Calcium Influx Factor, Further Evidence It Is 5,6-Epoyxeicosatrienoic Acid*

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We present evidence in astrocytes that 5,6-epoyxeicosatrienoic acid, a cytochrome P450 epoxygenase metabolite of arachidonic acid, may be a component of calcium influx factor, the elusive link between release of Ca$^{2+}$ from intracellular stores and capacitative Ca$^{2+}$ influx. Capacitative influx of extracellular Ca$^{2+}$ was inhibited by blockade of the two critical steps in epoyxeicosatrienoic acid synthesis: release of arachidonic acid from phospholipid stores by cytosolic phospholipase A$_2$ and cytochrome P450 metabolism of arachidonic acid. AAOCF$_3$, which inhibits cytosolic phospholipase A$_2$ and blocked thapsigargin-stimulated release of arachidonic acid as well as thapsigargin-stimulated elevation of intracellular free calcium. Inhibition of P450 arachidonic acid metabolism with SKF525A, econazole, or N-methylsulfonyl-6-(2-propargyloxyphenyl)hexanamide, a substrate inhibitor of P450 arachidonic acid metabolism, also blocked thapsigargin-stimulated Ca$^{2+}$ influx. Nano- to picomolar 5,6-epoyxeicosatrienoic acid induced [Ca$^{2+}$]$_i$ elevation consistent with capacitative Ca$^{2+}$ influx. We have previously shown that 5,6-epoyxeicosatrienoic acid is synthesized and released by astrocytes. When 5,6-epoxyeicosatrienoic acid was applied to the rat brain surface, it induced vasodilation, suggesting that calcium influx factor may also serve a paracrine function. In summary, our results suggest that 5,6-epoxyeicosatrienoic acid may be a component of calcium influx factor and may participate in regulation of cerebral vascular tone.

One of the biochemical pathways for metabolism of arachidonic acid is the cytochrome P450 monoxygenase pathway, which results in formation of 4-regio- and stereoisomeric products; cis-5,6-, 8,9-, 11,12-, and 14,15-epoxyeicosatrienoic acids (EETs).\(^1\) Compared with our knowledge of the lipoygenase and cyclooxygenase pathways for arachidonic acid metabolism, relatively little is understood about the epoxygenase pathway. Numerous physiological roles have been suggested for the EETs and collectively, the EETs appear to have potent effects on ion channels (1–3).

One of the mechanisms for regulation of intracellular calcium dynamics in response to hormones and other agonists is through the “capacitative pathway” as originally described by Putney (4, 5). Activation of this pathway occurs through G protein receptor-mediated activation of phospholipase C, which catalyzes the hydrolysis of phosphatidylinositol bisphosphate yielding inositol trisphosphate (Ins(1,4,5)P$_3$) and diacylglycerol. Ins(1,4,5)P$_3$ binds to its receptor on the intracellular calcium stores, initiating release of stored calcium (6). As the stores are depleted of calcium, a second messenger termed “calcium influx factor” (CIF) is released (7). CIF induces influx of extracellular calcium through second messenger operated channels (SMOC) in the plasma membrane, thereby coupling calcium entry to depletion of internal stores. To date, the identity of CIF remains unknown.

In previous work from our laboratory (8), we reported that capacitative calcium influx was linked to arachidonic acid release by activation of the 85-kDa cytosolic phospholipase A$_2$ (cPLA$_2$) in human U937 lymphoma cells and in rat cortical astrocytes (9), suggesting that arachidonic acid, or a metabolite thereof, was a component of CIF. Recent work from several other groups has shown that the actions of cytochrome P450 monooxygenase may also be coupled to capacitative calcium influx (10, 11). Hoebel et al. (11) recently reported that functional P450 activity was critical to regulation of store-operated calcium influx and proposed that an EET may constitute the driving force for capacitative calcium entry in endothelial cells. Graier et al. (10) have presented evidence suggesting that 5,6-EET stimulates capacitative calcium influx in endothelial cells, consistent with CIF. However, if 5,6-EET is CIF or a component thereof, then it should have similar effects in all cell lines which signal through Ins(1,4,5)P$_3$.

Although CIF is hypothesized to have its primary actions at an intracellular level, it may also be released into the extracellular environment, acting as a paracrine signal for SMOC calcium influx independent of release of calcium from intracellular stores. In Jurkat cells, CIF was released to the extracellular medium upon stimulation with phytohemagglutinin (7). Endothelium-derived hyperpolarizing factor, which induces NO- and prostaglandin I$_2$-independent relaxation of vascular smooth muscle, is released in response to agonists operating through Ins(1,4,5)P$_3$-dependent signaling (12–14). Harder et al. (15) and Gebremedhin et al. (16) reported that cat brain converted arachidonic acid to EETs, which dilated cerebral arteries, implying that endothelium-derived hyperpolarizing factor may be an EET. Hecker et al. (17) reported an NO- and cyclooxygenase-independent relaxation of porcine aortic rings.

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\(^{1}\) The abbreviations used are: EET, epoxyeicosatrienoic acid; Ins(1,4,5)P$_3$, inositol 1,4,5-trisphosphate; CIF, calcium influx factor; SMOC, second messenger-operated channels; cPLA$_2$, cytosolic phospholipase A$_2$; AA, arachidonic acid; CSF, cerebrospinal fluid; DPBS, Dulbecco’s phosphate-buffered saline; [Ca$^{2+}$]$_i$, intracellular calcium.
which was mediated by calcium-activated K+ channels (KCa) and required the combined actions of PLA2 and P450, precisely those systems which may mediate CIF. Additional reports indicate that KCa are closely coupled to capacitative calcium influx (18, 19). Taken together, these reports lead to the speculation that endothelium-derived hyperpolarizing factor may represent a paracrine function of CIF. In the present report we present evidence suggesting that 5,6-EET is a CIF in astrocytes and when released into the extracellular environment may participate in regulation of local cerebrovascular tone.

**EXPERIMENTAL PROCEDURES**

**Materials**

Young adult rats and rat pups (1–2 days old) were purchased from Hilltop Lab Animals (Scottsdale, PA). Dulbecco’s modified essential medium and Dulbecco’s phosphate-buffered saline (DPBS) with or without calcium were purchased from Life Technologies, Inc. (Grand Island, NY). Fetal calf serum was from Hyclone (Logan, UT). Arachidonic acid and EETs (free acids) were purchased from Cayman Chemical (Ann Arbor, MI). Fura-2 AM and calcium calibration buffers were obtained from Molecular Probes (Eugene, OR). SKF96365, arachidonitrifluororomethyl ketone (AAOCF3), econazole, SKF525A, and thapsigargin were purchased from Calbiochem (San Diego, CA). Indomethacin was obtained from Sigma. N-Methylsulfonyl-6-(2-propargyloxyphenyl)hex-1-ene-2,4-dione (MS-PPOH) was synthesized by Dr. J. R. Palcik (University of Texas Southwestern Medical Center, Dallas, TX).

**Methods**

**Cell Culture—Astrocytes** were prepared from 1–2-day-old rat pups as described previously (20). In brief, cortices were isolated, cleaned of white matter and meninges, minced, and trypsin-digested for 10 min. The dissociated tissue was diluted into Dulbecco’s modified essential medium (supplemented with 10% fetal calf serum and 2 mM glutamine) and seeded into 75-cm² tissue culture dishes at an initial density of 10^6 cells grown in a 25-mm diameter pure as assessed by the presence of glial fibrillary acidic protein. Cells were used for experiments at a total of 4 weeks after removal from the rat.

**Fura-2 AM Loading—[Ca^{2+}]i was measured with the ratiometric dye, Fura-2, as described previously (21). Astrocytes were washed 3 times with DPBS supplemented with 2% fatty acid-free bovine serum albumin and 1 mM glucose and placed in 1.5 ml of this medium for 10 min. Cells were loaded with 5 µM Fura-2 AM for 50 min at room temperature. This loading procedure resulted in complete dye hydrolysis as determined by scanning the excitation spectra of loaded cells, with fluorescence at 350 and 380 nm. Emission was measured at 510 nm, via a microphotometer. The entire system for data collection and analysis was computer driven.**

**After loading, astrocytes were washed twice and placed in 2 ml of DPBS supplemented with 1 mM glucose and 1% fatty acid-free bovine serum albumin. For measurement of [Ca^{2+}]i, a field of 1–2 astrocytes was selected using slit width adjustments on the microphotometer. Basal [Ca^{2+}]i was recorded for several seconds, at a sampling rate of 1 frame/s.**

For experiments using agonists (arachidonic acid, 5,6-EET, and thapsigargin), inhibitors (econazole, SKF96365, SKF525A, AAOCF3, and MS-PPOH), these agents were added to the tissue culture dish in a volume of 200 µl. This volume assured almost immediate mixing with the buffer, as determined by dye diffusion. For experiments using indomethacin, Fura-2-loaded astrocytes were incubated with 10 µM indomethacin for 15 min prior to measurement of [Ca^{2+}]i. Each experiment was performed on a separate culture of astrocytes.

**[Ca^{2+}]i was calculated as described previously (25) using a correction for intracellular viscosity (23). Autofluorescence at 350 and 380 was recorded from wells of astrocytes not loaded with Fura-2 and was subtracted from all measurements. Agonist-stimulated [Ca^{2+}]i concentrations were normalized to the basal [Ca^{2+}]i level in each experiment and are expressed as a percent of basal. Leakage of Fura-2 into the medium was monitored by measurement of intracellular Fura-2 concentration at the isobestic wavelength, 362 nm (25).**

For calcium-free experiments, cells were placed in calcium-free DPBS containing 1 mM glucose and 1% fatty acid-free bovine serum albumin immediately prior to the experiment. EGTA (0.5 mM) was added to chelate any residual calcium. Astrocytes were incubated for 10 min to allow calcium-free conditions for longer than 15 min. As described previously, this treatment did not result in lifting of cells from the monolayer.

**Arachidonic Acid Release—Arachidonic acid release was measured as described previously (8, 26). Briefly, 1 x 10^6 cells grown in a 25-mm diameter well were radiolabeled by addition of 0.5 µCi of [3H]arachidonic acid to the growth medium for 24 h prior to the assay. Incorporation of radiolabel was 95%. After labeling, the cells were washed three times and placed in DPBS supplemented with 1 mM glucose and 1% fatty acid-free bovine serum albumin. Next, cells were preincubated with 10 µM econazole, 5 µM AAOCF3, 1 µM MS-PPOH, or 10 µM SKF525A for 2 min in a final volume of 1.0 ml. Following preincubation, cells were stimulated with 1 µM thapsigargin for 2 min. Radioactivity released into the medium after thapsigargin stimulation was determined by scintillation counting. Controls consisted of unstimulated cells, or cells treated with inhibitors alone. Econazole, AAOCF3, MS-PPOH, and SKF525A did not significantly alter basal arachidonic acid release as compared with untreated controls. For experiments conducted under calcium-free conditions, calcium-free DPBS supplemented with 1 mM glucose, 1% fatty acid-free bovine serum albumin, and 0.5 mM EGTA was utilized for all incubations. Results are expressed as percent of control (unstimulated) cells.**

**Preparation and Handling of EETs—EETs are highly labile in the aqueous environment. Concentrated EET stock solutions were stored in aliquots at −70 °C in acetoniitrile. A fresh aliquot was used for each experiment. Just prior to use, the acetoniitrile solution was dried under N2 and EETs were resuspended in ethanol and kept on ice. Aliquots were stored as aliquots of DPBS as required for agonist stimulation. The final concentration of ethanol in all experiments did not exceed 0.1%.**

**Cranial Window—The acute cranial window technique and in vivo microscopy were utilized to examine the effect of 5,6-EET on rat cerebral arteriolar diameter, as described previously (27). Briefly, young adult male Sprague-Dawley rats were anesthetized with xipentobarbital (75 mg/kg) and supplemented with pentobarbital. After completion of a tracheotomy, each rat was ventilated with room air. The end-expiratory CO₂ of each rat was continuously monitored with a capnometer (Transverse Medical Monitors, model 2200) and was maintained at approximately 30 mm Hg by adjusting the respiratory rate and volume. Arterial blood pressure was measured via a cannula inserted into the right femoral artery. Arterial samples were periodically analyzed with a Corning Blood Gas Analyzer to ensure normal P O₂, P CO₂, and blood pH. A cannula was also inserted into the right femoral vein for systemic administration of supplemental anesthetic.**

Pial arteries were visualized using a cranial window implanted into the scalp through a midline incision. The skin and fascia were retracted and a 3-mm diameter craniotomy was made over the left parietal cortex using a stereotaxic drill. With the aid of a surgical microscope, microscissors were used to remove the dura and expose the pial surface of the brain. A 12-mm cranial window frame with a 6.5-mm diameter glass window was implanted over the craniotomy. The cranial window was equipped with three openings. Two openings were used as an inlet and outlet for filling the space under the cranial window with test solutions. The inlet and outlet valves were positioned such that the test solutions
flowed over the cortical surface as viewed through the cranial window. The third opening of the cranial window was connected to a Statham pressure transducer for continuous measurement of intracranial pressure. The outlet of the window was connected to plastic tubing whose pressure of 5 mm Hg throughout the experiment. The space under the cranial window was filled with artificial CSF. This fluid was equilibrated with gas containing 5.9% CO2, 6.6% O2, and 87.5% N2, which produces pH and gas tensions in a normal range for CSF. The vehicle for all agents applied under the cranial window was artificial CSF. The diameter responses of three to five arterioles were studied in a given rat using a Vickers image-splitting device as described previously (28). The responses of the arterioles in a given rat were averaged, and this single number was used to compute the average for a group of rats. After implantation of the cranial window, baseline arteriolar diameter was established by washing the window with 1 ml of artificial CSF at 5-min intervals. At the end of each 5-min interval, baseline measurements of pial arteriolar diameter were recorded. Next, 5,6-EET was added at the indicated time point. The broken line, cells were pretreated with 10 μM indomethacin for 15 min, followed by stimulation with 10 μM AA. Indomethacin had no effect on AA-induced elevation of [Ca2+]i. In B (solid line), separate astrocyte cultures were treated with 10 μM ecaonazole, followed by 10 μM arachidonic acid. The broken line, astrocytes were stimulated with 10 μM AA in Ca2+-free DPBS. Tracings are representative of six separate experiments performed on different days.

**RESULTS**

Exogenous Arachidonic Acid Stimulates Calcium Influx in Astrocytes—Basal [Ca2+]i, in astrocytes was 88 ± 5 nM, consistent with published reports (21). Exogenous arachidonic acid (1–100 μM) stimulated a dose-dependent elevation in [Ca2+]i in astrocytes (data not shown). A representative tracing of the [Ca2+]i elevation stimulated by arachidonic acid is shown in Fig. 1A. Arachidonic acid (10 μM) stimulated an elevation in [Ca2+]i in rat cortical astrocytes to 265 ± 14% of basal (n = 6). The elevation in [Ca2+]i was not immediate, but appeared to require approximately 175 s for initiation. Although lag time after application of an agonist is often difficult to compare accurately, the lag time for the arachidonic acid response greatly exceeded the usual lag time for other agonists and was consistently observed in all experiments. This delay suggests that metabolism of arachidonic acid may be necessary to produce an elevation in [Ca2+]i. Pretreatment with the cyclooxygenase inhibitor indomethacin (10 μM, 15 min) had no effect on either basal or arachidonic acid-induced elevation of [Ca2+]i (Fig. 1A, broken line), suggesting that a cyclooxygenase metabolite was not involved in arachidonic acid-induced elevation of [Ca2+]i.

Removal of extracellular [Ca2+]i, (Fig. 1B, broken line) completely blocked AA-induced elevation of [Ca2+]i, suggesting that AA-induced [Ca2+]i elevation in astrocytes is due to influx of extracellular Ca2+. Addition of the cytochrome P450 inhibitor econazole (10 μM) had no effect on basal [Ca2+]i (Fig. 1B). However, a 2-min econazole pretreatment dramatically attenuated the arachidonic acid-induced elevation of [Ca2+]i, to 130 ± 9% of basal (n = 6). Similar results were observed with a second inhibitor of P450, SKF525A (data not shown). These results suggest that both arachidonic acid-induced elevation in astrocyte [Ca2+]i may require cytochrome P450 metabolism of arachidonic acid.

**Arachidonic Acid Release Is Coupled to Thapsigargin-stimulated [Ca2+]i Elevation in Astrocytes**—If a metabolite of arachi-
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donic acid is a component of CIF, then arachidonic acid should be released in response to depletion of intracellular calcium stores. Using [3H]arachidonic acid-labeled astrocytes, we investigated the total release of arachidonic acid and metabolites in response to a 2-min stimulation with thapsigargin. Activation of the capacitative pathway with thapsigargin induced release of arachidonic acid (and/or metabolites) of 399 \pm 43% of basal (Fig. 2A, filled bars). In calcium-free medium supplemented with EGTA, thapsigargin-stimulated release of arachidonic acid (and/or metabolites) was reduced to 233 \pm 13% of basal. Similar to our observations in U937 (8), these results suggest that a portion of the thapsigargin-stimulated arachidonic acid release is coupled to influx of extracellular calcium, while a portion is also coupled to depletion of intracellular calcium stores. Thus, PLA2 activity may be associated with release of calcium from intracellular stores. Inhibition of cytochrome P450 with econazole, SKF525A, or MS-PPOH did not inhibit thapsigargin-induced arachidonic acid release. Inhibition of cPLA2 with AAOCF3 inhibited thapsigargin-stimulated arachidonic acid release to control levels.

Thapsigargin-induced Elevation of [Ca2+]i Is Blocked by Inhibitors of Cytochrome P450 and cPLA-

We have previously shown that capacitative calcium influx requires the action of cPLA2 and release of arachidonic acid in U937 cells (8). The results above suggest that arachidonic acid elevates [Ca2+]i, in the astrocyte, possibly through a P450 metabolite. Therefore, we investigated whether cPLA2 and P450 were also linked to capacitative calcium influx in the astrocyte.

Thapsigargin is a pharmacological agent that inhibits the Ca2+-ATPase of the intracellular calcium store and activates capacitative calcium influx independent of phospholipase C (29). The thapsigargin-stimulated elevation of [Ca2+]i is dependent on 2 sources of calcium, release from intracellular stores followed by capacitative influx of extracellular calcium. If capacitative calcium influx is mediated by release of arachidonic acid and subsequent metabolism via P450, then capacitative calcium influx should be inhibited by blockade of either cPLA2 or P450. To test this we utilized econazole, SKF525A (30), and MS-PPOH (31), inhibitors of cytochrome P450 and AAOCF3, a selective inhibitor of cPLA2 (32). A summary of these experiments appears in Fig. 2A and representative tracings in Fig. 2B.

In the astrocytes, thapsigargin elevated [Ca2+]i to 432 \pm 62% of basal (Fig. 2, A and B, trace 1). To further dissect this response, the relative size of thapsigargin-releaseable intracellular calcium stores in astrocytes was determined by stimulation with thapsigargin in calcium-free DPBS supplemented with 0.5 mM EGTA. Under these conditions, thapsigargin elevated [Ca2+]i by only 196 \pm 23% of basal (Fig. 2, A and B, trace 2), approximately half that observed in experiments where extracellular calcium was 1 mM.

To assess the effect of cPLA2 or P450 inhibitors on capacitative calcium influx, astrocytes were pretreated with 5 \mu M AAOCF3, 10 \mu M econazole, 10 \mu M SKF525A, or 1 \mu M MS-PPOH for 2 min. Inhibition of cPLA2 with AAOCF3 or inhibition of cytochrome P450 with econazole or SKF525A had no effect on thapsigargin-stimulated elevation of [Ca2+]i, in calcium-free medium, indicating that these compounds had no effect on release of calcium from intracellular stores (data not shown). In DPBS containing 1 mM extracellular calcium (Figs. 2, A and B, trace 5) inhibition of cPLA2 with AAOCF3 blocked capacitative calcium influx and produced [Ca2+]i levels consistent with release of calcium from intracellular stores alone. In calcium replete medium, inhibition of cytochrome P450 with 10 \mu M econazole or 10 \mu M SKF525A also inhibited capacitative calcium influx (Fig. 2, A and B, traces 3 and 4). These two P450 inhibitors blocked the maximum thapsigargin-stimulated [Ca2+]i elevation to the same extent as calcium-free medium, suggesting that capacitative Ca2+ influx was inhibited. However, the sustained phase of [Ca2+]i elevation was only partially inhibited. The cytochrome P450 enzymes comprise a large family of isozymes (33) of which econazole and SKF525A are general inhibitors which block many isozymes. We therefore used an additional inhibitor of cytochrome P450, MS-PPOH (31). MS-PPOH is a “suicide substrate” inhibitor of P450 arachidonic acid epoxygenase, designed to resemble the substrate arachidonic acid and inactivate the enzyme. In rat renal microsomes, MS-PPOH was a potent and selective inhibitor of arachidonic acid epoxygenase activity (31). In the astrocyte, 1 \mu M MS-PPOH inhibited thapsigargin-stimulated capacitative calcium influx to a level consistent with depletion of intracellular calcium stores (Fig. 2, A and B, trace 6). Taken together, these results suggest that formation of CIF requires the combined actions of cPLA2 and cytochrome P450 and may involve an epoxide of arachidonic acid.

5,6-EET Elevates [Ca2+]i in Astrocytes—If an EET is a component of CIF, then application of exogenous EET should stimulate capacitative calcium influx directly through SMOC, without affecting intracellular calcium stores. Therefore, we examined the effect of all 4 EETs on [Ca2+]i in astrocytes. At a concentration of 10^{-4} M, 8,9-(n = 3), 11,12-(n = 3), and 14,15-EET (n = 6) had no effect on [Ca2+]i (data not shown). As shown in Fig. 3, 5,6-EET induced a dose-dependent increase in [Ca2+]i, in astrocytes, which was elevated to 150% of basal by 10^{-10} M 5,6-EET and to a maximum of 310% of basal by 10^{-7} M. The nanomolar to picomolar activity of 5,6-EET suggests that this response was not a result of lipid-induced alterations in membrane fluidity. However, the dose-response curve for 5,6-EET was bell-shaped, and at higher concentration (10^{-6} M), 5,6-EET consistently produced a submaximal response in all experiments. However, this decreased response was not statistically significant. Consistent with reports by Graier et al. (10), we have observed similar 5,6-EET-induced increases in [Ca2+]i, in cultured endothelial cells (data not shown). The hydration products of 14,15- and 5,6-EET, 14,15- and 5,6-dihydroxyeicosatrienoic acid, had no effect on [Ca2+]i, in the astrocyte.

5,6-EET Induces Influx of Extracellular Calcium, and Not Depletion of Intracellular Calcium Stores—If 5,6-EET is CIF, then it should activate influx of extracellular calcium through
SMOC, and should not induce release of calcium from intracellular stores. When astrocytes were placed in calcium-free DPBS supplemented with 0.5 mM EGTA to chelate residual calcium, 5,6-EET did not elevate \([\text{Ca}^{2+}]_i\), suggesting that 5,6-EET-induced influx of extracellular calcium without effect on intracellular calcium stores (Fig. 4A, broken line).

In addition to SMOC calcium influx channels, many cells also have receptor-operated channels (34). Receptor-operated channels are activated by a ligand binding to its receptor, which directly controls the opening of a calcium channel. In contrast, SMOC are controlled by a second messenger (CIF). To discriminate between receptor-operated channels and SMOC we first utilized the inhibitor of SMOC, SKF96365 (35). Fig. 4A shows a typical elevation in \([\text{Ca}^{2+}]_i\) observed in response to 156 nM 5,6-EET in astrocytes. The 5,6-EET-induced elevation in \([\text{Ca}^{2+}]_i\) was completely inhibited by pretreatment with 1 \(\mu\)M SKF96365 (Fig. 4B). Nimodipine (50 \(\mu\)M) had no effect on 5,6-EET-stimulated elevation of \([\text{Ca}^{2+}]_i\), indicating 5,6-EET did not activate voltage-gated calcium channels. Additionally, pretreatment with 100 \(\mu\)M neomycin for 15 min also had no effect on 5,6-EET-stimulated elevation of \([\text{Ca}^{2+}]_i\), suggesting that 5,6-EET did not require phospholipase C activation to elevate \([\text{Ca}^{2+}]_i\) (data not shown).

Further support for 5,6-EET acting directly on SMOC is provided in Fig. 5A. In these experiments, astrocytes were treated with 1 \(\mu\)M thapsigargin in 1 mM calcium-containing DPBS. After the maximum elevation in \([\text{Ca}^{2+}]_i\), was attained in response to thapsigargin, 156 nM 5,6-EET was added. If 5,6-EET activated receptor-operated channels, it is likely that an additional increase in \([\text{Ca}^{2+}]_i\), would be produced (4). However, there was no further elevation in \([\text{Ca}^{2+}]_i\), providing further evidence that 5,6-EET acts directly on SMOC.

Inhibition of Thapsigargin-stimulated \([\text{Ca}^{2+}]_i\) Elevation by Econazole Is Overcome by Addition of 5,6-EET—If 5,6-EET is a CIF, then inhibition of calcium influx with the P450 inhibitor econazole should be reversed by addition of exogenous 5,6-EET. To test this, Fura-2 loaded astrocytes were pretreated with 10 \(\mu\)M econazole for 2 min as shown in Fig. 5B. Next, cells were stimulated with 1 \(\mu\)M thapsigargin. As described previously, in the presence of econazole the thapsigargin-stimulated elevation in \([\text{Ca}^{2+}]_i\), was reduced to a level consistent with depletion of intracellular calcium stores alone. Upon attaining the maximum sustained \([\text{Ca}^{2+}]_i\), in response to thapsigargin, 156 nM 5,6-EET was added. As can be seen in Fig. 5B, 5,6-EET rapidly increased \([\text{Ca}^{2+}]_i\), in the presence of econazole.
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**FIG. 6.** 14,15-EET inhibits 5,6-EET-stimulated elevation of [Ca\(^{2+}\)]. Fura-2 loaded astrocytes were stimulated with 156 nM 14,15-EET followed by 156 nM 5,6-EET as indicated. [Ca\(^{2+}\)], was measured as described in the legend to Fig. 1. Results are representative of six separate experiments performed on different days.

**FIG. 7.** The effect of 5,6-EET on in vivo rat cerebral arteriolar diameter. 5,6-EET was diluted with artificial CSF and cumulative doses were applied under the rat cranial window chamber. Diameter responses were recorded at 3 and 5 min after application. The maximal response was at 3 min and is shown in the figure. Values are the average ± S.E. for responses in 4 rats. a, p < 0.01 versus control for 10\(^{-5}\)–10\(^{-6}\) M; b, p < 0.01 versus 10\(^{-7}\), 10\(^{-8}\) M. Please note that S.E. for 10\(^{-7}\) is too small to show graphically.

**in Astrocytes**—Our previous work (20, 36) and that of others (37, 38) indicates that the primary EET metabolites in astrocytes are 5,6- and 14,15-EET. As described above, 14,15-EET had no effect on [Ca\(^{2+}\)], in astrocytes. However, in six separate experiments, we found that pretreatment of astrocytes with 156 nM 14,15-EET blocked 5,6-EET-induced elevation of [Ca\(^{2+}\)]. A representative tracing of these results is shown in Fig. 6. Subsequent addition of a second dose of 156 nM 5,6-EET counteracted the inhibitory effect of 14,15-EET.

5,6-EET May Also Act as a Paracrine Signal—Our present results in astrocytes suggest that 5,6-EET may be the elusive CIF of the capacitative calcium influx pathway. We have previously shown that 5,6-EET causes dilation of cerebral arterioles when applied topically under an in vivo cranial window in rabbits (36). Leffler et al. (39) have recently shown that 5,6-EET is also a potent dilator of piglet cerebral arterioles. However, the effects of EETs may differ with species. Since the astrocytes used in the above tissue culture experiments were of rat cortical origin, we tested the activity of 5,6-EET on vascular astrocytes used in the above tissue culture experiments were of rat cortical origin, we tested the activity of 5,6-EET on vascular astrocytes used in the above tissue culture experiments were of rat cortical origin, we tested the activity of 5,6-EET on vascular astrocytes used in the above tissue culture experiments were of rat cortical origin.

As shown in Fig. 7, 5,6-EET caused dilation of cerebral arterioles when topically applied to the brain surface. Curiously, the dose-response of 5,6-EET on pial arteriolar diameter, like 5,6-EET-induced [Ca\(^{2+}\)], elevation in astrocytes (Fig. 3), was bell-shaped. 5,6-EET produced a linear increase in arteriolar diameter over the concentration range 10\(^{-9}\)–10\(^{-6}\) M. However, a higher dose of 10\(^{-5}\) M produced a very significantly reduced dilator response. A similar trend at the higher concentration of 5,6-EET has been reported by Leffler et al. (39) in the piglet cerebral microcirculation. This reduced dilator response at 10\(^{-5}\) M suggests inhibition of the dilator stimulus or activation of an opposing constrictor response.

**DISCUSSION**

Since the first reports identifying store-controlled or capacitative calcium influx, the link between intracellular calcium stores and influx of calcium through the plasma membrane has remained obscure. Randriamampita and Tsien (7) isolated a CIF-like substance from stimulated Jurkat cells, which was of molecular weight less than 500 and moderately hydrophobic. Parekh et al. (40) reported that CIF formation involved a phosphatase and a diffusible second messenger. Pasolato et al. (41) have presented evidence suggesting that formation of CIF involved hydrolysis of GTP and possibly a low molecular weight G protein. It has been hypothesized that CIF represents a complex of several different biochemical components (4, 42). Our previous results suggest that the link to CIF requires the activation of cPLA\(_2\) and release of arachidonic acid from cellular phospholipids in U937 cells (8). Other groups have reported similar correlations between arachidonic acid release and calcium influx (43, 44). Cytochrome P450 activity has also been coupled to the formation of CIF (45, 46) and is one of the major pathways through which arachidonic acid is metabolized. In the present report, we present evidence suggesting that the link to CIF involves 5,6-EET, a cytochrome P450 metabolite of arachidonic acid.

Several lines of evidence support a role for 5,6-EET as a component of CIF. First, CIF appears to be ephemeral in nature, having a short half-life. This property has made its identification difficult (4, 7). 5,6-EET is a relatively short-lived metabolite of arachidonic acid, which is rapidly degraded in the aqueous environment, consistent with the properties of CIF. Second, in order to function as CIF, arachidonic acid must first be released from its intracellular phospholipid storage pool through the action of PLA\(_2\), followed by metabolism via cytochrome P450. We have previously reported a coupling between cPLA\(_2\), arachidonic acid release, and depletion of calcium from intracellular stores (8). In the present report, we demonstrate that thapsigargin-stimulated arachidonic acid release is coupled to release of calcium from intracellular stores in the astrocytes. Consistent with our data from U937 cells, inhibition of cPLA\(_2\) activity in the astrocyte effectively blocked capacitative calcium influx, but not release of intracellular calcium stores. Similar to reports from other laboratories (10, 11, 45), inhibition of cytochrome P450 with econazole or SKF525A also inhibited capacitative calcium influx. Furthermore, MS-PFOH, a specific inhibitor of the P450 system that metabolizes arachidonic acid to 5,6-EET, also inhibited capacitative calcium influx, to a level consistent with release of calcium from intracellular stores alone. Thus, the two enzymatic systems which produce 5,6-EET are both coupled to capacitative calcium influx.

We have also shown that 5,6-EET dose dependently activates calcium influx in the astrocyte at nano- to picomolar concentrations. The influx of calcium initiated by 5,6-EET is consistent with CIF for several reasons. First, 5,6-EET did not induce calcium influx in calcium-free medium, suggesting that it has no effect on intracellular calcium stores. Second, 5,6-EET-stimulated calcium influx was blocked by inhibition of SMOC with SKF96365. Third, the elevation in [Ca\(^{2+}\)], produced by 5,6-EET was not additive to that produced by thapsigargin. Fourth,
exogenous 5,6-EET could rapidly overcome econazole inhibition of thapsigargin-stimulated [Ca\(^{2+}\)]\(_i\) elevation. Finally, the 5,6-EET-stimulated [Ca\(^{2+}\)]\(_i\) elevation was unaffected by blockade of voltage-gated channels with nimodipine or inhibition of phospholipase C with neomycin.

Graier et al. (10) have elaborately demonstrated that 5,6-EET is consistent with CIF in endothelial cells. This group has shown that inhibition of P450 blocked capacitative calcium influx, while induction of P450 with dexamethasone/clofibrate enhanced thapsigargin-stimulated [Ca\(^{2+}\)]\(_i\) elevation. Thus, reports from endothelial cells (10), U937 cells (8), and our present report in astrocytes all support the hypothesis that 5,6-EET is coupled to CIF.

In addition to the effects of 5,6-EET on elevation of [Ca\(^{2+}\)]\(_i\), we have also found that 14,15-EET may also participate in 5,6-EET-mediated [Ca\(^{2+}\)]\(_i\) regulation. 14,15-EET effectively blocked 5,6-EET-induced elevation of [Ca\(^{2+}\)]\(_i\). This effect could be overcome with additional doses of 5,6-EET. However, loss of 14,15-EET’s ability to prevent 5,6-EET-induced calcium influx could alternatively be explained by a time-dependent inactivation of 14,15-EET due to its metabolism to the vicinal diol by epoxide hydrolase (20). Malcolm et al. (47) have previously reported that 14,15-EET inhibited capacitative calcium entry in platelets. We (20) and others (15, 48) have previously reported that 14,15-EET inhibited capacitative calcium entry (51, 52). Thus, we speculate that as 5,6-EET activates SMOC calcium influx and [Ca\(^{2+}\)]\(_i\), rises above a certain level, additional opposing intracellular mechanisms may be activated, such as reduced Ins(1,4,5)P\(_3\)-receptor sensitivity (53–55). At the in vivo level we speculate that as intracellular calcium is initially elevated in vascular smooth muscle by 5,6-EET, calcium-activated K\(^+\) channels induce hyperpolarization and smooth muscle relaxation. However, as [Ca\(^{2+}\)]\(_i\), rises above a certain point, calcium-mediated contractile mechanisms may be activated, which counteract the initial dilatation.

In summary, we report that 5,6-EET may be a component of CIF in the astrocyte. Furthermore, our results suggest that 5,6-EET released by astrocytes may regulate pial arteriole diameter. Thus, Ins(1,4,5)P\(_3\)-mediated signaling in astrocytes may be coupled to regulation of cerebral blood flow.

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