20-Hydroxyeicosatetraenoic Acid-induced Vasoconstriction and Inhibition of Potassium Current in Cerebral Vascular Smooth Muscle Is Dependent on Activation of Protein Kinase C

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20-Hydroxyeicosatetraenoic acid (20-HETE), a cytochrome P450 metabolite of arachidonic acid, is a potent vasoconstrictor, and has been implicated in the myogenic activation of renal and cerebral arteries. We examined the role of protein kinase C (PKC) in the signal transduction pathway by which 20-HETE induces vasoconstriction and inhibition of whole-cell K⁺ current in cat cerebral vascular smooth muscle. 20-HETE induced a concentration-dependent constriction in isolated pressurized cat middle cerebral arteries (~20 ± 8% at 1 μM). However, in the presence of an N-myristoylated PKC pseudosubstrate inhibitor peptide (MyrPKC-I₁₉₋₂₇), 20-HETE induced a concentration-dependent vasodilation (26 ± 4% at 1 μM). In whole-cell voltage clamp studies, application of 20-HETE inhibited whole-cell K⁺ current recorded in cat cerebral vascular smooth muscle cells, an effect that was attenuated by MyrPKC-I₁₉₋₂₇. Further evidence for the role of PKC activation in response to 20-HETE is the finding that 20-HETE increased the phosphorylation of myristoylated, alamine-rich PKC substrate in cultured cat cerebral vascular smooth muscle cells in a concentration- and PKC-dependent manner. These data provide evidence that PKC is an integral part of the signal transduction pathway by which 20-HETE elicits vasoconstriction of cerebral arteries and inhibition of whole-cell K⁺ current in cat cerebral vascular smooth muscle.

Arachidonic acid metabolites of the cytochrome P450 monoxygenase pathway have recently been found to play a major role in modulating vascular tone in the renal and cerebral circulations (1–3). The major cytochrome P450 metabolite of arachidonic acid produced in the cerebral and renal vasculature is 20-hydroxyeicosatetraenoic acid (20-HETE)1 (4–6). 20-HETE is a potent vasoconstrictor in isolated cat cerebral and rat renal microvessels over the concentration range of 10⁻¹¹ to 10⁻⁹ M (4, 5). The underlying cellular-ionic mechanism of this vasoconstrictor response appears to be depolarization-induced influx of calcium secondary to inhibition of large conductance calcium-activated potassium channels (KCa) (4, 6, 7). Independent of the depolarization induced by inhibitory effects on KCa, recent data indicate that 20-HETE also activates L-type calcium channels in a concentration-dependent manner, an effect that is antagonized by nifedipine (8, 9).

Several reports identify a role for 20-HETE in the regulation of renal tubular ion transport. In cells of the thick ascending limb of the rat kidney, 20-HETE decreases the open state probability of an apical 70 pS K⁺ channel (10), thus regulating K⁺ recycling across the membrane and Na⁺ resorption. In the medullary thick ascending limb of the loop of Henle, Na⁺/K⁺-(NH₄)₂CO₃ transport activity is reduced by 20-HETE (11). In proximal tubular epithelial cells, the activity of the Na⁺/K⁺-ATPase is reduced by 20-HETE, an effect that is dependent upon activation of protein kinase C (PKC) (12–14). These observations implicate 20-HETE in a diverse array of effector functions. However, the exact signal transduction pathway by which 20-HETE exerts these effects is unknown. Most of the effects described above could be related to an increased activity of PKC (15–17). Because several cis-unsaturated fatty acids, including arachidonic acid and its metabolites, activate PKC (18–20), and activation of PKC decreases the activity of KCa (21–24) and promotes vasoconstriction (25), we hypothesize that the effects of 20-HETE on cerebral arterial tone and whole-cell K⁺ channel current involve activation of PKC. In this report, we provide functional evidence indicating that 20-HETE promotes cerebral vasoconstriction and inhibition of whole-cell K⁺ current via a pathway that involves PKC. We also provide biochemical evidence that 20-HETE increases the phosphorylation of myristoylated, alamine-rich PKC substrate (MARCKS) in cultured cat cerebral vascular smooth muscle cells (VSMCs) in a concentration-related and PKC-dependent manner.

EXPERIMENTAL PROCEDURES

Isolated Pressurized Vessel Studies—Isolated cat middle cerebral arteries (outside diameter, 200–400 μm; length, 10–12 mm) were placed in a perfusion chamber, cannulated with glass micropipettes, and secured in place with 8-O polyethylene suture (Ethicon, Somerville, N.J.), and side branches were tied off with 10-0 polyethylene suture using a stereomicroscope (Carl Zeiss, Inc., Berlin, Germany). The arterial segments were bathed in physiological salt solution (PSS) equilibrated with a 95% O₂-5% CO₂ gas mixture at 37 °C. During the experiment, the outflow cannula was clamped off, and the vessels were pressurized to 80 mm Hg. The inflow cannula was connected in series with a volume reservoir and a pressure transducer (Gould Instruments Division, Cleveland, Ohio) to monitor intraluminal pressure. Internal diameters of the vessels were measured with a video system composed of a CCTV camera (KP-130AU, Hitachi, Tokyo, Japan), a TV monitor (CV-1271, Sony, Tokyo, Japan), and a videomicroimeter system (model 303, Colorado Video, Inc., Boulder, CO). After an equilibration period of...
30 min, the arterial segments were preconstricted with 5 μM serotonin, and the vasodilator response to acetylcholine (1 μM) was determined. Vessels that exhibited no response to either serotonin or acetylcholine were excluded from the study. Cumulative concentration-response curves for 20-HETE (0.1–1000 nM) were obtained, in the absence or presence of the PKC inhibitor Myr-yPKC-I(19–27), by adding it to the bath and allowing a 10-min equilibration period. Internal diameters were measured 2–5 min after application of 20-HETE.

Isolation of Vascular Muscle Cells from Cerebral Microvessels—Cerebral microvessels were isolated according to a protocol published previously (4). Briefly, adult mongrel cats were anesthetized as described above and vessels were isolated by microdissection. Isolated vessels were minced and placed in a low-Ca²⁺ PSS containing 134 mM NaCl, 5.4 mM KCl, 1.2 mM MgSO₄, 0.24 mM KH₂PO₄, 0.05 mM CaCl₂, 11 mM glucose, and 10 mM HEPES, pH adjusted to 7.4 with NaOH. Vessel fragments were transferred to a vial containing 88.5 units/ml collagenase type II, 2 mM dithiothreitol, and 1 mM trypsin inhibitor in low-Ca²⁺ PSS. The vial was placed in a water-jacketed beaker on a microstirrer, and the tissue was stirred (12 rpm) at 37 °C for a total of 1 h in the enzyme solution. At 5-min intervals, the supernatant fractions were collected and checked for appearance of dispersed cells under a microscope; fresh enzyme solution was added to the vessels for continued digestion and fraction collection. Pieces of vessel were disrupted mechanically by forcing them repeatedly through a Pasteur pipette. Fractionation of the cell suspension was transferred to a test tube and diluted with normal PSS and placed on ice. Aliquots of cells were removed from the suspension for immunofluorescence staining with anti-smooth muscle α-actin antibody (Cy3 conjugate, Sigma) and anti-factor VIII antibody (FITC conjugate, Atlantic) to confirm vascular smooth muscle origin of the cells and to assess possible contamination with endothelial cells. VSMCs thus isolated were found to be free of endothelial cell contamination and were used for seeding of cultures and for electrophysiological experiments.

Whole-cell K⁺ Current Recording—Outward whole-cell K⁺ current were recorded at room temperature from cerebral arterial muscle cells using pipette or intracellular solution containing 145 KCl mM, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM EGTA, 2 mM magnesium adenosine triphosphate, 0.1 mM GTP, and 10 mM HEPES, with the final pH adjusted to 7.2 with KOH. The external solution bathing the cells was composed of 140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES, and 10 mM glucose, with pH adjusted to 7.4 with NaOH. Outward whole-cell K⁺ current were elicited every 1 s by depolarizing pulses of 300-ms duration from a holding potential of −70 mV to 80 mV in 10-mV increments. The effect of increasing concentrations of 20-HETE was studied 1 day after application of PKC inhibitor, Myr-yPKC-I(19–27). (Calbiochem). The effect of the PKC activator phorbol 12-myristate 13-acetate (PMA; Sigma) on whole-cell K⁺ current was studied by addition to the bath. The identification of this outward current as Ca²⁺-activated K⁺ current was justified by its sensitivity to blockade by low concentration (1 mM) of tetraethylammonium chloride or charybdotoxin (50 nM) and activation by the calcium ionophore A23187 (1 μM) (data not shown).

Culture of Cerebral Vascular Smooth Muscle—Freshly dissociated cat cerebral VSMCs were prepared as detailed above. Cells were washed three times in RPMI 1640 containing 20% fetal bovine serum and 1% penicillin-streptomycin, then plated onto 35-mm tissue culture dishes, and incubated at 37 °C, 5% CO₂. Cell culture medium was changed twice daily for the first 3 days of culture. After 6 days of culture, the cells were suspended by treatment with trypsin-EDTA in PBS and transferred to 75-cm² tissue culture flasks and grown in media as described above for 1 week. After 1 week, cells were split into 12-well plates for MARCKS assay or frozen in liquid nitrogen for future use.

MARCKS Extraction—Cat cerebral VSMCs were maintained in RPMI 1640 (Life Technologies, Inc.) supplemented with 20% fetal bovine serum (Sigma) and 1% penicillin-streptomycin (Life Technologies, Inc.) and incubated at 37 °C with 95% humidity and 5% CO₂. Cells were seeded into 12-well plates at equal density (10³ cells/well) and allowed to reach 80% confluence (10³ cells/well). MARCKS extraction was performed based on a modification of a protocol published previously and normalized to cell number prior to lysis (26). Prior to [³²P]orthophosphate labeling, cells were starved for 48 h in DMEM F-12/Ham’s medium supplemented with 1% penicillin-streptomycin. Cells were washed once with phosphate-free DMEM (Life Technologies, Inc.), and 100 μCi of [³²P] (DuPont NEN) were added in 1 ml of phosphate-free DMEM per well and incubated for 6 h at 37 °C, 5% CO₂. After 6 h, test compounds 20-HETE, PMA, and PKC inhibitor were added, followed by incubation for 5 min at 37 °C. The medium was removed rapidly, the wells were washed once with PBS, and 175 μl of lysis buffer (10 mM Tris-HCl, pH 7.4, 1 mM NaSO₄, 1 mM Na₂VO₄, 5 mg/ml saponin, 0.2% glycerol, and 0.5% Triton X-100) were immediately added. After 5 min of incubation in lysis buffer at room temperature, cells were scraped into a 1.5-ml microcentrifuge tube, vortexed 20 s, and centrifuged at 20,000 × g for 5 min to pellet debris. The supernatant was removed to a 1.5-ml tube, and 700 μl of ice-cold methanol, 175 μl of ice-cold chloroform, and 550 μl of deionized H₂O were added, vortexed for 30 s, and centrifuged at 9000 × g for 2 min at room temperature. The aqueous phase was removed, and 600 μl of ice-cold methanol were added, followed by centrifugation at 20,000 × g for 5 min to pellet precipitated protein. The supernatant was removed, and the samples were dried in a vacuum concentrator for 5 min to remove residual chloroform/methanol. The pellets were resuspended in 130 μl of 2 × SDS Laemmli sample buffer by vigorous vortexing. 130 μl of 80% acetic acid were added, and the samples were incubated on ice for 30 min. The acid-insoluble material was pelleted by centrifugation at 14,000 × g at 4 °C for 10 min. Acid-soluble proteins (MARCKS) in the supernatant were removed to a fresh tube and dried in a vacuum concentrator for 3 h, washed by resuspension in 500 μl of distilled water, and dried for an additional 3 h under high heat. The final sample was resuspended in 50 μl of 0.25 × SDS sample buffer by vigorous vortexing, boiled 3 min, and loaded along with molecular weight standards (Bio-Rad) onto 3.5% stacking/10% resolving SDS-polyacrylamide gels electrophoresis gels (Bio-Rad Ready Gels). Following SDS-polyacrylamide gel electrophoresis, the gels were electro-photographically transferred to nitrocellulose membranes. membranes were washed once with PBS, wrapped in plastic, and autoradiographed using DuPont Reflections film. Western blotting was performed to confirm equal loading and the identity of MARCKS by probing the membranes with a monoclonal antibody directed against human MARCKS (Upstate Biotechnology, Inc.) (data not shown). Quantitation was achieved by scanning densitometry of autoradiograms (Molecular Dynamics, Personal Densitometer) and verified by scintillation counting of excised nitrocellulose squares corresponding to MARCKS identified by Western blotting. Data are presented as the mean percentage of change in autoradiogram density for experimental treatments (PMA, 20-HETE) from the mean vehicle-treated autoradiogram density.

Statistics—Data are presented as mean ± S.E. where appropriate.
Significant differences represent a $p < 0.05$ using a paired Student's $t$ test.

**Chemicals and Supplies**—20-HETE was purchased from BioMol (Plymouth Meeting, PA), MyrPKC-I(19–27) was from Calbiochem (San Diego, CA), and [32P]orthophosphate was from DuPont NEN (Boston, MA). DMEM, RPMI 1640, and antibiotics were from Life Technologies, Inc. (Bethesda, MD). All other chemicals were supplied by Sigma unless otherwise noted.

**RESULTS**

**Effect of PKC Inhibition on the Vasoconstrictor Actions of 20-HETE**—The effects of increasing concentrations of 20-HETE on the inner diameter of isolated pressurized (80 mm Hg) cat middle cerebral arteries, in the absence or presence of MyrPKC-I(19–27) (50 μM), are depicted in Fig. 1. Inhibition of PKC by this pseudosubstrate peptide has been demonstrated previously to be potent and highly specific (27, 28). Under control conditions, cumulative addition of increasing concentrations of 20-HETE (1 nM, 100 nM, 300 nM, and 1 μM) to the bath resulted in a concentration-dependent reduction in inner diameter. The percentage of change in diameter from baseline averaged $-6 \pm 3\%$ (mean ± S.E.) at 1 nM, $-13 \pm 3\%$ at 100 nM, $-21 \pm 4\%$ at 300 nM, and $-29 \pm 7\%$ at 1 μM ($n = 6$ for all concentrations studied). The vasoconstrictor effect of 20-HETE reached a maximum within 2–5 min. After washout of 20-HETE from the bath, the cannulated arterial segment was pretreated for 10 min with MyrPKC-I(19–27) (50 μM) by addition to the bath. No significant changes in the baseline diameter of the arterial segment were observed for a 10-min period after addition of the inhibitor alone. Cumulative addition of 20-HETE (1 nM to 1 μM) in the presence of MyrPKC-I(19–27) resulted in a concentration-dependent increase in diameter; the percentage of change in diameter averaged $4 \pm 3\%$ (mean ± S.E.) at 1 nM, $13 \pm 6\%$ at 100 nM, $20 \pm 7\%$ at 300 nM, and $27 \pm$
11% at 1 μM (n = 6 for all concentrations studied). The arterial preparations were washed repeatedly with fresh PSS for 30 min, after which time the effect of cumulative addition of 20-HETE (1 nM to 1 μM) to the bath was redetermined. The vasoconstrictor response to 20-HETE returned to the pre-inhibitor treatment level; the percentage of change in diameter from baseline averaged -13 ± 6% (mean ± S.E.) at 1 nM, -19 ± 11% at 100 nM, -28 ± 16% at 300 nM, and -34 ± 19% at 1 μM (n = 6 for all concentrations studied). These results demonstrate that the vasoconstrictor action of 20-HETE can be reversibly abolished by inhibition of PKC and indicates that this response is dependent on a signal transduction pathway in which PKC plays an integral role.

Inhibition of Whole-cell K⁺ Current by 20-HETE Is Dependent on PKC Activation—The effects of 20-HETE on whole-cell K⁺ current in freshly dispersed cat cerebral VSMCs were studied using the whole-cell voltage clamp technique (29). Addition of increasing concentrations of 20-HETE (100–300 nM) to the bathing solution resulted in significant inhibition of peak whole-cell K⁺ current by 38 ± 4% (n = 5) at 100 nM and by 58 ± 9% (n = 5) at 300 nM (p < 0.001 compared with control) (Fig. 2). 20-HETE did not appear to shift the current-voltage relationship, thus suggesting that 20-HETE decreases whole-cell current amplitude by decreasing either the probability of channel opening or the number of active channels. Addition of the MyrPKC-I(19–27) (100 nM) alone did not change the amplitude of the whole-cell K⁺ current as compared with control (Fig. 3). In the presence of MyrPKC-I(19–27) (100 nM), addition of 20-HETE (300 nM) to the bath failed to inhibit whole-cell K⁺ current (Fig. 3), indicating that inhibition of PKC prevents the inhibitory action of 20-HETE on whole-cell K⁺ current. In a separate series of experiments, activation of PKC by addition of 100 nM PMA resulted in a diminution of peak whole-cell outward current (Fig. 4), indicating that known activators of PKC, such as PMA (30), reduce the amplitude of whole-cell K⁺ current in cat cerebral VSMCs. The inhibitory effects of PMA on
whole-cell $K^+$ current were prevented by prior addition of 100 nM MyrPKC-I(19–27). Taken together, these results indicate that 20-HETE inhibits whole-cell $K^+$ current in cat cerebral VSMCs by a mechanism that involves PKC activation.

### 20-HETE Increases PKC-dependent MARCKS Phosphorylation in Cerebral VSMCs

MARCKS is a ubiquitously expressed substrate that has been shown to be the major in vivo target for phosphorylation by PKC (31–33). MARCKS is a heat-stable, acid-soluble protein that migrates at 80–87 kDa on SDS-polyacrylamide gel electrophoresis gels (26). Previous studies in rat aortic smooth muscle cells have demonstrated expression of MARCKS, and the phosphorylation of MARCKS in these cells can be modulated by activators of PKC such as PMA and angiotensin II (26). We exploited these properties of MARCKS to determine if 20-HETE induces a PKC-mediated phosphorylation of MARCKS in primary cultures of cat cerebral VSMCs.

Acetic acid extraction of proteins from $^{32}$P-labeled VSMCs treated with PMA (100 nM) in the absence or presence of MyrPKC-I(19–27) (100 nM) or 20-HETE (100 nM and 1 μM) demonstrated a PKC-dependent effect of PMA and a concentration-related effect of 20-HETE on MARCKS phosphorylation (Fig. 5). Treatment with PMA (100 nM) increased MARCKS phosphorylation by 73 ± 6%, as assessed by scanning densitometry of autoradiograms. The relative number of moles of $^{32}$P incorporated into MARCKS in response to the different treatments was assessed by scintillation counting of excised nitrocellulose squares corresponding to MARCKS. Baseline incorporation was 2.08 ± 0.14 fmol $^{32}$P, whereas treatment with PMA increased incorporation to 9.72 ± 0.53 fmol $^{32}$P, and treatment with 20-HETE increased incorporation to 6.23 ± 0.49 fmol and 7.47 ± 0.65 fmol at 100 nM and 1 μM, respectively ($p < 0.05$ for all with respect to baseline, $n = 6$). To determine if the 20-HETE induced increase in MARCKS phosphorylation was dependent on PKC activation, we treated cells with 1 μM 20-HETE and examined the effect of increasing concentrations of MyrPKC-I(19–27). These results are depicted in Fig. 6. 20-HETE (1 μM)-induced MARCKS phosphorylation was inhibited by MyrPKC-I(19–27) in a concentration-dependent manner and averaged 15 ± 3% (mean ± S.E.) at 1 μM, 26 ± 6% at 10 μM, 79 ± 2% at 50 μM, and 93 ± 0.4% at 100 μM. A fit of this data by a single exponential yielded a value for an IC$_{50}$ of 30.64 ± 13.11 μM MyrPKC-I(19–27).

### DISCUSSION

Recent reports have described several potential roles for 20-HETE in mediating an array of cellular functions, ranging from regulation of vascular tone and myogenic reactivity (4–8, 34, 35) and ion transport in the kidney (10–14) to promotion of mitogenesis and tumorigenesis (36–38). However, there are presently few reports addressing the signal transduction pathways by which 20-HETE exerts these effects. The ability of cis-unsaturated fatty acids, such as arachidonic acid, to serve...
as potent activators of PKC has been well established (18–20). The fact that 20-HETE is a cis-unsaturated fatty acid, which closely resembles arachidonic acid, compelled us to investigate the role of PKC in the signal transduction pathway of this system.

The vasoconstrictor effects of 20-HETE are similar to the previously well-described effects of PKC-activating phorbol esters in isolated pressurized vessels. PMA is a potent vasoconstrictor in feline (39, 40), bovine (41, 42), rat (43), and rabbit cerebral arteries (44), and these effects are reversed by inhibitors of PKC (39–43). The results reported here demonstrate that inhibition of endogenous PKC using a highly specific cell-permeable pseudosubstrate peptide inhibitor (Myr\(^{\gamma}\)PKC-I(19–27)) (27, 28) abolishes the vasoconstrictor actions of 20-HETE in isolated pressurized cat middle cerebral arteries. In the presence of this inhibitor, the vasoconstriction normally induced by 20-HETE becomes a profound vasodilation. The underlying mechanism of this vasodilatory response to application of exogenous 20-HETE observed after blockade of PKC is unknown. However, it could be the result of unmasking of a normally less predominant vasodilatory response to 20-HETE secondary to PKC inhibition. Evidence for the existence of a vasodilatory response to 20-HETE has been documented by several studies in which 20-HETE acted as an endothelium- and cyclooxygenase-dependent vasodilator in the human pulmonary circulation (45), rabbit renal (46, 47), and mesenteric circulations (48).

In the present study, using whole-cell voltage clamp recording, we also demonstrated that inhibition of PKC attenuates the inhibitory effect of 20-HETE on whole-cell \(K^+\) current recorded from cat cerebral VSMCs. Supporting evidence for the involvement of PKC activation in the 20-HETE-induced inhibition of whole-cell \(K^+\) current is the use of PMA, a known activator of PKC (30). PMA attenuated whole-cell \(K^+\) current in a Myr\(^{\gamma}\)PKC-I(19–27)-sensitive manner, similar to the effect of 20-HETE. This finding unequivocally demonstrates that activation of PKC is one of the intracellular signaling pathways involved in the 20-HETE-induced inhibition of whole-cell \(K^+\) current in cat cerebral VSMCs. These findings are consistent with previous reports that demonstrated that activation of PKC in porcine vascular smooth muscle (24), as well as in other tissues such as pancreatic beta cells (22) and pituitary tumor cells (21), results in decreased activity of type-I large conductance calcium-activated potassium channels (\(K_{Ca}\)). Although the exact site of phosphorylation is not clear, analysis of published rat, human, and mouse (GenBank accession nos. U55995, U13913, and U09383, respectively) \(K_{Ca}\) channel alpha subunit sequences using Prosise data base search engines\(^2\) demonstrates the existence of 16 potential phosphorylation sites for PKC. It is unknown whether the \(K_{Ca}\) channel subunits are directly phosphorylated by PKC, or whether another membr

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\(^2\) http://dot.imagen.bcm.tmc.edu:9331/pssprediction/pssp.html

A. Lange, D. Gebremedhin, J. Narayanan, and D. Harder, unpublished observation.
brane bound effector is the target of this phosphorylation. Further studies are required to examine this mechanism at the molecular level.

Other membrane-bound, ion-transporting proteins have been shown to be modulated by 20-HETE (10–14, 49) and by activation of PKC (12, 14, 50). Ominato et al. (12) demonstrated that inhibition of the Na\(^+\)-K\(^+\)-ATPase in rat proximal tubular epithelium by dopamine and parathyroid hormone is dependent on 20-HETE and the phospholipase C-PKC signal transduction pathway. In their study, inhibition of PKC or inhibition of 20-HETE formation could attenuate the inhibition of Na\(^+\)-K\(^+\)-ATPase activity by dopamine or parathyroid hormone. Although no direct analysis of the effect of PKC inhibition on 20-HETE modulation of this transporter was undertaken, 20-HETE and PKC have been implicated in the signal transduction pathway for both of these hormonal effector agents. Recently, Nowicki et al. (14) have demonstrated that the rat renal Na\(^+\)-K\(^+\)-ATPase is directly phosphorylated by PKC in response to 20-HETE, and that the inhibitory effect of 20-HETE on this transporter was abolished when the PKC phosphorylation site of the alpha subunit was mutated. Because both 20-HETE and PKC are known modulators of other membrane ion transport systems, it is logical to ponder whether 20-HETE mediates its effects via PKC in systems other than vascular smooth muscle and renal epithelium.

The data presented in this study for MARCKS phosphorylation in intact cerebral VSMCs provide additional evidence for a role of activation of PKC in the response to 20-HETE. Phosphorylation of MARCKS has been shown to be a specific and sensitive indicator of the level of PKC activity in several different cell types and several different species (26, 30–32). Investigation of the effects of 20-HETE on MARCKS phosphorylation in intact cat cerebral VSMCs revealed that application of exogenous 20-HETE increased phosphorylation of MARCKS, and that this increase was sensitive to inhibition of PKC by MyrPKC-I\(19-27\). Similar effects on MARCKS phosphorylation were observed using PMA as a stimulus for PKC activation. A recent report by Nowicki et al. (14) demonstrated that 20-HETE increases the phosphorylation of both histone and purified Na\(^+\)-K\(^+\)-ATPase protein by purified PKC in vitro, and increases the calcium sensitivity of the kinase at physiological calcium concentrations. In the same study, it was also shown that 20-HETE increased the translocation of PKC\(\alpha\) from the cytosol to the membrane in COS cells. Thus, it is possible that PKC\(\alpha\) may be the target PKC isozyme for the actions of 20-HETE. It is known that several isozymes of PKC including conventional (cPKCs) and novel (nPKCs) isozymes can phosphorylate MARCKS in intact cells (32, 51). Given that the effect of 20-HETE on other PKC isozymes has not been investigated and that different cells express different profiles of PKC isozymes (15), it is possible that several different isozymes may be involved in the response of PKC to 20-HETE. Further investigation to determine which PKC isozymes are activated by 20-HETE are needed.

In summary, we have demonstrated that the vasoconstrictor effects of 20-HETE in cerebral arteries and inhibition of whole-cell K\(^+\) current in cat cerebral VSMCs involve a pathway that is dependent upon activation of PKC. Furthermore, 20-HETE increases PKC activity in cat cerebral VSMCs, as assessed by assay of MARCKS phosphorylation. The elucidation of the underlying pathways by which 20-HETE mediates its vascular, membrane ionic, and mitogenic effects will aid in the determination of the role that 20-HETE plays in the mediation of complex responses such as myogenic activation of cerebral and renal arteries.

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