Cytochrome P450 Pathway Contributes to Methanandamide-induced Vasorelaxation in Rat Aorta

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Abstract
Purpose The generation of hyperpolarising vasorelaxant endothelial cytochrome P450 epoxygenase (CYP)—derived metabolites of arachidonic may provide beneficial effects for the treatment of cardiovascular diseases in which the bioavailability of NO is impaired. The cannabinoid methanandamide has vasodilatory properties linked to hyperpolarisation. The aim of the present work was to investigate the vasorelaxant effects of methanandamide in rat aorta, focusing on the role of cytochrome P450 pathway.
Methods Changes in isometric tension in response to a cumulative concentration-response curve of methanandamide (1 nM–100 μM) were recorded in aortic rings from male Wistar rats. The involvement of cannabinoid receptors, endothelial nitric oxide (NO)-, prostacyclin- and some hyperpolarising-mediated pathways were investigated. The activation of large-conductance Ca^{2+}-activated K^+ (BKCa) channels have also been evaluated.
Results Methanandamide provoked an endothelium-dependent vasorelaxation in rat aorta, reaching a maximal effect (Rmax) of 67%±2.6%. This vasorelaxation was clearly inhibited by the combination of CB_1 and CB_2 cannabinoid antagonists (Rmax: 21.6%±1.3%) and by the combination of guanylate cyclase and CYP inhibitors (Rmax: 16.7%±1.1%). The blockade induced separately by guanylate cyclase (31.3%±2.8%) or CYP (36.3%±6.6%) inhibitors on methanandamide vasorelaxation was not significantly modified by either CB_1 or CB_2 inhibition. BKCa channels inhibition caused a partial and significant inhibition of the methanandamide vasorelaxation (Rmax: 39.9%±3.3%).
Conclusions Methanandamide endothelium-dependent vasorelaxation is mediated by CB_1 and CB_2 cannabinoid receptors. The NO- and CYP-mediated pathways contribute in a concurrent manner in this vascular effect. Stimulation of both cannabinoid receptor subtypes is indistinctly linked to NO or CYP routes to cause vasorelaxation.

Key words Rat aorta · Vasorelaxation · Methanandamide · Cytochrome P450 epoxygenase pathway

Introduction

The endothelium releases nitric oxide (NO), prostacyclin and endothelium-derived hyperpolarizing factors to regulate arterial tone. Epoxyeicosatrienoic acids (EET) are endothelial cytochrome P450 epoxygenase-derived metabolites of arachidonic acid that activate large-conductance Ca^{2+}-activated K^+ channels (BKCa), causing hyperpolarisation of vascular smooth muscle and vasorelaxation [1, 2].

The formation of EET in endothelial cells is an important step in the NO- and prostacyclin-independent vasodilation of several vascular beds [3]. In fact, EET seem to be of major importance in the regulation of vascular tone in cases of endothelial dysfunction in which the bioavailability of NO is impaired [4]. So, therapies that increase the generation of EET or prevent their degradation may provide

beneficial effects for the treatment of diseases such as hypertension [5]. Furthermore, it has recently been pointed out that EET mimetics open up new and potentially fruitful research directions for the identification of novel therapeutic approaches for treating cardiovascular diseases [6].

The cardiovascular effects of both synthetic and endogenous cannabinoids have been examined and reviewed extensively, indicating they are vasodilators and hypotensive agents [7, 8]. Besides, endothelium is involved in the vasorelaxant properties of cannabinoids [9–11]. In relation to this, there is evidence that the endocannabinoids anandamide, virodhamine and 2-arachidonoylglycerol cause vasorelaxation through arachidonic acid metabolism via cyclooxygenase and/or epoxygenase pathways [11–15].

Methanandamide, the metabolic stable analog of the endocannabinoid anandamide, causes hypotension in rats [16–18]. In relation to vasodilator properties, methanandamide causes substantial hyperpolarisation (involving ATP-dependent potassium channels) of smooth muscle cells from isolated rat resistance vessels, resulting in vessel dilation [19, 20], and significantly increases BKCa channel currents in mouse aortic myocytes [21]. However, there have been no studies investigating the possible involvement of cytochrome P450-derived metabolites in methanandamide-induced vasorelaxation in resistance or conduit vessels.

Recent studies suggest that therapies that increase EET generation may provide beneficial effects in the treatment of diseases such as hypertension. Methananadamide shows an important hypotensive effect that may be linked to hyperpolarisation. Based on these data, we sought to investigate the mechanisms involved in the methanandamide vasorelaxant effect in the rat aorta, focusing on the involvement of cytochrome P450 enzymatic pathway in inducing this effect.

Material and methods

The experiments were designed and performed in strict accordance with the EC regulations for care and use of experimental animals (EEC Nº 86/609) and were approved by the Ethical Committee at the Rey Juan Carlos University.

Tissue preparation

Male Wistar rats (250–300 g body weight) were anaesthetised with sodium pentobarbital (50 mg/kg, i.p.). The abdomen was opened by a midline incision, and the aorta was carefully excised and placed in ice-cold Krebs-Henseleit solution (118 mM NaCl; 4.75 mM KCl; 1.2 mM $MgSO_4$; 1.19 mM KH_2PO_4; 2.54 mM $CaCl_2$; 25 mM $NaHCO_3$; 11 mM glucose). All connective and perivascular adipose tissue was removed, and care was taken to ensure the endothelium was not disrupted. Transverse vascular rings (3–4 mm long) were prepared. Some of the rings were deliberately denuded by rubbing and rolling them around with stainless steel forceps before being mounted [11].

Afterwards, rings were fixed vertically between two stainless steel hooks, suspended in a 5-ml jacketed glass organ bath containing Krebs-Henseleit 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 (Grass FT07), and tension measurements were recorded by computer (PowerLab/4e program). 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.

Experimental protocol

The effects of methanandamide on submaximal phenylephrine (Phe) (1 μM)-induced tone in endothelium intact rings was examined as described below. After an equilibration period, arteries were precontracted with Phe. When a stable level of tone was established, cumulative concentration-response curves to methanandamide were constructed (1 nM–100 μM). Control rings were similarly treated with 1 μM Phe, but corresponding vehicle additions (dimethylsulfoxide (DMSO) 0.5%) [22] were included. At the end of each cannabinoid concentration-response curve, carbachol (10 μM) was added to verify the existence or absence of a functional endothelium in the preparation. Arteries that relaxed more than 70% in response to carbachol were designated as endothelium-intact preparations.

To evaluate the role of the endothelium in the vascular effects of methanandamide, a cumulative concentration-response curve to cannabinoid (1 nM–100 μM) was constructed in endothelium-denuded preparations. In our studies, we considered rubbed rings that relaxed to carbachol less than 10% as endothelium-denuded arteries.

All subsequent experiments were performed using intact endothelium preparations. When antagonists/inhibitors were used in the experimental procedure, they were administered in the organ bath prior to Phe-induced contraction; the concentrations and time incubations for the experiments are specified in the following paragraphs.

As previously described by our group [10, 11], to test the involvement of the cannabinoid receptors CB_1 and CB_2 in the vascular effects of methanandamide, the following experiments were performed: 1) to evaluate the implication of $G_{i/0}$ protein-coupled receptors, aorta rings were pretreated with 300 ng/ml of pertussis toxin for 3 h; 2) to selectively evaluate CB_1 participation, aorta rings were pretreated with 1 μM of the selective CB_1 receptor antagonist, N- (piperidin-

1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (rimonabant) for 15 min; 3) to selectively evaluate CB_2 participation, aorta rings were pretreated with 1 μM of the selective CB_2 receptor antagonist, N-((1S)-endo-1,3,3-trimethyl bicycle (2.2.1) heptan-2yl)-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl) pyrazole-3 carboxamide (SR144528) for 15 min.

The involvement of NO pathway in the vascular response elicited by methanandamide was examined by pretrating aorta rings for 30 min with 1 μM of the guanylate cyclase inhibitor 1H-[1, 2, 4]oxadiazolo[4,3-a] quinoxalin-1-one (ODQ) [23]. Prostacyclin pathway contribution was measured by pretreating the preparations for 30 min with 10 μM indomethacin (cycloxygenase (COX) inhibitor) [10, 11]. Lastly, to test the involvement of EDHF, cytochrome P450 epoxygenase-derived metabolites and BKCa in the vascular effects of methanandamide, aorta rings were pretreated for 30 min with apamin plus charybdotoxin (EDHF inhibitor) at 10 nM each [10, 11], 5 μM 17-octadecynoic acid (17-ODYA) (cytochrome P450 epoxygenase and ω/ω-1-hydroxylase inhibitor) [10, 11] or 100 nM of iberiotoxin (BKCa blocker) [24].

When it was required, experiments with an analogous experimental protocol but with combined administrations of antagonists/inhibitors in the pretreatment period were also performed.

Besides, to evaluate the selective effect of the pretreatment with pertussis toxin on cannabinoid vascular responses, parallel concentration-response curve of a non-G protein vasorelaxant agent, sodium nitroprusside (1 nM–1 μM) [25], in the presence or absence of pertussis toxin (300 ng/ml for 3 h) were constructed. Similarly, to evaluate the nule effect of 17-ODYA on NO-mediated pathway, parallel concentration-response curve of the NO donor, sodium nitroprusside (1 nM–1 μM), in the presence or absence of 17-ODYA (5 μM for 30 min) were also carried out.

Parallel control antagonist/inhibitor experiments were also contructed preincubating the preparations with the corresponding antagonist/inhibitor or their combination prior to Phe precontration followed by vehicle (DMSO 0.5%) concentration-response curve. When the vehicle used to dissolve the antagonists or inhibitors was ethanol, its effects were also evaluated. For each aorta ring, only one experiment was carried out.

Statistical analysis

All relaxation responses were expressed as a percentage of the initial 1 μM Phe-evoked tone. R_{max} represents the maximal response obtained for the methanandamide concentration-response curve.

pEC_{50} values refer to the negative logarithm of the concentration of methanandamide that produced half (50%) the maximal relaxation obtained (EC_{50}). Cannabinoids are highly lipophilic compounds, and methanandamide was supplied as a 5 mg/ml solution in ethanol (Tocris Cookson). The maximun methanandamide concentration that could be solubilised in 0.5% DMSO for administration in the bath was 100 μM. For this reason, we decided to use 100 μM methanandamide in the present work and for the vascular effect to fit the R_{max} in the logistic equation for pEC_{50} calculation, even though this concentration could not provoke the maximal vasorelaxant effect that could be reached with this cannabinoid. EC_{50} values were obtained from each concentration-relaxation curve by fitting the normalised data to the four-parameter logistic equation (GraphPad PRISM V4.00):

$$Y = \text{Bottom} + (\text{Top} - \text{Bottom})/1 + 10^{(\text{LogEC50}-X)\cdot\text{HillSlope}}$$

where X is the logarithm of the methanandamide concentration, and Y is the response; Bottom is the lower response plateau, and Top is the upper response plateau, equivalent to the percent maximal relaxation response R_{max}; EC50 is the X value when Y is halfway between Bottom and Top, and HillSlope is the slope factor that describes the steepness of the curve.

Data are expressed as the mean ± the standard error of the mean (s.e.m.), obtained from 8 to 12 preparations taken from, at least, five different animals. Each experimental protocol was carried out on a maximum of two different preparations from the same animal. The number of animals in each group is expressed by *n*. Methanandamide concentration-response curves in the different experimental groups were compared using an analysis of variance (ANOVA), followed by a Bonferroni/Dunn post-hoc test. *P* values <0.05 were considered significant.

Methanandamide concentration–response curve is identical in all figures of the manuscript. This curve is obtained from all the data collected through out all the experimental design. From all animals, 1–2 preparations (aortic rings) were always kept for a control curve.

Drugs

Phe, carbachol, ODQ, indomethacin, apamin, charibdotoxin, 17-ODYA, sodium nitroprusside and iberiotoxin were obtained from Sigma (Sigma Chemical Company, Poole, Dorset U.K.). Pertussis toxin was supplied by Research Biochemicals International (RBI, USA). Methanandamide was supplied by Tocris Cookson (Bristol, U.K.). Rimonabant and SR144528 were obtained from Sanofi Recherche (Montpellier, France).

Phe, carbachol, apamin, charibdotoxin, sodium nitroprusside, iberiotoxin and pertussis toxin were dissolved in distilled water. Indomethacin, ODQ, rimonabant, SR144528 and 17-

Table 1 Phenylephrine (Phe)-induced contraction, Carbachol (Ca)-induced relaxation and maximal decrease in Phe-induced tone (Rmax) in the different experimental time, vehicle and antagonist/inhibitor control groups

Experimental group	Phe 1μM-induced tone (mN)	Ca 10μM relaxation (%)	Rmax (%)
Time (control)	9.00±0.64	88.21±4.34	16.87±2.83
0.5% DMSO (intact arteries)	8.84±1.10	91.70±6.72	14.37±2.87
0.5% DMSO (denuded arteries)	11.09±0.82	2.59±0.47***	14.90±2.11
Ethanol	9.60±1.16	94.85±12.01	18.37±4.97
300 ng/ml pertussis toxin	11.13±0.88	79.45±7.48	12.29±1.96
1 μM rimonabant	10.49±1.09	73.66±7.32	16.57±5.70
1 μM SR144528	8.83±0.86	76.98±3.57	16.96±2.94
1 μM rimonabant + 1 μM SR144528	9.44±1.49	79.02±12.16	25.51±5.15*
10 μM indomethacin	10.71±0.85	75.22±3.13	12.42±1.48
100 nM apamin + 100 nM charybdotoxin	10.16±0.89	87.88±12.82	22.94±3.61*
5 μM 17-ODYA	9.49±0.70	73.73±3.64	13.30±2.18
1 μM ODQ	15.56±0.70***	2.12±0.35***	8.35±1.48
1 μM ODQ + 5 μM 17-ODYA	16.12±1.15***	4.38±2.33***	6.31±2.23
1 μM rimonabant + 1 μM ODQ	15.41±1.15***	2.33±1.06***	11.91±2.29
1 μM SR144528 + 1 μM ODQ	16.42±0.58***	0.62±0.27***	11.49±2.76
1 μM rimonabant + 5 μM 17-ODYA	10.69±0.94	71.98±2.15	11.68±4.91
1 μM SR144528 + 5 μM 17-ODYA	11.14±0.54	70.92±4.75	14.99±3.14
100 nM iberiotoxin	10.83±0.70	72.31±6.82	16.19±3.71

Values are expressed as mean ± s.e.m. for 4–12 animals. *$P<0.05$; ***$P<0.001$ vs control

ODYA were dissolved in ethanol (Panreac Química S.A., Barcelona, Spain). A cannabinoid stock solution (10^{-2} M) was prepared daily in 0.5% DMSO (v/v). Additional dilutions were made by mixing 1 volume of stock solution with up to nine volumes of distilled water.

Results

As Table 1 shows, except in the experimental groups which include ODQ in the pretreatment period, 1 μM Phe caused similar increases in arterial tone in all preparations of the different experimental groups. Analogous results were obtained when carbachol response were tested in the preparations of the different experimental groups at the end of the protocol. Finally, all vehicle concentration-response curves resulted similar in the different experimental groups.

When ethanol was used as the vehicle for the antagonists or inhibitors, the maximum volume administered in the organ bath was 5 μl, in order to prevent any modification of the functionality of the preparation (Table 1) [22].

Vasorelaxation caused by methanandamide in rat aorta ring: involvement of endothelium

Methanandamide caused a concentration-dependent vasorelaxation in intact rat aorta rings, resulting in a R_{max} value of 67%±2.6% and a pEC_{50} value of 5.7±0.1 (n=12) (Fig. 1). This vasorelaxation was gradual in onset and took 7–10 min to reach a plateau at each concentration step.

Endothelial denudation resulted in the complete inhibition of the vasorelaxation induced by methanandamide (R_{max}=10.9%±1.9% (n=7)) resulting a maximal effect similar to that obtained in the corresponding (denuded rings) vehicle (0.5% DMSO) group (R_{max}=14.9%±2.1% (n=4)) (Fig. 1).

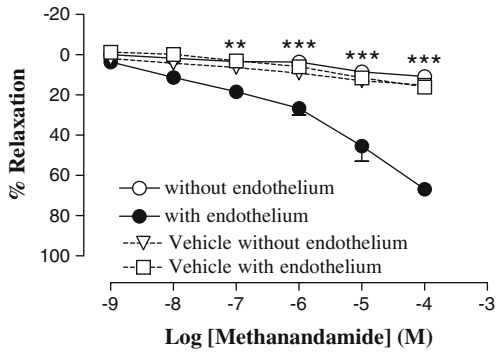

Fig. 1 Concentration-response curve for the relaxation of methanandamide in isolated rat aorta rings precontracted by phenylephrine (1 μM) in the presence or in the absence of functional endothelium. Statistical significance: **$P<0.01$, ***$P<0.001$ vs with endothelium. The 0.5% dimethylsulphoxide (DMSO) *curve* represents the effect of the vehicle alone in the presence and absence of endothelium

Involvement of $G_{i/0}$ protein-coupled receptors in vasorelaxation caused by methanandamide in rat aorta rings

Incubation of the rat aorta ring preparations with 300 ng/ml pertussis toxin for 3 h abolished the vasorelaxation caused by methanandamide (R_{max}=19.5%±3.6% (n=5) P<0.001 vs metanandamide: R_{max}=67%±2.6% (n=12)) (Fig. 2a), resulting in a maximal effect similar to that obtained in the corresponding control group (concentration-response curve of vehicle in preparations previously pretreated with pertussis toxin) (R_{max}=12.3%±2.0% (n=4)).

Involvement of CB_1 and CB_2 receptors in vasorelaxation caused by methanandamide in rat aorta rings

The vasorelaxation caused by methanandamide in endothelium-intact aorta rings was significantly reduced due to pretreatment with 1 μM of the CB_1 antagonist rimonabant for 15 min (R_{max}=31.9%±4.3% (n=6), P<0.001 vs methanandamide: R_{max}=67%±2.6% (n=12)) (Fig. 2b) and after pretreatment with 1 μM of the CB_2 antagonist SR144528 for 15 min (R_{max}=42.1±5.0% (n=5), P<0.001 vs methanandamide: R_{max}=67%±2.6% (n=12)) (Fig. 2c). Furthermore, when the preparation was pretreated with a combination of rimonabant and SR144528 at 1 μM each for 15 min, the methanandamide-induced vasorelaxation was clearly abolished (R_{max}=21.6%±1.3% (n=5), P<0.001 vs methanandamide: R_{max}=67%±2.6% (n=12)) (Fig. 2d), resulting in similar values to those obtained in the corresponding control arteries (concentration-response curve of vehicle in preparations previously pretreated with rimonabant plus SR144528) (R_{max}=21.2%±2.9% (n=5)).

Involvement of EDHF, prostacyclin, NO and cytochrome P450 epoxygenase pathways in vasorelaxation caused by methanandamide in rat aorta rings

Pretreatment with 10 μM of the COX inhibitor indomethacin for 30 min did not cause any modification of the methanandamide-induced vasorelaxation in rat aorta tissue (R_{max}=56.6%±5.9%, pEC_{50}=5.7±0.04 (n=9) P>0.05 vs methanandamide: R_{max}=67%±2.6%, pEC_{50}=5.7±0.05 (n=12)) (Fig. 3a). In addition, pretreatment with apamin plus charibdotoxin at 10 nM each for 30 min did not also provoke any modification of the methanandamide-induced vasorelaxation in rat aorta (R_{max}=61.3%±5.1%, pEC_{50}=5.4±0.06 P>0.05 (n=6) vs methanandamide: R_{max}=67%±2.6%, pEC_{50}=5.7±0.05 (n=12)) (Fig. 3a).

However, pretreatment with 1 μM ODQ for 30 min or pretreatment with 5 μM of the cytochrome P450 epoxygenase and ω/ω-1-hydroxylase inhibitor, 17-ODYA, for 30 min provoked similar and significant inhibitions of the methanandamide-induced vasorelaxation in rat aorta rings (ODQ: R_{max}=31.3%±2.9% (n=7), P<0.001 vs methanandamide: R_{max}=67%±2.6% (n=12)) (Fig. 3b); 17-ODYA: R_{max}=36.3%±6.6% (n=5) P<0.001 vs methanandamide: R_{max}=67%±2.6% (n=12)) (Fig. 3c)). Furthermore, when

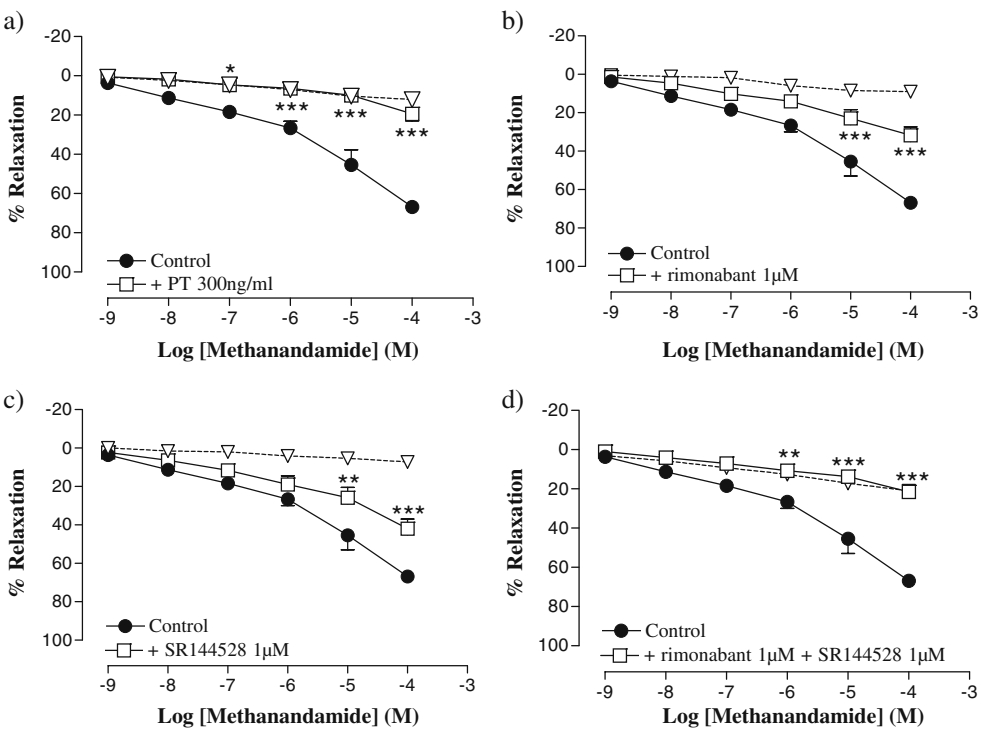

Fig. 2 Effect of pertussis toxin (*PT*) (**a**), rimonabant (**b**), SR144528 (**c**) and rimonabant plus SR144528 (**d**) on the relaxation produced by methanandamide in intact rat aorta rings precontracted by phenylephrine (1 μM). Statistical significance: **P*<0.05, ***P*<0.01, ****P*< 0.001 vs control. *Dotted line* in each graph represents the concentration-response curve of vehicle in preparations previously pretreated with the corresponding antagonist/inhibitor alone or their combinations

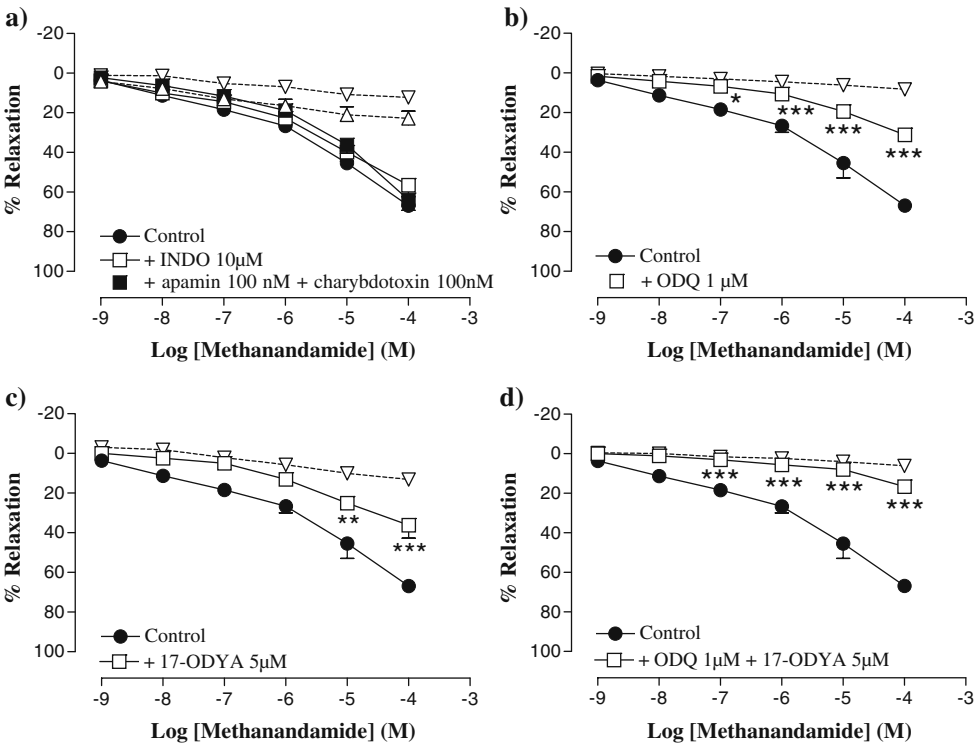

Fig. 3 Effect of apamin plus charibdotoxin and indomethacin (INDO) (**a**), ODQ (**b**), 17-ODYA (**c**), and 17-ODYA plus ODQ (**d**) on the relaxation produced by methanandamide in intact isolated rat aorta rings precontracted by phenylephrine (1 μM). Statistical significance: **P*<0.05, ***P*<0.01, ****P*< 0.001 vs control. *Dotted line* in each graph represents the concentration-response curve of vehicle in preparations previously pretreated with the corresponding antagonist/inhibitor alone or their combinations. In figure (**a**) upper triangles corresponds to apamin plus charibdotoxin and botton triangles correspondes to INDO

preparations were simultaneously incubated with ODQ 1 μM plus 17-ODYA 5 μM, a complete inhibition of methanandamide vasorelaxant effect was obtained (R_{max}= 16.7%±1.1% (*n*=7), *P*<0.001 vs methanandamide: R_{max}= 67%±2.6% (*n*=12)), resulting in similar values to those obtained in the corresponding control arteries (concentration-response curve of vehicle in preparations previously pretreated with ODQ 1 μM plus 17-ODYA 5 μM) (R_{max}= 6.3%±2.2% (*n*=5)) (Fig. 3d).

Contribution of CB_1 and CB_2 receptors in activating NO vs cytochrome P450 pathways involved in the vasorelaxation caused by methanandamide in rat aorta rings

In order to clarify whether there is a specific role for each cannabinoid receptor subtype in activating cytochrome P450 or NO pathways involved on methanandamide-induced vasorelaxant effect on rat aorta, experiments with dual blockades were performed.

Pretreatment with 17-ODYA 5 μM plus rimonabant 1 μM for 30 min provoked a similar inhibition of the methanandamide-induced vasorelaxation in rat aorta rings than those caused by both drugs separately (17-ODYA plus rimonabant: R_{max}=39.9%±2.4% (*n*=7), *P*>0.05 vs 17-ODYA: R_{max}=36.3%±6.6% (*n*=5); vs rimonabant: R_{max}= 31.9%±4.3% (*n*=7) (Fig. 4c). Furthermore, pretreatment with 17-ODYA 5 μM plus SR144528 1 μM for 30 min also provoked a similar inhibition of the methanandamide-induced vasorelaxation in rat aorta rings than those caused by both drugs separately (17-ODYA plus SR144528: R_{max}=37.0%±6.6% (*n*=7), *P*>0.05 vs 17-ODYA: R_{max}= 36.3%±6.6% (*n*=5); vs SR144528: R_{max}=42.1%±5.0% (*n*=7) (Fig. 4d).

However, pretreatment with ODQ 1 μM plus rimonabant 1 μM for 30 min provoked a significant increase in the inhibition of the methanandamide-induced vasorelaxation in rat aorta rings in relation to those caused by both drugs separately (ODQ plus rimonabant: R_{max}=21.4%±2.0% (*n*=7), *P*<0.05 vs ODQ: R_{max}=31.3%±2.8% (*n*=5); vs rimonabant: R_{max}=31.9%±4.3% (*n*=7) (Fig. 4a).

In addition, pretreatment with ODQ 1 μM plus SR144528 1 μM for 30 min also provoked a significant increase in the inhibition of the methanandamide-induced vasorelaxation in rat aorta rings than that caused by SR144528 alone (ODQ plus SR144528: R_{max}=28.5%±1.4% (*n*=6), *P*<0.05 vs SR144528: R_{max}=42.1%±5.0% (*n*=5)), but it caused a similar percentage of inhibition of the methanandamide-induced vasorelaxation in rat aorta rings than that caused by ODQ alone: R_{max}=31.3%±2.8% (*n*=7) (Fig. 4b).

Involvement of BKCa in vasorelaxation caused by methanandamide in rat aorta rings

Incubation of the preparations with 100 nM of the BKCa channel blocker, iberiotoxin, for 30 min significantly inhibited the methanandamide-induced vasorelaxant effect (R_{max}=39.9%±3.3% (*n*=6) *P*<0.001 vs methanandamide: R_{max}=67%±2.6% (*n*=12)) (Fig. 5).

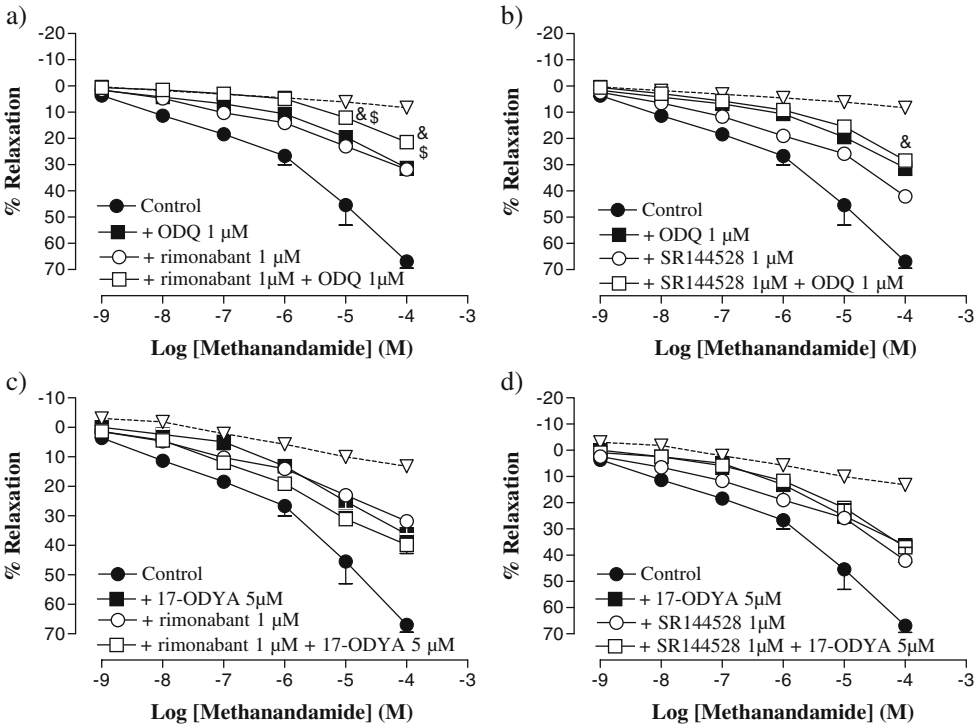

Fig. 4 Contribution of CB_1 and CB_2 cannabinoid receptors in activating NO (**a**, **b**) and cytochrome P450 (**c**, **d**) pathways involved in the vasorelaxation caused by methanandamide in rat aorta rings. Statistical significance: & $P<0.05$ vs rimonabant or SR144528 alone; $ $P<0.05$ vs 17-ODYA or ODQ alone. *Dotted line* in each graph represents the concentration-response curve of vehicle in preparations previously pretreated with the corresponding antagonist/inhibitor alone or their combinations

Involvement of Gi/0 protein-coupled receptors and cytochrome P450 epoxygenase pathway in vasorelaxation caused by sodium nitroprusside in rat aorta

Incubation of the rat aorta ring preparations with 300 ng/ml pertussis toxin for 3 h did not affect the vasorelaxation caused by sodium nitroprusside (R_{max}=113.3%±3.1% (n=5) $P>0.05$ vs sodium nitroprusside: R_{max}=109.1%±1.4% (n=5)) (Fig. 6a).

In addition, incubation of the rat aorta ring preparations with 17-ODYA 5 μM did not affect the vasorelaxation caused by sodium nitroprusside (R_{max}=115.2%±3.5% (n=5) $P>0.05$ vs sodium nitroprusside: R_{max}=126.7%±5.7% (n=5)) (Fig. 6b).

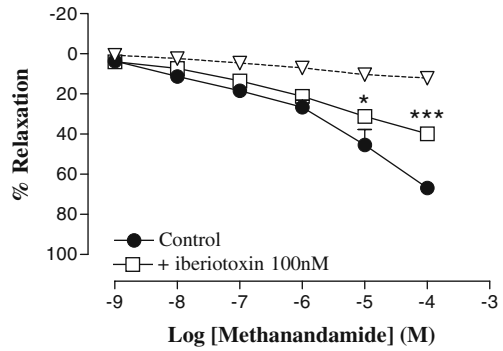

Fig. 5 Effect of iberiotoxin on the relaxation produced by methanandamide in intact rat aorta rings precontracted by phenylephrine (1 μM). Statistical significance: *$P<0.05$, ***$P<0.001$ vs control. *Dotted line* in this graph represents the concentration-response curve of vehicle in preparations previously pretreated with the corresponding inhibitor alone

Discussion

The present study demonstrates in rat aorta, that methanandamide causes an important vasorelaxant effect (about 70%) that is mediated by the CB_1 and CB_2 receptors. Nitric oxide (NO) and cytochrome P450 epoxygenase pathways contribute to methanandamide vasorelaxant properties in a concurrent manner. In addition, stimulation of both cannabinoid receptors is linked to these two intracellular pathways to mediate the vasorelaxant effect of methanandamide in rat aorta. Moreover, methanandamide through both subtypes of cannabinoid receptors can stimulate indistinct NO or cytochrome P450 epoxygenase routes to cause vasorelaxation in function of the bio-availability of these pathways. This work also demonstrates the contribution of BKCa channels on methanandamide vasorelaxant properties in rat aorta.

Methanandamide is the stable analogue of the endocannabinoid, anandamide, and as many cannabinoid agonists, it is a vasorelaxant agent [8]. Studies using isolated rat resistance vessels have shown that methanadamide provokes vasorelaxation in gastric, coronary, cerebral and mesenteric arteries [26–29]. The present study corroborates the methanandamide vasorelaxant effect in rat aorta. The

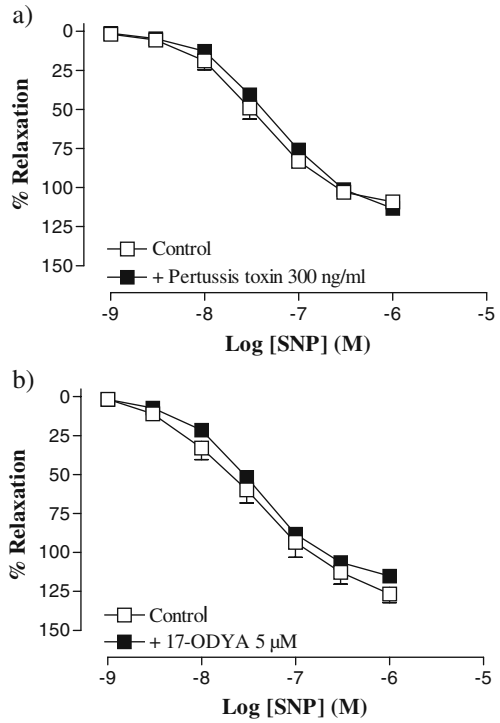

Fig. 6 Effect of pertussis toxin (**a**) and 17-ODYA (**b**) on the relaxation produced by sodium nitroprusside in intact isolated rat aorta rings precontracted by phenylephrine (1 μM)

methanandamide efficacy and potency observed (about 70% of maximal effect and an EC_{50} of 1.4 μM, respectively) are both lower than those described in rat resistance vessels (around 90% of efficacy, with an EC_{50} of 0.3 μM for potency). Mukhopadhyay et al. described the vasorelaxant effect of methanandamide in rabbit aorta, obtaining values of around 80% of efficacy, and EC_{50} of 0.6 μM for potency [30]. Species differences could explain this discrepancy [31].

Two $G_{i/0}$ coupled-cannabinoid receptors, CB_1 and CB_2, have been proposed to mediate the vascular actions of many cannabinoids in different vessels [7–9]. Our data confirm the selective implication of $G_{i/0}$-protein coupled receptors in the vasorelaxation effects caused by methanandamide in the rat aorta, since the cannabinoid vascular effect were significantly reduced with treatment with pertussis toxin, a $G_{i/0}$-protein blocker; but it did not affect the non-G-protein mediated vasorelaxation caused by sodium nitroprusside (Fig. 6). These data are in agreement with those described by other authors in conduit or resistance vessels [30, 32, 33].

A role has been demonstrated for CB_1, or rimonabant-sensitive receptors in the vasorelaxant effects of methanandamide in resistance arteries from rats [27, 34] and in rabbit aorta [30]. However, there are little data available addressing the role of CB_2 receptors in methanandamide-mediated vascular effects, and the few existing data discard a role for CB_2 receptors in rat mesenteric and gastric arteries [29, 34]. In the rat aorta, the vasorelaxant effect of methanandamide was sensitive to CB_1 and CB_2 receptor antagonists. Furthermore, the combination of rimonabant plus SR144528 resulted in a higher and complete inhibition of methanandamide vasorelaxation than that obtained using both antagonists separately. These data confirm that both, CB_1 and CB_2 receptors are involved in the methanandamide-induced vasorelaxation in rat aorta.

There is a general consensus regarding the pivotal role of the endothelium in the methanandamide vasorelaxant effect in rat resistance vessels [35–38]. Our results confirm the importance of endothelium in methanandamide vascular effect in the rat aorta, since denudation of the ring preparations led to the complete abolishment of the cannabinoid-induced vasorelaxation.

With respect to the involvement of endothelial derived relaxing factors in methanandamide-induced aorta vasorelaxation, it has been demonstrated the role of NO production and hyperpolarisation of smooth muscle cells in the methanandamide vasorelaxant effect in rat resistance vessels [14, 19, 20, 35–38]. However, in relation to prostacyclin production, there are insufficient data available to confirm or reject this possibility in conduit vessels. Besides, there have been no studies investigating the possible involvement of endothelilum-derived vasorelaxant cytochrome P450-derived metabolites (that could mediate hyperpolarisation) in methanandamide-induced vasorelaxation in resistance or conduit vessels. Our results confirm the involvement of NO mediated-pathway in methanandamide vasorelaxant effect in the rat aorta since pretreatment of the ring preparations with the guanylate cyclase inhibitor, ODQ, provoked a significant reduction (47% of maximal effect) of this vascular response. Besides, they also show that COX-derived metabolites are not involved in the vasorelaxant effect of methanandamide in rat aorta, since no modification of the methanandamide-induced vascular reponse was observed in the presence of indomethacin. Similar results have been described in studies using different resistance isolated arteries [14, 29].

In relation to the third endothelium-dependent pathway associated with hyperpolarisation, mention that it has been attributed to a noncharacterised endothelial factor, endothelium-derived hyperpolarising factor (EDHF), which could include different candidates as EETs (cytochrome P450 epoxygenase-derived metabolites of arachidonic acid AA), potassium ions or hydrogen peroxide (H_2O_2) [39–41], among others. The hyperpolarization caused by these mediators could imply intermediate and small conductance calcium-activated potassium channels, IK_{Ca} or SK_{Ca} respectively, or large-conductance calcium-activated potassium channels (BKCa channels) [42–44]. When the involvement of potassium channels in the vasorelaxant effect induced by

methanandamide was evalauted, two different results were obtained. Pretreatment with apamin plus charibdotoxin did not affect the methanandamide concentration-relaxation curve in rat aorta, but incubation with iberiotoxin resulted in significant inhibition (43% of maximal effect) of the methanandamide vascular effect in rat aorta, indicating that BKCa channels-mediated hyperpolarisation might be mainly involved in vasorelaxation caused by methanandamide in rat aorta. Similar results have been demonstrated in mouse aortic myocytes [21].

Since it has been demonstrated that EET provoke vascular smooth muscle hyperpolarisation by the direct activation of BKCa channels [43, 44], and that methanandamide is able to activate BKCa channels in rat aorta to caused vasorelaxation, the possible involvement of cytochrome P450 epoxygenase pathway in methanandamide vascular effect has been evaluated in the present work. Our experimental data show that treatment with 17-ODYA, the cytochrome P450 epoxygenase inhibitor, resulted in a significant reduction (48% of maximal effect) of the methanandamide vascular effect. Moreover, the level of inhibition was similar to that obtained with iberiotoxin. In summary, our results suggest that methanandamide, via the cytochrome P450 epoxygenase, generates endothelial mediators that stimulate BKCa channels, thereby provoking hyperpolarisation and vasorelaxation. We would like to point out that in this work has not been identified the possible cytochrome P450 epoxygenase derivated metabolite that can be able to stimulate BKca channels. Some candidates as EETs have already mentioned but other as H_2O_2 could be also involved. This latter mediator is an important endothelial vasorelaxant byproduct of oxidative cytochrome P450 metabolism of arachidonic acid, and that it is also able to cause a hyperpolarization-mediated vasorelaxation through potassium channels [45]. More research is needed to definitively establish the implication of this novel pathway in methanandamide vasorelaxation in rat aorta.

In summary, two different endothelium-dependent vasorelaxant pathways are involved in metahandnamide vasorelaxant response: NO-mediated and cytochrome P450-mediated pathways. Furthermore, these two intracellular pathways are independent and additive for methanandamide-induced vasorelaxation, since the pretreatment of the preaparations with ODQ and 17-ODYA simultaneously, completely blocked the methanandamide vascular response. Moreover, in rat aorta preparations the vasorelaxation caused by the NO donor, sodium nitroprusside, was not affected by 17-ODYA, confirming the independence of NO-mediated and cytochrome P450-mediated pathways (Fig. 6).

To clarify the role of the individual CB receptors in activating NO vs cytochrome P450 pathways on methahandamide-induced vasorelaxation in rat aorta, experiment with dual blockades rimonabant or SR144528 plus 17-ODYA; or rimonabant or SR144528 plus ODQ were also performed. Although slight significant increase in the inhibition of methanandamide vasorelaxant effect was noted with dual blockades respect to individual inhibition of NO-mediated pathway, our data suggest that both receptors are involved in either activating NO and in activating cytochrome P450 pathways, since dual blockades did not subtantially modified the inhibition caused by antagonists or inhibitors separately. Some studies have also demonstrated that CB receptor stimulation are linked to NO-related vasorelaxation [11, 12, 15], but there is not many published studies to confirm or reject the relationship between CB cannabinoid receptors and cytochrome P450 pathway on mediate vasorelaxant effect of cannabinoids. Watanabe et al. [13] demonstrated in endothelial cell cultures that anandamide and its metabolite arachidonic acid activate TRPV4 in an indirect way involving the cytochrome P450 epoxygenase-dependent formation of epoxyeicosatrienoic acids, provoking intracellular calcium influx which could lead to vasorelaxation. Recently, it has been described that novel lipid species such as 2-epoxyeicosatrienoylglycerols, which contain 11,12-EET or 14,15-EET produced in kidney, spleen and brain turned out to be potent activators of cannabinoid receptors [46], suggesting a functional link between cytochrome P450 pathways and endocannabinoid system. More research is needed to evaluate this possibility.

Finally, we also mentioned in the introduction that the formation of cytochrome P450 epoxygenase-derived products seems to be of major importance in the regulation of vascular tone in circumstances of an endothelial dysfunction in which

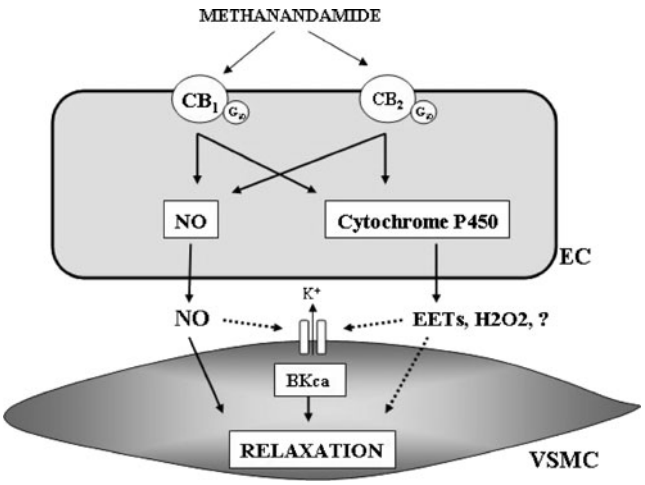

Fig. 7 Schematic diagram showing the proposed mechanisms of the endothelial dependent vasorelaxation to methanandamide in rat aorta. *EC*, endothelial cell; *VSMC*, vascular smooth muscle cell.; *NO*, nitric oxide; *EETs*: epoxyeicosatrienoic acids; *H2O2*: hydrogen peroxide; *BKCa*: large-conductance calcium-dependent potassium channels

the bioavailability of NO is impaired [4]. Thus, the importance of this vasorelaxant pathway in methanandamide vasorelaxation in resistance arteries (which much contribute to total peripheral resistance) and in cardiovascular disease models in which NO production is deficient, should be evaluated in order to validate this synthetic cannabinoid compound for use as new therapeutical agent.

Conclusions

Here, we demonstrated that methanandamide causes an important vasorelaxant effect in a conduit vessel. The activation of CB_1 and CB_2 cannabinoid receptors through the NO synthase and cytochrome P450 epoxygenase pathways (linked to activation of BKCa channels), are important and contributing routes in the methanandamide vasorelaxant effect reported in rat aorta. The link between methanandamide vasorelaxation and cytochrome P450 epoxygenase pathway could be exploited to develop cardiovascular therapeutical agents in circumstances of NO production deficiency (Fig. 7).

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Conflict of interest The authors declare that there are no conflicts of interest.

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