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

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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, 39). 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 (38). The anandamide-induced prolonged hypotension was blocked by the CB1 antagonist SR141716A (19, 20, 38), suggesting that this effect of anandamide is mediated by the CB1 receptor. The failure of anandamide and other potent cannabinoid receptor agonists to elicit the long-lasting depressor effect in CB1 knockout (−/−) mice provides further support for the involvement of the CB1 receptor in this component of the response (18). Recent studies have suggested that anandamide and other cannabinoid agonists induce hypotension by presynaptic inhibition of norepinephrine (NE) release (23, 26, 39) in a SR141716A-sensitive manner (16, 23). These findings suggest the involvement of a neuronal CB1 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 (38), suggesting that this particular component of the anandamide response may not involve the CB1 receptor.

In addition to the effect on blood pressure, anandamide evoked vascular smooth muscle relaxation in various endothelium-intact and -denuded arterial preparations (for a review, see Ref. 41). A SR141716A-sensitive, anandamide-induced vasorelaxation that does not respond to classical cannabinoid receptor agonists has been demonstrated by Wagner et al. (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 CB1 agonists) in isolated rat hepatic, rat mesenteric, and guinea pig basilar arterial preparations have been explained by a noncannabinoid...
receptor mechanism involving the VR1 vanilloid receptor (46). In that study, anandamide-induced vasodilation was shown to be blocked either by the VR1 antagonist capsazepine or the calcitonin gene-related peptide (CGRP) antagonist CGRP-(8–37) but not by SR141716A (46). Results of these studies suggest that anandamide activates VR1 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-CB1 receptor-mediated activation of pertussis toxin-sensitive G proteins and involves endothelium-derived nitric oxide (NO) for vasorelaxation. The other component was due to anandamide-activated VR1 receptors and CGRP-mediated vasorelaxation, which involves non-endothelium-derived NO and is independent of pertussis toxin-sensitive G proteins.

MATERIALS AND METHODS

Reagents. Methanandamide was purchased from Research Biochemicals (Natick, MA). Desacetyllevenantradol (DALN) was a gift from Pfizer (Groton, CT). WIN55212-2 was obtained from Sigma-RBI (St. Louis, MO). SR141716A was purchased from BioMol (Plymouth Meeting, CA). NE, indomethacin, N\textsuperscript{6}-nitro-L-arginine methyl ester (L-NAME), acetylcholine chloride (ACh), CGRP, CGRP-(8–37), anandamide, and arachidonic acid were obtained from Sigma. Capsaicin and capsazepine were purchased from Tocris (Ballwin, MO). Glyceryl trinitrate (GTN); Nitrostat, 0.4-mg tablets was a product of Parke-Davis (Ann Arbor, MI). All other chemicals used were of the highest analytic grade and were obtained from Sigma.

Tissue preparation. Male New Zealand White rabbits (2.5–3.5 kg) were anesthetized with ketamine (3 mg/kg im) plus xylazine (1 mg/kg im), followed by pentobarbital sodium (15 mg/kg iv). A cannula was placed into the carotid artery, and heparin (200 U/kg iv) was administered. After 10 min, the xylazine (1 mg/kg im), followed by pentobarbital sodium (15 mg/kg iv). A cannula was placed into the carotid artery, and heparin (200 U/kg iv) was administered. After 10 min, the

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 re-
response in endothelium-intact rings was ~60% relaxation (Fig. 1A). Methanandamide, a metabolically stable analog of anandamide (1), also produced a concentration-dependent relaxation of aortic rings (Fig. 1, A and B). No significant differences were observed when relaxations 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 ~80% was obtained with 20 μM methanandamide and the EC50 was 0.6 μM (Fig. 2B). The onset of relaxation began within 15 s after the addition of methanandamide to the KRB buffer.

The methanandamide-induced relaxation was markedly attenuated after mechanical denudation of the same ring (compare Fig. 1, C with B; see also Figs. 3B, 4C, and 5B). The non-endothelial-dependent relaxation achieved a maximum of 18–20% at high concentrations of methanandamide (20 μM). Anandamide-induced vasorelaxation was also abrogated (80–85%) after endothelial denudation (data not shown). To control for the integrity of the smooth muscle, addition of the NO donor GTN (0.1 μM) 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 μM) (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 18α-glycerrhetinic acid and connexin43 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 18α-glycerrhetinic acid (50 μM for 1 h). 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) reported that anandamide-induced vasorelaxation in rat coronary arteries was not affected by the gap junction inhibitor 18α-glycerrhetinic acid.

Methanandamide produces vasorelaxation in a SR141716A-sensitive manner but not via the CB1 receptor. To address the issue of whether the anandamide-induced vasorelaxation in rabbit aortic rings could be due to stimulation of the CB1 receptor, we evaluated the vasoactivity of DALN and WIN55212-2, two potent and efficacious CB1 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 μM; Fig. 1A). Additionally, the CB1 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 CB1 pharmacological profile.

To further address the hypothesis that anandamide and methanandamide-induced relaxation is mediated...
by the CB₁ receptor, studies were performed using the potent and selective CB₁ antagonist SR141716A. Anandamide and methanandamide produced relaxation in endothelium-intact aortic rings at all concentrations tested with EC₅₀ 0.7 and 0.6 μM, respectively (Fig. 2, A and B). The anandamide response was reduced in the presence of 1 μM SR141716A (Fig. 2A). It was not possible to properly determine the EC₅₀ because of concerns about lack of solubility at high concentrations of anandamide. The methanandamide-induced percent relaxation was significantly different (*P < 0.02 and ++P < 0.05) between control and capsazepine- or CGRP-(8–37)-treated rings.

Fig. 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 μM) for 30 min before the addition of anandamide, methanandamide, or ACh, which were added to the Krebs-Ringer bicarbonate (KRB) buffer 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 means ± SE of observations obtained from 6–8 separate aortic rings from different rabbits.

Fig. 3. Effect of capsazepine and the calcitonin gene-related peptide (CGRP) antagonist CGRP-(8–37) on methanandamide-induced vasorelaxation of NE-contracted endothelium-intact (A) and endothelium-denuded rabbit aortic rings (B). Capsazepine (3 mM), CGRP-(8–37) (2 mM), or vehicle was added to the KRB buffer 30 min before the addition of methanandamide. Data are presented as means ± SE of 4–6 separate aortic rings from different rabbits. The methanandamide-induced percent relaxation was significantly different (*P < 0.02 and ++P < 0.05) between control and capsazepine- or CGRP-(8–37)-treated rings.
Induce a further antagonism because this drug has been reported to evoke miscellaneous cellular effects at a concentration >3 μM (31, 43). The CB2 cannabinoid receptor antagonist SR144528 did not block the anandamide- or methanandamide-induced vasorelaxation in the rabbit aortic ring preparation. This suggests that the anandamide- or methanandamide-induced response in rabbit aortic rings was not mediated through CB2 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

![Graph A](image1.png)

**Fig. 4.** Effect of N^G^-nitro-L-arginine methyl ester (L-NAME) on methanandamide- and capsaicin-induced vasorelaxation of NE-contracted endothelium-intact (A) and endothelium-denuded rabbit aortic rings (B). 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 before the contraction with NE, and methanandamide, capsaicin, or CGRP was then added at the indicated cumulative final concentrations. Data are presented as means ± SE of the percent relaxation from 4 separate aortic rings from different rabbits. The percent relaxation was significantly different (*P < 0.02 and ++P < 0.05) between the absence and presence of L-NAME.

![Graph B](image2.png)

**Fig. 5.** Effects of pertussis toxin (PTX) on methanandamide-induced vasorelaxation of NE-contracted endothelium-intact (A) and endothelium-denuded rabbit aortic rings (B). Rings were pretreated with PTX (250 ng/ml) for 2 h at 37°C, and methanandamide was then added at the indicated cumulative final concentrations. Data are presented as means ± SE of the percent relaxation from 3 separate aortic rings from different rabbits. A: methanandamide-induced percent relaxation was significantly different (*P < 0.02) between vehicle- and PTX-treated endothelium-intact aortic rings at all the concentrations of methanandamide. B: no significant differences were observed between vehicle- and PTX-treated aortic rings after endothelium denudation.
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 glyceryl trinitrate (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 VR<sub>1</sub> receptor-dependent neuromediator release. Pretreatment (30 min at 37°C) of endothelium-intact rabbit aortic rings with either the VR<sub>1</sub> antagonist capsazepine (3 μM) or the CGRP receptor antagonist CGRP-(8–37) (2 μM) 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 μM methanandamide (Fig. 3B). These data suggest that the endothelium-independent component of the methanandamide-induced relaxation can be attributed to the activation of VR<sub>1</sub> receptors known to be present on the primary sensory neurons embedded in the smooth muscle cell layer in the aortic vessel wall (46). Furthermore, these results suggest that the methanandamide-stimulated VR<sub>1</sub> 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> of 1 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 NO synthase (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 after L-NAME treatment in endothelium-intact and -denuded rings (Fig. 4C). These results indicate that methanandamide, capsaicin, and CGRP produced vasorelaxation via a NO-mediated mechanism.

**Pertussis toxin treatment blocks methanandamide-induced relaxation in endothelium-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 a review, see Ref. 14). We performed a study to determine whether 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 endothelium-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 endothelium-intact rings (data not shown). The methanandamide-induced relaxation in pertussis toxin-treated endothelium-intact rings is equivalent in magnitude to that produced in endothelium-denuded rings (20%). This observation suggests that the endothelium-dependent component of the methanandamide-induced relaxation in the 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 endothelium-dependent, SR141716A-sensitive component of anandamide- or methanandamide-induced relaxation in the 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 NO 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>i/o</sub> protein 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 failed to evoke relaxation of the rabbit aortic ring preparation. Antagonism of the anandamide- or methanandamide-evoked vasorelaxation,
induced relaxation was measured in the presence of these substances, in those studies, anandamide- or carbachol-mediated relaxation by SR141716A in isolated, buffer-perfused rat mesenteric and coronary vasculature as well as in mesenteric arterial segments implicated the CB1 receptor in these events (32). Unlike our experiments, in those studies, anandamide- or carbachol-induced relaxation was measured in the presence of L-NAME (300 μM) and indomethacin (10 μM) 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 NO, obviating the notion of anandamide as an EDHF. Furthermore, 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: 1) anandamide and its metabolically stable analog methanandamide produced an identical profile of relaxation; 2) arachidonic acid itself did not produce any response in the rabbit aortic ring preparation (data not shown); 3) epoxyeicosatrienoic acid inhibitor 17-octadecanoic acid did not alter anandamide- or methanandamide-induced relaxation (data not shown); and 4) participation of cyclooxygenase products in methanandamide-induced vasorelaxation can be ruled out because indo-0060377Z (to S. Mukhopadhyay) and by National Institute on Drug Abuse Grants R01-DA-03690 and K05-DA-00182 (to A. C. Howlett).

In summary, the present findings indicate that anandamide and methanandamide induce vasorelaxation in the rabbit aorta by the activation of a non-CB1 “anandamide” receptor coupled to G_{i/o} protein(s) via an endothelium-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 VR1 receptor and subsequent release of the vasodilator CGRP.

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