

**Anandamide-induced vasorelaxation in rabbit aortic rings has two components:  
G protein-dependent and G protein-independent**

**Somnath Mukhopadhyay<sup>#%</sup>, Barry M. Chapnick and Allyn C. Howlett<sup>%</sup>**

Department of Pharmacological and Physiological Science  
Saint Louis University School of Medicine, St. Louis, Missouri, USA

<sup>#</sup>Corresponding author

Somnath Mukhopadhyay

Program in Neuroscience /Drug Abuse Research

J. L. Chambers Biomedical/Biotechnology Research Institute

North Carolina Central University, Durham, NC 27707, USA

Phone # (919) 530-7762; Fax # (919) 530-7760

Email: smukhopadhyay@wpo.nccu.edu

<sup>%</sup>Current address

Program in Neuroscience /Drug Abuse Research

J. L. Chambers Biomedical/Biotechnology Research Institute

North Carolina Central University, Durham, NC 27707, USA

## **Abstract**

The endogenous cannabinoid, anandamide (arachidonylethanolamide), produces vasorelaxation in different vascular beds. In the present study, we have found that anandamide and the metabolically stable analog, methanandamide, produced dose-dependent (10 nM - 10 :M) vasorelaxation of about 80% in the rabbit aortic ring preparation in an endothelium-dependent manner. Non-endothelium-dependent vasorelaxation was observed to be a maximum of 20-22% at > 10 :M methanandamide. The efficacious CB<sub>1</sub> receptor analogs desacetyllevonantradol (10 :M) and WIN55212-2 (10 :M) failed to produce vasorelaxation; however, the endothelium-dependent vasorelaxation evoked by methanandamide was partially (75%) blocked by the CB<sub>1</sub> receptor antagonist SR141716A. The VR<sub>1</sub> vanilloid receptor antagonist capsazepine or the calcitonin gene related peptide (CGRP) antagonist CGRP-(8-37) partially attenuated (25%) the vasorelaxation in endothelium-intact preparations, and greatly reduced the response in endothelium-denuded preparations. Pretreatment of the aortic rings with N<sup>G</sup> nitro-L-arginine methyl ester completely blocked the methanandamide-, capsaicin-, and CGRP-induced vasorelaxation. Pretreatment of aortic rings with pertussis toxin attenuated the methanandamide-induced vasorelaxation in endothelium-intact aortic rings, indicating the involvement of Gi/o-proteins in the vasorelaxation; however, pertussis toxin treatment failed to block the endothelium-independent response. Thus, in rabbit aorta, methanandamide-induced vasorelaxation exhibits two components: 1) in endothelium-intact rings, an SR141716A-sensitive, non-CB<sub>1</sub> receptor component that requires pertussis toxin-sensitive G proteins and NO production; and 2) in endothelium-denuded rings, a component that is mediated by VR<sub>1</sub> vanilloid receptors and possibly by the subsequent release of CGRP, that requires NO production but is independent of pertussis toxin-sensitive G proteins.

## **Keywords**

Cannabinoid receptors, vanilloid receptors, pertussis toxin, endothelium, nitric oxide

Cannabinoid drugs are known to produce profound cardiovascular effects in humans and animals (2,13,15). Recent findings have demonstrated that some cardiovascular effects are produced by the eicosanoid anandamide (arachidonylethanolamide) and its analogs in various animal models including conscious or anesthetized normotensive or spontaneously hypertensive rats (19,20,38). In anesthetized rats, anandamide produced sequential, triphasic changes consisting of a transient bradycardia and hypotension followed by a brief pressure increase and finally a relatively long-lasting depressor effect (39). The anandamide-induced prolonged hypotension was blocked by the CB<sub>1</sub> antagonist SR141716A (19,20,39), suggesting that this effect of anandamide is mediated by the CB<sub>1</sub> receptor. The failure of anandamide and other potent cannabinoid receptor agonists to elicit the long lasting depressor effect in CB<sub>1</sub> knockout (-/-) mice provides further support for the involvement of the CB<sub>1</sub> receptor in this component of the response (17). Recent studies have suggested that anandamide and other cannabinoid agonists induce hypotension by presynaptic inhibition of norepinephrine release (23, 26, 38) in a SR141716A-sensitive manner (16, 23). These findings suggest the involvement of a neuronal CB<sub>1</sub> receptor signaling mechanism for certain cardiovascular effects of cannabinoid drugs. In contrast, SR141716A failed to attenuate the anandamide-induced activation of both initial bradycardia and subsequent pressure changes (39), suggesting that this particular component of the anandamide response may not involve the CB<sub>1</sub> receptor.

In addition to the effect on blood pressure, anandamide evoked vascular smooth muscle relaxation in various endothelium-intact and denuded arterial preparations (for review, see 40). An SR141716A-sensitive, anandamide-induced vasorelaxation that does not respond to classical cannabinoid receptor agonists has been demonstrated by Kunos' laboratory (40, 41), who proposed that a novel "anandamide receptor" was involved. However this interpretation is not compatible with the findings of Pratt et al. (31), who proposed that metabolism of anandamide to other eicosanoid products could be responsible for the vasorelaxation.

More recently, the vasorelaxing effects of anandamide (but not classical CB<sub>1</sub> agonists) in isolated rat hepatic, rat mesenteric and guinea pig basilar arterial

preparations have been explained by a non-cannabinoid receptor mechanism involving the VR<sub>1</sub> vanilloid receptor (46). In that study, anandamide-induced vasodilation was shown to be blocked either by the VR<sub>1</sub> antagonist capsazepine or calcitonin gene-related peptide (CGRP) antagonist CGRP-(8-37), but not by SR141716A (46). Results of these studies suggest that anandamide activates VR<sub>1</sub> receptors on the perivascular sensory neurons to release CGRP, which would then evoke vascular relaxation.

In the present study, we found that in rabbit arterial ring preparations, methanandamide produced vasorelaxation by two different signal transduction pathways. The major component of the relaxation was attributed to a SR141716A-sensitive non-CB<sub>1</sub> receptor-mediated activation of pertussis toxin-sensitive G proteins and involves endothelial-derived NO for vasorelaxation. The other component was due to anandamide-activated VR<sub>1</sub> receptors and CGRP-mediated vasorelaxation, which involves non-endothelial-derived NO and is independent of pertussis toxin-sensitive G proteins.

## **Materials and Methods:**

**Reagents:** Methanandamide was purchased from Research Biochemicals Inc. (Natick, MA). Desacetyllevonantradol (DALN) was a gift from Pfizer, Inc. (Groton, CT). WIN55212-2 was obtained from Sigma-RBI (St. Louis, MO). SR141716A was purchased from BIOMOL (Plymouth Meeting, CA). Norepinephrine (NE), indomethacin, N<sup>G</sup> nitro-L-arginine methyl ester (L-NAME), acetylcholine chloride (ACh), CGRP, CGRP (8-37), anandamide and arachidonic acid were obtained from Sigma Chemical Co. (St. Louis, MO). Capsaicin and capsazepine were purchased from Tocris Co. (Ballwin, MO). Glyceryl trinitrate (Nitrostat, 0.4 mg tablets) was a product of Parke-Davis, Inc. (Ann Arbor, MI). All other chemicals used were of the highest analytical grade and were obtained from Sigma Chemical Co. (St. Louis, MO).

**Tissue Preparation:** Male New Zealand white rabbits (2.5-3.5 kg) were anesthetized with ketamine (3 mg/kg, i.m.) plus xylazine (1mg/kg, i.m.) followed by sodium pentobarbital (15 mg/kg, i.v.). A cannula was placed into the carotid artery and heparin (200/kg, i.v.) was administered. After 10 min, animals were exsanguinated. The abdomen

was opened by a midline incision and the aorta was carefully excised and placed in ice-cold Krebs-Ringer bicarbonate (KRB) buffer (118.3 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl<sub>2</sub>, 1.2 mM MgSO<sub>4</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 25 mM NaHCO<sub>3</sub>, 0.026 mM Na-EDTA and 11.1 mM glucose, pH 7.4) at 4° C. All connective and perivascular adipose tissues were removed, with caution being taken not to disrupt the endothelial cell lining. Aortas were then refrigerated in KRB buffer for use either the same or the following day. Storage for 24 h did not affect endothelium-dependent vasomotor responses. Transverse vascular rings 3-4 mm long were prepared from the aorta and suspended from an isometric force transducer (Grass FT .03) according to methods previously described (27,28). Briefly, the rings were mounted horizontally between an L-shaped fixed stainless steel rod and a freely moving stainless steel triangle attached to an isometric force transducer by an S hook in a 25 ml jacketed glass organ bath. Rings were bathed in 20 ml KRB buffer (37° C) and continuously aerated with 95% O<sub>2</sub>/5% CO<sub>2</sub>. The KRB buffer also contained indomethacin (10 µM) to inhibit cyclooxygenase activity. The rings were then gradually stretched over a period of 45-60 min until the optimum basal tension (2 g, as predetermined from tension-force studies) was achieved. The tissues were washed with 20 ml fresh KRB every 15-20 min. After an initial equilibration period of 20 min, rings were contracted with KCl (60 mM) to stabilize and enhance subsequent submaximal contraction (28). The tissues were washed with fresh KRB to remove KCl, and thereafter, submaximal tone (60-70 %) was induced in the rings with NE (100 nM). Following the submaximal NE-induced contraction of the rings, the test compounds were added into the incubation medium to achieve the final indicated concentrations in a cumulative manner. When aortic rings were treated with SR141716A, capsazepine or CGRP-(8-37), these drugs were included for 30 min at 37° C and then anandamide- or methanandamide-induced relaxation was determined. Data are presented as percent relaxation, calculated as a percent of the difference between the NE-contracted tone and the basal tone. After each series of additions, the rings were washed several times with fresh KRB and allowed to re-equilibrate for 20-30 min.

***Measurement of vasorelaxing effects of drugs under endothelium-denuded conditions:***

For the endothelium-denuded preparation, the endothelium was removed by rubbing the

intimal surface of the rings with the roughened shaft of a 23-gauge needle (27,28). The successful removal of endothelium was ascertained by the absence or markedly reduced (90%) relaxation produced in response to ACh. These same rings were also tested with the endothelium-independent relaxing agent glyceryl trinitrate to assure that the lost response to ACh was due to the loss of endothelium and not due to damage to the underlying smooth muscle structure. After the removal of the endothelium, the rings were allowed to re-equilibrate to basal tension for 30-45 min in KRB buffer, following which, the vasorelaxing effects of the indicated drugs were determined.

***Treatment with pertussis toxin:*** In order to determine the involvement of G<sub>i/o</sub> proteins in vasorelaxation, rabbit aortic rings were treated with pertussis toxin (250 ng/ml) or its vehicle for 2 h at 37° C (26). After the incubation period, the rings were washed with KRB, submaximally contracted with NE, and vasomotor responses to agonists and/or antagonists were determined. Experiments were performed such that drug-induced vasorelaxation was determined both before and after pertussis toxin treatment in order to compare the responses in a paired manner.

***Treatment with L-NAME:*** To determine the role of NO in agonist-induced vasorelaxation, endothelium-intact or endothelium-denuded rings were pretreated with L-NAME (30 µM for 45 min at 37° C). The rings were then washed with KRB, allowed to equilibrate for an additional 20 min, contracted with NE, and vasomotor responses to the test compounds were measured. Agonist-induced vasorelaxation was determined both before and after L-NAME treatment in the same rings in order to make paired comparisons.

***Data analysis:*** All values are expressed as mean ± sem of multiple, separate experiments. Data were analyzed by ANOVA followed by a Bonferroni *posthoc* test. The log dose-response curves were analyzed by nonlinear regression analysis of a sigmoidal curve in order to determine concentrations at half-maximal response and the slope factors (Graphpad Inplot).

## Results:

### *Methanandamide-induced vasorelaxation has two components: endothelium-dependent and independent*

Anandamide (100 nM-10  $\mu$ M) produced a vasorelaxation of NE-contracted aortic rings. The maximal response in endothelial-intact rings was approximately 60% relaxation (Fig. 1A). Methanandamide, a metabolically stable analog of anandamide (1), also produced a concentration-dependent relaxation of aortic rings (Fig. 1A, B). No significant difference was observed when relaxation produced by anandamide and methanandamide were compared, suggesting that anandamide degradation is not prevalent under these experimental conditions. In endothelium-intact aortic rings the maximum relaxation of about 80 % was obtained with 20  $\mu$ M methanandamide, and the EC<sub>50</sub> was 0.6  $\mu$ M (Fig. 2B). The onset of relaxation began within 15 sec after the addition of methanandamide to the KRB.

The methanandamide-induced relaxation was markedly attenuated following mechanical denudation of the same ring (compare Fig. 1C with 1B; see also Figs. 3B, 4C, 5B). The non-endothelial-dependent relaxation achieved a maximum of 18-20 % at high concentrations of methanandamide (20  $\mu$ M). Anandamide-induced vasorelaxation was also abrogated (80-85%) following endothelial denudation (data not shown). To control for the integrity of the smooth muscle, addition of the NO-donor GTN (0.1  $\mu$ M) to the endothelium-denuded ring produced almost complete relaxation (95-100%) (Fig. 1C). No significant difference was observed in GTN-induced relaxation before or after endothelium denudation or with a higher concentration of GTN (1  $\mu$ M) (data not shown).

Chaytor and colleagues (7) reported that in rabbit superior mesenteric arterial preparations, gap junctions are involved in anandamide-induced endothelium-dependent relaxation because the response could be blocked by the gap-junction inhibitor 18 $\alpha$ -glycyrhetinic acid and the connexin-43 mimetic peptide. We tested for a gap junction involvement in rabbit aortic ring preparations by preincubation of the rabbit aortic rings with the gap junction inhibitor 18 $\alpha$ -glycyrhetinic acid (50  $\mu$ M for 1 hr). This treatment did not block the anandamide- or methanandamide-induced relaxation (data not shown). This suggests that anandamide/methanandamide produced vasorelaxation in the rabbit

aortic rings through a gap junction-independent mechanism and therefore does not involve transfer of small molecules between cells. A recent study by White and colleagues (44) have reported that anandamide-induced vasorelaxation in rat coronary arteries was not affected by gap junction inhibitor 18 $\alpha$ -glycyrhethinic acid.

***Methanandamide produces vasorelaxation in a SR141716A-sensitive manner but not via the CB<sub>1</sub> receptor***

To address the issue of whether the anandamide-induced vasorelaxation in rabbit aortic rings could be due to stimulation of the CB<sub>1</sub> receptor, we evaluated the vasoactivity of DALN and WIN55212-2, two potent and efficacious CB<sub>1</sub> receptor agonists of the cannabinoid and aminoalkylindole chemical classes. Neither the classical cannabinoid agonist DALN nor the aminoalkylindole WIN55212-2 were able to produce a significant relaxation of the endothelium-intact aortic rings at any of the concentrations tested (100 nM-10  $\mu$ M) (Fig. 1A). Additionally, the CB<sub>1</sub> agonists produced no response whether the endothelium was functionally intact or not (data not shown). Thus, the anandamide and methanandamide response is not consistent with a CB<sub>1</sub> pharmacological profile.

To further address the hypothesis that anandamide and methanandamide-induced relaxation is mediated by the CB<sub>1</sub> receptor, studies were performed using the potent and selective CB<sub>1</sub> antagonist SR141716A. Anandamide and methanandamide produced relaxation in endothelium-intact aortic rings at all concentrations tested with EC<sub>50</sub> 0.7  $\mu$ M and 0.6  $\mu$ M, respectively (Fig. 2A and 2B). The anandamide response was reduced in the presence of 1  $\mu$ M SR141716A (Fig. 2A). It was not possible to properly determine the EC<sub>50</sub> because of concern about lack of solubility at high concentrations of anandamide. The methanandamide log concentration-response curve also underwent a rightward parallel shift (EC<sub>50</sub> = 4.2  $\mu$ M) in the presence of 1  $\mu$ M SR141716A without any alteration in the maximum response, consistent with competitive antagonism (Fig. 2B). When added to the incubation medium following methanandamide-induced relaxation, SR141716A partially reversed the response (data not shown). Higher concentrations of SR141716A were not used to induce a further antagonism because this

drug has been reported to evoke miscellaneous cellular effects at a concentration greater than 3  $\mu\text{M}$  (31,43). The  $\text{CB}_2$  cannabinoid receptor antagonist SR144528 did not block the anandamide- or methanandamide-induced vasorelaxation in the rabbit aortic ring preparation. This suggest that anandamide or methanandamide-induced response in rabbit aortic rings were not mediated through  $\text{CB}_2$  cannabinoid receptors.

SR141716A did not alter ACh-induced relaxation under conditions identical to those used for the anandamide and methanandamide responses (Fig. 2C), suggesting specificity of the antagonist for the anandamide-induced response. The endothelium-independent component of the methanandamide-induced relaxation, which was observed in denuded rings, was not attenuated in the presence of SR141716A (data not shown). Furthermore, SR141716A did not affect the vasorelaxation evoked by GTN (data not shown), indicating that the antagonism is not at the level of the smooth muscle.

#### ***VR<sub>1</sub> receptor and CGRP antagonists block methanandamide-induced endothelium-independent relaxation***

The role of vascular innervation on the methanandamide-induced relaxation of rabbit aortic rings was addressed by examining the effects of  $\text{VR}_1$  receptor-dependent neuromediator release. Pretreatment (30 min at 37° C) of endothelium-intact rabbit aortic rings with either the  $\text{VR}_1$  antagonist capsazepine (3  $\mu\text{M}$ ) or the CGRP receptor antagonist CGRP-(8-37) (2  $\mu\text{M}$ ) partially blocked (15-20%) methanandamide-induced relaxation (Fig. 3A). In endothelium-denuded rings, pretreatment with both capsazepine and CGRP-(8-37) totally blocked the relaxation evoked by 20  $\mu\text{M}$  methanandamide (Fig. 3B). These data suggest that the endothelium-independent component of the methanandamide-induced relaxation can be attributed to the activation of  $\text{VR}_1$  receptors known to be present on the primary sensory neurons embedded in the smooth muscle cell layer in the aortic vessel wall (46). Further, these results suggest that the methanandamide-stimulated  $\text{VR}_1$  receptor evokes vasorelaxation primarily by the release of the vasodilator CGRP from the prearterial nerve endings.

The methanandamide-induced, endothelium-independent vasorelaxation is only a small fraction of the vasorelaxation that can be produced by VR<sub>1</sub> receptors in the rabbit aortic ring preparation. Capsaicin and CGRP (10 μM) produced robust relaxation of endothelium-intact rabbit aortic rings (70 % (Fig. 4A) and 60 % (Fig. 4C), respectively). This occurs in a concentration-dependent manner with EC<sub>50</sub>'s of 1 μM and 0.3 μM, respectively (data not shown). Of importance, the capsaicin- and CGRP-mediated relaxation in endothelium-denuded rings was significantly less than that observed with endothelium-intact rings (25 % and 15%, respectively (Fig. 4)). This shows that capsaicin- and CGRP-evoked relaxation in the rabbit aortic ring preparation exhibits an endothelium-dependent component that is not related to the response that was produced by methanandamide.

#### ***L-NAME blocks both methanandamide- and capsaicin induced relaxation***

Pretreatment (30 min at 37° C) of endothelial intact rings with the NOS- inhibitor *L-NAME* (30 μM), almost completely blocked methanandamide- and capsaicin-induced relaxation in endothelium-intact (Fig. 4A) and denuded (Fig. 4B) rabbit aortic rings. CGRP-mediated relaxation was also significantly blocked following *L-NAME* treatment in endothelium intact and denuded rings (Fig. 4C). These results indicate that methanandamide, capsaicin, and CGRP produced vasorelaxation via an NO-mediated mechanism.

#### ***Pertussis toxin treatment blocks methanandamide-induced relaxation in endothelial intact rings***

The CB<sub>1</sub> and CB<sub>2</sub> cannabinoid receptors are coupled to signal transduction pathways that are mediated via the pertussis toxin-sensitive G<sub>i/o</sub> proteins (for review see 14). We performed a study to determine if the methanandamide-induced vasorelaxation is mediated via G<sub>i/o</sub> proteins. Treatment of rabbit aortic rings with pertussis toxin (250 ng/ml for 2 h at 37° C) significantly blocked the methanandamide-induced relaxation in endothelial-intact rings (Fig. 5A). However, in endothelial-denuded rings, pertussis toxin treatment had no effect on methanandamide-induced relaxation (Fig. 5B). Pertussis

toxin treatment also blocked the anandamide-induced vasorelaxation in endothelial-intact rings (data not shown). The methanandamide-induced relaxation in pertussis toxin treated endothelial-intact rings is equivalent in magnitude to that produced in endothelium-denuded rings (20%). This observation suggests that the endothelial-dependent component of the methanandamide-induced relaxation in rabbit aorta is mediated by a pertussis toxin-sensitive G protein, whereas the endothelium-independent, VR<sub>1</sub>-mediated component of the relaxation is not.

### **Discussion:**

The present study demonstrates that anandamide and methanandamide can activate at least two different pathways to produce vasorelaxation in the rabbit aortic ring model. When taken together, the data indicate that the endothelial-dependent, SR141716A-sensitive component of anandamide- or methanandamide-induced relaxation in rabbit aorta is regulated by a pertussis toxin-sensitive G protein. The results of the present study have also provided evidence in support of the dual involvement of pertussis toxin-sensitive G proteins and nitric oxide in the vasoregulatory effects of anandamide and methanandamide. In contrast, the endothelium-independent, VR<sub>1</sub>-mediated component of the relaxation is G<sub>o</sub>-independent. This represents the first report of the involvement of pertussis toxin sensitive G proteins with the endothelium-dependent component of anandamide/methanandamide relaxation, and is important because it implicates a G protein coupled receptor in the endothelium-dependent response.

The anandamide-induced relaxation was found to be endothelium-dependent in bovine coronary arteries (31) but not in rat mesenteric (42), coronary (44) and hepatic (45) arteries. The finding of CB<sub>1</sub> mRNA and protein in rat and human vascular endothelial cells (9,21) allows for the possible involvement of CB<sub>1</sub> receptors in vasoregulation at the level of endothelial cells. However, mediation by the CB<sub>1</sub> receptor in the present studies is made untenable by the observation that very potent and efficacious cannabinoid receptor agonists DALN and WIN55212-2 fail to evoke the relaxation of the rabbit aortic ring preparation. Antagonism of the anandamide or

methanandamide-evoked vasorelaxation response by SR141716A is in agreement with previous findings in rat coronary (33) and mesenteric (34) arteries. In the present studies, a greater concentration of SR141716A (1 $\mu$ M) was necessary to shift the concentration-response curve 8-fold for methanandamide in the aortic ring relaxation compared with a response believed to be mediated via the CB<sub>1</sub> receptor. For example, for the inhibition by WIN55212-2 of the guinea pig myenteric plexus longitudinal muscle twitch response, a shift of one order of magnitude could be produced by SR141716A at a concentration of 100 nM (8). Similar to the observations made in this study anandamide-induced relaxation in rat coronary arteries was attenuated by SR141716A at relatively higher concentration (3  $\mu$ M; 44) These findings suggest that a novel non-cannabinoid receptor that nevertheless exhibits sensitivity to SR141716A exists in this preparation. Our studies support the existence of a SR141716A-sensitive "anandamide" receptor, which has been previously proposed by Kunos and colleagues (18, 41).

Anandamide has previously been proposed to be an endothelial-derived hyperpolarization factor (EDHF) (32). Blockade of carbachol- and anandamide-mediated relaxation by SR141716A in isolated, buffer-perfused rat mesenteric and coronary vasculature as well as in mesenteric arterial segments implicated the CB<sub>1</sub> receptor in these events (32-34). Unlike our experiments, in those studies anandamide- or carbachol-induced relaxation was measured in the presence of L-NAME (300  $\mu$ M) and indomethacin (10  $\mu$ M) to detect a non-NO, EDHF response. However, these findings were not confirmed by other laboratories (5,30). The "EDHF"-like response of anandamide measured in the presence of L-NAME was found to be pertussis-toxin sensitive (42). Results of our study suggest that anandamide-induced vasorelaxation involves a pertussis-toxin sensitive G protein but that the final mediator is nitric oxide, obviating the notion of anandamide as an EDHF. Further, our finding that SR141716A fails to alter the response to ACh but exhibits a competitive inhibition for methanandamide would eliminate the possibility that the ACh response occurred via the release of endogenously synthesized anandamide in the rabbit aorta.

Anandamide-derived arachidonic acid released by the action of fatty acid aminohydrolase and subsequently formed eicosatrienoic acid derivatives was held responsible for the vasodilatory action of anandamide in studies by Pratt et al. (31). The

involvement of eicosanoid metabolites can be ruled out in the present study for the following reasons: a) anandamide and its metabolically stable analog methanandamide produced an identical profile of relaxation; b) arachidonic acid itself did not produce any response in rabbit aortic ring preparation (data not shown); c) the epoxyeicosatrienoic acid inhibitor 17-octadecanoic acid did not alter anandamide or methanandamide-induced relaxation (data not shown); and d) participation of cyclooxygenase products in methanandamide-induced vasorelaxation can be ruled out because indomethacin was used in all the experiments reported in the present study.

Zygmunt and colleagues (46) demonstrated that in isolated rat hepatic, rat mesenteric, and guinea pig basilar arterial preparations, anandamide-induced relaxation was almost completely blocked by the VR<sub>1</sub> vanilloid receptor antagonist capsazepine, but not by SR141716A. They suggested that the VR<sub>1</sub>-mediated release of neuromediators is responsible for the anandamide-evoked vasorelaxation (46). The response observed by Zygmunt et al. (46) appears to be comparable to that which we observed in the endothelial-denuded rings, which is consistent with a mechanism methanandamide activates the VR<sub>1</sub> receptor to cause vasorelaxation via CGRP. In mesenteric arteries of rabbits (25), splenic, gastric and hepatic arteries of rats (4), splenic arteries of pigs (29), cerebral arteries of cats (10, 35), and pulmonary arteries of guinea-pigs (22) and humans (24), CGRP-induced vasorelaxation is endothelium-independent. The relaxation that we observed in the rabbit aortic ring preparation was not pertussis toxin-sensitive, which would be consistent with the actions of CGRP occurring via a non-G<sub>i/o</sub> subtype of G proteins (6).

The significant attenuation by *L*-NAME of both components of the methanandamide-evoked vasorelaxation, as well as the capsaicin- and CGRP-evoked vasorelaxation suggests the involvement of NO in both endothelium-dependent and independent vasorelaxation. Anandamide has been reported to activate endothelial nitric oxide synthase (NOS) in human saphenous vein, and this effect was blocked by SR141716A (37). Although there is no consensus regarding the role of NO in capsaicin and CGRP-induced vasorelaxation, evidence suggests that capsaicin causes the release of CGRP which in turn activates endothelial NOS activity to produce NO to evoke vasodilation (11, 12). In vascular smooth muscle cells (36) and in rat thoracic aorta (12),

CGRP increased NOS activity via a cAMP-dependent pathway. Results from other laboratories suggest that NO is involved in the capsaicin-stimulated release of CGRP (3).

In summary, the present findings indicate that anandamide and methanandamide induce vasorelaxation in the rabbit aorta by the activation of a non-CB<sub>1</sub> "anandamide" receptor coupled to G<sub>i/o</sub> protein(s) via an endothelial-dependent mechanism requiring NO synthesis. To the best of our knowledge, this is the first report of the involvement of a pertussis toxin sensitive G protein in methanandamide-induced vasodilation. A less prominent endothelium-independent component of the vasorelaxation results from anandamide's stimulation of the VR<sub>1</sub> receptor and subsequent release of the vasodilator CGRP.

### **Acknowledgements**

We thank Drs. Randy Sprague and Alan Stephenson for help in rabbit surgery and aortic ring preparation. This work was supported by an American Heart Association grant 0060377Z to S. M. and NIDA grants R01-DA03690 and K05-DA00182 to A.C.H.

## Reference

1. **Abadji V, Lin S, Taha G, Griffin G, Stevenson LA, Pertwee RG, And Makriyannis A.** (R) -methanandamide: a chiral novel anandamide possessing higher potency and metabolic stability *J Med Chem* 37:1899-1893, 1994.
2. **Benowitz N and Jones RT.** Cardiovascular and metabolic considerations in prolonged cannabinoid administration in man *J Clin Pharmacol* 21:214S-223S, 1981.
3. **Brain SD, Hughes SR, Cambridge H, and O'Driscoll G.** The contribution of CGRP to neurogenic vasodilator responses. *Agents and Actions* 38: C19-C21, 1993.
4. **Bratveit M, Haugan A, and Helle KB.** Effects of CGRP on regional haemodynamics and on selected hepato-splanchnic arteries from the rat: a comparison with VIP and atriopeptin II. *Scand. J Clin Lab. Invest* 51:167-174, 1991.
5. **Chataigneau T, Feletou M., Thollon C, Villeneuve N, Vilaine J-P, Duhault J, and Vanhoutte PM.** Cannabinoid CB<sub>1</sub> receptor and endothelium-dependent hyperpolarization in guinea pig carotid, rat mesenteric and porcine coronary arteries. *Brit J Pharmacol* 123: 968-974, 1998.
6. **Chatterjee TK, Fisher RA.** Multiple affinity and guanine nucleotide sensitive forms of the calcitonin gene related peptide (CGRP) receptor. *Can J Physiol Pharmacol* 1995 73: 968-73, 1995.
7. **Chaytor AT, Martin PM, EvansWH, Randall MD, and Griffith TM.** The endothelial component of cannabinoid-induced relaxation in rabbit mesenteric artery depends on gap junctional communication. *J Physiol* 520: 539-550, 1999.
8. **Coutts AA, and Pertwee RG.** Inhibition by cannabinoid receptor agonists of acetylcholine release from the guinea-pig myenteric plexus. *Brit J Pharmacol* 121:1557-66, 1997.
9. **Deutsch DG, Goligorsky MS, Schmid PC, Krebsbach, RJ, Schmid HHO, Das SK, Dey SK, Arreaza G, Thorup C, Stefano G, and Moore LC.** Production and physiological actions of anandamide in the vasculature of the rat kidney. *J. Clin Invest* 100: 1538-1546, 1997.
10. **Edvinsson L, Fredholm B, Hamel E, Jansen I, and Verrechia C.** Perivascular peptides relax arteries concomitant with stimulation of cyclic AMP accumulation or release of an endothelium derived relaxing factor. *Neurosci Lett* 58:213-220, 1985.
11. **Elhawary AM, and Pang CCY.** Renal vascular and tubular actions of CGRP: effect of NG-Nitro L Arginine methyl ester. *J Pharmacol Exp Therp* 273: 56-63, 1995.

12. **Gray DW, and Marshall I.** NO synthesis inhibitors attenuate CGRP endothelium-dependent vasorelaxation in rat aorta. *Eur J Pharmacol* 212:37-42, 1992.
13. **Hillard CJ.** Endocannabinoids and vascular function. *J Pharmacol Exp Therp* 294: 27-32, 2000.
14. **Howlett AC.** Pharmacology of cannabinoid receptors. *Annu Rev Pharmacol Toxicol* 35: 607-634, 1995
15. **Howlett AC and Mukhopadhyay S.** Cellular signal transduction by anandamide and 2-arachidonoylglycerol. *Chem Phys Lipids* 108: 53-70, 2000.
15. **Ishac E, Jiang L, Lake KD, Varga, K, Abood ME, and Kunos G.** Inhibition of exocytotic noradrenaline release by presynaptic cannabinoid CB<sub>1</sub> receptors on peripheral sympathetic nerves. *Brit J Pharmacol* 118: 2023-2028, 1996.
17. **Jarai Z, Wagner JA, Varga K, Lake KD, Compton D, Martin BR, Zimmer AM, Bonner TI, Buckley NE, Mezey E, Razdan RK, Zimmer A, and Kunos G.** Cannabinoid-induced mesenteric vasodilation through an endothelial site distinct from CB<sub>1</sub> or CB<sub>2</sub> receptors. *Proc Natl Acad Sci USA* 96:14136-14141, 1999.
18. **Jarai Z, Wagner JA, Goparaju SK, Wang L, Razdan RK, Sugiura T, Zimmer M, Bonner T., Zimmer A, and Kunos G.** Cardiovascular effects of 2-arachidonoylglycerol in anesthetized mice. *Hypertension* 35: 679-690, 2000.
19. **Lake KD, Compton DR, Varga K, Martin BR, and Kunos G.** Cannabinoid-induced hypotension and bradycardia in rats is mediated by CB<sub>1</sub>-like cannabinoid receptors. *J Pharmacol Expt Therp* 281: 1030-1037, 1997a.
20. **Lake KD, Martin BR, Kunos G, and Varga K.** Cardiovascular effects of anandamide in anesthetized and conscious normotensive and hypertensive rats. *Hypertension* 29:1204-1210, 1997b.
21. **Liu J, Gao B, Mirshahi F, Sanyal AJ, Khanolkar AD, Makriyannis A, and Kunos G.** Functional CB<sub>1</sub> cannabinoid receptors in human vascular endothelial cells. *Biochem J* 346: 835-840, 2000.
22. **Maggi CA, Patacchini R, Perretti F, Tramontana M, Manzini S, Geppetti P, and Santicioli P.** Sensory nerves, vascular endothelium and neurogenic relaxation of guinea pig isolated pulmonary artery. *Naunyn-Schiedeberg's Arch Pharmacol* 342:78-84, 1990.
23. **Malinowska B, Godlewski G, Bucher B and Schlicker E.** Cannabinoid CB<sub>1</sub> receptor-mediated inhibition of neurogenic vasorepressor response in the pithed rat. *Naunyn Schmiedebergs Arch Pharmacol* 356:197-202, 1997.

24. **McCormack DG, Mak JCW, Coupe MO, and BarnesPJ.** CGRP vasodilation of human pulmonary vessels. *J. Appl. Physiol* 67:1265-1270, 1989.
25. **Nelson MT, Huang, Y, Brayden JE, Hescheler J, and Standen NB.** Arteriolar dilations in response to CGRP involve activation of K<sup>+</sup> channels. *Nature* 344:770-773, 1990.
26. **Niederhoffer N, and Szabo B.** Effect of the cannabinoid receptor agonist WIN55212-2 on sympathetic cardiovascular regulation. *Br J Pharmacol* 126: 457-466, 1999
27. **Pawloski JR, and Chapnick BM.** Antagonism of LTD<sub>4</sub>-evoked relaxation in canine renal artery and vein. *Am J Physiol* 265: H980-H985, 1993.
28. **Pawloski JR, and Chapnick BM.** LTD<sub>4</sub> and bradykinin evoke endothelium-dependent relaxation of the renal vein: dissimilar mechanisms. *Am J Physiol* 261: H88-H95, 1991.
29. **Pernow J.** Actions of constrictor (NPY and endothelin) and dilator (Substance P, CGRP and VIP) peptides on pig splenic and human skeletal muscle arteries: involvement of the endothelium. *Brit J Pharmacol* 97: 983-989, 1989.
30. **Plane F, Holland M, Waldron GJ, Garland CJ, and Boyle JP.** Evidence that anandamide and EDHF act via different mechanisms in rat isolated mesenteric arteries. *Brit. J Pharmacol* 121: 1509-1511, 1997.
31. **Pratt PF, Hillard CJ, Edgmond WS, and Campbell WB.** N-arachidonylethanolamide relaxation of bovine coronary artery is not mediated by CB<sub>1</sub> cannabinoid receptor. *Am. J Physiol* 274: H375-H381, 1998.
32. **Randall MD, Alexander SP, H., Bennet T, Boyd EA, Fry JR, Gardiner SH, Kemp PA, McCulloch AI, and Kendall DA.** An endogenous cannabinoid as an endothelium-derived vasorelaxant. *Biochem Biophys Res Comm* 229: 114-120, 1996.
33. **Randall MD, and Kendall DA.** Involvement of a cannabinoid in endothelium-derived hyperpolarizing factor-mediated coronary vasorelaxation. *Eur J Pharmacol* 335: 205-209, 1997a.
34. **Randall MD, McCulloch, AI, and Kendall DA.** Comparative pharmacology of endothelium-derived hyperpolarizing factor and anandamide in rat isolated mesentery. *Eur J Pharmacol* 333: 191-197, 1997b.

35. **Saito A, Masaki T, Uchiyama Y, Lee TJF, and Goto K.** CGRP and vasodilator nerves in large cerebral arteries of cats. *J Pharmacol. Expt. Therp* 248: 455-462, 1989.
36. **Schini-Kerth VB, Fisslthaler B, and Busse R.** CGRP enhances induction of NO synthase in vascular smooth muscle cells via a cyclic AMP-dependent mechanism. *Am J Physiol* 267: H2483-H2490, 1994.
37. **Stefano GB, Salzet M, Magazine HI, and Bilfinger TV.** Antagonism of LPS and IFN- $\gamma$  induction of iNOS in human saphenous vein endothelium by morphine and anandamide by nitric oxide inhibition of adenylate cyclase. *J Cardiovasc Pharmacol* 31: 813-820, 1998.
38. **Varga K, Lake KD, Huangfu D, Guyenet PG, and Kunos G.** Mechanism of the hypotensive action of anandamide in anesthetized rats. *Hypertension* 28: 682-686, 1996.
39. **Varga K, Lake K, Martin BR, and Kunos G.** Novel antagonist implicates the CB<sub>1</sub> cannabinoid receptor in the hypotensive action of anandamide. *Eur J Pharmacol* 278: 279-283, 1995.
40. **Wagner JA, Varga K, and Kunos G.** Cardiovascular actions of cannabinoids and their generation during shock. *J Mol Med* 76: 824-36, 1998.
41. **Wagner JA, Varga K, Jarai Z, and Kunos G.** Mesenteric vasodilation mediated by endothelial anandamide receptors. *Hypertension* 33: 429-434, 1999.
42. **White R, and Hiley CR.** A comparison of EDHF-mediated and anandamide-induced relaxations in the rat isolated mesenteric artery. *Brit J Pharmacol* 121: 1573-1584, 1997.
43. **White R, and Hiley CR.** The actions of the cannabinoid receptor antagonist, SR141716A, in the rat isolated mesenteric artery. *Brit J Pharmacol* 125: 689-696, 1998.
44. **White R, Venessa Ho W, Bottrill FE, Ford Wr, and Hiley CR.** Mechanism of anandamide-induced vasorelaxation in rat isolated coronary arteries. *Brit J Pharmacol* 134: 921-929, 2001
45. **Zygmunt PM, Edwards G, Weston AH, Larsson B, and Hogestatt ED.** Involvement of voltage-dependent potassium channels in the EDHF-mediated relaxation of rat hepatic artery. *Brit J Pharmacol* 121:141-149, 1997.
46. **Zygmunt PM, Petersson J, Andersson DA, Chuang HH, Sorgard M., DiMarzo V, Julius D, and Hogestatt ED.** Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. *Nature* 400: 452-457, 1999.

## Figure legends

Figure 1. Vasorelaxant effect of anandamide and methanandamide and cannabinoid receptor agonists on NE-contracted rabbit aortic rings. (A) Anandamide, methanandamide, desacetyllevonantradol (DALN) and WIN55212-2 were applied to rabbit aortic rings at the indicated concentrations and % relaxation was determined. Data are presented as mean  $\pm$  SEM of 4 separate aortic rings from different rabbits. (B) Representative polygraph tracing illustrating the vasorelaxant effects of methanandamide on NE-contracted endothelium intact rabbit aortic ring preparation with endothelium intact, and (C) after the endothelium was disrupted. Each arrow represents the time of addition and numbers correspond to final concentrations of methanandamide.

Figure 2. Dose-dependent vasorelaxation of NE-contracted rabbit aortic rings by anandamide (A), methanandamide (B) and acetylcholine (ACh) (C) in the presence and absence of SR141716A. Rings were incubated with SR141716A (1  $\mu$ M) for 30 min prior to addition of anandamide, methanandamide or ACh, which were added to the KRB by incrementing the concentration at 2 min intervals. Each point on the graph represents the cumulative final concentration of the vasorelaxant compounds. Data are presented as the mean  $\pm$  SEM of observations obtained from 6-8 separate aortic rings from different rabbits.

Figure 3. Effect of capsazepine and CGRP-(8-37) on methanandamide-induced vasorelaxation of NE-contracted (A) endothelium-intact and (B) endothelium-denuded rabbit aortic rings. Capsazepine (3 mM), CGRP-(8-37) (2 mM) or vehicle was added to the KRB 30 min before the addition of methanandamide. Data are presented as mean  $\pm$  SEM of 4-6 separate aortic rings from different rabbits. The methanandamide-induced % relaxation was significantly different (\* $p$ <0.02 and ++ $p$ <0.05) between control and capsazepine- or CGRP-(8-37)- treated rings.

Figure 4. Effect of L-NAME on methanandamide- and capsaicin-induced vasorelaxation of NE-contracted (A) endothelium-intact and (B) endothelium-denuded rabbit aortic rings. (C) Effect of L-NAME on CGRP-induced vasorelaxation of endothelium-intact and endothelium-denuded rabbit aortic rings. Rings were pretreated with L-NAME (30 mM) or its vehicle

(water) for 30 min prior to the contraction with NE and then methanandamide, capsaicin or CGRP was added at the indicated cumulative final concentrations. Data are presented as the mean  $\pm$  SEM of % relaxation from 4 separate aortic rings from different rabbits. The % relaxation was significantly different (\* $p < 0.02$  and ++ $p < 0.02$ ) between the absence (hatched bar) and presence (filled bar) of L-NAME.

Figure 5. Effects of pertussis toxin on methanandamide-induced vasorelaxation of NE-contracted (A) endothelium-intact and (B) endothelium-denuded rabbit aortic rings. Rings were pretreated with pertussis toxin (250 ng/ml) for 2 h at 37 °C and then methanandamide was added at the indicated cumulative final concentrations. Data are presented as mean  $\pm$  SEM of % relaxation from 3 separate aortic rings from different rabbits. (A) The methanandamide-induced % relaxation was significantly different (\* $p < 0.02$ ) between vehicle-(hatched bars) and pertussis toxin-treated (filled bars) endothelium-intact aortic rings at all the concentrations of methanandamide. (B) No significant difference was observed between vehicle- (hatched bars) and pertussis toxin-treated (filled bars) aortic rings after endothelium denudation.

**Figure 1**

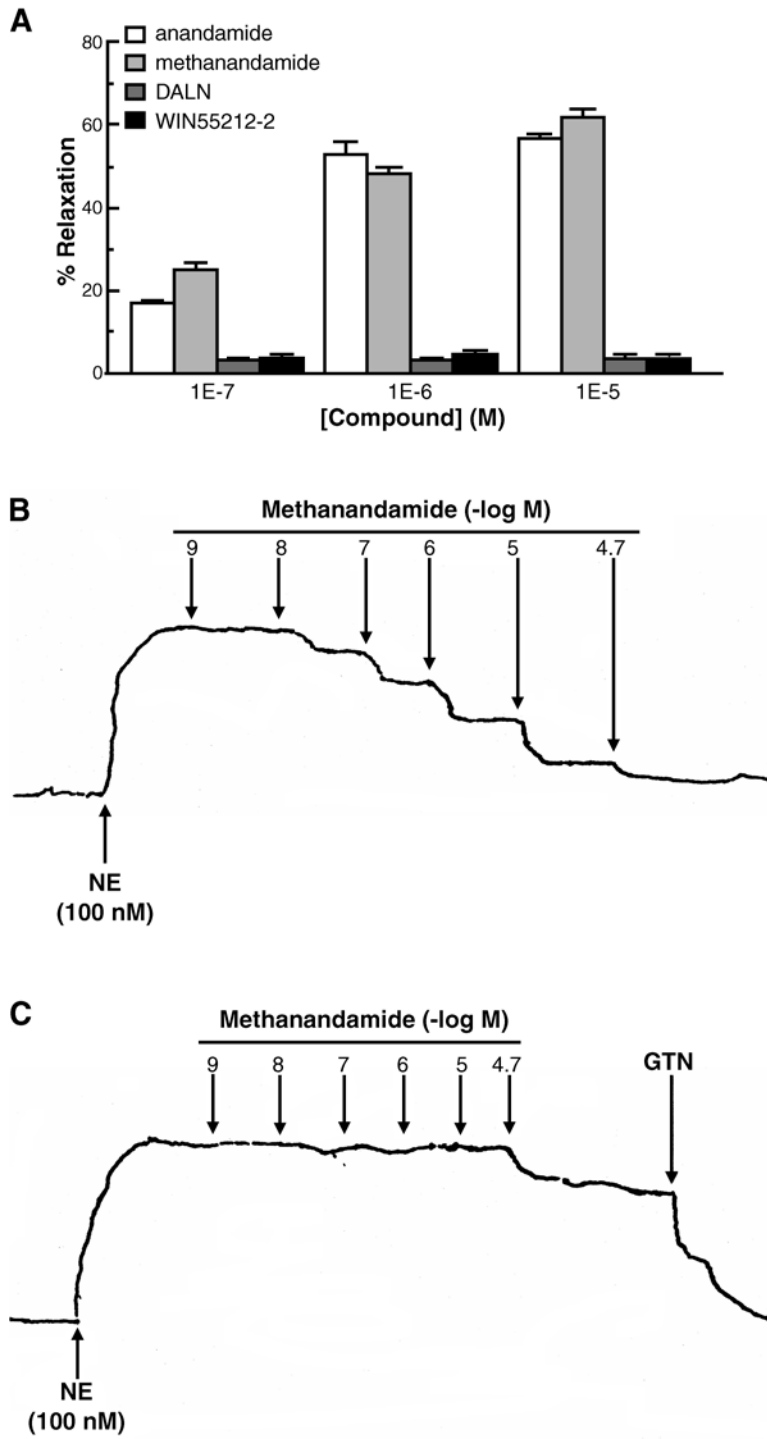


Figure 2

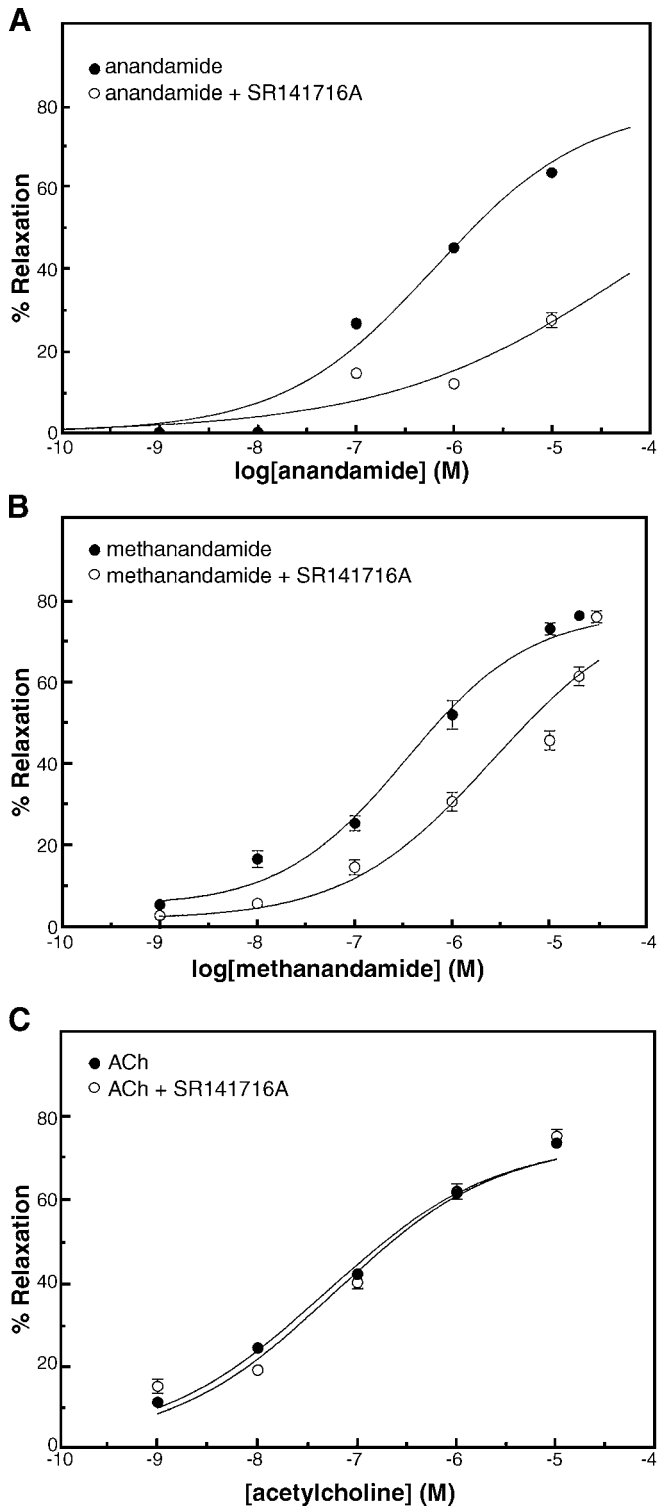
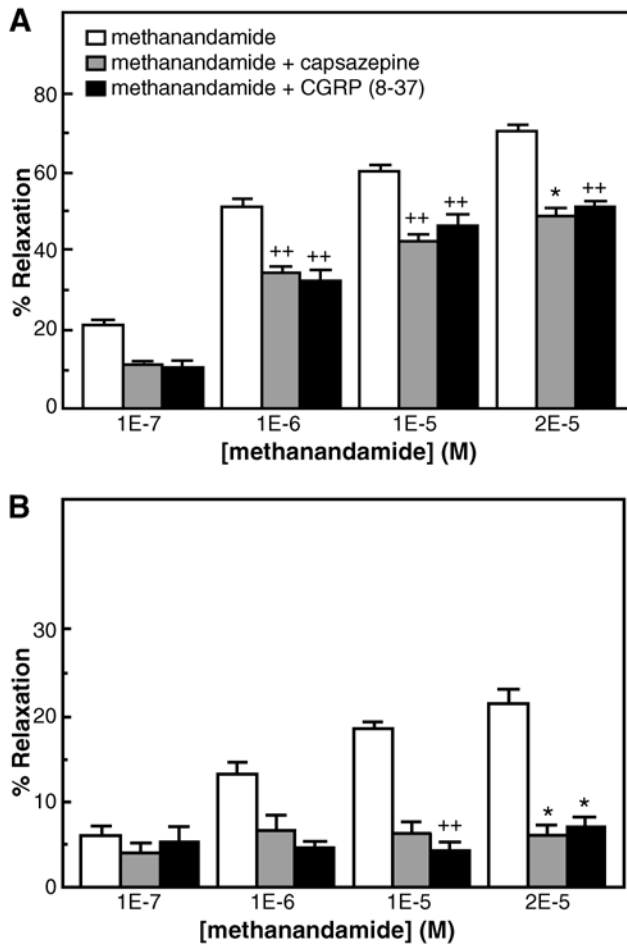


Figure 3



**Figure 4**

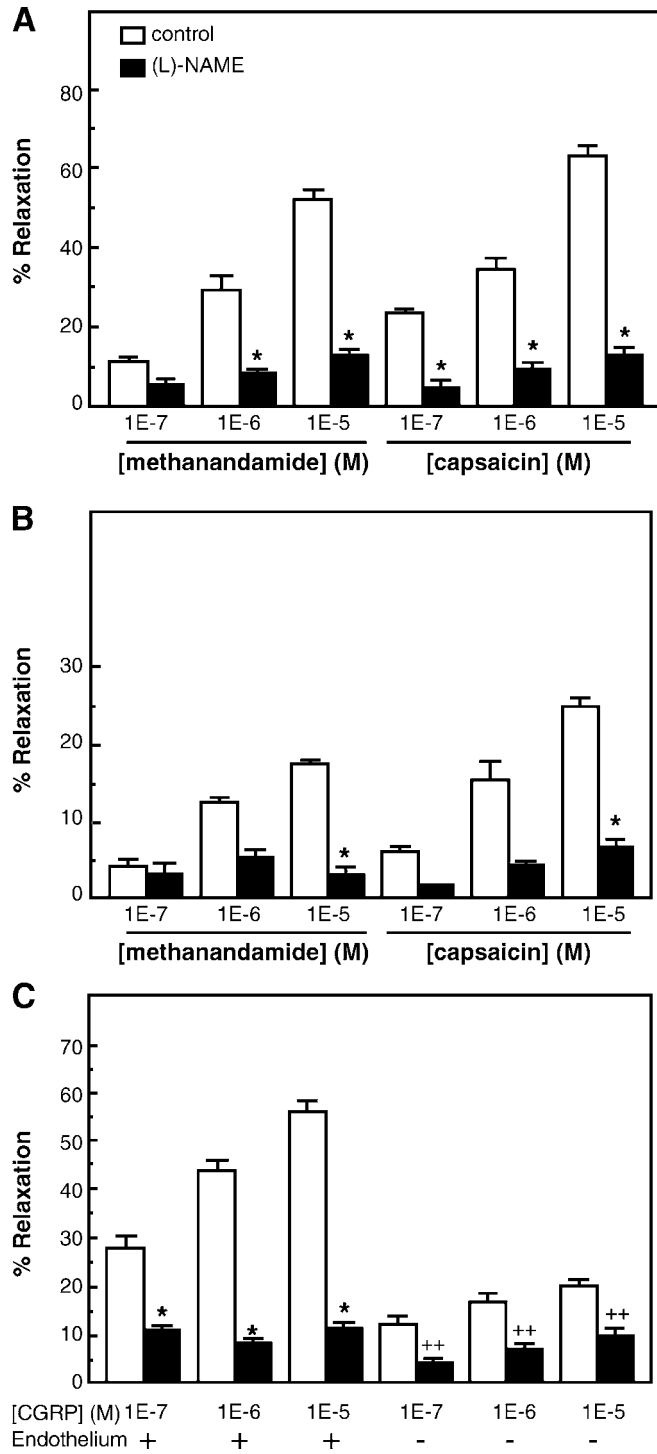


Figure 5

