Title
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Journal
Journal of Biological Chemistry, 263(32)

ISSN
0021-9258

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Publication Date
1988-11-01

DOI
10.1016/s0021-9258(18)37431-3

THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Vol. 263, No. 32, Issue of November 15, pp. 16591-16596, 1988
Printed in U.S.A.

Formation and Biological Activity of 12-Ketoeicosatetraenoic Acid in the Nervous System of *Aplysia**

(Received for publication, June 24, 1988)

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12-Hydroperoxy-5,8,10,14-eicosatetraenoic acid (12-HPETE), a lipoxygenase product, simulates the synaptic responses produced by the modulatory transmitter, histamine, and the neuroactive peptide, Phe-Met-Arg-Phe-amide (FMRFamide), in identified neurons of the marine mollusk, *Aplysia californica* (Piomelli, D., Shapiro, E., Feinmark, S. J., and Schwartz, J. H. (1987) *J. Neurosci.* 7, 3675–3886; Shapiro, E., Piomelli, D., Feinmark, S., Vogel, S., Chin, G., and Schwartz, J. H. (1988) *Cold Spring Harbor Symp. Quant. Biol.* 53, in press). The 12-lipoxygenase pathway has not yet been fully characterized, but 12-HPETE is known to be metabolized further. We therefore began to search for other metabolites in order to investigate whether the actions of 12-HPETE might require its conversion to other active products. Here we report the identification of 12-keto-5,8,10,14-eicosatetraenoic acid (12-KETE), a metabolite of 12-HPETE formed by *Aplysia* nervous tissue. This product was identified in incubations of the tissue with arachidonic acid using high performance liquid chromatography, UV spectrometry, and gas chromatography/mass spectrometry. [^{3}H]12-KETE was formed from endogenous lipid stores in nervous tissue, labeled by incubation with [^{3}H]arachidonic acid, when stimulated by application of histamine. In L14 and L10 cells, identified neurons in the abdominal ganglion, applications of 12-KETE elicit changes in membrane potential similar to those evoked by histamine. 12(*S*)-Hydroxy-5,8,10,14-eicosatetraenoic acid, another metabolite of 12-HPETE, is inactive. These results support the hypothesis that 12-HPETE and its metabolite, 12-KETE, participate in transduction of histamine responses in *Aplysia* neurons.

Evidence has been presented that lipoxygenase metabolites of arachidonic acid act as second messengers in neurons (Piomelli *et al.*, 1987a and b). In nervous tissue of *Aplysia*, the modulatory neurotransmitter histamine and the neuroactive tetrapeptide FMRFamide[1] stimulate release of 12-hydroxyeicosatetraenoic acid (12-HETE), a stable end product of the 12-lipoxygenase pathway (Piomelli *et al.*, 1987a, 1987b). 12-HPETE, the short-lived precursor of 12-HETE, mimics the increase in outward K^+ current produced by Phe-Met-Arg-Phe-amide in sensory neurons (Piomelli *et al.*, 1987b; Belardetti *et al.*, 1987) and the change in membrane potential elicited by histamine in L14 cells (Shapiro *et al.*, 1988). 12(*S*)-HETE is inactive (Piomelli *et al.*, 1987b; Shapiro *et al.*, 1988). These results suggest that 12-HPETE, or a metabolite derived from it, serves as an intracellular signal that mediates the synaptic actions of Phe-Met-Arg-Phe-amide and histamine. The recent identification of several novel metabolites derived from 12-HPETE in mammalian tissues (Pace-Asciak *et al.*, 1983; Glasgow *et al.*, 1986; Fruteau de Laclos *et al.*, 1987) raises the possibility that modulation of membrane ionic conductances produced by 12-HPETE in *Aplysia* neurons might result from its conversion to an active metabolite.

In this study, we report that: 1) nervous tissue of *Aplysia* converts arachidonate to 12-KETE, a metabolite of 12-HPETE that was recently identified in human platelets (Fruteau de Laclos *et al.*, 1987); 2) synthesis of 12-KETE is stimulated by applying histamine to *Aplysia* nervous tissue; and 3) application of 12-KETE, like 12-HPETE, mimics the electrophysiological responses produced by histamine in two identified *Aplysia* neurons, L10 and L14. Our experiments support the idea that these metabolites of arachidonic acid participate in mediating some of the synaptic actions of histamine.

EXPERIMENTAL PROCEDURES

Aplysia weighing 70–200 g (Howard Hughes Medical Institute Mariculture Research Facility, Woods Hole Oceanographic Institution, Woods Hole, MA, and Marinus, Sand City, CA) were kept in aquaria at 15 °C. Central ganglia with nerves attached were removed through an incision in the foot from animals anaesthetized by injection of isotonic $MgCl_2$. The ganglia were homogenized in cold supplemented artificial seawater (Eisenstadt *et al.*, 1973) using a Polytron (Brinkmann Instruments) after removal of the connective tissue sheath. Alternatively, after the sheath was removed, isolated neural components (cell bodies and neuropil) were incubated for 2 h in seawater (0.2 ml) containing [^{3}H]arachidonic acid (25 μCi/ml, Amersham Corp., 85–135 Ci/mmol). This incubation resulted in the incorporation of labeled arachidonic acid into cellular phospholipids (Piomelli *et al.*, 1987a).

Extraction of Lipids—Acetone (0–4 °C) was added to homogenates (1:1, v/v), and the resulting precipitate was removed by low-speed centrifugation. The supernatant was acidified to pH 3.5 and metabolites extracted twice with ethyl acetate (2 volumes). The organic layers were combined, dried over sodium sulfate, and evaporated under vacuum. Samples from experiments with prelabeled nervous tissue were extracted with ethyl acetate without prior addition of acetone.

High Performance Liquid Chromatography (HPLC)—Analytical normal-phase HPLC was carried out using a silica column (250 × 4.6 mm, 5 μm, Supelco, Bellefonte, PA) eluted isocratically with hex-

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[1] The abbreviations used are: FMRFamide, Phe-Met-Arg-Phe-amide; 12-HETE, 12-hydroxyeicosatetraenoic acid; 12-HPETE, 12-hydroperoxy-5,8,10,14-eicosatetraenoic acid; 12-KETE, 12-keto-5,8,10,14-eicosatetraenoic acid; 12-ODTE, 12-oxo-5,8,10-dodecatrienoic acid; PFB, pentafluorobenzyl; NDGA, nordihydroguaiaretic acid; HPLC, high performance liquid chromatography; GC/MS, gas chromatography/mass spectrometry; 12(*S*)-HETE, 12(*S*)-hydroxy-5,8,10,14-eicosatetraenoic acid.

ane:isopropyl alcohol:acetic acid (98:2:0.1, v/v/v) at a flow rate of 1 ml/min. Absorbance was monitored continuously at 270 nm, and full UV spectra were taken using a diode-array spectrophotometer (Hewlett-Packard 1090M, Palo Alto, CA); 30-s fractions were collected and radioactivity measured by liquid scintillation. Purifications on a preparative scale were carried out using a Polygosil silica column (500 × 10 mm, 10 μm, Alltech) eluted with the same solvent system at a flow rate of 3 ml/min. Reversed-phase HPLC was performed with a Nucleosil C18 column (250 × 4.6 mm, 5 μm, Alltech) eluted isocratically with methanol:water:acetic acid (65:35:0.1) at a flow rate of 1 ml/min; absorbance was monitored at 280 nm. In some experiments, carbonyl groups were reduced by adding 1–2 mg of sodium borohydride to samples dissolved in ethanol (0.1 ml) and by incubating for 15 min at 0–4 °C. Samples were then filtered through glass wool and dried under nitrogen. The resulting alcohols were separated by normal-phase HPLC as described above with the UV detector set at 235 nm.

Gas Chromatography/Mass Spectrometry (GC/MS)—Arachidonate metabolites were purified by preparative normal-phase HPLC (see above), and methyl esters prepared by treating the purified material with an excess of ethereal diazomethane for 2 min. To prepare the pentafluorobenzyl (PFB) esters, samples were incubated with pentafluorobenzyl bromide (35% in 10 μl of acetonitrile) and diisopropylethylamine (10 μl) diluted with acetonitrile (30 μl) for 10 min at room temperature. To prepare methoxime derivatives, esterified samples were exposed to methoxylamine hydrochloride (1% in pyridine, 20 μl) for 1 h at 60 °C.

Analyses were performed on a Hewlett-Packard 5987A GC/MS fitted with an HP-1 capillary column (12 m, Hewlett-Packard) using helium as carrier gas. For electron impact analyses the column temperature was programmed from 150 to 250 °C at a rate of 30 °C/min. The injector was kept at 250 °C and the source at 200 °C. Carrier flow was regulated by a constant head pressure of 52 kPa. The electron voltage was kept at 25 eV. Negative ion chemical ionization analyses were carried out using methane as the ionizing gas (source pressure approximately 2×10^{-4} torr). The injector was kept at 250 °C and the source at 150 °C. Oven temperature was kept at 60 °C for 1 min and then raised to 320 °C at a rate of 30 °C/min.

Preparation of Standards—12-KETE and 12-oxo-5,8,10-dodecatrienoic acid (12-ODTE) were prepared as described by Fruteau de Laclos *et al.* (1987). Briefly, 12-HPETE (50–100 μg) was incubated for 15 min at 37 °C with hemoglobin (2 ml, 1 mg/ml in phosphate-buffered saline, pH 7.2). The reaction was stopped by adding an equal volume of cold acetone, and the products were extracted and purified by normal-phase HPLC as described above. Alternatively, 12-KETE was prepared by oxidation of 12-HETE with activated manganese dioxide (Fruteau de Laclos *et al.*, 1987). 13-Keto-9,11-octadecadienoic acid was prepared enzymatically from linoleic acid (Garssen *et al.*, 1971). Briefly, linoleic acid (1.5 mg) was incubated anaerobically with soybean lipoxygenase (4,000,000 units) in 0.02 M sodium borate buffer (pH 7.8, 200 ml) for 2 h at room temperature with continuous stirring. The reaction mixture was acidified, and the products extracted with ethyl acetate and purified by preparative normal-phase HPLC as described above. Both geometric isomers of 13-keto-9,11-octadecadienoic acid (9-cis, 11-trans, and 9,11-trans, ratio, 3:2), which are stable when stored in hexane at −20 °C for up to 2 months, were identified by HPLC, UV spectrometry, and GC/MS (Dix and Marnett, 1985).

Intracellular Recordings—Abdominal ganglia were pinned ventral side up to silicone plastic (Sylgard, Dow Chemical Corp., Midland, MI) in a chamber superfused continuously with supplemented artificial seawater at room temperature. The connective tissue sheath was removed by dissection and L14 neurons, identified as described previously (Carew and Kandel, 1977; Byrne *et al.*, 1979), were impaled with one or two glass microelectrodes filled with potassium citrate (1–5 MΩ resistance). Compounds to be tested were administered by pressure ejection from a glass micropipette situated approximately 0.5 mm from the cell body. Samples from stock solutions of 12-HPETE, 12-KETE, 13-keto-octadecadienoic acid, and 12(*S*)-HETE (kept in hexane or ethanol at −20 °C) were dried under nitrogen, reconstituted in seawater, and sonicated for 15 s.

Materials—We obtained histamine, nordihydroguaiaretic acid (NDGA), soybean lipoxygenase, hemoglobin, and acetylsalicylic acid from Sigma, St. Louis, MO; arachidonic acid and linoleic acid from Nu-Chek-Prep (Elysian, MN); 12-HPETE and 12 (*S*)-HETE from Biomol (Plymouth Meeting, PA). Samples of 12-KETE and 12-ODTE were the generous gift of Dr. Alan Brash (Vanderbilt University).

RESULTS

Identification of 12-KETE: Formation from Exogenous Arachidonate and 12-HPETE—Homogenates of *Aplysia* nervous tissue were incubated for 30 min with arachidonic acid (50 μM), and the metabolites formed were analyzed by normal-phase HPLC (Fig. 1*A*). We observed several unidentified components with absorption maxima at 270 nm (compounds a_1, a_2, and *b*).

The UV spectra of compounds a_1 and a_2 (Fig. 1*A*, *inset*) revealed the presence of a dienone or dienal chromophore

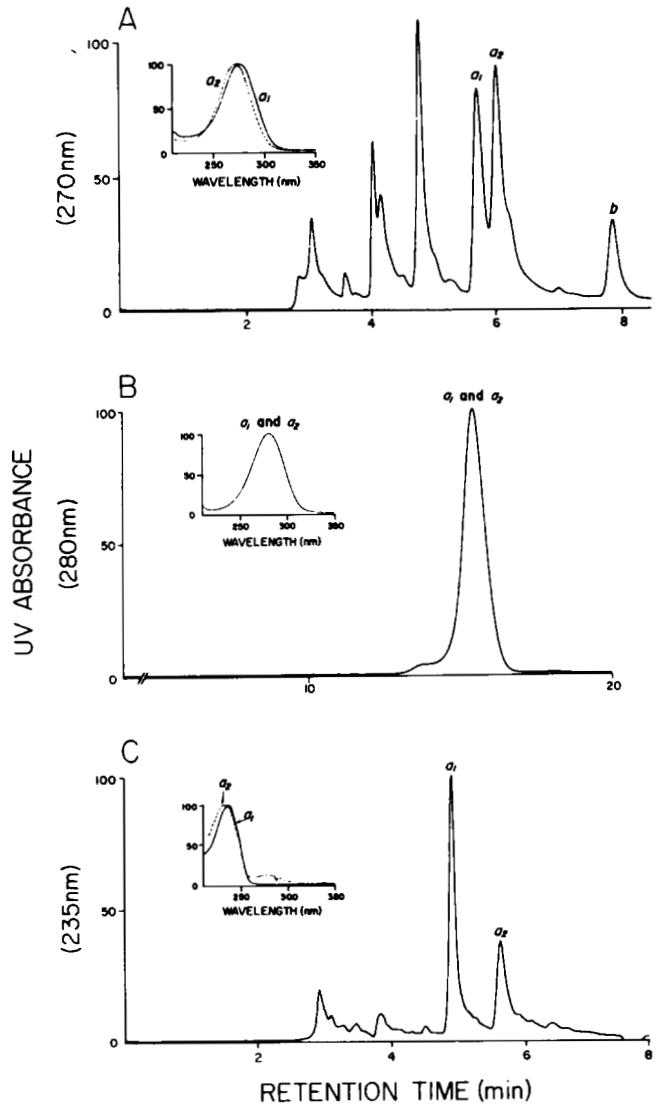

FIG. 1. **Isolation and characterization of 12-KETE from incubations of *Aplysia* nervous tissue with arachidonic acid (100 μM).** *A*, normal-phase HPLC. Extracted lipids were fractionated on a silica column eluted with hexane:isopropyl alcohol:acetic acid (98:2:0.1, v/v/v) at 1 ml/min. UV absorbance (*relative intensity*) was monitored at 270 nm. *B*, reversed-phase HPLC analysis of compounds a_1 and a_2 after they had been purified by normal-phase HPLC. Fractions containing 12-KETE, reduced to dryness and redissolved in the mobile phase, were applied to a Nucleosil C18 column eluted with methanol:water:acetic acid (65:35:0.1, v/v/v) at 1 ml/min. UV absorbance (*relative intensity*) was monitored at 280 nm. *C*, normal-phase HPLC of the alcohols resulting from reduction of a_1 and a_2 with sodium borohydride. These alcohols were fractionated on a silica column as described above. UV absorbance (*relative intensity*) was monitored at 235 nm. *Insets* show spectra obtained with a flow-through diode-array spectrophotometer of the compounds in the HPLC mobile phase (see "Experimental Procedures").

with maximal absorbance at 273 nm for a_1 and 271 nm for a_2 (measured by diode-array detection in the solvent used for normal-phase HPLC). After they were purified by normal-phase HPLC, compounds a_1 and a_2 were also analyzed by reversed-phase HPLC, where they eluted as a single component (Fig. 1*B*). UV spectral analysis (Fig. 1*B*, *inset*), carried out in the solvent used for the reversed-phase HPLC, showed a pronounced bathochromic shift in absorbance (λ_{max} = 280 nm) caused by the increased polarity of the solvent. This spectral shift is characteristic of conjugated dienones and dienals (Glasgow *et al.*, 1986; Fruteau de Laclos *et al.*, 1987).

We confirmed the presence of a conjugated carbonyl group by reducing the methyl esters of compounds a_1 and a_2 with sodium borohydride. Analysis of the reduced methyl esters of a_1 and a_2 by HPLC revealed two components with UV absorbance near 235 nm (Fig. 1*C*): the first (a_1) eluted with the retention time of 12-HETE methyl ester and had an absorption maximum at 235 nm (Fig. 1*C*, *inset*) typical of cis-trans conjugated dienes. The second component (a_2) had a maximal absorbance near 231 nm, compatible with a trans-trans diene. These results suggest that the treatment of compounds a_1 and a_2 with sodium borohydride resulted in the reduction of a conjugated carbonyl moiety, yielding two alcohols, 12-hydroxy-5,8,10,14(*ZZEZ*)-eicosatetraenoic acid methyl ester (12-HETE methyl ester) and its geometric isomer, 12-hydroxy-5,8,10,14(*ZEEZ*)-eicosatetraenoic acid.

Radiolabeling experiments indicated that compounds a_1 and a_2 are derived from arachidonic acid. In experiments similar to that shown in Fig. 1*A*, lipids were extracted and analyzed by normal-phase HPLC: two major peaks of radioactive material appeared (Fig. 2). The first contained the [^{3}H] arachidonate added as substrate as well as [^{3}H]12-HETE. The second corresponded to compounds a_1 and a_2. This trace is typical of four experiments: on average, the radioactivity associated with a_1 and a_2 was 18,949 ± 4,960 cpm (mean ± S.E.), about 23% of total counts in the chromatogram. Furthermore, formation of these products was inhibited (>95%, $n = 2$) by incubation of the homogenates with the lipoxygenase inhibitor, NDGA (30 μM), but not by acetylsalicylic acid, a cyclooxygenase blocker (0.5 mM).

In accord with the idea that the compounds are produced from arachidonic acid through the 12-lipoxygenase pathway, we found that a_1 and a_2 could also be formed when nervous tissue was incubated with 12-HPETE (50 μM, 10 min, data not shown). Boiling the tissue did not affect the conversion of exogenous 12-HPETE to a_1 and a_2, however, which confirmed that significant amounts of exogenous material can be converted nonenzymatically (Fruteau de Laclos *et al.*, 1987; Fruteau de Laclos and Borgeat, 1988).

Compounds a_1 and a_2 have the HPLC retention values and UV spectra of authentic 12-KETE prepared by incubating 12-HPETE with hemoglobin or by oxidation of 12-HETE with manganese dioxide. Identification of compounds a_1 and a_2 as 12-KETE was further strengthened by negative ion chemical ionization GC/MS. The pentafluorobenzyl (PFB) esters of metabolites a_1 and a_2 both eluted together and produced a mass spectrum identical to that of authentic 12-KETE with only one prominent ion at m/z 317 (M − 181, loss of PFB) (Fig. 3*A*). Moreover, conversion of the PFB esters of a_1 and a_2 to the corresponding methoximes produced the expected

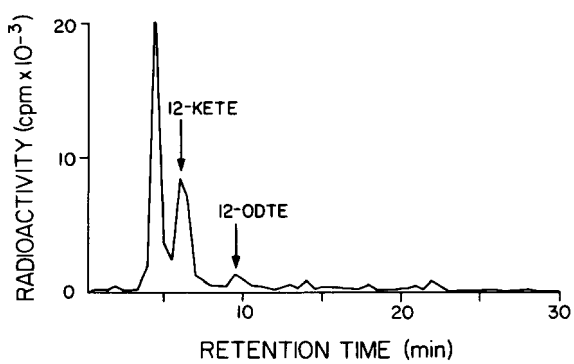

FIG. 2. **HPLC purification of radioactive compounds a_1 and a_2.** Nervous tissue was incubated for 30 min with [^{3}H]arachidonic acid (12.5 μCi). The radioactive products were extracted and fractionated by normal-phase HPLC as described in the legend to Fig. 1. Radioactivity in fractions (0.5 min) was counted by liquid scintillation.

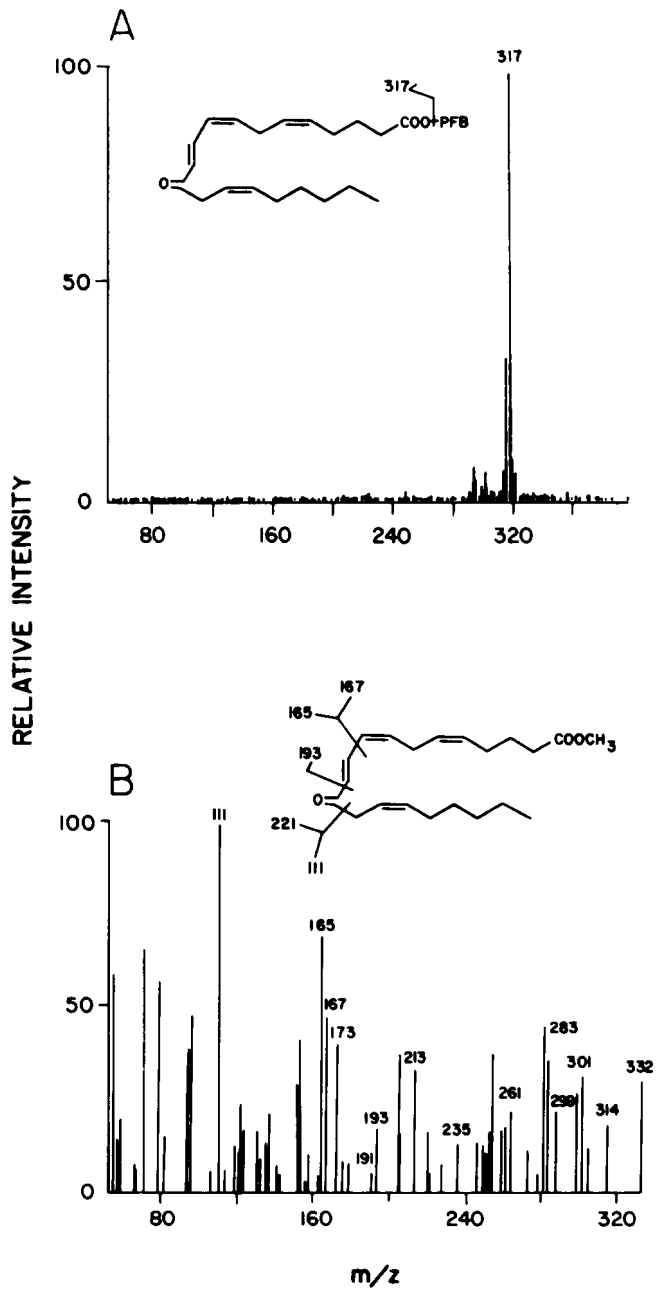

FIG. 3. **GC/MS analyses of compounds a_1 and a_2.** *A*, negative ion chemical ionization mass spectrum of the PFB esters of a_1 and a_2. The MS source was held at 150 °C and methane (2×10^{-4} torr) was the ionizing gas. The base peak in the spectrum represents the loss of the PFB ester group leaving the carboxylate anion. *B*, mass spectrum of the methyl esters of a_1 and a_2. The MS source was held at 200 °C and the ionizing voltage was set to 25 eV.

shift in the base peak to m/z 346 (data not shown), confirming the presence of a carbonyl group in these metabolites.

Additional structural analysis was carried out by electron impact GC/MS. The methyl esters of compounds a_1 and a_2 were eluted from the GC with a carbon chain value of 21.8 (Fig. 3*B*), as previously reported for 12-KETE methyl ester (Fruteau de Laclos *et al.*, 1987). Ions of high intensity were observed at m/z 332 (M^+), 314 ($M^+ - 18$, loss of H_2O), 301 ($M^+ - 31$, loss of CH_3O), 299 ($M^+ - [18 + 15]$), 283 ($M^+ - [31 + 18]$), 261 (loss of $CH_2-(CH_2)_3CH_3$), 235 (β-cleavage, with loss of C14–C20), 221 (loss of $CH_2-CH=CH-(CH_2)_4-CH_3$), 193 (loss of C12–C20), 167, 165, and 111 (base peak).

Formation of [^{3}H]12-KETE from Endogenous [^{3}H]Arachidonate—To show that 12-KETE also can be formed from endogenous stores, nervous tissue was prelabeled with [^{3}H] arachidonic acid. After unincorporated label was removed, the tissue was stimulated mechanically to induce the release of radioactive products (Piomelli *et al.*, 1987a). In seven experiments, radioactivity in the HPLC fractions corresponding to 12-KETE was 1302 ± 514 cpm/ganglion (10% of counts on chromatogram). In the same experiments the fractions containing [^{3}H]arachidonic acid and [^{3}H]12-HETE (which were not well resolved under these conditions) were 2559 ± 791 cpm.

Identification of 12-ODTE—In addition to 12-KETE, a compound with the retention time of 12-ODTE on normal-phase (compound *b* in Fig. 1*A*) and on reversed-phase HPLC (not shown), is formed by nervous tissue incubated with either 12-HPETE (50 μM) or exogenous arachidonic acid (50 μM). The UV spectrum of this material in nonpolar and polar solvents is identical to that of authentic 12-ODTE (λ_{max} of 272 in nonpolar and 280 in polar solvents) (Glasgow *et al.*, 1986; Fruteau de Laclos *et al.*, 1987). Furthermore, reduction with sodium borohydride shifted the absorbance maximum to 235 nm, as previously reported for this 12-carbon conjugated dienal (Glasgow *et al.*, 1986). GC/MS analysis by negative ion chemical ionization of the PFB ester-methoxime derivative produced a mass spectrum identical to that of authentic 12-ODTE-PFB ester-methoxime with a prominent ion at m/z 236 ($M - 181$, loss of PFB). This material was never detected in experiments with tissue prelabeled with [^{3}H]arachidonic acid. Because 12-ODTE is not formed in detectable amounts from endogenous fatty acid stores, we did not characterize this compound further.

Stimulation of [^{3}H]12-KETE Production by Neurotransmitter—Application of histamine to *Aplysia* nervous tissue results in the generation of 12-HETE (Piomelli *et al.*, 1987a). We used a similar experimental protocol to test whether 12-KETE could be released by activating histamine receptors. Products, extracted from the incubation media, were purified by normal-phase HPLC (Fig. 4*A*). Histamine stimulated the production of two major radioactive components; these eluted at the retention times of 12-HETE (unresolved from unreacted arachidonic acid) and 12-KETE. Application of histamine caused nearly a 10-fold increase in radioactivity associated with 12-KETE compared to controls ($p < 0.05$, Student's *t* test) (Fig. 4*B*). Histamine did not evoke formation of radioactive material eluting at the retention time of 12-ODTE, or of the two epoxy alcohols, 8-hydroxy-11,12-epoxyeicosatrienoic acid and 10-hydroxy-11,12-epoxyeicosatrienoic acid (Walker *et al.*, 1979; Hamberg, 1986; Shapiro *et al.*, 1988).

Biological Activity of 12-KETE on Identified Aplysia Neurons—The biological activity of authentic 12-KETE was tested on L14 and L10 cells. L14 cells are a group of motor neurons in the abdominal ganglion that control inking, a defensive behavior of the animal. In these cells, application of histamine or stimulation of L32 neurons cause a response that typically consists of an early depolarization followed by a slow hyperpolarization (Kretz *et al.*, 1986). In the majority of neurons tested, applications of 12-HPETE or 12-KETE (1–2 nmol) from an extracellular puff micropipette were found to mimic the histamine response (Fig. 5, Table I). Similar puffs of 12(*S*)-HETE were without effect, whereas applications of 13-keto-9,11-octadecadienoic acid, a carbonyl derivative of linoleic acid, produced responses similar to 12-KETE. This suggests that a carbonyl moiety may be important in these actions.

L10 is a mixed action neuron involved in cardiac and kidney function (Koester and Koch, 1987). Its response to histamine is a slow hyperpolarization caused by an increase in K^+ conductance and a decrease in Ca^{2+} conductance (Kretz *et al.*, 1986). We found that 12-KETE and 13-keto-octadecadienoic acid (data not shown) produced similar inhibitory responses

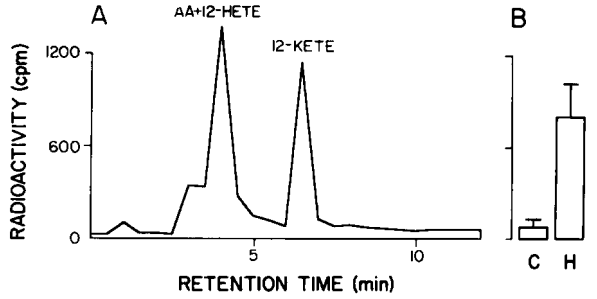

FIG. 4. **Stimulation of [^{3}H]12-KETE formation by histamine.** Nervous tissue, labeled in artificial seawater for 2 h with [^{3}H] arachidonic acid (25 μCi/ml), was exposed for 1 min to histamine (100 μl, final concentration 50 μM). Products were then extracted from 50-μl samples of the incubation medium and subjected to normal-phase HPLC as described in the legend to Fig. 1. Radioactivity in fractions (0.5 min) was counted by liquid scintillation. *A*, representative chromatogram typical of four experiments. Analysis of material in the peak eluting before 12-KETE by reversed-phase HPLC reveals that it contains arachidonic acid (*AA*) (88%) and 12-HETE (12%). *B*, amounts of [^{3}H]12-KETE formed during exposure to histamine (*H*) or artificial seawater (control, *C*). Error bars represent the S.E., $n = 4$.

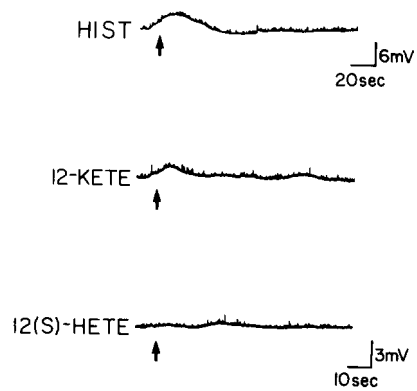

FIG. 5. **Effect of histamine and 12-lipoxygenase products on the membrane potential of L14.** 1–2 nmol of a test substance were ejected by pressure (5 s, 6 p.s.i.) from a glass micropipette situated about 0.5 mm from the cell body of an L14 impaled with a microelectrode for recording voltage. Histamine (10^{-4} M in the pipette) and 12-KETE elicited early depolarizing responses followed by a small slow hyperpolarization. 12(*S*)-HETE was ineffective in changing the membrane potential. The histamine (*HIST*) response measured in this particular specimen was larger and longer lasting than the response to 12-KETE (note difference in calibration of the electrophysiological traces). The calibration for the 12-KETE response is the same as that for 12(*S*)-HETE.

TABLE I

Responses of identified Aplysia neurons to histamine and metabolites of arachidonic acid

Compounds were applied to cells L14 and L10 as described in the legend to Fig. 5 and "Experimental Procedures." Responses observed were depolarizing, hyperpolarizing, or dual-action (depolarizing/hyperpolarizing). A, Biological activity of metabolites. Within an experiment, the total number of which are listed, each cell was tested as many as four times. The number of observations refers to the total number of times each of the compounds was applied. All three types of response are tabulated (total responses). B, types of response. The types of response obtained in the experiments tabulated in A are given as percentages of the total responses.

A. Biological activity of metabolites

Treatment	Experiments	Observations	Total responses
		number	
Cell L14			
Histamine	28	58	54
12-KETE	6	18	16
12(*S*)-HETE	7	20	4
12-HPETE	15	34	28
Cell L10			
Histamine	15	35	33
12-KETE	3	11	8
12(*S*)-HETE	5	12	1
12-HPETE	9	18	6

B. Types of response

Treatment	Depolarizing	Hyperpolarizing	Dual-action
		% responses	
Cell L14			
Histamine	11	2	87
12-KETE	12	25	62
Cell L10			
Histamine	0	100	0
12-KETE	0	100	0

(Table I). 12-HPETE was effective in only 30% of the cells tested, however, and 12(*S*)-HETE did not mimic the hyperpolarization produced by histamine (Table I).

DISCUSSION

Metabolites of 12-HPETE: Possible Intracellular Modulators—We have proposed that lipoxygenase metabolites of arachidonic acid may act as intracellular second messengers in neurons of *Aplysia* (Piomelli *et al.*, 1987a, 1987b). This idea was prompted by the observation that two modulatory neurotransmitters, histamine and FMRFamide, stimulate release of [^{3}H]12-HETE from nervous tissue labeled by incubation with [^{3}H]arachidonic acid. Furthermore, application of 12-HPETE, the precursor of 12-HETE, simulates the changes in cell membrane conductance produced by these transmitter substances both in *Aplysia* sensory cells and in L14 neurons (Piomelli *et al.*, 1987b; Belardetti *et al.*, 1987; Shapiro *et al.*, 1988). The physiological actions of 12-HPETE, which are not shared either by 12(*S*)-HETE, or by the 5-lipoxygenase product, 5-HPETE, suggest that this short-lived hydroperoxide participates in the synaptic responses. Does 12-HPETE carry out this signaling function directly, or rather does it require conversion to other metabolic products?

In plant and animal cells, the metabolism of unsaturated hydroperoxides derived from arachidonic, linoleic, or linolenic acids is complex (Zimmermann, 1966; Little and O'Brien, 1968; Christophersen, 1968; Galliard and Phillips, 1972; Zimmermann and Coudron, 1979; Gardner, 1980; Veldinck *et al.*, 1970; Hamberg, 1986; Vick and Zimmermann, 1987). Increasing evidence indicates that many biologically active molecules are generated from 12-HPETE. Metabolites thus far identified include 12-KETE (Fruteau de Laclos *et al.*, 1987), 12-ODTE (Glasgow *et al.*, 1986; Fruteau de Laclos *et al.*, 1987) and several isomeric epoxy alcohols (Walker *et al.*, 1979; Pace-Asciak *et al.*, 1983; Bryant and Bailey, 1979). The possibility that 12-HPETE must be metabolized to produce its actions in *Aplysia* neurons is suggested by an observation of Belardetti *et al.* (1987): the increased opening of K^+ channels produced by this hydroperoxide occurs only in cell-attached, but not in cell-free patches of sensory neuron membrane. This is consistent with the idea that a cytosolic component, possibly a soluble metabolizing enzyme, is required.

Identification of 12-KETE—In this study, we describe a bioactive metabolite formed in nervous tissue of *Aplysia*, the keto acid, 12-KETE. This compound was identified by HPLC, UV spectrometry, and GC/MS, in lipid extracts of the nervous tissue incubated with exogenous arachidonic acid or 12-HPETE. Our identification is in agreement with a previous report describing the formation of 12-KETE by human platelets (Fruteau de Laclos *et al.*, 1987). The presence of a compound, which we have tentatively identified as the Δ^8-trans isomer of 12-KETE, was not described in platelets, however. We have not determined whether this isomer is formed biologically in *Aplysia* nervous tissue or whether it is produced artifactually during preparation of the samples.

Formation of 12-KETE from arachidonate was inhibited by treatment with NDGA, supporting the idea that 12-lipoxygenase catalyzes the biosynthesis of this metabolite. When 12-HPETE was used as precursor, however, generation of 12-KETE was not affected either by inhibiting the lipoxygenase or by boiling the tissue. In accord with these results, previous studies have shown that the conversion of fatty acid hydroperoxides to keto acids and aldehydes can be catalyzed by hematin or by heme-containing proteins (Fruteau de Laclos *et al.*, 1987; Fruteau de Laclos and Borgeat, 1988; Dix and Marnett, 1985). Cellular hemoglobin and other iron-containing proteins are abundant in neurons and glial cells of mollusks (Ghiretti and Ghiretti-Magaldi, 1972; Kraus *et al.*, 1988) and may catalyze the formation of 12-KETE when exogenous 12-HPETE is used as substrate. Whether this mechanism operates in *Aplysia* neurons during the synthesis of 12-KETE from endogenous arachidonic acid is not yet known. The existence of a nonenzymatic pathway does not rule out enzymatic synthesis in the cell, however. It is possible that 12-lipoxygenase sequentially catalyzes the addition of molecular oxygen and then the conversion of the hydroperoxide to a carbonyl, as Glasgow *et al.* (1986) suggested for 12-ODTE.

In addition to 12-KETE, we have found that several other products of 12-HPETE can be formed in *Aplysia* nervous tissue including the polyunsaturated aldehyde, 12-ODTE (this paper) and two epoxy alcohols, 8-hydroxy-11,12-epoxyeicosatrienoic and 10-hydroxy-11,12-epoxyeicosatrienoic acids (Shapiro *et al.*, 1988; Feinmark *et al.*, 1988). Release of these metabolites appears to be regulated differentially. For example, prelabeled abdominal ganglia exposed to histamine selectively release [^{3}H]12-HETE and [^{3}H]12-KETE. On the other hand, after intracellular stimulation of the identified neuron L32, prelabeled abdominal ganglia release [^{3}H]12-HETE (Piomelli *et al.*, 1987a) and [^{3}H]8-hydroxy-11,12-epoxyeicosatrienoic acid (Shapiro *et al.*, 1988). These observations suggest that activation of specific receptors can cause the release of characteristic metabolites. A possible explanation is that the receptors activated by the application of histamine differ from those activated by the transmitter released endogenously by L32. Although all the known actions of L32 cells are simulated by histamine (Kretz *et al.*, 1986), it is still uncertain whether L32 cells actually use histamine as their transmitter

(Schwartz *et al.*, 1986). Whether truly histaminergic or not, L32 cells may synapse on follower neurons with a distinctive subset of histamine receptors.

In preliminary experiments in the cerebral ganglion of *Aplysia*, we found that intracellular stimulation of an identified histaminergic neuron, C2 (Weinreich *et al.*, 1975; Schwartz *et al.*, 1986) results in release of [^{3}H]12-KETE. Stimulation of C2 did not evoke the formation of [^{3}H]12-ODTE or of the epoxy alcohols, however. These results further support the idea that activation of specific histamine receptors at some synapses leads to the formation of 12-KETE. Whatever the explanation for the differences in metabolites generated, the selective release of products observed suggest that different metabolites of arachidonic acid might play distinctive roles in specific neurons.

12-KETE Simulates Physiological Responses to Histamine—The possibility that 12-KETE participates in the intracellular transduction of some actions of histamine under physiological conditions is supported by our pharmacological experiments with L14 and L10, two identified neurons in the abdominal ganglion. Each shows different and characteristic electrophysiological responses to histamine. Application of histamine to L14 results in a depolarization of the membrane potential typically followed by a longer lasting hyperpolarization (Kretz *et al.*, 1986). In these neurons, 12-KETE, like 12-HPETE (Shapiro *et al.*, 1988), evokes a response similar to that caused by histamine. In L10, on the other hand, 12-KETE simulates the hyperpolarization produced by histamine in about 70% of tests, but 12-HPETE is effective only in 30% (Table I). A possible explanation is that the puffed metabolites are not completely accessible to critical sites in L10 at the concentrations applied. Further experiments, using L10 neurons in culture, will be useful to test this idea.

The physiological activity of 12-KETE observed is in accord with the idea that conversion of 12-HPETE to the keto acid is necessary for some of the biological actions of 12-HPETE. To show this definitively, however, further work is required. In this study, we have shown that the responses of L14 and L10 cells to 12-HPETE and 12-KETE are similar to those produced by histamine. The similarity of actions observed, however, does not exclude the possibility that 12-HPETE and 12-KETE act independently. Moreover, similar physiological responses might be produced by different mechanisms. Proof that these 12-lipoxygenase products affect the same ionic channels modulated by the endogenous transmitter will require further electrophysiological work using voltage-clamp and patch-clamp studies.

Acknowledgments—We are grateful to Wayne Glasgow and Alan R. Brash, Vanderbilt University, for providing 12-ODTE and 12-KETE and for helpful suggestions, and to Jillayn Lindahl for her expert and cheerful assistance in typing the manuscript.

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