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Vascular effects of anandamide and *N*-acylvanillylamines in the human forearm and skin microcirculation

[1]Pouya Movahed, [2]Vladimir Evilevitch, [1]Tomas L.G. Andersson, [3]Bo A.G. Jönsson, [2]Per Wollmer, *,[1]Peter M. Zygmunt & [1]Edward D. Högestätt

[1]Department of Clinical and Experimental Pharmacology, Lund University Hospital, SE-221 85 Lund, Sweden; [2]Department of Clinical Physiology, Malmö University Hospital, SE-205 02 Malmö, Sweden and [3]Department of Occupational and Environmental Medicine, Lund University Hospital, SE-221 85 Lund, Sweden

1 The endocannabinoid anandamide is an emerging potential signalling molecule in the cardiovascular system. Anandamide causes vasodilatation, bradycardia and hypotension in animals and has been implicated in the pathophysiology of endotoxic, haemorrhagic and cardiogenic shock, but its vascular effects have not been studied in man.

2 Human forearm blood flow and skin microcirculatory flow were recorded using venous occlusion plethysmography and laser-Doppler perfusion imaging (LDPI), respectively. Each test drug was infused into the brachial artery or applied topically on the skin followed by a standardized pin-prick to disrupt the epidermal barrier.

3 Anandamide failed to affect forearm blood flow when administered intra-arterially at infusion rates of 0.3–300 nmol min^{-1}. The highest infusion rate led to an anandamide concentration of approximately 1 μM in venous blood as measured by mass spectrometry.

4 Dermal application of anandamide significantly increased skin microcirculatory flow and coapplication of the transient receptor potential vanilloid 1 ($TRPV_1$) antagonist capsazepine inhibited this effect. The $TRPV_1$ agonists capsaicin, olvanil and arvanil all induced concentration-dependent increases in skin blood flow and burning pain when administered dermally. Coapplication of capsazepine inhibited blood flow and pain responses to all three $TRPV_1$ agonists.

5 This study shows that locally applied anandamide is a vasodilator in the human skin microcirculation. The results are consistent with this lipid being an activator of $TRPV_1$ on primary sensory nerves, but do not support a role for anandamide as a circulating vasoactive hormone in the human forearm vascular bed.
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Abbreviations: AUC, area under the curve; CB, cannabinoid; LDPI, laser-Doppler perfusion imaging; PU, perfusion units; ROI, region of interest; $TRPV_1$, transient receptor potential vanilloid 1

Introduction

N-acylethanolamines belong to a growing family of endogenous signalling molecules (Schmid, 2000), acting on a variety of receptors and ion channels (Devane *et al*., 1992; Felder *et al*., 1995; Poling *et al*., 1996; Zygmunt *et al*., 1999; Chemin *et al*., 2001; Maingret *et al*., 2001; Ahern, 2003; Fu *et al*., 2003; Nicholson *et al*., 2003). In 1992, Mechoulam and co-workers reported that the *N*-acylethanolamine anandamide is present in brain and activates the central cannabinoid (CB) receptor (Devane *et al*., 1992). Anandamide and other *N*-acylethanolamines are considered to be generated on demand following receptor activation (Stella & Piomelli, 2001) and hydrolysis of membrane phospholipids, a reaction catalysed by an *N*-acylphosphatidylethanolamine-selective phospholipase D (Okamoto *et al*., 2004). The tissue level of anandamide increases during myocardial and cerebral ischaemic injury (Natarajan *et al*., 1981; Schabitz *et al*., 2002; Berger *et al*., 2004) and inflammation (McVey *et al*., 2003; Dinis *et al*., 2004), and hydrolytic cleavage of the amide bond by fatty acid amide hydrolase is responsible for the elimination of anandamide and other *N*-acylethanolamines (Cravatt *et al*., 2001). Several *N*-acylethanolamines have been detected in plasma from both animal and man, and the level of anandamide increases in patients with endotoxic shock (Giuffrida *et al*., 2000; Wang *et al*., 2001). Macrophages and monocytes (Varga *et al*., 1998), endothelial cells (Deutsch *et al*., 1997) and sensory nerves (Ahluwalia *et al*., 2003) are possible cellular sources of anandamide in the vascular system. Interestingly, endocannabinoid-induced activation of SR141716A-sensitive CB receptors have been implicated in the hypotension occurring during endotoxic, haemorrhagic and cardiogenic shock (Wagner *et al*., 1997; 2001; Varga *et al*., 1998; Wang *et al*., 2001).

Studies on whole animals or isolated tissues have shown that anandamide induces a variety of effects in the cardiovascular system, including vasodilatation, bradycardia and hypotension (Högestätt & Zygmunt, 2002). The mechanisms behind these effects are not fully understood but seem to depend on the bioassay and the mode of administration of anandamide

*Author for correspondence; E-mail: peter.zygmunt@med.lu.se

(Högestätt & Zygmunt, 2002). In isolated arterial segments, anandamide produces vasorelaxation *via* activation of transient receptor potential vanilloid 1 ($TRPV_1$) on perivascular sensory nerves and subsequent release of the potent vasodilator calcitonin gene-related peptide (Zygmunt *et al*., 1999; 2002). $TRPV_1$ is a nonselective cation channel, belonging to the transient receptor potential ion channel superfamily, and the receptor for capsaicin, the pungent ingredient in hot chilli peppers (Caterina *et al*., 1997). The mechanism behind the anandamide-induced vasodilatation in isolated vascular beds and whole animals is, however, controversial, and both $TRPV_1$ (Ralevic *et al*., 2000; Mendizabal *et al*., 2001; Smith & McQueen, 2001; Harris *et al*., 2002; Akerman *et al*., 2004; Pacher *et al*., 2004), CB_1 receptors (Varga *et al*., 1996; Jarai *et al*., 1999; Wagner *et al*., 1999; Pacher *et al*., 2004) and yet undefined molecular mechanisms (Jarai *et al*., 1999; Wagner *et al*., 1999; White *et al*., 2001) have been implicated. Species differences add further complexity to the interpretation of animal data (Högestätt & Zygmunt, 2002; Randall *et al*., 2004). Although the effects of anandamide have been extensively studied in animals, there are no reports on its biological activity in man.

In the present study, we examined the effect of anandamide on blood flow in the human forearm using venous occlusion plethysmography and laser-Doppler perfusion imaging (LDPI). The former technique measures blood flow mainly in skeletal muscle and test drugs are administered *via* an intravascular route. The latter method measures microcirculatory flow in skin and test drugs are applied topically and hence reach the blood vessels from the adventitial side. We also examined the effects of the potent $TRPV_1$ agonists olvanil (Hughes *et al*., 1992; Janusz *et al*., 1993) and arvanil (Di Marzo *et al*., 2000) on skin blood flow. These compounds share structural features with both capsaicin and anandamide, but are considered less pungent than capsaicin (Janusz *et al*., 1993; Di Marzo *et al*., 2000).

Methods

The studies were approved by the regional ethics committee in Lund and performed according to the declaration of Helsinki. Written informed consent was obtained from all subjects prior to inclusion.

Venous occlusion plethysmography

Forearm blood flow was measured in both arms simultaneously in six healthy nonsmoking male subjects, aged 22–31 years, using strain gauge plethysmography (Whitney, 1953) at an ambient temperature of 24–26°C. A 27-gauge needle was inserted into the left brachial artery under sterile conditions after local anaesthesia with lignocaine gel. A stock solution of anandamide (1 mM) in ethanol was prepared by the local hospital pharmacy. Anandamide was diluted in sterile saline and the solution was infused at a constant rate of 1 ml min^{-1}. Anandamide was infused at doses of 0.3, 1, 3, 10, 30, 100 and 300 nmol min^{-1}. The corresponding ethanol concentrations in the infusion solutions were 0.03, 0.1, 0.3, 1, 3, 10 and 30%, respectively. Assuming a forearm blood flow of 50 ml min^{-1}, the concentrations of anandamide and ethanol was diluted by a factor of 50 in arterial blood. Basal blood flow was recorded during an 18-min saline infusion prior to administration of the test substances. Two and four subjects were exposed to five (0.3, 1, 3, 10 and 30 nmol min^{-1}) and four (10, 30, 100 and 300 nmol min^{-1}) incremental doses of anandamide, respectively. Each dose of anandamide was infused during 6 min. After washout with saline, the ethanol vehicle, corresponding to the highest anandamide concentration used, was infused for 6 min. Blood pressure (sphygmomanometry) and heart rate (peripheral pulse) were measured manually in the right arm at the end of each 6-min infusion period. Venous blood samples for quantification of anandamide were collected in Vacutainer® tubes from an antecubal vein in the left arm at the end of each infusion period. In separate experiments with four of the subjects, the vasodilator responses to sodium nitroprusside (Nitropress®, Abbott, Abbott Park, IL, U.S.A.) and acetylcholine (Miochol®, Novartis, Basel, Switzerland) were also measured. Both sodium nitroprusside and acetylcholine were diluted in saline and infused at a rate of 1 ml min^{-1}. After measuring of basal blood flow during infusion of saline, sodium nitroprusside was infused over 6-min periods at rates of 10 and 40 nmol min^{-1}. After a 12 min washout period with saline infusion, acetylcholine was infused over 6-min periods at rates of 40, 80 and 160 nmol min^{-1}. Forearm blood flow was measured for 10 s every 15 s during the last 3 min of each 6-min infusion. The last five measurements in each recording were used for analysis. Blood flow was expressed in ml per min per 100 ml of forearm volume (Whitney, 1953) and converted to the ratio between the infused arm and the control arm. Changes in blood flow were presented as % change from baseline.

LDPI

A total of 28 healthy nonsmoking subjects (17 female), aged 21–57 years, participated in the study. Subjects were lying down and the left or right arm was extended. Small droplets (20 μl) of the test solutions were applied topically on the volar side of the forearm at a maximum of seven test spots. An adhesive tape marker was placed beside each spot. A sterile lancet (ALK Lancet, ALK-Abelló A/S, Hørsholm, Denmark) with a tip length of 100 μm was used to pin-prick the skin at the site of the test solution. After approximately one min, the droplets were dried off and LDPI of the forearm skin was performed over a period of 35 min in darkened environment (PeriScan PIM II Perfusion Imager, Perimed AB, Stockholm, Sweden). The sampling depth of the laser beam depends on the optical properties of the surface, but is normally 300–500 μm in skin (Jakobsson & Nilsson, 1993). The intensity of the backscatter under basal conditions was set to 8.0 V by adjusting the distance between the scanner and the skin. Spatial differences in skin blood flow were displayed on a PC monitor using a six colour-coded image. The regions of interest (ROI) on the images were defined as circles, each covering 25 picture elements, and positioned with the guidance of the tape markers. The mean perfusion of the skin within each ROI was expressed in arbitrarily units (perfusion units; PU). Area under the curve (AUC) was calculated for the entire recording period of 35 min. The subjects were asked to report pain or other discomfort experienced immediately after the pin-prick.

Anandamide (30 mM) was provided in Tocrisolve (Tocris, Bristol, U.K.), whereas capsaicin, olvanil and arvanil (each 100 mM) were dissolved in ethanol. The stock solutions were

diluted with polyoxyethylene sorbitan mono-oleate (Tween80) in saline (1 : 48) to obtain the final test solutions. When the effects of capsazepine and its vehicle (dimethylsulphoxide) were studied, capsazepine (100 mM) or vehicle was added to the anandamide stock solution, whereas capsazepine or vehicle together with olvanil, arvanil or capsaicin (each dissolved at 100 mM in dimethylsulphoxide) were added to Tween80 in saline (1:24) to obtain the final test solutions. Histamine was dissolved in and diluted with saline. Control experiments with appropriate vehicle compositions were always run in parallel. Arvanil were purchased from Cayman chemicals (Ann Arbor, MI, U.S.A.). Capsaicin, olvanil and Tocrisolve were obtained from Tocris (Bristol, U.K.), while anandamide was obtained from either Cayman chemicals or Tocris. Histamine, capsazepine, Tween 80 and dimethylsulphoxide were purchased from Sigma (St Louis, MO, U.S.A.).

Quantification of anandamide

Venous blood samples were centrifuged to obtain plasma. Aliquots (200 μl) of blood plasma were precipitated with 1 ml ice-cold acetone, containing 1 μM $^2H_8$-labelled anandamide as internal standard. After centrifugation at 3000 r.p.m. for 10 min (5°C), the supernatant was collected in polypropylene tubes and kept on ice until vacuum evaporated. The extraction residue was reconstituted in 100 μl methanol with 0.3 mM ascorbic acid and stored at −20°C until analysed. Standards for quantifications were obtained by addition of different amounts of anandamide to the blood samples. A Perkin-Elmer Series 200 liquid chromatography system with autosampler (Applied Biosystems, Norfolk, CT, U.S.A.), coupled to an API 3000 LC-MS-MS (Applied Biosystems/MDS-SCIEX, Toronto, Canada), was used for the anandamide analysis. The column was a Genesis C_8 (20 × 2.1 mm) with a particle size of 4 μm (Jones, Lakewood, CO, U.S.A.). Aliquots of 5 μl were injected by the autosampler. Mobile phase was a water-methanol gradient, containing 0.5% acetic acid, and the initial mobile flow was 75% methanol. A linear gradient to 100% methanol was applied in 6 min. The mobile flow rate was 0.2 ml min^{-1}. The turbo ion spray interface was set to 370°C, the declustering potential 40 V and collision energy 35 V. The analyses were performed in the positive ion multiple reaction monitoring mode and the mass fragment used for anandamide was m/z 348.2/62.0, while m/z 356.4/63.0 were chosen for $^2H_8$-anandamide. The peak area ratios between the analytes and the internal standards were used for quantification. The limit of detection for anandamide was below 1 nM.

Calculations and statistics

For evaluation of drug effects, maximum skin perfusion and AUC were calculated in GraphPad Prism 3.0 (GraphPad Software Inc., San Diego, CA, U.S.A.). Data in text and figures are presented as mean ± s.e.m. and n indicates the number of experiments performed (number of subjects). Statistical analysis was performed using Student's paired t-test when comparing two groups and ANOVA followed by Bonferroni's *post hoc* test for multiple comparisons (GraphPad Prism). Fisher's exact test followed by Bonferroni's *post hoc* test was used for comparing pain responses. Statistical significance was accepted when $P < 0.05$.

Results

Effect of anandamide on forearm blood flow

Anandamide given intra-arterially at doses between 0.3 and 300 nmol min^{-1} had no significant effect on forearm blood flow when compared with baseline or vehicle (Figure 1). Higher doses of anandamide could not be used, because of vehicle-induced pain and limitations in solubility of anandamide. There were no significant changes in blood pressure or heart rate during the infusion of anandamide or vehicle in any of the subjects. Four of the six subjects experienced local

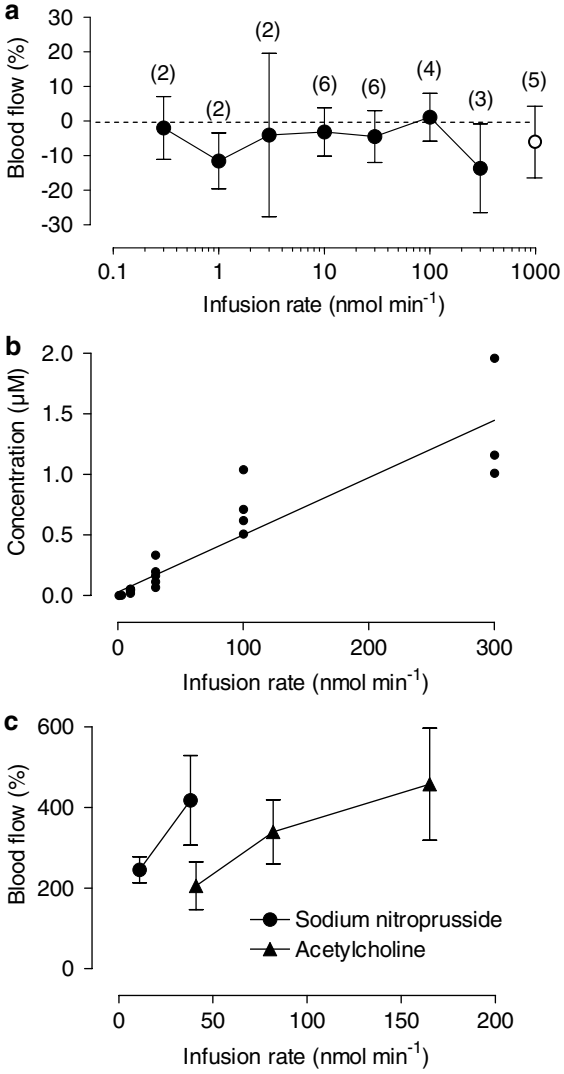

Figure 1 Anandamide at concentrations between 0.3 and 300 nmol min^{-1} (filled circles) and vehicle (unfilled circle), corresponding to the highest anandamide dose, did not significantly affect forearm blood flow when infused into the brachial artery of the left arm (a, $n = 2$–6). The number of patients (n) exposed to each dose are given above the bars. There was a close correlation between the amount of anandamide infused and the concentration of anandamide in venous blood drawn from the left antecubal vein at the end of each infusion period (b, $n = 2$–6). Anandamide could not be detected in control samples. In separate experiments, sodium nitroprusside (10 and 40 nmol min^{-1}) and acetylcholine (20, 80 and 160 nmol min^{-1}) caused pronounced increases in forearm blood flow (c, $n = 4$).

symptoms during infusion of the highest dose of anandamide. The reactions ranged from a discreet feeling of warmth in one subject to a sharp pain, leading to premature termination of the infusion in one subject. All symptoms disappeared immediately on switching the infusion solution to saline. Similar responses were observed after vehicle infusion. No other adverse events or reactions were reported. There was a close correlation between the amount of anandamide infused and the concentration of anandamide in venous blood obtained from the ipsilateral arm at the end of the infusion period (Figure 1). The change in blood flow and plasma concentration of anandamide after the 30 nmol min^{-1} dose were 4.5±7.5% and 161±40 nM, respectively ($n=6$). The concentration of anandamide after the highest dose (300 nmol min^{-1}) was 1.4±0.3 μM ($n=3$). Anandamide could not be detected in venous blood during the initial infusion with saline. Infusion of sodium nitroprusside and acetylcholine caused concentration-dependent increases in forearm blood flow in all subjects investigated (Figure 1).

Effect of anandamide and other TRPV$_1$ agonists on skin blood flow

Histamine applied topically at a concentration of 1 mM induced a transient increase in blood flow, reaching a maximum after approximately 2 min (Figure 2). Anandamide at a concentration of 30 mM induced a long-lasting increase in skin blood flow (Figure 2). A lower concentration of anandamide (1 mM) had no effect. In five subjects, showing robust responses to anandamide, the TRPV$_1$ antagonist capsazepine (10 mM) given together with anandamide almost abolished the increases in skin blood flow (Figure 2), while capsazepine did not inhibit responses to histamine ($n=3$, data not shown). None of the subjects exposed to anandamide or vehicle reported any adverse effects, whereas two and four out of 19 subjects exposed to histamine reported burning pain and itching immediately after the pin-prick, respectively.

The TRPV$_1$ agonists capsaicin, olvanil and arvanil all induced concentration-dependent increases in skin blood flow (Figure 3). Both olvanil and arvanil at a concentration of 1 mM produced consistent and sustained responses, which in the case of olvanil did not decline over the observation period of 35 min (Figure 3). Olvanil and arvanil produced a larger increase in blood flow than capsaicin at equimolar concentrations ($P<0.001$; Figure 3). Capsazepine (10 mM) given together with capsaicin, olvanil or arvanil inhibited the increases in skin blood flow (Figure 4). Olvanil (Figure 5) and histamine ($n=3$, data not shown) had no effect on skin blood flow unless the epidermal barrier was disrupted with a pin-prick.

All 17 subjects exposed to 1 mM capsaicin and nine out of 10 subjects exposed to 10 mM capsaicin reported burning pain immediately after the pin-prick, whereas only four out of 10 subjects exposed to capsaicin (1 mM) together with capsazepine (10 mM) reported burning pain. Among those

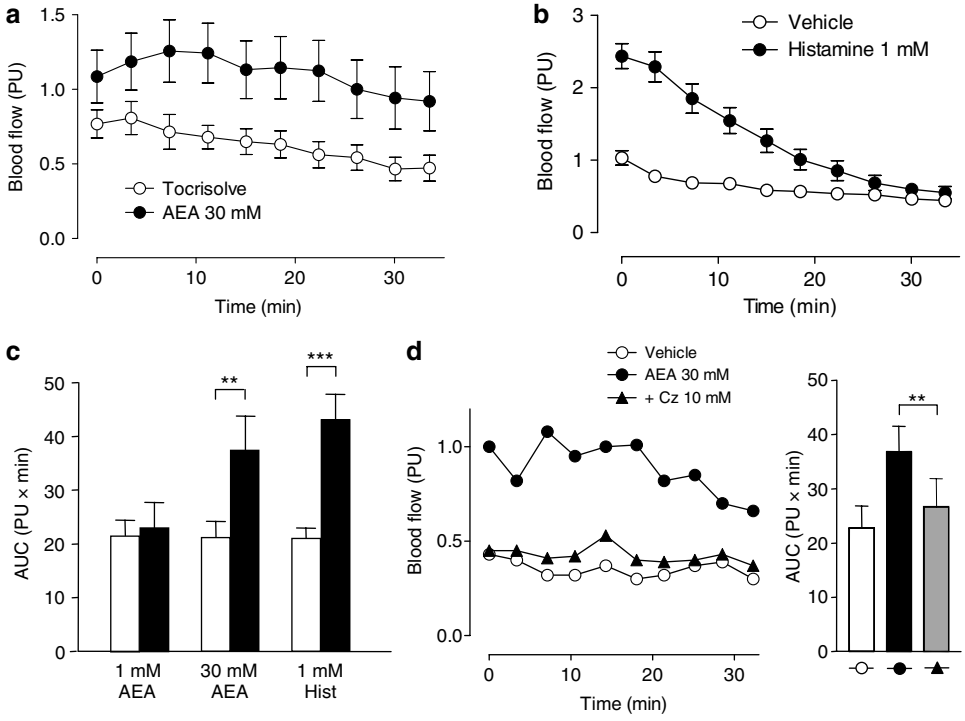

Figure 2 Anandamide (AEA) at a concentration of 30 mM produced a long-lasting increase in skin blood flow (a; $n=10$). Histamine (Hist) at a concentration of 1 mM produced a transient increase in skin blood flow (b; $n=19$). The areas under the curves (AUC) for the test substances (filled bars) and vehicles (unfilled bars) are shown in (c). Capsazepine (Cz) at a concentration of 10 mM inhibited the anandamide-induced increase in skin blood flow (d; $n=5$). Curves in (d) show responses in one of the subjects. Column bars in (d) show AUC for AEA (filled bar), AEA + Cz (grey) and vehicle (unfilled bar). Please note that the concentrations indicated in the figure refer to the concentrations in the droplet (20 μl), which was applied topically on the volar side of the forearm. A sterile lancet was then used to pin-prick the skin at the site of the test solution to disrupt the epidermal barrier. After approximately 1 min, the solution was dried off and LDPI of the forearm skin was performed over a period of 35 min. Blood flow is given as perfusion units (PU). $^{**}P<0.01$, $^{***}P<0.001$.

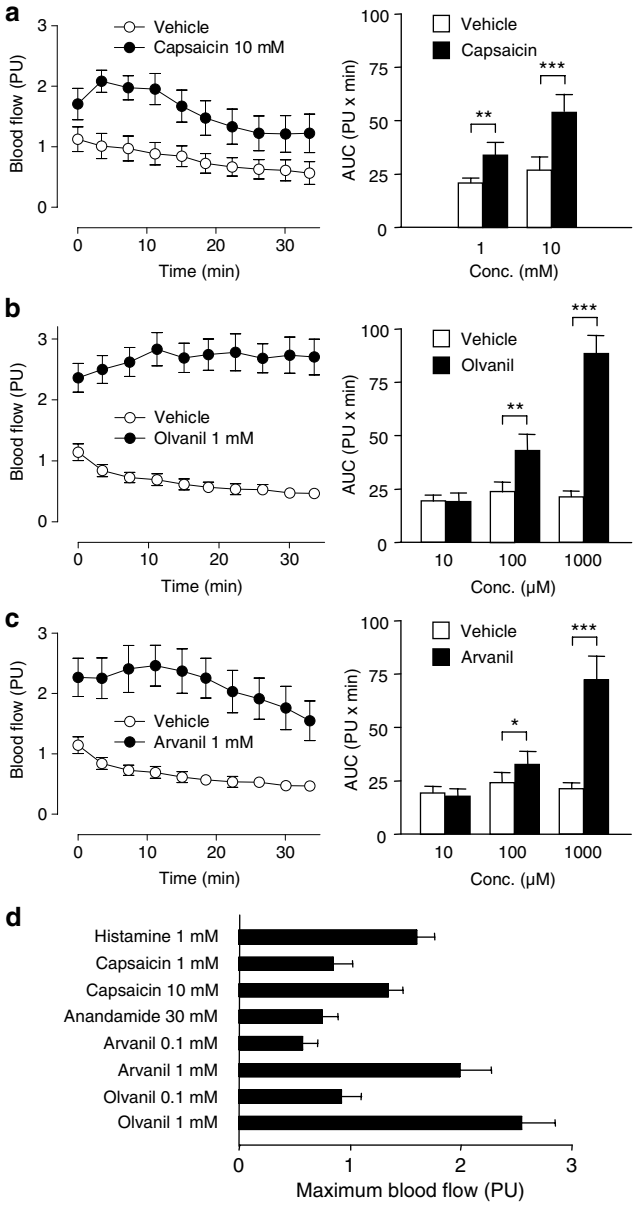

Figure 3 The $TRPV_1$ agonists capsaicin (a; $n=10$–19), olvanil (b; $n=10$) and arvanil (c; $n=10$) caused concentration-dependent increases in skin blood flow. Column bars show areas under the curves (AUC) for the test substances (filled bars) and vehicles (unfilled bars). The maximum blood flow for each test substance, adjusted for time-matched effects of the vehicles, is shown in (d). Please note that the concentrations indicated in the figure refer to the concentrations in the droplet (20 μl), which was applied topically on the volar side of the forearm. A sterile lancet was then used to pin-prick the skin at the site of the test solution to disrupt the epidermal barrier. After approximately 1 min, the solution was dried off and laser-Doppler perfusion imaging of the forearm skin was performed over a period of 35 min. Blood flow is given as perfusion units (PU). $*P<0.05$, $**P<0.01$, $***P<0.001$.

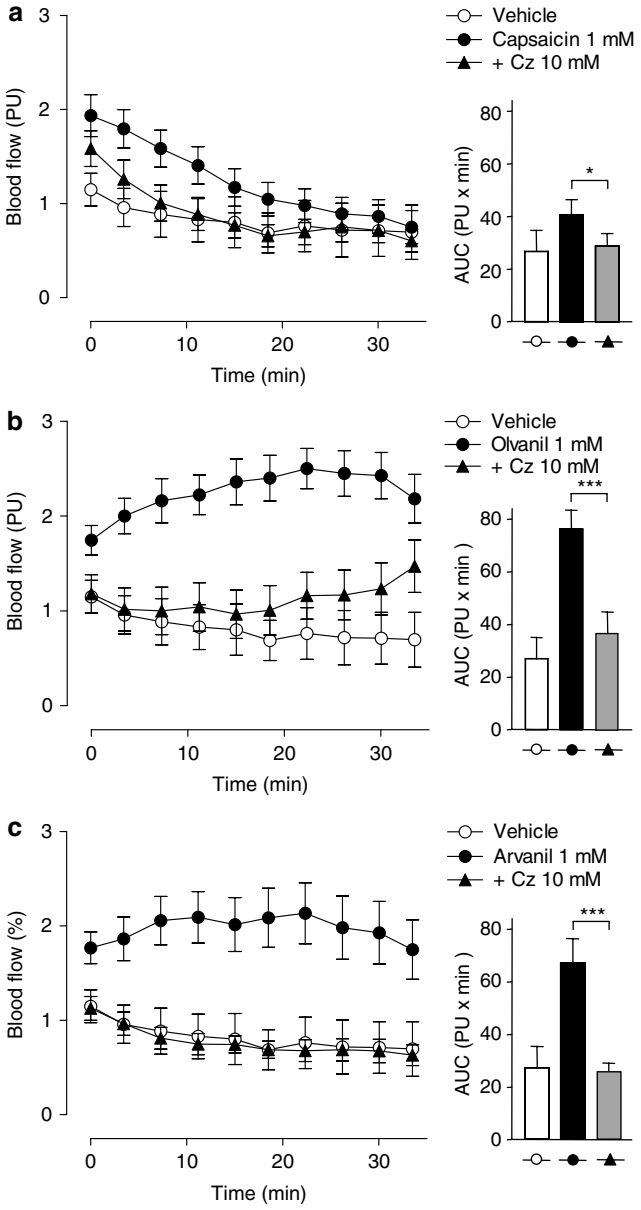

Figure 4 Capsazepine (Cz) at a concentration of 10 mM inhibited the increase in skin blood flow induced by the $TRPV_1$ agonists capsaicin (a), olvanil (b) and arvanil (c), each given at a concentration of 1 mM ($n=10$). Column bars show areas under the curves (AUC) for the agonists given alone (black bars) or together with Cz (grey bars), and vehicles (unfilled bars). Please note that the concentrations indicated in the figure refer to the concentrations in the droplet (20 μl), which was applied topically on the volar side of the forearm. A sterile lancet was then used to pin-prick the skin at the site of the test solution to disrupt the epidermal barrier. After approximately 1 min, the solution was dried off and LDPI of the forearm skin was performed over a period of 35 min. Blood flow is given as perfusion units (PU). $*P<0.05$, $***P<0.001$.

exposed to olvanil, three out of 10 subjects exposed to 0.1 mM and eight out of 15 subjects exposed to 1 mM of the drug reported burning pain. Among those exposed to arvanil, one out of 10 subjects exposed to 0.1 mM and seven out of 15 subjects exposed to 1 mM of the drug reported burning pain. When the drugs were added together with capsazepine (10 mM), only one out of 10 subjects exposed to olvanil (1 mM) and none of the 10 subjects exposed to arvanil (1 mM) reported burning pain. The capsazepine-induced inhibition of burning pain was statistically significant for all three agonists ($P=0.001$ for capsaicin, $P=0.04$ for olvanil, $P=0.02$ for arvanil).

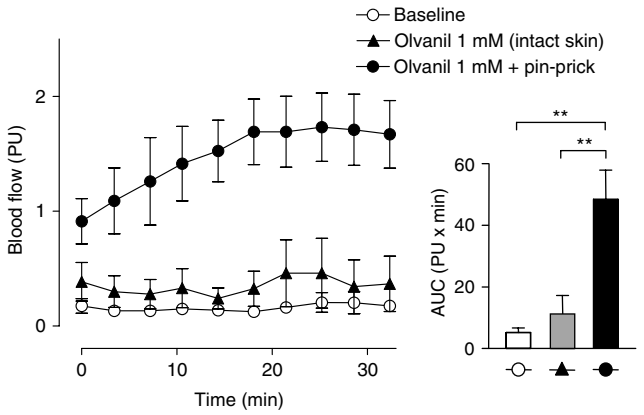

Figure 5 Olvanil at a concentration of 1 mM did not increase skin blood flow unless the epidermal barrier was disrupted with a pin-prick. Topical application of olvanil with (filled circles) and without a standardized pin-prick (filled triangles). Baseline (unfilled circles) indicates blood flow in surrounding intact skin. Please note that the concentrations indicated in the figure refer to the concentrations in the droplet (20 μl), which was applied topically on the volar side of the forearm. A sterile lancet was then used to pin-prick the skin at the site of the test solution. After approximately 1 min, the solution was dried off and LDPI of the forearm skin was performed over a period of 35 min. Column bars show areas under the curves (AUC) for the different measurements. Blood flow is given as perfusion units (PU). $^{**}P<0.01$.

Discussion

This study shows that anandamide as well as the $TRPV_1$ agonists capsaicin, olvanil and arvanil cause vasodilatation in the human skin microcirculation and provides the first evidence for biological activity of anandamide in man. Coapplication of the $TRPV_1$ antagonist capsazepine attenuates the blood flow responses to these compounds, but not that to histamine. This is consistent with anandamide being an activator of the cloned human $TRPV_1$ (Smart *et al*., 2000) and extends previous observations in animals that anandamide produces vasodilatation *via* activation of $TRPV_1$ on primary sensory nerves (Zygmunt *et al*., 1999; 2002; Ralevic *et al*., 2000; Harris *et al*., 2002; Li *et al*., 2003; Akerman *et al*., 2004). Although $TRPV_1$-containing nerve endings are the most likely cellular target for anandamide, keratinocytes and mast cells, which also express $TRPV_1$ in human skin (Denda *et al*., 2001; Stander *et al*., 2004), may also be involved in the anandamide response.

To induce a similar increase in skin blood flow, a higher concentration in the test solution was required of anandamide than of capsaicin, olvanil and arvanil. However, this is expected as the latter compounds are more potent than anandamide as $TRPV_1$ activators in isolated arteries and cells heterologously expressing $TRPV_1$ (Zygmunt *et al*., 1999; 2002; De Petrocellis *et al*., 2000; Smart *et al*., 2000; Andersson *et al*., 2002; Jerman *et al*., 2002). As shown with olvanil and histamine, topical application *per se* did not affect skin microcirculation unless the epidermal barrier was disrupted with a pin-prick. Considering the small size of the wound and that the test solutions were wiped away from the skin shortly after the pin-prick, it is reasonable to assume that only a small fraction of test substances reach the microcirculation.

Anandamide is known as a ligand at CB_1 and CB_2 receptors (Devane *et al*., 1992; Felder *et al*., 1995), and activation of such receptors on, for example, the endothelium, smooth muscle or sympathetic nerve terminals, could contribute to the vasodilator effect of anandamide in human skin. However, capsazepine almost completely inhibited the responses to anandamide in all five subjects examined. Furthermore, intradermal administration of the synthetic CB receptor agonist HU210, using microdialysis fibres, did not *per se* affect skin blood flow, while reducing the itching and blood flow responses to histamine (Dvorak *et al*., 2003). It is therefore unlikely that activation of CB receptors contributes to the anandamide-induced increase in skin blood flow.

It is interesting to note that none of the subjects exposed to anandamide experienced pain, whereas all subjects reported burning pain following application of capsaicin. The *N*-acylvanillylamines olvanil and arvanil also evoked less pain than capsaicin at equipotent doses in terms of blood flow increase ($P<0.01$). The mechanism behind this dissociation of local vasodilatation (efferent function) and burning pain (afferent function) is unclear, but could be related to $TRPV_1$-independent effects of anandamide, olvanil and arvanil on ion channels involved in action potential generation and propagation (Poling *et al*., 1996; Chemin *et al*., 2001; Maingret *et al*., 2001; Lo *et al*., 2003; Nicholson *et al*., 2003).

Anandamide did not alter blood flow in the human forearm when the lipid was infused into the brachial artery at doses of 3–300 nmol min^{-1}, leading to concentrations in venous blood substantially exceeding those in patients with endotoxic shock (Giuffrida *et al*., 2000; Wang *et al*., 2001). At an intermediate dose of 30 nmol min^{-1}, the concentration of anandamide in plasma was about 10 times higher than the serum concentration of anandamide in endotoxic shock (Giuffrida *et al*., 2000; Wang *et al*., 2001). Since anandamide could not be detected in venous blood during the initial saline infusion, the basal level of anandamide must have been lower than 1 nM (limit of detection), which is well below the concentrations needed to activate $TRPV_1$ (Zygmunt *et al*., 1999) or the CB_1 receptor (Devane *et al*., 1992). It is therefore unlikely that the results were confounded by high levels of circulating endogenous anandamide. In contrast to anandamide, sodium nitroprusside and the endothelium-dependent vasodilator acetylcholine caused a pronounced and consistent vasodilatation in this physiologically relevant setting. Since anandamide has a high affinity to albumin and displays more than 99% protein binding in human plasma (Bojesen & Hansen, 2003), the free concentration of anandamide may not be high enough to activate a putative endothelial anandamide receptor. High plasma protein binding would also prevent anandamide from reaching the smooth muscle and perivascular nerves. Animal studies showing cardiovascular effects of anandamide when administered *via* an intravascular route have used either protein-free perfusion solutions or high bolus doses of anandamide, probably exceeding the anandamide-binding capacity of albumin (Varga *et al*., 1996; Jarai *et al*., 1999; Wagner *et al*., 1999; Smith & McQueen, 2001; Ford *et al*., 2002; Harris *et al*., 2002; Akerman *et al*., 2004).

Taken together, our findings do not support a role for anandamide as a circulating vasoactive hormone in the human forearm vascular bed. However, this may not apply to nonhealthy subjects, who might respond differently to anandamide. Our results also do not exclude that anandamide

produced in the vascular wall or in the surrounding tissue may act as a local vasodilator, for example, during inflammation and tissue ischaemia (Natarajan *et al*., 1981; Schabitz *et al*., 2002; McVey *et al*., 2003; Berger *et al*., 2004; Dinis *et al*., 2004). Both endothelial cells and resident macrophages are potential sources of anandamide (Deutsch *et al*., 1997; Varga *et al*., 1998). Preliminary results have indicated substantial levels of *N*-acylethanolamines in atherosclerotic lesions of apolipoprotein E-deficient mice (Movahed *et al*., 2002). Circulating monocytes and macrophages adhering to the endothelium may also provide high local concentrations of anandamide, contributing to peripheral vasodilatation and hypotension during endotoxic, haemorrhagic and cardiogenic shock (Wagner *et al*., 1997; 2001; Varga *et al*., 1998; Wang *et al*., 2001). Anandamide may also be formed within primary sensory neurones and function as an intracellular messenger in $TRPV_1$-containing nerves (Ahluwalia *et al*., 2003). Although the physiological role of anandamide in the cardiovascular system remains elusive, this study clearly shows that anandamide is able to cause vasodilatation in human skin when an extravascular route of administration is used.

Many vascular beds, including the skin, receive a rich supply of sensory nerves, forming a network of fibres containing calcitonin gene-related peptide and/or substance P in the adventitial-medial border of arteries (Holzer, 1992; Zygmunt *et al*., 1999). During inflammation and tissue ischaemia, these nerves may influence local blood flow through $TRPV_1$-mediated sensing of the chemical environment (Holzer, 1992; Franco-Cereceda *et al*., 1993; Caterina *et al*., 1997; Strecker *et al*., 2005). Capsaicin-sensitive primary afferents have also been implicated in myocardial preconditioning (Li & Peng, 2002; Hu *et al*., 2003), blood pressure regulation during high sodium intake (Vaishnava & Wang, 2003) and other conditions associated with high levels of circulating calcitonin gene-related peptide (Brain & Grant, 2004). Drugs targeting $TRPV_1$ on primary afferents may therefore provide novel opportunities for treatment of disorders of the cardiovascular system besides their obvious use as pain relievers. Since species differences have been demonstrated for $TRPV_1$ (Nagy *et al*., 2004), it is important to evaluate the effects of new drugs on the human orthologue of this ion channel. Topical application of drugs on the skin followed by standardized pin-pricking and LDPI provides a simple and safe method for studying the pharmacology of drugs on native $TRPV_1$ in man. Using this method, we show for the first time that capsazepine is active on capsaicin-induced responses in humans. Furthermore, the $TRPV_1$ agonists olvanil and arvanil induce consistent and long-lasting increases in skin blood flow, making them suitable pharmacological tools for testing the effects of novel $TRPV_1$ antagonists in this bioassay. These agonists may also provide advantages over capsaicin when targeting $TRPV_1$ for treatment of neuropathic pain and microvascular skin disorders, because of their longer duration of action and a lower tendency to evoke burning pain.

The present study shows for the first time that anandamide has biological activity in man, an action most likely involving activation of $TRPV_1$ on perivascular sensory nerves. The lack of effect of intravascular anandamide on human forearm blood flow does not support a role for anandamide as a hormonal regulator of vascular tone.

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