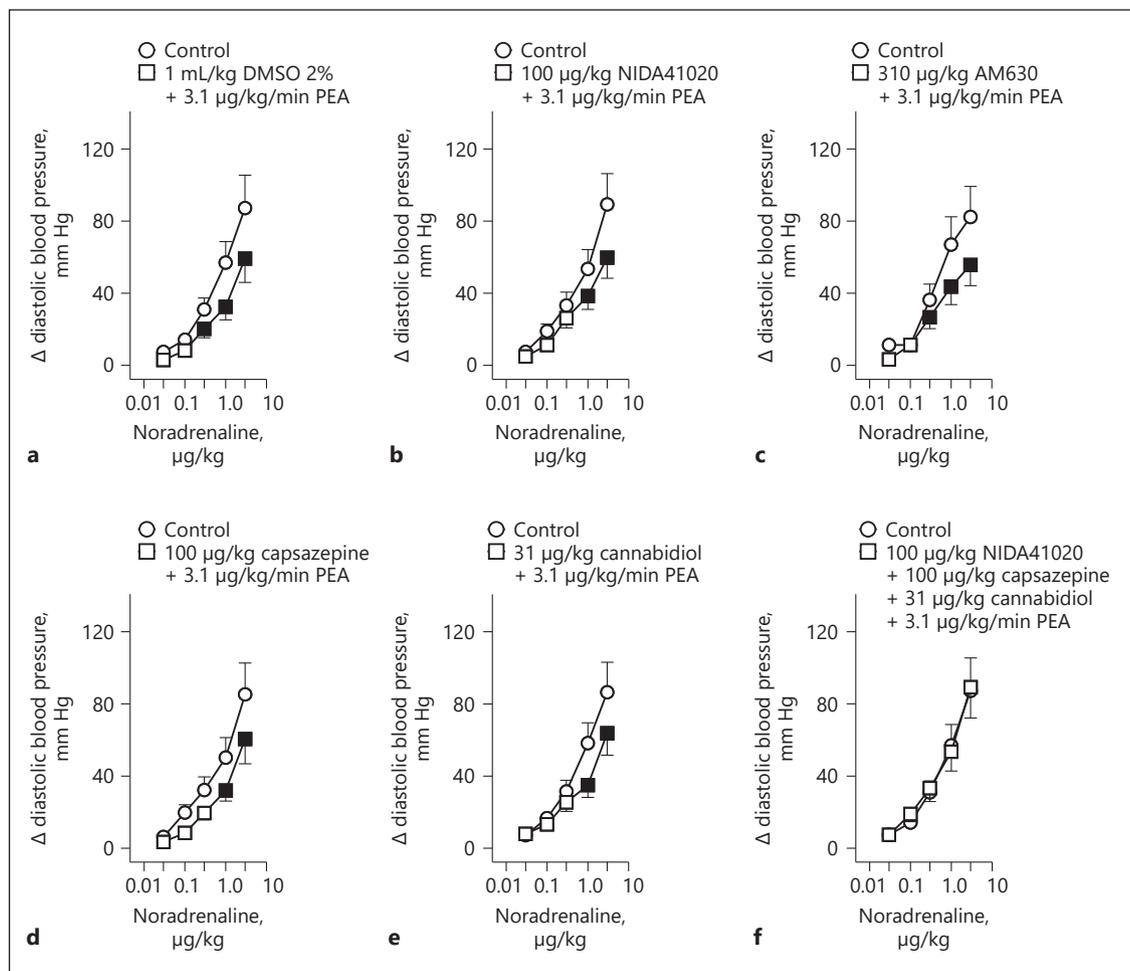
Fig. 5. Effect of i.v. bolus injections of: DMSO 2% (1 mL/kg each; **a**); 100 μg/kg NIDA41020 (**b**); 310 μg/kg AM630 (**c**); 100 μg/kg capsazepine (**d**); 31 μg/kg cannabidiol (**e**), and the combination 100 μg/kg NIDA41020 + 100 μg/kg capsazepine + 31 μg/kg cannabidiol (**f**) on palmitoylethanolamide (PEA)-induced inhibition of the vasopressor responses induced by noradrenaline in pithed rats. Empty symbols depict either control responses (○) or non-significant ($p > 0.05$) responses (□) versus the control. Solid symbols (■) represent significantly different responses ($p < 0.05$) versus the control.

not explore is the potential interference of palmitoylethanolamide with the RAAS, in view that in pithed rats a proportion of blood pressure seems to be maintained by activation of the RAAS, as reported by Schlicker et al. [30]. Consistent with this possibility, infusions of angiotensin II produce tachycardic and vasopressor responses [33, 34]. Clearly, this is a hypothetical speculation that requires other experimental approaches that include the use of i.v. bolus injections of palmitoylethanolamide in pithed rats whose low resting blood pressure is restored by an i.v. continuous infusion of a vasoconstrictor agent (with the RAAS pharmacological approach implemented by Schlicker et al. [30]).

Possible Mechanisms Involved in the Inhibition by Palmitoylethanolamide of the Vasopressor Responses Induced by Sympathetic Stimulation or Exogenous Noradrenaline

Our results show that the vasopressor responses induced by electrical sympathetic stimulation and exogenous noradrenaline were dose-dependently inhibited by palmitoylethanolamide. These inhibitory responses cannot be attributed to tachyphylaxis as the control responses remained unchanged after vehicle (Fig. 1a, 2a). An additional fact drawn from this finding is that no time-dependent changes occurred in our experiments. Moreover, it is worth emphasising that we did not measure electri-

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cally induced perivascular sympathetic nerve activity directly, but the electrically induced neurotransmitter release in the systemic vasculature could be estimated indirectly by measurement of the evoked vasopressor response. In this respect, it may be noted that there was some degree of variability in the control vasopressor responses of the different (sub)groups (Fig. 1–5), as similarly observed in other studies [25, 22, 26, 27]. This is most likely due to individual differences in each animal, but this biological variability certainly has no bearing on the results obtained with the different compounds, as each effect was analysed within the same group of animals, as previously reported [22, 25–27].

The inhibition of the vasopressor responses by sympathetic stimulation seems to be predominantly mediated by TRPV1 receptors, as capsazepine induced a more pronounced blockade of these responses (Fig. 4e) as compared to that produced for the vasopressor responses to exogenous noradrenaline (Fig. 5d). These results suggest that TRPV1 receptors may be mainly located prejunctionally (i.e., on perivascular sympathetic neurons). As TRPV1 receptors are cationic channels related with the excitation and transmission of action potentials, its activity on sympathetic nerves would result in noradrenaline release rather than inhibition. Interestingly, activation of prejunctional TRPV1 receptors on sensory CGRPergic perivascular fibres is classically associated with CGRP release and vasodilatation [35]. Moreover, it has been reported that palmitoylethanolamide activates TRPV1 channels [36]; on the other hand, part of its entourage effect is mediated via TRPV1 [14]. Thus, one possibility for explaining the inhibition by palmitoylethanolamide sensitive to capsazepine is by its possible inhibition of CGRP release from perivascular sensory CGRPergic nerves. Clearly, this speculation needs to be further investigated with additional experiments that fall beyond the scope of the present study.

It is important to highlight that NIDA141020 (a CB_1 receptor antagonist) and cannabidiol (a GPR55 antagonist with other complex pharmacological properties) induced a slight blockade of the inhibition by palmitoylethanolamide on the sympathetic vasopressor responses (Fig. 4b, f, respectively). This blockade suggests the involvement of CB_1 and GPR55 receptors, respectively. Indeed, CB_1 receptors have been associated with the inhibition of noradrenaline release in the systemic vasculature of pithed rats [8]. However, as the affinity of palmitoylethanolamide for CB_1 receptors is rather low (Table 1), it is possible that this effect is also induced by the entourage effect classically described for palmitoylethanolamide

rather than a direct interaction [37]. Finally, the partial blockade by cannabidiol (GPR55 antagonist; Table 1) of the inhibition induced by palmitoylethanolamide on the vasopressor responses is probably mediated by endothelial GPR55 receptors, which have been reported to induce vasodilatation [8, 10].

In the case of the vasopressor responses induced by exogenous noradrenaline, the antagonists NIDA16020046 (CB_1), capsazepine (TRPV1), and cannabidiol (GPR55), but not AM630 (CB_2), induced a partial blockade of the inhibition induced by palmitoylethanolamide when given separately. These results suggest that the inhibition of noradrenaline-induced vasopressor responses involves the sum of a combination of effects mediated by CB_1, TRPV1, and GPR55 receptors. This idea is further supported by the fact that only the combination of CB_1, TRPV1 and GPR55 antagonists could abolish this vasopressor response (Fig. 5f). The same pharmacological profile was observed in the sympathetic vasopressor responses (Fig. 4g). Similar results have been reported where palmitoylethanolamide induces its effects via a complex interaction with CB_1, CB_2, TRPV1, GPR55 and PPAR receptors and channels [38].

Potential Clinical Implications for the Use of Palmitoylethanolamide

Palmitoylethanolamide is commercially available and has recently been suggested for therapeutic use as an anti-inflammatory agent and against retinopathy [39]. Its chronic use has also been suggested to increase the quality of life in patients with certain types of pain [40] and to decrease pain symptoms in patients with fibromyalgia [41]. Nevertheless, these studies seem to be devoid of a careful analysis on the potential side effects, particularly the cardiovascular consequences of the use of palmitoylethanolamide. Our results in pithed rats, an experimental model predictive of systemic cardiovascular side effects [42] that cannot be obtained from in vitro studies [43], suggest that palmitoylethanolamide produces hypotension and an attenuation of the sympathetic vasopressor outflow. These potential vascular side effects should be considered in future clinical studies analysing the therapeutic uses of palmitoylethanolamide.

Conclusion

Taken together, our results suggest that palmitoylethanolamide induced hypotension and an inhibition of the noradrenergic vasopressor responses via complex inter-

actions that involve CB$_1$ (sympathetic), TRPV1 (sensory) and GPR55 (endothelial) receptors. Hence, it seems very likely that the functional activation of these prejunctional and endothelial receptors by palmitoylethanolamide would explain, at least in part, the hypotension produced by this compound.

Statement of Ethics

All experimental protocols in this study were approved by our Institutional Ethics Committee (CICUAL-Cinvestav; permission protocol No. 507-12) in accordance with the guide for the Care and Use of Laboratory Animals in the USA [44] and with the ARRIVE guidelines [45].

Disclosure Statement

All authors declare that they have no competing interests.

Author Contributions

B.A.M.-C. performed the experiments and participated in the design of the study with C.M.V.; E.R.-S.J. and B.A.M.-C. performed the data analysis and produced the graphs. A.M., A.G.-H., C.M.V., and B.A.M.-C. participated in the data interpretation and writing of the manuscript. All authors read and approved the final manuscript.

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