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Studies on the effects of anandamide in rat hepatic artery

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1 The effects of anandamide on K^+ currents and membrane potential have been examined in freshly-isolated smooth muscle cells from rat hepatic artery and the results compared with the effects of this arachidonic acid derivative on tension and membrane potential changes in segments of whole artery.

2 In the presence of 0.3 mM L-NOARG and 10 μM indomethacin, anandamide (0.1–100 μM) and endothelium-derived hyperpolarizing factor (EDHF; liberated by acetylcholine, 0.01–10 μM) each relaxed endothelium-intact segments of hepatic artery precontracted with phenylephrine. These effects of anandamide, but not those of EDHF, were antagonized by the cannabinoid receptor antagonist, SR141716A (3 μM).

3 The relaxant effects of anandamide were unaffected by a toxin combination (apamin plus charybdotoxin, each 0.3 μM) which abolishes EDHF relaxations and were essentially unchanged in endothelium-denuded arteries. The relaxant effects of anandamide in endothelium-intact arteries were significantly reduced in a physiological salt solution containing 30 mM KCl and abolished when the K^+ concentration was raised to 60 mM.

4 Anandamide (10 μM), acetylcholine (1 μM, via release of EDHF) and levcromakalim (10 μM) each markedly hyperpolarized the membrane potential of the smooth muscle cells of endothelium-intact arteries. However, when the endothelium was removed, the hyperpolarizing effects of both anandamide (10 μM) and acetylcholine were essentially abolished whereas those of levcromakalim (10 μM) were unaffected.

5 Under voltage-clamp conditions, anandamide (10 μM) abolished spontaneous transient outward currents (STOCs) in freshly-isolated single hepatic artery cells held at 0 mV but had no effect on the holding current at this potential. In current-clamp mode, the spontaneous hyperpolarizing potentials which corresponded to the STOCs were abolished with no significant change in basal membrane potential.

6 Anandamide (10 μM) abolished the iberiotoxin-sensitive K^+ current ($I_{BK(Ca)}$) produced by caffeine and the corresponding hyperpolarizations generated by this xanthine derivative in current-clamp mode. In contrast, anandamide had no effect on $I_{BK(Ca)}$ generated on exposure to NS1619 (30 μM).

7 It was concluded that anandamide is not EDHF in the rat hepatic artery. Anandamide-induced hyperpolarization is exerted indirectly and requires the presence of the endothelium. Anandamide also acts on the smooth muscle cells to inhibit processes which require functional intracellular calcium stores. This direct action seems more important than membrane hyperpolarization in relaxing phenylephrine-contracted vessels.

Keywords: Anandamide; potassium currents; smooth muscle cells; arteries, hepatic; endothelium-derived hyperpolarizing factor (EDHF); spontaneous transient outward currents; intracellular calcium

Introduction

The existence of an endothelium-derived hyperpolarizing factor (EDHF) which causes vasodilatation through activation of smooth muscle potassium (K^+) channels was proposed ten years ago (see Taylor & Weston, 1988). Although many studies now confirm the existence of EDHF as an entity distinct from nitric oxide and cyclo-oxygenase products (see Cohen & Vanhoutte, 1995; Garland *et al*., 1995), there is no consensus view on the identity of this factor. In some vascular preparations it has been proposed that EDHF is a cytochrome P450 metabolite of arachidonic acid, such as an epoxyeicosatrienoic acid (Bauersachs *et al*., 1994; Hecker *et al*., 1994; Lischke *et al*., 1995), whereas in other vessels this seems unlikely (Corriu *et al*., 1996; Zygmunt *et al*., 1996; Fukao *et al*., 1997).

On the basis of recent studies in the rat isolated perfused mesenteric arterial bed, Randall *et al*. (1996) have proposed that EDHF is the arachidonic acid derivative anandamide and that its effects are exerted via cannabinoid receptors. These workers showed that the reductions in perfusion pressure produced by both EDHF and anandamide were inhibited by the cannabinoid receptor antagonist, SR141716A (Randall *et al*., 1996). Furthermore, these effects were also reduced when the extracellular K^+ concentration was increased to prevent hyperpolarization and subsequent relaxation of the smooth muscle cells (Randall *et al*., 1996). However, these workers did not directly demonstrate hyperpolarization and activation of K^+ currents in the presence of anandamide.

In the present study, we have investigated the 'anandamide hypothesis' (Randall *et al*., 1996) in the rat hepatic artery, a preparation in which the effects of EDHF have been extensively characterized (Zygmunt *et al*., 1994a, b, c; 1996; 1997; Zygmunt & Högestätt 1996). In this vessel, the actions of EDHF can be totally abolished by a combination of the K^+ channel inhibitors apamin plus charybdotoxin, a property which is a characteristic marker for the actions of this factor (Zygmunt & Högestätt, 1996).

The present study involved the use of the whole-cell configuration of the patch-clamp technique in both voltage- and current-clamp modes together with recordings of tension and of membrane potential in intact arteries. These varied approaches were used to characterize the effects of anandamide

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and to highlight any differences between the effects of EDHF and this arachidonic acid derivative.

Methods

Whole-tissue experiments

Female Sprague-Dawley rats (250–300 g) were killed by CO_2 asphyxia followed by exsanguination. The hepatic artery was removed and divided into ring segments, 1–2 mm long, which were suspended between metal wires in organ baths containing warmed (37°C) physiological salt solution (PSS) of the following composition (mM): NaCl 119, $NaHCO_3$ 15, KCl 4.6, NaH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 1.5 and (+)-glucose 6.0. The PSS was bubbled with a mixture of 95% O_2 and 5% CO_2, resulting in a pH of 7.4. During an equilibration period of about one hour, the resting wall tension was adjusted to approximately 2 mN mm^{-1} vessel length.

Tension recording

Isometric tension was measured by a force-displacement transducer (Grass Instruments FT03C, U.S.A.), connected to a polygraph (for details, see Högestätt *et al*., 1983). Relaxations induced by acetylcholine and anandamide were studied in vessels contracted by phenylephrine. The concentration of phenylephrine was titrated for each vascular segment to give a contraction amounting to 70–90% of an initial response to 10 μM phenylephrine (Zygmunt *et al*., 1994a). When stable contractions were obtained, acetylcholine or anandamide were added cumulatively to determine concentration-response relationships. The incubation time with the modifying agents, indomethacin, L-NOARG, SR141716A and charybdotoxin plus apamin was at least 20 min and each vessel segment was exposed to only one such treatment. When necessary, the K^+ concentration of the PSS was raised to 30 mM by adding KCl (1 M) directly into the organ bath without correcting for changes in osmolarity. Isotonic 60 mM K^+ solution was prepared by exchanging NaCl in the PSS with KCl in equimolar amounts.

Whole-tissue electrophysiology

The membrane potential was recorded as previously described (Taylor *et al*., 1988; Zygmunt *et al*., 1994c). Vessel segments with intact endothelium were superfused with PSS at 37°C from thermostatically-controlled reservoirs after either lightly pinning to a Sylgaard strip in a thermostatically-controlled bath or mounting in a single-vessel myograph (Model 400A, J.P. Trading, Denmark). Glass microelectrodes filled with 0.5 M KCl (tip resistance between 80–150 MΩ) were advanced from the adventitial side of the artery at resting tension. A successful impalement was characterized by a sudden negative shift in voltage followed by a stable negative potential for 5 min. Acetylcholine, anandamide or levcromakalim was added directly into the organ bath to achieve the desired concentration. In some experiments, the vascular endothelium was removed by perfusing the artery with distilled water for 10 s after the vessel had been mounted in the bath. In these investigations, vessels were exposed first to acetylcholine (1 μM) to test for endothelium removal followed by anandamide (10 μM) and then by levcromakalim (10 μM, to assess K^+ channel opening).

Single-cell electrophysiology

Production of isolated cells Hepatic arteries were removed from male Sprague-Dawley rats (200–300 g) into a 'Ca^{2+}-free' PSS and carefully cleaned of fat and connective tissue with fine scissors in conjunction with a dissecting microscope. The artery was opened along its longitudinal axis and cut into four segments. Cells were dispersed in a collagenase/pronase enzyme solution originally described by Klöckner and Isenberg (1985). Segments of hepatic arteries were agitated in enzyme solution at 37°C for 30 min. They were then washed in the same solution free of enzyme and subsequently triturated with a wide bore, smooth-tipped pipette before a further 5 min agitation at 37°C in (pre-warmed) enzyme solution. The partially-digested tissue was then washed and triturated in Kraftbrühe (KB-medium; Klöckner & Isenberg, 1985). Cells were stored at 8°C in KB-medium and used within 9 h of separation.

Whole-cell current and voltage recordings

Amphotericin B was used to produce whole-cell 'perforated' patches (Rae *et al*., 1991). A fresh amphotericin B stock solution (30 mg ml^{-1}) was made each day by dissolving this agent in dimethylsulphoxide (DMSO). Immediately before experimentation, an amphotericin B-containing pipette solution (300 μg ml^{-1}) was prepared and used for up to 90 min. Pipette tips were briefly dipped into pipette solution and then back-filled with the amphotericin B-containing solution. After formation of giga-seals, 15–25 min were allowed for amphotericin B to diffuse into the cell membrane. Recordings were made with an Axopatch-1C amplifier (Axon Instruments) under either voltage- or current-clamp modes. The settling time of this system was less than 500 μs. Patch pipettes were pulled from Pyrex glass (687–055, Jencons, U.K.) and had resistances of 3–4 MΩ when filled with the internal (intracellular) solution. Voltage commands and data acquisition were performed on-line with an AT-compatible computer equipped with an appropriate interface, the sampling frequency of which was 15 kHz (Axon TL-1, Axon Instruments). For cell stimulation and for recording, the pCLAMP 5.5 programme was used (Axon Instruments). Data were stored on a digital audio tape recorder (Sony; cut-off frequency 20 kHz) and the evoked membrane currents were monitored on a Gould Windograf recorder (−3 dB cut-off frequency 460 Hz). Analysis was performed with Axograph 3.5.5 (Axon Instruments).

The effects of the compounds were investigated by adding the appropriate amount of each agent to the main reservoir containing the external solution to ensure that responses were obtained under steady-state conditions. The bath (1 ml) was continuously perfused (1 ml min^{-1}) with fresh external solution by use of a pump (Microperpex, Pharmacia LKB, Freiburg, Germany); a second identical pump was used to remove excess solution from the recording chamber. Caffeine (10 mM) was applied by pressure ejection from borosilicate pipettes (1B100F-4, World Precision Instruments, U.K.) of tip diameter 2–4 μm for a period of 1 s. The tips of application pipettes were positioned 40–80 μm from the cell surface and pneumatic pressures of 14 psi, generated with a PV820 Pneumatic Picopump (World Precision Instruments, U.K.), were applied. Electrophysiological responses were examined only in those cells which contracted in response to the application of caffeine. All experiments were performed at room temperature (22°C–24°C).

Solutions

The collagenase/pronase solution for separation of hepatic arterial cells comprised (mM): KOH 130, $CaCl_2$ 0.05, taurine 20, pyruvate 5, creatine 5, HEPES 10, buffered with methanesulphonic acid to pH 7.4, collagenase (Type VIII) 1.0 mg ml^{-1}, pronase (Calbiochem) 0.2 mg ml^{-1} and fatty acid-free albumin 1 mg ml^{-1}. KB-medium comprised (mM): KCl 85, KH_2PO_4 30, $MgSO_4$ 5, Na_2ATP 5, K-pyruvate 5, creatine 5, taurine 20, β-OH-butyrate 5, fatty acid-free albumin 1 mg ml^{-1}, pH adjusted to 7.20 at 6°C with KOH. The composition of the Ca^{2+}-containing bath solution was (mM): NaCl 125, KCl 4.8, $MgCl_2$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 1.2, (+)-glucose 6.0 and HEPES 10. The Ca^{2+}-free PSS was identical to this bath solution except that calcium was excluded and the Mg^{2+} concentration was raised to 3.7 mM. The bath solutions were

buffered with NaOH to pH 7.30 and aerated with O_2. The Ca^{2+}-free pipette (internal) solution comprised (mM): NaCl 5, KCl 120, $MgCl_2$ 1.2, K_2HPO_4 1.2, (+)-glucose 6.0, HEPES 10, oxalacetic acid 5, sodium pyruvate 2, sodium succinate 5, buffered with KOH to pH 7.30 at 24°C.

Drugs

Anandamide (arachidonylethanolamide) was purchased from RBI as a solution in ethanol (5 mg ml^{-1}). NS1619 (1-(2′-hydroxy-5′-trifluoromethylphenyl)-trifluoromethyl-2-(3H)benzimidazolone; Neurosearch, Denmark) was dissolved in tissue-culture grade DMSO. SR141716A (*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride; Sanofi-Winthrop) and levcromakalim (SmithKline Beecham) were each dissolved in absolute ethanol. Apamin (Alomone), synthetic charybdotoxin (Latoxan) and iberiotoxin (Alomone) were each dissolved in saline. Acetylcholine chloride, (−)-phenylephrine hydrochloride, L-NOARG (N^{ω}-nitro-L-arginine), caffeine and indomethacin (Confortid, Dumex) were dissolved in distilled water. Stock solutions of the substances were stored at −20°C. Unless otherwise stated, all drugs and reagents were obtained from Sigma.

Calculations and statistics

Tension changes are expressed as a percentage of the phenylephrine-induced contraction immediately before addition of acetylcholine or anandamide. The maximal relaxation induced by each concentration of the drugs was used in subsequent calculations. The negative logarithm of the drug concentration eliciting 50% of the maximal relaxation (pEC_{50}) was determined by linear regression analysis by use of the data points immediately above and below the half-maximal response. E_{max} refers to the maximal relaxation achieved (100% denotes a complete reversal of the phenylephrine-induced contraction). Values are presented as mean ± s.e.mean, and *n* indicates the number of vascular segments or cells (from different animals) examined. Statistical analysis was performed by using Student's *t* test (two-tailed) or multiple analysis of variance (MANOVA) followed by Bonferroni Dunn's *post hoc* test (Statview 4.12). Statistical significance was accepted when $P<0.05$.

Results

Whole-tissue experiments

Effects of SR141716A on relaxations mediated by EDHF and anandamide In the presence of 0.3 mM L-NOARG and 10 μM indomethacin, acetylcholine and anandamide each induced relaxations of endothelium-intact rat hepatic artery precontracted with phenylephrine (Figure 1). Under these conditions, the cannabinoid CB_1 receptor antagonist SR141716A (3 μM) significantly reduced the pEC_{50} value for anandamide from 6.1 ± 0.1 to 5.4 ± 0.2 but not its maximal response (E_{max}; 97 ± 1% versus 84 ± 10%, $n=10$, Figure 1). In contrast, acetylcholine-induced relaxations were unaffected by 3 μM SR141716A (Figure 1). Anandamide also relaxed arteries without endothelium ($pEC_{50}=6.0\pm0.1$; $E_{max}=98\pm1\%$; $n=6$).

Effects of charybdotoxin plus apamin and high KCl on anandamide-induced relaxations A combination of the K^+ channel blockers charybdotoxin and apamin (each 0.3 μM), which completely inhibits EDHF-mediated relaxations in the rat hepatic artery (Zygmunt & Högestätt 1996), had no effect on anandamide-induced relaxations (Figure 2). Exposure to 30 mM KCl, which almost abolished EDHF-mediated responses (present study, data not shown; see Zygmunt *et al.*, 1994c), attenuated relaxations in response to anandamide (Figure 3a). Under these conditions, the pEC_{50} and E_{max} values

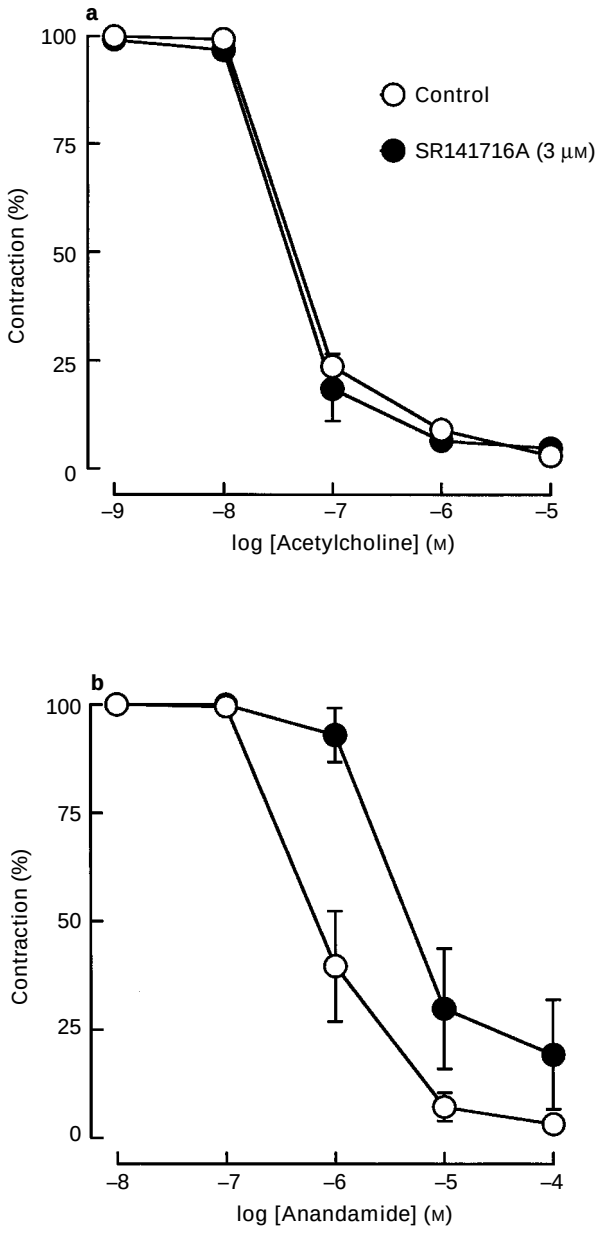


Figure 1 Effects of SR141716A on (a) EDHF-mediated relaxation ($n=6$) and (b) anandamide-induced relaxation ($n=10$) in phenylephrine-contracted hepatic arteries. All experiments were performed in the continuous presence of L-NOARG (0.3 mM) and indomethacin (10 μM); 100% on the y-axis denotes the amplitude of the phenylephrine-induced contraction before addition of acetylcholine or anandamide. Data are presented as means and vertical lines show s.e.mean.

for anandamide were significantly reduced from 6.2 ± 0.2 to 5.5 ± 0.1 and from 96 ± 2% to 47 ± 12%, respectively ($P<0.05$, $n=6$). Anandamide was unable to relax arteries contracted by an isosmolar 60 mM K solution ($n=4$, data not shown).

Membrane potential changes in whole arteries In PSS containing 0.3 mM L-NOARG and 10 μM indomethacin, the basal membrane potential in hepatic arteries mounted at resting tension and with an intact endothelium was −55 ± 2 mV ($n=9$). Under these conditions and as shown previously (Zygmunt *et al.*, 1994c), acetylcholine (1 μM) elicited a hyperpolarization of −11 ± 2 mV ($n=5$; Figure 3b). In arteries with an intact endothelium, anandamide hyperpolarized the cells by −17 ± 3 mV ($n=5$; Figure 3b). However, when the endothelium was removed and in the presence of 10 μM indomethacin (but not 0.3 mM L-NOARG), only tiny hyperpo-

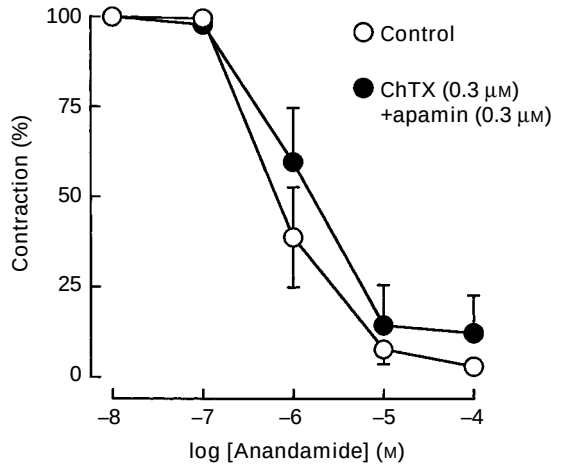


Figure 2 Relaxations induced by anandamide in the absence (control) or presence of 0.3 μM charybdotoxin (ChTX) plus 0.3 μM apamin in phenylephrine-contracted hepatic arteries. All experiments were performed in the continuous presence of L-NOARG (0.3 mM) and indomethacin (10 μM); 100% on the y-axis denotes the amplitude of the phenylephrine-induced contraction before addition of anandamide. Data are presented as means and vertical lines show s.e.mean ($n=8$).

larizing shifts in membrane potential were produced by 1 μM acetylcholine (1 ± 2 mV, $n=4$) and 10 μM anandamide (3 ± 0.5 mV, $n=4$), whereas levcromakalim (10 μM) hyperpolarized the cells by 28 ± 2 mV (Figure 3c; $n=4$). Ethanol (0.1%), the vehicle for anandamide, had no effect on the membrane potential (data not shown).

Single-cell electrophysiology

Effects of anandamide on basal K^+ currents The essential absence of any hyperpolarizing effect of anandamide following endothelium removal indicated that the opening of smooth muscle K^+ channels was unlikely to underlie the actions of this arachidonic acid derivative. To consolidate this view, membrane currents were measured by use of the whole-cell mode of the patch-clamp technique. Since the tension experiments suggested that anandamide might interact with cannabinoid receptors, whole-cell conditions were achieved with amphotericin-generated perforated patches in order to minimize the loss of intracellular contents into the recording pipette.

When cells were held at 0 mV in a calcium-containing PSS, spontaneous transient outward currents (STOCs) were usually observed. These occurred singly or in bursts and could be inhibited by iberiotoxin (Figure 4c). Exposure to anandamide (10 μM) always abolished STOCs within 5 min ($n=9$) and often within a much shorter time (Figure 4a). However, anandamide produced no detectable change in the level of the basal current at the holding potential of 0 mV (Figure 4). In time-matched vehicle controls, ethanol did not obviously modify STOC discharge (Figure 4b).

Effects of anandamide on caffeine-induced potassium currents Since STOCs are believed to result from the release of calcium from intracellular calcium stores and the subsequent activation of large conductance calcium-sensitive K^+ channels (BK_{Ca}; for reviews see Bolton & Imaizumi, 1996; Large & Wang, 1996), a possible inhibitory action of anandamide on such stores was further investigated with caffeine. This xanthine derivative is capable of releasing calcium from intracellular stores, an action which can be monitored by measuring the subsequent activation of current flow through BK_{Ca} (see Bolton & Imaizumi, 1996; Large & Wang, 1996). When cells were held at 0 mV in a calcium containing PSS, a 1 s pressure ejection of caffeine from an electrode close to the cell induced a large iberiotoxin-sensitive current which lasted for several seconds

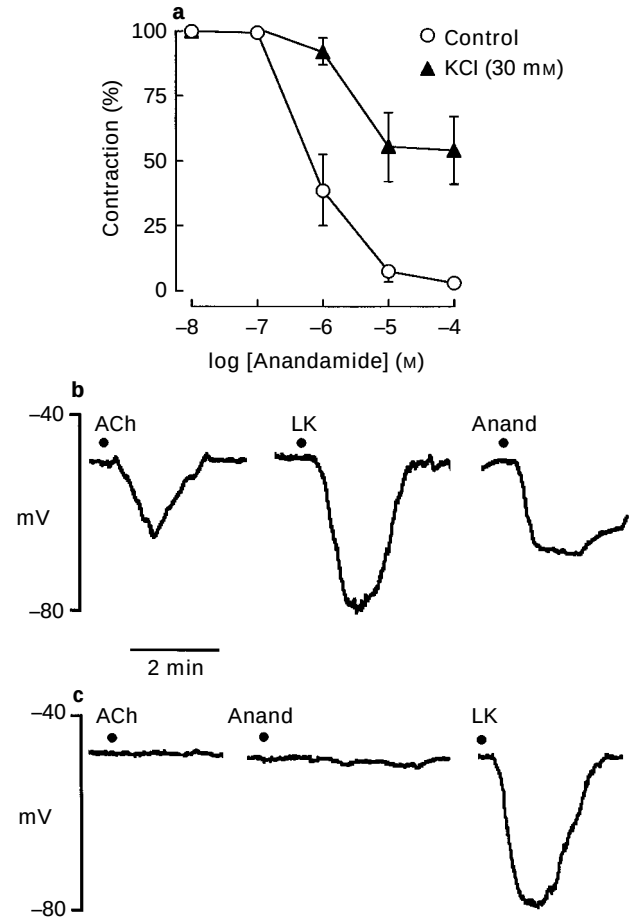


Figure 3 (a) Relaxations induced by anandamide in the absence (control) or presence of 30 mM KCl in phenylephrine-contracted hepatic arteries. All experiments were performed in the continuous presence of L-NOARG (0.3 mM) and indomethacin (10 μM); 100% on the y-axis denotes the amplitude of the phenylephrine-induced contraction before addition of anandamide. Data are presented as means and vertical lines show s.e.mean ($n=6$). (b) Hyperpolarizations induced by acetylcholine (ACh, 1 μM) followed by levcromakalim (LK, 10 μM) and anandamide (Anand, 10 μM) in a continuous recording from a single hepatic artery cell in a vessel with an intact endothelium. The time intervals between the 3 traces were 5.5 and 6 min, respectively, and indomethacin (10 μM) was present throughout. (c) Changes in membrane potential evoked by acetylcholine (ACh, 1 μM) followed by anandamide (Anand, 10 μM) and levcromakalim (LK, 10 μM) in a continuous recording from a single hepatic artery cell in a vessel from which the endothelium had been removed. Responses in (b) and (c) were recorded with KCl-filled sharp microelectrodes in the continuous presence of indomethacin (10 μM) and the time intervals between the 3 traces in (c) were 6 min and 6.5 min, respectively.

(Figure 5c). In 5 experiments, these currents (755 ± 61 pA, $n=5$) were always abolished after 10 min exposure to 10 μM anandamide (Figure 5a). In contrast, the effects of repeated exposure to caffeine were not reduced by ethanol, as shown in time-matched control experiments (control 690 ± 140 pA; ethanol, 687 ± 160 pA; $n=4$; Figure 5b).

Effects of anandamide on $I_{BK(Ca)}$ induced by NS1619 As previously shown in rat portal vein (Edwards *et al.*, 1994), the benzimidazolone derivative NS1619 markedly enhanced the current ($I_{BK(Ca)}$) flowing through BK_{Ca}, even in calcium-free conditions. To determine whether anandamide abolished the caffeine-induced increase in $I_{BK(Ca)}$ by inhibiting BK_{Ca} directly, hepatic artery cells were exposed to NS1619 (30 μM). In parallel with the data from an earlier portal vein study (Edwards *et al.*, 1994), NS1619 markedly enhanced $I_{BK(Ca)}$ which was evoked when cells were held at -10 mV under calcium-free

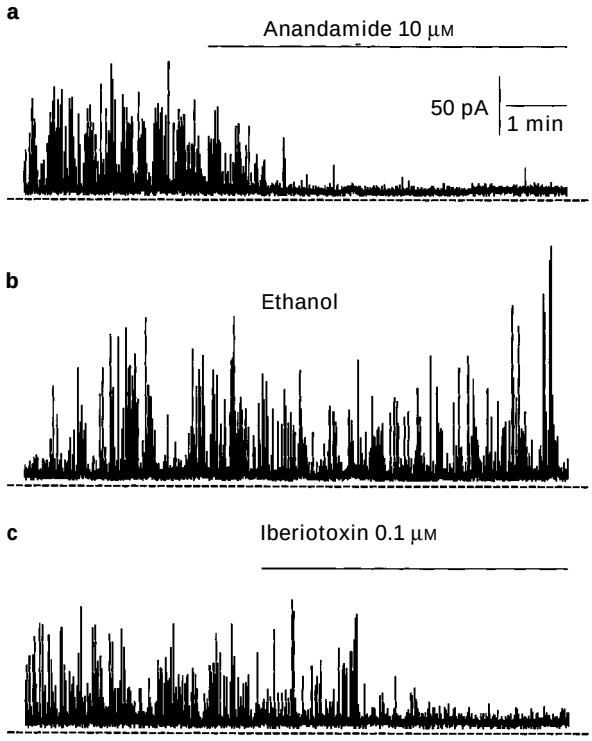


Figure 4 Effects of (a) anandamide, (b) 0.07% ethanol (the vehicle for anandamide) and (c) the BK_{Ca} inhibitor iberiotoxin on spontaneous transient outward currents (STOCs). Hepatic cells were held at a potential of 0 mV in a calcium-containing PSS. Ethanol was present throughout the whole experimental period shown in (a) and (b). The records were obtained under voltage-clamp conditions from different cells and dashed lines refer to the zero current level.

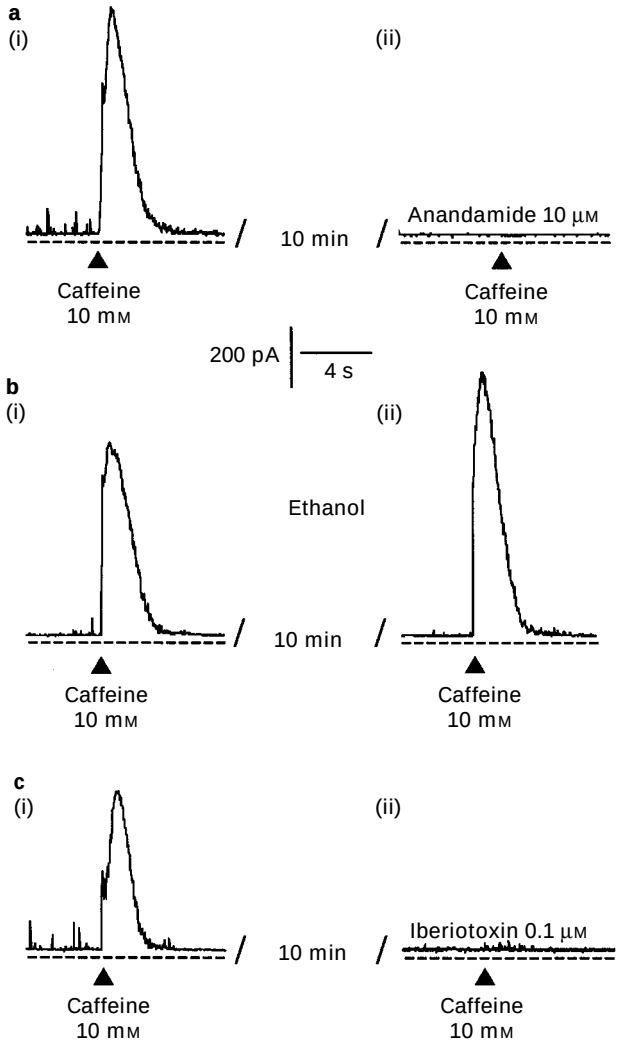


Figure 5 Effects of (a) anandamide, (b) 0.07% ethanol (the vehicle for anandamide) and (c) the BK_{Ca} inhibitor iberiotoxin on caffeine-induced potassium currents. Hepatic cells were held at a potential of 0 mV in a calcium-containing PSS. After the first caffeine application (i), cells were exposed to test drugs and the response to caffeine was determined 10 min later (ii). Ethanol was present throughout the whole experimental period shown in (a) and (b). Caffeine was applied by pressure ejection (14 psi) for 1 s. Traces were obtained under voltage-clamp conditions from different cells and dashed lines refer to the zero current level.

conditions and then stepped to potentials more positive than -10 mV (Figure 6a, b). The action of NS1619 on BK_{Ca} was confirmed with iberiotoxin (Figure 6b). A holding potential of -10 mV was used to inactivate other voltage-sensitive channels such as the delayed rectifier, K_V, current flow, which would have prevented the easy analysis of drug action on BK_{Ca}. After 10 min exposure to 10 μM anandamide, the ability of NS1619 to activate $I_{BK(Ca)}$ was unaffected (Figure 6a).

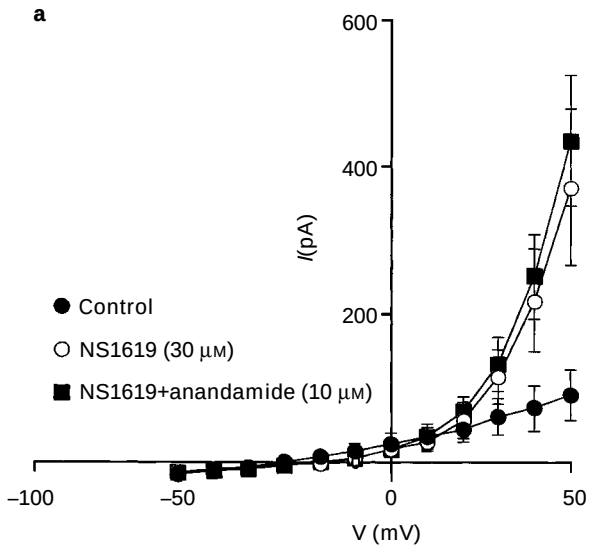
Effects of anandamide on $I_{K(V)}$ To determine any effects of anandamide on delayed rectifier currents ($I_{K(V)}$), hepatic artery cells were stepped from -90 mV to a range of more depolarized test potentials. Under these conditions, the predominant current activated at approximately -30 mV, indicating that it was carried by K_V (Figure 7). After 10 min exposure to anandamide (10 μM), $I_{K(V)}$ was essentially abolished (Figure 7).

Effects of anandamide and caffeine on the membrane potential As already stated, most hepatic artery cells generated STOCs when clamped at 0 mV (see Figure 4). When recording conditions were switched to current-clamp, the mean basal membrane potential was -42 ± 1 mV ($n=17$) and in some cells, frequent hyperpolarizing bursts were superimposed upon the recorded basal potential. These hyperpolarizations corresponded in frequency and relative magnitude to the STOCs which were observed under voltage-clamp conditions (Figure 4). Exposure to anandamide (10 μM) for 5 min abolished the spontaneous transient hyperpolarizations and although the membrane potential tended to hyperpolarize in some cells, this trend was not significant (control: -38 ± 2 mV; anandamide: -41 ± 3 mV; $n=8$). The vehicle for anandamide (ethanol) was also without effect (data not shown; $n=2$). Exposure of cells to caffeine by pressure ejection produced a transient hyperpolarization to a near theoretically-maximum membrane potential of -91 ± 4 mV ($n=17$) and cessation of any spontaneous hyperpolarizations for up to several minutes. After 10 min exposure to anandamide (10 μM), caffeine-induced hyperpolarizations were totally abolished whereas no such inhibition was seen in time-matched control experiments ($n=5$).

Discussion

Is anandamide EDHF?

In the present study, anandamide caused a concentration-dependent relaxation in both intact and endothelium-denuded hepatic arteries of the rat, which is in agreement with data obtained with the rat perfused mesenteric bed (Randall *et al.*, 1996). However, consistent with the findings of White and Hiley (1997) in rat mesenteric artery, the anandamide-induced relaxation of rat hepatic artery was unaffected in the presence of apamin plus charybdotoxin, a combination of K^+ channel inhibitors which totally abolishes the relaxant effects of EDHF in these tissues (Zygmunt & Högestätt, 1996; White & Hiley, 1997). Furthermore, an axiomatic feature of the action of



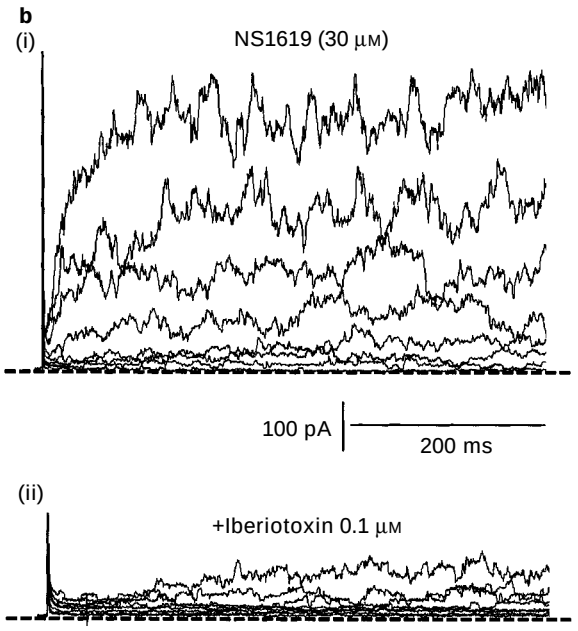


Figure 6 Effect of anandamide (a) on $I_{BK(Ca)}$ induced by NS1619 in hepatic arterial cells under 'calcium-free' conditions. Currents were generated by stepping for 500 ms from a holding potential of −10 mV to test potentials between −60 mV to +50 mV in 10 mV increments. Current (*I*)-voltage (V) realtionships were determined before (control), after 10 min exposure to NS1619 and after 10 min exposure to anandamide in the continuing presence of NS1619. Each point represents the mean and vertical lines show s.e.mean ($n=5$). (b) NS1619-induced $I_{BK(Ca)}$ at test potentials ranging from 0 to +70 mV before (i) and after (ii) 10 min exposure to iberiotoxin, an inhibitor of BK_{Ca}. Traces were obtained under voltage-clamp conditions and dashed lines indicate the zero current level.

EDHF is that it hyperpolarizes the smooth muscle cells in a variety of vascular beds (for reviews, see Cohen & Vanhoutte, 1995; Garland *et al*., 1995) including those of the rat hepatic artery (present study; Zygmunt *et al*., 1994c). In the present study, anandamide markedly hyperpolarized the smooth muscle cells of rat hepatic artery with an intact endothelium but only minor changes were observed in endothelium-denuded vessels. Additionally, no significant hyperpolarization of single hepatic artery cells was detected under current-clamp conditions nor was any outward K^+ current generated in voltage-clamp mode when these cells were exposed to anandamide.

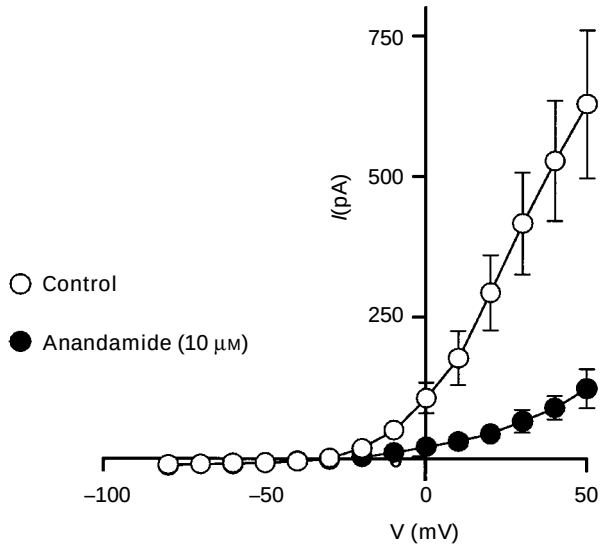


Figure 7 Effect of anandamide on evoked K^+ currents in hepatic arterial cells under voltage-clamp conditions. Currents were generated by stepping for 500 ms from a holding potential of −90 mV to test potentials between −80 mV to +50 mV in 10 mV increments and the current (*I*)-voltage (V) relationships shown were determined before (control) and after 10 min exposure to anandamide. Each point represents the mean and vertical lines show s.e.mean ($n=5$).

Collectively, these data provide powerful evidence that anandamide is not EDHF, at least in the rat hepatic artery and they also question the role which membrane hyperpolarization plays in the relaxant actions of this arachidonic acid derivative.

Endothelial actions of anandamide

Recent microelectrode experiments have shown that anandamide produces a marked hyperpolarization in intact vessels, an effect tacitly assumed to represent a direct action of this agent on the vascular smooth muscle cells (Chataigneau *et al*., 1997; Plane *et al*., 1997). As already described, anandamide-induced hyperpolarization was also detected in the present study but this effect was only seen in the presence of the vascular endothelium, a clear indication that such membrane potential changes were mediated indirectly, presumably by an action on the endothelial cells.

The exact nature of this indirect action of anandamide was beyond the scope of the present study. However, it could represent the incorporation of anandamide into endothelial cell metabolic pathways with the subsequent liberation of a hyperpolarizing substance. Alternatively, it could result from the direct modulation of endothelial cell ion channels (see Ordway *et al*., 1991) and the subsequent release of a hyperpolarizing mediator.

Smooth muscle actions of anandamide

One of the most striking effects of anandamide was its ability to inhibit STOCs in isolated hepatic artery myocytes. Much evidence now indicates that these reflect Ca^{2+} release from intracellular calcium stores and the subsequent opening of BK_{Ca} (Bolton & Imaizumi, 1996; Large & Wang, 1996) and possibly other calcium-sensitive channels (Mironneau *et al*., 1996). Inhibition of STOCs by anandamide could be caused by the direct inhibition of BK_{Ca}. However, anandamide had no effect on NS1619-induced current flow through BK_{Ca}, a strong indication that the intracellular calcium store, and not BK_{Ca} itself, is the site of action of the arachidonic acid derivative. To confirm this view, hepatic artery cells were exposed to caffeine, the ability of which to release calcium from intracellular stores and to activate calcium-sensitive conductances, including $I_{BK(Ca)}$, has been widely described (Bolton & Imaizumi, 1996;

Kirkup *et al*., 1996; Large & Wang, 1996). In these experiments anandamide totally suppressed the ability of caffeine to activate BK_{Ca}.

Unsaturated fatty acids, which are structurally very close to anandamide, inhibit the influx of calcium via Ca^{2+} release-activated Ca^{2+} channels following emptying of intracellular calcium stores (Gamberucci *et al*., 1997). Furthermore, anandamide and related cannabinoids also modulate the intracellular Ca^{2+} concentration in certain cell lines (Felder *et al*., 1995; Sugiura *et al*., 1996). Which, if any, of these mechanisms is affected by anandamide in vascular smooth muscle is the subject of an on-going study.

Anandamide exerts a dual action

On the basis of the present study, it seems clear that anandamide exerts a dual action in the rat hepatic artery. Membrane hyperpolarization is produced indirectly via an unknown intermediate. The contribution that this makes to the observed relaxation of vessels precontracted with phenylephrine seems insignificant since the position of the concentration-effect curve to anandamide is essentially endothelium-independent. However, hyperpolarization could be the mediator of any decrease in the spontaneous tone of a vessel. Additionally, anandamide exerts a direct inhibitory effect on intracellular calcium stores in the hepatic artery and we suggest that this represents the more important mechanism by which anandamide relaxes vascular smooth muscle. Such an action would inhibit the calcium-sensitive Cl^- current ($I_{Cl(Ca)}$) induced by agonists such as phenylephrine and which is a key excitatory trigger event in vascular smooth muscle contraction (Large & Wang, 1996). Thus exposure to anandamide before an agonist like phenylephrine would prevent the generation of $I_{Cl(Ca)}$ or inhibit this current and repolarize the vascular smooth muscle cell if applied during a phenylephrine-induced contraction (see Plane *et al*., 1997). However, the extent of any anandamide-induced hyperpolarization (ie. a shift of the membrane potential to levels more negative than the basal potential) would depend on the integrity of the vascular endothelium in that vessel.

If the extracellular $[K^+]$ is raised, the relaxant effects of anandamide are inhibited or abolished (present study; Randall *et al*., 1996; Plane *et al*., 1997) and in rat mesenteric artery, Plane *et al*. (1997) have shown that the relaxation of phenylephrine-induced contractions is antagonized by either iberiotoxin or charybdotoxin. Such observations have led to the conclusion that the underlying mechanism of anandamide-induced relaxation must be 'K^+ channel opening' (Randall *et al*., 1996; Plane *et al*., 1997). However, this interpretation of the data may be too simplistic. High-K^+ contractions (unlike those produced by phenylephrine) essentially occur without the involvement of intracellular calcium stores (Karaki *et al*., 1997). Thus, if the relaxant effects of anandamide are the result of an inhibitory action at such stores, the effects of this arachidonic acid derivative should indeed be reduced or abolished when vascular tone is induced by high-K^+. Furthermore, a greater and/or longer-lasting phenylephrine-induced depolarization could be expected in the presence of either charybdotoxin or iberiotoxin. These agents block BK_{Ca}, the Ca^{2+}-sensitive K^+ channel of major importance in terminating such agonist-induced electrical changes. In the presence of such toxins, the resulting contraction probably becomes less influenced by the status of anandamide-sensitive Ca^{2+} stores and more dependent on the influx of this ion through L-type voltage-sensitive Ca^{2+} channels. Thus the antagonism of anandamide by charybdotoxin or iberiotoxin may reflect a complex indirect action, as just described, rather than an indication that anandamide acts as an opener of BK_{Ca}.

Are cannabinoid (CB) receptors involved in the actions of anandamide?

Anandamide interacts with both CB_1 and CB_2 receptors (Felder *et al*., 1995; see Lake *et al*., 1997) and the CB_1 receptor antagonist, SR141716A, partially inhibited the relaxant and vasodilator actions of anandamide in both the present study and in that carried out by Randall *et al*. (1996). However, the CB_1 receptor agonist HU-210 (Felder *et al*., 1995) only marginally reduced tension in isolated mesenteric arteries (Plane *et al*., 1997) and WIN 55,212-2, a cannabinoid receptor agonist which has a higher affinity for CB_2 than CB_1 receptors (Felder *et al*., 1995) failed to relax rat mesenteric arteries (Plane *et al*., 1997). Collectively, these findings certainly question the role of CB_1 receptors in the relaxant actions of anandamide. The relatively high concentrations of SR141716A required to inhibit anandamide could indicate that CB_2 receptors are somehow involved (Showalter *et al*., 1996), but the lack of effect of WIN 55,212-2 (Plane *et al*., 1997) does not favour this view. Further studies are clearly necessary to establish the extent to which the direct and indirect effects of anandamide are mediated by cannabinoid receptor subtypes.

Conclusions

The data obtained in the present study do not confirm the proposal that EDHF and anandamide are the same entity, at least in the rat hepatic artery. Importantly, no evidence of direct K^+ channel opening in vascular smooth muscle cells by anandamide or of membrane hyperpolarization was found. These are both axiomatic properties of 'EDHF'. In fact, anandamide actually inhibited current flow through K^+ channels (K_V; present study), an action also shown for polyunsaturated fatty acids which are structurally very similar to anandamide (Gilbertson *et al*., 1997). The major relaxant effect of anandamide in intact hepatic arteries seems to be associated with an inhibitory action at Ca^{2+} stores. Some evidence in favour of the involvement of CB receptors in this response was obtained, although further studies are required to confirm this.

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