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12(*R*)-Hydroxyicosatetraenoic acid: A cytochrome P450-dependent arachidonate metabolite that inhibits Na^+,K^+-ATPase in the cornea

(mass spectrometry/deuterium/SKF 525A)

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ABSTRACT **When corneal microsomes were incubated with arachidonic acid in the presence of an NADPH-generating system, four polar metabolites (compounds A–D) were formed. Synthesis of these metabolites could be inhibited by carbon monoxide, SKF 525A, and anti-cytochrome *c* reductase antibodies. One of the metabolites, compound C, was found to inhibit partially purified Na^+,K^+-ATPase from the corneal epithelium in a dose-dependent manner with an ID_{50} of ≈50 nM. After compound C was purified by TLC and HPLC, it was found to have a UV absorption spectrum with a maximum absorbance at 236 nm suggesting the presence of a conjugated diene. Mass spectrometric analysis using positive- and negative-ionization modes was carried out on derivatized compound C that had been synthesized from a mixture of specifically labeled ([5,6,8,9,11,12,14,15-$^2H_8$]arachidonic acid) and unlabeled arachidonic acid. Abundant fragment ions were consistent with compound C being a monooxygenated derivative of arachidonic acid with a hydroxyl substituent at carbon-12 of the icosanoid backbone; all deuterium atoms from [$^2H_8$]arachidonate were retained in the structure. Oxidative ozonolysis yielded products indicating double bonds between carbons at positions 10 and 11 and positions 14 and 15 of the 20-carbon chain. Compound C was, therefore, characterized as a 12-hydroxyicosatetraenoic acid. However, only 12(*R*) isomer was found to be an inhibitor of the Na^+,K^+-ATPase from the corneal epithelium, suggesting that the biologically active compound C was 12(*R*)-hydroxy-5,8,10,14-icosatetraenoic acid. Such an inhibitor of Na^+,K^+-ATPase synthesized in the cornea may have an important role in regulating ocular transparency and aqueous human secretion.**

Cytochrome P450-dependent monooxygenase represents a third pathway by which arachidonic acid can be metabolized in animal tissues. The cytochrome P450 system converts arachidonic acid to several oxygenated metabolites: monohydroxyicosatetraenoic acids, epoxyicosatrienoic acids (which can undergo hydrolysis by epoxide hydrolase to form the diol derivatives), and the ω, ω-1, and ω-2 hydroxylation products (1–4). The formation of these metabolites is strictly dependent on NADPH and on molecular oxygen and is inhibited by carbon monoxide and SKF 525A (5).

The epithelium is the outermost layer of the cornea and generally constitutes 10–15% of the total corneal thickness. The cornea, which consists of five or six layers of cells and is organized in a functional syncytium, plays a major role in the secretion of chloride ions from the aqueous or endothelial side towards the tear side (6). This active transport of chloride in the epithelium assists the endothelium in maintaining stromal dehydration and, thus, corneal transparency (7). The movement of chloride occurs against an electrochemical gradient across the basolateral membrane of the epithelium by way of a Na^+,K^+-carrier. Essential for the operation of this carrier system is the transmembrane sodium gradient maintained by the activity of the Na^+,K^+-ATPase (8).

We have demonstrated (9, 10) the presence of the cytochrome P450 system in the corneal epithelium and shown that this system, in the presence of NADPH and molecular oxygen, metabolized arachidonic acid to several oxygenated metabolites. Two cytochrome P450-dependent metabolites (compounds C and D) of the cornea were found to possess biological activities, namely inhibition of the corneal Na^+, K^+-ATPase and vascular reactivity (10).

In the present study, we described the structural elucidation of compound C, the Na^+,K^+-ATPase inhibitor.

EXPERIMENTAL PROCEDURES

Materials. Arachidonic acid was from NuCheck (Elysian, MN), and [1-^{14}C]arachidonic acid (56 μCi/mmol; 1 Ci = 37 GBq) was obtained from Amersham. [5,6,8,9,11,12,14,15-^{3}H(N)]Arachidonic acid (220 Ci/mmol) was obtained from New England Nuclear. [5,6,8,9,11,12,14,15-$^2H_8$]Arachidonic acid was a gift from Howard Sprecher, Ohio State University. NADPH, NADP, and glucose 6-phosphate were obtained from Sigma. Glucose 6-phosphate dehydrogenase was purchased from Boehringer Mannheim. 12(*S*)- and 12(*R*)-hydroxy-5,8,10,14-icosatetraenoic acid [12(*S*)-HETE and 12(*R*)-HETE, respectively] were from Biomol, Research Laboratories (Philadelphia, PA). Bis(trimethylsilyl) trifluoroacetamide was obtained from Supelco. Diazald, which was used to prepare ethereal solutions of diazomethane, and 5% Rh/95% Al_2O_3 by weight were obtained from Aldrich. All solvents were HPLC grade.

Preparation of Corneal Microsomes. Fresh bovine eyes were obtained from the local abattoir. They were collected within 10 min after slaughtering and immediately immersed in an ice-chilled saline solution and brought to the laboratory on ice within 1–2 hr. The eyes were washed twice with 0.9% saline, and the corneal epithelium was gently scraped off into isotonic phosphate-buffered saline, pH 7.4 (PBS), and homogenized by using a glass tissue grinder operated at low speed. The homogenate was centrifuged at 1500 × *g* for 10 min, and the supernatant was centrifuged at 10,000 × *g* for 20 min. The 10,000 × *g* supernatant was further centrifuged at 105,000 × *g* for 90 min, and the resulting microsomal pellet was resuspended in PBS.

Arachidonate Metabolism. The incubation mixture contained 3 mg of microsomal protein, 7 μM [^{14}C]arachidonic

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Abbreviations: 12(*R*)-HETE, 12(*R*)-hydroxy-5,8,10,14-icosatetraenoic acid; 12(*S*)-HETE, 12(*S*)-hydroxy-5,8,10,14-icosatetraenoic acid.

acid, and an NADPH-generating system (0.1 mM glucose-6-phosphate/0.4 μM NADP/1 unit of glucose-6-phosphate dehydrogenase). The incubation was carried out for 30 min at 37°C. In some experiments, corneal microsomes (3 mg of protein per ml) were incubated with a mixture of [^{14}C]arachidonic acid diluted with unlabeled arachidonic acid (30,000 cpm/μg) and [^{2}H]arachidonic acid in a molar ratio of 3:1. The incubations were carried out for 30 min at 37°C in the presence of an NADPH-generating system. The reaction was terminated by acidification to pH 4.0, and the arachidonate metabolites were extracted with ethyl acetate. Extraction efficiency was 60–70%. The final extract was resuspended in 200 μl of methanol, and arachidonate metabolites were separated by reverse-phase HPLC.

Pulse-Labeling Experiment in Epithelial Cells. Intact corneal epithelial cells were isolated as described above and washed twice with PBS. Approximately 1–2 × 10^7 cells were incubated with [^{3}H]arachidonic acid (2 μCi; 220 Ci/mmol) for 5 min at 37°C. The reaction was terminated by acidification; radiolabeled metabolites were extracted and separated by reverse-phase HPLC as described below.

Separation and Purification of Arachidonate Metabolites. Reverse-phase HPLC was performed on C_{18} Microsorb column (250 × 4.6 mm, Rainin Instrument, Woburn, MA) using a linear gradient of 1.25% (vol/vol) increase in acetonitrile per min from acetonitrile/water/acetic acid, 50:50:0.1 (vol/vol), to acetonitrile/acetic acid, 100:0.1 (vol/vol), at a flow rate of 1 ml/min. Radioactivity was monitored by a flow detector (Radiometric Instrument and Chemical, Tampa, FL), and 0.5-ml fractions were collected.

Fractions containing compound C were pooled, evaporated, and resuspended in methanol. Methylation was performed with freshly prepared ethereal solution of diazomethane. The methylated fraction was further purified by TLC on silica gel G (Analtech, Newark, DE) using a mixture of hexane/ethyl acetate, 3:1 (vol/vol), as the solvent system. The methylated compound was reextracted with methanol and subjected to further derivatization.

Spectrometric Analysis. Gas chromatography/mass spectrometry was carried out on a Nermag 1010C gas chromatograph/mass spectrometer using either electron-impact (70 eV; 1 eV = 1.602 × 10^{-19} J) ionization conditions or chemical ionization with methane as reagent gas (0.1 torr; 1 torr = 133.3 Pa) and measuring either positive or negative ions. Gas chromatographic separations were carried out on a 10-m capillary DB-1 column (20 μm, J&W Scientific, Rancho Cordova, CA). Helium was used as carrier gas at a flow rate of 50 cm/sec. The methyl ester trimethylsilyl ether derivatives of compound C with and without prior catalytic reduction were prepared as described for electron-impact gas chromatography/mass spectrometry (11). These derivatives were also studied by positive ion-chemical ionization. Electron-capture negative-ion chemical-ionization mass spectrometry was used to analyze the pentafluorobenzyl ester trimethylsilyl ether derivative of compound C which was prepared essentially following the method of Strife and Murphy (12). Ultraviolet spectroscopy was carried out using a Hewlett–Packard model 8452A photodiode array spectrophotometer (Palo Alto, CA) using 20% (vol/vol) methanol in water as solvent.

Preparation and Measurement of Na^+,K^+-ATPase Activity. Partially purified corneal epithelial Na^+,K^+-ATPase was prepared as described by Jorgenson (13). Briefly, corneal epithelial microsomes were solubilized with $NaDodSO_4$ as follows: microsomes (1.4 mg/ml) were incubated with $NaDodSO_4$ (0.56 mg/ml) in 2 mM EDTA/50 mM imidazole/3 mM ATP, pH 7.5, for 45 min at room temperature with continuous stirring. The solubilized microsomes were then applied on discontinuous density gradients and centrifuged at 110,000 × *g* for 90 min; the gradient consisted of three successive layers of sucrose 29.4%, 15%, and 10% (wt/vol). The pellet was resuspended in 25 mM imidazole/1 mM EDTA, pH 7.5, and stored at 20°C. The activity of Na^+,K^+-ATPase was measured as the rate of release of inorganic phosphate in 30 mM histidine/20 mM KCl/130 mM NaCl/3 mM $MgCl_2$/3 mM ATP, pH 7.5 (13). After equilibration for 5 min at 37°C, 10 μl of the enzyme preparation (1–5 μg of protein) was added to 0.5 ml of reaction mixture. The reaction was allowed to proceed for 30 min at 37°C. The compounds to be tested were dissolved in PBS and added to the enzyme 10 min prior to the addition of the reaction mixture. Ouabain was dissolved in distilled water. The reaction was terminated by the addition of 0.8 ml of color reagent containing ammonium molybdate/malachite green/sterox as described by Candia *et al.* (14). After 1 min, 100 μl of 34% (wt/vol) sodium citrate solution was added and mixed. The final solution was then read at 660 nm in a Beckman DB spectrophotometer. All solutions were read against references containing the same concentration of ATP as the incubation tubes. Enzyme activity was expressed as μmol of P_i released per hr per mg of protein.

RESULTS

Production of Compound C by Epithelial Microsomes. Incubation of corneal epithelial microsomes with arachidonic acid in the presence of an NADPH-generating system resulted in the formation of several oxygenated metabolites (Fig. 1). The major polar metabolites were compounds A and B with retention times of 8.5 and 11.0 min, respectively. The two nonpolar metabolites C and D had retention times of 23.0 and 24.8 min, respectively.

As summarized in Table 1, the corneal microsomes had high activity of NADPH-dependent metabolism of arachidonic acid, whereas the non-NADPH-dependent metabolism was low and probably represents cyclooxygenase activity that could be inhibited by indomethacin. Formation of the NADPH-dependent metabolites (compounds A, B, C, and D, Fig. 1) was inhibited by preincubating the microsomes for 5 min with carbon monoxide (70% inhibition) or SKF 525A (60% inhibition); both are inhibitors of cytochrome P450-

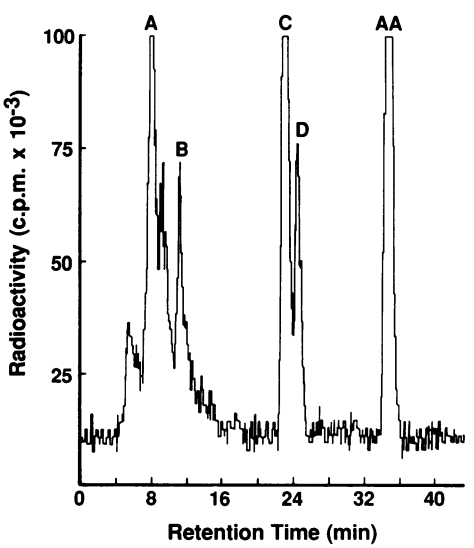

FIG. 1. Reverse-phase HPLC separation of arachidonate metabolites formed by corneal epithelial microsomes in the presence of NADPH. Microsomes (3 mg of protein) were incubated with 7 μM [^{14}C]arachidonic acid in the presence of NADPH-generating system for 30 min at 37°C. The reaction was terminated by acidification, and radiolabeled metabolites were extracted and separated by reverse-phase HPLC. A–D, compounds A–D; AA, arachidonic acid.

Table 1. Effect of inhibitors of arachidonate metabolism on the NADPH-dependent conversion in bovine corneal microsomes

Inhibitor	Activity, nmol of AA converted per mg of protein per 30 min
None	0.33 ± 0.10
NADPH	2.65 ± 0.25
NADPH/carbon monoxide	0.72 ± 0.17
NADPH/SKF 525A (100 μM)	1.05 ± 0.28
NADPH/anti-cyt *c* IgG (1 mg)	0.63 ± 0.21
NADPH/indomethacin (10 μM)	2.78 ± 0.30

Microsomes (3 mg/ml) were incubated with 7 μM [^{14}C]arachidonic acid in the presence of the NADPH-generating system. SKF 525A, dissolved in water, and indomethacin, dissolved in 4.2% (wt/vol) $NaHCO_3$, were added to the microsomes 10 min prior to the addition of [^{14}C]arachidonic acid. The antibodies against cytochrome *c* reductase (anti-cyt *c* IgG) were prepared as described (15) and incubated with the microsomes 30 min prior to the addition of [^{14}C]arachidonic acid. Carbon monoxide was bubbled into the microsomal solution for 5 min prior to the addition of the substrate. No conversion of arachidonic acid (AA) was seen when boiled microsomes were incubated in the presence or absence of NADPH (data not shown). The results are the mean ± SEM, $n = 3$.

dependent enzymes. In addition, antibodies raised against cytochrome *c* reductase, the rate-limiting component of the cytochrome P450, inhibited the reaction by 70%, an inhibition that was dependent on the weight ratio of antibodies to microsomal protein (15). Thus, it was clear that these arachidonic acid metabolites were derived from an NADPH-dependent cytochrome P450 pathway, a system shown (9, 10) to be present in this tissue. Compounds C and D were both formed by the microsomes from exogenous arachidonic acid in an arachidonic acid/compound molar ratio of 3:1 or 2:1 for compound C and compound D, respectively. However, in pulse-labeling experiments in which a very low concentration of 5 nM [^{3}H]arachidonic acid was incubated with intact corneal epithelial cells, compound C was the only one to be formed (Fig. 2). (Since this amount of [^{3}H]arachidonic acid was not sufficient to stimulate the enzyme but only to label the pool of free arachidonate in the cell, these results represent the basal endogenous conversion of arachidonic acid.)

Structural Studies. When compound C was analyzed by ultraviolet spectroscopy, a smooth absorption curve with a UV maximum at 236 nm was observed (data not shown). Furthermore, analysis of the effluent with an HPLC photodiode array detector revealed that this UV spectrum was observed uniquely

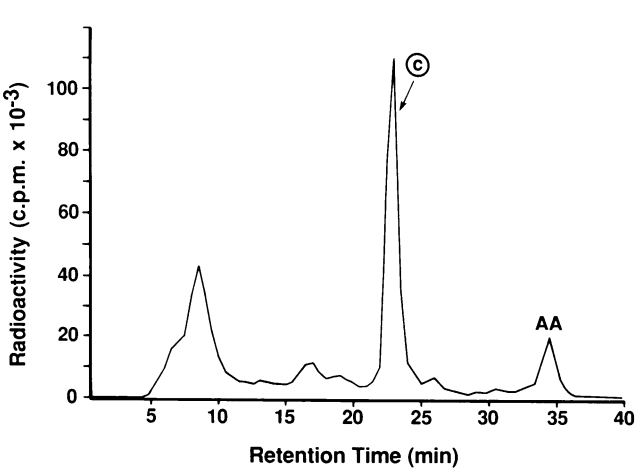

FIG. 2. Conversion of [^{3}H]arachidonate by corneal epithelial cells. Cells were incubated with 1 μCi of [^{3}H]arachidonate for 5 min at 37°C. The reaction was terminated by acidification, and arachidonate metabolites were extracted and separated by reverse-phase HPLC. C, compound C; AA, arachidonic acid.

for the radioactive component C. Spectroscopic analysis of compound D indicated that this metabolite had no UV absorbing characteristics.

When experiments were carried out using the mixture of [$^2H_8$]arachidonic acid and unlabeled arachidonic acid, gas chromatography/mass spectrometry analysis of the methyl ester trimethylsilyl derivative of compound C under chemical-ionization conditions revealed a major ion at m/z 295 with the ^{2}H-labeled isotope peak 6 mass units higher at m/z 301 (Fig. 3*A*). Further, ions in the mass spectra of the methyl ester trimethylsilyl ether derivative were at m/z 407 (MH^+), m/z 391 (M − 15), m/z 317 (M − Me_3SiO) all of which had ^{2}H satellite peaks. Thus, this data indicated that the metabolite that was being observed had origins from arachidonate as well as [$^2H_8$]arachidonate. The most abundant ion at m/z 295 was consistent with fragmentation adjacent to a trimethylsiloxy group and is the most abundant ion in the chemical ionization mass spectrum of the methyl ester trimethylsilyl ether derivative of 12-HETE. Further substantiation of the position and number of hydroxyl groups in compound C was obtained by catalytic hydrogenation followed by derivatization and electron-impact ionization mass spectrometry. The mass spectra of the trimethylsilyl ether methyl ester derivative of hydrogenated compound C had a base peak at m/z 301 and another major ion at m/z 215, which would correspond to cleavage adjacent to the trimethylsilyl ether oxygen (data not shown). Other significant ions were observed at m/z 399 (M − 15), m/z 367 (M − 47), and a trimethylsilyl migration ion at m/z 272 (11). Since neither of these positive ion mass spectra resulted in significant ions containing the entire 20-carbon chain of compound C, electron-capture negative-ion chemical-ionization mass spectrometry of the pentafluorobenzyl ester trimethylsilyl ether derivative compound C was determined as shown in Fig. 3*B*. This mass spectrum was dominated by the carboxylate anion due to the loss of the pentafluorobenzyl group and charge retention on the carboxyl anion. The ion at m/z 391 was consistent with a monohydroxy-substituted icosatetraenoic acid and supported the structure of compound C as a 12-hydroxyicosatetraenoic acid.

The position of the double bonds, in part, was carried out by formation of the menthoxycarbonyl derivative followed by oxidative ozonolysis. The products were analyzed by gas chromatography/mass spectrometry following methylation of the resultant dicarboxylic acids. From degradation of compound C, a compound corresponding to the menthoxycarbonyl derivative of dimethyl 2-hydroxymalate was observed (16). This data was consistent with double bonds between positions 14 and 15 and positions 10 and 11. This data, combined with the UV spectrum of compound C that indicated the presence of a conjugated diene, led to the structural identification of this molecule as 12-hydroxy-5,8,10,14-icosatetraenoic acid. Compound C was furthermore found to have the identical gas chromatographic retention time and HPLC retention time as 12-hydroxy-5,8,10,14-icosatetraenoic acid derived from platelets.

We have demonstrated (10) the ability of compound C to inhibit the Na^+,K^+-ATPase activity of membrane fractions from bovine corneal epithelium. In this study we prepared a partially purified Na^+,K^+-ATPase from the corneal epithelium. The preparation was inhibited (20–85%) by ouabain (0.01–1 mM). The inhibitory effects of compound D and compound C were compared to those of the chemically synthesized 12(*R*)-HETE and 12(*S*)-HETE. As seen in Fig. 4, 12(*R*)-HETE was the only stereoisomer that inhibited Na^+,K^+-ATPase to the same degree as the purified compound C. The isomer 12(*S*)-HETE had no significant effect on Na^+,K^+-ATPase activity. Thus, compound C could correspond to 12(*R*)-hydroxy-8,10,14-icosatetraenoic acid. The potency to inhibit Na^+,K^+-ATPase of compound D was at least 50% of that of compound C. Unfortunately, insufficient material was available for direct

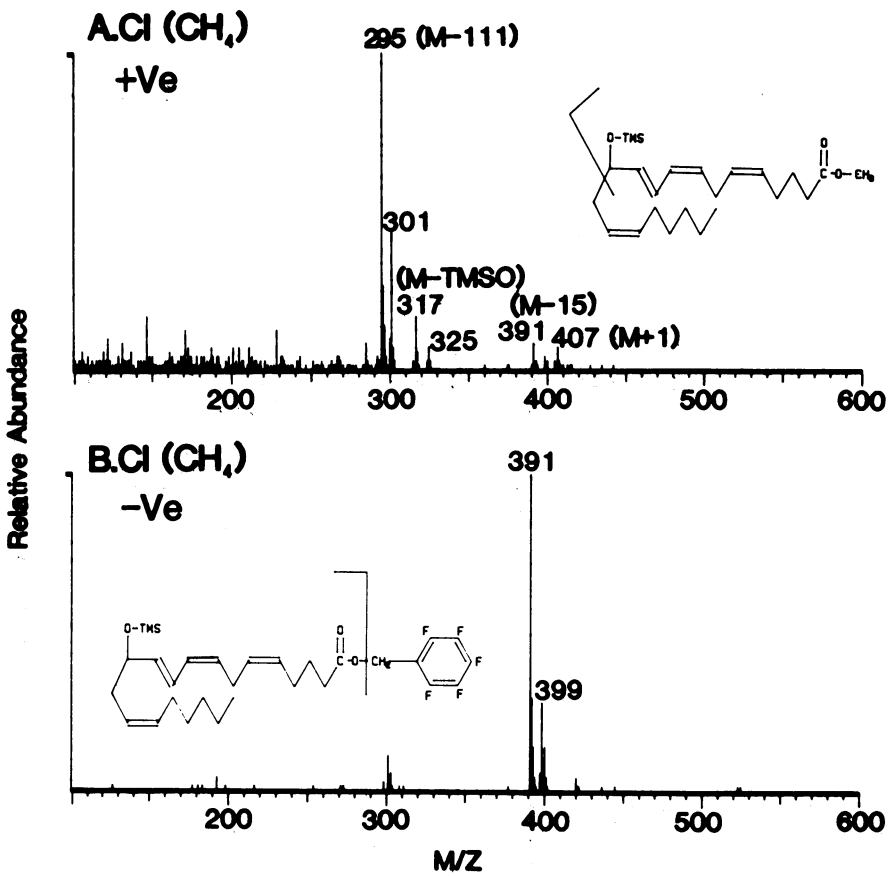

FIG. 3. (*A*) Positive-ion chemical-ionization mass spectrum of the methyl ester trimethylsilyl ether derivative of compound C obtained following incubation of corneal microsomes with a molar mixture of unlabeled and ^{2}H-labeled arachidonic acid, 3:1. (*B*) Negative-ion chemical-ionization mass spectrum of the pentafluorobenzyl trimethylsilyl ether derivative of compound C obtained as described in *A*.

stereochemical analysis of compound C or assessment of optical purity.

DISCUSSION

The present study described the formation of 12(*R*)-HETE as one of the major cytochrome P450-dependent arachidonate metabolites in the corneal epithelium and as an endogenous inhibitor of Na^+,K^+-ATPase. Capdevila *et al.* (17) demonstrated that hepatic microsomal cytochrome P450 converted arachidonic acid to the monohydroxyicosatetraenoic acid and that synthesis of the 12(*R*) isomer was highly preferred to that of the 12(*S*) isomer. In addition, Woolard *et al.* (18) demonstrated that human skin from psoriatic lesions converted arachidonate to 12(*R*)-HETE and suggested that a cytochrome P450-dependent enzyme was the metabolic pathway responsible for the formation of the 12(*R*)-HETE. In this study, we provide further evidence that 12(*R*)-HETE is a cytochrome P450-dependent metabolite of arachidonate based on NADPH dependency, inhibition by carbon monoxide, inhibition by SKF 525A, and inhibition of synthesis by antibodies directed against cytochrome *c* reductase. We further demonstrate the comparable biological properties of synthetic 12(*R*)-HETE to the biologically derived metabolite compound C and provide evidence for the potential endogenous formation of this compound.

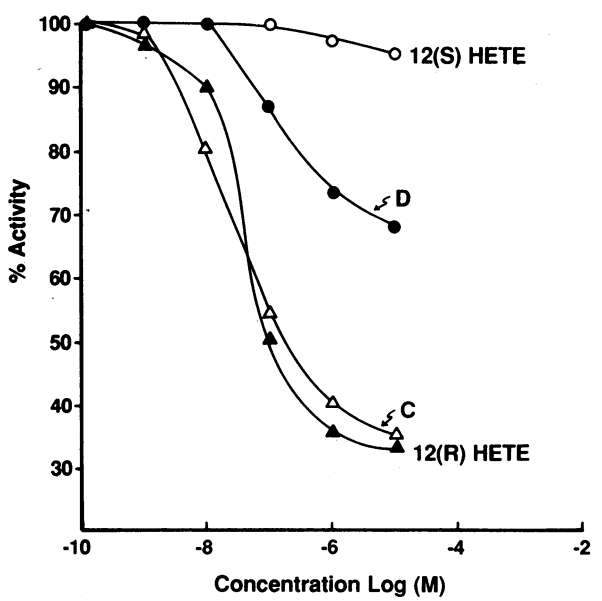

FIG. 4. Effects of arachidonic acid metabolites on the corneal Na^+,K^+-ATPase activity. Na^+,K^+-ATPase, partially purified from bovine corneal epithelium, was preincubated with the various compounds for 10 min before the addition of the reaction mixture containing ATP. ATPase activity was determined as the release of inorganic phosphate. The specific activity of the partially purified Na^+,K^+-ATPase was 28.8 ± 3.7 μmol per hr per mg, and 85% of this activity was inhibited by 1 mM ouabain. Results are the mean ± SEM, $n = 3$.

Such an endogenous inhibitor of Na^+,K^+-ATPase synthesized in the cornea may have fundamental importance in ocular-transporting epithelia that rely on this pump mechanism. Ocular-transporting epithelia include the corneal epithelium and endothelium, the epithelia of the ciliary body, the lens subcapsular epithelium, and the retinal pigment epithelium. Deturgescence of the cornea mediated by the corneal endothelium and secretion of aqueous humor by ciliary body are examples of basic physiological functions dependent upon metabolic pumps that are readily inhibited by digitalis glycosides such as ouabain. Product(s) of cytochrome P450-dependent metabolism of arachidonic acid may modulate these and other important functions in the eye (e.g., maintenance of lenticular transparency and integrity of visual receptor–retinal pigment epithelium interrelationships) and may be important for normal aqueous humor formation. Perturbations of this pathway may play a role in the pathogenesis of certain diseases such as Fuch corneal dystrophy,

which is characterized by failure of the endothelial pump causing stromal and epithelial edema and perhaps even the development of spontaneous cataract formation. Kinoshita (19) has shown that in mice of the Nakano cataract strain that develop a spontaneous cataract after birth there is a failure of lens Na^+,K^+-ATPase due to the presence of an unidentified inhibitor (or inactivator) of Na^+,K^+-ATPase. If indeed the pathway functions in the modulation of aqueous humor secretion, then manipulation of the pathway might be useful in the management of ocular hypertension.

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