Formation and Biological Activity of 12-Ketoeicosatetraenoic Acid in the Nervous System of \textit{Aplysia}\textsuperscript{*}

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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, \textit{Aplysia californica} (Piomelli, D., Shapiro, E., Feinmark, S. J., and Schwartz, J. H. (1987) \textit{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 \textit{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. \textsuperscript{\textsuperscript{1}}H\textsuperscript{12}-KETE was formed from endogenous lipid stores in nervous tissue, labeled by incubation with \textsuperscript{\textsuperscript{1}}H\textsuperscript{12}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 metabolites, 12-KETE, participate in transduction of histamine responses in \textit{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 \textit{Aplysia}, the modulatory neurotransmitter histamine and the neuroactive tetrapeptide FMRFamide\textsuperscript{1} stimulate release of 12-hydroxyeicosatetraenoic acid (12-HPETE), 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\textsuperscript{+} 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 Lacos et al., 1987) raises the possibility that modulation of membrane ionic conductances produced by 12-HPETE in \textit{Aplysia} neurons might result from its conversion to an active metabolite.

In this study, we report that: 1) nervous tissue of \textit{Aplysia} converts arachidonate to 12-KETE, a metabolite of 12-HPETE that was recently identified in human platelets (Fruteau de Lacos et al., 1987); 2) synthesis of 12-KETE is stimulated by applying histamine to \textit{Aplysia} nervous tissue; and 3) application of 12-KETE, like 12-HPETE, mimics the electrophysiological responses produced by histamine in two identified \textit{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**

\textit{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\textsubscript{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 \textsuperscript{\textsuperscript{1}}H\textsuperscript{12}arachidonic acid (25 \textmu{C}/ml, Aramsham 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 \textmu{m}, Supelco, Bellefonte, PA) eluted isocratically with hex-
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An episopropyl 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 esters (PFB), esters were incubated with pentafluorobenzyl bromide (35% in 10 ml of acetonitrile) and disoprolylamine (10 ml) with acetonitrile (30 ml) for 10 min at room temperature. To prepare methoxime derivatives, esterified samples were exposed to methoxylamine hydrochloride (1% in pyridine, 20 ml) for 1 h at 60°C.

Analyses were performed on a Hewlett-Packard 5897A 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 × 10⁻⁵ torr). The injector was kept at 250°C and the source at 150°C. Oven temperature was kept at 60°C for 4 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 Lamnos 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 Lamnos et al., 1987). 13-Keto-9,11-octadecadienoic acid was prepared enzymatically from linoleic acid (Garssen et al., 1979). Briefly, linoleic acid (1.5 mg) was incubated anaerobically with soybean lipoygenase (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 Mannett, 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, 12-oxo-5,8,10-dodecatrienoic acid, and 12(S)-HETE were kept in hexane or ethanol at -20°C and were dried under nitrogen, reconstituted in seawater, and sonicated for 15 s.

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

Identification of 12-KETE: Formation from Exogenous Arachidonate and 12-HPETE—Homogenates of Aplysia nervous tissue were incubated for 15 min with arachidonic acid (50 μM), and the metabolites formed were analyzed by normal-phase HPLC (Fig. 1A). We observed several unidentified components with absorption maxima at 270 nm (compounds a₁, a₂, and b). The UV spectra of compounds a₁ and a₂ (Fig. 1A, inset) revealed the presence of a diene or dienal chromophore.

UV spectra were taken using a diode-array spectrophotometer (Hewlett-Packard 1090M, Palo Alto, CA); 30-5 fractions were collected and UV absorbance (relative intensity) was monitored at 280 nm. C, normal-phase HPLC (Fig. 1B). 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 a₁ and a₂ after they had been purified by normal-phase HPLC. Fractions containing 12-KETE, reduced to dryness and reconstituted 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 235 nm. C, normal-phase HPLC of the alcohols resulting from reduction of a₁ and a₂ with sodium borohydride. These alcohols 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 280 nm. C, normal-phase HPLC of the alcohols resulting from reduction of a₁ and a₂ with sodium borohydride. These alcohols 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 235 nm.

UV spectra of compounds a₁ and a₂ (Fig. 1A, inset) revealed the presence of a diene 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 a₁ and a₂ after they had been purified by normal-phase HPLC. Fractions containing 12-KETE, reduced to dryness and reconstituted 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 235 nm. C, normal-phase HPLC of the alcohols resulting from reduction of a₁ and a₂ with sodium borohydride. These alcohols 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 235 nm.
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. 1B). UV spectral analysis (Fig. 1B, inset), carried out in the solvent used for the reversed-phase HPLC, showed a pronounced bathochromic shift in absorbance (\( \lambda_{max} = 280 \) 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. 1C): the first (\( a_1 \)) eluted with the retention time of 12-HETE methyl ester and had an absorption maximum at 235 nm (Fig. 1C, 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.

Radio-labeling experiments indicated that compounds \( a_1 \) and \( a_2 \) are derived from arachidonic acid. In experiments similar to that shown in Fig. 1A, lipids were extracted and analyzed by normal-phase HPLC: two major peaks of radioactive material appeared (Fig. 2). The first contained the \([\text{H}]\) arachidonate added as substrate as well as \([\text{H}]\)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 \( \mu \)M), but not by acetylsalicylic acid, a cyclooxygenase blocker (0.5 \( \mu \)M).

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-HETE (50 \( \mu \)M, 10 min, data not shown). Boiling the tissue did not affect the conversion of exogenous 12-HETE 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 \( m/z = 317 \) (\( M^- \), loss of PFB) (Fig. 3A). 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 \([\text{H}]\)arachidonic acid (12.5 \( \mu \)Ci). 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 \( \times \) \( 10^{-4} \) 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.
produced a mass spectrum identical to that of authentic 12-ODTE—produced by sodium borohydride shifted the absorbance maximum to a phase (compound b in Fig. 1). The UV spectrum of this material in nonpolar and polar solvents is identical to that of authentic 12-ODTE (Amax 236 nm, as previously reported for this 12-carbon conjugated material). 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. 1A) 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 (Amax of 272 in nonpolar and 280 in polar solvents) (Glasgow et al., 1986; Fruteau de Lacos 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 [3H]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 [3H]12-KETE Production by Neurotransmitter—Application of histamine to Aplysia nervous tissue results in the generation of 12-HETE (Piromelli 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. 4A). Histamine stimulated the production of two major radioactive components; these eluted at the retention times of 12-HETE (unresolved from unresolved 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. 4B). 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 carboxyl 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.
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.

| Treatment | Experiments | Observations | Total responses |
|-----------|-------------|--------------|----------------|
| 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 1). 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 [1^3H]12-HETE from nervous tissue labeled by incubation with [1^3H]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 Couchond, 1979; Gardner, 1980; Veldink 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 Llaclos et al., 1987), 12-ODTE (Glasgow et al., 1986; Fruteau de Llaclos 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 Llaclos et al., 1987). The presence of a compound, which we have tentatively identified as the Δ⁵-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 Llaclos et al., 1987; Fruteau de Llaclos and Borget, 1988; Dix and Marnett, 1985). Cellular hemoglobin and other iron-containing proteins are abundant in neurons and glial cells of mollusks (Ghirettii and Ghirettii-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 [1^3H]12-HETE and [1^3H]12-KETE. On the other hand, after intracellular stimulation of the identified neuron L32, prelabeled abdominal ganglia release [1^3H]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 1). 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.

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