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Anandamide vehicles: a comparative study

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Abstract

Among the studies that investigate the vasorelaxation induced by anandamide, one of the most frequent differences is the use of distinct solvents that could modify vascular function and explain the controversial results described. The aims of this study were: to evaluate the influence of different cannabinoid vehicles in vascular function of rat aorta, and to compare the vasorelaxation induced by anandamide dissolved in different vehicles. Vehicles were: ethanol (70%), Tween 80/ethanol (2:1 and 1:1), 1:1:18 (Tween 80/ethanol/saline) and dimethylsulphoxide (DMSO) 0.5%. All the vehicles tested, except DMSO 0.5%, modified the vascular and/or the endothelial function in rat aorta rings. Anandamide caused a time- and concentration-dependent vasorelaxation in all the experimental groups except in ethanol group, but the mechanisms involved in its vasorelaxation appear to be different depending on the vehicle used. The results obtained with vehicles containing Tween 80 suggest a non-endothelial component in the vasorelaxation caused by anandamide, while those obtained with DMSO at 0.5% suggest an endothelial component in this vasorelaxation.

Keywords: Anandamide; Vehicle; Vasorelaxation; aorta, rat

1. Introduction

Since 1992, when anandamide was identified as the first endogenous cannabinoid receptor ligand, many studies have been carried out to establish the physiological role of this fatty acid amine. Studies in isolated blood vessels have provided evidence of a vasodilator action of anandamide and its analogs, however the precise mechanisms by which these compounds produce vasodilatation remain to be determined (Randall and Kendall, 1998; Kunos et al., 2000; Hogestatt and Zygmunt, 2002). Some authors propose endothelium-dependent mechanisms in the vasorelaxation induced by anandamide while other researchers describe endothelium-independent mechanisms implicated in this vascular effect. Randall et al. (1996) proposed that anandamide may be the endothelium-derived hyperpolarizing factor (EDHF). More recently, other authors have demonstrated that anandamide stimulates the release of EDHF, which may involved myoendothelial gap junctions (Chaytor et al., 1999; Harris et al., 2002). Anandamide has also been shown to cause activation of nitric oxide formation in cultured vascular endothelial cells (Deutsch et al., 1997; Liu et al., 2000; Fimiani et al., 1999; Mombouli et al., 1999) and bovine coronary arteries (Pratt et al., 1998). However, other authors described that vasorelaxant effects of anandamide are largely insensitive to inhibition of nitric oxide synthase in rat mesenteric arteries (White and Hiley, 1997; Jarai et al., 1999). So, the vasorelaxation caused by anandamide seems to be partly endothelium-dependent, in some, but not all preparations (White and Hiley, 1997 Chaytor et al., 1999; Jarai et al., 1999).

Vascular smooth muscle cells and sensory nerves have also been proposed as potential cellular targets for ananda-

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mide in the vascular system. In fact, this cannabinoid inhibits L-type calcium channels, the delayed rectifier potassium channel, the forskolin-induced cAMP formation and the release of calcium from intracellular stores in vascular smooth muscle cells (Holland et al., 1999; Gebremedhin et al., 1999; Van Den Bossche and Vanheel, 2000). Furthermore, other researchers have indicated that the vasodilatory effect of anandamide is endothelium-independent and that this endocannabinoid acts on sensory nerves within the vascular wall, causing the release of a potent vasodilator neuropeptide, the calcitonin gen-related peptide (CGRP) (Zygmunt et al., 1997, 1999; Ralevic et al., 2000; Ho and Hiley, 2003).

On the other hand, it has been suggested that vasodilatation by anandamide would be able to have two components. Wagner et al. (1999) have described in mesenteric arteries that anandamide induced vasorelaxation partially by a *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (SR141716A)-sensitive non-cannabinoid CB_1 receptor site located on the endothelium and partially by a SR141716A-resistant direct action on vascular smooth muscle. Mukhopadhyay et al. (2002) also demonstrated that anandamide can activate at least two different pathways to produce vasorelaxation in rabbit aortic rings: one is endothelium-dependent, SR141716A-sensitive and regulated by a pertussis toxin-sensitive G protein and the other is endothelium-independent, vanilloid VR_1-mediated and G protein-independent.

Regardless, most proposals or demonstrations have been accompanied by subsequent reports that do not support the initial findings leading to the proposal that methodological differences may exist between studies (Randall, 2003).

Non-ionic surface active agents, such as polysorbates, other surface active detergent substances and ethanol are frequently used as solubilization aids in a wide variety of experiments to evaluate the role of water-insoluble substances, such as cannabinoids, in biological processes, such as cardiovascular responses. Ideally, solvents used for these purposes should have no pharmacological effect. In spite of the large variety of ionic or non-ionic surface active agents that could be used to dissolve water-insoluble substances, there are few studies on the modifications that these solvents can provoke in vascular function. To date, the existing results have described that some organic solvents as Tweens, cremophor, ethanol or DMSO, depending on the concentration used and time of exposure, can induce deleterious effects in vascular properties or cause damage in vascular tissue (Zengil et al., 1995; Uluoglu et al., 1996; Lawrence et al., 1998; Rosenblum et al., 2001; Bogman et al., 2003).

Since a methodological difference in the solubilization of cannabinoids could be one of the influential factors in the conflicting results obtained in vasorelaxation by anandamide and, since Tween 80, DMSO and ethanol are commonly included in vehicles used for cannabinoid solubilization, the aim of the present study was firstly to study the modifications that some vehicles, containing Tween 80, ethanol and DMSO, provoke on vascular function in rat aorta, and secondly to examine the vasorelaxation caused by anandamide in rat aorta when it was dissolved in different vehicles. The selected vehicles were: ethanol (700 ml l^{-1}), Tween 80/ethanol (w/v) (1.3 and 0.65 $ml \cdot l^{-1}$), 1:1:18 (Tween 80/ethanol/saline) and DMSO (0.5%).

2. Material and methods

This study was in accordance with European Community Guidelines for the use of experimental animals. Male Wistar rats (250–300 g body weight) were anaesthetised with sodium pentobarbital (50 mg kg^{-1}, i.p.) and the thoracic aorta was carefully removed and placed in ice-cold Krebs–Henseleit solution with the following mM composition: (118 NaCl; 4.75 KCl; 1.2 $MgSO_4$; 1.19 KH_2PO_4; 2.54 $CaCl_2$; 25 $NaHCO_3$; 11 glucose). All connective and perivascular adipose tissues were removed with caution in order to avoid disruption of the endothelium. Transverse vascular rings 3–4 mm long were prepared and fixed vertically between two stainless steel hooks and suspended in a 5-ml jacketed glass organ bath, containing buffer at 37 °C and continuously bubbled with 95% O_2 and 5% CO_2. The upper wire was connected to an isometric force transducer for tension measurements. The rings were mounted with a resting tension of 2 g. Tissues were equilibrated for 90 min, during which time the medium was replaced every 15 min.

In all vessels, the presence of a functional endothelium was tested by precontracting with phenylephrine 10^{-7} M and adding 10 μM of carbachol. Arteries which relaxed to carbachol more than 80% were designated as endothelium-intact preparations. When denuded preparations were used, the endothelium was destroyed by rubbing before being mounted in the organ bath. Arteries which relaxed to carbachol less than 10% were designated as endothelium-denuded preparations.

2.1. Protocol for the study of the modifications caused by the vehicles on vascular function

The experiments were carried out both in intact and in denuded arteries. After 90 min equilibration, the preparations were randomized to a different vehicle experimental group. Arteries were submaximal precontracted with phenylephrine 10^{-6} M and when a stable level of tone was established, cumulative concentration–response curves of each vehicle were constructed. Only one vehicle curve was carried out in each aorta ring. Ethanol, Tween 80/ethanol at two different concentration (1.3 and 0.65 $ml \cdot l^{-1}$, respectively), 1:1:18 (Tween 80/ethanol/saline) and DMSO

0.5% were used. The increasing vehicle concentrations were added at 7–10 min intervals. Control rings were similarly treated with phenylephrine 10^{-6} M but no further additions were made. At the end of each vehicle concentration–response curve, 10 μM of carbachol was added to verify the existence of functional endothelium in the corresponding preparation.

In intact preparations, only after each series of additions, the rings were washed several times with Krebs–Henseleit buffer for a 30–40 min reequilibration period. In the experiments where it was necessary to determine if the vehicle administered produced an inhibition or a destruction of the endothelial cell lining, a third contraction was generated after the reequilibration period by administration of 10^{-6} M of phenylephrine followed by 10 μM-carbachol relaxation.

Fig. 1 shows a scheme of the protocol followed for the investigation of modifications that selected vehicles induced in vascular tone and in endothelium-dependent relaxation.

2.2. Protocol for the study of the vasorelaxation caused by anandamide when dissolved in the different vehicles

The experiments were also carried out both in intact and in denuded arteries. After 90 min equilibration, the preparations were randomized to different experimental groups. Arteries were precontracted with phenylephrine 10^{-6} M and when a stable level of tone was established, cumulative concentration–response curves of anandamide dissolved in the different vehicles were constructed. Only one anandamide curve was carried out in each aorta ring. The increasing anandamide concentrations were added at 7–10 min intervals. Control rings were similarly treated with phenylephrine 10^{-6} M but corresponding vehicle additions were made. At the end of each anandamide concentration–response curve, 10 μM of carbachol was added to verify the existence of functional endothelium in the corresponding preparation.

2.3. Drugs

Phenylephrine and carbachol (Sigma, Poole, Dorset UK) were dissolved in distilled water. Tween 80 (Merck, Schuchardt, Germany), DMSO and ethanol (Panreac Química, Barcelona, Spain) were used for the vehicle mixture. Anandamide (anhydrous ethanol) was obtained from Tocris Cookson, UK.

Tween 80 at concentration of 1.3 $ml \cdot l^{-1}$ was made by mixing one part of ethanol with two parts of Tween 80 by weight, removing the ethanol by evaporation and then adding distilled water to form a 1 ml dispersion. To ensure that the dispersion was homogeneous, the distilled water was added in a series of aliquots of increasing volume (50×2, 100×2, 200 and 500 μl). The mixture was shaken between additions with a vortex mixer (Pertwee et al., 1992). Tween 80 at the concentration of 0.65 $ml \cdot l^{-1}$ was made mixing equal volumes of the previous Tween 80 solution and distilled water to form 1 ml of dispersion.

The vehicle 1:1:18 was made by mixing one part of ethanol, one part of Tween 80 and eighteen parts of saline (9‰ NaCl). A solution of 1 ml was prepared.

The vehicle DMSO 0.5% was made by mixing 5 μl of pure DMSO and 995 μl of distilled water. A solution of 1 ml was prepared.

Ethanol solution was prepared by mixing 700 μl of absolute ethanol and 300 μl of distilled water. A solution of 1 ml was prepared.

Dilutions were made serially. Each dilution step involved the mixture of 1 volume of dispersion with up to 9 volumes of distilled water. All solutions were prepared on the day of the experiment and were protected from light.

The dilutions of the different vehicles selected in this study were the equivalent to those required to dissolve anandamide for a concentration–response curve in a range between 10^{-9} M and 10^{-4} M. Table 1 shows the concentrations, reached in the organ bath, of the different vehicles used to construct the corresponding concentration–response curves.

2.4. Data and statistical analysis

Relaxation responses are expressed as the percentage relaxation of the tone induced by phenylephrine 10^{-6} M. R_{max} is the maximal response obtained when the vehicle or anandamide concentration–response curve is carried out. Data are given as the mean±S.E.M. for n=8–12 rings from, at least, four different animals. Statistical comparisons of concentration–response curves were made by a two-way analysis of variance followed by the Bonferroni/Dunn post-hoc test. Statistical comparisons of carbachol-induced relaxation were made by a Student's

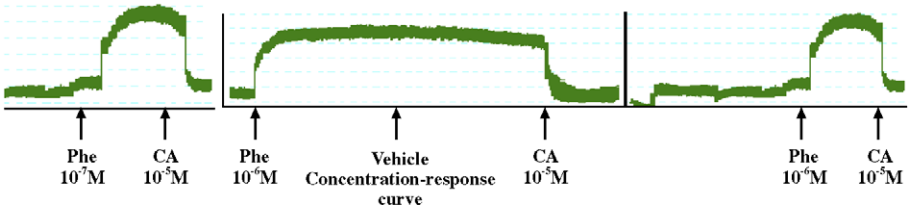

Fig. 1. Protocol for the study of the modifications caused by the vehicles on vascular function. Phe: phenylephrine; CA: carbachol.

Table 1
Concentrations of the vehicles (ml/l) used to carry out the corresponding concentration–response curve in the different experimental groups

Drug	Concentrations of the vehicles administered					
AEA	1:1:18 (50 ml·l^{-1})	"modified 1:1:18" (52.6 ml·l^{-1})	Tween 80 1.3 ml·l^{-1}	Tween 80 0.65 ml·l^{-1}	DMSO 0.5%	Etanol (700 ml·l^{-1})
In bath (M)	In bath (ml/l)	In bath (ml/l)	In bath (ml/l)	In bath (ml/l)	In bath (ml/l)	In bath (ml/l)
10^{-4}	0.5	0.53	0.013	$6.5\cdot10^{-3}$	0.05	6.3
10^{-5}	0.05	0.053	$1.3\cdot10^{-3}$	$6.5\cdot10^{-4}$	$5\cdot10^{-3}$	0.63
10^{-6}	$5\cdot10^{-3}$	$5.3\cdot10^{-3}$	$1.3\cdot10^{-4}$	$6.5\cdot10^{-5}$	$5\cdot10^{-4}$	$6.3\cdot10^{-2}$
10^{-7}	$5\cdot10^{-4}$	$5.3\cdot10^{-4}$	$1.3\cdot10^{-5}$	$6.5\cdot10^{-6}$	$5\cdot10^{-5}$	$6.3\cdot10^{-3}$
10^{-8}	$5\cdot10^{-5}$	$5.3\cdot10^{-5}$	$1.3\cdot10^{-6}$	$6.5\cdot10^{-7}$	$5\cdot10^{-6}$	$6.3\cdot10^{-4}$
10^{-9}	$5\cdot10^{-6}$	$5.3\cdot10^{-6}$	$1.3\cdot10^{-7}$	$6.5\cdot10^{-8}$	$5\cdot10^{-7}$	$7\cdot10^{-5}$

The concentrations administered are the equivalent for carrying out a cumulative-concentration response curve of anandamide (AEA) from 10^{-9} to 10^{-4} M. In the vehicles 1:1:18 and "modified 1:1:18" concentration refer to Tween 80.

t-test. In all cases, a P value≤0.05 was considered to be statistically significant.

3. Results

Phenylephrine 10^{-6} M caused an increase in arterial tone that was similar in all the experimental groups (data not shown).

Control (time-matched) intact and denuded arteries had a similar maximum falldown in the phenylephrine-precontracted tone within the duration of the experiment, approximately 1 h (Table 2). The 10 μM carbachol endothelium-dependent relaxation in intact preparations figures also in Table 2. The 10 μM carbachol endothelium-dependent relaxation in denuded preparations was 6.16±0.82%.

3.1. Effect of ethanol on the vascular function of rat aorta rings

Ethanol provoked a biphasic modification on phenylephrine-induced vascular tone either in intact or in denuded rat aorta rings. It caused a significant maximal decrease in vascular tone by the second-highest concentration used (0.63 ml·l^{-1}) (Table 2), but it provoked an increase in vascular tone by the highest dilution administered (6.3 ml·l^{-1}) (intact arteries: 2.98±7.75%; denuded arteries: 4.53±4.83%).

Administration of ethanol did not modify the endothelium-dependent vasorelaxation in the arteries (Table 2).

3.2. Effect of Tween 80 on vascular function of rat aorta rings

The spontaneous decrease in vascular tone in intact arteries was significantly increased by Tween 80 1.3 ml·l^{-1}, but it was not influenced by Tween 80 0.65 ml·l^{-1} (Table 2).

Neither Tween 80 1.3 ml·l^{-1} nor Tween 80 0.65 ml·l^{-1} modified the phenylephrine-induced vascular tone in endothelium-denuded arteries (Table 2).

Tween 80 at the two concentrations used (1.3 and 0.65 ml·l^{-1}), caused a significant inhibition of the endothelium-dependent relaxation (Table 2).

To identify if the modification in the endothelium-dependent relaxation caused by these vehicles was reversible, a third 10-μM carbachol relaxation was generated in these phenylephrine-precontracted arteries after a new 30–40 min reequilibration period. As shown in Table 2, the third 10 μM-carbachol administration caused a vasorelaxation that reached values no significantly different from those obtained in the control preparations (Table 2).

Table 2
Maximal decreases in vascular tone (R_{max}, %) caused by the different vehicles tested and relaxations (%) induced by 10 μM of carbachol after the vehicle concentration–response curve followed by the 30–40 min reequilibration period in intact arteries

Vehicle	R_{max} (%)		10 μM carbachol relaxations in intact arteries (%)	
	With endothelium	Without endothelium	After vehicle curve	After reequilibration period
Control (time-matched)	14.28±2.89	16.28±3.49	85.97±4.22	89.60±5.94
Ethanol (7×10^{-5}–6.3 ml·l^{-1})	42.60±7.91[a]	13.58±3.44	95.54±8.97	
Tween 80 1.3 ml·l^{-1} (1.3×10^{-7}–1.3×10^{-2}ml·l^{-1})	29.64±4.93[a]	13.77±3.69	9.93±6.4[a]	92.71±2.61
Tween 80 0.65 ml·l^{-1} (6.5×10^{-8}–6.5×10^{-3} ml·l^{-1})	15.01±2.40	10.49±1.61	60.65±5.46[b]	97.49±1.28
1:1:18 (5×10^{-6}–0.5 ml·l^{-1})	16.81±4.45	24.75±9.45	−0.2±0.88[c]	82.21±4.81
"modified 1:1:18" (5.3×10^{-6}–0.53 ml·l^{-1})	16.07±4.84	12.04±2.11	−0.28±.71[c]	84.01±3.71
DMSO 0.5% (5×10^{-7}–5×10^{-2} ml·l^{-1})	19.12±3.91	18.34±2.74	91.70±6.72	

Data are expressed as mean±S.E.M. for 8–12 experiments. A two-way ANOVA followed by the Bonferroni/Dunn post-hoc test was used for statistical comparisons of vehicles concentration–response curves, and a Student's *t*-test was used for statistical comparisons of carbachol-induced relaxation. In both cases: [a]P<0.05 vs. control; [b]P<0.01 vs. control; [c]P<0.001 vs. control.

3.3. Effect of 1:1:18 on the vascular function of rat aorta rings

The vehicle 1:1:18 did not influence the spontaneous decrease in the vascular tone of endothelium intact or denuded phenylephrine-precontracted aorta rings (Table 2).

As we mentioned in the Introduction, ethanol might influence vascular function at both the smooth muscle and the endothelium (Lawrence et al., 1998). To prove if the ethanol present in the vehicle mixture could influence the vascular tone, a modification in this vehicle was introduced: the ethanol was removed by evaporation. This new vehicle, called "modified 1:1:18", did not also modified the spontaneous decrease in vascular tone of endothelium intact or denuded phenylephrine-precontracted aorta rings (Table 2).

When the effect of "unmodified" and "modified" 1:1:18 were compared in intact or denuded arteries, there were no differences between the modifications in vascular tone caused by each vehicle (Table 2).

The vehicles 1:1:18 and "modified 1:1:18" caused a complete inhibition of the endothelium-dependent relaxation in rat aorta rings with respect to the control time-matched preparations (Table 2). The third 10 μM-carbachol administration after a new 30–40 min reequilibration period caused a vasorelaxation that reached values similar to those obtained in the control untreated preparations (Table 2).

3.4. Effect of DMSO 0.5% on the vascular function of rat aorta rings

DMSO 0.5% did not modify the spontaneous in phenylephrine-induced vascular tone in intact or denuded arteries (Table 2).

When the endothelium-dependent relaxation was tested by administration of 10 μM of carbachol at the end of the DMSO 0.5% concentration–response curve in intact arteries, the endothelium-dependent vasorelaxation reached values no significantly different from those obtained when endothelial function was tested in the control time-matched preparations (Table 2).

3.5. Effect of anandamide in rat aorta rings

In intact arteries, and except in the ethanol group, anandamide caused a concentration-dependent vasorelaxation. The relaxant response was slow in onset and took about 7–10 min to peak. The magnitude of the response was similar in all the experimental groups (ethanol: R_{max} of 42.60±7.91%; Tween 80 1.3 ml·l^{-1}: R_{max} of 43.91±6.13%; Tween 80 0.65 ml·l^{-1}: R_{max} of 34.03±5.93%; 1:1:18: R_{max} of 34.1±11.16%; "modified 1:1:18": R_{max} of 43.39±16.87% and DMSO 0.5%: R_{max} of 56.63±9.66%).

Similarly, in denuded preparations, and except in the ethanol group, anandamide also caused a concentration-dependent relaxation. However, in these preparations the magnitude of the response caused by anandamide was different depending on the vehicle used (ethanol: R_{max} of 24.64±6.10%; Tween 80 1.3 ml·l^{-1}: R_{max} of 48.96%±7.57%; Tween 80 0.65 ml·l^{-1}: R_{max} of 60.84±4.27%; 1:1:18: R_{max} of 30.47±8.16%; "modified 1:1:18": R_{max} of 38.92±5.24% and DMSO 0.5%: R_{max} of 20.43±3.14%).

3.6. Vasorelaxation induced by anandamide when it was dissolved in ethanol

Fig. 2 shows the response induced by anandamide (10^{-9}–10^{-5} M) dissolved in ethanol in phenylephrine-precontracted intact and denuded rat aorta rings. In the presence of ethanol, anandamide did not affect the spontaneous decrease in vascular tone, regardless of whether intact or endothelium-denuded vessels were examined.

The maximal response caused by anandamide dissolved in ethanol in intact and in denuded preparations was similar.

3.7. Vasorelaxation induced by anandamide when it was dissolved in Tween 80 1.3 ml·l^{-1} and Tween 80 0.65 ml·l^{-1}

Fig. 3 shows the vasorelaxation induced by anandamide (10^{-9}–10^{-4} M) dissolved in Tween 80 1.3 ml·l^{-1} in phenylephrine-precontracted intact and denuded rat aorta rings. When this solvent was used, the vasorelaxation

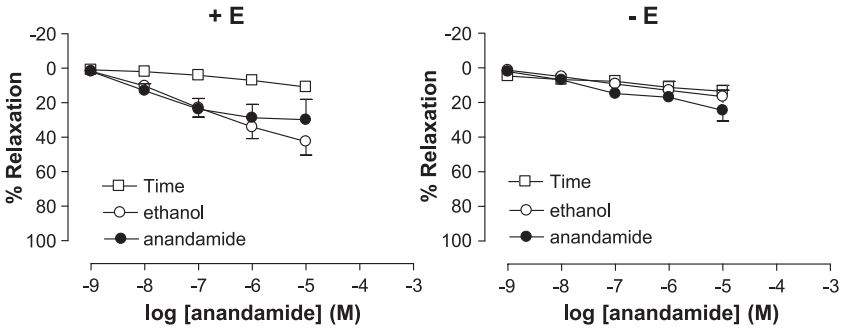

Fig. 2. Concentration-dependent vasorelaxation of Phe 10^{-6} M-precontracted rat aorta rings induced by anandamide dissolved in ethanol in intact and denuded rat aorta rings. Data are expressed as mean±standard error of observations obtained from 8 to 12 preparations. A two-way ANOVA followed by Bonferroni/Dunn post hoc test was used for statistical analysis. +E: with endothelium; −E: without endothelium.

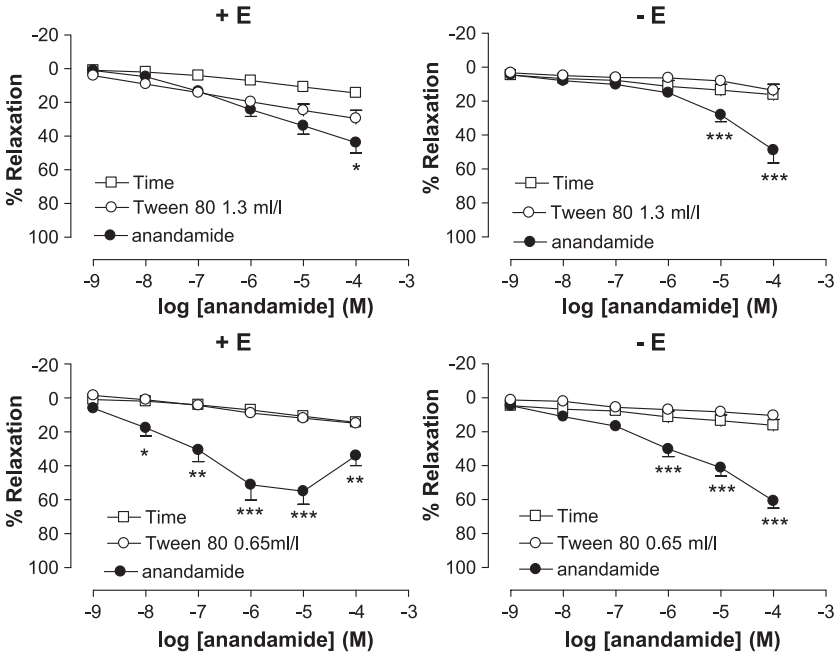

Fig. 3. Concentration-dependent vasorelaxation of Phe 10^{-6} M-precontracted rat aorta rings induced by anandamide dissolved in Tween 80 1.3 $ml \cdot l^{-1}$ (upper graphs) and Tween 80 0.65 $ml \cdot l^{-1}$ (lower graphs) in intact and denuded rat aorta rings. Data are expressed as mean±standard error of observations obtained from 8 to 12 preparations. A two-way ANOVA followed by Bonferroni/Dunn post hoc test was used for statistical analysis (*$P \leq 0.05$ vs. Tween 80 1.13 or 0.65 $ml \cdot l^{-1}$; **$P \leq 0.01$ vs. Tween 80 1.13 or 0.65 $ml \cdot l^{-1}$; ***$P \leq 0.001$ vs. Tween 80 1.13 or 0.65 $ml \cdot l^{-1}$). +E: with endothelium; −E: without endothelium.

induced by anandamide in intact arteries was significantly greater than that obtained in the vehicle-treated arteries. Endothelial denudation also resulted in a significantly greater vasorelaxation induced by anandamide with respect to that observed in denuded vehicle-treated control rings.

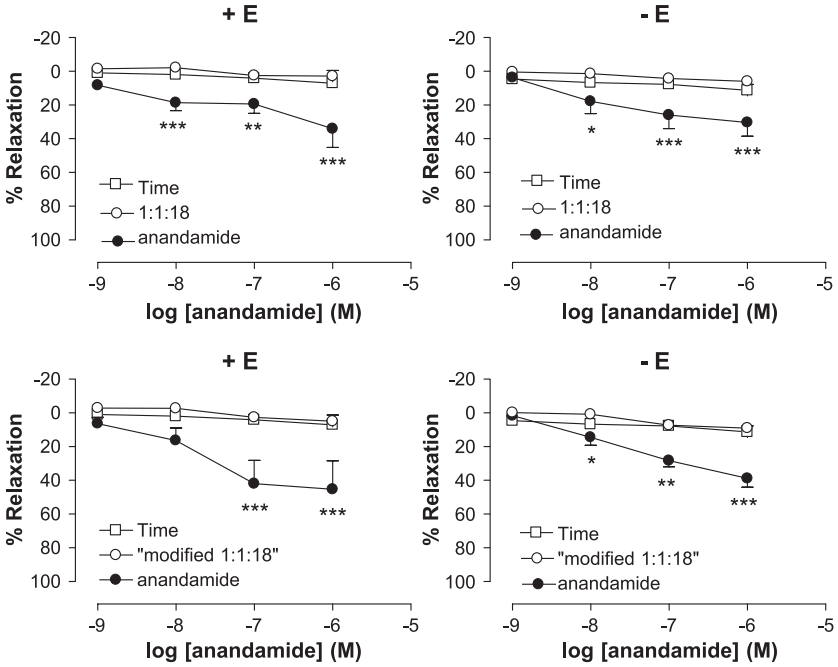

Fig. 4. Concentration-dependent vasorelaxation of Phe 10^{-6} M-precontracted rat aorta rings induced by anandamide dissolved in 1:1:18 (upper graphs) and "modified 1:1:18" (lower graphs) in intact and denuded rat aorta rings. Data are expressed as mean±standard error of observations obtained from 8 to 12 preparations. A two-way ANOVA followed by Bonferroni/Dunn post hoc test was used for statistical analysis (*$P \leq 0.05$ vs. 1:1:18 or "modified 1:1:18"; **$P \leq 0.01$ vs. 1:1:18 or "modified 1:1:18"; ***$P \leq 0.001$ vs. 1:1:18 or "modified 1:1:18"). +E: with endothelium; −E: without endothelium.

The maximal vasorelaxation caused by anandamide dissolved in Tween 80 1.3 ml·l^{-1} in intact and in denuded preparations was similar (Fig. 3, upper graphs).

Fig. 3 also shows the vasorelaxation induced by anandamide (10^{-9}–10^{-4} M) dissolved in Tween 80 0.65 ml·l^{-1} in phenylephrine-precontracted intact and denuded rat aorta rings. When this solvent was used, either in intact or in denuded preparations the vasorelaxation caused by anandamide was significantly greater than that observed in the corresponding vehicle-treated control preparations.

The maximal vasorelaxation caused by anandamide dissolved in Tween 80 0.65 ml·l^{-1} in intact and in denuded preparations was also similar (Fig. 3, lower graphs).

3.8. Vasorelaxation induced by anandamide when it was dissolved in "unmodified" and "modified" 1:1:18 (v:v:v) (Tween 80/ethanol/saline)

Fig. 4 shows the vasorelaxation induced by anandamide (10^{-9}–10^{-6} M) dissolved in 1:1:18 in phenylephrine-precontracted intact and denuded rat aorta rings.

When anandamide was dissolved in this vehicle mixture, it was able to produce, both in intact and in denuded aorta preparations, a relaxation in the vascular tone that was greater than and statistically different from the vasorelaxation caused by the corresponding vehicle-treated control preparations.

The maximal vasorelaxation caused by anandamide dissolved in 1:1:18 in intact and in denuded preparations was similar (Fig. 4, upper graphs).

Fig. 4 also shows the vasorelaxation induced by anandamide (10^{-9}–10^{-6} M) dissolved in "modified 1:1:18" in phenylephrine-precontracted intact and denuded rat aorta rings. When anandamide was dissolved in this vehicle, a similar pattern to that obtained in the 1:1:18 groups was observed. Anandamide dissolved in "modified 1:1:18" was able to produce, either in intact or in denuded aorta preparations, a vasorelaxation that was greater than and statistically different from that obtained in the corresponding vehicle-treated control preparations.

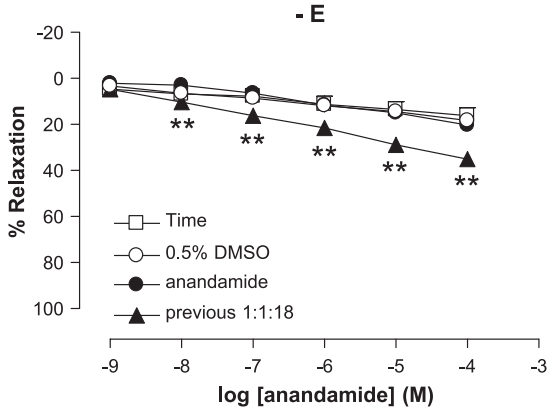

Fig. 6. Concentration-dependent vasorelaxation of Phe 10^{-6} M-precontracted rat aorta rings induced by anandamide dissolved in DMSO 0.5% in denuded rat aorta rings in presence (▲) or absence (●) of a previous administration of the vehicle 1:1:18 that contains 50 ml/l of Tween 80. Data are expressed as mean±standard error of observations obtained from 8 to 12 preparations. A two-way ANOVA followed by Bonferroni/Dunn post hoc test was used for statistical analysis (*P<0.05 vs anandamide; **P≤0.01 vs. anandamide).

The maximal vasorelaxation caused by anandamide dissolved in "modified" 1:1:18 in intact and in denuded preparations was also similar (Fig. 4, lower graphs).

3.9. Vasorelaxation induced by anandamide when it was dissolved in DMSO 0.5%

Fig. 5 shows the vasorelaxation induced by anandamide (10^{-9}–10^{-4} M) dissolved in DMSO 0.5% in phenylephrine-precontracted intact and denuded rat aorta rings.

Anandamide dissolved in DMSO 0.5% caused a vasorelaxation significantly greater than the relaxation produced by the vehicle alone in intact rat aorta rings. Endothelial denudation resulted in the disappearance of the vasorelaxant effect of this cannabinoid, resulting in relaxant values similar to those obtained in the corresponding vehicle-treated control rings.

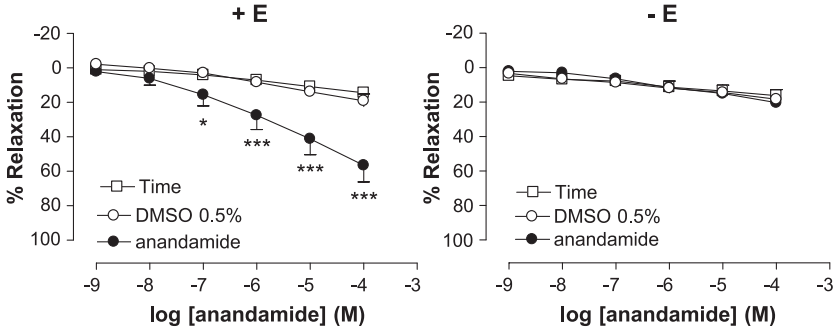

Fig. 5. Concentration-dependent vasorelaxation of Phe 10^{-6} M-precontracted rat aorta rings induced by anandamide dissolved in DMSO 0.5% in intact and denuded rat aorta rings. Data are expressed as mean±standard error of observations obtained from 8 to 12 preparations. A two way ANOVA followed by Bonferroni/Dunn post hoc test was used for statistical analysis (*P≤0.05 vs. DMSO 0.5%; ***P≤0.001 vs. DMSO 0.5%). +E: with endothelium; −E: without endothelium.

3.10. Vasorelaxation induced by anandamide dissolved in DMSO 0.5% in denuded arteries and previous administration of the vehicle 1:1:18

Fig. 6 shows the vascular response induced by anandamide (10^{-9}–10^{-4} M) dissolved in DMSO 0.5% in phenylephrine-precontracted denuded rat aorta rings, previous administration of the vehicle 1:1:18. Under these conditions, anandamide was able to induce a significant vasorelaxation that was not observed without the addition of 1:1:18.

4. Discussion

When a general review of the scientific literature on vascular actions of anandamide is carried out, it can be observed that a large number of different, non-reproducible and opposite results exist. A more detailed review of these articles reveals that the vehicles used for endocannabinoid solubilization are different and that, in most cases, their influence on vascular function has not been evaluated. Could this fact be the reason for the controversial and non reproducible results obtained?

Our study provides evidence on the influence of different vehicles, frequently used as cannabinoid solvents, on vascular tone and endothelial function in rat aorta rings. In addition, this study demonstrates for the first time that the vasorelaxant effect of anandamide varies depending on the vehicle in which this drug is dissolved.

There is no chemical company that offers anandamide in a native form. Anandamide is supplied dissolved, normally in ethanol and in a solution of 5 mg/ml. Ethanol might influence vascular function at both the level of smooth muscle and the endothelium. Lawrence et al. (1998) described that ethanol causes an increase in intracellular calcium levels in endothelial cells and in smooth muscle provoking vasorelaxation and vasoconstriction, respectively. The vasoconstriction is only observed in endothelium-denuded arteries. We have also described that ethanol, in intact arteries, caused a biphasic modification in vascular tone, resulting an initial marked vasorelaxation following by a vasoconstriction. When ethanol was tested in endothelium-denuded arteries, we did not observed a significant decrease in the vascular tone of the preparations, but the highest concentration tested (6.3 $ml \cdot l^{-1}$) also caused an increase in the vascular tone, confirming the results obtained by Lawrence et al.

On the contrary, the endothelium relaxant response to carbachol was not influenced by ethanol administration, supporting the findings of other authors that have also described that ethanol does not modify the acetylcholine induced vasorelaxation in rat aorta when it is used at low concentrations (Hatake et al., 1993).

Tween 80 at 1.3 $ml \cdot l^{-1}$, but not at 0.65 $ml \cdot l^{-1}$, caused a significant concentration-dependent decrease in vascular tone. It has been described that Tween 80 inhibits the contractile effects of vasoactive agents such phenylephrine, serotonin and bradykinin (Zengil et al., 1995). Our results are have also demonstrated that Tween 80, at the highest concentration used, was not able to maintain the vascular tone in phenylephrine-precontracted arteries, provoking a significant decrease in the vascular tone of intact arteries. On the other hand, the Tween 80 vasorelaxant effect in intact arteries was endothelial-dependent, because it disappeared when arteries were denuded of endothelium. It has been described that non-ionic surfactants alter cellular membrane and cells become nonspecifically permeable to substances that ordinarily do not penetrate intact cells. Tween 80 and Tween 60 act on similar fashion, and the effect is reversible (Hards and Wright, 1984; Hards and Patterson, 1986; Stano et al., 1997). Recently, some authors have concluded that surfactants demonstrate a transporter-specific interaction, rather than unspecific membrane permeabilization (Bogman et al., 2003). Although further investigations should be carried out, the results in this study would be able to support this possibility. Tween 80 at 1.3 $ml \cdot l^{-1}$ could permeabilize endothelial cells and the ion influx between intracellular and extracellular medium could trigger vasodilatory effects. In denuded arteries, the same cell permeabilization occurred in vascular smooth muscle cells, but the mechanisms involved are only able to maintain the vascular tone.

Both Tween 80 vehicles provoked a partial, but significant, inhibition in endothelium-dependent relaxation of phenylephrine-precontracted intact arteries. Although we can not deduce the precise mechanism of these effects on the vascular function, our results are in agreement with other authors that have demonstrated that higher concentrations of Tween 80 (10^{-1}–10^{-3} $ml \cdot l^{-1}$) inhibit the potency of Ach, an endothelium-dependent vasorelaxant, by a direct effect on endothelial integrity causing a marked desquamation of vascular endothelium (Zengil et al., 1995, Uluoglu et al., 1996). This study has demonstrated a reversible inhibition of the endothelium-dependent vasorelaxation, resulting in the total recovery of the carbachol response after the reequilibration period in Tween 80 experimental groups, which bears little correlation to histological damage of this cell lining.

The 1:1:18 and "modified 1:1:18" mixtures produced parallel results: while they did not cause a modification in the vascular tone, they provoked a total inhibition of carbachol-mediated vasorelaxation. The presence or absence of ethanol ("unmodified" or "modified" forms respectively) in this vehicle mixture was not responsible for the modifications in endothelial function in our preparations. The high concentration of Tween 80 presents in this vehicle mixture (50 $ml \cdot l^{-1}$), much higher than in the Tween 80 vehicles (1.3 and 0.65 $ml \cdot l^{-1}$), could explain the total inhibition of carbachol vasorelaxation produced. Indeed, different experiments carried out in our laboratory using two different concentrations of Tween 80 diluted in water or saline (50 and 25 $ml \cdot l^{-1}$) have confirmed these data,

resulting 10 μM carbachol relaxations after the vehicle concentration response curve less than 5% in all the cases. As we previously mentioned, other authors have also described modifications of endothelium-dependent vasodilatation by concentrations of Tween 80 even lower than those (Zengil et al., 1995, Uluoglu et al., 1996).

DMSO 0.5% was the only vehicle that was able to maintain vascular tone and endothelium-dependent relaxation at values similar to those obtained at the beginning of the experiment and in control arteries. We carried out experiments with DMSO at other concentrations (pure, 50%, 20%, 10%, 5%, 1%, 0.8%), and all of them produced a significant decrease in vascular tone although they did not modify the endothelium-dependent relaxation in phenylephrine-precontracted arteries (data not shown). Some studies have described that DMSO (0.01–0.2%) inhibited the concentration dependent dilation caused by pinacidil in arteries from cats and rats, interacting with an oxygen-sensitive site on the channel (Rosenblum et al., 2001). We did not test this possibility in our preparations, but since no modification was produced by this vehicle in vascular function we can assume that, at least, in our preparation, DMSO 0.5% is an innocuous solvent that maintains both smooth muscle and endothelial function. Besides, it has been described that DMSO has a membrane penetrate action. Studies on membrane penetration and carrier effect of DMSO have been carried out in agriculture, basic biology, animals and man. All of them describe that when substances are combined with DMSO these compounds have an absolute rate constant for penetration greater than their rate when they were administered alone (Jacob and Herschler, 1986). This property gives DMSO an advantage with respect to the other vehicles tested in our studies.

Anandamide supplied by the chemical company Tocris (solution in ethanol 5 mg/ml) could be solubilized for administration in the bath in a concentration range between 10^{-9} and 10^{-4} M in the following vehicles: ethanol, Tween 80 1.3 $ml \cdot l^{-1}$, Tween 80 0.65 $ml \cdot l^{-1}$ and DMSO 0.5%. When ethanol was used, the maximal concentration of anandamide administered in the experiments was 10^{-5} M. As we mentioned previously, at the concentration of 10^{-4} M this vehicle provoked an increase in the vascular tone of endothelium intact and denuded arteries. This contractile effect of the vehicle could mask the vasodilatory effect of anandamide in our results. For this reason, we decided not to include this concentration in the assays with the endocannabinoid.

It was impossible to dissolve anandamide to be administered in the same concentration range when 1:1:18 and "modified 1:1:18" were used because of the commercial concentration supplied. In that case, the anandamide concentration range administered was 10^{-9}–10^{-6} M.

Surprisingly, anandamide did not cause vasorelaxation when it was dissolved in ethanol. On the contrary, when anandamide was dissolved in whatever of the other vehicles, it induced a time- and concentration-dependent relaxation of phenylephrine-contracted intact rat aorta rings. This is partially consistent with previous findings in a variety of resistant vessels from the rat (Chaytor et al., 1999; Grainger and Boachie-Ansah, 2001; Hogestatt and Zygmunt, 2002; Randall et al., 2002). There are not many studies on the vasorelaxant effect of anandamide in conduit vessels, only one, in rabbit aorta rings (Mukhopadhyay et al., 2002). The vasorelaxation caused by anandamide in rabbit aorta was similar to that obtained in this study in all the experimental groups with intact arteries. In general, the vasorelaxation caused by anandamide in conduit vessels is around 50–60% of the precontacted tone (Mukhopadhyay et al., 2002), and this relaxation is usually greater in resistance arteries, coronary, hepatic and mesenteric, reaching relaxant values close to 100% of precontracted tone (Randall and Kendall, 1998; Wagner et al., 1999; Zygmunt et al., 1999; Harris et al., 2002).

Apart from the description of anandamide vasorelaxation in rat aorta, the most important contribution of this study is to demonstrate that for the same vascular bed, the vasorelaxant effect of anandamide depends on the vehicle used.

When anandamide was dissolved in ethanol, no vasorelaxation was observed in intact and in denuded rat aorta rings. When this endocannabinoid was solubilized in DMSO 0.5%, relaxation was induced only in intact arteries but not in denuded arteries. In addition and as we described previously, it must be pointed out that DMSO 0.5% did not cause an inhibition in endothelium-dependent relaxation, suggesting these results that anandamide dissolved in DMSO 0.5% caused an endothelium-dependent relaxation in rat aorta.

On the contrary, when Tween 80 is one the components of the vehicle mixture, anandamide caused a relaxation similar in intact and denuded arteries. Furthermore, both the Tween 80 vehicle mixtures, at the two different concentrations tested, and the vehicles 1:1:18 and "1:1:18 modified" provoked an inhibition of endothelium-dependent relaxation. These data seem to indicate that the vasorelaxation caused by this cannabinoid dissolved in Tween 80 vehicles is endothelium-independent. Besides, the magnitude of the vasorelaxation induced by anandamide was similar in intact and in denuded arteries when the cannabinoid was dissolved in the same vehicle mixture.

Is it possible that the vasorelaxation caused by anandamide has two components: an endothelial and a non-endothelial component, and only when one of them is inhibited the other one became evident? As Tween 80 is the main difference between the vehicle mixtures in which non-endothelial component of anandamide vasorelaxation is noted, we have carried out experiments in which the vehicle 1:1:18 (in which the concentration of Tween 80 is the highest used) was administered previously to perform a concentration–response curve of anandamide dissolved in DMSO 0.5% in denuded arteries. Anandamide was able to cause a concentration-dependent relaxation greater than that induced without the previous 1:1:18 administration. This

preliminary data could confirm that Tween 80 is able to permeabilize the vascular smooth muscle and permits that anandamide causes vasorelaxation by an internal mechanism. In fact, other authors have used Tween 80 as a permeabilization agent for different types of cells (Hards and Wright, 1984; Hards and Patterson, 1986; Stano et al., 1997; Bogman et al., 2003). Further investigations are necessary to evaluate the mechanisms implicated in the vasorelaxation induced by anandamide when it is dissolved in distinct vehicles.

To conclude, this study shows the influence of the vehicle used to dissolve anandamide in the characterization of the mechanisms involved in the vasorelaxation induced by anandamide. The vehicles in which Tween 80 is present would suggest a non- endothelial component in the vasorelaxation caused by anandamide, while the vehicle DMSO at 0.5% would suggest an endothelial component in the vasorelaxation induced by this endocannabinoid. Subsequent studies with other cannabinoid substances have to be carried out to confirm if it exist parallel results.

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