U-46619 but not serotonin increases endocannabinoid content in middle cerebral artery: evidence for functional relevance

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REGULATION OF CEREBRAL VASCULAR TONE is a primary determinant of cerebral blood flow, which in turn, is vital for the maintenance of local neuronal activity and cerebral hydrostatic pressure. A host of diverse mediators regulate cerebral vascular tone in response to local and global changes in the metabolic demands from brain tissue. Cerebrovascular autoregulation of flow is achieved via balanced actions of relaxing and contracting mediators that arise from a variety of cellular sources. In addition, compounds are formed in pathological conditions such as atherosclerosis, vasospasm, thrombosis, and inflammation that are potent and efficacious regulators of cerebrovascular tone.

Several years ago, Gebremedhin et al. (17) made the observation that vascular smooth muscle cells (VSMC) of cat and rat small cerebral arteries express the CB1 cannabinoid receptor. Activation of the CB1 receptor of cerebral VSMC results in nearly complete inhibition of the L-type calcium channel to produce vasodilation of isolated arterial segments. CB1 receptor agonists also vasodilate human (30, 31), rabbit (13), and rat (52) cerebral arteries.

These results have led us to explore the physiological and pathophysiologival conditions during which the VSMC CB1 receptor is activated. There are two well-studied putative endogenous agonists of the CB1 cannabinoid receptor [endocannabinoids (eCBs)], N-arachidonylethanolamine (AEA) (9) and 2-arachidonylglycerol (2-AG) (32, 50). The eCBs, like other lipid mediators, are not stored in vesicles but are synthesized “on demand” in response to either elevations in intracellular Ca2+ and/or activation of phospholipases by signal transduction cascades (20). In particular, increased intracellular Ca2+ and thrombin have been shown to increase 2-AG content and release in human umbilical vein endothelial cells (49).

Because vasorelaxation is often accompanied by both elevation in intracellular Ca2+ and activation of phospholipases, we tested the hypothesis that vasorelaxation in small cerebral arteries is due to the role of this signaling pair in neuronal communication where eCBs are elevated during times of high synaptic activity and function as presynaptic inhibitors of neurotransmission (15).

Occlusion of a cerebral artery by thrombotic or atherothrombotic embolic material is found in 80–90% of all strokes. Atherosclerotic alterations of the cerebral endothelium are associated with platelet hyperreactivity and, thus, thrombosis. Stimulated platelets release two major classes of vasoactive mediators during the secretion reaction: arachidonic acid metabolites and biogenic amines (42, 47). Of these compounds, thromboxane A2 (TXA2) and 5-hydroxytryptamine (5-HT) are released in the greatest quantities and are potent constrictors of human cerebral arteries in vitro (39, 51).

We have determined the effects of 5-HT and the TXA2 receptor agonist U-46619 on the eCB content of rat middle cerebral arteries. Interestingly, we find that U-46619 produces a concentration-related increase in both AEA and 2-AG.

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whereas 5-HT significantly reduces both eCBs. These effects did not require an intact endothelium, suggesting a nonendothelial site for the agonist stimulus and eCB production. In isolated, perfused cerebral arteries, we found that the CB1 receptor antagonist SR-141716 significantly increased U-46619 vasoconstriction but not 5-HT constriction. These results support the hypothesis that U-46619-induced vasoconstriction is opposed by the concurrent synthesis of a vasodilatory eCB. Interestingly, the vasoconstriction produced by 5-HT does not share this intrinsic feedback mechanism.

**MATERIALS AND METHODS**

**Materials.** Buffers and salts were purchased from Sigma (St. Louis, MO). Papain, dithioerythritol, collagenase, soybean trypsin inhibitor, phenylmethylsulfonfluoride (PMSF), and 5-HT were also purchased from Sigma. Elastase was purchased from CalBiochem (La Jolla, CA). U-46619 and the AEA and 2-AG deuterated standards were purchased from Research Biochemicals International (Natick, MA). SR-141716 was obtained from the National Institute on Drug Abuse Drug Invention Supply Program (Bethesda, MD). AM-251 was purchased from Toecris Cookson (Ellisville, MO). Cannabinoid receptor ligands were added in dimethylsulfoxide (DMSO); the volume of DMSO never exceeded 0.3% of the total bath volume. Control incubations utilized an equal amount of DMSO. All organic solvents were high-performance liquid chromatography grade and were purchased from either Fisher Scientific (Pittsburgh, PA) or Sigma.

**Animals.** Male Sprague-Dawley rats (Harlan, Madison, WI) were maintained on a 12:12-h light-dark schedule with food and water available ad libitum. The Animal Care and Use Committee of Medical College of Wisconsin approved all experimental protocols. All studies were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**Isolation of MCA.** Rats were deeply anesthetized with isoflurane (Abbott, North Chicago, IL) followed by swift decapitation. Brains were removed, and the proximal portion of the MCA was dissected and placed in ice-cold physiological salt solution (PSS) containing (in mM): 119 NaCl, 4.7 KCl, 1.6 CaCl2, 1.17 MgSO4, 5.5 glucose, 24 NaHCO3, 1.18 NaH2PO4, 5.8 HEPES, and 0.026 EDTA.

**Isolation of cerebral VSMC.** VSMC were enzymatically isolated from rat MCA according to a published method with minor modifications (24). Briefly, arteries were placed for 20 min at room temperature in a culture tube containing 1 ml of dissociation solution of the following composition (in mM): 137 NaCl, 5.6 KCl, 1.0 MgCl2, 0.10 CaCl2, 0.42 Na2HPO4, 0.44 NaH2PO4, 4.2 NaHCO3, 10.0 HEPES, 5.5 glucose, and 1 mg/ml bovine serum albumin. All but 100–200 μl of this solution was removed and replaced with 1 ml of dissociation solution that contained 26 U/ml papain and 1 mg/ml dithioerythritol, and the mixture was incubated for 30 min in a dry-bath heater at 37°C. At the end of this interval, the solution was further incubated for 10 min at 37°C with 1 ml of dissociation solution containing 1.95 U/ml collagenase, 75 U/ml elastase, and 1 mg/ml soybean trypsin inhibitor. Subsequently, the incubation solution was removed without disturbing the arteries, the culture tube was held at a 45° angle, and 2 ml of room temperature dissociation solution were slowly added. The arteries were allowed to settle in the culture tube for 5 min before the solution was exchanged for 500 μl of dissociation solution, and the solution was gently triturated to disperse VSMC from the digested arteries. The VSMC were transferred to a microcentrifuge tube containing PSS at 4°C and used for immunocytochemistry on the same day.

**Immunocytochemistry.** Immunocytochemistry was used to determine CB1 receptor expression in single cerebral VSMC. Single drops of VSMC suspensions were placed on glass slides, air-dried, and fixed with 95% ethanol. The fixed cells were washed twice with phosphate-buffered saline (PBS) and then permeabilized by immersion in PBS-Triton (PBS-T) for 15 min. VSMC were incubated overnight in a humidified chamber with a 1:50 dilution of anti-CB1 receptor polyclonal antibody raised in rabbits (Affinity BioReagent, Golden, CO) in PBS-Triton/normal goat serum (PBS-TNGS). As a cell-specific marker, VSMC were incubated in parallel with a 1:25 dilution of anti-α-actin monoclonal antibody raised in mice (Abcam Bioreagents, Cambridge, UK). After being washed three time in PBS-T, the cells were incubated in the dark for 2 h at room temperature with appropriate fluorescently conjugated secondary antibodies consisting of either a 1:200 dilution of goat anti-rabbit Alexa-Fluor 488 (Molecular Probes, Eugene, OR) or a 1:500 dilution of goat anti-mouse Cy3 (Jackson ImmunoResearch, West Grove, PA). Fluorescent images were captured on the same day using a Nikon Eclipse E600 microscope and Spot Advanced Software.

**Vascular reactivity.** MCA were dissected and cleaned of adhering fat and connective tissue, and the endothelium was removed by the intraluminal introduction of an air bolus. Endothelium removal was verified by the complete elimination of the vasodilator response to acetylcholine. The MCA were cannulated by using tapered glass micropipettes in a heated (37°C) lucite perfusion and superfusion chamber according to a published method (16). Arteries were maintained at a constant perfusion pressure of 60 mmHg in PSS. All solutions were equilibrated with 21% O2–5% CO2–balance N2 to maintain a pH of 7.4 and PO2 of 140 mmHg. Constrictors and Win-55212-2 were added in a cumulative manner. At the end of each experiment, all arteries were perfused and superfused with Ca2+-free PSS to determine the amount of Ca2+-dependent active tone. Internal diameters were obtained by using a Nikon SMZ-800 inverted microscope/Spot RT camera (Diagnostic Instruments, Sterling Heights, MI) with images captured and analyzed with the use of Spot/Metaview acquisition-analysis-graphics software. Diameters were determined as the mean of 20 measurements made along the length of the vessel. Diameters were determined when the vessel reached equilibrium, usually 7–10 min after the addition of drug. The percent constriction is calculated by using the formula [(predrug diameter – postdrug diameter/predrug diameter) × 100].

**Biochemical detection of AEA and 2-AG.** In experiments in which MCA were analyzed for eCB content, the endothelium was either left intact or mechanically disrupted by gentle rubbing with moistened cotton. Endothelium-intact and -denuded arteries were incubated for 30 min at room temperature in PSS containing PMSF (100 μM) and other agents as noted. The incubations were carried out in the presence of PMSF, an efficacious inhibitor of fatty acid amide hydrolase (FAAH) (7, 23) and monoacylglycerol lipase (44). After incubation, eCBs were extracted from the arteries as previously described (40). Briefly, MCAs were weighed and placed into borosilicate glass culture tubes containing 1 ml of acetonitrile with 84 pmol of [3H]AEA and 186 pmol 2-[3H]2-AG. MCAs were homogenized and sonicated in an ice-cold water bath for 1 h. Samples were incubated overnight at −20°C to precipitate proteins. Samples were centrifuged at 1,500 g, and supernatants were removed to a new glass tube and evaporated to dryness under N2 gas. The samples were resuspended in 500 μl methanol and redried and resuspended in 20 μl of methanol, 5 μl of which were analyzed by liquid chromatography mass spectrometry.

Endocannabinoid concentrations were determined by using isotopic-dilution, liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (LC-APCI-MS). Samples (5 μl) were separated on a reverse-phase C18 column (Kromasil, 250 × 2 mm, 5 μm diameter) using solvent A (deionized water, 1 mM ammonium acetate, and 0.005% acetic acid) and solvent B (methanol, 1 mM ammonium acetate, and 0.005% acetic acid). Samples were eluted at 300 μl/min by a linear gradient. The percentage of solvent B increased linearly from 85% solvent B to 100% solvent B in 25 min and then was held at 100% solvent B for 10 min. Detection was made in a positive ion mode using an Agilent 1100 LC-MSD, SL model. Selective ion...
monitoring was used to detect \(^{1}H\)AEA, AEA, 2-\(^{2}H\)AG and 1(3)-AG, and 2-AG and 1(3)-AG. 2-AG is often observed as a doublet as it isomerizes to 1(3)-AG during extraction (48), therefore, the area of both peaks was added to yield total 2-AG (19). Endocannabinoid contents were normalized to arterial weight.

**Statistical analyses.** Data are expressed as means ± SE. Differences in the mean eCB content between treatment groups were determined by one-way analysis of variance followed by post hoc Dunnett’s comparisons. When appropriate, the EC\(_{50}\) and maximal effect (\(E_{\text{max}}\)) values were calculated from the concentration-response curves by fitting data to a logistic sigmoidal equation using GraphPad Prism software. An \(\alpha\) level of 0.05 was used for all statistical tests.

**RESULTS**

**CB\(_{1}\) receptor expression by VSMC.** Freshly isolated cerebral VSMC were subjected to immunocytochemical analysis and were individually examined for fluorescence. A representative Nomarski image of VSMC isolated from rat MCA is shown (Fig. 1A). 4',6-Diamidino-2-phenylindole dihydrochloride staining indicates cell nuclei (Fig. 1B). The fluorescent signals obtained from cerebral VSMC (Fig. 1C) labeled with anti-CB\(_{1}\) indicated the expression of CB\(_{1}\) receptor protein in VSMC of the rat cerebral circulation, concurring with previous reports of their expression in small cerebral arteries of mice, rats, and cats (1, 17, 38). Strong fluorescence also was detected after incubation of the same VSMC with the smooth muscle specific \(\alpha\)-actin, confirming the origin of this cell from arterial muscle (Fig. 1D). A merged image is shown in Fig. 1E.

**Endocannabinoid content in cerebral arteries.** Isolated rat MCAs, with mean weights of 2.85 ± 0.09 mg, contain measurable amounts of AEA and 2-AG (\(n = 141\); Tables 1 and 2). AEA and 2-AG contents were both significantly greater in endothelium-denuded compared with endothelium-intact MCA (Tables 1 and 2; \(P < 0.05\)). If we assume that 1 gram is approximately 1 ml, then the apparent concentrations in the unstimulated vessels were 8–14 nM AEA and 7–14 \(\mu\)M 2-AG. Incubation of the vessels for 30 min with U-46619 (\(n = 36\)) increased AEA and 2-AG content in both endothelium-intact and in endothelium-denuded MCA (Table 1; \(P < 0.05\)). At a concentration of 100 nM, U-46619 increased the apparent concentrations of AEA and 2-AG by 3 nM and 3.6 \(\mu\)M, respectively, in the endothelium-intact MCA and by 4.8 nM and 6.4 \(\mu\)M in the endothelium-denuded MCA.

Conversely, incubation of the rat MCA with 5-HT (\(n = 33\)) significantly decreased AEA and 2-AG content in both endothelium-intact and in endothelium-denuded MCA compared with control vessels incubated in buffer alone (Table 2; \(P < 0.05\)).

**Effects of CB\(_{1}\) receptor antagonists on U-46619-induced contractions.** If the production of eCBs contributes to the overall effect of U-46619 on MCA diameter, we predict that the presence of the CB\(_{1}\) receptor antagonist SR-141716 should enhance its vasoconstriction. U-46619 (10–300 nM) produced a concentration-dependent contraction of endothelium-denuded MCA. The presence of SR-141716 (1 \(\mu\)M) in the superfusion chamber resulted in a significant leftward shift in the concentration-contraction curve for U-46619 but had no effect on maximum contraction (Fig. 2A). The mean EC\(_{50}\) for U-46619 before treatment with SR-141716 (121 nM (115–127)) was significantly different compared with the EC\(_{50}\) after treatment with SR-141716 (42 nM (41,44)); the \(E_{\text{max}}\) was not altered by the presence of SR-141716 (56% compared with 53%). An equivalent volume (4.2 \(\mu\)l) of DMSO vehicle in the superfusion chamber had no effect on either the EC\(_{50}\) or the \(E_{\text{max}}\) values for U-46619. SR-141716 (10–1,000 nM) had no effect on arterial diameter compared with vehicle control (\(n = 7\)).

In a second study, we determined the effects of both SR-141716 and a second CB\(_{1}\) receptor antagonist AM-251 (27), on U-46619-induced contractions of MCA to confirm that the

| Table 1. AEA and 2-AG content in +Endo and –Endo MCA-treated with U-46619 |
|------------------|------------------|
|                  | +ENDO            | –ENDO            |
| Control          | 100 (20)         | 100 (17)         |
| U-46619          |                  |                  |
| 10 nM            | 127.7±8.1 (9)*   | 121.3±7.3 (10)*  |
| 100 nM           | 136.5±9.7 (10)*  | 133.0±12.4 (7)*  |
| 2-AG Content (% Control) |                  |                  |
| Control          | 100 (20)         | 100 (17)         |
| U-46619          |                  |                  |
| 10 nM            | 138.3±19.4 (10)* | 132.0±9.0 (9)*   |
| 100 nM           | 152.4±14.1 (9)*  | 143.6±15.9 (8)*  |

Values are as means ± SE; number of animals is indicated in parentheses. +Endo, endothelium intact; –Endo, endothelium denuded. Control N-arachidonylthanolamine (AEA) content was 8.1 ± 0.6 pmol/g (+Endo) and 14.5 ± 0.9 pmol/g (–Endo). Control 2-arachidonylglycerol (2-AG) content was 7.3 ± 0.3 nmol/g (+Endo) and 14.7 ± 0.9 nmol/g (–Endo). *\(P < 0.05\) compared with control.
Table 2. AEA and 2-AG content in +Endo and −Endo MCA treated with 5-HT

<table>
<thead>
<tr>
<th></th>
<th>+ENDO</th>
<th>−ENDO</th>
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<tbody>
<tr>
<td><strong>AEA Content (% Control)</strong></td>
<td>100 (17)</td>
<td>100 (20)</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-HT 0.1 nM</td>
<td>83.2±4.5 (9)*</td>
<td>75.2±3.5 (8)*</td>
</tr>
<tr>
<td>10 nM</td>
<td>65.6±5.1 (9)*</td>
<td>73.7±1.9 (7)*</td>
</tr>
<tr>
<td><strong>2-AG Content (% Control)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-HT 0.1 nM</td>
<td>79.9±5.3 (9)*</td>
<td>80.2±9.6 (8)*</td>
</tr>
<tr>
<td>10 nM</td>
<td>68.5±5.6 (9)*</td>
<td>79.1±5.0 (7)*</td>
</tr>
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</table>

Values are means ± SE; number of animals is indicated in parentheses. Control AEA content was 8.3 ± 0.4 pmol/g (+Endo) and 13.8 ± 1.0 pmol/g (−Endo). Control 2-AG content was 8.3 ± 0.2 nmol/g (+Endo) and 16.6 ± 0.7 nmol/g (−Endo). *P < 0.05 compared with control.

effects of SR-141716 are CB1 receptor specific. In this set of experiments, the vessels exhibited an increased sensitivity to U-46619; however, the effects of SR-141716 were qualitatively similar to the first study. In particular, the EC50 for U-46619 in the presence of either SR-141716 or AM-251 was significantly lower compared with the EC50 for U-46619 in the presence of DMSO vehicle (Table 3). The Emax for U-46619 was not affected by the presence of either AM-251 or SR-141716.

The pattern of effect of CB1 receptor antagonists to affect the EC50 value for U-46619 without affecting the Emax suggests that eCBs exert a greater influence on vessel diameter at low concentrations of U-46619 than concentrations that produce maximal constriction. To explore this issue, a moderate concentration of Win-55212-2 (30 nM) was used to activate directly CB1 receptors in vessels exposed to low (30 nM) and high (300 nM) concentrations of U-46619 (Fig. 2B). As expected, Win-55212-2 decreased the constriction produced by a low concentration of U-46619 (30 nM) but had no effect on the constriction produced by a supramaximal concentration of U-46619 (300 nM). These data are consistent with the results obtained using CB1 receptor antagonists and suggest that CB1 receptor-mediated vasodilatation is overcome when TXA2 receptors are maximally activated.

Effects of CB1 receptor antagonists on 5-HT-induced contractions. 5-HT (0.1–1,000 nM) also produced a concentration-dependent constriction of endothelium-denuded (n = 4) rat MCA. The presence of SR-141716 (1 μM) in the superfusion chamber had no effect on either the maximum constriction or the concentration-construction curve for 5-HT (Table 3, Fig. 3A). An equivalent volume (4.2 μl) of DMSO vehicle in the superfusion chamber had no effect on either the EC50 values or the Emax values for 5-HT (Table 3).

The lack of effect of SR-141716 on 5-HT-mediated vasoconstriction is consistent with our finding that incubation of the MCA with 5-HT did not elevate eCB content. However, this finding could also reflect a lack of influence of CB1 receptor activation on 5-HT-mediated constriction. To rule out this explanation, we determined the effects of an exogenously administered CB1 receptor agonist Win-55212-2 on 5-HT-mediated vasoconstriction (Fig. 3B). In agreement with our earlier studies, we found that Win-55212-2 produced a concentration-dependent dilation of endothelium-denuded rat MCA with an EC50 (95% confidence interval) of 28 nM (25 nM, 31 nM) and an Emax (95% confidence interval) of 31% dilation (30%, 32%).

Table 3. EC50 and Emax values for U-46619-induced contractions of endothelium-denuded MCA after treatment with SR-141716, AM-251, or DMSO vehicle

<table>
<thead>
<tr>
<th>Constrictor</th>
<th>Treatment</th>
<th>EC50 (μM)</th>
<th>Emax (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>U-46619</td>
<td>DMSO</td>
<td>18.3 (15.1, 22.0)</td>
<td>44.9 (42.0, 48.8)</td>
</tr>
<tr>
<td>U-46619</td>
<td>SR-141716</td>
<td>8.0 (7.3, 8.9)</td>
<td>49.7 (48.4, 51.1)</td>
</tr>
<tr>
<td>U-46619</td>
<td>AM-251</td>
<td>12.5 (11.0, 14.1)</td>
<td>49.9 (48.1, 51.8)</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 4 animals. MCA, middle cerebral artery; CI, confidence interval. The EC50 and maximal effect (Emax) values were calculated from the concentration-response curves by fitting data to a logistic sigmoidal equation. †Emax and 95% CI data are expressed as percentage of the MCA diameter after the vessel had equilibrated. ‡Statistically significant compared with DMSO treated.
The MCA is extremely sensitive to exogenously added cannabinoid receptor agonists. In the present study, the vasodilatory EC50 for Win-55212-2 was 28 nM. This suggests that the CB1 receptor of the cerebral VSMC exhibits high signaling efficiency, particularly compared with cerebellar membranes where the EC50 for Win-55212-2 to induce GDP/GTP exchange is 100 nM (26). The CB1 receptor antagonist/inverse agonist SR-141716 (5) had no effect on arterial diameters when added alone, which suggests that the cerebral VSMC CB1 receptor is neither tonically activated by eCBs nor constitutively active.

We used LC-MS to determine the endogenous eCB content in MCAs. Interestingly, we found that these arteries contain both AEA and 2-AG after incubation in buffer containing PMSF to inhibit eCB catabolism by FAAH (7) and monoacylglycerol lipase (44). If we assume a correspondence between gram wet weight and milliliter volume, then the apparent basal concentration in the vessel of AEA is 8–14 nM and 2-AG is 7–14 μM. Because CB1 receptor antagonists did not alter MCA diameter in the absence of U-46619, it is possible that this represents an eCB pool that is not able to activate CB1 receptors. U-46619 increased the apparent concentrations of AEA by 3–5 nM and 2-AG by 4–6 μM. The dissociation constant (KD) value of AEA for the CB1 receptor is approximately 100 nM (22); whereas 2-AG is about 3 μM (46). Therefore, the increase in 2-AG evoked by U-46619 is in the concentration range that is likely to activate the CB1 receptor. Of course, the critical concentrations of the eCBs are those at the CB1 receptor sites, but our current assay methods do not allow for determination of this concentration.

AEA and 2-AG contents were greater in endothelium-denuded compared with endothelium-intact MCA. It is possible that process of mechanical disruption of MCA endothelium caused tissue damage that resulted in increased eCB content in endothelium-denuded MCA. In support of this possibility, brain trauma and ischemia increase eCB content (35, 37). It is also possible that mechanical disruption of the endothelium, which would eliminate the release of endothelium-derived hyperpolarizing factors, resulted in depolarization of VSMC. This depolarization would increase the likelihood of eCB synthesis (10). Further studies will elucidate these possibilities.

Although data suggest that U-46619 and 5-HT both contract VSMC via inositol trisphosphate-dependent intracellular Ca2+ mobilization and Ca2+ influx through voltage-operated L-type Ca2+ channels (11, 14), opposite effects of the two vasoconstrictors on AEA and 2-AG production were observed. This difference could be due to differences in the magnitude of increase of intracellular calcium concentration ([Ca2+]i) because AEA synthesis in particular requires a large increase in [Ca2+]i (10). In support of this possibility, treatment of rat aortic VSMC with the TXA2 receptor agonists carboyclic TXA2 and U-46619 resulted in a 2.5- and 1.5-fold increase in [Ca2+]i, at 25 s, respectively; these increases returned to basal values at 3 min (45). By contrast, treatment of rat cerebrovascular VSMC with 5-HT resulted in a small and transient increase in intracellular Ca2+, which was dependent on 5-HT2 receptor activation and occurred in only 67% of the cells (55). It is also possible that other downstream signaling events oppose the effects of 5-HT on eCBs synthesis. For example, 5-HT induces the release of norepinephrine from cerebral arteries and part of the vasoconstriction produced by 5-HT can

**DISCUSSION**

In agreement with earlier reports that cat cerebral VSMC express the CB1 receptor (17) and that the CB1 receptor is expressed in the VSMC layer in rat cerebral arteries (1), we find that freshly isolated VSMC from rat cerebral arteries express the CB1 receptor as evaluated by immunocytochemistry, suggesting that VSMC is a target of eCBs. The primary findings of this report are that AEA and 2-AG are present in isolated rat MCAs and that their production is increased by the TXA2 mimetic U-46619. Although SR-141716 had no effect on arterial diameter in the absence of U-46619, SR-141716 and a second antagonist AM-251 produced leftward shifts in the U-46619 concentration-contraction curve. By contrast, 5-HT decreased the MCA content of both AEA and 2-AG, and the presence of SR-141716 in the superfusion chamber had no effect on the 5-HT concentration-contraction curve. These data suggest that TXA2 receptor activation results in the synthesis of eCBs, which activate VSMC CB1 receptors to dampen its vasoconstriction. This feedback mechanism is not involved in the vasoconstriction produced by 5-HT. These data support a role of eCBs in the regulation of cerebral vascular tone.

**Fig. 3. A:** effects of SR-141716 on 5-hydroxytryptamine (5-HT)-induced vasoconstriction. Rat MCA were isolated and, following equilibration, were incubated with either DMSO (control; 4.2 μM) or SR-141716 (1 μM final concentration delivered in 4.2 μl DMSO) for 10 min. Increasing concentrations of 5-HT were added, and vessel diameter was determined after equilibrium had been reached. Each point is the mean of 5 determinations; vertical lines represent means ± SE. **B:** effect of Win-55212-2 on 5-HT-induced vasoconstriction. Rat MCA were isolated and, following equilibration, were incubated with 10–5 M 5-HT for 10 min. Increasing concentrations of Win-55212-2 were added in a cumulative manner, and vessel diameter was determined after equilibrium had been reached. Each point is the mean of 5 determinations; vertical lines represent means ± SE.

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be inhibited by adrenergic receptor antagonists (29). In any event, the striking difference in effect of these two potent cerebral vasoconstrictors on eCB content suggests that eCB synthesis requires more than an elevation in VSMC intracellular calcium content.

There are only a few other reports of the regulation of eCB content by activation of G protein-coupled receptors in tissues or cells. Thrombin has been shown to increase 2-AG content in human umbilical endothelial cells (49); incubation of whole rat aorta with carbachol results in an endothelium-dependent increase in vessel 2-AG content (33), and endothelin-1 enhances the production of AEA by astrocytes (54). Platelet-activating factor also has been reported to increase the content of AEA in RAW-264.7 mouse macrophage cells (41) and to increase the synthesis of 2-AG in P388D1 macrophages by a process that appears to involve phospholipase C activation (2). At present, there are too few studies to allow for generalizations to be made regarding the tissue-specific regulation of eCB synthesis by different GPCRs, but our study provides important new evidence that physiological stimuli can dynamically regulate AEA and 2-AG synthesis in arteries involved in cerebral blood flow regulation.

Because TXA2 is a potent vasoconstrictor released by stimulated platelets (51), our finding that the TXA2 mimetic U-46619 increases MCA eCB content and is a more effective constrictor when the VSMC CB1 receptor is blocked by SR-141716 suggests a role for the eCBs in the regulation of thrombosis. We hypothesize that activation of the VSMC CB1 receptor by eCBs should attenuate the vasoconstriction and prothrombotic activity of platelet-derived TXA2. Thus pharmacological augmentation of cerebral vessel eCB signaling could serve as a novel approach to the treatment of thrombotic stroke. Cerebral ischemia has more severe effects in CB1 knockout than in wild-type mice (38). In particular, dysregulation of regional cerebral blood flow during reperfusion in the ischemic penumbra of CB1 receptor knockout mice suggests that eCB signaling through CB1 receptors acts to enhance blood flow and promote cell survival in the cerebral vasculature after stroke (38). On the other hand, infarct volume was significantly reduced in SR-141716-treated rats following permanent middle cerebral artery occlusion (3, 34). Thus, it appears that the role of eCBs and the CB1 receptor during cerebral ischemia is dependent on the model and includes contributions from vascular and parenchymal CB1 receptors.

The current data suggest that vascular smooth muscle cells of cerebral arteries both produce and respond to eCBs. Data from other studies indicate that endothelial cells can also be eCB transmitting and targeted cells. Sugiuira and colleagues (49) have demonstrated that human umbilical vein endothelial cells generate 2-AG in response to calcium ionophore and thrombin. Endothelial cells from the brain express CB1 receptors (6) as well as two other targets of eCBs, CB2 receptors, and TRPV1 vanilloid receptors (18). 2-AG (6) and AEA (18) produce an increase in cerebrovascular endothelial cell Ca2+ content, which is partially sensitive to inhibition by SR-141716. 2-AG also produces phosphorylation of vasodilator-stimulated phosphoprotein through TRPV1 receptors (18). The eCBs also produce endothelium-dependent effects in other vascular beds that exhibit complex receptor relationships (21). AEA has been reported to produce endothelium-dependent relaxations in bovine coronary arteries by metabolism of exogenous AEA to vasodilatory eicosanoids (43). AEA vasodilates renal arteries though the production of nitric oxide by endothelial cells (8). In addition to the CB and TRPV1 receptors, a novel G protein-coupled “anandamide receptor” distinct from CB1 or CB2 receptors has been proposed to exist on the vascular endothelium (25, 36, 53) that could mediate AEA-induced vasodilation of mesenteric arteries.

Our finding that vasoactive agents regulate the eCB content of rat cerebral arteries is of considerable importance in light of the experimental evidence demonstrating that cannabinoids affect the outcome of cerebral ischemia and trauma in vivo (35, 37). A survey of the literature indicates a paradox in that both CB1 receptor agonist and antagonist treatment can protect the brain from trauma and ischemia. Perhaps the paradox is explained by the diverse cell types that express the CB1 receptor in brain, including neurons (12), astrocytes (4), endothelial cells (28), and cerebral arterial VSMC (17).

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