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Biologically Active Metabolites of the 12-Lipoxygenase Pathway Are Formed by *Aplysia* Nervous Tissue

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**INTRODUCTION**

*Aplysia californica* is a marine mollusk that has been the subject of extensive neurobiological study. Its simple nervous system, composed of large neurons, has permitted the identification and mapping of many specific cell connections. In addition to electrophysiologic observations of these cells, the biochemical definition of the neurotransmitter content of many neurons also has been accomplished. In spite of this wealth of detail, little data have been reported on the lipid biochemistry in this species. Polyunsaturated fats, an important component of membrane phospholipids, are especially prominent in marine animals. Nevertheless, as recently as 1973, Komai *et al.* suggested that these lipids were absent from a related species of *Aplysia*. We have reevaluated the fatty acid composition of *Aplysia* nervous tissue and specifically investigated the metabolism of an important polyunsaturated fat, arachidonic acid. In addition to its role as a phospholipid component, this fatty acid is the presursor of a variety of biologically active metabolites. One pathway, initiated by the 12-lipoxygenase enzyme, converts arachidonate into 12-hydroperoxy-eicosatetraenoic acid (12-HPETE), which is rapidly reduced to 12(S)-hydroxy-eicosatetraenoic acid (12(S)-HETE). Although these products were first discovered in 1974, no clear biological function had yet been assigned to them. Our studies of lipid metabolism in *Aplysia* have led to the identification of a 12-lipoxygenase pathway that is activated by physiological stimuli. Furthermore, the production of several biologically active metabolites will be described in this volume. The metabolism of 12-HPETE to 12-keto-eicosatetraenoic acid is discussed elsewhere; the identifica-
tion and activity of an epoxy alcohol, 8-hydroxy-11,12-epoxy-eicosatrienoic acid (8-HEpETE), is the subject of this report.

METHODS

Preparation of Tissue: Biochemical Analyses

Ganglia were dissected from *Aplysia* (70–200 g; Howard Hughes Medical Institute Mariculture Resource Facility, Woods Hole, MA or Marinus, Sand City, CA), and trimmed of connective tissue. In some experiments, the ganglia were exposed to [*H]*arachidonic acid (2.5–25 μCi for 2–20 h) in an artificial seawater. The tissue was then washed and stimulated in order to study the metabolism of its endogenous lipids as described below. In other experiments, the nervous tissue was homogenized and incubated with exogenous arachidonate (50 μM) or histamine (50 μM) in the seawater.

Preparation of Tissue: Electrophysiologic Analyses

The trimmed abdominal or cerebral ganglia were pinned to silicone plastic in a superfusion chamber (0.3–0.4 ml) with flowing artificial seawater (1–5 ml/min). Identified cell bodies were impaled with glass microelectrodes (10–20 MΩ resistance filled with 2 M potassium citrate) and identified by previously defined *in situ* methods. In some experiments, cells were impaled with two electrodes, one for recording voltage and the other to pass current. In a series of experiments designed to examine the stimulation of arachidonic acid metabolism by synaptic stimulation, ganglia were incubated with [*H]*arachidonate (see above) before the identified presynaptic (L32) and postsynaptic (L14) cells were impaled. To test for biological activity, compounds were pressure-ejected from micropipettes (10–20 μm tip diameter) positioned approximately 0.5 mm above the cell body of L14. Pressure pulses lasted 1–10 s and ejected volumes of 1–10 μl of the solutions to be tested. Solutions of 12-HPETE, 12(S)-HETE, 8-HEpETE sodium salt in alcohol were dried under nitrogen and resuspended in seawater by sonication.

Lipid Extractions and Analysis

Lipids were extracted from homogenates of nervous tissue with hexane/isopropanol (3:2; v/v) as previously described. Complex lipids were transesterified by treatment with 12% boron trifluoride in methanol at 70 °C for 1 h. The recovered fatty acid methyl esters were measured by gas chromatography (HP5840 GC, SP2340 fused silica capillary, 30 m × 0.32 mm).

Unextracted bath samples were analyzed for the presence of 12-HETE by chromatography on a Novapak C18 column (150 × 3.9 mm) eluted with methanol/water/acetic acid (73:27:0.1; v/v) flowing at 1 ml/min.

In other experiments, the bath medium was acidified with formic acid (pH 3.6–4.0) and extracted twice either with ethyl acetate or with diethyl ether. The dried extracts were fractionated by normal-phase HPLC (Silicar LC Si, 250 × 4.6 mm) eluted with hexane/isopropanol/acetic acid (98:2:0.1; v/v) at 1 ml/min. Purified fractions were used for further analysis by GC/MS, scintillation counting, enzymatic hydrolysis, or reversed-phase HPLC.
Preparation of Standard Epoxy Alcohols: Enzymatic Hydrolysis

Synthetic 8(R,S)-hydroxy-11,12-trans-epoxy-5,14-cis-9-trans-eicosatrienoic acid methyl ester was prepared by the method of Corey and Wei-Guo and saponified to produce the sodium salt just before use. Standard epoxy alcohols were prepared from rat lung and purified as reported. Rat lung epoxide hydrolase was prepared according to Pace-Asciak.

Purified epoxy alcohols as well as the products of their enzymatic hydrolysis were chromatographed by reversed-phase HPLC (Nucleosil C18; 250 × 4.6mm) eluted with methanol/water/acetic acid (75:25:0.1; v/v) at 1 ml/min.

TABLE 1. Fatty Acids of Total Lipids in Aplysia Nervous System*

| Fatty Acid | Relative Abundance (Area %) |
|------------|-----------------------------|
| 14:0       | 12.2 ± 6.2                  |
| 16:0       | 10.4 ± 3.9                  |
| 18:0       | 10.4 ± 3.9                  |
| 18:1       | 9.8 ± 1.6                   |
| 18:2       | 5.3 ± 3.2                   |
| 20:1*      | 4.3 ± 1.2                   |
| 20:2       | 5.5 ± 1.9                   |
| 20:4       | 9.9 ± 1.5                   |
| 20:5       | 2.9 ± 1.5                   |
| 22:2       | 4.9 ± 1.3                   |
| 22:4       | 24.3 ± 5.9                  |
| 22:6       | 4.2 ± 3.3                   |
| Others     | 10.7 ± 4.1                  |

*From Piomelli et al.

RESULTS AND DISCUSSION

Fatty Acid Composition of Aplysia Nervous Tissue

Aplysia neural components (neuronal cell bodies and neuropil) were isolated and homogenized, and their total cellular lipids were extracted. In some experiments, the lipid extract was fractionated into phospholipid subclasses before the fatty acid content of the extract or subclass was determined. Polyunsaturated fatty acids as a group made up over 57% of the total fatty acid content of the neural lipids, and arachidonic acid alone comprised nearly 10% of the total (Table 1). The distribution of fatty acids within each phospholipid subclass did not differ greatly from that found in the total lipid extract. When isolated neural components were incubated with [3H]arachidonic acid, the fatty acid was rapidly incorporated into membrane phospholipids, from which it could be released by a variety of stimuli.
Identification of 12-HETE

Neural components were homogenized and incubated with exogenous arachidonic acid. After 30 min, lipids were extracted and fractionated by normal-phase HPLC (FIG. 1A). A major peak eluted at the retention time of authentic 12-HETE. The identity of the material was confirmed to be 12-HETE by ultraviolet spectrophotometry (FIG. 1B) and by GC/MS using negative-ion chemical ionization of the pentafluorobenzyl ester–trimethylsilyl ether (PFB-TMS) derivative and electron impact mass fragmentography of the methyl ester–TMS derivative. The stereochemistry of the 12-hydroxyl is tentatively assigned to be (S) based upon the cross-reactivity of the material derived from Aplysia with antiserum raised against 12(S)-HETE.

Neurotransmitter-induced Synthesis of 12-HETE

Neural components were first labeled with [3H]arachidonate and then exposed to histamine, a neurotransmitter in Aplysia. Products isolated from the bathing...
FIGURE 3. Formation of 12-HETE produced by intracellular stimulation of L32 neurons. Labeled abdominal ganglia were stimulated as described in the legend to Figure 4. HPLC chromatograms of bath samples taken before (B) and after (A) stimulation. Stimulation of L32 evoked production of labeled 12-HETE. Samples were analyzed by reversed-phase HPLC for 12-HETE (see Methods). (Modified from Piomelli et al.)

medium were fractionated by reversed-phase HPLC, and each fraction was tested for radioactivity. A single peak of labeled material was detected at a retention time identical to that of synthetic 12-HETE (Fig. 2A). Release of [3H]12-HETE was significantly less during control incubations or in the presence of the histamine antagonist cimetidine (Fig. 2B).

Similar results were obtained when the putative histaminergic neuron L32 was stimulated to release its endogenous transmitter. L32 cells were identified in ganglia that had been prelabeled with [3H]arachidonate. The neuron was impaled and driven electrically by three 2-s pulses, which produced 40 spikes. This protocol induced the synthesis of [3H]12-HETE (450 ± 85 cpm/ganglion). No product could be detected during control periods (n = 5) (Fig. 3).

Production of 12-HETE after treatment with histamine or intracellular stimulation of L32 neurons suggests a potential physiologic role for metabolites of the 12-lipoxygenase pathway. When 12(S)-HETE was applied to L14 neurons, which are follower cells of L32, no effect was observed (Fig. 4). In contrast, 12-HPETE, the immediate precursor of 12-HETE, induced a response similar to that caused by histamine; both 12-HPETE and histamine cause a dual-action response of L14, similar to the response of L14 after intracellular stimulation of L32 (Fig. 4). Furthermore, L14's dual-action response to histamine could be blocked by applying bromophenacyl bromide, an inhibitor of phospholipase.17

FIGURE 4. Response of L14. L14 was impaled for intracellular recording of membrane potential. L32 stimulation: The presynaptic cell, L32, was driven with a 5-s train of depolarizing pulses of 200 ms at 5 Hz. Each pulse produced three spikes in L32 (not shown). The resting potential of L14 was -60 mV. This response is typical but was not recorded from the same L14 used in the other recordings. Histamine (100 pmol over 1 s) was applied to L14 (resting potential -70 mV). 12-HPETE (100 pmol over 3 s) was applied to L14 (resting potential -70 mV). 12(S)-HETE (250 pmol over 5 s) was applied to L14 (resting potential -64 mV). Histamine and 12-HPETE records are from the same L14 cell. The arrow indicates the beginning of drug application. (From Piomelli et al. Reprinted by permission from Proceedings of the National Academy of Sciences of the United States of America.)
Although the activity found with 12-HPETE may be inherent to this molecule, the reactive hydroperoxide is known to undergo complex metabolism (Fig. 5). In the presence of iron-containing proteins, 12-HPETE undergoes an intramolecular rearrangement to yield two diastereomeric epoxy alcohols 8(R,S)-hydroxy-11,12-epoxy-5,9,14-eicosatrienoic acid (8-HEpETE) and 10(R,S)-hydroxy-11,12-epoxy-5,8,14-eicosatrienoic acid (10-HEpETE). The epoxy alcohols are converted to trihydroxy acids (THETE) by an epoxide hydrolase. In addition, 12-HPETE can be converted to carbonyl-containing molecules, for example, the keto acid 12-keto-5,8,10,14-eicosatetraenoic acid (12-KETE). In this paper, we have studied the synthesis and physiological activity of the epoxy alcohols in Aplysia nervous tissue. Data on 12-KETE are reported elsewhere.

**FIGURE 5.** Metabolic pathways of 12-HPETE.

Homogenates of Aplysia nervous tissue were incubated with [3H]arachidonate and the products purified by extractive isolation followed by normal-phase HPLC. A major product was detected at the retention time reported for 8-HEpETE and had the same elution characteristics as biosynthetically prepared standard 8-HEpETE. When HPLC-purified Aplysia-derived 8-HEpETE and standard 8-HEpETE were reanalyzed by reversed-phase HPLC, they again showed identical retention values (Fig. 6B). In addition, incubation of purified and standard 8-HEpETE with rat lung epoxide hydrolase converted both compounds to products (isomeric THETE) that were indistinguishable by HPLC analysis (Fig. 6C, D). This biochemical evidence that the Aplysia metabolite is 8-HEpETE was confirmed by GC/MS both in negative-ion
chemical ionization (pentafluorobenzyl ester, trimethylsilyl ether) and mass fragmentography (methyl ester, trimethylsilyl ether).  

In order to test for the physiologic production of the metabolite, L32 neurons in abdominal ganglia prelabeled with [3H]arachidonic acid were driven electrically. The labeled products isolated from the bath were fractionated by normal-phase HPLC

![Figure 6](image_url)

**FIGURE 6.** Reversed-phase HPLC analysis of purified 8-HEpETE before and after hydrolysis. A: [3H]8-HEpETE, synthesized with rat lung 12-lipoxygenase, was applied to the HPLC. C: Some of this material was incubated with epoxide hydrolase before analysis. Purified Aplysia [3H]8-HEpETE (B) and its enzymatic hydrolysis product (D) were similarly analyzed. One-min fractions of the HPLC effluent were assayed for radioactivity by liquid scintillation counting. (From Piomelli et al. Reprinted by permission from Proceedings of the National Academy of Sciences of the United States of America.)

![Figure 7](image_url)

**FIGURE 7.** Production of metabolites from prelabeled abdominal ganglia. Ganglia were labeled with [3H]arachidonate before L32 neurons were impaled and driven as described in the legend to FIGURE 4. A: L32 was stimulated to fire 40 spikes. The bath medium was analyzed for radioactive products by normal-phase HPLC; 1-min fractions were collected. B: Bath medium was collected as a control before impaling L32. C: [3H]epoxy alcohols were quantified after L32 (n = 4) in the abdominal ganglion or C2 (n = 5) in the cerebral ganglion were stimulated intracellularly. Solid bar: controls; open bar: stimulated. Values are mean ± SEM. (From Piomelli et al. Reprinted by permission from Proceedings of the National Academy of Sciences of the United States of America.)
In addition to previously identified compounds, a major peak of material eluted at the retention time of 8-HEpETE. This material was absent in controls (FIG. 7B). Although both epoxy alcohols are produced in nearly equal amounts by the nonenzymatic rearrangement of 12-HPETE, no 10-HEpETE could be detected in the experimental samples that contained significant amounts of 8-HEpETE (FIG. 7A). Interestingly, 10-HEpETE is chemically stable in contrast to its isomer, which contains an allylic epoxide and is, therefore, quite labile. Thus the appearance of

![Diagram](https://example.com/diagram.png)

**FIGURE 8.** L14 response to 8-HEpETE. L14 cell was impaled with two intracellular electrodes, one to pass current and the other to record voltage. One-second hyperpolarizing current pulses were delivered at 0.2 Hz to monitor L14's input resistance. A: A 2-s application of histamine (1 μl; 0.1 nmol) to L14 causes a dual-action, fast depolarizing–slow hyperpolarizing response in L14 (top trace, HIST). Membrane potential of L14 was -60 mV. A 10-s application of 8-HEpETE (4 μl; 24 nmol) elicits a hyperpolarizing response and decreased resistance in L14 (middle trace, 8-HEpETE). Membrane potential of L14 was -40 mV. A 10-s application of vehicle (10 μl; prepared in parallel to the 8-HEpETE hydrolysate) to L14 causes no response and no change in input resistance (lower trace, VEHICLE). Membrane potential of L14 was -60 mV. B: In the same experiment, a second 10-s application of 8-HEpETE (3 μl; 18 nmol) again caused a hyperpolarization of L14 (dashed line) associated with decreased input resistance. Conductance pulses are shown at faster sweep speed to demonstrate the decrease in input resistance. L14 resting membrane potential was -60 mV.

8-HEpETE after L32 stimulation without concomitant production of 10-HEpETE suggests that this conversion is enzymatically controlled. Additional support for this idea comes from the observation that a different neural circuit (C2 and its followers in the cerebral ganglion) that is known to possess 12-lipoxygenase activity does not generate epoxy alcohols (FIG. 7C). Thus, the presence of the 12-lipoxygenase alone is not sufficient to yield 8-HEpETE in an Aplysia neuron.
Biological Activity of 8-HEpETE

The physiologic activity of 8-HEpETE was assessed by applying the epoxy alcohol to L14 neurons. Histamine produces a dual-action response in L14 (Figs. 4 and 8A). In contrast, 8-HEpETE induces marked hyperpolarization that appears similar to the late hyperpolarizing phase of the histamine response. No effect was detected with the vehicle control. The hyperpolarization induced by 8-HEpETE is accompanied by an increased ionic conductance (Fig. 8B). This effect has a calculated reversal potential of $-77 \text{ mV}$ (data not shown), which is similar to the conductance change and reversal potential of the slow inhibitory postsynaptic potential caused by L32 stimulation. The similarities of the reversal potential and conductance changes between the hyperpolarizing effects of 8-HEpETE and synaptic activation (activation of L32) suggests a common ionic mechanism. Moreover, the differences between the response of L14 neurons to 8-HEpETE and the effect of histamine and L32 stimulation may be due to the compound nature of the dual-action synaptic response. 8-HEpETE may be responsible for the hyperpolarizing phase, while another metabolite (perhaps 12-KETE; see References 7, 22) induces the initial depolarization. Further electrophysiological and patch-clamp analysis is underway to provide direct evidence for the identity of the conductance pathways modulated by L32, histamine, and specific metabolites of arachidonic acid in L14.

We have demonstrated an active 12-lipoxygenase pathway in Aplysia nervous tissue. Physiologic stimulation leads to the production of several biologically active compounds, including the epoxy alcohol 8-HEpETE. Furthermore, there is some indication that the production of this epoxy alcohol is under enzymatic control. The synthesis and activity of 8-HEpETE lead us to propose that this compound may serve as a novel second messenger in the nervous system of Aplysia.

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