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Long-term inhibition of nitric oxide synthase potentiates effects of anandamide in the rat mesenteric bed

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Abstract

In rat isolated mesenteric beds, anandamide induced a concentration-dependent reduction (0.01–50 μM) of the contractile responses elicited by bolus administration of noradrenaline. The anandamide-induced reductions of noradrenaline responses were unmodified by the in vitro exposure to the nitric oxide synthase (NOS) inhibitor, 100 μM L-N^G-nitro-L-arginine methyl ester (L-NAME), whereas they were significantly potentiated after the long-term in vivo administration of L-NAME (70 mg/kg/day during 4 weeks). Responses to anandamide were not potentiated and even reduced in mesenteric beds from rats made hypertensive by aortic coarctation. In mesenteric beds isolated from either untreated or in vivo L-NAME treated rats, concentration–response curves to anandamide were significantly attenuated by the non-selective K^+ channel blocker tetraethylammonium (TEA) but were not modified by either endothelium removal, or the soluble guanylate cyclase inhibitor 1*H*-[1,2,4] oxadiazolo [4,3-*a*] quinoxalin-1-one (ODQ) or the cannabinoid receptor antagonists 6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1*H*-indol-3-yl] (4-methoxyphenyl) methanone (AM630) and 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-4-morpholinyl-1*H*-pyrazole-3-carboxamide (AM281). On the other hand, the vanilloid receptor agonist (*E*)-*N*-[4-hydroxy-3-methoxyphenyl)methyl]-8-methyl-6-nonenamide (capsaicin) induced a concentration-dependent inhibition of noradrenaline-induced vasoconstriction, and the vanilloid receptor antagonist *N*-[2-(4-chlorophenyl)ethyl]-1,3,4,5-tetrahydro-7,8-dihydroxy-2*H*-2-benzazepine-2-carbothioamide (capsazepine) caused a significant reduction of anandamide-induced responses in mesenteric beds isolated from both control and chronic L-NAME treated rats. The non-metabolizable analogue of anandamide, methanandamide, produced higher reductions of noradrenaline responses than anandamide in mesenteric beds isolated from controls but not from the L-NAME treated rats. Moreover, in mesenteric beds from untreated but not from L-NAME treated rats, the effects of anandamide were significantly potentiated by the inhibitor of endocannabinoid degradation, 200 μM phenylmethylsulphonyl fluoride (PMSF), and by the inhibitor of anandamide uptake, 5 μM (all *Z*)-*N*-(4-hydroxyphenyl)-5,8,11,14-eicosatetraenamide (AM404). It is concluded that long-term inhibition of NOS potentiates anandamide-induced relaxations probably through changes in either endocannabinoid metabolism or uptake. A possible compensatory role for endocannabinoids in vascular function in situations in which nitric oxide (NO) synthesis is long-term impaired arises from the present results.

Keywords: Endocannabinoid; Anandamide; Nitric oxide (NO); Hypertension; L-N^G-nitro-L-arginine methyl ester (L-NAME); Mesenteric bed, rat

1. Introduction

Although a great advance in cannabinoid research within the central nervous system has been attained within the last few years, the physiological role of endocannabinoids in the cardiovascular system still remains elusive. In this regard, the fact that the prototypic endocannabinoid anandamide has been found to relax smooth muscles through the activation of K^+ channels in mesenteric arteries (Randall et al., 1996; White and Hiley, 1997) lead to the proposal that endocannabinoids could act as an endothelium-derived hyperpolarizing factor (EDHF) in the rat mesenteric bed (Randall et al., 1996).

A link between EDHF and nitric oxide (NO), i.e. inhibition of EDHF release by NO, has been reported in rabbit carotid and porcine coronary arteries (Bauersachs et al., 1996). This observation raised the possibility that whenever NO synthesis is impaired, EDHF may act as a compensatory mechanism to maintain the endothelial vasodilator function, as proposed by Corriu et al. (1998) in coronary arteries taken from dogs treated with the NO synthase (NOS) inhibitor L-nitroarginine. Hence, the possibility exists that if endocannabinoids act in the vasculature

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as an EDHF, the effects of anandamide could be potentiated after the chronic inhibition of NOS.

The aim of the present work was to study the effects of anandamide on transient noradrenaline-induced contractile responses in the rat mesenteric bed. This experimental approach was selected on the basis that whereas the action of cannabinoids in the regulation of tone maintenance has been extensively studied (for review, see Hillard, 2000), little is known about the possible role of endocannabinoids in the regulation of vascular reactivity to contractile agents such as noradrenaline. In addition, we tested the hypothesis that the vascular effects of anandamide could be potentiated in mesenteric beds isolated from rats made hypertensive through the chronic administration of L-N^G-nitro-L-arginine methyl ester (L-NAME) to cause NOS inhibition.

2. Materials and methods

2.1. Animal treatment and blood pressure measurements

L-NAME was daily dissolved in tap water at a concentration of 30 mg/100 ml and orally administered ad libitum during 4 weeks to male Wistar rats of 230–260 g body weight. The daily intake of the drug that was recorded through the measurement of fluid intake was approximately 70 mg/kg.

Aortic coarctation was carried out according to the method described by Rojo-Ortega and Genest (1968). Briefly, the rats were anaesthetized with ether and a dorsal incision was performed in order to reach the abdominal cavity and to identify the abdominal aorta. The vessel was ligated with a cotton thread between the renal arteries and below the superior mesenteric artery. In the sham group, the surgery was simulated and no ligation of the aorta was performed. The animals were employed 7 days after the surgery.

The systolic arterial blood pressure that consisted in the mean of four consecutive determinations per rat, was measured by using the tail-cuff method and was controlled before and at the end of L-NAME administration. Animals that achieved a systolic blood pressure higher than 155 mm Hg were considered as hypertensive.

Control rats were kept and their systolic blood pressure was measured as for the L-NAME group and aortic coarctated group.

2.2. Mesenteric bed preparation

The animals were anaesthetized with ether, the abdomen was opened and the mesenteric bed was cannulated and removed according to the method described by McGregor (1965).

The isolated mesenteric bed was transferred to a perpex chamber at 37 °C and perfused with Krebs solution bubbled with 95% O_2/5% CO_2 (in mM: NaCl 118; KCl 4.7; $MgCl_2$ 1.2; NaH_2PO_4 1.0; $CaCl_2$ 2.6; $NaHCO_3$ 25.0; glucose 11.1; final pH 7.4), at a constant flow rate of 2 ml/min maintained by a peristaltic pump. Changes in

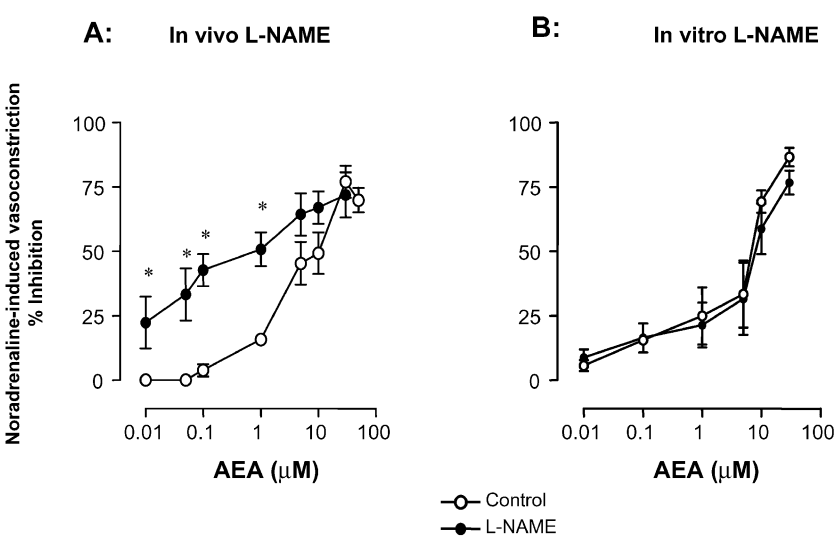

Fig. 1. Effects of anandamide on the noradrenaline-induced vasoconstriction in the rat mesenteric bed. Increasing concentrations of anandamide (AEA) were perfused 10–15 min before and during a bolus injection of the dose of noradrenaline that caused an increase in the perfusion pressure of 60–80 mm Hg, i.e. 10–30 nmol in the controls and 3–10 nmol in the L-NAME treated groups. Controls are depicted in open circles. L-NAME (filled circles) was administered either in vivo (Panel A, 70 mg/kg/day during 4 weeks) or in vitro (Panel B, 100 μM in the organ bath, perfused 15 min before and simultaneously with the increasing concentrations of anandamide). Results are means ± S.E.M. ($n = 6–8$) of the percent reductions of an initial contraction to noradrenaline that was performed after the perfusion of either Krebs solution during 60 min (Panel A) or Krebs solution during 45 min followed by 100 μM L-NAME in the forthcoming 15 min (Panel B). $^*P < 0.05$ when compared to the corresponding control values.

vascular resistance were measured as changes in perfusion pressure and recorded by a Statham transducer connected to a Grass polygraph. The mesenteric bed was allowed a settling period of 60 min after mounting, before starting the experiments.

2.3. Experimental protocols

After an equilibration period of 60 min at 37 °C, the mesenteric beds isolated from L-NAME treated as well as from age-matched control rats were constricted with bolus injections of noradrenaline at a dose that induced responses of approximately 70% of the maximum for the controls and 50% of the maximum for the in vivo L-NAME treated rats. The absolute values for the pressor responses were, in both cases, between 60 and 80 mm Hg and were obtained with 10–30 nmol for the controls and 3–10 nmol for the L-NAME treated groups. Bolus administrations of noradrenaline were performed up to nine times in each preparation at intervals of 15–20 min. Consecutive responses attained equal values throughout the control experiments as reported previously (Mendizabal et al., 1999). Noradrenaline was administered as bolus injections because the short contractile responses induced by this agent are highly reproducible.

Concentration–response curves to either anandamide (0.01–50 μM) or methanandamide (0.01–50 μM) or capsaicin (0.01–10 μM) were performed by evaluation of the reductions of noradrenaline-induced contractions. Cannabinoids and capsaicin were present in the perfusion buffer at a constant concentration, which was then rampered up to generate cumulative concentration–response effects. The corresponding concentrations of either cannabinoids or capsaicin were maintained since 15 min before the bolus administration of noradrenaline and up to the end of the contractile response, usually attained 3–5 min after the addition of noradrenaline. The results were expressed as the percent inhibition of the pressor effect of noradrenaline. The basal tone was unmodified by the concentrations of either anandamide, methanandamide or capsaicin employed.

The endothelial layer of the mesenteric bed was removed by perfusion with saponin 0.1% v/v for 45 s (Peredo and Enero, 1993). To evaluate the effects of endothelial removal on anandamide-induced inhibition of contractile responses, concentration–response curves were performed 45 min after endothelium removal. The effectiveness of this procedure was evaluated at the end of the experiment by examination of the lack of relaxant effects of an infusion of 0.1 μM acetylcholine on mesenteric beds previously contracted by infusion of 1 μM noradrenaline.

In order to test the effects of the in vitro inhibition of NOS as well as of soluble guanylate cyclase, the concentration–response curves to anandamide were performed in the presence of their respective inhibitors, 100 μM L-NAME and 1 μM 1*H*-[1,2,4] oxadiazolo [4,3-*a*] quinoxalin-1-one (ODQ), added 15 min before and during the concentration–response curve to anandamide.

Since both endothelial removal, in vitro inhibition of NOS with L-NAME and in vitro inhibition of soluble guanylate cyclase with ODQ, produced per se an enhancement of contractile responses to noradrenaline, the corresponding dose of noradrenaline in these experimental groups was reduced by a factor of three in order to obtain a pressor response of the same magnitude as that attained in the controls. The doses of noradrenaline employed were in the exact same position on the dose–response curve before and after each treatment as reported in a previous work (Mendizabal et al., 1999).

To study the participation of K^+ channels, the responses to anandamide were assayed in the presence of the non-specific K^+ channel blocker tetraethylammonium (TEA), added 30 min before and during the concentration–response curve to the endocannabinoid.

To study the participation of cannabinoid CB1 receptors and vanilloid VR1 receptors on anandamide-induced relaxations, the responses to cannabinoids were assayed in the presence of either the cannabinoid receptor antagonists, 5 μM 6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1*H*-indol-3-yl] (4-methoxyphenyl) methanone (AM630) and 1 μM 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-4-morpholinyl-1*H*-pyrazole-3-carboxamide (AM281), or the vanilloid receptor antagonist, 1 μM *N*-[2-(4-chlorophen-

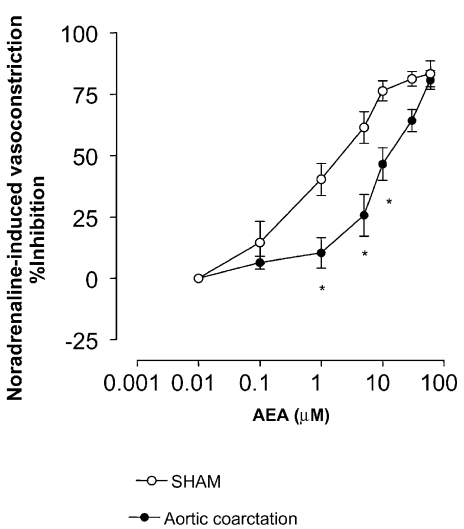

Fig. 2. Effects of aortic coarctation on the reduction of noradrenaline-induced vasoconstriction elicited by anandamide in the rat mesenteric bed. Increasing concentrations of anandamide (AEA) were perfused 10–15 min before and during a bolus injection of the dose of noradrenaline that caused an increase in the perfusion pressure of 60–80 mm Hg, i.e. 10–30 nmol in the sham-operated group and 3–10 nmol in the aortic coarctation group. Results are means ± S.E.M. ($n = 6$) of the percent reductions of an initial contraction to noradrenaline that was performed in mesenteric beds isolated from either rats with aortic coarctation (filled circles) or the corresponding sham-operated control group (open circles). * $P < 0.05$ when compared to the corresponding control values.

yl)ethyl]-1,3,4,5-tetrahydro-7,8-dihydroxy-2*H*-2-benzazepine-2-carbothioamide (capsazepine), added, respectively, 20 and 45 min before and during the concentration–response curves to anandamide.

To evaluate the effects of the inhibition of anandamide degradation and of anandamide uptake, the concentration–response curves to anandamide were performed in the presence of either 200 μM phenylmethylsulphonyl fluoride (PMSF) or 5 μM (all *Z*)-*N*-(4-hydroxyphenyl)-5,8,11,14-eicosatetraenamide (AM404), added 15 min before and up to the end of the experiments.

2.4. Animals and drugs

Male Wistar rats were bred in the animal facilities of the Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires. Experiments were conducted in accordance with the Helsinki declaration on research involving animals and human beings.

(−)-Noradrenaline bitartrate, L-N^G-nitro-L-arginine methyl ester (L-NAME), saponin, 1*H*-[1,2,4] oxadiazolo [4,3-a] quinoxalin-1-one (ODQ), tetraethylammonium acetate (TEA) and phenylmethylsulphonyl fluoride (PMSF) were purchased from Sigma. (all *Z*)-*N*-(2-hydroxyethyl)-5,8,11,14-eicosatetraenamide (anandamide), [*R*-(all *Z*)]-*N*-(2-hydroxy-1-methylethyl)-5,8,11,14-eicosatetraenamide (*R*-(+)-methanandamide), 6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1*H*-indol-3-yl] (4-methoxyphenyl) methanone (AM630), 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-4-morpholinyl-1*H*-pyrazole-3-carboxamide (AM281), (all *Z*)-*N*-(4-hydroxyphenyl)-5,8,11,14-eicosatetraenamide (AM404), (*E*)-*N*-[4-hydroxy-3-methoxyphenyl)methyl]-8-methyl-6-nonenamide ((*E*)-capsaicin) and *N*-[2-(4-chlorophenyl)ethyl]-1,3,4,5-tetrahydro-7,8-dihydroxy-2*H*-2-benzazepine-2-carbothioamide (capsazepine) were obtained from Tocris Cookson. Anandamide and methanandamide were dissolved in ethanol; AM630, AM281, AM404, capsaicin and capsazepine were dis-

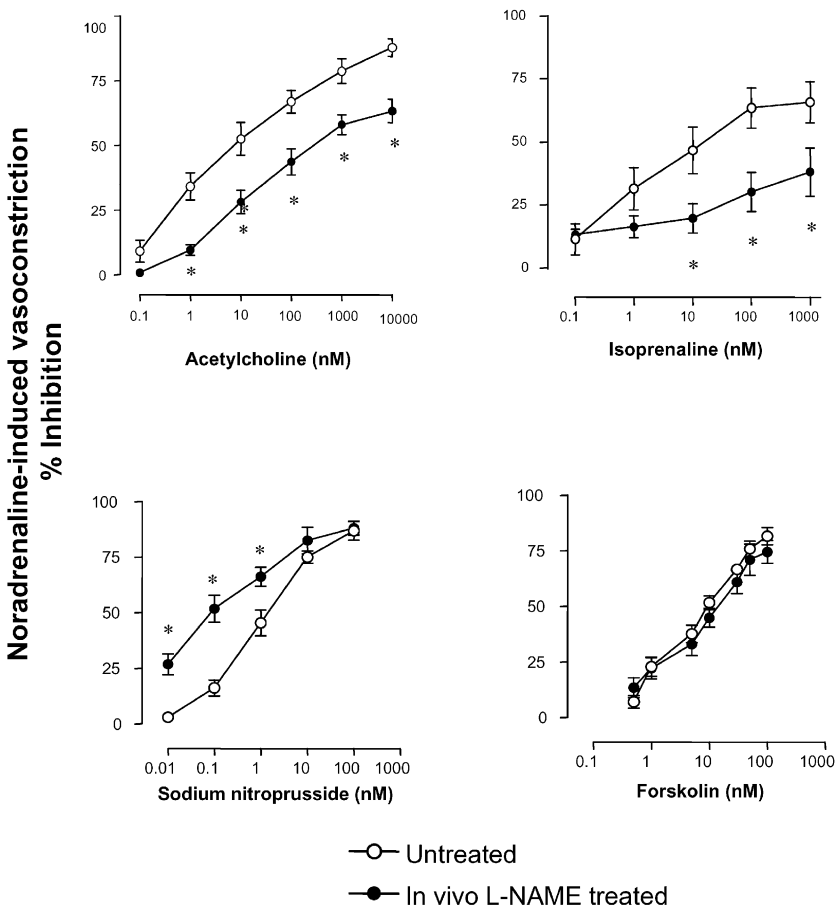

Fig. 3. Effects of acetylcholine, isoprenaline, sodium nitroprusside and forskolin on the noradrenaline-induced vasoconstriction of the rat mesenteric bed. Increasing concentrations of the drugs tested were added 10–15 min before and during a bolus injection of the dose of noradrenaline that caused an increase in the perfusion pressure of 60–80 mm Hg, i.e. 10–30 nmol in the controls (open circles) and 3–10 nmol in the L-NAME treated group (70 mg/kg/day during 4 weeks, filled circles). Results are expressed as the mean ± S.E.M. ($n = 6–8$) of the percent reductions of an initial contraction to noradrenaline that was performed 60 min after the onset of the perfusion with Krebs solution. $^{*}P < 0.05$ when compared to the corresponding control values.

solved in dimethyl sulphoxide. The remaining drugs were dissolved in distilled water. Neither AM630, nor AM281, nor capsazepine, nor AM404, nor PMSF, nor TEA, nor the maximal concentrations of ethanol and dimethyl sulphoxide that were added to the mesenteric beds, 0.1% and 0.05%, respectively, had any effect on the contractile responses elicited by noradrenaline in the mesenteric bed.

2.5. Statistical analysis

Results are expressed as the means ± S.E.M. Statistical comparisons were made by analysis of variance (ANOVA) with Newman–Keuls posteriori analysis and Student's *t*-test for paired and unpaired data, as appropriate. A *P*-value smaller than 0.05 was considered as significant.

3. Results

The effects of the endocannabinoid anandamide on noradrenaline-induced vasoconstriction were tested in mesenteric beds isolated from untreated as well as from in vivo L-NAME treated rats (70 mg/kg/day during 4 weeks). As shown in Fig. 1 Panel A, anandamide induced a concentration-dependent inhibition of the contractile responses elicited by the bolus administration of noradrenaline, which started at 1 μM and reached a maximum at 30 μM anandamide. The effect of anandamide was significantly potentiated after the chronic in vivo administration of L-NAME, where the reduction of noradrenaline-induced vasoconstriction started at 0.01 μM anandamide and reached a maximum equal to that attained in the controls. Different to that observed after the chronic treatment with L-NAME, the in vitro exposure of the mesenteric beds to 100 μM L-NAME did not modify the anandamide-induced inhibition of the contractions elicited by noradrenaline compared to the controls (Fig. 1, Panel B).

In order to determine if the potentiation of the inhibitory effects of anandamide on noradrenaline-induced contraction were the consequence of the increase in blood pressure induced by chronic L-NAME treatment, responses to anandamide were tested in mesenteric beds isolated from animals made hypertensive by a means other than the inhibition of NOS, such as aortic coarctation. Different to that observed in rats made hypertensive by long-term L-NAME administration, the effects of anandamide on noradrenaline-induced contractile responses were not potentiated, and even reduced, in mesenteric beds isolated from rats made hypertensive by aortic coarctation (Fig. 2).

The reductions on noradrenaline-induced vasoconstriction caused by anandamide were compared with the effects of other substances known to induce vasodilation, namely the cholinergic agonist acetylcholine, the β-adrenoceptor agonist isoprenaline, the nitric oxide donor sodium nitroprusside (SNP) and the adenylate cyclase activator forskolin. As shown in Fig. 3, the contractile responses to noradrenaline were reduced in a concentration-dependent manner by acetylcholine, isoprenaline, sodium nitroprusside and forskolin in mesenteric beds isolated from untreated rats. Moreover, whereas both acetylcholine and isoprenaline effects on noradrenaline-induced vasoconstriction were diminished after the chronic in vivo administration of L-NAME, the SNP-induced inhibition of the con-

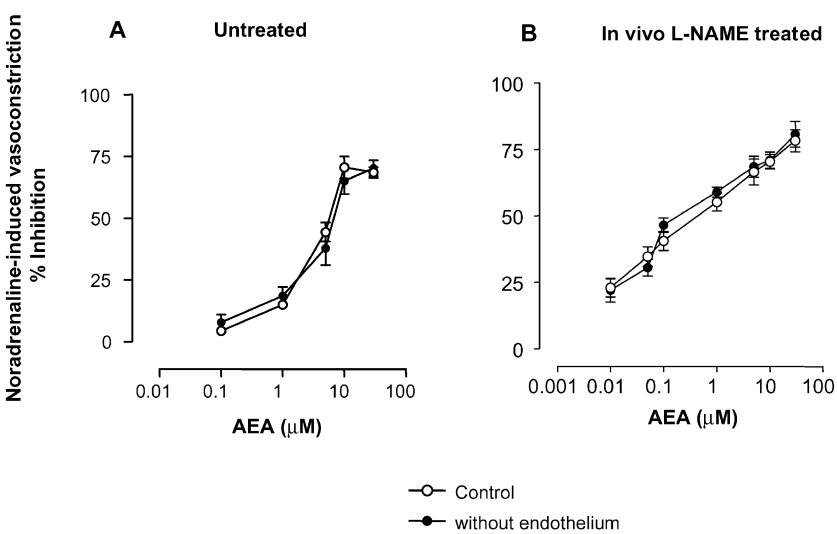

Fig. 4. Effects of endothelium removal on anandamide-induced inhibition of contractile responses to noradrenaline in the rat mesenteric bed. Increasing concentrations of anandamide (AEA) were added 10–15 min before and during a bolus injection of the dose of noradrenaline that caused an increase in perfusion pressure of 60–80 mm Hg in mesenteric beds isolated from untreated (Panel A) and L-NAME treated rats (Panel B). For details, see Fig. 1. Endothelium was removed through a 45-s infusion with 0.1% saponin. Results are means ± S.E.M. ($n = 6–8$) of the percent reductions of an initial contraction to noradrenaline, performed 60 min after the perfusion with Krebs solution of the mesenteric beds with either intact endothelium (controls, open circles) or in endothelium-denuded preparations (filled circles).

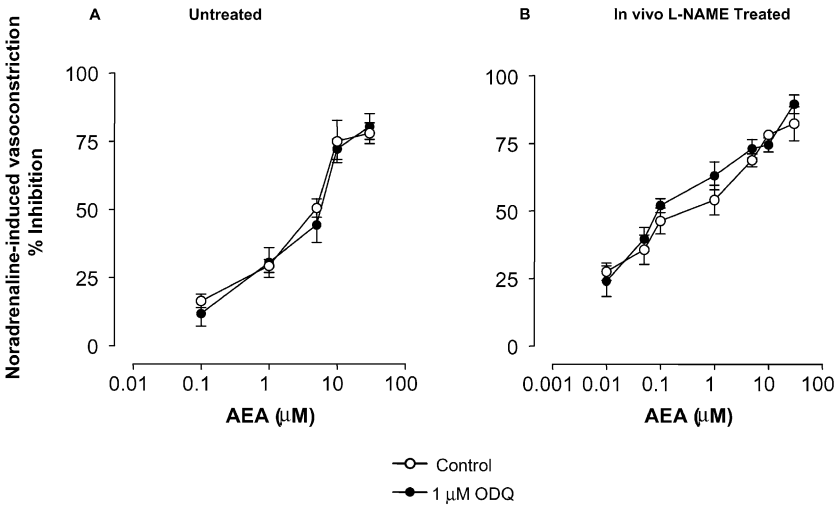

Fig. 5. Effects of the soluble guanylate cyclase inhibitor ODQ on anandamide-induced inhibition of contractile responses to noradrenaline in the rat mesenteric bed. Increasing concentrations of anandamide (AEA) were added 10–15 min before and during a bolus injection of the dose of noradrenaline that caused an increase in perfusion pressure of 60–80 mm Hg in mesenteric beds isolated from untreated (Panel A) and L-NAME treated rats (Panel B). For details, see Fig. 1. The guanylate cyclase inhibitor 1 μM ODQ was perfused 15 min before and simultaneously with the increasing concentrations of anandamide. Results are means ± S.E.M. ($n = 6–8$) of the percent reductions of an initial contraction to noradrenaline, performed 15 min after the onset of the perfusion with 1 μM ODQ.

tractile responses to noradrenaline was potentiated, as observed with anandamide, after in vivo NOS inhibition. No differences on the effects of forskolin were observed between the controls and the L-NAME treated group.

In order to test whether the effect of anandamide on noradrenaline-induced contractions could involve the participation of the vascular endothelium, concentration–response curves to the endocannabinoid were performed in

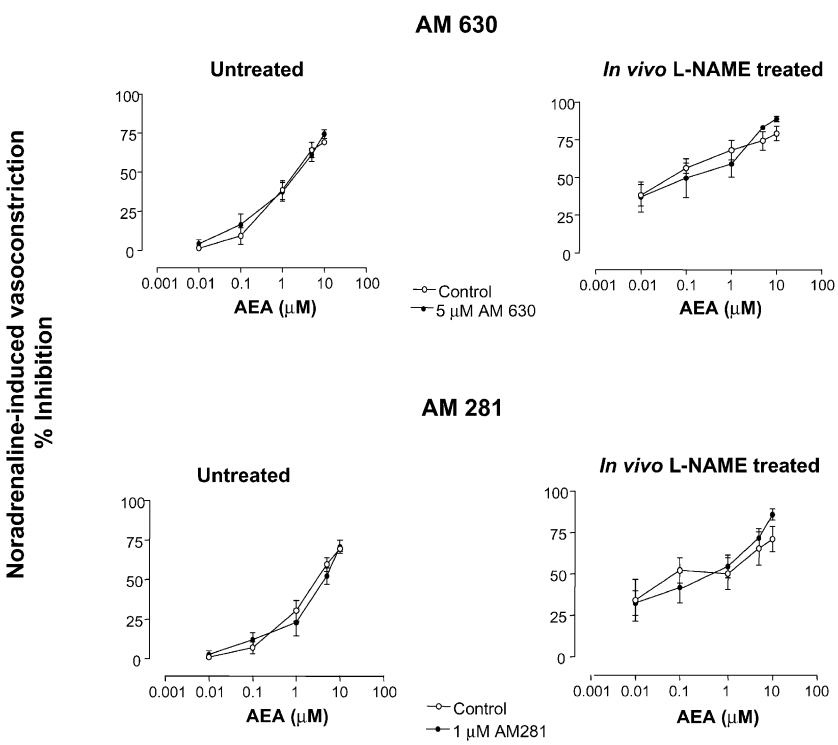

Fig. 6. Effects of the cannabinoid receptor antagonists AM 630 and AM 281 on the anandamide induced inhibition of contractile responses to noradrenaline in the rat mesenteric bed. Increasing concentrations of anandamide (AEA) were added 10–15 min before and during a bolus injection of the dose of noradrenaline that caused an increase in the perfusion pressure of 60–80 mm Hg in mesenteric beds isolated from untreated and L-NAME treated rats (for details, see Fig. 1). The cannabinoid receptor antagonists 5 μM AM630 (upper panel) and 1 μM AM281 (lower panel) were perfused 15 min before and simultaneously with the increasing concentrations of anandamide. Results are means ± S.E.M. ($n = 6–8$) of the percent reductions of an initial contraction to noradrenaline, performed 15 min after the onset of the perfusion with either 5 μM AM630 or 1 μM AM281.

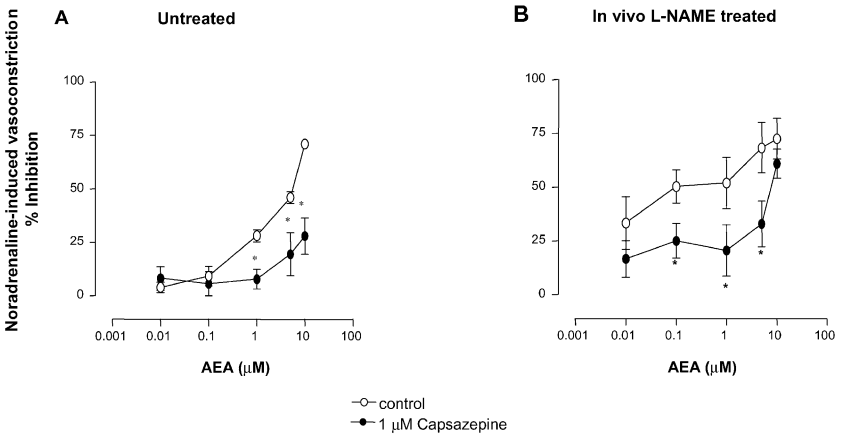

Fig. 7. Effects of the vanilloid receptor antagonist capsazepine on the anandamide-induced inhibition of contractile responses to noradrenaline in the rat mesenteric bed. Increasing concentrations of anandamide (AEA) were added 10–15 min before and during a bolus injection of the dose of noradrenaline that caused an increase in the perfusion pressure of 60–80 mm Hg in mesenteric beds isolated from either untreated (Panel A) or L-NAME treated rats (Panel B). For details, see Fig. 1. The vanilloid receptor antagonist 1 μM capsazepine, was perfused 45 min before and simultaneously with the increasing concentrations of anandamide. Results are means ± S.E.M. ($n = 6–8$) of the percent reductions of an initial contraction to noradrenaline, performed 15 min after the onset of the perfusion with 1 μM capsazepine. * $P < 0.05$ when compared to the corresponding control values.

either mesenteric beds with intact endothelium or in endothelium-denuded preparations. Fig. 4 shows that endothelium removal did not modify the anandamide-induced reductions of contractile responses to noradrenaline either in the untreated (Panel A) or in the in vivo L-NAME treated rats (Panel B).

The effects of the soluble guanylate cyclase inhibitor ODQ on anandamide-induced inhibition of contractile responses were studied in mesenteric beds isolated from untreated as well as from L-NAME treated rats. As shown in Fig. 5, the inhibition of the soluble guanylate cyclase with ODQ did not modify the concentration–response curves to anandamide either in the untreated (Panel A) or in the in vivo L-NAME treated group (Panel B).

To study the possibility that the action of anandamide in the vasculature could be mediated by specific receptors, the effects of anandamide on the contractile responses to noradrenaline were tested in the presence of cannabinoid as well as vanilloid receptor antagonists. As shown in Fig. 6, the cannabinoid receptor antagonists AM630 and AM281 were unable to modify concentration–response curves to anandamide either in the untreated or in the L-NAME treated group. On the contrary, the vanilloid receptor antagonist capsazepine (Fig. 7) caused a significant reduction of anandamide-induced responses in mesenteric beds isolated from both untreated (Fig. 7, Panel A) and L-NAME treated rats (Fig. 7, Panel B).

In order to study if, as observed for anandamide, the specific activation of vanilloid receptors was able to reduce noradrenaline-induced contractions, the effects of the VR1 receptor agonist capsaicin on noradrenaline responses were studied in the rat mesenteric bed. As shown in Fig. 8, capsaicin induced a concentration-dependent inhibition of the contractile responses elicited by the bolus administration of noradrenaline, which started at 0.01 μM and reached a maximum at 5 μM capsaicin. Different to that observed for anandamide, the chronic treatment with L-NAME did not modify the capsaicin-induced inhibition of the contractions elicited by noradrenaline compared to the controls.

To test whether anandamide-induced responses could be mediated by the opening of K^+ channel, the effects of the non-selective K^+ channel blocker tetraethylammonium (TEA) were studied in mesenteric beds isolated from either untreated or L-NAME treated rats. As shown in Fig. 9, the

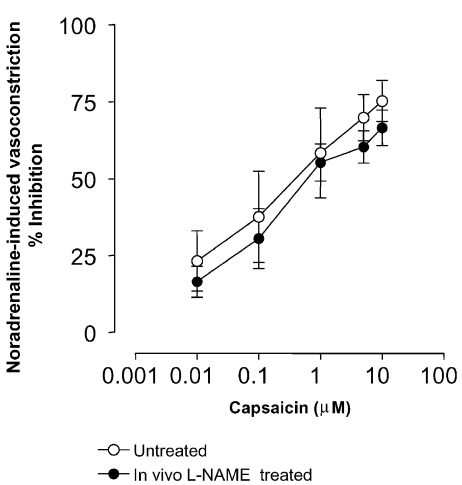

Fig. 8. Effects of the vanilloid receptor agonist capsaicin on contractile responses elicited by noradrenaline in the rat mesenteric bed. Increasing concentrations of capsaicin were perfused 10–15 min before and during a bolus injection of the dose of noradrenaline that caused an increase in the perfusion pressure of 60–80 mm Hg, i.e. 10–30 nmol in the controls and 3–10 nmol in the L-NAME treated rats. For details, see Fig. 1. Results are means ± S.E.M. ($n = 6$) of the percent reductions of an initial contraction to noradrenaline that was performed in mesenteric beds isolated from either controls (open circles) or L-NAME treated rats (filled circles).

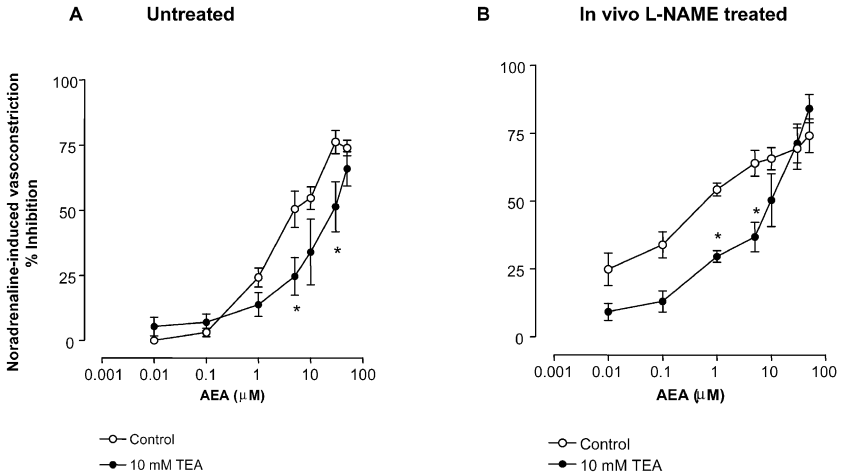

Fig. 9. Effects of the non-selective K^+-channel blocker tetraethylammonium (TEA) on the anandamide-induced inhibition of contractile responses to noradrenaline in the rat mesenteric bed. Increasing concentrations of anandamide (AEA) were added 10–15 min before and during a bolus injection of the dose of noradrenaline that caused an increase in the perfusion pressure of 60–80 mm Hg in mesenteric beds isolated from either untreated (Panel A) or L-NAME treated rats (Panel B). For details, see Fig. 1. The non-selective K^+-channel blocker, 10 mM TEA, was perfused 30 min before and simultaneously with the increasing concentrations of anandamide. Results are means ± S.E.M. ($n = 6–7$) of the percent reductions of an initial contraction to noradrenaline, performed 30 min after the onset of the perfusion with 10 mM TEA.

non-selective K^+ channel blocker 10 mM TEA significantly attenuated the anandamide-induced inhibition of contractile responses to noradrenaline in mesenteric beds isolated from either untreated (Fig. 9, Panel A) or L-NAME treated rats (Fig. 9, Panel B).

The effects of anandamide on noradrenaline-induced vasoconstriction were compared with those obtained with its non-metabolizable analogue methanandamide and also studied in the presence of either the non-specific inhibitor of endocannabinoid degradation PMSF or the specific inhibitor of anandamide uptake AM404. As shown in Fig. 10, the responses induced by methanadamide were significantly higher than those induced by anandamide in mesenteric beds isolated from untreated rats (Fig. 10, Panel A). Unexpectedly, no differences were found between anandamide and methanandamide when these drugs were assayed in mesenteric beds isolated from L-NAME treated rats (Fig. 10, Panel B). In addition, Fig. 11 shows that both the non-specific inhibitor of endocannabinoid degradation, 200 μM PMSF, and the specific inhibitor of anandamide

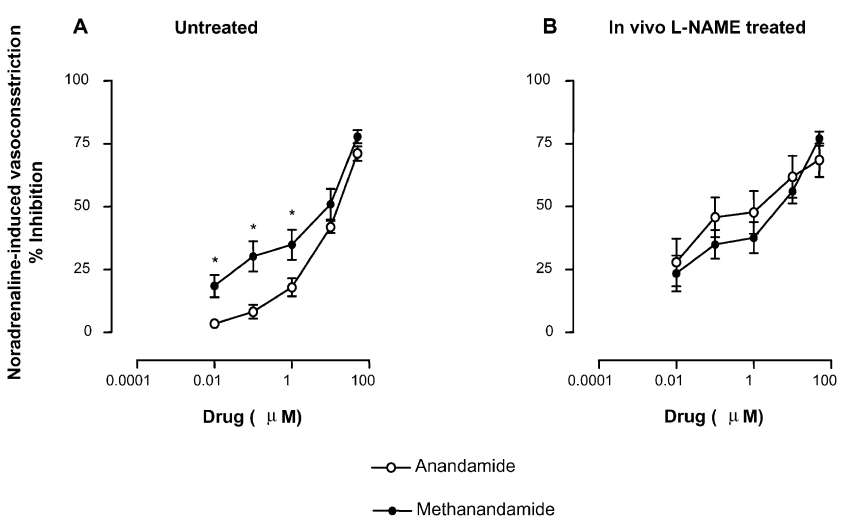

Fig. 10. Comparison of the effects of anandamide and methanandamide on the noradrenaline-induced vasoconstriction in the rat mesenteric bed. Increasing concentrations of anandamide and of its non-metabolizable analogue methanandamide were added 10–15 min before and during a bolus injection of the dose of noradrenaline that caused an increase in the perfusion pressure of 60–80 mm Hg in mesenteric beds isolated from untreated (Panel A) and L-NAME treated rats (Panel B). For details, see Fig. 1. Results are means ± S.E.M. ($n = 6–8$) of the percent reductions of an initial contraction to noradrenaline, performed 60 min after the onset of the perfusion with Krebs solution. $^* P < 0.05$ when compared to the corresponding control values.

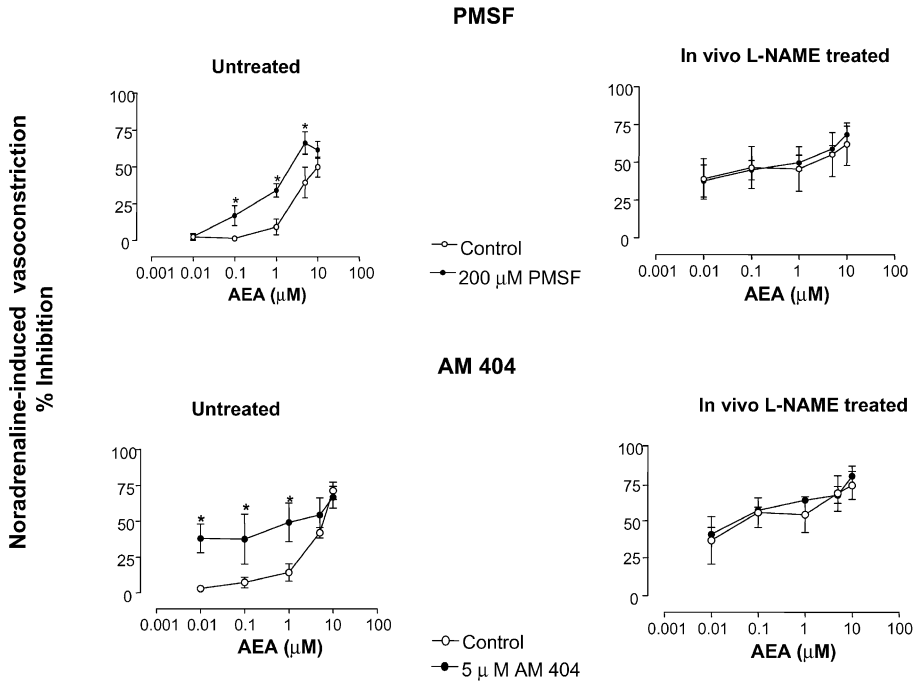

Fig. 11. Effects of the non-specific anandamide amidase inhibitor PMSF and of the specific anandamide uptake inhibitor AM404 on the anandamide-induced inhibition of contractile responses to noradrenaline in the rat mesenteric bed. Increasing concentrations of anandamide were added 10–15 min before and during a bolus injection of the dose of noradrenaline that caused an increase in the perfusion pressure of 60–80 mm Hg in mesenteric beds. For details, see Fig. 1. Both the non-specific anandamide amidase inhibitor 200 μM PMSF (upper panel) and the specific anandamide uptake inhibitor 5 μM AM404 (lower panel) were perfused 15 min before and simultaneously with increasing concentrations of anandamide in mesenteric beds isolated from either untreated or L-NAME treated rats. Results are means ± S.E.M. ($n = 6–8$) of the percent reductions of an initial contraction to noradrenaline, performed 15 min after the onset of the perfusion with either 200 μM PMSF or 5 μM AM404. $^{*}P < 0.05$ when compared to the corresponding control values.

uptake, 5 μM AM404, significantly potentiated anandamide-induced responses in mesenteric beds isolated from untreated rats. The inhibitor of anandamide uptake caused that concentrations of anandamide as low as 0.01 μM were now effective to reduce, by about 40%, the noradrenaline-induced vasoconstriction (Fig. 11). On the contrary, no differences were observed when either the anandamide uptake blocker, AM404, or the endocannabinoid degradation inhibitor, PMSF, were tested in mesenteric beds isolated from L-NAME treated rats (Fig. 11).

4. Discussion

In the majority of previous studies, the effects of cannabinoids in the vascular system were linked to an inhibitory action not only in the release of noradrenaline from sympathetic nerves (Ishac et al.,1996; Niederhoffer and Szabo, 1999) but also in the basal tone of vascular preparations. Relaxations produced by cannabinoids were reported for sustained contractions induced by different agents, administered as to mimic the mechanisms involved in tone maintenance, i.e. either as a constant infusion or at a fixed concentration in the organ bath. Among the preparations whose vascular tone is reduced by cannabinoids are the rat mesenteric bed (Randall et al., 1996; Plane et al., 1997), the vasculature of the rat kidney (Deutsch et al., 1997), the bovine coronary artery (Pratt et al., 1998) and the guinea-pig isolated basilar artery (Zygmunt et al., 2000).

The present work shows that, in addition to the reductions reported for the vascular tone, the endocannabinoid anandamide, as well as its non-metabolizable analogue methanandamide, induce a concentration-dependent inhibition of the transient contractions elicited by bolus injections of noradrenaline in the rat mesenteric bed. This latter finding would suggest that endocannabinoids could be of physiological relevance not only in the local regulation of basal tone but also in physiological situations in which plasma concentrations of catecholamines are increased, such as during an adrenergic discharge, or under pathological conditions in which sympathetic activity is enhanced, such as in some types of human essential hypertension.

In addition, the present observation that the inhibitory effect of anandamide on noradrenaline-induced contractions is potentiated after the long-term in vivo administration of L-NAME could suggest a link between NO and cannabinoids. Nevertheless, the in vitro exposure to the NOS inhibitor was unable to modify the anandamide-induced reductions of the contractile responses to noradrenaline in the mesenteric bed, in spite of the fact that both in vivo and in vitro administration of L-NAME are equally

effective to inhibit NOS activity in this tissue (Mendizabal et al., 2000). Hence, the possibility exists that the enhancement of anandamide-induced reductions of contractile responses observed after the in vivo L-NAME treatment had been the consequence of an adaptive change resulting from the prolonged NOS inhibition.

Since the chronic inhibition of NOS induces hypertension, it is highly possible that the potentiation of the effects of anandamide observed in mesenteric beds isolated from L-NAME treated rats could have been the consequence of the increase in blood pressure produced by the treatment. Nevertheless, this latter possibility appears to be unlikely on the basis that the inhibitory effects of anandamide on noradrenaline-induced contractile responses were not potentiated, and even reduced, in mesenteric beds isolated from rats made hypertensive by aortic coarctation.

Moreover, the potentiation of the inhibitory effects of anandamide on noradrenaline-induced contractions after the chronic in vivo administration of L-NAME is not likely to be the consequence of an unspecific alteration of the contractile machinery caused by the long-term inhibition of NOS. This is because after L-NAME treatment, the inhibition of contractile responses was in fact reduced for acetylcholine and isoprenaline, unmodified for forskolin and enhanced for SNP. Furthermore, even in the latter case, the mechanism involved is not likely to be the same as that underlying anandamide effects; i.e. whereas the relaxation caused by SNP is linked to cGMP production and prevented by the guanylate cyclase inhibitor ODQ (Hwang et al., 1998; Garcia-Pascual et al., 1999), this latter drug was unable to modify anandamide inhibition of contractile responses either in the untreated or in the L-NAME treated rats.

It is of interest to note that anandamide was able to reduce contractions elicited by other contractile agents, such as KCl (Mendizabal et al., unpublished observations). This finding precludes the possibility that the effects of anandamide had been the consequence of a functional antagonism with adrenoceptors.

On the other hand, the fact that anandamide-induced inhibition of contractile responses to noradrenaline was unmodified after endothelial removal either in untreated or in L-NAME treated rats suggests that the inhibitory effect of this cannabinoid is independent not only of NO but also of other endothelium-derived relaxing factors in the rat mesenteric bed. Whereas this observation agrees with most of the studies performed in the rat mesenteric vasculature, which show that anandamide-induced relaxations are not endothelium-dependent (Randall et al., 1996; White and Hiley, 1997), it differs from the results obtained by Chaytor et al. (1999) and Jarai et al. (1999), who found a small endothelium-dependent component of the effect of anandamide. This discrepancy could be due to the different experimental models employed. In this regard, whereas Chaytor et al. tested the relaxant effects of anandamide in rings of rabbit superior mesenteric arteries precontracted with phenylephrine and Jarai et al. measured reductions in vascular tone induced by anandamide in rat mesenteric beds perfused with phenylephrine, we studied the effects of anandamide on contractile responses elicited by bolus injections of noradrenaline in the rat mesenteric bed.

It has been suggested that anandamide could act as an EDHF in the mesenteric bed (Randall et al., 1996). The fact that the reductions in contractile responses to noradrenaline induced by anandamide were significantly attenuated by the exposure to 10 mM of the non-specific K^+ channel blocker, tetraethylammonium, is likely to support the view that anandamide had acted in the rat mesenteric bed, at least partially, through the activation of K^+ channels and the consequent hyperpolarization of the vascular smooth muscle. This is in agreement with most of the results obtained when different K^+ channel blockers were tested in mesenteric preparations; i.e. several authors have found that anandamide-induced relaxations were abolished when apamin and caribdotoxin were employed either in the perfused mesenteric bed (Randall et al., 1996) or in segments of mesenteric arteries (Plane et al., 1997) and White and Hiley (1997) reported that 10 mM TEA attenuated anandamide-induced relaxations in the rat perfused mesenteric bed.

Regarding the possible involvement of specific cannabinoid CB1 receptors, their participation is tentatively precluded by the observation that the cannabinoid receptor antagonists AM630 and AM281 did not antagonize the reductions of the noradrenaline-induced contractions caused by anandamide in the mesenteric bed. Although our results differ from the observation that the relaxant effects of anandamide can be reduced, in the same preparation, by CB1 receptor antagonist SR 141716 A (Randall et al., 1996), this CB1 receptor antagonist was not tested by us because in our hands, it also reduced per se the noradrenaline-induced contractions of the mesenteric bed (Mendizabal, unpublished observations). In addition, our results are in agreement with studies that reported the lack of inhibitory effect of CB1 antagonists on the actions of anandamide (Chaytor et al., 1999; Jarai et al., 1999).

The lack of effect of the CB1 receptor antagonists on anandamide-induced relaxations could have resulted from the long-lasting and poorly reversible nature of anandamide effects (Plane et al., 1997). Nevertheless, this possibility is precluded by the finding that the VR1 receptor antagonist capsazepine was indeed able to prevent the inhibitory effects of anandamide on noradrenaline-induced contractions. This latter finding agrees with the observations that anandamide elicits vasodilation of isolated hepatic and mesenteric small arteries by activation of vanilloid receptors (Zygmunt et al., 1999). Moreover, its non-metabolizable analog methanandamide induces relaxation through vanilloid receptors on capsaicin-sensitive nerves in the isolated mesenteric arterial bed and small mesenteric arteries (Ralevic et al., 2000). Furthermore, anandamide and methanandamide were shown to be agonists at recom-

binant rat VR1 receptor (Zygmunt et al., 1999) and in the human VR1 clone (Smart et al., 2000).

Regarding the possible link between cannabinoids and vanilloid receptors, positive interactions appears to arise both from the observation that the vanilloid receptor agonist capsaicin was effective to reduce noradrenaline-induced contractions and that the vanilloid receptor antagonist capsazepine was able to inhibit the action of anandamide. Nevertheless, whereas the effective concentrations of anandamide that were antagonized by capsazepine in the controls are within the affinity range reported for this cannabinoid, this was not the case for the L-NAME treated group, in which the concentrations of anandamide effective to reduce contractile responses to noradrenaline (0.01–0.1 μM) were much below the affinity constant. The latter finding suggests that VR1 receptor could be supersensitive under pathophysiological conditions such as L-NAME hypertension. Nevertheless, this latter possibility appears to be unlikely since no differences were observed for the effects of capsaicin between controls and L-NAME treated rats. Hence, the possibility exists that the inhibition of anandamide effects induced by capsazepine could very well be due to an action of capsazepine unrelated to VR1 receptors or, alternatively, that the effects of anandamide had resulted from the interaction with a different subsite of the VR1 receptor than that activated by capsacin.

The fact that methanandamide was more potent than anandamide to reduce noradrenaline-induced contractions, and that both the inhibition of anandamide degradation with PMSF and of anandamide uptake with AM404 potentiated its inhibitory action, suggests that metabolism and uptake could be physiological mechanisms for the regulation of cannabinoid-induced effects in the vasculature. Potentiation of anandamide effects has also been reported for the uptake inhibitor AM404, tested in vivo on the anandamide-induced hypotension of anesthesized guinea pigs (Calignano et al., 1997) and for the anandamide aminohydrolase inhibitor PMSF assayed in vitro on anandamide-induced relaxations of rat isolated mesenteric arteries (White and Hiley, 1997).

A link between anandamide uptake and NO has been proposed for human neuroblastoma and lymphoma cell lines (Maccarrone et al., 1998) and for human umbilical vein endothelial cells (Maccarrone et al., 2000), where the anandamide carrier was found to be activated by NO. Hence, it is tempting to suggest that in the opposite condition, i.e. in the absence of NO, a diminution of the effectiveness of anandamide uptake by its specific carrier might occur. Nevertheless, since the acute in vitro inhibition of NOS did not modify anandamide-induced reductions of contractile responses, the possibility exists that its long-term inhibition could have induced changes in anandamide metabolism. In this regard, the fact that neither the anandamide degradation enzyme inhibitor PMSF nor the anandamide uptake inhibitor AM404 had potentiated the effects of anandamide in mesenteric beds isolated from L-NAME treated rats is likely to indicate that anandamide metabolism as well as anandamide uptake are less active in hypertensive L-NAME treated than in control rats. In further support of this view is the observation that methanadamide was equipotent to anandamide to reduce contractions to noradrenaline in mesenteric beds isolated from L-NAME treated rats.

In summary, although it has been previously reported that the hypotensive effects of anandamide on arterial blood pressure are higher in spontaneously hypertensive than in normotensive rats (Lake et al., 1997), the present work is, to our knowledge, the first study that provides evidence that the vascular effects of endocannabinoids might be potentiated in a model of hypertension caused by long-term suppression of NO synthesis. Whether or not an EDHF, anandamide is likely to play an important role in the control of vascular function, as suggested also by the observation that both endothelial cells and macrophages release this and other endocannabinoid, 2-arachidonylglycerol (Deutsch et al., 1997; Di Marzo et al., 1999). Hence, although further studies are necessary to evaluate the physiological relevance of the present findings, they could suggest a possible compensatory role for endocannabinoids in vascular function in situations in which NO synthesis is chronically impaired, such as in human essential hypertension (Forte et al., 1997).

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