

Regional Differences in Anandamide- and Methanandamide-Induced Membrane Potential Changes in Rat Mesenteric Arteries

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ABSTRACT

The possibility that anandamide is an endothelium-derived hyperpolarizing factor was explored in the rat mesenteric vasculature by use of conventional microelectrode techniques. In the main mesenteric artery, anandamide and its more stable analog methanandamide hardly caused a measurable change in membrane potential of the smooth muscle cells, which promptly hyperpolarized to EDHF liberated by acetylcholine. Inhibition of endogenous anandamide breakdown by phenylmethylsulfonyl fluoride did not increase membrane responses to acetylcholine. The CB₁ receptor antagonist SR141716 did not significantly influence EDHF-mediated hyperpolarization except at extremely high concentrations. Smooth muscle cells of third to fourth order branches of the mesenteric artery, which have a more negative resting membrane potential and show smaller responses to acetylcholine, hyperpolarized by about 6 mV to both anandamide and methanandamide, whereas another CB₁ receptor agonist, WIN 55,212-2, had no effect. Mechanical

endothelium removal or pre-exposure to SR141716A did not affect anandamide- and methanandamide-induced hyperpolarizations. However, in the presence of capsazepine, a selective vanilloid receptor antagonist, these membrane potential changes were reversed to a small depolarization, whereas EDHF-induced hyperpolarizations were not affected. Pretreating small vessels with capsaicin, causing desensitization of vanilloid receptors and/or depletion of sensory neurotransmitter, completely blocked methanandamide-induced hyperpolarizations. These findings show that anandamide cannot be EDHF. In smooth muscle cells of small arteries, anandamide-induced changes in membrane potential are mediated by vanilloid receptors on capsaicin-sensitive sensory nerves. The different membrane response to the cannabinoids between the main mesenteric artery and its daughter branches might be explained by the different density of perivascular innervation.

In response to various relaxing agonists, the vascular endothelium releases several factors that decrease the tone of the underlying smooth muscle. Besides nitric oxide (NO) and prostacyclin, an endothelium-derived hyperpolarizing factor (EDHF) assists endothelium-dependent vasodilation. The chemical nature of EDHF is as yet unclear, although several studies suggest that a nonprostanoid metabolite of arachidonic acid is likely involved (for review, see Cohen and Vanhoutte, 1995).

The potent vasodilatory effect of arachidonylethanolamide (anandamide), an endogenous arachidonic acid derivative (Fig. 1) that selectively binds to the brain type cannabinoid (CB₁) receptor (Devane et al., 1993), has been shown in a variety of isolated vascular preparations. Its mechanism of action, however, is complex and the subject of intense investigation (Ellis et al., 1995; Deutsch et al., 1997; Pratt et al.,

1998; Jarai et al., 1999; Wagner et al., 1999). In the perfused mesenteric vascular bed of the rat, exogenous application of anandamide caused vasodilation (Randall et al., 1996). This influence was reported to be endothelium-independent. In addition, the endothelium-dependent, NO- and prostanoid-independent dilation induced by carbachol was inhibited by the CB₁ receptor antagonist SR141716A (Randall et al., 1996). Anandamide, therefore, has been proposed as EDHF (Randall et al., 1996). A number of later studies, however, could not confirm this proposal (Plane et al., 1997; Zygmunt et al., 1997, 2000; Chataigneau et al., 1998; Fulton and Quilley, 1998). Furthermore, the vasodilator response to anandamide of isolated rat hepatic and small mesenteric arteries was recently shown to be caused by stimulation of vanilloid receptors on the perivascular sensory nerves, most likely causing the release of vasodilator neuropeptides such as calcitonin gene related peptide (CGRP) (Zygmunt et al., 1999).

In the present study, we investigated the influence of anan-

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ABBREVIATIONS: NO, nitric oxide; EDHF, endothelium-derived hyperpolarizing factor; CB₁, type 1 cannabinoid receptor; L-NNA, N^G-nitro-L-arginine; PMSF, phenylmethylsulfonyl fluoride; CB₂, type 2 cannabinoid receptor; CGRP, calcitonin gene related peptide.

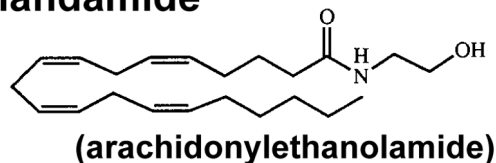
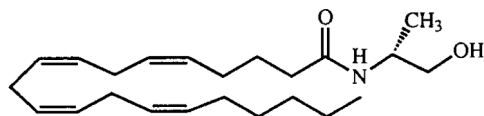
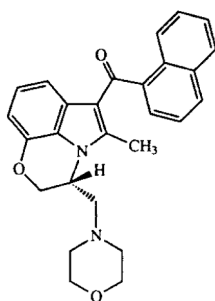
Anandamide**(R+)-Methanandamide****(R+)-WIN 55,212-2**

Fig. 1. Structures of the cannabinoid receptor agonists used in this study.

damide and of its synthetic, stable derivative (Pertwee et al., 1995) methanandamide (Fig. 1) on the resting membrane potential of smooth muscle cells of the main mesenteric artery of the rat, and compared it with the effect of these cannabinoids in small (third to fourth order) daughter branches of these arteries. In addition, we measured the endothelium-dependent hyperpolarization elicited by acetylcholine in the main artery, and investigated the influence of SR141716A on the membrane potential response to this EDHF-liberating vasodilator. In other experiments, we tested the influence of a selective inhibitor of vanilloid receptors, capsazepine, and of prolonged pre-exposure to the vanilloid receptor agonist capsaicin, which causes desensitization and/or neurotransmitter depletion, on cannabinoid-induced hyperpolarizations in small arteries. Also the influence on the resting membrane potential of the structurally unrelated CB₁ receptor agonist WIN 55,212-2 (Fig. 1) was assessed.

Experimental Procedures

Preparations. From 4- to 6-week-old Wistar rats anesthetized with an intraperitoneal injection of a lethal dose (200 mg kg⁻¹) of pentobarbitone, the mesentery was excised and placed in cold normal Krebs-Ringer solution with composition 135 mM NaCl, 5 mM KCl, 20 mM NaHCO₃, 2.5 mM CaCl₂, 1.3 mM MgSO₄·7H₂O, 1.2 mM KH₂PO₄, 0.026 mM EDTA, and 10 mM glucose. This solution was continuously gassed with a 95% O₂, 5% CO₂ gas mixture. The main superior mesenteric artery was dissected free of adherent connective tissue and cut into 4- to 6-mm-long ring segments. For the electrophysiological measurements, a ring was carefully slit along the longitudinal axis, taking care not to injure the endothelium. The opened vascular strip was pinned down, intimal side upward, to the bottom of a small recording chamber kept at 35°C, in which the tissue was

continuously superfused (3 bath volumes min⁻¹) with warmed (35°C) and oxygenated Krebs-Ringer bicarbonate fluid (pH 7.4). In other experiments, third to fourth order branches of the mesenteric artery were selected. Segments of these vessels (length 3–4 mm) were pinned down in the experimental chamber to penetrate from the adventitial side. After mounting and incision at both sides of the impalement site, the preparations were allowed to equilibrate for at least 60 min before starting the microelectrode impalements. At the end of the experiments, small vessels were moved to an automated wire myograph (model 500A; JP Trading, Aarhus, Denmark) to calculate their internal diameter. Two stainless steel wires were guided through the lumen, one was connected to a force transducer and the other fixed to a micrometer. From the passive wall tension-internal circumference characteristics, the mean internal diameter of these vessels at a transmural pressure of 100 mm Hg was calculated according to the method of Mulvany and Halpern (1977). In some experiments, the endothelium was removed from small arteries by rubbing the intimal surface of the vessel with an inserted cat's whisker. All experiments were performed in the presence of high concentrations of nitro-L-arginine and indomethacin to exclude interference from NO and prostanoids, respectively.

Electrophysiological Measurements. Transmembrane potentials were measured as described previously (Vanheel and Van de Voorde, 1997). Briefly, conventional microelectrodes were pulled with a vertical pipette puller (David Kopf, Tujunga, CA) from 1-mm-o.d. filamented glass tubings (Hilgenberg, Malsfeld, Germany). Microelectrodes were filled with 1 M KCl. Their electrical resistance, measured in the normal Krebs-Ringer solution, ranged from 40 to 80 MΩ. The measured potential was followed on an oscilloscope and traced with a pen recorder at low speed. Absolute values of membrane potential were taken as the difference of the stabilized potential after cell impalement and the zero potential upon withdrawal of the microelectrode from the cell. Changes in membrane potential produced by application of acetylcholine, anandamide, or cromakalim in control conditions and after experimental intervention were measured in the same smooth muscle cell from continuous recordings after addition of these agents from the appropriate stock solution. The recorded pen traces were digitized off-line with a digitizing tablet connected to a PC.

Materials. Acetylcholine chloride, indomethacin, N^G-nitro-L-arginine (L-NNA), cromakalim, and capsazepine were obtained from Sigma Chemical Co. (St. Louis, MO). (*E*)-Capsaicin was obtained from Calbiochem (La Jolla, CA). Anandamide was purchased from three different sources: Research Biochemicals International (Natick, MA), ICN Pharmaceuticals (Costa Mesa, CA), and Sigma Chemical Co. *R*-(+)-Methanandamide [*R*-(+)-arachidonyl-1'-hydroxy-2'-propylamide] and *R*-(+)-WIN 55,212-2 mesylate were obtained from Research Biochemicals International. Phenylmethylsulfonyl fluoride (PMSF) was obtained from Fluka Chemie AG (Buchs, Switzerland). SR141716A [*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide HCl] was kindly provided by Research Biochemicals International as part of the Chemical Synthesis Program of the National Institute of Mental Health (contract number N01 MH30003). These substances were added from the appropriate stock solutions a few minutes before use. All concentrations are expressed as final molar concentrations in the superfusion chamber. Acetylcholine was dissolved in 50 mM potassium hydrogen phthalate buffer, pH 4.0. L-NNA was dissolved in water; indomethacin, anandamide, methanandamide, cromakalim, capsazepine, capsaicin, and PMSF in anhydrous ethanol; and SR141716A and WIN 55212-2 in dimethyl sulfoxide.

Statistics. Results are expressed as means ± S.E.M. Statistical significance was evaluated using Student's *t* test for paired or unpaired observations, as appropriate, a *p* value <0.05 indicating a significant difference; *n* indicates the number of preparations, each obtained from a different rat.

Results

Main Mesenteric Arteries. In the continuous presence of L-NNA and indomethacin, the mean resting membrane potential was -51.4 ± 3.1 mV ($n = 75$). Direct application of high concentrations of anandamide (10 – 100 μ M) had only a minor influence on the membrane potential (Fig. 2). With 10 μ M cannabinoid, the mean change in membrane potential was -0.5 ± 1.4 mV. During the same cell impalements, responses to acetylcholine were always observed, indicating that the smooth muscle cells were able to hyperpolarize in response to EDHF (Fig. 2). The application of 1 μ M acetylcholine produced a transient hyperpolarization with a mean amplitude of 19.0 ± 4.3 mV ($n = 8$). A smaller concentration (0.3 μ M) elicited smaller peak hyperpolarizations (Fig. 2), which averaged -13.5 ± 4.4 mV ($n = 43$). In the continuous presence of anandamide, submaximal membrane potential responses to acetylcholine were not significantly influenced (Fig. 2). In four experiments, hyperpolarizations averaged 14.1 ± 2.7 mV in the absence and 12.8 ± 3.0 mV in the presence of the endocannabinoid.

Similar to what was observed with anandamide, application of the more stable derivative of the cannabinoid, *R*-(+)-methanandamide (5 – 10 μ M), produced only minor changes of the resting potential, while during the same cell impalement substantial electrical responses to acetylcholine were observed (Fig. 3). In five experiments, 10 μ M methanandamide changed the membrane potential by -1.0 ± 0.6 mV.

In the next series of experiments, the influence on EDHF-mediated hyperpolarization of cumulative concentrations of the antagonist of the cannabinoid CB_1 receptor SR141716A was assessed. Figure 4 shows traces from two representative experiments. Due to the compressed time scale used to construct the figures from these long-term recordings, acetylcholine-induced hyperpolarizations merely appear as inverted peaks. The application of 1 μ M antagonist did not significantly influence the resting membrane potential. After pre-exposing the vessel strip for at least 10 min to this concentration of SR141716A, the acetylcholine (0.3 μ M)-induced endothelium-dependent hyperpolarization was not signifi-

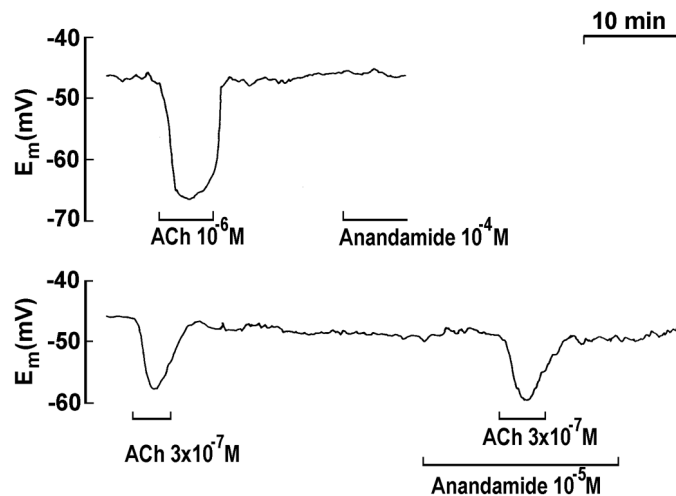


Fig. 2. Influence of anandamide in the main mesenteric artery. Original recordings of the membrane potential (E_m) recorded in a smooth muscle cell from the main mesenteric artery during application of acetylcholine (ACh, 1 or 0.3 μ M) or/and anandamide (10 or 100 μ M). Upper and lower traces from two different experiments.

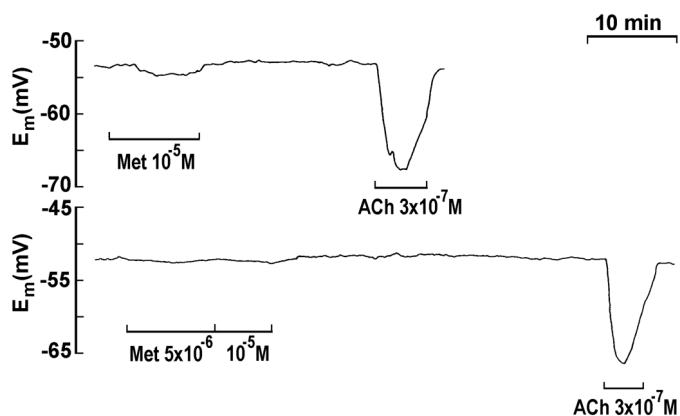


Fig. 3. Influence of methanandamide in the main mesenteric artery. Original traces of the membrane potential (E_m) recorded in smooth muscle cells from resting main mesenteric arteries during application of methanandamide (Met, 5 or 10 μ M) and acetylcholine (ACh, 0.3 μ M).

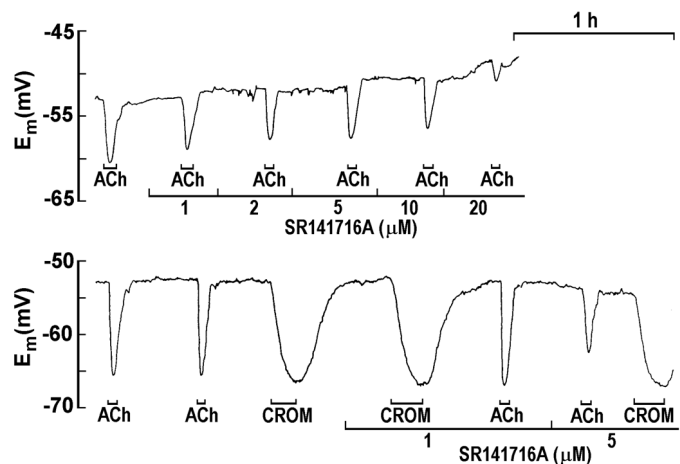


Fig. 4. Influence of SR141716A on membrane potential responses to ACh and cromakalim in the main mesenteric artery. Original long-term recordings of the resting membrane potential (E_m) in a smooth muscle cell of the main mesenteric artery during repeated application of acetylcholine (ACh, 0.3 μ M) (top) or of ACh (0.3 μ M) and cromakalim (CROM, 0.4 μ M) (bottom) in the absence and the presence of increasing micromolar concentrations of SR141716A.

cantly affected. With larger concentrations of the antagonist, however, decreased responses to acetylcholine were observed. In some preparations, 20 μ M the antagonist was necessary to decrease acetylcholine-induced hyperpolarization, whereas in other preparations inhibition was apparent at 5 μ M (Fig. 4). From all experiments ($n = 5$), a statistically significant ($p < 0.02$) inhibition of the mean response to acetylcholine was obtained by 10 μ M SR141716A. Endothelium-independent hyperpolarizations induced by the K_{ATP} channel opener cromakalim were not inhibited by high concentrations of SR141716A (Fig. 4).

Endogenous anandamide is metabolically degraded by an anandamide amidohydrolase enzyme. If acetylcholine would release endothelial anandamide as EDHF, it might be expected that inhibition of anandamide breakdown would increase the endothelium-dependent hyperpolarization elicited by acetylcholine. In another series of experiments, therefore, we investigated the influence of the amidase inhibitor PMSF (Pertwee et al., 1995) on EDHF-induced hyperpolarizations. In this series of experiments, low concentrations of acetylcholine (0.1 μ M) were applied, hyperpolarizing the smooth

muscle cells submaximally by 4.2 ± 1.4 mV. In none of the five preparations, however, an increase of the endothelium-dependent hyperpolarization was observed in the presence of PMSF. Conversely, the mean membrane potential response was significantly ($p < 0.05$) reduced to -2.6 ± 0.4 mV. A typical example is depicted in Fig. 5.

Small Mesenteric Arteries. The small mesenteric arteries used in this study had an average normalized diameter (at a transmural pressure of 100 mm Hg) of 206 ± 14 μ m, as determined in six preparations at the end of the experiments (under *Experimental Procedures*). The mean resting membrane potential of the smooth muscle cells was -64.6 ± 0.7 mV ($n = 27$), significantly ($p < 0.01$) more negative than that of smooth muscle cells of the main artery. The addition of acetylcholine (1 or 3 μ M) hyperpolarized the cells by 7.1 ± 1.1 mV ($n = 7$) or 11.1 ± 0.5 mV ($n = 20$), respectively. Application of anandamide (10 μ M) hyperpolarized the cells by 6.0 ± 0.5 mV ($n = 6$). This membrane potential change occurred substantially slower than the hyperpolarization produced by acetylcholine (Fig. 6). Moreover, the membrane potential recovered very slowly from anandamide exposure (Fig. 6). A second exposure to anandamide induced much smaller changes in membrane potential ($n = 3$; data not shown). In preparations from which the endothelium was removed and which no longer responded to 3 μ M acetylcholine, anandamide induced comparable hyperpolarizations (-6.8 ± 1.7 mV, $n = 4$; Fig. 6).

Similar results were obtained with the more stable analog methanandamide, which displays somewhat higher affinity for the CB₁ receptor. A representative experiment is shown in Fig. 7. In the experiment depicted, the first exposure to methanandamide (10 μ M) hyperpolarized the smooth muscle cell slowly by 5.4 mV, whereas acetylcholine (3 μ M) caused 10.5 mV hyperpolarization. After the second application of the cannabinoid, a transient depolarization was noted before the membrane potential became more negative (to less extent than during the first exposure), whereas acetylcholine still induced fully reproducible hyperpolarizations. Furthermore, as in the main artery (Fig. 5), PMSF decreased acetylcholine responses ($n = 3$). The mean response to a first methanandamide (10 μ M) exposure was 6.1 ± 1.4 mV ($n = 6$). A first exposure to another CB₁ receptor agonist WIN 55,212-2 (Fig. 1), however, failed to significantly change the resting mem-

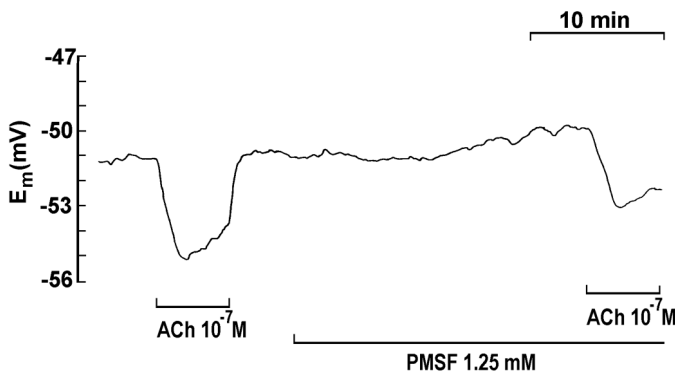


Fig. 5. Influence of inhibition of anandamide hydrolysis on endothelium-dependent hyperpolarization in the main mesenteric artery. Original traces of the membrane potential (E_m) recorded in a smooth muscle cell from the main mesenteric artery during application of a submaximal concentration (0.1 μ M) of acetylcholine (ACh) in the absence and the presence of the amidase inhibitor PMSF (1.25 mM).

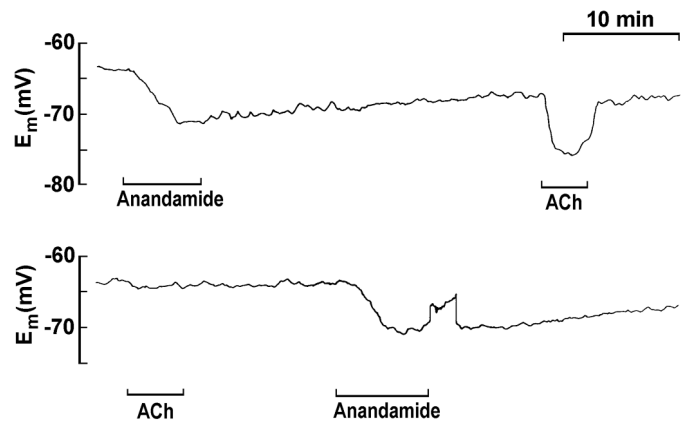


Fig. 6. Influence of anandamide on small mesenteric arteries. Original recordings of the membrane potential (E_m) recorded in smooth muscle cells from small mesenteric arteries during application of anandamide (10 μ M) and acetylcholine (ACh). Top, vessel with intact endothelium, 1 μ M ACh was applied. Bottom, vessel with mechanically damaged endothelium, 3 μ M ACh was applied. Shortly after washout of anandamide, the microelectrode was temporarily partly dislodged from this cell.

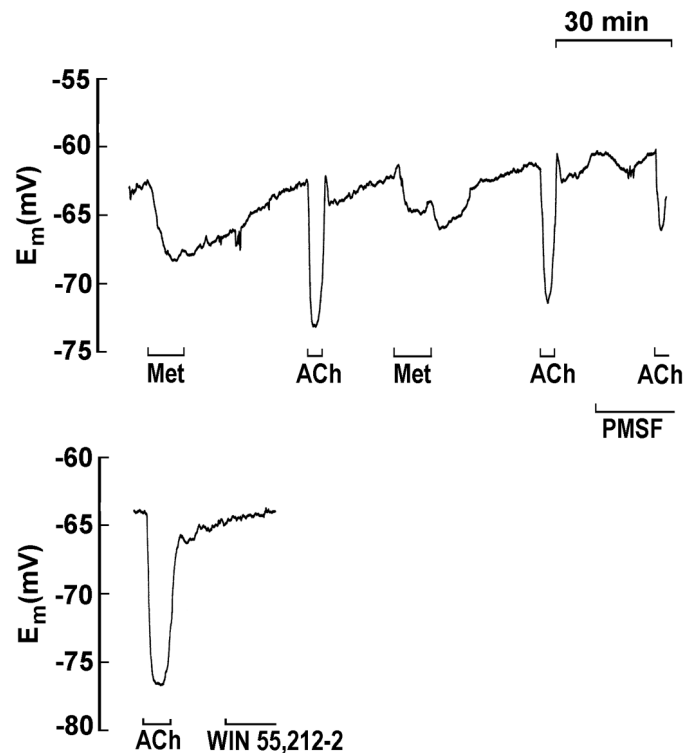


Fig. 7. Influence of methanandamide and WIN 55,212-2 on small mesenteric arteries. Top, original long-term recording of the membrane potential (E_m) recorded in a smooth muscle cell from a small mesenteric artery during repeated applications of methanandamide (Met, 10 μ M) and acetylcholine (ACh, 3 μ M). Near the end of the experiment, the influence of pre-exposure to PMSF (1 mM) was also tested. Bottom, influence of acetylcholine (ACh, 3 μ M) and of WIN 55,212-2 (10 μ M) on the E_m .

brane potential of these arteries ($+0.3 \pm 0.4$ mV, $n = 4$; Fig. 7).

In the next series of experiments, the influence of SR141716A on methanandamide-induced hyperpolarization was tested. Given the small reproducibility of cannabinoid action on the membrane potential, this subset of experiments was performed on vessels never pre-exposed to a cannabinoid before testing its influence in the presence of the antagonist.

After pre-exposure to SR141716A (2 μ M), the hyperpolarization induced by methanandamide was not significantly changed (5.8 ± 1.1 mV, $n = 6$; Fig. 8).

In the next series of experiments, we investigated the influence of capsazepine (3 μ M), a selective vanilloid receptor antagonist, on anandamide- or methanandamide-induced hyperpolarizations in small vessels never pre-exposed to a cannabinoid. Treatment with the vanilloid receptor antagonist depolarized smooth muscle cells by about 3 mV (Fig. 8). In the presence of capsazepine, the anandamide (10 μ M)-induced hyperpolarization was completely abolished ($+0.2 \pm 1.7$ mV, $n = 3$). In the experiment depicted, the cannabinoid even depolarized the smooth muscle cell. In contrast, the acetylcholine-induced hyperpolarization was not influenced ($n = 3$, Fig. 8). Similar findings were obtained with methanandamide. In vessels pre-exposed to capsazepine (3 μ M), methanandamide (10 μ M) significantly ($p < 0.05$) depolarized the cells by 2.1 ± 0.4 mV ($n = 4$).

Finally, pretreatment of small mesenteric arteries for at least 1 h with the vanilloid receptor agonist capsaicin, causing desensitization and/or depletion of sensory neurotransmitter, completely abolished hyperpolarizations induced by 10 μ M methanandamide (-0.1 ± 0.3 mV, $n = 5$) but not those caused by EDHF liberated by acetylcholine (Fig. 8).

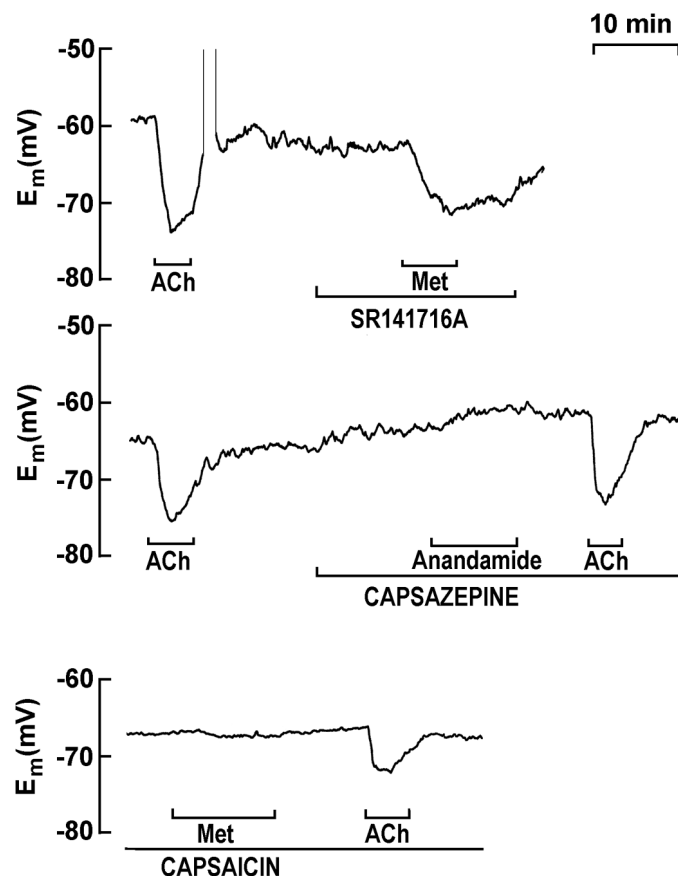


Fig. 8. Influence of SR141716A, capsazepine, and prolonged capsaicin pretreatment on cannabinoid-induced hyperpolarizations. Original recordings of the membrane potential (E_m) recorded in a smooth muscle cell from small mesenteric arteries during application of acetylcholine (ACh, 3 μ M) and methanandamide (Met, 10 μ M) in the presence of SR141716A (2 μ M) (top), of ACh (3 μ M) and anandamide (10 μ M) in the presence of capsazepine (3 μ M) (middle), and of methanandamide (Met, 10 μ M) and ACh (3 μ M) after 60-min pretreatment with capsaicin (10 μ M) (bottom).

Discussion

The main new findings of the present study are that 1) in contrast to their effect in small mesenteric arteries, neither anandamide nor its metabolically more stable analog *R*-(+)-methanandamide produce significant hyperpolarization of smooth muscle cells of the rat main mesenteric artery, whereas the same cells promptly respond to EDHF liberated by acetylcholine; 2) EDHF-mediated membrane potential responses in this artery are unaffected by 1 to 5 μ M of the cannabinoid receptor antagonist SR141716A, and not increased but rather decreased by an inhibitor of anandamide hydrolysis; 3) in small mesenteric arteries, both anandamide and methanandamide-induced hyperpolarizations are, unlike the response to acetylcholine, not affected by endothelium removal; 4) in these arteries, the CB₁ receptor agonist WIN 55,212-2 does not change the resting membrane potential; and 5) in these small vessels, the hyperpolarizations elicited by anandamide (10 μ M) and methanandamide (10 μ M) are totally abolished by pre-exposure to 3 μ M capsazepine or by pretreatment with capsaicin.

In the main mesenteric artery, neither anandamide nor methanandamide significantly influenced the membrane potential of the smooth muscle cells. Since the same cells showed a clear response to EDHF liberated by acetylcholine, these findings directly show that the endocannabinoid cannot act as EDHF in this artery. Further support for this was found in the experiments in which the influence of PMSF on the EDHF-mediated hyperpolarization was tested. The serine protease blocker is known to inhibit anandamide amidase, the enzyme responsible for hydrolysis of anandamide to arachidonic acid and ethanolamine (Deutsch and Chin, 1993; Pertwee et al., 1995) and to potentiate the vascular responses to exogenous anandamide (Pertwee et al., 1995; White and Hiley, 1997; Ishioka and Bukoski, 1999). However, after pre-exposure of main mesenteric arteries to PMSF, no increase of EDHF-mediated hyperpolarization was found. Moreover, in the presence of cannabinoid receptor antagonizing concentrations of SR141716A [K_i is approximately 10 and 700 nM for CB₁ and CB₂, respectively (Pertwee, 1997)], the hyperpolarization induced by acetylcholine was not significantly influenced. The concentration of the antagonist had to be increased to extremely high (unselective) levels to observe significant inhibition. This suggests that EDHF does not act on cannabinoid receptors. In addition, stimulation of these receptors presumably does not influence the resting membrane potential of the smooth muscle cells, as indicated both by the absence of any significant change upon application of another type of CB₁ receptor agonist, WIN 55,212-2, and by the lack of influence of SR141716A on methanandamide-induced hyperpolarization as observed in the smaller mesenteric arteries. Actually, in various cells both CB₁ and CB₂ receptor stimulation is known to be coupled via pertussis toxin-sensitive G-proteins to decreases in cellular cAMP levels (Childers and Deadwyler, 1996). In several vascular preparations, however, the reverse change in intracellular cAMP concentration is often associated with smooth muscle cell hyperpolarization, as indicated by the hyperpolarizing influence of dibutyryl-cAMP and of the stable analog of prostacyclin iloprost (Parkington et al., 1993).

In small mesenteric arteries of the rat, anandamide was shown to cause repolarization of precontracted (Plane et al.,

1997) and hyperpolarization of resting vessels (Chataigneau et al., 1998). In resting preparations, this membrane potential change was reported to be endothelium-dependent and sensitive to glibenclamide (Chataigneau et al., 1998). Since the EDHF-mediated hyperpolarization induced by acetylcholine in most vessels is not affected by the K_{ATP} channel inhibitor but is sensitive to the combined application of the K^+ channel blockers charybdotoxin and apamin (Corriu et al., 1996; Chen and Cheung, 1997), this observation ruled out anandamide as EDHF in the arteries (Chataigneau et al., 1998). Similar indications were obtained in tension studies, in which a differential sensitivity of anandamide- and EDHF-induced vasorelaxation to charybdotoxin (Plane et al., 1997) or to the charybdotoxin and apamin combination (White and Hiley, 1997; Zygmunt et al., 1997) was found. Recently, it was shown that the anandamide-induced inhibition of phenylephrine- or prostaglandin $F_{2\alpha}$ -induced contraction of isolated rat hepatic and mesenteric arteries was caused by activation of perivascular nerve vanilloid receptors, releasing vasodilator neuropeptides such as CGRP (Zygmunt et al., 1999). Indeed, the vasorelaxation was abolished by capsaicin-pretreatment, which causes desensitization and/or neurotransmitter depletion in perivascular sensory nerves, and was blocked by the vanilloid receptor antagonist capsazepine. In the present membrane potential measurements, we found that the hyperpolarization of the smooth muscle cells of rat small mesenteric arteries induced by $10 \mu\text{M}$ anandamide was totally unaffected by endothelium removal and was completely inhibited by capsazepine ($3 \mu\text{M}$). This further supports the involvement of activation of perivascular nerve vanilloid receptors by the endocannabinoid, as shown in tension measurements (Zygmunt et al., 1999). Capsazepine pre-exposure did not affect the acetylcholine-induced endothelium-dependent hyperpolarization. Also the hyperpolarizing influence of the stable analog methanandamide, which in control conditions was similar to that of anandamide, was fully blocked by capsazepine and by pretreatment with capsaicin, suggesting that this structurally closely related substance might similarly activate perivascular sensory nerves. The small depolarization observed by application of the cannabinoids in the presence of capsazepine might reflect their direct inhibitory action on the K_v current, as recently shown in freshly isolated vascular smooth muscle cells (Van den Bossche and Vanheel, 2000). The lack of influence of mechanical endothelium removal on cannabinoid-induced smooth muscle cell hyperpolarization in rat small mesenteric arteries, as observed in the present study, agrees with the largely endothelium-independent nature of the vasodilation they produce in this preparation, as reported in several studies (Randall et al., 1996; White and Hiley, 1997; Ishioka and Bukoski, 1999; Wagner et al., 1999). It contrast, however, with previous membrane potential measurements in rat small mesenteric (Chataigneau et al., 1998) and hepatic arteries (Zygmunt et al., 1997). Eventually, this might be related to differences in the method of endothelial cell denudation, implying that a short perfusion of isolated vessels with saponin or distilled water might in some cases perhaps also destruct some perivascular nerves.

In the present study, the different nature of EDHF and anandamide is further indicated by the dissimilar membrane potential responses to acetylcholine and to the cannabinoids in main and in small arteries. Thus, whereas smooth muscle

cells of small arteries slowly (and not reproducibly) hyperpolarize in response to anandamide or methanandamide, their membrane potential changes considerably less to $1 \mu\text{M}$ acetylcholine than does that of the smooth muscle cells of the main mesenteric artery, which fail to hyperpolarize in response to the cannabinoids. Several explanations are possible for the lack of influence of anandamide and methanandamide in the main mesenteric artery. Apart from differences in density of perivascular sensory innervation between the main mesenteric artery and its daughter branches (Ralevic et al., 1996), regional differences in vanilloid receptor density, in quantity or in nature of the released neuropeptides and/or of the smooth muscle cell receptors, or the smooth muscle cell electrophysiological responses to these substances might all contribute.

In summary, we have shown that in the resting main mesenteric artery of the rat, in which the generation of NO and prostanoids was inhibited, anandamide and methanandamide cause only negligible changes of the membrane potential of the smooth muscle cells, unlike the EDHF liberated by acetylcholine. In addition, neither cannabinoid nor vanilloid receptors are involved in hyperpolarizations mediated by this EDHF. These findings provide additional evidence that anandamide and EDHF are two different substances. Moreover, this work has demonstrated substantial heterogeneity in the response to anandamide and methanandamide between the main mesenteric artery and its daughter branches. In the latter, hyperpolarizations insensitive to SR141716A and to endothelium removal but sensitive to a low concentration of capsazepine and to pretreatment with capsaicin support the involvement of perivascular sensory nerves, at least in the membrane electrical response to these cannabinoids. The contribution of this endothelium- and CB_1 receptor-independent hyperpolarization to the vasorelaxation that these compounds elicit, however, remains to be determined.

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