# Cannabinoid agonists induce relaxation in the bovine ophthalmic artery: evidences for CB$_1$ receptors, nitric oxide and potassium channels

[1]Maria Rosaria Romano & *,[1]Marcello D. Lograno

[1]Department of Pharmacobiology, Section of Pharmacology, University of Bari, Via Orabona 4, 70125 Bari, Italy

1 Glaucoma pathophysiology appears to involve vascular deficits, which may contribute to initiation and progression of the disease.

2 Anandamide, the endogenous cannabinoid ligand, and WIN55212-2, a synthetic cannabinoid agonist, are able to evoke concentration-dependent relaxations in bovine ophthalmic artery rings, precontracted with 5-hydroxytryptamine (5-HT) (1 $\mu$M). Endothelium removal reduces cannabinoid agonist potency and efficacy.

3 The selective cannabinoid 1 (CB$_1$) receptor antagonists SR141716A (100 nM) and AM251 (100 nM) cause a shift to the right in the concentration–response curves to anandamide and WIN55212-2 in arterial rings both in the presence and in the absence of endothelium.

4 In endothelium-intact arteries, the nitric oxide synthase inhibitor, $N^G$-monomethyl-L-arginine (L-NMMA, 300 $\mu$M), completely blocked the anandamide- and WIN55212-2-relaxant responses; by contrast, the nitric oxide donor $S$-nitroso-$N$-acetylpenicillamine (SNAP, 100 $\mu$M) induced an increase in vasorelaxant responses to cannabinoid agonists.

5 Relaxations to anandamide and WIN55212-2 were inhibited by iberiotoxin (IbTX, 200 nM), a blocker of large conductance, Ca$^{2+}$-activated K$^+$ channel (BK$_{Ca}$), and by 4-aminopyridine (4-AP; 1 mM), a blocker of delayed rectifier K$^+$ channel, whereas the blockade of $K_{ATP}$ channels by glibenclamide (5 $\mu$M) and of small conductance Ca$^{2+}$-activated K$^+$ channels (SK$_{Ca}$) by apamin (100 nM) did not produce any effects.

6 These data suggest that anandamide and WIN55212-2 relax the bovine ophthalmic artery by involving CB$_1$ the cannabinoid receptor-sensitive pathway. In endothelium-intact arteries, relaxation occurs through activation of nitric oxide synthase cyclic GMP and Ca$^{2+}$-activated K$^+$ channels. They also cause endothelium-independent relaxation by involving potassium channel opening.
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## Introduction

Glaucoma is a progressive optic neuropathy, which induces characteristic structural modifications to the optic nerve head and typical alteration of the visual field. Elevated intraocular pressure clearly remains the major risk factor, even if there is an increasing acceptance in the current literature showing that the ischaemic component contributes to the pathophysiology of glaucoma (Flammer, 1994; Chung *et al.*, 1999; Flammer *et al.*, 2002). Subsequent, to various clinical observations, research has recently been oriented towards the study of vascular risk factors that could contribute both to initiation and progression of disease. It has been observed that glaucomatous eyes show an impaired ocular blood flow; the causative factors remain unknown (Flammer *et al.*, 2002). All drugs currently used to treat glaucoma aim at lowering intraocular pressure, therefore an evaluation of the vasoactive effects of drugs utilized in glaucoma pharmacological therapy might be particularly relevant. In addition, glaucoma is a chronic disease and the quest for new ocular hypotensive agents able to improve ocular blood flow is important for its treatment.

The presence of a functional endocannabinoid system in the eye demonstrates an important role for endocannabinoid in ocular physiology. Numerous studies support the intraocular pressure-lowering effects of cannabinoid (Pate *et al.*, 1996; Song & Slowey, 2000; Porcella *et al.*, 2001) through actions

*Author for correspondence; E-mail: mdlograno@farmbiol.uniba.it

that are mostly mediated by cannabinoid 1 ($CB_1$) receptors. Recently, we have found $CB_1$ receptors in bovine ciliary muscle, supporting a direct role for the $CB_1$ receptors in controlling intraocular pressure (Lograno & Romano, 2004).

Cannabinoids and, in particular, the endocannabinoid anandamide are involved in potent dilator responses in various vascular beds (for review see Randall *et al.*, 2002). Some of the most exciting studies show that anandamide is able to mediate vasorelaxant responses, probably *via* coupling to nitric oxide release, metabolism to vasoactive arachidonic metabolites, prostanoid involvement, endothelium-derived hyperpolarizating factor release and inhibition of calcium channels (Deutsch *et al.*, 1997; Pratt *et al.*, 1998; Liu *et al.*, 2000; Grainger & Boachie-Ansah, 2001; Moezi *et al.*, 2004; O'Sullivan *et al.*, 2004). Indeed, although there are a variety of putative mechanisms that could explain these events, in the literature there is still considerable discrepancy. The anandamide vasorelaxant responses appear to be both endothelium dependent and independent according to the vascular bed (White & Hiley, 1997; Randall & Kendall, 1998). In cat cerebral artery and rat mesenteric artery, ananadamide-induced relaxations were independent of the endothelium (Plane *et al.*, 1997; White & Hiley, 1997; Gebremedhin *et al.*, 1999); by contrast, in rabbit cerebral and aortic arteries, rat renal arterioles and bovine coronary arteries, anandamide-evoked relaxations were endothelium dependent (Deutsch *et al.*, 1997; Pratt *et al.*, 1998; Fleming *et al.*, 1999; Gebremedhin *et al.*, 1999; Mukhopadhyay *et al.*, 2002). In addition, studies in isolated preparations of vascular tissues have also demonstrated that vasorelaxant effects of anandamide were sensitive to the selective cannabinoid antagonist SR141716A, supporting the involvement of the $CB_1$ receptors (White & Hiley, 1997; Gebremedhin *et al.*, 1999).

Still, very little is known on the vascular actions of cannabinoids in ocular tissues; however, cannabinoid vaso-relaxant properties might be able to increase ocular blood flow (Tomida *et al.*, 2004). Porcella *et al.* (1998) have proposed that cannabinoids can act as vasodilators on blood vessels of the anterior uvea, thus improving aqueous humour uveoscleral outflow.

The aim of the present study was to provide pharmacological evidence for the existence of functional cannabinoid receptors in the bovine ophthalmic artery and to investigate the vasoactive properties of endogenous and synthetic cannabinoid agonists. For this purpose, we have detected the possible mechanism(s) underlying the relaxant effects of cannabinoids by examining the involvement of vascular endothelium, the possible role of nitric oxide and the putative role of potassium channels.

## Methods

### Myograph studies

Bovine eyes, including the immediate retro-orbital structures, obtained from a slaughterhouse, were enucleated within 5 min after death and immediately put in ice-cold oxygenated modified Krebs solution (composition, mM: NaCl 136.8, KCl 5.4, $MgSO_4$ 0.8, $NaH_2PO_4$ 1.4, $NaHCO_3$ 12, $CaCl_2$ 2.7, D-glucose 5, Na-ascorbate 0.2, pH 7.4) to transport to the laboratory. The main ophthalmic artery running along the

optic nerve to the eye was dissected free of adherent connective and adipose tissue. Two adjacent rings were cut from each artery (0.7–1.2 mm in diameter, 2–3 mm in length) and were mounted on fine tungsten wires (50 $\mu$M in diameter) on a myograph system (Fort 10, WPI, Sarasota, FL, U.S.A.). Tension was measured and recorded on a powerLab 4/20 recorder (ADInstruments, Castle Hill, NSW, Australia). Once mounted, samples were kept at 37°C in Krebs solution continuously gassed with 5% $CO_2$ in $O_2$. The ophthalmic arteries were stretched to an optimal passive tension of 5 mN and allowed to equilibrate for at least 90 min during which the Krebs solution was changed several times; all experiments were carried out in the presence of indomethacin (10 $\mu$M). In all vessels, the integrity of the endothelium was assessed by precontracting the vessel with 1 $\mu$M 5-hydroxytryptamine (5-HT), followed by relaxation with 10 $\mu$M carbachol; relaxations greater than 90% were designated as endothelium intact. When endothelium was not required, it was removed by rubbing the intima with a human hair; carbachol-induced relaxation of <10% indicated successful removal. Removal of the endothelium had no significant effect on 5-HT-induced tone in the same preparations (with endothelium, $14.7 \pm 0.7$ mN; after endothelial removal, $14.5 \pm 0.7$ mN; $n = 34$).

### Experimental protocol

The effects of anandamide and WIN55212-2 on 5-HT-induced tone were examined in paired rings derived from the same arteries. Ophthalmic arteries were contracted with 5-HT (1 $\mu$M), and once a stable contraction was achieved and maintained, the vasorelaxant effects of anandamide and WIN55212-2 were assessed as cumulative concentration–response curves by the addition of stock solutions to the buffer. All protocols were carried out in both the presence and the absence of endothelium. The steady-state response to anandamide and WIN55212-2 was taken at each concentration and expressed as a percentage relaxation of the imposed 5-HT contraction. The involvement of cannabinoid receptors was assessed using the selective $CB_1$ receptor antagonists SR141716A and AM251, each at 100 nM (Rinaldi-Carmona *et al.*, 1996; Lan *et al.*, 1999). These antagonists were added to the preparations 15 min before preconstriction and were present during construction of the concentration–response curves.

The vasorelaxant action to cannabinoid agonists was also evaluated in the presence of pertussis toxin (400 ng/ml), the nitric oxide synthase inhibitor $N^G$-monomethyl-L-arginine (L-NMMA; 300 $\mu$M, Rees *et al.*, 1990), the nitric oxide donor S-nitroso-N-acetylpenicillamine (SNAP, 100 $\mu$M; Ferrero *et al.*, 1999), the guanylyl cyclase inhibitor 1H-[1,2,4] oxadiazolo-[4,3-a] quinoxalin-1-one (ODQ, 1 $\mu$M; Garthwaite *et al.*, 1995) and capsaicin (10 $\mu$M). All these were added 30 min before and remained present throughout the construction of the concentration–response curves. In line with the findings of White *et al.* (2001), capsaicin was also added 30 min before determination of concentration–response curves. However, in some of the experiments with capsaicin, a 60 min preincubation was also used to evaluate possible time-dependent effects.

Finally, the potassium channel role in the vasorelaxant effects to cannabinoid agonists was evaluated. A pretreatment for 30 min with potassium channel blockers, such as iberiotoxin

(IbTX, 200 nM; Galvez *et al.*, 1990), glibenclamide (5 μM), apamin (100 nM) and 4-aminopyridine (4-AP, 1 mM), was assayed on arterial rings to provide further insight for the identification of the potassium channel(s) involved. Thereafter, rings were precontracted with 5-HT and maximally relaxed to cannabinoid agonists as cited above.

## Data and statistical analysis

All relaxation responses are expressed as percentage relaxation of the tone induced by 1 μM 5-HT. Values are given as mean ± s.e.m. and *n* refers to the number of animals. The concentration of vasorelaxation, giving a half-maximal response ($EC_{50}$), was obtained by fitting four-parameter sigmoidal concentration–response curves using Prism Graph-Pad (version 3.0, San Diego, CA, U.S.A.), and expressed as its negative logarithm, $pEC_{50}$. $R_{max}$ refers to the maximal response achieved. Statistical analysis was performed by analysis of variance (ANOVA), followed by Bonferroni's *post hoc* test (Prism 3.0). Student's *t*-test was used when appropriate. *P*-values of < 0.05 were considered as statistically significant.

## Drugs

All drugs were supplied by Tocris Cookson (Bristol, U.K.), except where indicated. 5-HT, creatinine sulphate, IbTX, pertussis toxin (Sigma Aldrich, St Louis, MO, U.S.A.), L-NMMA acetate and SNAP were dissolved in distilled water to a concentration of 10 mM. Anandamide was supplied as a water-soluble emulsion and dissolved in distilled water. SR141716A and SR144528 (granted by Sanofi Recherche, Montpellier, France), AM251, WIN55212-2 and ODQ were dissolved in dimethylsulphoxide (Sigma) to a concentration of 10 mM. Serial dilutions were prepared daily in Krebs solution. All reagents were of analytical grade. The final bath concentration of dimethylsulphoxide did not exceed 0.1%, which we have found elsewhere to have little or no effect on mechanical activity.

# Results

Anandamide (0.01–50 μM) and WIN55212-2 (1 nM–50 μM) evoked concentration-dependent relaxant responses in the

bovine ophthalmic arteries precontracted with 5-HT. Both the potency (*P* < 0.001) and reactivity (*P* < 0.001) of cannabinoid agonists were significantly greater in endothelium-intact arterial rings compared with endothelium-denuded (Figure 1, Table 1) rings. Relaxation to cannabinoid agonists was unaffected by the presence or the absence of indomethacin.
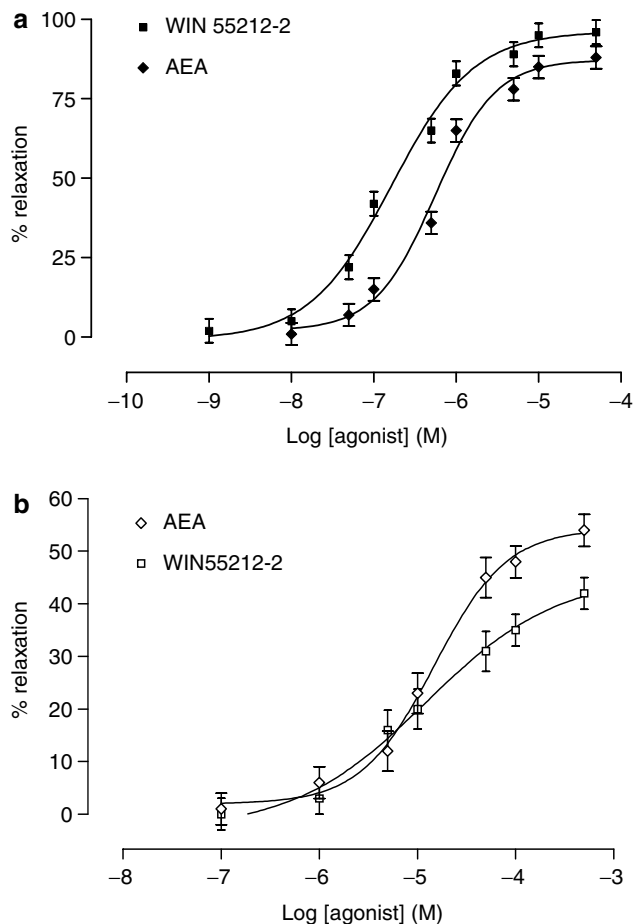


**Figure 1** The vasorelaxant responses of anandamide and WIN55212-2 in bovine ophthalmic artery precontracted to 5-HT with (a) endothelium intact and (b) after removal of endothelium. Data are given as means with error bars representing s.e.m.

**Table 1** Effects of cannabinoid receptor antagonists on the vasorelaxation responses to anadamide and WIN55212-2 in the endothelium-intact bovine ophthalmic artery compared with endothelium-denuded precontracted with 5-HT

| Treatment | Endothelium denuded | | | Endothelium intact | | |
|---|---|---|---|---|---|---|
| | $pEC_{50}$ | $R_{max}$ (%) | n | $pEC_{50}$ | $R_{max}$ (%) | n |
| Anandamide control | 6.38 ± 0.08 | 87.3 ± 4.1 | 12 | 4.89 ± 0.09 | 54.3 ± 3.9 | 10 |
| SR141716A (100 nM) | 5.23 ± 0.11** | 59.1 ± 5.7* | 10 | 4.08 ± 0.22* | 39.1 ± 7.8* | 10 |
| AM 251 (100 nM) | 5.79 ± 0.12* | 65.4 ± 3.5* | 10 | 4.17 ± 0.14* | 32.9 ± 4.6* | 10 |
| SR144528 (1 μM) | 6.41 ± 0.02 | 86.1 ± 3.6 | 8 | 4.78 ± 0.06 | 54.9 ± 6.8 | 8 |
| WIN55212-2 control | 6.78 ± 0.07 | 96.2 ± 3.0 | 12 | 5.11 ± 0.09 | 46.0 ± 2.6 | 12 |
| SR141716A (100 nM) | 5.13 ± 0.52** | 83.2 ± 29.7 | 10 | 4.26 ± 0.24* | 29.7 ± 8.0* | 8 |
| AM 251 (100 nM) | 5.84 ± 0.17* | 57.4 ± 5.9* | 10 | 4.31 ± 0.08* | 27.7 ± 1.4* | 8 |
| SR144528 (1 μM) | 6.77 ± 0.18 | 94.1 ± 11.0 | 8 | 5.10 ± 0.1 | 49 ± 3.8 | 8 |

All experiments were performed in the presence of 10 μM indomethacin. Data are presented as mean ± s.e.m. $pEC_{50}$ and $R_{max}$ values were obtained directly from individual log concentration–response curves.
*n* indicates the number of animals.
*\*P* < 0.05, \*\**P* < 0.001 compared to control values.

*The involvement of cannabinoid CB₁ receptors*

In endothelium-intact arteries, the $CB_1$ receptor antagonist SR141716A (100 nM) produced a significant shift to the right in the concentration–response curve to anandamide ($P < 0.001$; Figure 2a; Table 1). Similarly, SR141716A (100 nM) significantly reduced the vasorelaxant response to WIN55212-2

($P < 0.001$; Table 1; Figure 2b); again, in the absence of endothelium, SR141716A (100 nM) yielded small but significant inhibition of relaxation to cannabinoid agonists ($P < 0.05$; Table 1; Figure 3a, b). Another $CB_1$ receptor antagonist AM251 (100 nM) inhibited relaxation to anandamide ($P < 0.05$; Table 1; Figure 2c) and WIN55212-2 ($P < 0.05$; Table 1; Figure 2d). Similarly, in the absence of endothelium, AM251
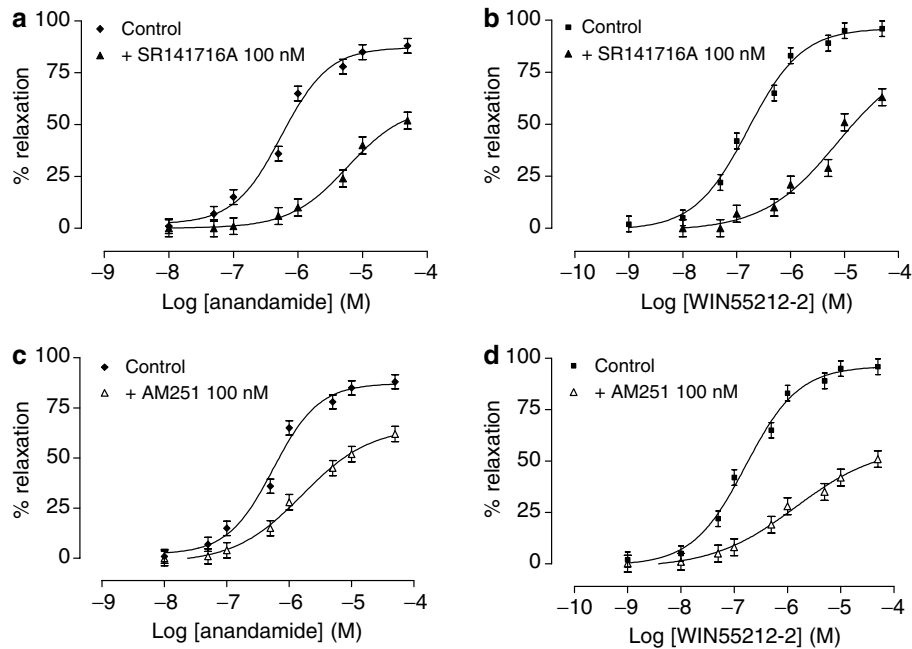


**Figure 2** Effects of the $CB_1$ receptor antagonists SR141716A 100 nM (a and b) and AM251 100 nM (c and d) on vasorelaxant responses to anandamide and WIN55212-2 in endothelium-intact bovine ophthalmic artery precontracted with 5-HT. Data are given as means with error bars representing s.e.m.
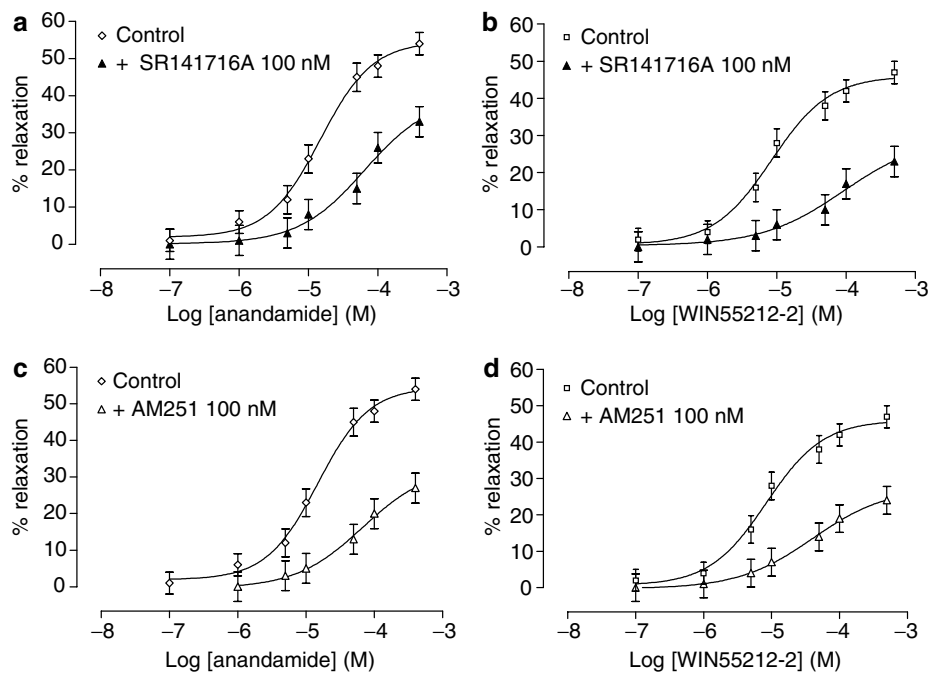


**Figure 3** Effects of the $CB_1$ receptor antagonist SR141716A 100 nM (a and b) and AM251 100 nM (c and d) on vasorelaxant responses to anandamide and WIN55212-2 in endothelium-denuded bovine ophthalmic artery precontracted with 5-HT. Data are given as means with error bars representing s.e.m.

produced slight displacement to the right of the concentration–response curves to anandamide and WIN55212-2 ($P < 0.05$; Table 1; Figure 3c, d). The $CB_2$ receptor antagonist SR144528 (1 $\mu$M) was without any effect on relaxation elicited by cannabinoid agonists (Table 1). In endothelium-denuded arteries, pretreatment with capsaicin (for 30 min; 10 $\mu$M), which causes functional desensitization of the vanilloid receptor system, had no effect on relaxation evoked by cannabinoid agonists (in the presence of capsaicin: anandamide $pEC_{50} = 4.91 \pm 0.07$, $R_{max} = 53.6 \pm 2.8$; $n = 6$; WIN55212-2 $pEC_{50} = 5.10 \pm 0.06$, $R_{max} = 46.2 \pm 3.0$; $n = 6$). The same lack of effects by capsaicin was observed with preincubation up to 60 min (data not shown).

## Involvement of the $G_i/G_o$ protein

$CB_1$ receptors have been shown to couple to $G_i$ as well as $G_o$ protein (Porter & Felder, 2001). To analyse the effector pathway responsible for activation of vasorelaxation to cannabinoid agonists in bovine ophthalmic artery, we determined the effect of pertussis toxin. Bovine ophthalmic arterial rings were incubated with 400 ng ml$^{-1}$ pertussis toxin for a period of 30 min at 37°C. As shown in Figure 4, vasorelaxation with 50 $\mu$M anandamide was completely abolished both in the presence and the absence of endothelium. The same results were obtained with pertussis toxin and WIN55212-2 (50 $\mu$M; data not shown), suggesting the involvement of the $G_{i/o}$ protein-coupled signalling pathway in vasorelaxation to anandamide and WIN55212-2.

## Involvement of nitric oxide in mediating cannabinoid vasorelaxations

In endothelium-intact arteries, the nitric oxide synthase inhibitor L-NMMA (300 $\mu$M) completely blocked relaxation to anandamide and WIN55212-2 ($P < 0.001$, Figure 5a, b). In endothelium-denuded arteries, L-NMMA (300 $\mu$M) had no effect on anandamide and WIN55212-2 vasorelaxations (control anandamide: $pEC_{50} = 6.16 \pm 0.04$, $R_{max} = 90.3 \pm 3.4$, $n = 6$; in the presence of inhibitor: $pEC_{50} = 6.16 \pm 0.08$, $R_{max} = 89.1 \pm 2.9$, $n = 6$; control WIN55212-2: $pEC_{50} = 6.80 \pm 0.08$, $R_{max} = 96.3 \pm 3.0$, $n = 6$; in the presence of inhibitor: $pEC_{50} = 6.81 \pm 0.11$, $R_{max} = 94.8 \pm 2.9$, $n = 6$).

The nitric oxide donor SNAP (100 $\mu$M) caused a small but significant increase in vasorelaxation to anadamide ($pEC_{50} = 6.24 \pm 0.06$, $R_{max} = 112.1 \pm 3.9\%$, $P < 0.05$; $n = 8$; Figure 5a), most notably at high concentrations of anandamide. It is noteworthy that in the presence of SNAP,

WIN55212-2 raised maximal relaxation, which was not significant ($pEC_{50} = 6.63 \pm 0.18$, $R_{max}$ 118.4 $\pm$ 9.9%; $P > 0.05$, $n = 10$; Figure 5b).

## Involvement of cyclic GMP

To investigate the involvement of guanylyl cyclase in the cannabinoid-evoked response, endothelium-intact ophthalmic arteries were pretreated with the selective guanylyl cyclase inhibitor ODQ (1 $\mu$M) for 30 min. ODQ completely abolished
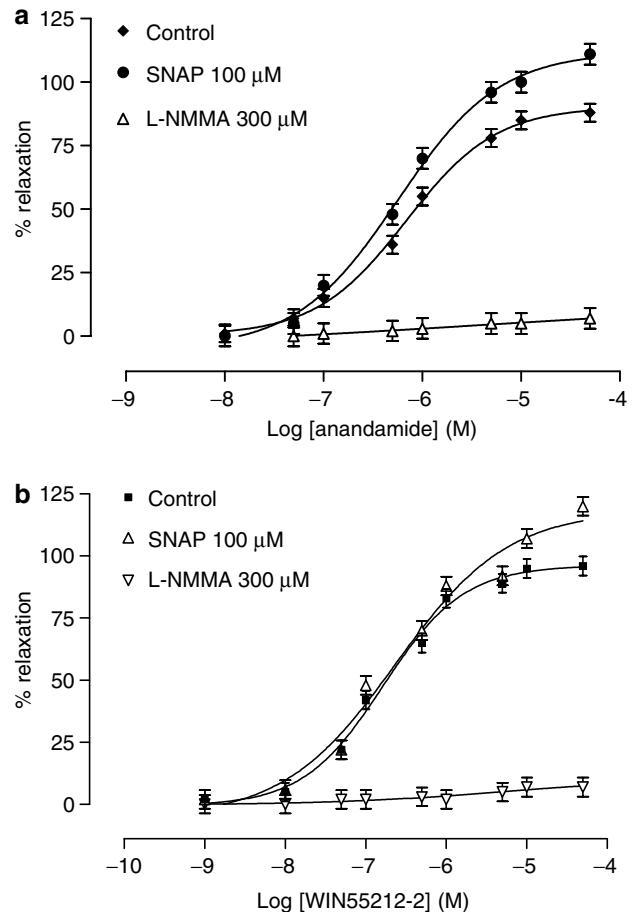
**Figure 5** Effects of L-NMMA (300 $\mu$M) and SNAP (100 $\mu$M) on (a) anandamide- and (b) WIN55212-2-induced relaxation in endothelium-intact bovine ophthalmic artery precontracted with 5-HT. Data are given as means with error bars representing s.e.m.
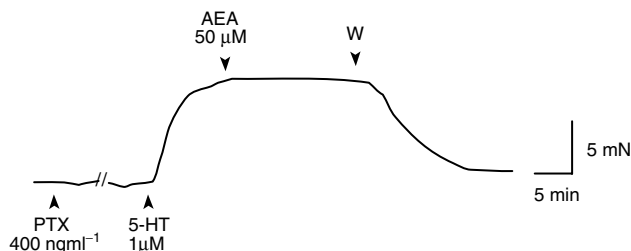
**Figure 4** Typical tracing showing one experiment in which the anandamide (50 $\mu$M) response was blocked by pretreatment (20 min) with pertussis toxin (400 ng/ml).
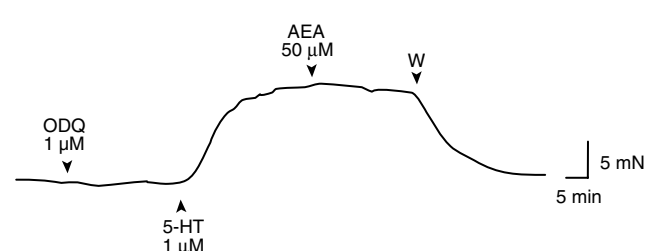
**Figure 6** Typical tracing showing one experiment in which the anandamide (50 $\mu$M) response was blocked by pretreatment with ODQ (1 $\mu$M) for 30 min.

the vasorelaxant effects evoked by $50\,\mu M$ anandamide (Figure 6, $n = 8$) but did not alter the 5-HT response. Interestingly, the same results were obtained with WIN55212-2 ($50\,\mu M$) in the presence of ODQ ($1\,\mu M$).

### Role of potassium channel in vasorelaxant responses to cannabinoid agonists

In endothelium-intact arterial rings, the selective blocker of large conductance $Ca^{2+}$-activated $K^+$ ($BK_{Ca}$) channels IbTX ($200\,nM$) significantly reduced the vasorelaxant effect of anandamide and WIN55212-2 ($P < 0.01$;. Figure 7a, b; $n = 6$). Similarly, 4-AP ($1\,mM$), a delayed rectifier $K^+$ current blocker, slightly attenuated the vasorelaxant responses caused by anandamide and WIN55212-2 (Figure 7a, b; $n = 5$). By contrast, pretreatment of arterial rings with the small conductance $Ca^{2+}$-activated $K^+$ ($SK_{Ca}$) channel blocker apamin ($100\,nM$) and the selective blocker of ATP-sensitive ($K_{ATP}$) $K^+$ channels glibenclamide ($5\,\mu M$) failed to modify the relaxation induced by anandamide and WIN55212-2. Interestingly, in endothelium-denuded arteries, IbTX ($200\,nM$) caused a substantial reduction of relaxation induced by anandamide and WIN55212-2 (Figure 8a, b; $n = 6$); likely, the relaxant responses to cannabinoid agonists were also affected by the presence of 4-AP ($1\,mM$), whereas apamin ($100\,nM$) and glibenclamide ($5\,\mu M$) did not induce any effects (Figure 8a, b; $n = 6$).

### Role of $CB_1$ receptor in $K^+$ channel activation on relaxation to cannabinoid agonists

To further evaluate the role of $CB_1$ receptor in $K^+$ channel activation in endothelium-independent relaxation evoked by cannabinoid agonists, we have assessed the combination of IbTX ($200\,nM$) with AM251 ($100\,nM$), in endothelium-denuded arteries, which caused complete inhibition of anandamide relaxations (Figure 9; $n = 6$). The same result was obtained in the presence of WIN55212-2 (data not shown).

## Discussion

The major finding in this study is the presence of functional cannabinoid receptors in the bovine ophthalmic artery. Our results show that relaxation to cannabinoid agonists such as anandamide and WIN55212-2 involves activation of $K^+$ channels *via* a nitric oxide/cyclic GMP-sensitive pathway, which is apparently dependent on the $CB_1$ cannabinoid receptor.

Anandamide was able to affect concentration-dependent dilation of the ophthalmic arteries precontracted with 5-HT. This effect was greater in ophthalmic arterial rings with intact endothelium. In endothelium-denuded arteries, the ability of anandamide to provoke relaxations was greatly reduced; the maximal effect yielded was about 50%, indicating that
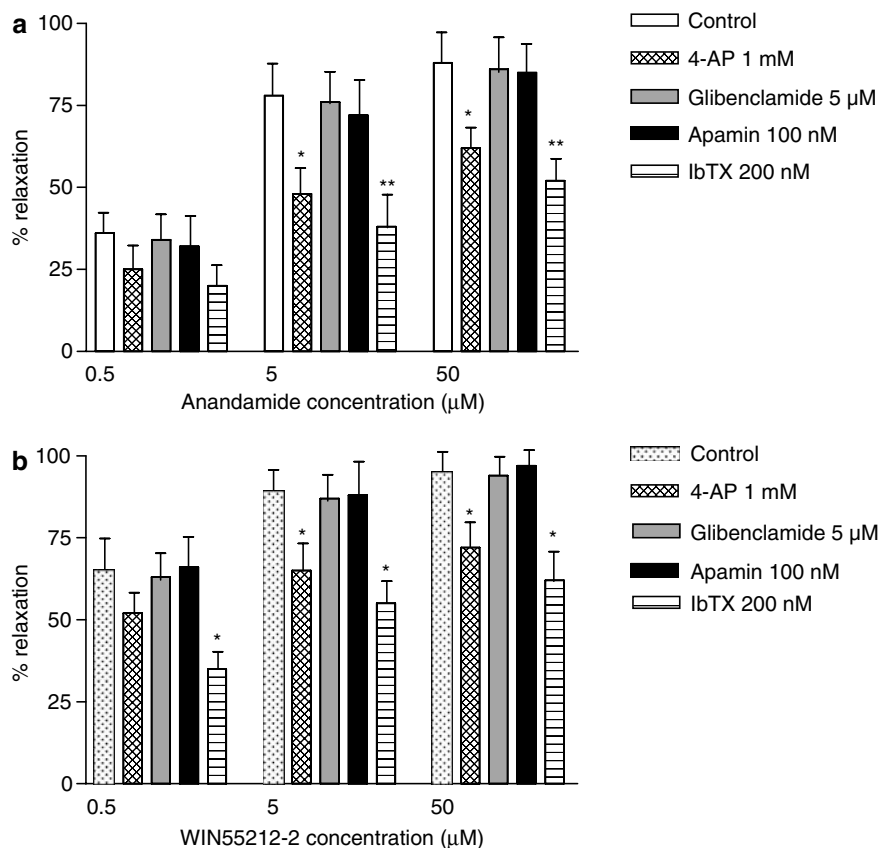


**Figure 7** Effects of potassium channel blockers on vasorelaxation to (a) anandamide and (b) WIN55212-2 in bovine ophthalmic artery with intact endothelium. Data are given as means with error bars representing s.e.m. *$P < 0.05$, **$P < 0.001$ significance when compared with the control relaxation using one-way ANOVA followed by Bonferroni's *post hoc* test.
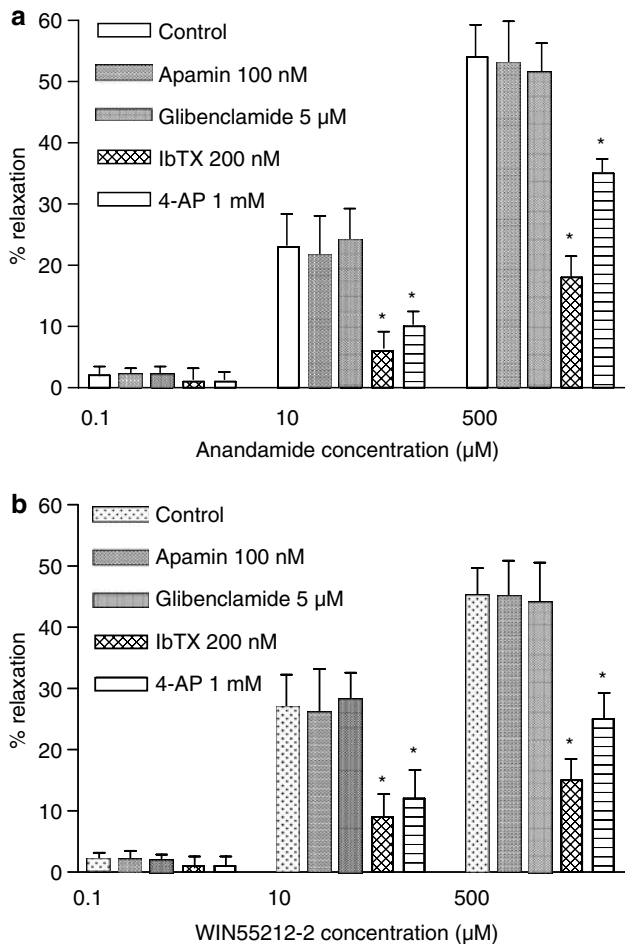
**Figure 8** Effects of potassium channel blockers on vasorelaxation to anandamide (a) and WIN55212-2 (b) in bovine ophthalmic artery with endothelium denuded. Data are given as means with error bars representing s.e.m. $*P < 0.05$ significance when compared with control relaxation using one-way ANOVA followed by Bonferroni's *post hoc* test.
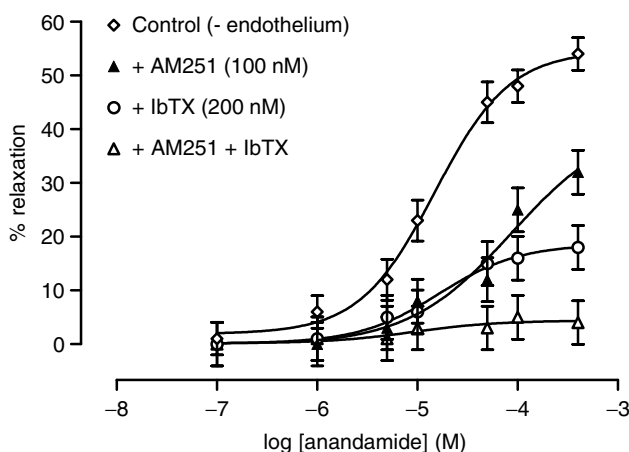


**Figure 9** Effect of coadministration of IbTX (200 nM) and AM251 (100 nM) on vasorelaxation to anandamide in endothelium-denuded bovine ophthalmic artery precontracted with 5-HT. Data are given as means with error bars representing s.e.m.

endothelial integrity plays a significant role in mediating vasorelaxation. Surprisingly, the anandamide-induced vasorelaxant effect was mimicked by WIN55212-2 in arterial rings

with both intact and denuded endothelium. These data are consistent with those of a previous study conducted in cat cerebral artery, which has demonstrated the involvement of $CB_1$ cannabinoid receptors in relaxation to anandamide and that WIN55512-2 was able to mimic the relaxant effect (Gebremedhin *et al.*, 1999).

To examine the role of cannabinoid receptors in relaxation to cannabinoid agonists, the effects of anandamide and WIN55212-2 were tested in the presence of cannabinoid receptor antagonists. At a low concentration, SR141716A (100 nM) caused a rightward displacement of the concentration–response curve of anandamide and WIN55212-2, both in endothelium-intact and -denuded arteries, showing that the antagonism was not exclusively through the endothelial receptor. In addition, an alternative $CB_1$ receptor antagonist AM251 produced a small but significant shift to the right of the concentration–response curve to anandamide and WIN55212-2, both in the presence and absence of endothelium. The $CB_2$ cannabinoid receptor antagonist SR144528 did not block the relaxation of ophthalmic arteries induced by anandamide and WIN55212-2, both in the presence and absence of endothelium. This suggests that $CB_2$ cannabinoid receptors were not involved in anandamide- or WIN55212-2-induced relaxation.

The important role of $CB_1$ cannabinoid receptor in cannabinoid agonist-induced relaxation has been supported by investigation with pertussis toxin, which blocked vasorelaxation to anandamide and WIN55212-2 in endothelium-intact and -denuded arteries, indicating that the coupling of cannabinoid receptor to $G_i/G_o$ proteins is essential to initiate the vasorelaxant responses. Sugiura *et al.* (1998) reported that $CB_1$ receptor mRNAs are highly expressed in human aortic smooth muscle cells as well as endothelial cells; although the expression of $CB_1$ receptor proteins in ocular vascular cells has not yet been investigated, one could hypothesize their presence.

The endothelium-dependent relaxation induced by cannabinoid agonists in bovine ophthalmic artery could be mediated *via* a cannabinoid agonist-evoked release of endothelium-derived relaxant factors such as nitric oxide. To test this hypothesis, in arteries with endothelium, the nitric oxide synthase inhibitor L-NMMA has been assessed inhibiting the vasorelaxation to anandamide and WIN55212-2; this supports the hypothesis that the cannabinoid agonist effects in bovine ophthalmic artery are mediated *via* release of nitric oxide from endothelium. These data are consistent with previous results obtained in a variety of vascular tissues (Deutsch *et al.*, 1997; Bilfinger *et al.*, 1998; Maccarrone *et al.*, 2000; Mukhopadhyay *et al.*, 2002). Moreover, the nitric oxide donor SNAP increased relaxation to anandamide, especially at a high concentration; by contrast, in the presence of SNAP, WIN55212-2 raised the maximal relaxant response but not significantly. This is likely after due to the different stereochemistry of WIN55212-2 compared with anandamide, which could activate a synergic process of slower nitric oxide-dependent transduction. It is known that after nitric oxide has been produced, it links to the haeme group of soluble guanylyl cyclase (Ignarro, 1990a) stimulating the production of cyclic GMP. For this reason, the generation of cyclic GMP is widely used as an index of nitric oxide biosynthesis (Ignarro, 1990b). It is intriguing that a guanylyl cyclase inhibitor ODQ almost completely eliminated of vasorelaxation to cannabinoid agonists in bovine ophthalmic artery. These findings clearly indicate that the

increase in nitric oxide production/release and the activation of guanylyl cyclase to generate cyclic GMP are involved in $CB_1$ receptor-mediated endothelium-dependent relaxation, emphasizing the role of PLC in the activation of nitric oxide synthase and in the accumulation of cGMP. Indeed, it is observed that cannabinoid $CB_1$ agonists are able to evoke a rapid and transient increase in intracellular $Ca^{2+}$ by a mechanism whereby a receptor-mediated release of $G_{i/o}$ protein $\beta\gamma$ subunits might activate PLC, leading to inositol-1,4,5-triphosphate release (Sugiura et al., 1998); this, in turn, triggers cascade reactions involving the stimulation of constitutive nitric oxide synthase activity (Maccarrone et al., 2000). It is interesting to note that pretreatment with L-NMMA (300 $\mu$M) on endothelium-denuded arteries did not have an impact on the relaxant effects of cannabinoid agonists, suggesting a limited role for nitric oxide. This lack of response is unlikely due to insufficient treatment as we have found the same protocol to be effective at completely inhibiting nitric oxide-dependent relaxation in the presence of endothelium.

In endothelium-intact arteries, the involvement of nitric oxide is known to activate $K^+$ channels. Indeed, we have tested the highly selective blocker of $BK_{Ca}$ channels, IbTX (200 nM), which caused potent inhibition of vasorelaxant responses to cannabinoid agonists. In addition, 4-AP (1 mM), glibenclamide (5 $\mu$M) and apamin (100 nM) did not cause any inhibition of the relaxation to cannabinoid agonists. This indicates that the activation of $Ca^{2+}$-activated $K^+$ channels is involved in relaxation mediated by cannabinoid receptors present at the endothelial level. Our results suggest that the endothelial component of cannabinoid agonist-induced relaxation involves a nitric oxide/cyclic GMP-sensitive and $Ca^{2+}$-activated $K^+$ channel pathway.

In addition to endothelial effects, the cannabinoid agonists can produce endothelium-independent relaxation by stimulating the vanilloid receptor (Zygmunt et al., 1999; White et al., 2001). Our observations have shown that pretreatment of endothelium-denuded arteries with capsaicin, which is an agonist of vanilloid receptors and provokes functional desensitization of capsaicin-sensitive sensory nerves, had no effect on relaxation induced by anandamide or WIN55212-2.

To establish the factors involved in cannabinoid agonist-evoked vasorelaxation in endothelium-denuded bovine ophthalmic arterial rings, we have examined the contribution of $K^+$ channels. IbTX (200 nM) significantly reduced vasorelaxation to the cannabinoid agonists examined. This is in agreement with the work by Plane et al. (1997) who found that in isolated mesenteric arterial segments, the relaxation responses to anandamide were blocked by selective inhibitors

of $BK_{Ca}$ channels. However, in other vascular tissues, the role of $BK_{Ca}$ channel activation in anandamide-induced relaxation is quite controversial (White & Hiley, 1997; Randall & Kendall, 1998). In addition, 4-AP (1 mM) attenuated the vasorelaxation, even if the inhibitor effect was lower when compared with that of IbTX. This might be due to the presence of a minor amount of delayed rectifier $K^+$ current or to the possibility that it could not be the main mechanism underlying the vasorelaxation of cannabinoid agonists in bovine ophthalmic artery.

Apamin (100 nM), a $SK_{Ca}$ channel blocker, and glibenclamide (5 $\mu$M), a $K_{ATP}$-channel blocker, failed to modify cannabinoid agonist-induced relaxation in bovine ophthalmic arteries, in agreement with various findings in other vascular beds (Randall et al., 1997; White & Hiley, 1997). The role of potassium channels in the vasorelaxant response modulation to cannabinoid agonists is conflictual within the literature. The explanation for these conflicts could be provided by tissue specificity or species specificity, as well as by the varied distribution of cannabinoid receptors in several vascular tissues.

To shed light on the relevance of $CB_1$ receptors in $K^+$ channel activation, the AM251/IbTX combination was tested in denuded arteries. This produced additional inhibitory effects on anandamide relaxation, suggesting that they utilize different pathways. Therefore, it is not speculative to hypothesize the presence of a heterogenous cannabinoid receptor population and/or multiple relaxation mechanisms.

In conclusion, these data demonstrate that the relaxant effects of anandamide and WIN55212-2 in bovine ophthalmic arteries involve $CB_1$ cannabinoid receptors. In particular, endothelium-dependent relaxation involves the $CB_1$ cannabinoid receptor-sensitive pathway that might involve activation of nitric oxide synthase, cyclic GMP and $Ca^{2+}$-activated $K^+$ channels. Additionally, we have shown that anandamide and WIN55212-2 also cause endothelium-independent relaxation by involving the activation of potassium channels. Exploring the role of cannabinoid agonists in ophthalmic artery is an important goal for understanding the pharmacology of ocular blood flow. It is not speculative to assert that ophthalmic artery vasorelaxation may increase the supply of oxygen to the retina, thus preventing ischaemic injury.

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