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Involvement of a cannabinoid in endothelium-derived hyperpolarizing factor-mediated coronary vasorelaxation

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Abstract

We have recently proposed that an endocannabinoid is the endothelium-derived hyperpolarizing factor (EDHF) and have now tested this hypothesis in the rat isolated perfused heart. In this preparation bradykinin gave rise to nitric oxide- and prostanoid-independent relaxations, assessed as reductions in coronary perfusion pressure ($ED_{50} = 14.9 \pm 5.9$ pmol; $R_{max} = 25.2 \pm 2.2\%$), which are thought to be mediated by EDHF. These relaxations were antagonised by both the highly selective cannabinoid antagonist, SR141716A (1 μM) ($R_{max} = 8.3 \pm 1.2\%$, $P < 0.001$) and by the calcium-dependent potassium channel blocker tetrabutylammonium (300 μM) ($R_{max} = 6.7 \pm 3.4\%$, $P < 0.01$) and were abolished by the EDHF inhibitor clotrimazole (3 μM). The endogenous cannabinoid, anandamide, similarly caused coronary vasorelaxation ($R_{max} = 32.3 \pm 2.3\%$), which was abolished by clotrimazole (3 μM) and antagonised by both 300 μM tetrabutylammonium ($R_{max} = 18.2 \pm 2.8\%$, $P < 0.01$) and 1 μM SR141716A ($R_{max} = 16.4 \pm 3.3\%$, $P < 0.01$). Accordingly, these results suggest that EDHF-mediated responses in the rat coronary vasculature are due to an endogenous cannabinoid and that anandamide causes vasorelaxation through potassium channel activation. These findings are, therefore, consistent with our recent proposal that EDHF is an endogenous cannabinoid. © 1997 Elsevier Science B.V.

Keywords: Endothelium; EDHF (endothelium-derived hyperpolarizing factor); Anandamide; SR141716A; Heart, rat; Cannabinoid (endocannabinoid), endogenous

1. Introduction

We have recently proposed that anandamide, an endogenous cannabinoid derived from arachidonic acid (Di Marzo et al., 1994), represents an endothelium-derived hyperpolarizing factor, EDHF (Randall et al., 1996). This proposal was based on the finding that, in the rat isolated mesentery and in the conscious rat, the highly selective cannabinoid receptor antagonist SR141716A (Rinaldi-Carmona et al., 1994) inhibits EDHF-mediated relaxations (Randall et al., 1996). Furthermore, under conditions which evoke EDHF release, we have detected an arachidonic acid metabolite which is similar to or identical with anandamide. Exogenous anandamide also causes endothelium-independent relaxations in the mesentery, which were blocked by raising extracellular potassium, consistent with these responses being mediated via potassium channel activation (Randall et al., 1996). These effects of anandamide were not mimicked by arachidonic acid and were also insensitive to cyclo-oxygenase inhibition, suggesting that anandamide does not undergo metabolism to yield other vasoactive eicosanoids. Furthermore, relaxations to anandamide are also sensitive to both potassium channel blockade (with tetraethylammonium) and EDHF inhibitors (proadifen and clotrimazole, Randall et al., 1997b). These EDHF inhibitors are also cytochrome *P*-450 inhibitors and their ability to inhibit EDHF-mediated relaxations has been taken to support the proposal the EDHF is an epoxide of arachidonic acid produced via a cytochrome *P*-450-dependent mono-oxygenase (Singer et al., 1984; Pinto et al., 1987; 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 potassium channel blockers and

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have been shown to inhibit potassium channel activation (Zygmunt et al., 1996) and may therefore inhibit EDHF at its site of action rather than its synthesis. Accordingly they are, at best, regarded as EDHF inhibitors.

In view of the above proposal we have now investigated the effects of the cannabinoid receptor antagonist, SR141716A, on EDHF-mediated relaxations to bradykinin in the rat coronary vasculature (Baydoun and Woodward, 1991; Bauersachs et al., 1994; Fulton et al., 1995). In the rat heart Bauersachs et al. (1994) reported that the EDHF-mediated relaxations were sensitive to tetrabutylammonium, at a concentration which is relatively selective for inhibition of calcium-dependent potassium channels and we have now examined the effects of tetrabutylammonium against both EDHF-mediated and anandamide-induced relaxations. We have also investigated the effects of the EDHF inhibitor, clotrimazole (Fulton et al., 1995; McCulloch et al., 1997), which is thought to act through potassium channel inhibition (Zygmunt et al., 1996), on the responses to bradykinin and anandamide in the rat isolated perfused heart.

2. Materials and methods

2.1. Isolated perfused rat heart

Heparinized (1000 u/kg, i.p.) male Wistar rats (250–350 g; Bantin and Kingman, Hull, UK) were anaesthetized with sodium pentobarbitone (60 mg/kg, i.p.). Following a thoracotomy, the hearts were removed and perfused using the Langendorff technique with a constant flow of 15 ml/min with oxygenated modified Krebs–Henseleit solution (containing (in mM): NaCl, 118; KCl, 4.7; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 25; $CaCl_2$, 2; D-glucose, 10) also containing 2 mM sodium pyruvate and 10 μM indomethacin (Randall et al., 1997a). Coronary perfusion pressure was monitored to assess the vasorelaxant responses to bradykinin and anandamide, while a balloon catheter was inserted into the left ventricle to monitor mechanical performance in terms of left ventricular developed pressure and heart rate (Randall et al., 1997a).

2.2. Experimental protocol

To define the nitric oxide (NO)-independent component of relaxation to bradykinin and also to raise vascular tone, the NO synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, 100 μM) was added to the perfusion fluid (Bauersachs et al., 1994; Fulton et al., 1995). In preliminary experiments when either anandamide or bradykinin were added in the absence of L-NAME the vasorelaxant responses were small in magnitude, reflecting the lack of vascular tone. In all experiments the vasorelaxants were administered close-arterially as bolus doses in volumes less than 100 μl. In order to investigate the effects of tetrabutylammonium (potassium channel blocker), clotrimazole (EDHF inhibitor) and SR141716A (cannabinoid receptor antagonist) these agents, in separate experiments, were added to the perfusion fluid at the appropriate concentrations and dose–response curves were then constructed for the vasorelaxants.

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 reduction in coronary perfusion pressure occurred. The variables were determined by fitting the data to the logistic equation:

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

where R is the response, A the dose of vasorelaxant, R_{max} the maximum reduction in coronary perfusion pressure and n_H the slope function. 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.

Table 1
Baseline cardiac variables

	Control ($n = 22$)	NAME ($n = 22$)	NAME + 1 μM SR141716A ($n = 13$)	NAME + 300 μM TBA ($n = 5$)	NAME + 3 μM clotrimazole ($n = 3$)
Coronary perfusion pressure (mmHg)	81.0 ± 3.8	152 ± 7 [a]	143 ± 9	181 ± 16	129 ± 16
Left ventricular developed pressure (mmHg)	85.5 ± 4.8	86.9 ± 4.3	81.0 ± 8.7	51.2 ± 3.6 [b]	84.2 ± 1.0
Heart rate (beats/min)	267 ± 9	257 ± 11	253 ± 11	223 ± 27	224 ± 28

The effects of 100 μM L-NAME and also the effects of SR141716A, tetrabutylammonium (TBA) and clotrimazole in the presence of L-NAME on baseline cardiac variables in the rat isolated perfused heart are shown.
[a] $P < 0.001$ indicates significant differences between the absence and presence of L-NAME.
[b] $P < 0.001$ indicates significant differences between the presence of L-NAME and the presence of L-NAME plus inhibitor.

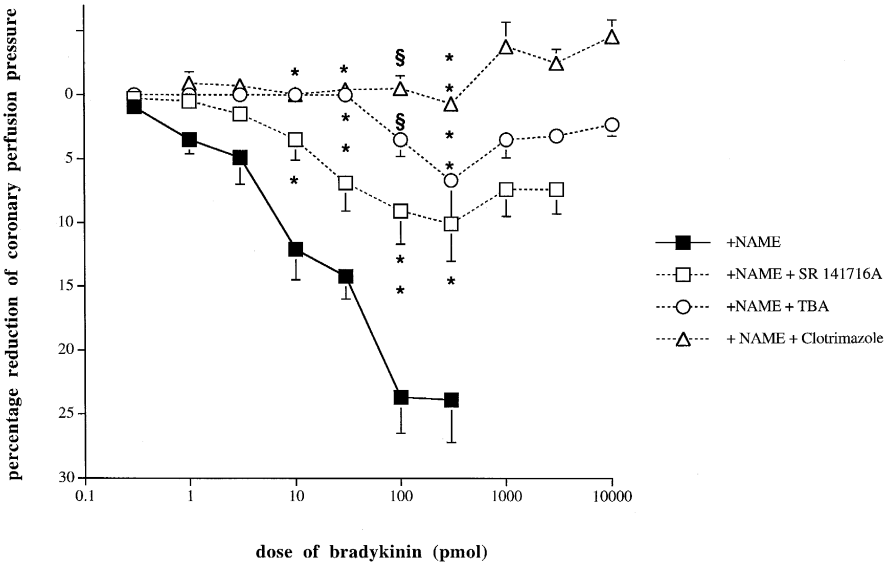

Fig. 1. The effects of SR141716A (1 μM, $n = 6$), tetrabutylammonium (TBA; 300 μM, $n = 5$) and clotrimazole (3 μM, $n = 3$) on the reduction of coronary perfusion pressure by bradykinin and in the rat isolated perfused heart in the presence of 100 μM L-NAME. The * ($P < 0.05$), * * ($P < 0.01$) and § ($P < 0.001$) indicate significant differences between responses in the presence of the inhibitor and L-NAME compared to the presence of L-NAME alone ($n = 22$). The data are given as means with vertical bars indicating the S.E.M.

2.4. Drugs

Bradykinin, clotrimazole, tetrabutylammonium, L-NAME and indomethacin were all obtained from Sigma (Poole, UK); anandamide was synthesised from arachidonoyl chloride and ethanolamine (Devane et al., 1992) and dissolved in an inert oil/water emulsion by Dr. E.A. Boyd, University of Nottingham; SR141716A (*N*-piperidino-5-(4-chlorophenyl)-1-(2,-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide) was also obtained from Dr.

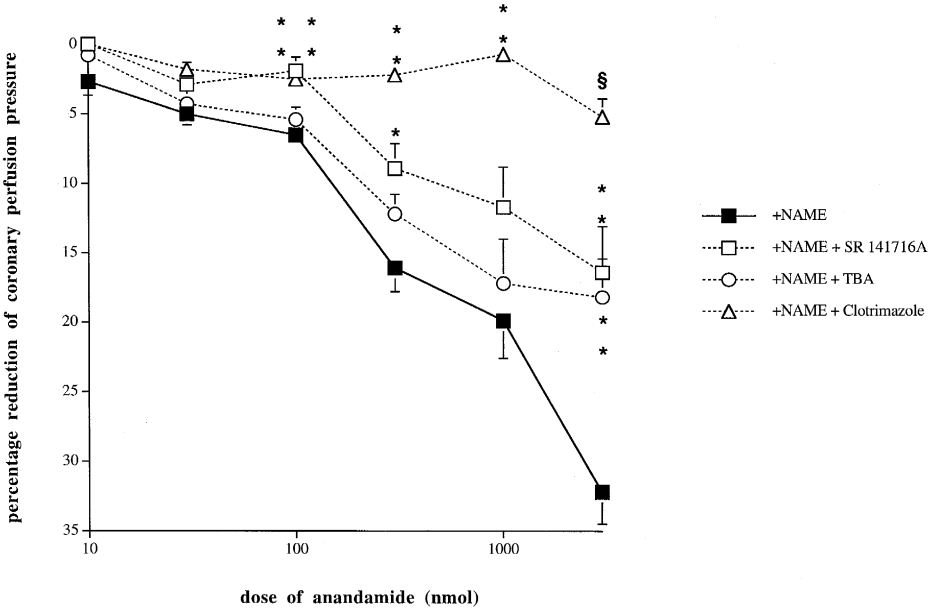

Fig. 2. The effects of SR141716A (1 μM, $n = 5$), tetrabutylammonium (TBA; 300 μM, $n = 5$) and clotrimazole (3 μM, $n = 3$) on the reduction of coronary perfusion pressure by anandamide in the rat isolated perfused heart in the presence of 100 μM L-NAME. The * ($P < 0.05$), * * ($P < 0.01$) and § ($P < 0.001$) indicate significant differences between responses in the presence of the inhibitor and L-NAME compared to the presence of L-NAME alone ($n = 13$). The data are given as means with vertical bars indicating the S.E.M.

Boyd. Clotrimazole, SR141716A, levcromakalim and indomethacin were all dissolved as stock solutions in ethanol. All drugs were then dissolved in buffer at the appropriate concentration.

3. Results

3.1. Baseline cardiac variables

In the 22 hearts studied L-NAME (100 μM) significantly ($P < 0.001$) increased coronary perfusion pressure from a baseline value of 81.0 ± 3.8 to 152 ± 7 mmHg. The subsequent addition of any of the inhibitors (SR141716A, tetrabutylammonium or clotrimazole) did not significantly affect coronary perfusion pressure and only in the case of tetrabutylammonium was there any significant depression of cardiac mechanical performance (Table 1).

3.2. Relaxant effects of bradykinin

In the presence of 100 μM L-NAME, bradykinin (0.3–300 pmol) caused reductions in coronary perfusion pressure ($ED_{50} = 14.6 \pm 5.9$ pmol and maximum reduction in coronary perfusion pressure (R_{max}) = 25.2 ± 2.1%; $n = 22$). These relaxations were significantly attenuated by 1 μM SR141716A, such that the R_{max} was 8.3 ± 1.2% ($P < 0.001$), while the $ED_{50} = 10.8 \pm 6.5$ pmol ($n = 7$) (Fig. 1). SR141716A did not, however, alter vasorelaxation to the potassium channel activator levcromakalim (35 nmol), which caused a 29.7 ± 6.7% reduction of coronary perfusion pressure in its absence and a 28.3 ± 5.4% relaxation in its presence ($n = 6$). In the presence of 300 μM tetrabutylammonium, vasorelaxation to bradykinin was attenuated ($R_{max} = 6.7 \pm 3.4\%$; $P < 0.01$; $n = 5$) (Fig. 1). While clotrimazole (3 μM) completely abolished relaxation to bradykinin ($n = 3$) (Fig. 1).

3.3. Relaxant effects of anandamide

Fig. 2 shows that anandamide (10 nmol-3 μmol) also caused dose-related reductions of coronary perfusion pressure in the presence of L-NAME ($n = 13$). Due to limited availability of anandamide the maximum response could not be fully defined, but at the maximum dose used (3 μmol) the relaxation was 32.3 ± 2.3%. These responses were antagonised by both 1 μM SR141716A (relaxation at 3 μmol = 16.4 ± 3.3%; $P < 0.01$; $n = 5$) and 300 μM tetrabutylammonium (relaxation at 3 μmol = 18.2 ± 2.8%; $P < 0.01$; $n = 5$) (Fig. 2). In the presence of 3 μM clotrimazole the vasorelaxant responses were attenuated with the only significant relaxation occurring at 3 μmol (5.2 ± 1.3%; $P < 0.001$; $n = 3$) (Fig. 2).

Arachidonic acid (10 μmol) had no effect on coronary perfusion pressure.

4. Discussion

In the present investigation we have shown that EDHF-mediated relaxations in the coronary vasculature are sensitive to cannabinoid receptor blockade, whilst the prototype cannabinoid, anandamide, causes vasorelaxation through potassium channel activation.

The finding that SR141716A opposes the EDHF-mediated responses agrees with our previous findings in the isolated mesentery and also in the conscious rat (Randall et al., 1996). In the present study we have further confirmed that this agent does not interfere with potassium channel activation or hyperpolarization in the coronary vasculature, as SR141716A did not affect the responses to the potassium channel activator levcromakalim. The present findings, therefore, extend to the coronary vasculature the possibility that an endogenous cannabinoid accounts for EDHF-mediated relaxations (Randall et al., 1996).

The relaxant responses to bradykinin showed the characteristics of being mediated by EDHF as they were NO- and prostanoid-independent, but were sensitive to tetrabutylammonium and clotrimazole. The sensitivity of bradykinin-induced relaxation to these EDHF inhibitors confirms the findings of Bauersachs et al. (1994) and Fulton et al. (1995), who respectively showed that tetrabutylammonium and clotrimazole blocked responses to bradykinin in the isolated perfused heart, without affecting responses to sodium nitroprusside. In the case of clotrimazole, recent findings suggest that it inhibits EDHF activity at the level of potassium channel activation (Zygmunt et al., 1996; Randall et al., 1997b) rather than through cytochrome *P*-450 inhibition. In common with the EDHF-mediated responses, anandamide also induced relaxations which were both NO- and prostanoid-independent. These responses were selectively antagonised by SR141716A, and so we have, for the first time, demonstrated that anandamide is a coronary vasodilator. Furthermore, the vasorelaxant responses to anandamide were sensitive to the EDHF inhibitors, tetrabutylammonium and clotrimazole. These findings point to anandamide causing vasorelaxation via potassium channel activation and are, therefore, in agreement with our previous findings in the rat isolated mesenteric arterial bed that vasorelaxation to anandamide is sensitive to potassium channel blockade and high extracellular potassium (Randall et al., 1996, 1997b). Indeed, subsequent electrophysiological studies have confirmed, in rat mesenteric arterial segments, that anandamide is a hyperpolarizing agent (Plane et al., 1997).

Anandamide may potentially be metabolised to yield arachidonic acid. However, this is unlikely to account for the actions of anandamide as administration of higher doses of arachidonic acid had no effects on coronary perfusion pressure. Furthermore, all of the present experiments were carried out in the presence of indomethacin, thereby ruling out any involvement of prostanoids.

In conclusion, we have shown that EDHF-mediated responses are sensitive to cannabinoid receptor blockade in the rat coronary vasculature and that vasorelaxation to anandamide and EDHF share several characteristics. Therefore, our findings support our recent proposal that an endogenous, endothelium-derived cannabinoid is an EDHF.

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