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Comparative pharmacology of endothelium-derived hyperpolarizing factor and anandamide in rat isolated mesentery

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Abstract

We have recently proposed that anandamide, or a related cannabinoid, is the endothelium-derived hyperpolarizing factor (EDHF) and have now compared EDHF-mediated responses (induced by carbachol in the presence of both nitric oxide and prostanoid synthesis inhibitors) with those induced by anandamide in the rat isolated superior mesenteric arterial bed. Both EDHF-mediated and anandamide-induced relaxations were inhibited in the presence of high K^+ (60 mM) and opposed by blockade of K^+ channels with 10 mM tetraethylammonium. The cytochrome P450 inhibitors, and putative EDHF inhibitors, clotrimazole (10 μM) and proadifen (SKF 525A) (10 μM), opposed both anandamide-induced and EDHF-mediated relaxations and also relaxant responses to the K^+ channel activator levcromakalim. Therefore, EDHF-mediated and anandamide-induced vasorelaxations show very similar pharmacological characteristics, with both responses being mediated via K^+ channel activation. Further, the actions of EDHF and anandamide are both sensitive to proadifen and clotrimazole, EDHF antagonists which appear to act through K^+ channel inhibition. Accordingly, these results support our proposal that an endocannabinoid is an EDHF. © 1997 Elsevier Science B.V.

Keywords: Endothelium; EDHF (endothelium-derived hyperpolarizing factor); Anandamide; K^+ channel; Cytochrome *P*-450 inhibitor; Cannabinoid

1. Introduction

In 1980 Furchgott and Zawadzki demonstrated that the endothelium, in response to agonist stimulation, released the endothelium-derived relaxant factor (EDRF) (Furchgott and Zawadzki, 1980). Nitric oxide (NO) was identified as an important mediator of endothelium-dependent relaxations by Palmer et al. (1987). However, it has become clear that NO does not account for all EDRF activity, and that there is an additional factor, the endothelium-derived hyperpolarizing factor (EDHF), which contributes to these responses by hyperpolarizing the vascular smooth muscle through K^+ channel activation (Taylor and Weston, 1988; Garland et al., 1995). Indeed, EDHF is now thought to assume greatest importance in resistance beds and may be upregulated in response to impairment of NO activity (Kilpatrick and Cocks, 1994; Kemp et al., 1995; McCulloch et al., 1997).

The identity of EDHF has so far remained elusive, but it is thought to be a non-prostanoid arachidonic acid metabolite (Cohen and Vanhoutte, 1995). It has been suggested that EDHF may be a cytochrome-*P*-450-derived arachidonate metabolite, as some inhibitors of this enzyme system oppose endothelium-dependent relaxations (Singer et al., 1984; Pinto et al., 1987) and in particular those mediated by EDHF (Bauersachs et al., 1994; Hecker et al., 1994; Fulton et al., 1995; Campbell et al., 1996). However, more recent evidence casts severe doubt on this contention, since not all cytochrome *P*-450 inhibitors inhibit EDHF-mediated responses (Corriu et al., 1996; Zygmunt et al., 1996). In addition, the selectivity of these agents must be questioned because some cytochrome *P*-450 inhibitors, which inhibit EDHF responses, are also K^+ channel blockers and have been shown to inhibit K^+ channel activation (Zygmunt et al., 1996) and may therefore inhibit EDHF at its site of action rather than its synthesis.

More recently we have proposed that arachidonoylethanolamide (anandamide), an endogenous cannabinoid derived from arachidonic acid (Di Marzo et al., 1994), may represent EDHF (Randall et al., 1996). This contention is based on the finding that in the rat isolated mesentery and the conscious rat, the highly selective cannabinoid receptor antagonist SR 141716A inhibits

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NO-independent endothelium-dependent relaxations mediated by EDHF. Furthermore, under conditions which evoke EDHF release, we have detected an arachidonic acid metabolite which is similar to or identical with anandamide. We have also reported that exogenous anandamide causes endothelium-independent relaxations which are blocked by raising extracellular K^+, consistent with these responses being mediated via K^+ channel activation (Randall et al., 1996).

In view of our proposal that anandamide, or a related substance, is EDHF we have now carried out a comparison of the vasorelaxant responses to carbachol, which releases both NO and EDHF, with those to anandamide. In order to define EDHF-mediated responses we have used carbachol to evoke endothelium-dependent relaxations in the presence of both nitric oxide synthase and cyclo-oxygenase inhibitors. In the present investigation we have compared the sensitivity of EDHF-mediated and anandamide-induced responses to K^+ channel blockade and elevation of extracellular K^+ (Adeagbo and Triggle, 1993). Furthermore, we have examined the effects of the cytochrome *P*-450 inhibitors proadifen (SKF 525A) and clotrimazole on EDHF-mediated and anandamide responses as there is now evidence that these agents are EDHF antagonists, acting through K^+ channel blockade (Zygmunt et al., 1996).

2. Materials and methods

2.1. Preparation of the isolated buffer-perfused superior mesenteric arterial bed

Male Wistar rats (250–350 g; Bantin and Kingman, Hull, UK) were anaesthetized with sodium pentobarbitone (60 mg/kg, i.p.: Sagatal, Rhône Mérieux, Harlow, UK) and following a mid-line incision the superior mesenteric artery was cannulated. The arterial vasculature was dissected away from the guts and placed in a jacketed organ bath as previously described (Randall and Hiley, 1988) and perfused at 5 ml/min with gassed ($95\%O_2/5\%CO_2$) Krebs–Henseleit solution (containing (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, $CaCl_2$ 2 and D-glucose 10) plus indomethacin (10 μM).

2.2. Experimental protocol

Perfusion pressure in the superior mesenteric arterial bed was continuously monitored by a pressure transducer coupled to a MacLab 4e recording system (ADInstruments, New South Wales, Australia). Following a 30 min equilibration period, methoxamine (1–30 μM) was added to the perfusion fluid to increase vascular tone by 80–100 mmHg. Once stable tone had been established the vasorelaxant effects of carbachol and anandamide were assessed in the absence and presence of the NO synthase inhibitor N^G-nitro-L-arginine methyl ester (100 μM). Vasorelaxants were administered close-arterially as bolus doses in random order in volumes less than 100 μl. In view of the augmented vasoconstrictor responses in the presence of N^G-nitro-L-arginine methyl ester, consistent with the blockade of basal NO synthase activity, the concentration of methoxamine used in these experiments was reduced (to 1–4 μM) to induce an equivalent level of tone. This methodology has previously been shown not to alter the responses to an endothelium-independent relaxant, papaverine (McCulloch and Randall, 1996).

In order to define EDHF-mediated responses, N^G-nitro-L-arginine methyl ester was incorporated in the subsequent experiments involving carbachol, so that the relaxant responses were entirely NO-independent, and therefore EDHF-mediated. Anandamide responses were found to be unaffected by the presence of N^G-nitro-L-arginine methyl ester and are therefore NO-independent, accordingly N^G-nitro-L-arginine methyl ester was not routinely included in the anandamide experiments. In preliminary experiments involving the various inhibitors the additional presence of N^G-nitro-L-arginine methyl ester had no effects on anandamide-induced relaxation. Sodium nitroprusside was used as an endothelium-independent vasorelaxant control. The responses to sodium nitroprusside are well known to be augmented in the presence of N^G-nitro-L-arginine methyl ester and so all responses to sodium nitroprusside were determined in the absence of N^G-nitro-L-arginine methyl ester.

The vasorelaxant effects of carbachol, anandamide and sodium nitroprusside were assessed in the absence and presence of the relatively non-selective K^+ channel blocker tetraethylammonium (10 mM). In order to investigate further the contribution of hyperpolarization to EDHF-mediated and anandamide-induced vasorelaxation, the K^+ concentration of the physiological buffer was increased to 60 mM by isotonic replacement of NaCl with KCl (Adeagbo and Triggle, 1993).

The effects of the cytochrome *P*-450 inhibitors, proadifen (SKF 525A) and clotrimazole, were also assessed against carbachol, anandamide and sodium nitroprusside. In these experiments the appropriate agent was added to the buffer at 10 μM for 30 min prior to the construction of the dose–response curve to the vasorelaxant. Both agents caused reductions in established tone and therefore it was found necessary to increase the methoxamine concentration (to approx. 100 μM) to induce comparable levels of tone to that in control experiments.

2.3. Data and statistical analysis

All data are given as the mean ± S.E.M. and were compared by analysis of variance with statistical significance being determined by Bonferroni's post-hoc test. ED_{50} values for vasorelaxant responses were obtained from individual dose–response curves as the dose at which

the half-maximal relaxant response occurred. These variables were determined by fitting the data to the logistic equation:

$$R = \frac{R_{max} \cdot A^{n_H}}{ED_{50}^{n_H} + A^{n_H}}$$

where R is the reduction in tone, A the dose of vasorelaxant, R_{max} the maximum reduction of established tone, n_H the slope function and ED_{50} the dose of the vasorelaxant giving half the maximal relaxation. The curve fitting was carried out using KaleidaGraph software (Synergy, Reading, PA, USA). The ED_{50} values were converted to the logarithmic values for statistical analysis. In some cases, due to availability of anandamide, the full dose–response curves could not be fully defined and in these cases the effects of the various treatments have been compared by analysis of variance of the individual responses with Bonferroni's post-hoc test.

2.4. Drugs

All solutions were prepared on the day of the experiment. Proadifen (SKF 525A), clotrimazole, tetraethylammonium acetate, N^G-nitro-L-arginine methyl ester, carbachol and indomethacin were all obtained from Sigma (Poole, UK); anandamide which was synthesised from arachidonoyl chloride and ethanolamine (Devane et al., 1992) and dissolved in an inert oil/water emulsion by Dr E.A. Boyd, Department of Pharmaceutical Sciences, University of Nottingham; levcromakalim was a generous gift from SKB. Proadifen, clotrimazole and indomethacin were all dissolved as stock solutions in absolute ethanol. All other drugs were then diluted to the required concentrations in the Krebs–Henseleit solution.

3. Results

3.1. Effects of N^G-nitro-L-arginine methyl ester on carbachol and anandamide-induced relaxations

Carbachol (5.5 pmol–546 nmol) caused dose-related relaxations of methoxamine-induced tone, described by $ED_{50} = 1.87 \pm 0.58$ nmol and $R_{max} = 87.5 \pm 4.3\%$ ($n = 11$). In the presence of 100 μM N^G-nitro-L-arginine methyl ester, carbachol (55 pmol–5.46 μmol; $n = 18$) similarly relaxed the established tone, but was significantly ($P < 0.001$) less potent ($ED_{50} = 13.7 \pm 4.0$ nmol), while the maximal response was unaffected ($R_{max} = 80.7 \pm 5.1\%$) (Fig. 1a).

Anandamide (1 nmol–1 μmol) caused dose-related relaxations of methoxamine-induced tone which were unaffected by the presence of N^G-nitro-L-arginine methyl ester ($n = 4$) (Fig. 1b).

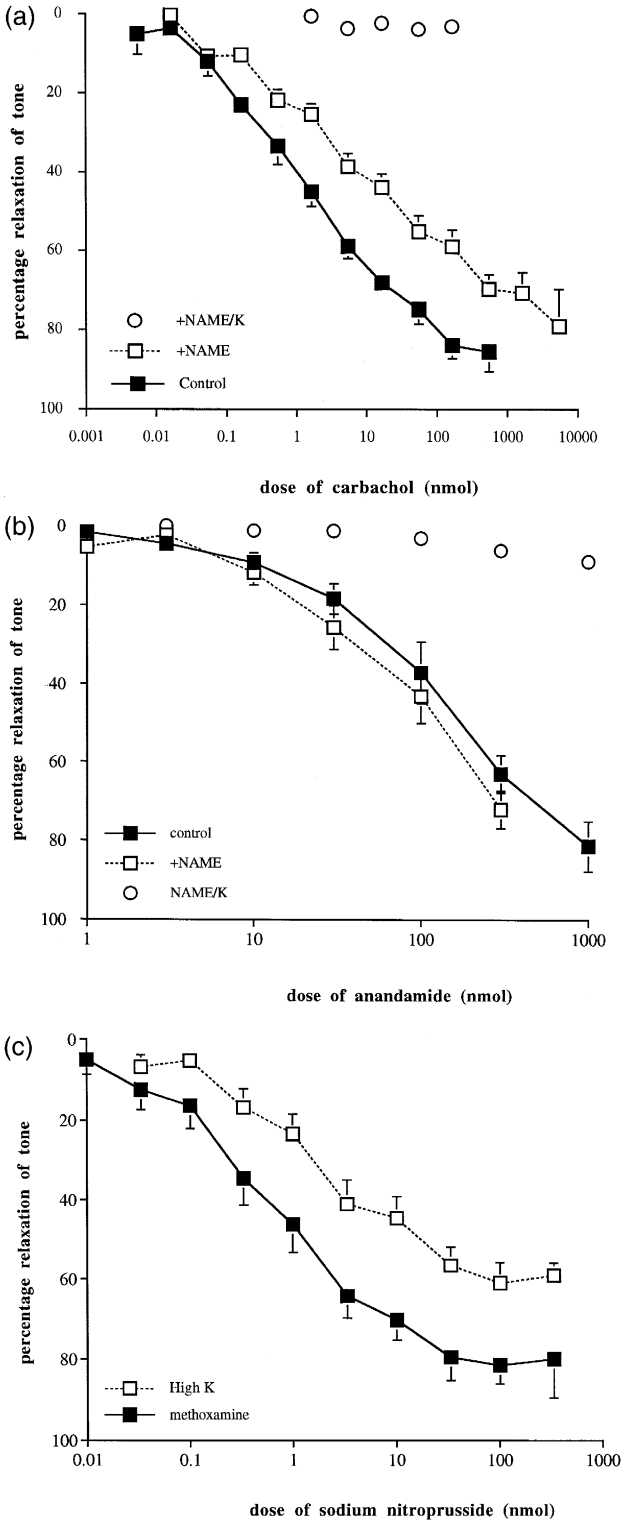

Fig. 1. The vasorelaxant effects of (a) carbachol, (b) anandamide and (c) sodium nitroprusside in the rat isolated perfused mesenteric arterial bed. In a and b the (– ■ –) indicates the control relaxant responses of methoxamine-induced tone, (– □ –) indicates the responses in the additional presence of 100 μM N^G-nitro-L-arginine methyl ester (L-NAME), and (○) shows the relaxant effects of the agents against tone raised by high extracellular K^+ (60 mM) in the presence of 100 μM L-NAME. In c (– □ –) indicates the responses in the presence of high K^+ (60 mM). In each case data are given as mean with the bars indicating S.E.M.

3.2. Effects of high K^+ on carbachol, anandamide and sodium nitroprusside-induced relaxations

In the presence of N^G-nitro-L-arginine methyl ester, raising tone with high K^+ abolished the relaxant responses to both carbachol (Fig. 1a; $n = 3$) and anandamide (Fig. 1b; $n = 5$), such that at the maximum doses used the relaxations of tone were $8.9 \pm 2.5\%$ (anandamide) and $3.1 \pm 2.1\%$ (carbachol).

Sodium nitroprusside (10 pmol–336 nmol) also relaxed high K^+-induced tone. In this respect maximum relaxation was depressed ($62.4 \pm 3.4\%$; $P < 0.001$; $n = 7$) relative to the maximum responses against methoxamine-induced tone ($83.1 \pm 3.1\%$; $n = 7$) (Fig. 1c). The ED_{50} values were 0.75 ± 0.19 nmol (against methoxamine) and 1.88 ± 0.64 nmol (against 60 mM K^+), which were not significantly different.

3.3. Effects of 10 mM tetraethylammonium on vasorelaxant responses to carbachol, anandamide and sodium nitroprusside

Fig. 2 shows that in the presence of N^G-nitro-L-arginine methyl ester (100 μM), the addition of tetraethylammonium (10 mM) caused the dose–response curve to carbachol to be shifted significantly ($P < 0.001$) to the right ($ED_{50} = 579 \pm 251$ nmol) with a significant ($P < 0.001$) depression of the maximum ($R_{max} = 47.7 \pm 9.7\%$), compared to that in the presence of N^G-nitro-L-arginine methyl ester alone (data given above).

Anandamide (1 nmol–1 μmol) also induced dose-related relaxations of tone which were described by $ED_{50} = 39.2 \pm 16.1$ nmol and $R_{max} = 94.7 \pm 9.8\%$ ($n = 8$). Following the addition of 10 mM tetraethylammonium the vasorelaxant responses to anandamide ($n = 8$) were significantly inhibited such that there was a reduction ($P < 0.05$) in the maximal response ($68.9 \pm 5.1\%$), while the ED_{50} was not significantly affected (56.7 ± 13.8 nmol) (Fig. 3).

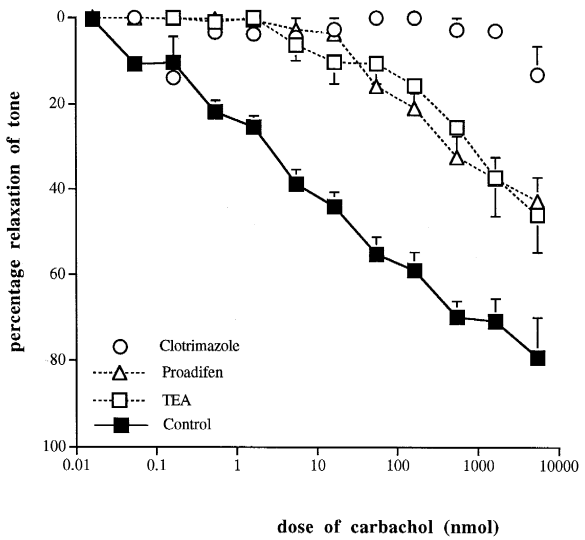

Fig. 2. The vasorelaxant effects of carbachol in the rat isolated perfused mesenteric arterial bed preconstricted with methoxamine in the presence of 100 μM L-NAME: under control conditions (– ■ –); in the presence of 10 mM tetraethylammonium (TEA) (– □ –); in the presence of 10 μM proadifen (– △ –); in the presence of 10 μM clotrimazole (○). In each case data are given as mean with the bars indicating S.E.M.

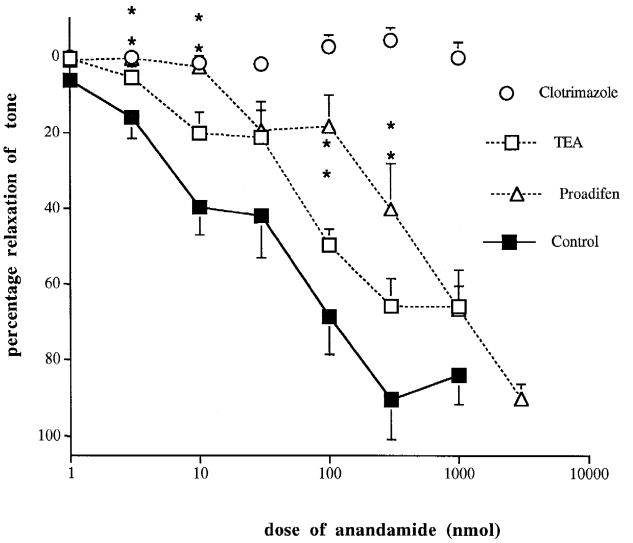

Fig. 3. The vasorelaxant effects of anandamide in the rat isolated perfused mesenteric arterial bed preconstricted with methoxamine: under control conditions (– ■ –); in the presence of 10 mM tetraethylammonium (TEA) (– □ –); in the presence of 10 μM proadifen (– △ –); in the presence of 10 μM clotrimazole (○). In the case of the proadifen data the responses have been compared with the control data for anandamide by analysis of variance and significant differences are indicated by ** ($P < 0.01$). In each case data are given as mean with the bars indicating S.E.M.

Addition of tetraethylammonium (10 mM) did not influence vasorelaxation to sodium nitroprusside (10.1 pmol–101 nmol) as the dose–response in its absence was described by $ED_{50} = 316 \pm 119$ pmol and $R_{max} = 84.7 \pm 2.1\%$ ($n = 4$), while in the presence of tetraethylammonium the values were not significantly different ($ED_{50} = 176 \pm 40$ pmol and $R_{max} = 89.6 \pm 2.9\%$; $n = 4$) (Fig. 4).

3.4. Effects of clotrimazole on vasorelaxation to carbachol, anandamide, levcromakalim and sodium nitroprusside

In the presence of 100 μM N^G-nitro-L-arginine methyl ester, clotrimazole (10 μM) substantially suppressed the relaxant responses to carbachol, so that at the maximum dose of carbachol used (5.46 μmol) the relaxation of tone was $13.1 \pm 6.6\%$ ($n = 3$), which is significantly ($P < 0.001$) less than that seen in the presence of N^G-nitro-L-arginine methyl ester alone ($79.2 \pm 9.4\%$ relaxation; $n = 12$) (Fig. 2).

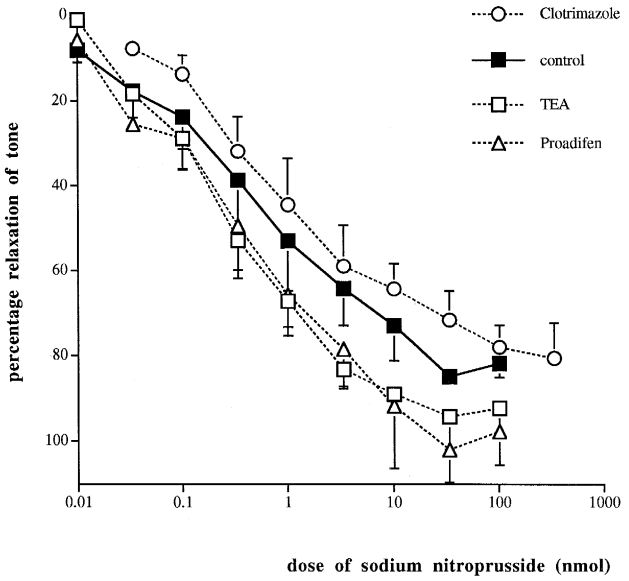

Fig. 4. The vasorelaxant effects of sodium nitroprusside in the rat isolated perfused mesenteric arterial bed preconstricted with methoxamine: under control conditions (– ■ –); in the presence of 10 mM tetraethylammonium (– □ –); in the presence of 10 μM proadifen (– △ –); in the presence of 10 μM clotrimazole (– ○ –). In each case data are given as mean with the bars indicating S.E.M.

In the presence of 10 μM clotrimazole the vasorelaxant responses to anandamide (1 nmol–1 μmol; $n = 3$) were completely abolished (Fig. 3).

Addition of clotrimazole (10 μM) also significantly ($P < 0.001$) and substantially reduced the relaxant responses to a single dose of levcromakalim (35 nmol) from 93.3 ± 2.4 ($n = 5$) to $11.6 \pm 7.7\%$ ($n = 3$).

Fig. 4 shows that the relaxant responses to sodium nitroprusside (20 pmol–1 μmol) were not significantly influenced by the presence of 10 μM clotrimazole ($ED_{50} = 1.08 \pm 0.46$ nmol and $R_{max} = 82.2 \pm 4.8\%$; $n = 4$), compared to the control data given above.

3.5. Effects of proadifen on vasorelaxation to carbachol, anandamide, levcromakalim and sodium nitroprusside

In the presence of 100 μM N^G-nitro-L-arginine methyl ester the responses to carbachol were substantially inhibited by the additional presence of 10 μM proadifen (Fig. 2) such that there was a 16-fold rightward shift in the dose–response curve ($ED_{50} = 217 \pm 61$ nmol ($P < 0.001$) and $R_{max} = 44.8 \pm 4.1\%$ ($P < 0.05$; $n = 5$)).

In 6 preparations which were treated with 10 μM proadifen the dose–response curve to anandamide (1 nmol–3 μmol) was significantly shifted to the right and the significant differences between individual doses are indicated in Fig. 3.

The vasorelaxant responses to a single dose of levcromakalim (35 nmol) were substantially ($P < 0.001$) depressed in the presence of proadifen with a value of $34.1 \pm 8.7\%$ ($n = 4$) relaxation compared to $93.3 \pm 2.4\%$ ($n = 5$) in its absence.

The presence of proadifen resulted in augmented responses to sodium nitroprusside such that, compared with the control data given above, there was an increase ($P < 0.05$) in the maximal relaxation in its presence ($R_{max} = 100 \pm 5\%$) while the ED_{50} (372 ± 131 pmol) was unaltered ($n = 4$) (Fig. 4).

4. Discussion

In the present investigation we have now shown that EDHF-mediated and anandamide-induced relaxations share common characteristics, adding further weight to our proposal that EDHF is an endogenous cannabinoid.

The relaxant responses to carbachol were only partly sensitive to blockade of the NO synthase and this would confirm our previous work (Randall et al., 1996; McCulloch et al., 1997), and that by many others (Adeagbo and Triggle, 1993; Parsons et al., 1994; for review see Garland et al., 1995; Zygmunt and Högestätt, 1996), that there is a substantial NO-independent component to endothelium-dependent relaxations. This component is ascribed to EDHF activity (Garland et al., 1995). In the present study we used the technique devised by Adeagbo and Triggle (1993) to demonstrate that in the presence of high extracellular K^+ these responses were abolished, confirming that EDHF-mediated relaxations occur via the activation of a K^+ conductance. In comparison, the relaxant responses to anandamide were insensitive to NO synthase blockade, consistent with our previous studies demonstrating that this agent is an endothelium-independent vasorelaxant (Randall et al., 1996). However, in common with EDHF-mediated relaxations, those to anandamide were also blocked by raising extracellular K^+, consistent with anandamide acting as a hyperpolarizing agent. Furthermore, non-selective blockade of K^+ channels with tetraethylammonium also antagonised both EDHF-mediated and anandamide-induced relaxations. Taken together these results clearly indicate that both agents induce vascular smooth muscle relaxation through the common pathway of K^+ channel activation.

The precise identity of the K^+ channels activated by EDHF and anandamide is unclear from the present study. However, we have previously shown that relaxations to both EDHF (McCulloch et al., 1997) and anandamide (Millns and Randall, unpublished observations) in the rat mesentery are insensitive to glibenclamide and are therefore not mediated by ATP-sensitive K^+ channels.

The vasorelaxant responses to anandamide occurred in the presence of the cyclo-oxygenase inhibitor, indomethacin, and in pilot experiments did not differ between the absence and presence of this agent. It would, therefore,

appear that anandamide-induced relaxation is also prostanoid-independent, which contrasts with an earlier report on rabbit cerebral vessels that anandamide-induced relaxation occurs via prostanoid release (Ellis et al., 1996).

The identity of EDHF has been a matter of controversy for almost the last decade, with many reports suggesting that it is an epoxide of arachidonic acid produced from the epoxygenase pathway by a cytochrome *P*-450 monooxygenase system (Singer et al., 1984; Pinto et al., 1987; Bauersachs et al., 1994; Hecker et al., 1994; Fulton et al., 1995; Campbell et al., 1996). This contention is largely based on the observation that some inhibitors of cytochrome *P*-450 inhibit EDHF-mediated relaxations in a variety of different vascular bed and species. In the present study we similarly report that the cytochrome *P*-450 inhibitors proadifen and clotrimazole inhibit substantially the EDHF component of relaxations to carbachol, without inhibiting relaxation to the endothelium-independent vasorelaxant sodium nitroprusside. However, others have shown that these agents are also K^+ channel inhibitors and therefore inhibit EDHF at its site of action rather than its synthesis (Zygmunt et al., 1996). In the present study we have also found that both clotrimazole and proadifen greatly inhibited the relaxant responses to the K^+ channel activator levcromakalim, and so have confirmed the work of Zygmunt et al. (1996). Although, EDHF and levcromakalim activate different classes of K^+ channel (Garland et al., 1995; Zygmunt and Högestätt, 1996), these results certainly suggest that both clotrimazole and proadifen are also K^+ channel inhibitors, and perhaps should best be regarded as EDHF antagonists. In the present investigation we also found that both proadifen and clotrimazole inhibited anandamide-induced relaxation, further suggesting that anandamide and EDHF act via a common mechanism, consistent with our proposal that EDHF is an endogenous cannabinoid.

The order of potency for the inhibitors was the same for their actions against EDHF compared to anandamide-induced relaxation. However, proadifen had greater effects against carbachol compared to anandamide-induced relaxation. The greater inhibitory effects against the responses to carbachol probably relate the fact that proadifen has appreciable antimuscarinic properties (Taylor et al., 1980).

It was somewhat surprising that tetraethylammonium and proadifen did not fully block the EDHF-mediated responses. However, similar observations have been made by others in respect to non-selective K^+ channel blockers (Zygmunt and Högestätt, 1996) and cytochrome *P*-450 inhibitors (Zygmunt et al., 1996) and this apparent inability to abolish these responses may relate to their poor selectivity for the precise K^+ channels activated by EDHF.

Although clotrimazole, proadifen and tetraethylammonium showed the same rank order of potency against EDHF-mediated and anandamide-induced responses, proadifen and tetraethylammonium were, in general, more effective at blocking the former. The explanation for this apparent discrepancy is not immediately clear but may relate to the fact that for carbachol to cause EDHF-mediated relaxations it must evoke EDHF release from the endothelium, whilst anandamide acts directly on the vascular smooth muscle. This difference introduces a second potential site of action for the inhibitors, which could potentially interfere with EDHF release. In this context a recent study has shown that the action of endothelium-dependent vasorelaxants on the endothelium is accompanied by hyperpolarization of the endothelium, involving calcium-activated K^+ channels, sensitive to the EDHF inhibitor charybdotoxin (Marchenko and Sage, 1996).

An alternative explanation for the observation that proadifen and clotrimazole opposed anandamide-induced relaxations could be that by inhibiting cytochrome *P*-450 these agents prevent the metabolism of arachidonic acid (derived from anandamide degradation) to vasoactive epoxides. However, this would seem highly unlikely as we have reported that exogenous arachidonic acid has minimal relaxant effects in the mesentery (Randall et al., 1996). Others have reported variable effects of arachidonic acid in the mesentery with both small vasoconstrictor and vasorelaxant responses being observed, which are endothelium-dependent but are not affected by cytochrome *P*-450 inhibitors (Adeagbo and Malik, 1991). By contrast, vasorelaxation to anandamide is endothelium-independent (Randall et al., 1996) and sensitive to the cytochrome *P*-450 inhibitors.

In order to control for non-specific actions of the EDHF inhibitors used, their effects were also assessed against responses to sodium nitroprusside, an endothelium-independent vasorelaxant. The inhibitors of EDHF activity, which appear to act through K^+ channel blockade, did not oppose vasorelaxation to sodium nitroprusside, and in the case of proadifen the responses were even enhanced. In the case of high extracellular K^+ the responses to sodium nitroprusside were slightly attenuated. However, taken together these results indicate that tetraethylammonium, proadifen and clotrimazole do not oppose vasorelaxation non-selectively and confirm the selectivity of the various treatments for inhibiting EDHF-mediated relaxations. Furthermore, given the lack of influence of K^+ channel blockade on sodium nitroprusside-induced relaxation, these findings rule out K^+ channel activation as a major vasorelaxant mechanism of nitric oxide. This observation contrasts the work of others which suggested that nitric oxide induces hyperpolarization via K^+ channel activation (Tare et al., 1990).

In summary, we have shown that EDHF-mediated and anandamide-induced vasorelaxation have very similar pharmacological characteristics, acting through K^+ channel activation. Further, the actions of EDHF and anandamide are both sensitive to proadifen and clotrimazole, EDHF antagonists which appear to act through K^+ channel inhibition. The results, therefore, support our proposal that anandamide, or a related cannabinoid, is and EDHF.

Acknowledgements

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References

Adeagbo, A.S.O., Malik, K.U., 1991. Contribution of K^+ channels to arachidonic acid-induced endothelium-dependent vasodilation in rat isolated perfused mesenteric arteries. J. Pharmacol. Exp. Ther. 258, 452–458.

Adeagbo, A.S.O., Triggle, C.R., 1993. Varying extracellular $[K^+]$: A functional approach to separating EDHF- and EDNO-related mechanisms in perfused rat mesenteric arterial bed. J. Cardiovasc. Pharmacol. 21, 423–429.

Bauersachs, J., Hecker, M., Busse, R., 1994. Display of the characteristics of endothelium-derived hyperpolarising factor by a cytochrome P450-derived arachidonic metabolite in the coronary microcirculation. Br. J. Pharmacol. 113, 1548–1553.

Campbell, W.B., Gebremedhim, D., Pratt, P.F., Harder, D.R., 1996. Identification of epoxyeicosatrienoic acids as endothelium-derived hyperpolarizing factors. Circ. Res. 78, 415–423.

Cohen, R.A., Vanhoutte, P.M., 1995. Endothelium-dependent hyperpolarization: Beyond nitric oxide and cyclic GMP. Circulation 92, 3337–3349.

Corriu, C., Feletou, M., Canet, E., Vanhoutte, P.M., 1996. Inhibitors of the cytochrome P450-mono-oxygenase and endothelium-dependent hyperpolarization in the guinea-pig isolated carotid artery. Br. J. Pharmacol. 117, 607–610.

Devane, W.A., Hanus, L., Breuer, A., Pertwee, R.G., Stevenson, L.A., Griffin, G., Gibson, D., Mandelbaum, A., Etinger, A., Mechoulam, R., 1992. Isolation and structure of a brain constituent that binds to the cannabinoid receptor. Science 258, 1946–1949.

Di Marzo, V., Fontana, A., Cadas, H., Schinelli, S., Cimino, G., Schwartz, J.C., Piomelli, D., 1994. Formation and inactivation of endogenous cannabinoid anandamide in central neurons. Nature 372, 686–691.

Ellis, E.F., Moore, S.F., Willoughby, K.A., 1996. Anandamide and Delta9-THC dilation of cerebral arterioles is blocked by indomethacin. Am. J. Physiol. 269, H1859–H1864.

Fulton, D., Mahboubi, K., McGiff, J.C., Quilley, J., 1995. Cytochrome P450-dependent effects of bradykinin in the rat heart. Br. J. Pharmacol. 114, 99–102.

Furchgott, R.F., Zawadzki, J.V., 1980. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 288, 373–376.

Garland, C.J., Plane, F., Kemp, B.K., Cocks, T.M., 1995. Endothelium-dependent hyperpolarization: A role in the control of vascular tone. Trends Pharmacol. Sci. 16, 23–30.

Hecker, M., Bara, A.T., Bauersachs, J., Busse, R., 1994. Characterisation of endothelium-derived hyperpolarising factor as a cytochrome P450-derived arachidonic acid metabolite in mammals. J. Physiol. 481, 407–414.

Kemp, B.K., Smolich, J.J., Ritchie, B.C., Cocks, T.M., 1995. Endothelium-dependent relaxation in sheep pulmonary arteries and veins; resistance to block by N^G-nitro-L-arginine in pulmonary hypertension. Br. J. Pharmacol. 116, 2457–2467.

Kilpatrick, E.V., Cocks, T.M., 1994. Evidence for differential roles of nitric oxide (NO) and hyperpolarisation in endothelium-dependent relaxation of pig isolated coronary artery. Br. J. Pharmacol. 112, 557–565.

Marchenko, S.M., Sage, S.O., 1996. Calcium-activated K^+ channels in the endothelium of intact rat aorta. J. Physiol. 492, 53–60.

McCulloch, A.I., Bottrill, F.E., Randall, M.D., Hiley, C.R., 1997. Characterisation and modulation of EDHF-mediated relaxations in the rat isolated superior mesenteric arterial bed. Br. J. Pharmacol. 120, 1431–1438.

McCulloch, A.I., Randall, M.D., 1996. Modulation of vasorelaxant responses to K^+ channel openers by basal nitric oxide in the rat isolated superior mesenteric arterial bed. Br. J. Pharmacol. 117, 859–866.

Palmer, R.M.J., Ferrige, A.G., Moncada, S., 1987. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature 327, 524–526.

Parsons, S.J.W., Hill, A., Waldron, G.J., Plane, F., Garland, C.J., 1994. The relative importance of nitric oxide and nitric oxide-independent mechanisms in the acetylcholine-evoked dilatation of the rat mesenteric bed. Br. J. Pharmacol. 113, 1275–1280.

Pinto, A., Abrahams, N.G., Mullane, K.M., 1987. Arachidonic acid-induced endothelial-dependent relaxation of canine coronary arteries: Contribution of a cytochrome P450 dependent pathway. J. Pharmacol. Exp. Ther. 240, 856–863.

Randall, M.D., Hiley, C.R., 1988. Effect of phenobarbitone pretreatment upon endothelium-dependent relaxation to acetylcholine in rat superior mesenteric arterial bed. Br. J. Pharmacol. 94, 977–983.

Randall, M.D., Alexander, S.P.H., Bennett, T., Boyd, E.A., Fry, J.R., Gardiner, S.M., Kemp, P.A., McCulloch, A.I., Kendall, D.A., 1996. An endogenous cannabinoid as an endothelium-derived vasorelaxant. Biochem. Biophys. Res. Commun. 229, 114–120.

Singer, H.A., Saye, J.A., Peach, M.J., 1984. Effects of cytochrome P450 inhibitors on endothelium-dependent relaxation in rabbit aorta. Blood Vessels 21, 223–230.

Tare, M., Parkington, H.C., Coleman, H.A., Neild, T.O., Dusting, G.J., 1990. Hyperpolarisation and relaxation of arterial smooth muscle caused by nitric oxide derived from the endothelium. Nature 346, 69–71.

Taylor, S.G., Weston, A.H., 1988. Endothelium-derived hyperpolarizing factor: A new endogenous inhibitor from vascular endothelium. Trends Pharmacol. Sci. 9, 272–274.

Taylor, W.J., Wolf, A., Young, J.M., 1980. The interaction of amine local anaesthetics with muscarinic receptors. Br. J. Pharmacol. 71, 327–335.

Zygmunt, P.M., Högestätt, E.D., 1996. Role of K^+ channels in endothelium-dependent relaxation resistant to nitroarginine in the rat hepatic artery. Br. J. Pharmacol. 117, 1600–1606.

Zygmunt, P.M., Edwards, G., Weston, A.H., Davies, S.C., Högestätt, E.D., 1996. Effects of cytochrome P450 inhibitors on EDHF-mediated relaxation in the rat hepatic artery. Br. J. Pharmacol. 118, 1147–1152.