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12-Keto-Eicosatetraenoic Acid

A Biologically Active Eicosanoid in the Nervous System of *Aplysia*

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INTRODUCTION

In the neural tissue of *Aplysia*, the neurotransmitter histamine and the peptide FMRFamide evoke release of 12-hydroxyeicosatetraenoic acid (12-HETE), a stable end product of the 12-lipoxygenase pathway.^{1,3} The short-lived precursor of 12-HETE, 12-HPETE, simulates electrophysiological responses induced by FMRFamide in sensory cells^{3,4} and by histamine in L14 cells,² suggesting that this hydroperoxide or one of its metabolites serves as intracellular signal to mediate the synaptic actions of FMRFamide and histamine. Several novel metabolites derived from 12-HPETE have recently been identified in mammalian tissues.⁵⁻⁸ Therefore, it is possible that 12-HPETE produces its effects in *Aplysia* neurons only after conversion to an active metabolite.

Here, we report that a metabolite of 12-HPETE, 12-KETE, is released when *Aplysia* nervous tissue is stimulated by applying histamine. We also show that application of 12-KETE, like 12-HPETE, produces electrophysiological responses similar to those evoked by histamine in two identified *Aplysia* neurons, L10 and L14.

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. Homogenates of nervous tissue and isolated neural components (cell bodies and neuropil) were prepared as described previously.¹

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Extraction of Lipids

Acetone (0–4 °C) was added to homogenates (1:1, v/v) and the precipitate removed by low-speed centrifugation. Metabolites were extracted twice with ethyl acetate (2 vol) after acidifying the supernatant to pH 3.5. 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

Analytical normal-phase HPLC was performed using a silica column (250 × 4.6 mm, 5 μm; Supelco, Bellefonte, PA) eluted isocratically with hexane:isopropanol: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 obtained with a diode-array spectrophotometer (Hewlett-Packard 1090M, Palo Alto, CA); 30-sec fractions were collected and radioactivity counted by liquid scintillation. For purifications on a preparative scale, we used a Polygosil silica column (500 × 10 mm, 10 μm; Alltech, Deerfield, IL) 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 to alcohols by adding 1–2 mg of sodium borohydride to samples dissolved in ethanol (0.1 ml) and incubating for 15 min at 0–4 °C. Samples were then filtered through glass wool and dried under nitrogen. The resulting products were separated by normal-phase HPLC as described above with the UV detector set at 235 nm.

Gas Chromatography/Mass Spectrometry

Metabolites were purified by preparative normal-phase HPLC (see above), and converted to the methyl ester by treating the purified material with an excess of ethereal diazomethane for 2 min. To prepare the pentafluorobenzyl (PFB) esters, we incubated samples with pentafluorobenzyl bromide (35% in 10 μl acetonitrile) and diisopropylethylamine (10 μl) diluted with acetonitrile (30 μl) for 10 min at room temperature. To prepare methoxime derivatives, the 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, Palo Alto, CA) using helium as the carrier gas. For electron impact analyses the column temperature was programmed from 150 to 250 °C at a rate of 30 °C/min. We kept the injector at 250 °C and the source at 200 °C. Carrier flow was regulated at a constant head pressure of 52 kPa and the voltage kept at 25 eV. Negative ion chemical ionization analyses were done using methane as the ionizing gas (source pressure approximately 0.8 torr). We kept the injector 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 from 12-HPETE as described by Fruteau de Laclos *et al.*⁷ 12-KETE was also prepared by oxidation of 12-HETE with activated manganese dioxide.⁷

Intracellular recordings

Abdominal ganglia were pinned ventral side up to Sylgard, a silicone plastic (Dow Chemical, Midland, MI), in a chamber continuously superfused with supplemented artificial seawater at room temperature. The connective tissue sheath was removed by dissection; L14 neurons, identified as previously described,^{9,10} were impaled with one or two glass recording microelectrodes filled with potassium citrate (1–5 M Ω resistance). Compounds to be tested were ejected with pressure from a glass micropipette placed approximately 0.5 mm from the cell body. Samples from stock solutions of 12-HPETE, 12-KETE, and 12(S)-HETE (kept in hexane or ethanol at –20 °C) were dried under nitrogen, reconstituted in the seawater, and sonicated for 15 sec.

RESULTS AND DISCUSSION

Several biologically active molecules can be formed from 12-HPETE. Among the metabolites that have been identified thus far are 12-KETE,⁷ 12-ODTE,^{6,7} and several isomeric epoxy alcohols.^{5,11,12} The possibility that 12-HPETE must be metabolized to produce its action in *Aplysia* neurons is suggested by an observation of Belardetti *et al.*:⁴ the increased opening of K $_s^+$ channels evoked by 12-HPETE occurs only in cell-attached (but not in cell-free) patches of sensory neuron membranes. This suggests that a cytosolic component, possibly an enzyme, is required to metabolize the hydroperoxy acid further.

We describe here a novel bioactive metabolite formed in nervous tissue of *Aplysia*, the keto-acid 12-KETE. Identification was carried out by HPLC, UV spectrometry, and GC/MS in lipid extracts of the nervous tissue incubated with exogenous arachidonic acid or 12-HPETE. Homogenates of *Aplysia* nervous tissue were incubated with arachidonic acid (50 μ M, 30 min), and the metabolites formed were analyzed by normal-phase HPLC (FIG. 1A). Several unidentified components with absorption maxima at 270 nm were observed (compounds a_1 , a_2 , and b).

The UV spectra of compounds a_1 and a_2 (FIG. 1A, inset) indicated the presence of a dienone or dialen chromophore, with maximal absorbance at 273 nm for a_1 , and 271 nm for a_2 . After they were purified by normal-phase HPLC, we also analyzed compounds a_1 and a_2 by reversed-phase HPLC, where they eluted as a single component (FIG. 1B). UV spectral analysis (FIG. 1B, inset) showed a pronounced bathochromic shift in absorbance (λ max = 280 nm). A spectral shift caused by the increased polarity of the solvent is characteristic of conjugated dienones and diens.^{6,7}

The presence of a conjugated carbonyl group was confirmed by reducing the methyl esters of the two compounds with sodium borohydride. Analysis of the reduced methyl esters of a_1 and a_2 by normal-phase 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. This suggests that the

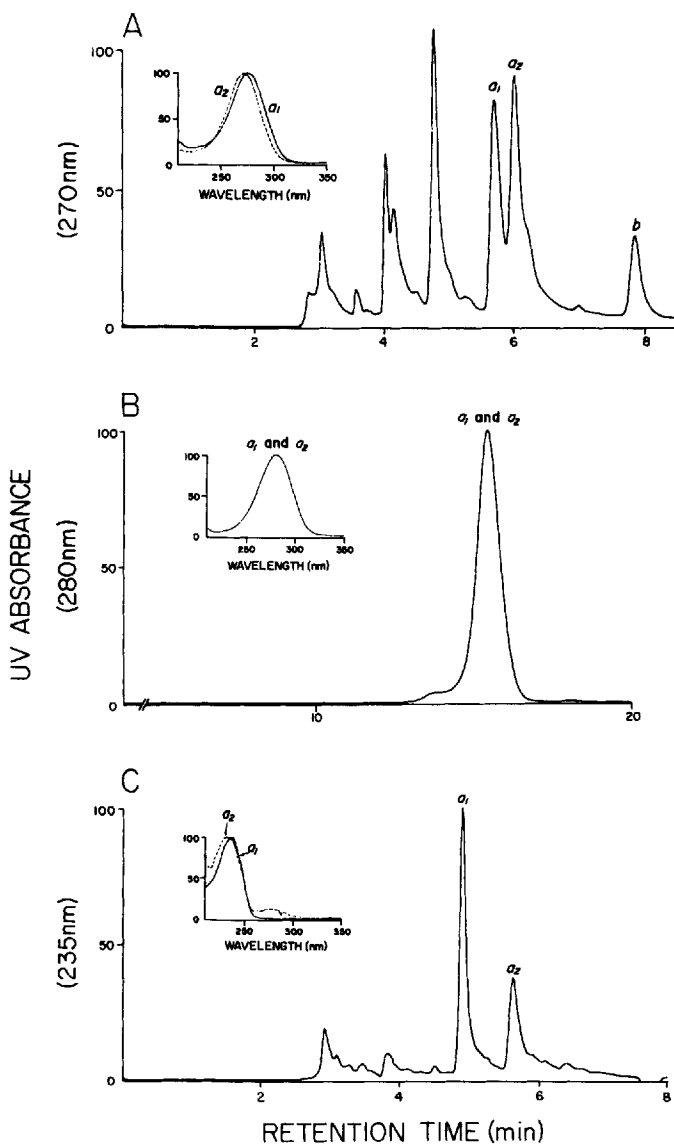


FIGURE 1. Isolation and characterization of 12-keto-5,8,10,14-eicosatetraenoic acid (12-KETE) from incubations of *Aplysia* nervous tissue with arachidonic acid (100 μ M). **A:** normal-phase HPLC. Extracted lipids were fractionated on a silica column eluted with hexane:isopropanol:acetic acid (98:2:0.1, v/v/v) at 1 ml/min. UV absorbance 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 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 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). (From Piomelli *et al.*¹⁵ Reprinted by permission from the *Journal of Biological Chemistry*.)

reduction of the compounds with sodium borohydride yields two alcohols, 12-hydroxy-5,8,10,14(*ZZZZ*)-eicosatetraenoic acid methyl ester (12-HETE methyl ester) and its geometric isomer, 12-hydroxy-5,8,10,14(*ZZEZ*)-eicosatetraenoic acid.

As shown by radiolabeling experiments, compounds a_1 and a_2 are derived from arachidonic acid. Normal-phase HPLC analysis resolved two major radioactive components (FIG. 2): the first contained [^3H]arachidonate added as substrate as well as [^3H]12-HETE, and the second corresponded to compounds a_1 and a_2 . Formation of these products was inhibited (>95%, $n = 2$) by incubation of the homogenates with the lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA) (30 μM), but not by aspirin, a cyclooxygenase blocker (0.5 mM). This suggests that a 12-lipoxygenase enzyme catalyzes the biosynthesis of this metabolite from arachidonic acid.

In accord with this idea, we found that compounds 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 these compounds, however, confirming previous reports that exogenous 12-HPETE can be converted nonenzymatically: conversion of fatty acid hydroperoxides to keto-acids and aldehydes can be catalyzed by hematin or by heme-containing proteins.^{7,13,14}

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. This identification was confirmed by GC/MS. Negative-ion chemical ionization analysis of pentafluorobenzyl (PFB) esters of metabolites a_1 and a_2 showed that these compounds 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. 3A).

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. 3B), as previously reported for 12-KETE methyl ester.⁷ Ions of high intensity were observed at m/z 332 (M^+), 314 ($M^+ - 18$, loss of H_2O), 301

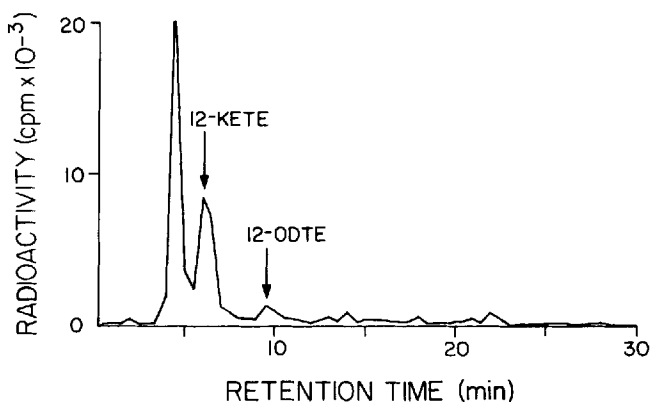


FIGURE 2. HPLC purification of radioactive compounds a_1 and a_2 . Nervous tissue was incubated for 30 min with [^3H]arachidonic acid (12.5 μCi). The radioactive products were extracted and fractionated by normal-phase HPLC as described in the legend to FIG. 1. Radioactivity in fractions (0.5 min) was counted by liquid scintillation. 12-KETE = 12-keto-5,8,10,14-eicosatetraenoic acid; 12-ODTE = 12-oxo-5,8,10-deodecatrienoic acid. (From Piomelli *et al.*¹⁵ Reprinted by permission from the *Journal of Biological Chemistry*.)

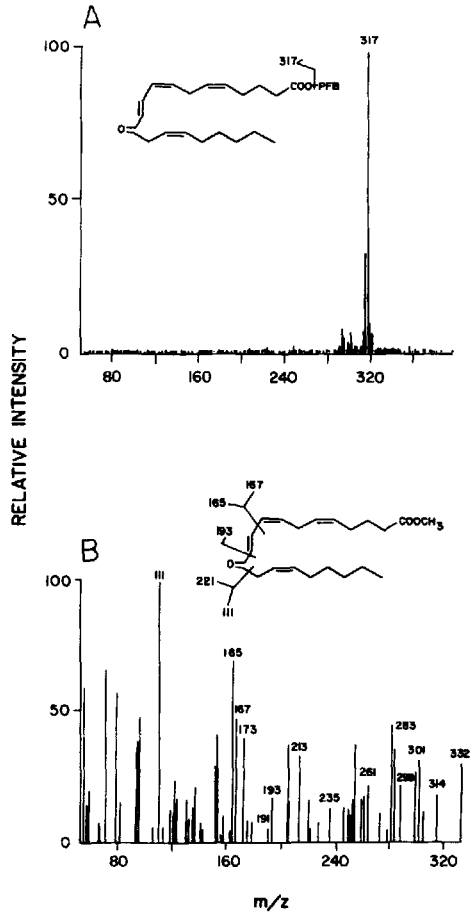


FIGURE 3. GC/MS analyses of compounds *a*₁ and *a*₂. **A:** Negative-ion chemical ionization mass spectrum of the pentafluorobenzyl (PFB) esters of *a*₁ and *a*₂. The MS source was held at 150 °C, and methane (0.8 torr) was the ionizing gas. The base peak in the spectrum represents the loss of the PFB-ester 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. (From Piomelli *et al.*¹⁵ Reprinted by permission from the *Journal of Biological Chemistry*.)

($M^+ - 31$, loss of CH_3O), 299 ($M^+ - [18 + 15]$), 283 ($M^+ - [31 + 18]$), 261 (loss of $CH_2 - (CH_2)_3CH_3$), 235 (beta-cleavage, with loss of C14 to C20), 221 (loss of $CH_2-CH = CH-(CH_2)_4-CH_3$), 193 (loss of C12-C20), 167 and 165 and 111 (base peak).

Stimulation of [³H]12-KETE Production by Neurotransmitter

Histamine stimulates the generation of [³H]12-HETE in *Aplysia* nervous tissue prelabeled with [³H]arachidonic acid.¹ Using a similar experimental protocol, we found that 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).

In addition to 12-KETE, other products of 12-HPETE are formed in *Aplysia* nervous tissue, including the short-chain aldehyde 12-oxododecatrienoic acid (12-ODTE) and two epoxy alcohols, 8-hydroxy-11,12-epoxyeicosatrienoic and 10-

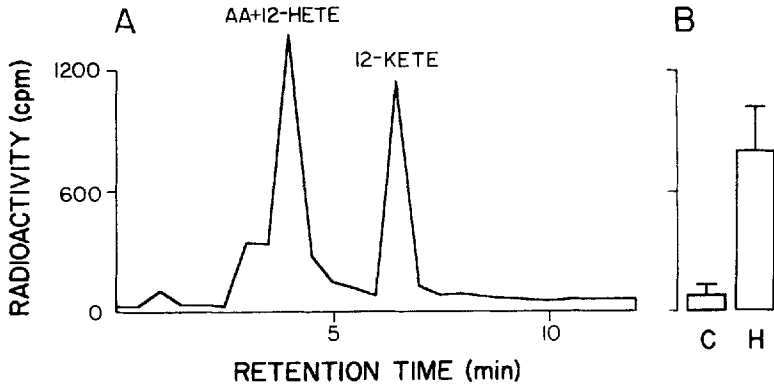


FIGURE 4. Stimulation of [^3H]12-KETE formation by histamine. Nervous tissue, labeled in artificial seawater for 2 h with [^3H]arachidonic acid (25 $\mu\text{Ci/ml}$), was exposed for 1 min to histamine (100 μl , final concentration 50 μM). Products were then extracted from 50- μl samples of the incubation 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-keto-5,8,10,14-eicosatetraenoic acid (12-KETE) by reversed-phase HPLC reveals that it contains arachidonic acid (AA) (88%) and 12-hydroxyeicosatetraenoic acid (12-HETE) (12%). **B:** Amounts of [^3H]12-KETE formed during exposure to histamine (H) or artificial seawater (control, C). Error bars represent the SEM, $n = 4$. (From Piomelli *et al.*¹⁵ Reprinted by permission from the *Journal of Biological Chemistry*.)

hydroxy-11,12, epoxy-eicosatrienoic acids.^{2,15,16} Application of histamine selectively releases [^3H]12-HETE and [^3H]12-KETE, however, On the other hand, after intracellular stimulation of the identified neuron L32, prelabeled abdominal ganglia release [^3H]12-HETE¹ and [^3H]8-hydroxy-11,12-epoxy-eicosatrienoic acid.² The reason for the difference in metabolites released by the two physiological treatments still remains to be determined. One possibility is that activation of specific receptors may result in the release of specific metabolites because the receptors activated by histamine might be a different subset of histaminergic receptors from those activated by the transmitter released endogenously by L32. Alternatively, while all the known actions of L32 cells are simulated by histamine and L32 PSPs are sensitive to cimetidine, a histamine antagonist in *Aplysia*,^{17,18} it is still uncertain whether L 32 cells are definitively histaminergic.¹⁹

In preliminary experiments, we found that intracellular stimulation of C2, an identified histaminergic neuron in the cerebral ganglion of *Aplysia*,^{19,20} results in release of [^3H]12-KETE. Stimulating C2 did not evoke the formation of [^3H]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.

Physiological Activity of 12-KETE on Identified Aplysia Neurons

Pharmacological experiments with L14 and L10, neurons of the abdominal ganglion, suggest that 12-KETE participates in the intracellular transduction of some

of the actions of histamine. Each identified cell shows different and characteristic electrophysiological responses to histamine. In L14, histamine rapidly depolarizes the membrane, which is typically followed by a longer-lasting hyperpolarization.¹⁸

In the majority of neurons tested, applications of 12-HPETE or 12-KETE (1–2 nmol) from an extracellular puff micropipette produced a response similar to that evoked by histamine (FIG. 5, TABLE 1). Similar puffs of 12(S)-HETE were ineffective.

L10, a mixed-action neuron regulating heart and kidney function,²¹ responds to histamine with a slow hyperpolarization, caused by increased K^+ conductance and decreased Ca^{2+} conductance.¹⁸ A similar inhibitory response in L10 cells was produced by 12-KETE (TABLE 1). In only 30% of the cells tested was 12-HPETE effective, however; and 12(S)-HETE was again ineffective (TABLE 1). A possible explanation is that, as applied by the puffing micropipette, the metabolites are not completely accessible to critical sites in L10 at the concentrations used. Further experiments, using L10 neurons in culture, would be useful to test this idea.

The biological actions of 12-KETE that we have observed are in agreement with the hypothesis that conversion of 12-HPETE to the keto-acid is necessary for some of the effects of the hydroperoxy acid. Voltage-clamp and patch-clamp studies would show whether these 12-lipoxygenase products affect the same ion channels modulated by the endogenous transmitter.

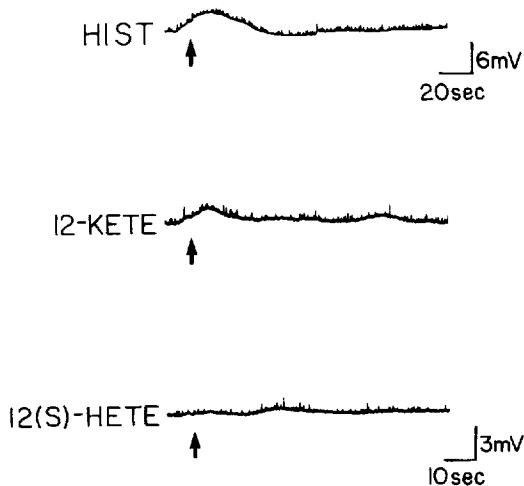


FIGURE 5. Effect of histamine and 12-lipoxygenase products on the membrane potential of L14. 1–2 nmoles of a test substance were ejected by pressure (5 sec, 6 psi) from a glass micropipette situated about 0.5 mm from the cell body of a L14 impaled with a voltage-sensitive microelectrode. Histamine (HIST) and 12-keto-5,8,10,14-eicosatetraenoic acid (12-KETE) elicited early depolarizing responses followed by a small slow hyperpolarization. 12(S)-hydroxy-5,8,10,14-eicosatetraenoic acid [12(S)-HETE] was ineffective in changing the membrane potential. The histamine 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). (From Piomelli *et al.*¹⁵ Reprinted by permission from the *Journal of Biological Chemistry*.)

TABLE 1. Responses of Identified *Aplysia* Neurons to Histamine and Metabolites of Arachidonic Acid^a

Treatment ^b	Experiments	Observations ^c	Responses ^d	Percent of Responses of Each Type		
				Depolarizing	Hyperpolarizing	Dual-Action ^e
Cell L14						
Histamine	28	58	54	11	2	87
12-KETE	6	18	16	12	25	62
12(S)-HETE	7	20	4	—	—	—
12-HPETE	15	34	28	—	—	—
Cell L10						
Histamine	15	35	33	0	100	0
12-KETE	3	11	8	0	100	0
12(S)-HETE	5	12	1	—	—	—
12-HPETE	9	18	6	—	—	—

NOTE: Compounds were applied to cells L14 and L10 as described in the legend to FIG. 5 and EXPERIMENTAL PROCEDURES.

^aFrom Piomelli *et al.*¹⁵^b12-KETE = 12-keto-5,8,10,14-eicosatetraenoic acid; 12(S)-HETE = 12(S)-hydroxy-5,8,10,14-eicosatetraenoic acid; 12-HPETE = 12-hydroperoxy-5,8,10,14-eicosatetraenoic acid.^cWithin an experiment, 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.^dTotal responses of all three types.^eDepolarizing/hyperpolarizing.

/ Ineffective.

§ Not determined.

SUMMARY

The lipoxygenase product 12-hydroperoxy-5,8,10,14-eicosatetraenoic acid (12-HPETE), 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*.^{1,2} The 12-lipoxygenase pathway has not yet been fully characterized, but 12-HPETE is known to be metabolized further. Therefore, we began to search for other metabolites in order to investigate whether the actions of 12-HPETE might require its conversion to other active products. We have identified 12-keto-5,8,10,14-eicosatetraenoic acid (12-KETE) as a metabolite of 12-HPETE formed by *Aplysia* nervous tissue. 12-KETE was identified in incubations of the tissue with arachidonic acid using HPLC, UV spectrometry, and gas-chromatography/mass spectrometry. [³H]12-KETE is formed from endogenous lipid stores in nervous tissue, labeled with [³H]arachidonic acid upon stimulation 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. Another metabolite of 12-HPETE, 12(s)-hydroxy-5,8,10,14-eicosatetraenoic acid [12(S)-HETE], is inactive. These results support the hypothesis that 12-HPETE and its metabolite, 12-KETE, participate in transduction of histamine responses in *Aplysia* neurons.

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