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N-arachidonylethanolamide relaxation of bovine coronary artery is not mediated by CB1 cannabinoid receptor

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Pratt, Phillip F., Cecilia J. Hillard, William S. Edgemond, and William B. Campbell. *N*-arachidonylethanolamide relaxation of bovine coronary artery is not mediated by CB1 cannabinoid receptor. *Am. J. Physiol.* 274 (*Heart Circ. Physiol.* 43): H375–H381, 1998.—It has been reported that the endogenous cannabinoid *N*-arachidonylethanolamide (AEA), commonly referred to as anandamide, has the characteristics of an endothelium-derived hyperpolarizing factor in rat mesenteric artery. We have carried out studies to determine whether AEA affects coronary vascular tone. The vasorelaxant effects of AEA were determined in isolated bovine coronary artery rings precontracted with U-46619 (3×10^{-9} M). AEA decreased isometric tension, producing a maximal relaxation of 51 ± 9% at a concentration of 10^{-5} M. Endothelium-denuded coronary arteries were not significantly affected by AEA. The CB1 receptor antagonist SR-141716A (10^{-6} M) failed to reduce the vasodilatory effects of AEA, suggesting that the CB1 receptor is not involved in this action of AEA. Because AEA is rapidly converted to arachidonic acid and ethanolamine in brain and liver by a fatty acid amide hydrolase (FAAH), we hypothesized that the vasodilatory effect of AEA results from its hydrolysis to arachidonic acid followed by enzymatic conversion to vasodilatory eicosanoids. In support of this hypothesis, bovine coronary arteries incubated with [^{3}H]AEA for 30 min hydrolyzed 15% of added substrate; ~9% of the radiolabeled product was free arachidonic acid, and 6% comigrated with the prostaglandins (PGs) and epoxyeicosatrienoic acids (EETs). A similar result was obtained in cultured bovine coronary endothelial cells. Inhibition of the FAAH with diazomethylarachidonyl ketone blocked both the metabolism of [^{3}H]AEA and the relaxations to AEA. Whole vessel and cultured endothelial cells prelabeled with [^{3}H]arachidonic acid synthesized [^{3}H]PGs and [^{3}H]EETs, but not [^{3}H]AEA, in response to A-23187. Furthermore, SR-141716A attenuated A-23187-stimulated release of [^{3}H]arachidonic acid, suggesting that it may have actions other than inhibition of CB1 receptor. These experiments suggest that AEA produces endothelium-dependent vasorelaxation as a result of its catabolism to arachidonic acid followed by conversion to vasodilatory eicosanoids such as prostacyclin or the EETs.

coronary circulation; eicosanoids; endothelial cells; epoxyeicosatrienoic acids; prostaglandins; fatty acid amide hydrolase; endothelium-derived hyperpolarizing factor; *N*-acylethanolamines

ACETYLCHOLINE, BRADYKININ, and A-23187 hyperpolarize and relax vascular smooth muscle by stimulating the synthesis and release of multiple endothelium-derived relaxing factors (6, 23, 24, 37). Both prostacyclin, a cyclooxygenase metabolite of arachidonic acid, and nitric oxide, a free radical formed from L-arginine (25), are potent endothelium-derived vasodilators. Additionally, evidence has accumulated that suggests that a third factor, apart from nitric oxide and prostacyclin, also contributes to vascular smooth muscle relaxation by hyperpolarizing the smooth muscle membrane (3, 9, 16, 21, 22, 35). This substance has been termed endothelium-derived hyperpolarizing factor (EDHF) (36) and in the broadest terms represents the remaining agonist-induced, endothelium-dependent hyperpolarization and vasorelaxation after complete inhibition of nitric oxide and prostacyclin synthesis and release.

The identity of EDHF may vary with the vascular bed. The majority of evidence in the coronary, mesenteric, and renal circulation suggests that epoxyeicosatrienoic acids (EETs), which are cytochrome *P*-450 metabolites of arachidonic acid, share the characteristics of EDHF (2, 4, 18, 27). We have reported that methacholine-induced relaxations and hyperpolarizations are blocked by inhibitors of cytochrome *P*-450, methacholine releases EETs from the coronary artery, and EETs relax, hyperpolarize, and open calcium-activated potassium channels in coronary arteries (4). Similarly, Chen and Cheung (5) demonstrated that acetylcholine-induced hyperpolarizations of rat mesenteric arteries were attenuated by the cytochrome *P*-450 inhibitor clotrimazole. In addition, acetylcholine-induced hyperpolarizations were significantly blocked in rats treated with cobalt chloride, a depleter of cytochrome *P*-450 enzymes. However, some preparations, such as guinea pig carotid artery, retain significant endothelium-dependent hyperpolarization after blockade by inhibitors of cyclooxygenase, nitric oxide synthase, and cytochrome *P*-450 epoxygenase (8). Therefore, it is possible that more than one EDHF exists.

Recently, Randall and co-workers (29) suggested that arachidonylethanolamide (AEA), also referred to as anandamide, may represent an EDHF in rat superior mesenteric arteries. AEA was originally described as an endogenous ligand for the cannabinoid receptor (13). Randall and co-workers reported that SR-141716A, an antagonist for the neuronal cannabinoid (CB1) receptor (14), attenuated the vasodilatory responses to the

endothelium-dependent vasodilators carbachol and A-23187 but had no effect on vasodilation produced by a nitric oxide donor or potassium-channel opener, which are endothelium-independent vasodilators. AEA induced vasodilation of mesenteric artery, an effect that was blocked by high potassium and SR-141716A but was unaffected by removal of endothelium. These results led them to suggest that AEA is an EDHF in rat mesenteric arteries.

In previous studies, we have isolated and identified all of the major metabolites of arachidonic acid in endothelial cells and isolated vessels (30–33). AEA synthesis was not detected in any of these studies. Additionally, in vessels prelabeled with [^{3}H]arachidonic acid, methacholine failed to stimulate [^{3}H]AEA release. These results are in opposition to the hypothesis that AEA is an endogenous endothelial mediator. To clarify this issue, we have examined the relaxant effects of AEA on bovine coronary artery rings to determine whether AEA was an EDHF in this preparation.

MATERIALS AND METHODS

Measurement of Vascular Reactivity

Coronary artery rings (2- to 3-mm length) were used to study the effects of AEA and synthetic EETs as described by Rosolowsky et al. (32). Briefly, coronary artery rings (1- to 2-mm diameter) were suspended horizontally between two stainless steel hooks in individual organ chambers with Krebs solution containing (in mM) 119 NaCl, 5 KCl, 24 $NaHCO_3$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 11 glucose, 0.02 EDTA, and 1.8 $CaCl_2$ at 37°C, and bubbled with 95% O_2-5% CO_2. In some cases, the endothelium was deliberately removed by physical rubbing (denuded vessels). The rings were equilibrated for 90 min under a resting tension of 2 g. After equilibration, the rings were challenged with KCl (4×10^{-2} M) until reproducible contractions were elicited. The vessels were washed between exposures to KCl and were allowed to return to baseline tension before the next addition of KCl. The thromboxane mimetic U-46619 (3×10^{-9} M) was used to contract the vessels to 50–80% of the maximal KCl-induced contraction. Indomethacin (10^{-5} M), SKF-525a (10^{-5} M), SR-141716A (10^{-6} M) or diazomethylarachidonyl ketone (DAK; 2.5×10^{-5} M) were given 20 min before precontraction with U-46619, and effects on basal tone were measured. After the contractions to U-46619 were stable (typically 20 min), AEA or 5,6-EET was added in increasing concentrations to the organ chamber and maximal relaxation was measured 5 min after the addition of each concentration of drug. Results are expressed as percent relaxation relative to the U-46619-induced contraction.

Endothelial Cell Culture

Endothelial cells were cultured according to a previously described method (31). Briefly, bovine hearts were obtained from the local slaughterhouse, and the large, epicardial left circumflex and anterior descending coronary arteries were dissected free from adherent fat and connective tissues. The vessels were rinsed with 5% fetal bovine serum in medium 199 containing 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) with a 1% solution of antibiotics (penicillin-streptomycin-amphotericin B), 0.3% gentamycin, and 0.3% nystatin. The vessels were cut into segments, and the lumen was filled with 0.4% collagenase in medium 199. After 30 min of incubation at 37°C, the vessels were flushed with medium 199, and the detached endothelial cells were collected in a sterile tube. The cells were sedimented by centrifugation, resuspended in RPMI 1640 with 25% fetal calf serum, 1% antibiotic solution, 0.3% nystatin, 0.3% gentamycin, 1% glutamine, and 0.1% tylosin, and plated in 25-cm^2 culture flasks. Cell cultures were incubated at 37°C in an atmosphere of 95% air-5% CO_2. Endothelial cells were identified by morphological appearance (i.e., cobblestone array) and by expression of angiotensin I-converting enzyme activity, acetylated low-density lipoprotein uptake, and positive staining for von Willebrand factor antigen. Culture medium was replaced every 3 days and/or the day before experiments were to be performed. On reaching confluence (7–10 days), cells were subcultured by detaching with 0.075% trypsin in Puck's-EDTA solution and replated in 20% fetal calf serum and RPMI 1640 with antibiotics. Experiments were performed on endothelial cells between *passages 2* and *4* and under subconfluent conditions.

Prelabeling of Cellular Lipids With [^{3}H]arachidonic Acid

Subconfluent monolayers of cultured bovine coronary artery endothelial cells (BCAECs) were labeled with [^{3}H]arachidonic acid according to a previously described method with minor modifications (1). Briefly, monolayers of BCAECs were incubated for 3 h at 37°C in RPMI 1640 with antibiotics containing 5% fetal calf serum and 0.5 μCi of [^{3}H]arachidonic acid in an atmosphere of 95% air-5% CO_2. At the end of the 3-h incubation, the final serum concentration was adjusted to 20%, and the cells were incubated overnight. The next day, the medium was removed, and the cells were washed twice with HEPES buffer containing fatty acid-free bovine serum albumin (2 mg/ml). The cells were then washed twice with protein-free HEPES and stimulated for 30 min with vehicle, A-23187 (5×10^{-6} M), or histamine (10^{-6} M). At the end of the incubation the medium was removed, purged with nitrogen, and frozen at −40°C until solid-phase extraction and reverse-phase high-performance liquid chromatography (HPLC) analysis were performed (see *Preparation of Samples for Reverse-Phase HPLC*).

Metabolism of [^{3}H]AEA by Bovine Coronary Arteries and Cultured Endothelial Cells

Bovine hearts were obtained from the local slaughterhouse, and the left anterior descending coronary artery was dissected from the heart. The vessels were cut into rings of ~3- to 4-mm width and were placed in prewarmed (37°C) HEPES buffer containing (in mM) 10 HEPES, 149 NaCl, 5 KCl, 1.8 $CaCl_2$, 1.0 $MgCl_2$, and 5.5 glucose. [^{3}H]AEA (1.0 μCi) was then added, and the incubation was continued for 30 min. Similar experiments were performed with subconfluent monolayers of cultured BCAECs. DAK (2.5×10^{-5} M) was added 30 min before the addition of [^{3}H]AEA to investigate the effects of the fatty acid amide hydrolase (FAAH) inhibitor on [^{3}H]AEA metabolism by whole vessels or cultured cells. At the end of the incubation period, the incubation medium was decanted, extracted, and analyzed by HPLC as described in *Reverse-phase HPLC*.

Preparation of Samples for Reverse-Phase HPLC

Solid-phase extraction. The samples were acidified to pH 3 with glacial acetic acid and adjusted with ethanol to a final concentration of 15%. The lipids were then extracted from the samples using an octadecylsilyl extraction column (Analytichem) using the following method. The columns were washed with 5 ml of ethanol followed by 20 ml of distilled H_2O. The sample was applied to the column followed by a

wash with 15% ethanol in H_2O and 20 ml of distilled H_2O. Ethyl acetate (6 ml) was applied to the column, and the eluate was collected and dried under nitrogen in preparation for reverse-phase HPLC.

Reverse-phase HPLC. The extracts were redissolved in 100 µl acetonitrile containing 0.1% glacial acetic acid and 100 µl distilled H_2O, and the radiolabeled products were resolved on a Nucleosil C_{18} reverse-phase column (5 µm, 4.5 × 250 mm, Phenomenex). *Solvent A* is distilled H_2O and *solvent B* is acetonitrile containing 0.1% glacial acetic acid. A linear gradient from 50% *solvent B* in *solvent A* to 100% *solvent B* over 40 min was used at a flow rate of 1 ml/min. The column effluent was collected in 0.2-ml fractions and analyzed for radioactivity by liquid scintillation spectrometry.

Labeling of Bovine Coronary Arteries With [^{3}H]ethanolamine for Determination of Synthesis of Endogenous AEA

Bovine coronary arteries were incubated with 1 µCi of [^{3}H]ethanolamine hydrochloride for 1 h at 37°C in HEPES buffer. The vessels were then incubated for 30 min with vehicle or A-23187 (5×10^{-6} M). The tissue was removed and weighed, and the buffer was saved for later solid-phase extraction of ^{3}H-labeled metabolites as described in *Solid-phase extraction*. ^{3}H-labeled metabolites were eluted from the column, dried under N_2, and analyzed by reverse-phase HPLC.

Thin-Layer Chromatographic Separation of AEA, EETs, and Arachidonic Acid

Arachidonic acid, AEA, and EETs were subjected to thin-layer chromatography as previously described (29). Briefly, arachidonic acid, AEA, and EET standards were spotted onto silica gel thin-layer chromatography plates (Whatman) and developed using a mobile phase of 95% methylene chloride-5% methanol and visualized with iodine.

Measurement of FAAH Activity in Bovine Coronary Artery Membranes

Coronary arteries were isolated, washed, and homogenized in tris(hydroxymethyl)aminomethane (Tris) buffer (50 mM Tris·HCl, 1.0 mM EDTA, and 3.0 mM $MgCl_2$, pH 7.4). Membranes were isolated by centrifugation at 12,000 *g* for 20 min. FAAH activity was determined in the membranes using a modification of previously described methods (20). Membranes were incubated in 0.5 ml of Tris buffer containing 1 mg/ml fatty acid-free bovine serum albumin and 9 nCi of [^{14}C]AEA labeled in the ethanolamine portion of the molecule. Incubations were stopped with the addition of 2 ml chloroform-methanol (1:2) followed by intermittent vortexing. After standing at room temperature for 30 min, the phases were separated with the addition of 0.67 ml of chloroform and 0.6 ml of water. After centrifugation at 1,000 revolutions/min for 10 min, the amount of ^{14}C in 1 ml each of the aqueous and organic phases was determined using liquid scintillation counting. The radioactivity in the aqueous phase was [^{14}C]-ethanolamine, whereas [^{14}C]AEA remained in the organic phase.

Materials

[^{3}H]AEA (221.0 Ci/mmol) and [^{3}H]arachidonic acid (211.8 Ci/mmol) were purchased from E.I. DuPont de Nemours (Boston, MA). [1-^{3}H]ethan-1-ol-2-amine hydrochloride (28 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). Arachidonyl [1′,2′-^{14}C]ethanolamide was the generous gift of Dr. David Ahern (NEN, Boston, MA). AEA (50 mg/ml in ethanol) was purchased from Cayman Chemical (Ann Arbor, MI) and was diluted in ethanol before addition to the organ chamber. 5,6-EET was synthesized according the method described by Corey et al. (see Refs. 4, 7). SR-141716A was generously donated by Sanofi (Montpellier, France). DAK was synthesized in our laboratory as described (23a). Briefly, arachidonic acid was dissolved in dry methylene chloride under N_2. The solution was cooled to 0°C and 5 molar equivalents of oxyalyl chloride, in methylene chloride, were added slowly. The reaction mixture was warmed to 25°C and stirred for 1 h. The reaction mixture was dried under N_2, and the residue was cooled to 0°C before the addition of ethereal diazomethane. The reaction was stirred for 1 h at 0°C, and the crude DAK was purified by normal-phase HPLC. Indomethacin and all reagents for buffers were obtained from Sigma Chemical (St. Louis, MO).

Statistical Analyses

Vessels were randomly assigned to a treatment group with at least one vessel each day serving as a control. The summarized data were analyzed by analysis of variance followed by Dunnett's modification of the *t*-test (see Ref. 38).

RESULTS

Figure 1 illustrates the effects of AEA on isometric tension in endothelium-intact and -denuded rings of precontracted bovine coronary arteries. AEA produced concentration-dependent relaxations in vessels with an intact endothelium [half-maximum effective concentration (EC_{50}) of 10^{-7} M]. Removal of the endothelium greatly reduced the relaxations to AEA except at the highest concentration tested (Fig. 1*B*). Cumulative additions of the ethanol vehicle were without effect. Therefore, AEA produces endothelium-dependent relaxations of bovine coronary arteries at concentrations between 10^{-9} and 10^{-6} M. To address the possible involvement of the cannabinoid CB1 receptor in mediating AEA-induced relaxations, vessels were pretreated for 20 min with SR-141716A (10^{-6} M), a CB1 receptor antagonist. SR-141716A was without effect on basal tone before the addition of U-46619 and failed to block AEA-induced relaxations of bovine coronary arteries (Fig. 1, *A* and *B*). 5,6-EET also relaxed the precontracted vessels, and its effect was also not blocked by SR-141716A (data not shown). Therefore, it appears that the cannabinoid receptor does not mediate the relaxations of coronary vessels to AEA.

AEA is hydrolyzed by FAAH in several tissues, including brain and liver, resulting in the production of arachidonic acid and ethanolamine (12, 20). We investigated the possibility that AEA hydrolysis to arachidonic acid could explain AEA-induced relaxations. Because arachidonic acid is easily converted by vascular tissue to PGI_2 and EETs (28, 32), we hypothesized that AEA-induced relaxation was caused by its conversion to arachidonate metabolites. In previous studies (32), we found that the combination of indomethacin and SKF-525a, to block both cyclooxygenase and cytochrome *P*-450 completely, inhibited arachidonic acid-induced relaxations. Pretreatment of vessels with indomethacin and SKF-525a blocked AEA-induced relaxations (Fig. 1*B*). Furthermore, AEA had no effect on contrac-

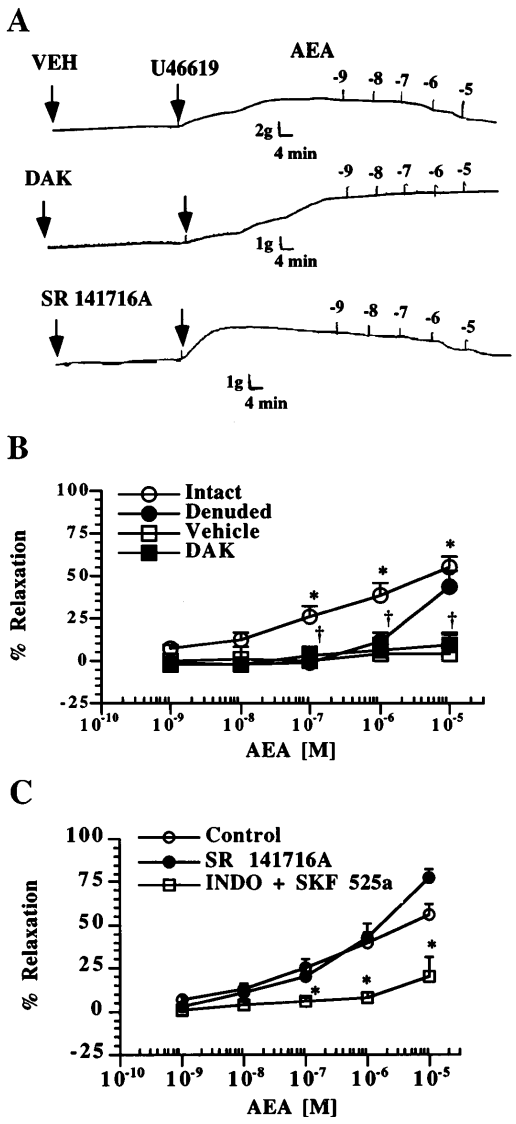

Fig. 1. Effects of *N*-arachidonylethanolamide (AEA) on vascular tone in bovine coronary arteries. *A*: original tracings of intact bovine coronary arteries pretreated with vehicle (VEH), diazomethylarachidonyl ketone (DAK), or SR-141716A before addition of U-46619 to induce contraction. *B*: rings of coronary artery, intact and denuded, were pretreated with vehicle (0.02% ethanol) or DAK (2.5×10^{-5} M) for 30 min before addition of U-46619 (3×10^{-9} M) to induce contraction. Once contractions were stable, increasing concentrations of AEA or its vehicle were added to organ chamber and maximal relaxations were recorded. Data are presented as percent relaxation (means ± SE); n = 4–15/group. * Significantly different from vehicle and DAK groups, $P < 0.05$; † significantly different from intact group, $P < 0.05$. *C*: rings of coronary artery were pretreated with SR-141716A (10^{-6} M) or indomethacin (INDO, 10^{-5} M) in combination with SKF-525a (10^{-5} M) for 20 min before addition of U-46619. Once contractions were stable, AEA was added in increasing concentrations to organ chambers and maximal relaxations were recorded; n = 3–6/group.* Significantly different from control, $P < 0.05$.

tions when the vessels were pretreated with an FAAH inhibitor, DAK (Ref. 23a; Fig. 1*A*). The effects of DAK on AEA-induced relaxations are specific, because sodium nitroprusside-induced relaxations were unimpaired by pretreatment with the same concentration of the FAAH inhibitor (EC_{50} 10^{-7} M, control, vs. 10^{-7} M, DAK treated). Indeed, membrane fractions obtained from bovine coronary artery exhibit protein- and time-dependent catabolism of AEA (Fig. 2*A*), which is completely inhibited by DAK (Fig. 2*B*). These results are consistent with the presence of FAAH in bovine coronary arteries.

To identify the metabolites of AEA, strips of bovine coronary artery were incubated with [^{3}H]AEA for 30 min, the lipids were extracted, and the extract was analyzed for ^{3}H-labeled metabolites by reverse-phase HPLC. Figure 3 illustrates that [^{3}H]AEA migrates at a retention time of 26.3 min on reverse-phase HPLC. There are no detectable peaks other than the [^{3}H]AEA in the incubation without vessels (Fig. 3*A*); however, in the presence of vessels, [^{3}H]AEA is converted to [^{3}H]arachidonic acid (retention time = 34 min), to the

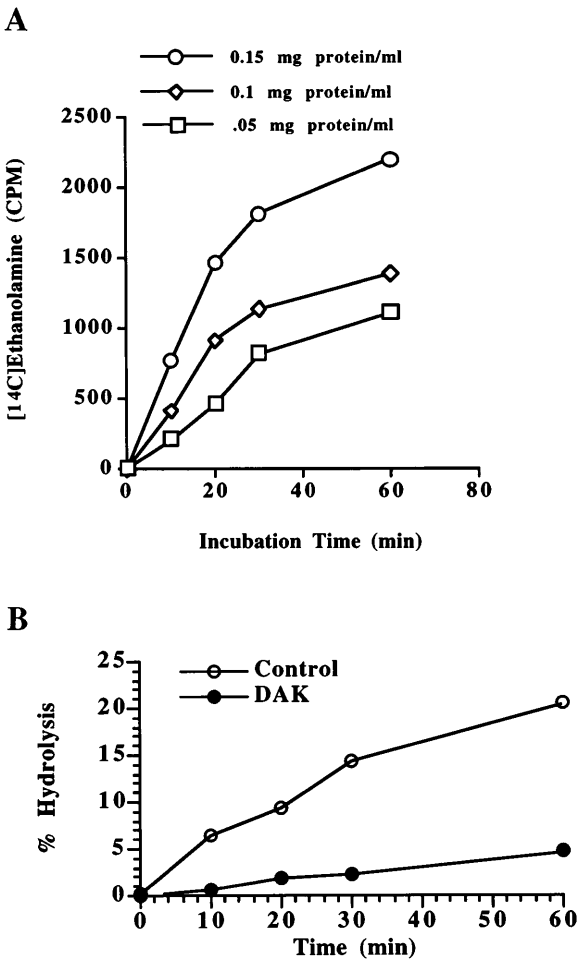

Fig. 2. Membranes from bovine coronary artery hydrolyze AEA to arachidonic acid (AA) and ethanolamine. A crude membrane fraction was isolated and incubated with [^{14}C]AEA for various times and at various protein levels (*A*) or in presence of fatty acid amide hydrolase (FAAH) inhibitor DAK (*B*; 2.5×10^{-5} M). Radiolabeled AEA and ethanolamine were separated by liquid-liquid extraction, and radioactivity of each was determined using liquid scintillation counting. *A*: data are expressed as counts per minute (cpm) of [^{14}C]ethanolamine. *B*: data are expressed as percent hydrolysis, which was calculated as 100 × cpm in aqueous phase divided by sum of cpm in aqueous phase + organic phase. All data are means from a single experiment carried out in triplicate.

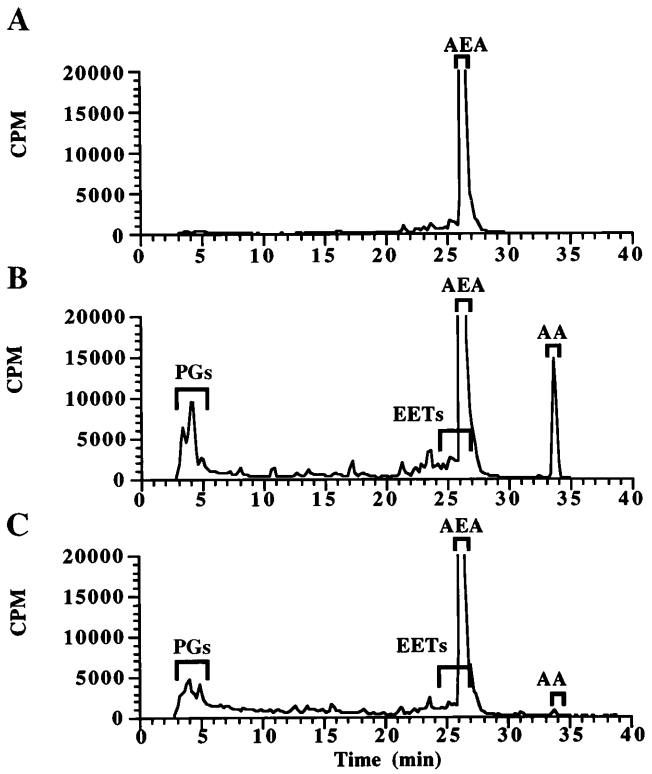

Fig. 3. Metabolism of [^{3}H]AEA by bovine coronary arteries. *A*: tissue-free control for nonenzymatic degradation of [^{3}H]AEA. Strips of coronary artery (300–700 mg wet wt) were pretreated with vehicle (*B*) or DAK (2.5×10^{-5} M; *C*) for 30 min in HEPES buffer before addition of 0.5 μCi of [^{3}H]AEA (10^{-6} M), and incubation was continued for 30 min at 37°C. Incubate was extracted, and metabolites were resolved by reverse-phase high-performance liquid chromatography (HPLC). Migration times of known standards are shown above chromatogram. EETs, epoxyeicosatrienoic acids; PGs, prostaglandins.

cyclooxygenase-derived [^{3}H]PGs (retention time = 3–11 min), and to [^{3}H]EETs (retention time = 24–28 min) (Fig. 3*B*). Similar results were obtained for BCAECs (data not shown). Pretreatment of vessels with DAK (2.5×10^{-5} M) prevented the hydrolysis of [^{3}H]AEA (Fig. 3*C*). Thus the vasorelaxant effects of AEA can be explained by its conversion to arachidonic acid and its subsequent metabolism to vasodilatory PGs and/or EETs. It is not known at this time whether hydrolysis is limited to the endothelium or whether smooth muscle may also contribute to hydrolysis of AEA.

An important criterion for the identification of an endothelium-derived vasoactive factor is the demonstration that the factor is synthesized by endothelial cells. Endothelial cells, prelabeled with [^{3}H]arachidonic acid, were stimulated with vehicle, the calcium ionophore A-23187, or histamine. The lipids were extracted, and the radiolabeled products were resolved by reverse-phase HPLC (Fig. 4). 14,15-[^{14}C]EET and 11,12-[^{14}C]-EET migrated with a retention time of 24.8 and 25.8 min, respectively, whereas [^{3}H]AEA migrated with a retention time of 26.3 min. Under nonstimulated or basal conditions, only a small amount of radiolabeled product was detected, which comigrated with the arachidonic acid standard (Fig. 4*A*). A-23187 stimulated the production of radiolabeled products that comigrated with [^{3}H]PGs, [^{3}H]EETs, and [^{3}H]arachidonic acid but none that comigrated with [^{3}H]AEA (Fig. 4, *B* and *C*). Similar results were obtained when endothelial cells were stimulated with 10^{-6} M histamine (data not shown). Interestingly, pretreatment of [^{3}H]arachidonic acid-labeled cells with SR-141716A (10^{-6} M) resulted in less [^{3}H]arachidonic acid released when stimulated with A-23187 than control cells (Fig. 4*C* vs. Fig. 4*B*). Additionally, when vessels were prelabeled with [^{3}H]-ethanolamine hydrochloride and subsequently treated with A-23187, no radiolabeled products comigrating with the [^{3}H]AEA standard were detected when analyzed by reverse-phase HPLC (Fig. 5). Finally, with the use of the same thin-layer chromatography system as reported by Randall and co-workers (29), we were unable to resolve the EETs [relative migration (R_f) = 0.34] from AEA (R_f = 0.34) but were able to separate arachidonic acid (R_f = 0.46) from the EETs and AEA (data not shown). From these data it appears that the PGs and EETs mediate the endothelium-dependent relaxations of bovine coronary arteries induced by AEA and that AEA is not synthesized by endothelial cells.

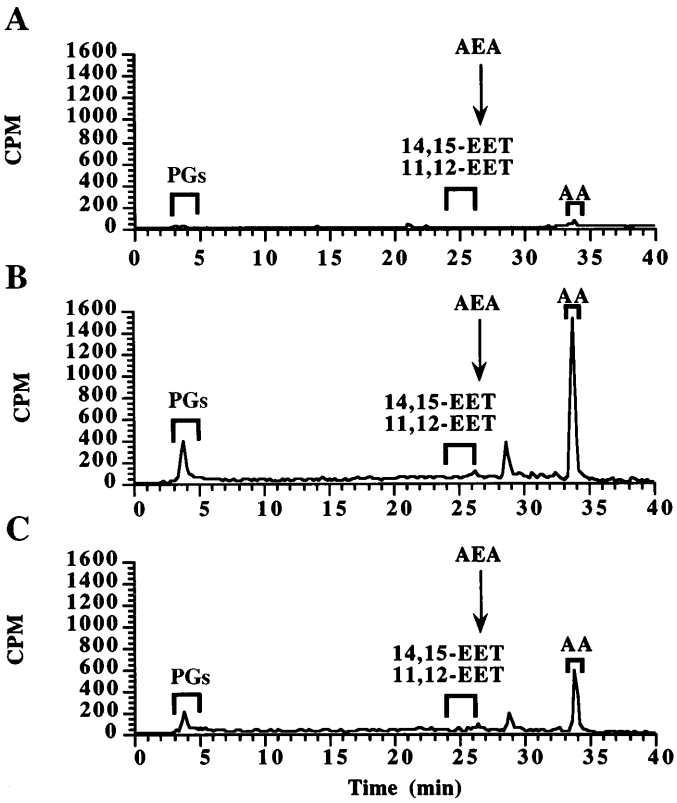

Fig. 4. Effects of SR-141716A on A-23187-stimulated release of [^{3}H]AA metabolites from coronary endothelial cells. Endothelial cells, prelabeled with [^{3}H]AA, were pretreated with vehicle (*B*) or SR-141716A (*C*) for 20 min at 37°C. Cells were stimulated with vehicle (*A*) or A-23187 (*B*, *C*) for 30 min at 37°C. At end of incubation, medium was removed, extracted, and analyzed by reverse-phase HPLC. Migration times of standards are shown above chromatogram.

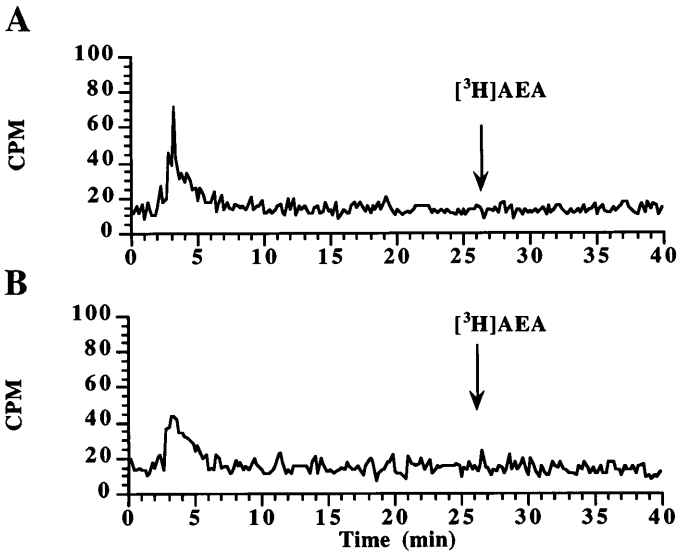

Fig. 5. Bovine coronary arteries fail to synthesize [^{3}H]AEA. Bovine coronary arteries were labeled with [^{3}H]ethanolamine hydrochloride for 1 h at 37°C. Vessels were then treated for 30 min with vehicle (0.3% ethanol) or A-23187 (5 μM). Incubation was terminated by removing tissue and freezing buffer at −40°C for later solid-phase extraction and reverse-phase HPLC analysis. Arrow indicates migration time of [^{3}H]AEA standard (26.3 min).

DISCUSSION

In the present study, we examined the effects of AEA on relaxations of precontracted bovine coronary arteries because AEA has been suggested to have the characteristics of an endothelium-dependent hyperpolarizing factor (29). Although we find that AEA elicits endothelium-dependent relaxation, our data do not support the hypothesis that AEA itself is an EDHF. AEA-induced vasodilation is not inhibited by the CB1 receptor antagonist SR-141716A, and endothelial cells do not synthesize AEA in response to stimulation with A-23187 or histamine. However, our data support the alternative hypothesis that AEA serves as an arachidonic acid donor in bovine coronary arteries and is converted to vasodilatory arachidonate metabolites, most likely prostacyclin and EETs. In support of this hypothesis, we have demonstrated that bovine coronary arteries and endothelial cells exhibit FAAH activity. FAAH is a membrane-associated enzyme that converts AEA and other fatty acyl amides to the free arachidonic acid and ethanolamine, in the case of AEA (10, 12, 20). Furthermore, blockade of FAAH with DAK results in loss of AEA-induced vasodilation and inhibition of the conversion of AEA to arachidonic acid and ethanolamine by the vessels. Our studies also demonstrate that [^{3}H]AEA is metabolized to ^{3}H-labeled metabolites that comigrate with [^{3}H]arachidonic acid, [^{3}H]PGs, and [^{3}H]EETs. Taken together, these experiments suggest that AEA is an indirect vasodilator of bovine coronary arteries.

Our results are strikingly different from those reported by Randall and co-workers (29), who studied AEA-induced relaxations of isolated, perfused rat mesenteric arteries. These investigators reported that AEA-induced relaxations were blocked by SR-141716A and that SR-141716A also inhibited carbachol-induced vasodilation when nitric oxide and cyclooxygenase were blocked. Furthermore, prelabeling of rat mesenteric arteries with [^{3}H]arachidonic acid followed by thin-layer chromatographic analysis of the effluent revealed the presence of an arachidonic acid metabolite that comigrated with authentic AEA. Therefore, they concluded that AEA may represent an EDHF. They did not determine the effects of AEA on the membrane potential of vascular smooth muscle cells. Interestingly, Randall and co-workers (29) reported an inability of arachidonic acid to relax the isolated, perfused mesentery artery. With the same conditions for thin-layer chromatography, we also demonstrated that the EETs comigrated with authentic AEA, which calls into question their conclusion that AEA is synthesized in endothelial cells. A possible explanation for their data that SR-141716A inhibits carbachol- and A-23187-induced vasodilation is our finding that SR-141716A inhibits arachidonic acid release and, thereby, would likely inhibit the synthesis of vasodilatory eicosanoids.

We and others (11, 26, 28, 32, 34) have demonstrated previously that arachidonic acid induces endothelium-dependent relaxations. In bovine coronary arteries, these relaxations are mediated by prostacyclin and EETs (32). The EDHF has been suggested to be a cytochrome *P*-450 metabolite of arachidonic acid (4), because inhibitors of cytochrome *P*-450 attenuate endothelium-dependent hyperpolarizations and relaxations (2, 4, 18, 19, 27). The specific cytochrome *P*-450 metabolites acting as EDHF(s) are the EETs. This is based on *1*) the ability of endothelial cells and vascular tissue to synthesize EETs in response to agonists (4, 31–33), *2*) the ability of the EETs to hyperpolarize and relax vascular smooth muscle (4, 17, 27), and *3*) the ability of potassium channel blockers to inhibit arachidonic acid and EET-induced relaxations (4, 19) and the ability of EETs to open calcium-activated potassium channels (4). Some investigators, however, indicate that either cytochrome *P*-450 inhibitors have no effect on endothelium-dependent hyperpolarizations (8) or the effects of cytochrome *P*-450 inhibitors on endothelium-dependent hyperpolarizations are the result of nonspecific actions (17).

In summary, we have demonstrated that AEA is a potent endothelium-dependent vasorelaxant in bovine coronary arteries. However, AEA appears to induce vasorelaxation not by activating the CB1 cannabinoid receptor but rather by its hydrolysis to arachidonic acid and the subsequent metabolism of arachidonic acid to vasodilatory eicosanoids. These data further support our conclusion that the EETs are EDHFs but that AEA probably is not.

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