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An Endogenous Cannabinoid as an Endothelium-Derived Vasorelaxant

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Since the identification of nitric oxide (NO) as an important mediator of endothelium-dependent relaxation, it has become clear that there is an additional endothelial relaxant factor, termed the endothelium-derived hyperpolarizing factor (EDHF). The identity of EDHF has remained elusive, but it is thought to be an arachidonic acid metabolite. We now report that EDHF-mediated relaxations in the rat mesenteric arterial bed are blocked by a highly selective cannabinoid receptor antagonist, SR141716A, consistent with EDHF being a cannabinoid-like substance. Furthermore, in conscious rats, the NO-independent depressor and regional vasodilator effects of bradykinin were inhibited by SR141716A. The relaxations in the isolated mesentery were accompanied by the accumulation of an arachidonic acid metabolite, which co-eluted on TLC separation with arachidonoylethanolamide (anandamide), an endogenous cannabinoid derived from arachidonate. We further report that anandamide is a potent vasorelaxant in the mesentery, acting via a hyperpolarizing mechanism. These findings suggest that an endogenous cannabinoid is an endothelium-derived vasorelaxant, which may be EDHF.

In 1980 Furchgott & Zawadzki (1) demonstrated that the endothelium, by releasing the endothelium-derived relaxant factor (EDRF), was a major site of vascular regulation. Nitric oxide (NO) was identified as an important mediator of endothelium-dependent relaxations in 1987 by Palmer *et al.* (2). However, it is now clear that NO does not mediate all the endothelium-dependent relaxations in a variety of blood vessels, and that there is an additional factor which contributes to these responses by hyperpolarizing the vascular smooth muscle (3-7). This factor is known as the endothelium-derived hyperpolarizing factor (EDHF), which is thought to be of greatest importance in resistance vessels (5), and may also be upregulated on loss of NO (8-10).

The identity of EDHF has so far remained elusive, but it is thought to be a non-prostanoid arachidonic acid metabolite (11). It has been suggested that EDHF may be a cytochrome P450-derived arachidonate metabolite, as some inhibitors of this enzyme system oppose NO-independent but endothelium-dependent relaxations (12-14). However, more recent evidence casts severe doubt on this contention, since not all cytochrome P450 inhibitors inhibit EDHF-mediated responses (15, 16). In addition, the selectivity of these agents must be questioned because many cytochrome P450 inhibitors are also potassium channel blockers (16) and therefore inhibit EDHF at its site of action rather than synthesis.

More recently an endogenous cannabinoid, arachidonoylethanolamide (anandamide), which is derived from arachidonic acid and ethanolamine (17), has been identified. Therefore, we tested the hypothesis that anandamide may represent EDHF. To address this important question, the effects of the highly selective cannabinoid antagonist, SR141716A

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(18), were investigated against NO-independent, but endothelium-dependent, relaxations both *in vitro* and *in vivo*. SR141716A was used at a concentration which is selective for cannabinoid CB1 receptors, with no activity at a variety of other receptors or ion channels, including potassium channels (18).

MATERIALS AND METHODS

Isolated perfused rat superior mesenteric arterial bed. Male Wistar rats (300-400g) were anaesthetized (pentobarbitone, 60mg kg^{-1}, i.p.), and the superior mesenteric artery was cannulated and perfused as the isolated arterial bed (19) at 5ml min^{-1} with Krebs-Henseleit buffer containing 10μM indomethacin. The relaxant responses were assessed against tone, which was raised (*ca* 100mmHg) by methoxamine (10-30μM). In some preparations NO synthesis was inhibited by inclusion of N^G-nitro-L-arginine methyl ester (L-NAME; 100μM) (20) in the buffer. In these experiments, the loss of basal NO production augments vasoconstrictor tone, hence the concentration of methoxamine was reduced to 1-2μM to induce a level of tone equivalent to that in the absence of L-NAME. In some preparations the vascular endothelium was selectively removed by perfusion with distilled water for 10min in place of buffer, and endothelial removal was confirmed by loss of endothelium-dependent relaxations to carbachol, but retention of endothelium-independent relaxations to sodium nitroprusside.

In order to assess the contribution of hyperpolarizing mechanisms to vasorelaxation caused by carbachol or anandamide, vascular beds were perfused with high K^+ (60mM) buffer to raise tone (ca. 100mmHg). This is an established technique to examine the contribution of K^+-dependent hyperpolarization to vasorelaxation (3).

Analysis of arachidonate metabolites in the mesenteric effluent. The isolated mesentery was perfused under recirculating conditions with Krebs-Henseleit buffer containing ^{3}H-arachidonic acid (1μCi per ml), together with 0.5% BSA, 50μM phenylmethylsulphonyl fluoride (to inhibit amidase activity (21)), 100μM L-NAME and 10μM indomethacin for 1h at which point carbachol (1μM) was included for a further hour. Perfusate fractions were extracted with dichloromethane and dried over $MgSO_4$. Analysis of these samples by TLC was carried out on silica, with 95% dichloromethane/5% methanol as the solvent, with visualisation using $KMnO_4$ solution. The ^{3}H content of each spot was determined by scraping the separated products from the TLC plates, adding scintillation cocktail and ^{3}H quantity was determined by liquid scintillation counting.

Regional haemodynamics in conscious rats. Male Long Evans rats (350-450g), under methohexitone anaesthesia, 40-60 mg kg^{-1}, i.p.), were chronically instrumented with miniaturised pulsed Doppler probes placed around the superior mesenteric and left renal arteries and the abdominal aorta, to allow measurements of regional blood flow as previously described (22). The abdominal aorta was catheterized for direct measurements of blood pressure and the jugular vein was also catheterized for drug administration. Following recovery from surgery and anaesthesia, blood pressure, heart rate and regional blood flows were continuously monitored in conscious, unrestrained rats and changes in response to a 3min infusion of bradykinin (36nmol kg^{-1} min^{-1}) were assessed in the absence and presence of L-NAME (11μmol kg^{-1} h^{-1}) and in the additional presence of SR141716A (21.6μmol kg^{-1}).

Data and statistical analysis. The individual responses for the *in vitro* experiments were analysed by ANOVA with Bonferroni's test. The haemodynamic data were compared by Wilcoxon's test, with comparisons being made between the responses to bradykinin in controls, or in the presence of L-NAME with those in the presence of both L-NAME and SR141716A.

Drugs. All drugs were obtained from Sigma Chemical Company, except levcromakalim (a generous gift from SKB), calcitonin gene-related peptide, bradykinin (Bachem (UK) Ltd), SR141716A (Tocris Cookson) and anandamide which was synthesised from arachidonoyl chloride and ethanolamine (23). Indomethacin, levcromakalim, arachidonic acid, and SR141716A were all dissolved as stock solutions in ethanol and diluted to the required concentrations in the buffer.

RESULTS

Carbachol caused dose-related relaxations in the mesenteric arterial bed which were only partly sensitive to 100μM L-NAME (n = 9) (Figure 1a). However, these NO- and prostanoid-independent relaxations were substantially inhibited by the selective cannabinoid receptor antagonist, SR 141716A (1μM) (Figure 1a).

Figure 1b show that the calcium ionophore, A23187 (n = 9), caused relaxations in the presence of 100μM L-NAME which were antagonized by 1μM SR141716A.

Figure 2 shows that the nitrovasodilator, sodium nitroprusside (29nmol, n = 5), the K^+ channel activator levcromakalim (35nmol, n = 5), human calcitonin gene-related peptide (CGRP; 30pmol, n = 3), in the presence of 100μM L-NAME, all caused relaxations of tone, which were unaffected by SR141716A (1μM).

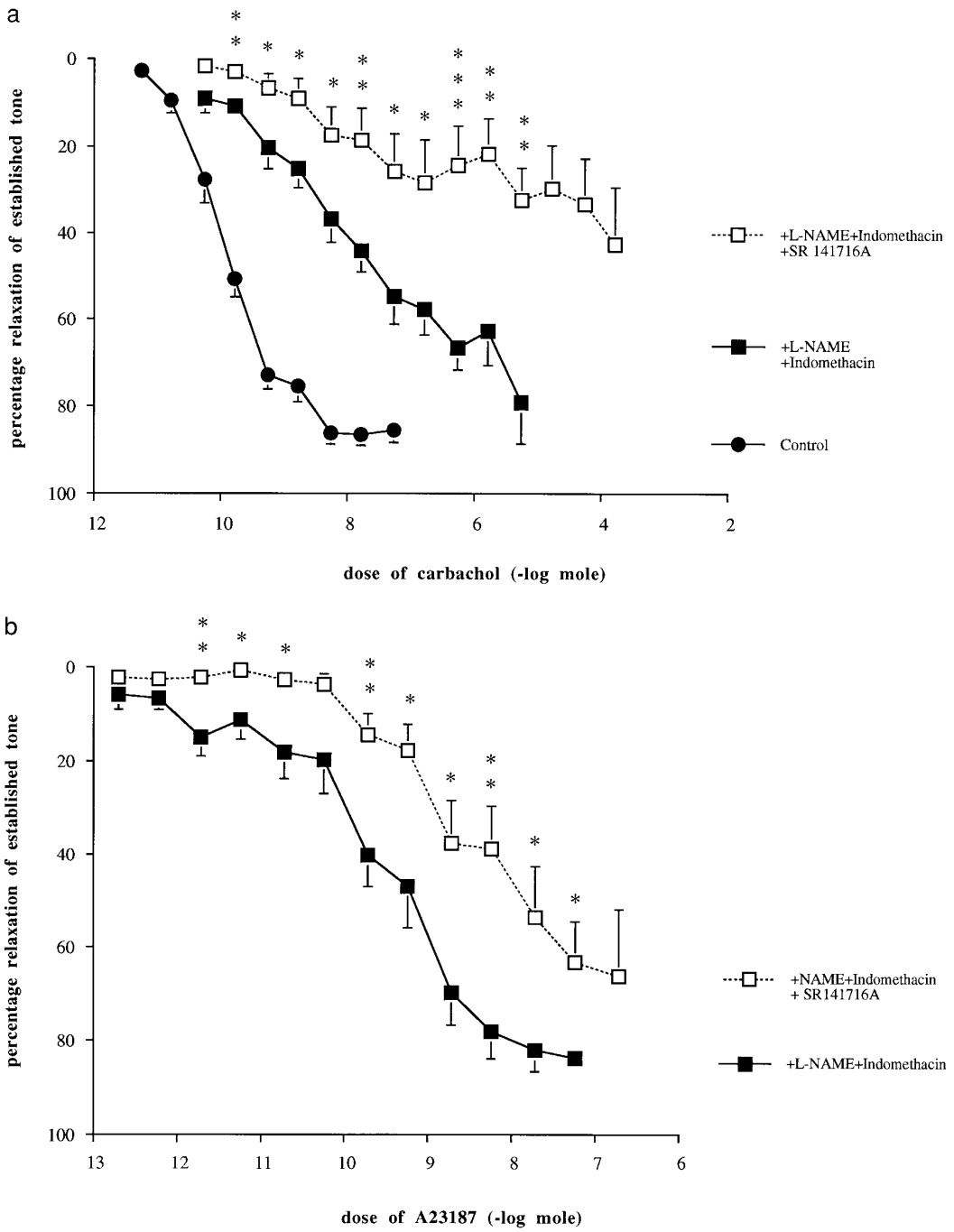

FIG. 1. The antagonism of endothelium-dependent relaxations to (a) carbachol and (b) A23187 by SR 141716A in the rat isolated mesentery. In both a & b the (■) shows the relaxant effects of carbachol (n = 9) or A23187 (n = 9) in the presence of 100μM L-NAME and 10μM indomethacin. In (a) control data for relaxant responses to carbachol in the absence of the inhibitors is shown (-●-; n = 8). In both a & b the results obtained following inclusion of 1μM SR141716A, in the presence of L-NAME and indomethacin are indicated by (· · · □ · · ·) for both carbachol (n = 8) and A23187 (n = 9). The relaxant responses in the presence of SR141716A have been compared with corresponding responses in the absence of the agent, but in the presence of L-NAME and indomethacin and the levels of significance are represented by * ($P < 0.05$), ** ($P < 0.01$) and *** ($P < 0.001$). As in all figures the data are given as mean ± s.e.mean.

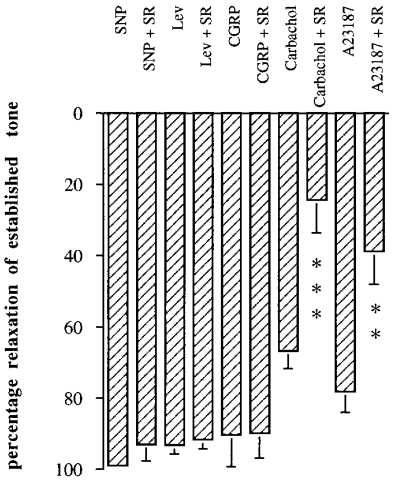

FIG. 2. Shows the selectivity of SR 141716A against endothelium-dependent relaxations. The graph shows the effects of 1μM SR 141716A (SR) against maximal vasorelaxant doses of sodium nitroprusside (SNP; 29nmol, n = 5), levcromakalim (Lev; 35nmol, n = 5), calcitonin gene-related peptide (CGRP; 30pmol, n = 3), carbachol (546nmol, n = 9) and A23187 (5.8nmol, n = 9) in the presence of 100μM L-NAME and 10μM indomethacin. In both cases the relaxant responses have been compared in the absence and presence of SR141716A and levels of significance are shown by ** ($P < 0.01$) and *** ($P < 0.001$).

In conscious rats (n = 6), infusion of bradykinin caused pronounced depressor effects, accompanied by regional vasodilatation as shown by increases in the renal, mesenteric and hindquarters vascular conductances (Figure 3). These haemodynamic effects were insensitive to NO synthase blockade with L-NAME. However, following the administration of SR141716A to L-NAME-treated rats, the hypotensive effects of bradykinin were significantly ($P < 0.01$) reduced, with the peak depressor response decreasing from 23.8 ± 5.8mmHg to 5.8 ± 3.7mmHg. The attenuation of the depressor response was accompanied by reduced vasodilatation to bradykinin in the renal, mesenteric and hindquarter vascular beds (Figure 3).

Thin layer chromatographic (TLC) analysis of the effluent from perfused mesenteries (n = 3) pre-incubated with ^{3}H-arachidonic acid revealed that there was an arachidonate metabolite (Rf = 0.21) which co-eluted with authentic anandamide (Rf = 0.21); ^{3}H-arachidonic acid had an Rf value of 0.27. The accumulation of the labelled metabolite and ^{3}H-arachidonate were both enhanced by inclusion of carbachol (1μM), such that the level of ^{3}H in the spots increased from 113 ± 2dpm to 235 ± 10dpm for the metabolite, and from 462 ± 81dpm to 1317 ± 133dpm for arachidonic acid (n = 3).

Anandamide (300nmol; n = 5) caused relaxations of the mesentery, but arachidonic acid (100nM-100μM) had no effect. Figure 4 shows that the responses to anandamide were unaffected by removal of the endothelium but were antagonized by 1μM SR141716A and reduced in the presence of high K^+ (60mM) buffer. In comparison, carbachol (5.46nmol) also caused relaxation of tone, and this was substantially impaired by removal of the endothelium or perfusion with high K^+ (60mM) buffer in the presence of 100μM L-NAME (Figure 4).

DISCUSSION

The results of the present investigation provide, for the first time, evidence that an endogenous cannabinoid may represent an EDHF.

The finding that endothelium-dependent relaxations were only partly sensitive to inhibition of NO synthesis agrees with our previous findings (24) and that of others (3, 5, 6) and clearly

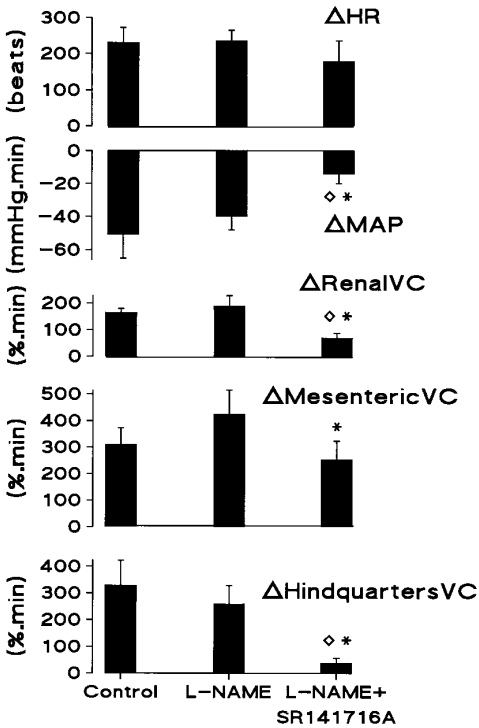

FIG. 3. The effects of SR141716A on the haemodynamic effects of bradykinin in conscious rats (n = 6). The data are shown as histobars indicating the integrated responses (i.e. areas under or over the curve for the plot of the haemodynamic response against time) for 3min infusions of bradykinin under control conditions, following treatment with L-NAME and after treatment with SR141716A in the presence of L-NAME. The results show the effects of bradykinin on mean arterial pressure (MAP), heart rate (HR), and renal, mesenteric and hindquarters vascular conductances (VC), where an increase in VC indicates vasodilatation. The data have been compared by Wilcoxon's test, with comparisons being made between the responses to bradykinin in controls ($\diamond$ indicating $P < 0.05$) or in the presence of L-NAME (* indicating $P < 0.05$) with those in the presence of both L-NAME and SR 141716A.

identifies a NO- and prostanoid-independent component to these relaxations. This component has been ascribed to the release of EDHF (5). We have now demonstrated that the highly selective cannabinoid antagonist, SR141716A (18), antagonizes these responses to the endothelium-dependent vasorelaxants, carbachol and A23187 in the isolated mesentery. These observations are consistent with an endogenous cannabinoid mediating these responses. In contrast, SR 141716A did not influence endothelium-independent vasorelaxation to sodium nitroprusside, levcromakalim (which activates ATP-sensitive K^+ channels) or CGRP, (also a K^+ channel activator, implicated in the small endothelium-independent relaxations to acetylcholine in the rat mesenteric arterial bed (25)). Therefore, SR 141716A does not oppose vasorelaxation non-specifically, nor does it directly interfere with K^+ channel activation leading to vasorelaxation via hyperpolarization. Indeed others have similarly found that SR141716A does not interact with potassium channels (18). Additionally, SR141716A does not inhibit endothelial cytochrome P450 systems involved in arachidonic acid metabolism (unpublished observations).

The findings in the isolated perfused mesentery were mirrored by similar observations *in vivo.* Specifically, infusion of the vasodilator, bradykinin, which acts in part via EDHF release (12, 26), caused reductions in blood pressure, accompanied by regional vasodilatations, which were insensitive to NO synthase inhibition. However, these responses where inhibited following administration of SR141716A, consistent with an endogenous cannabinoid mediating these responses *in vivo.*

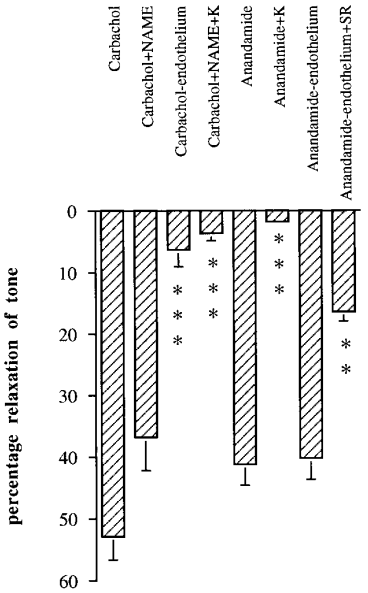

FIG. 4. Comparison of the vasorelaxant actions of anandamide (300nmol; n = 3-5) and the endothelium-dependent vasorelaxant carbachol (5.46nmol; n = 3-9) under various conditions: in the presence of 100μM L-NAME (+NAME), against tone raised by high K^+ (60mM: +K), following removal of the endothelium (−endothelium) and in the presence of SR 141716A (+SR) in endothelium-denuded preparations. These responses have been compared with the appropriate controls and the levels of significance are represented by ** ($P < 0.01$) and *** ($P < 0.001$).

The relaxant responses to carbachol in the mesentery were accompanied by the release of an arachidonic acid metabolite, which co-eluted on TLC with authentic anandamide. These findings indicate that anandamide or a closely related substance is released in response to the endothelium-dependent vasorelaxant. Furthermore, in the mesenteric arterial bed, exogenous anandamide was found to act as a vasorelaxant, whose action was independent of the release of endothelial autacoids, but sensitive to SR141716A. The relaxant responses to anandamide were also inhibited in the presence of high K^+ buffer, which also opposed EDHF-mediated vasorelaxation to carbachol, and is a standard technique (3) to assess EDHF responses, which are mediated via hyperpolarization due to potassium channel activation.

In summary, our results clearly show that the NO-independent component of endothelium-dependent relaxations, which is thought to be mediated via EDHF, is inhibited by the highly selective cannabinoid receptor antagonist SR 141716A, both *in vivo* and *in vitro*. Further, we also report, that under conditions which stimulate EDHF production, there is conversion of arachidonic acid to a metabolite which is similar to, or identical with, the newly described cannabinoid vasorelaxant, anandamide. Taken together our results indicate that an endothelium-derived cannabinoid, which may be anandamide or a related compound, is an endothelium-derived vasorelaxant, which may represent EDHF.

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