Metabolism of arachidonic acid in nervous system of marine mollusk *Aplysia californica*

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PIOMELLI, DANIELE. *Metabolism of arachidonic acid in nervous system of marine mollusk Aplysia californica.* Am. J. Physiol. 260 (Regulatory Integrative Comp. Physiol. 29): R844–R848, 1991.—Studies of the marine mollusk *Aplysia californica* indicate that products of the 12-lipoxygenase pathway may be involved in neuronal intracellular signaling. The nervous tissue of *Aplysia* has a 12-lipoxygenase activity that converts both exogenous and endogenous arachidonic acid to an array of products, which include 12-hydroperoxyeicosatetraenoic acid (12-HPETE) and its metabolites hepoxilin A_3, hepoxilin B_3, 12-ketoeicosatetraenoic acid, and 12-oxododecatrienoic acid. These eicosanoids were identified using a combination of high-performance liquid chromatography, ultraviolet spectrometry and gas chromatography-mass spectrometry. Generation of 12-lipoxygenase products was stimulated by application of the neurotransmitters, histamine and FMRF-amide, or by stimulation of identified neural cells. In electrophysiological studies of identified L14 and sensory neurons it was found that 12-HPETE and its metabolic products exert physiological actions that resemble those of histamine and FMRF-amide. These results suggest that products of 12-HPETE metabolism may act as second messengers in *Aplysia* neurons.

12-lipoxygenase; 12-hydroperoxyeicosatetraenoic acid; eicosanoids

THE MARINE OPISTOBRANCH MOLLUSK *Aplysia californica* provides a very useful model for neurobiological studies. Its relatively simple nervous system, composed of large, easily identifiable neurons, has allowed the characterization of many specific synaptic circuits. In addition, the membrane properties and the neurotransmitter content of many neurons have been determined (5). Despite this wealth of information, little is known of the biochemistry and physiology of bioactive lipids in this species. In this article the results of studies carried out on the metabolism of arachidonic acid (AA) in *Aplysia* neurons are summarized (10–14).

AA is the precursor of a group of biologically active molecules, the eicosanoids. In neurons, a major pathway of AA metabolism is initiated by 12-lipoxygenase (15). This enzyme catalyses the conversion of AA into 12-hydroperoxyeicosatetraenoic acid (12-HPETE), which can then be reduced to yield the alcohol 12-hydroxyeicosatetraenoic acid (12-HETE) or further metabolized to form two isomeric epoxy alcohols hepoxilin A_3 and B_3 as well as the carbonyl-containing compounds, 12-ketoeicosatetraenoic acid (12-KETE) and 12-oxododecatrienoic acid (Fig. 1; 3, 4, 8). The studies of *Aplysia* reviewed here indicate that the 12-lipoxygenase pathway may be activated by physiological stimuli and participate in neuronal intracellular signaling.

This paper was presented at the Symposium entitled "Comparative Physiology of Eicosanoids" held during the 74th Annual Meeting of the Federation of American Societies for Experimental Biology, Washington DC, April 4, 1990.

Fatty Acid Composition of Aplysia Nervous Tissue

Total cellular lipids were extracted from nervous tissue and analyzed by thin-layer chromatography and gas chromatography-mass spectrometry (GC/MS) (11). Polyunsaturated fatty acids comprised ~57% of the total fatty acid content of tissue lipids, and AA accounted for ~10% of the total. Other polyunsaturated fatty acids present included eicosapentaenoic acid (20:5) and docosatetraenoic acid (22:4).

Identification of 12-HETE

Neural tissue homogenates were incubated in the presence of exogenous AA (50 μM). Lipids were extracted and subjected to normal-phase high-performance chromatography (HPLC) (11). A major ultraviolet (UV)-absorbing peak (235 nm) eluted at the retention time of authentic 12-HETE. The identity of this material was confirmed by both UV spectrometry and GC/MS, using negative ion chemical ionization and electron-impact mass fragmentography.

Receptor-Dependent Release of 12-HETE

Nervous tissue of *Aplysia* was labeled by incubation with [^{3}H]AA. This procedure results in the rapid labeling of tissue phospholipids (11). The tissue was then exposed to histamine (50 μM), a well-characterized neurotransmitter in *Aplysia* (7) and released products were fractionated by reversed-phase HPLC. After addition of histamine, a major peak of radioactive material was detected at the retention time of authentic 12-HETE [1,710 ± 358 counts/min (cpm); $n = 4$]. Release of this labeled material was significantly reduced when, before addition of histamine, the nervous tissue was incubated in the presence of the histamine antagonist cimetidine. Release of lipoxygenase products from prelabeled nervous tissue was also seen with the neuropeptide, FMRF-amide (10 μM). In contrast, serotonin (100 μM), a neurotransmitter that activates the adenosine 3′,5′-cyclic monophosphate

(cAMP) cascade in *Aplysia* neurons (6), had no effect (13).

Electrical stimulation of L32 cells, a group of identified neurons in the abdominal ganglion, also resulted in the release of radioactive 12-HETE. L32 cells were identified in ganglia that had been prelabeled with [^{3}H]AA. The neurons were then impaled and stimulated electrically to produce action potentials (~40 spikes/stimulation). This caused the generation of 12-[^{3}H]HETE (450 ± 85 cpm/ganglion; $n = 5$), whereas no radioactive products could be detected in control, unstimulated samples.

Production of 12-HETE after treatment with neurotransmitters or after intracellular stimulation of L32 neurons suggests a potential physiological role for metabolites of the 12-lipoxygenase pathway. When 12-HETE was applied to L14 neurons, which are histaminoceptive followers of L32 cells, no effect was seen (Fig. 2). In contrast, 12-HPETE produced a response similar to that caused by histamine (Fig. 2; 12). Furthermore, the dual-action response to histamine could be blocked by incubating the ganglia in the presence of the phospholipase A inhibitor, *p*-bromophenacylbromide (14). In addition to these actions on histaminoceptive neurons, 12-HPETE mimicked the electrophysiological response of sensory cells to the tetrapeptide, FMRF-amide. FMRF-amide-induced opening of K^+ channels, membrane hyperpolarization, and shortening of the action potential were reproduced by applications of AA or 12-HPETE (1, 2, 13).

These findings suggest that 12-HPETE may be the second messenger underlying the response to histamine and FMRF-amide in *Aplysia* L14 and sensory cells.

12-HPETE is known to undergo a complex metabolism (Fig. 1). In the presence of hematin or iron-containing proteins, 12-HPETE gives rise to two diastereomeric epoxy alcohols, hepoxilin A_3 (8-hydroxy-11,12-epoxyeicosatrienoic acid) and hepoxilin B_3 (10-hydroxy-11,12-epoxyeicosatrienoic acid) (8). These epoxy alcohols are converted to trihydroxyacids by a specific epoxide hydrolase (9). In addition, 12-HPETE can be converted to the ketoacid 12-KETE and the short-chain aldehyde 12-oxododecatrienoic acid (3, 4). It is possible that the biological effects of 12-HPETE may be carried out by one or more of these products.

Formation of Hepoxilins in Aplysia Neurons

To begin to address this question, we studied the metabolism of 12-HPETE in *Aplysia* neurons. Homogenates of nervous tissue were incubated with [^{3}H]AA and

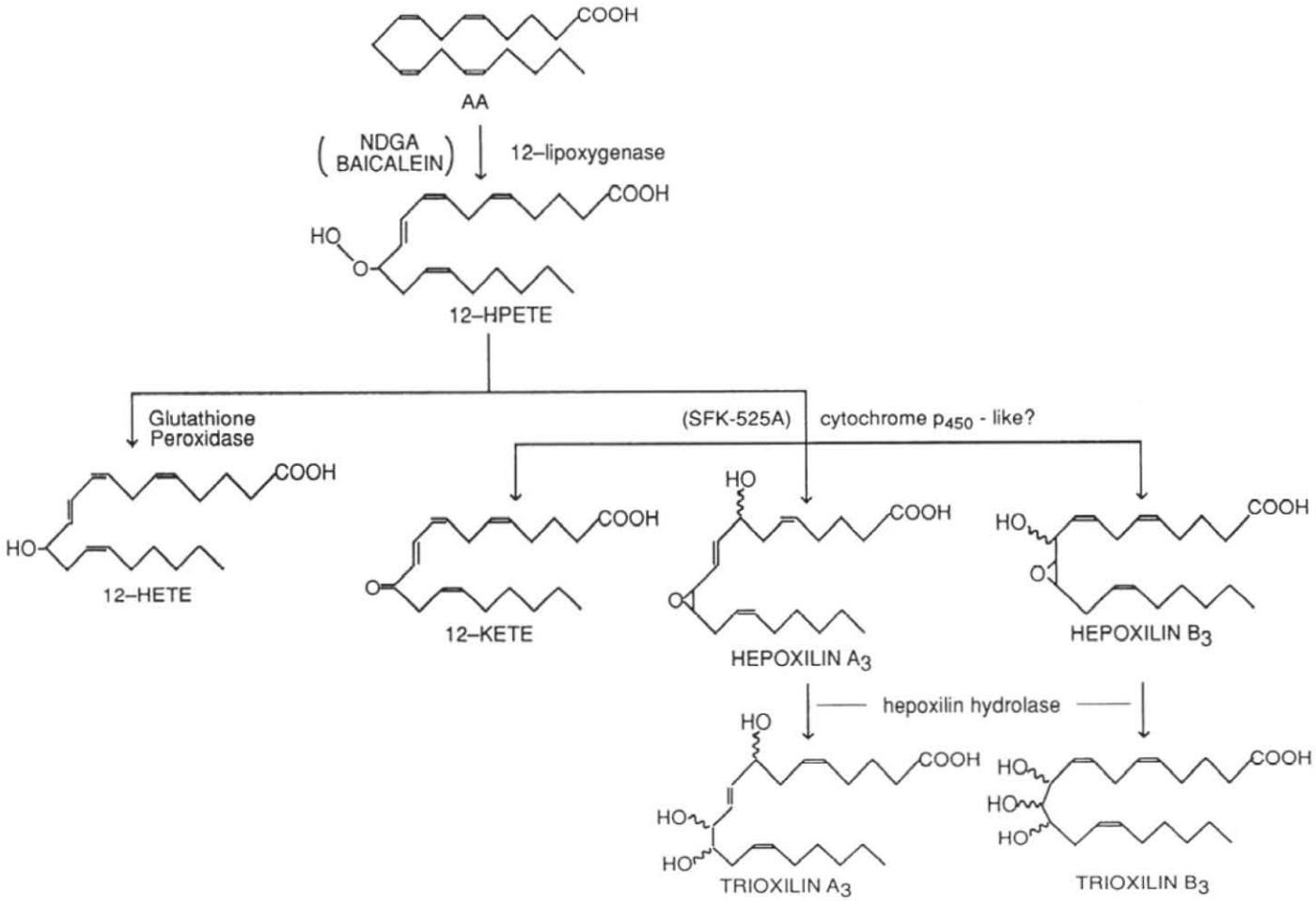

FIG. 1. 12-Lipoxygenase pathway in *Aplysia* nervous tissue. Enzyme inhibitors are indicated in parentheses. 12-HPETE, 12-hydroperoxyeicosatetraenoic acid; 12-HETE, 12-hydroxyeicosatetraenoic acid; 12-KETE, 12-ketoeicosatetraenoic acid; NDGA, nordihydroguaiaretic acid.

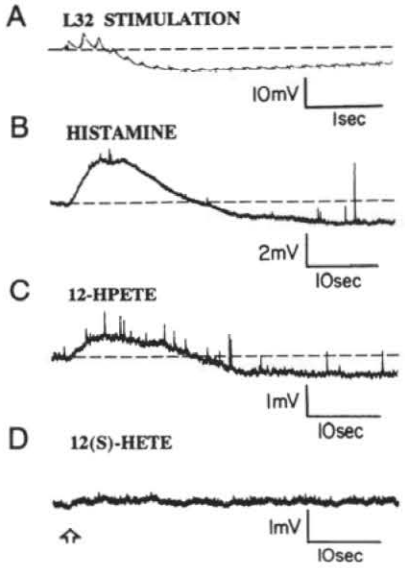

FIG. 2. Response of L14 neurons to stimulation of presynaptic L32 cells (*A*) or applications of histamine (*B*), 12-HPETE (*C*), and 12-HETE (*D*). [From Piomelli et al. (12).]

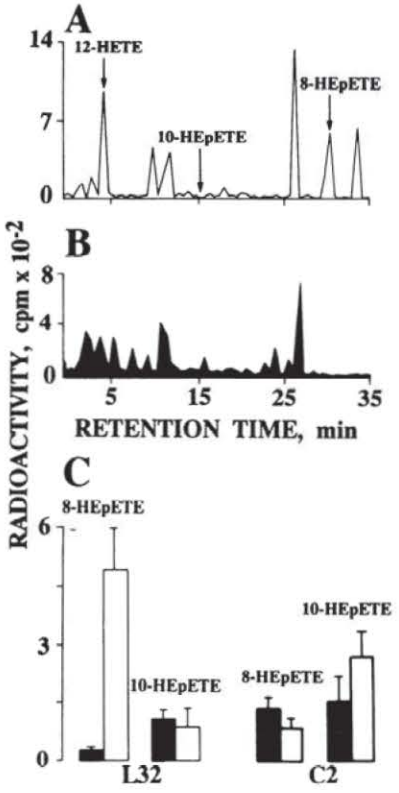

FIG. 3. Production of AA metabolites from radioactively labeled *Aplysia* ganglia. *A*: normal-phase high-performance liquid chromatography (HPLC) of metabolites formed by stimulation of L32 neurons. *B*: control, unstimulated sample. *C*: quantitation of hepoxilin formation after stimulation of L32 and C2 neurons. Values are means ± SE. [From Piomelli et al. (12).]

the lipid products were extracted and isolated by normal-phase HPLC (12). A major radioactive component had the same elution characteristics of standard [^{3}H]hepoxilin A_3 (prepared biosynthetically using a rat lung acetone extract). When *Aplysia*-derived material was further analyzed by reversed-phase HPLC, its retention time was again identical to that of [^{3}H]hepoxilin A_3. In addition,

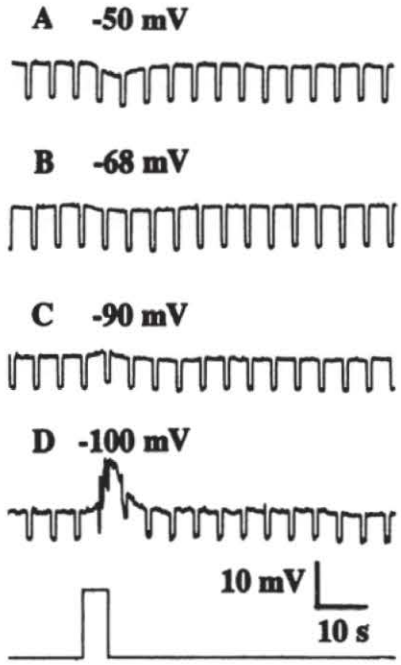

FIG. 4. Response of L14 neurons to application of hepoxilin A_3 at different membrane potentials (*A–D*). L14 neurons were impaled with 2 glass microelectrodes, one for passing current and the other for recording membrane potential. [From Piomelli et al. (12).]

incubation of standard hepoxilin A_3 and *Aplysia*-derived material with rat lung epoxide hydrolase resulted in the conversion of both compounds to products (isomeric trihydroxy acids) that were indistinguishable by HPLC analysis. This biochemical evidence was confirmed by GC/MS, using both negative ion chemical ionization and electron-impact mass fragmentography (12).

To test for the physiological production of hepoxilin A_3, L32 neurons were stimulated electrically in abdominal ganglia prelabeled with [^{3}H]AA. The radioactive products were extracted and analyzed by normal-phase HPLC (Fig. 3*A*). In addition to [^{3}H]AA and 12-[^{3}H]-HETE, a major radioactive peak eluted at the retention time of hepoxilin A_3. This material was absent in unstimulated control samples (Fig. 3*B*). Although both epoxy alcohols are produced in nearly equal amounts by the nonenzymatic rearrangement of 12-HPETE, no hepoxilin B_3 could be detected in the experimental samples that contained significant amounts of hepoxilin A_3. The specific appearance of hepoxilin A_3 after L32 stimulation, without apparent production of hepoxilin B_3, suggests that this reaction may be under enzymatic control. In agreement, stimulation of a different cell, the histaminergic neuron C2, did not result in the generation of hepoxilins (Fig. 3*C*; 12).

Biological Actions of Hepoxilin A_3

The physiological activity of hepoxilin A_3 was assessed on L14 neurons, which are synaptic followers of L32 cells (12). In L14, application of histamine or stimulation of L32 produced a dual action response (rapid membrane depolarization followed by longer lasting hyperpolarization) (Fig. 4*A*). In contrast, the application of hepoxilin A_3 (prepared by total chemical synthesis by Dr. Robert

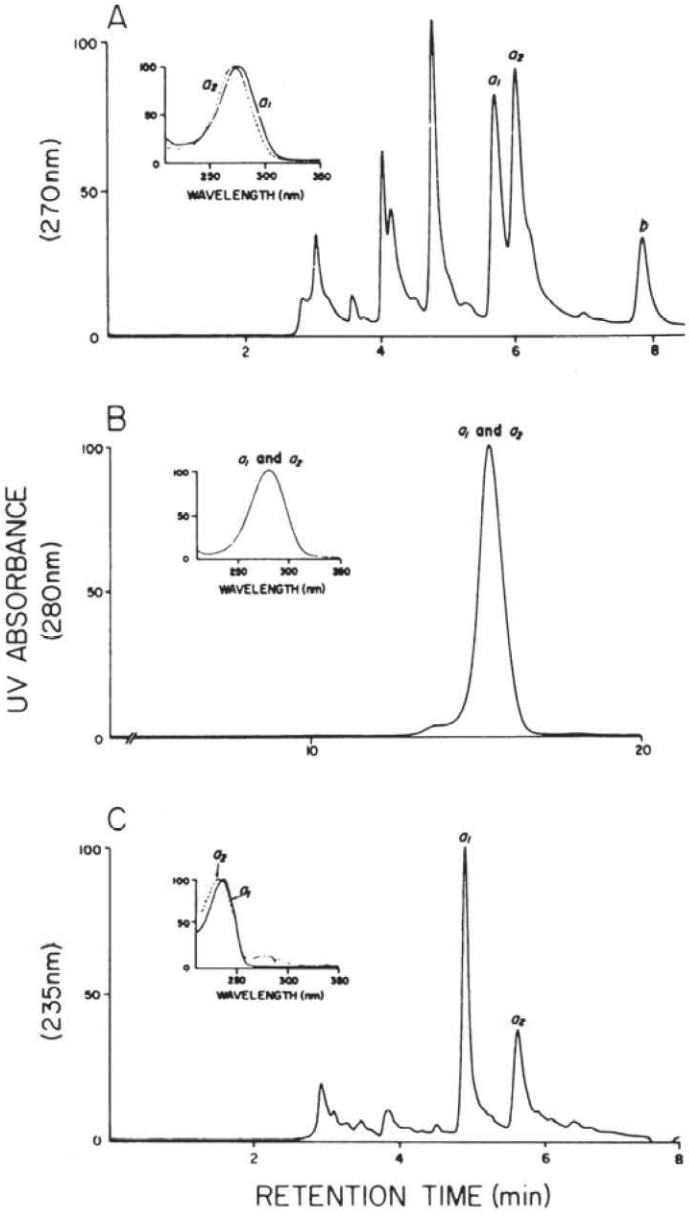

FIG. 5. Isolation and characterization of 12-KETE from *Aplysia* nervous tissue. *A*: normal-phase HPLC. *B*: reversed-phase HPLC. *C*: normal-phase HPLC of alcohols resulting from reduction of *compounds* a_1 and a_2 with sodium borohydride. *Insets* show spectra obtained using a flow-through diode-array spectrophotometer. UV, ultraviolet. [From Piomelli et al. (10).]

Zipkin, Biomol Research Laboratories) results in a marked membrane hyperpolarization, accompanied by an increased membrane ion conductance (Fig. 4*B*). No effect was seen with vehicle. The response to the hepoxilin had a calculated reversal potential of −77 mV, which is similar to the reversal potential of the slow inhibitory postsynaptic response caused by L32 stimulation. These similar conductance changes and reversal potential suggest a common ionic mechanism. The differences between these two responses may be due to the compound nature of the dual-action synaptic response to L32 stimulation. Possibly, the hepoxilin may be responsible for the hyperpolarizing phase, whereas another metabolite (perhaps 12-KETE, see below) may underlie the initial depolarization.

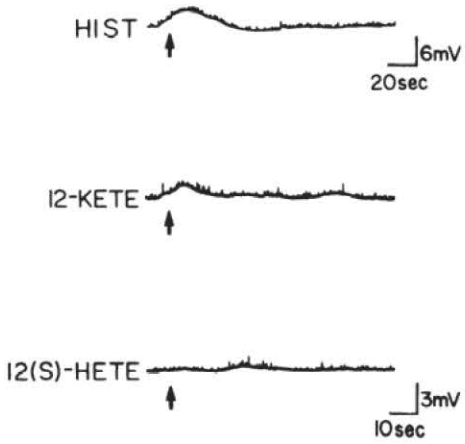

FIG. 6. Effects of histamine, 12-KETE, and 12-HETE on membrane potential of L14 neurons. L14 was impaled with a single microelectrode for voltage recording. [From Piomelli et al. (10).]

Identification of 12-KETE

Identification of 12-KETE was carried out by HPLC, UV spectrometry and GC/MS analysis of lipid extracts of nervous tissue incubated with exogenous AA (10). When *Aplysia* neural homogenates were incubated with AA and the metabolites analyzed by normal-phase HPLC, several unidentified components with absorption maxima at 270 nm were observed (Fig. 5*A*).

The UV spectra of *compounds* a_1 and a_2 (Fig. 5*A*, *inset*) suggested the presence of a dienone or dienal chromophore, with maximal absorption at 273 nm for a_1 and 271 nm for a_2. After purification by normal-phase HPLC, these compounds were also analyzed by reversed-phase HPLC, where they eluted as a single component (Fig. 5*B*). UV spectral analysis (Fig. 5*B*, *inset*) showed pronounced bathochromic shift in absorbance (lambda max = 280 nm). A spectral shift caused by increased polarity of the solvent is characteristic of conjugated dienones and dienals (4).

The presence of a conjugated carbonyl group was confirmed by reducing 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 absorbance near 235 nm (Fig. 5*C*). The first (a_1) eluted with the retention time of 12-HETE methyl ester and had an absorption maximum at 235 nm (Fig. 5*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. This suggests that reduction of the compounds with sodium borohydride yields two alcohols, 12-hydroxy-5,8,10,14-*ZZEZ*-eicosatetraenoic acid methyl ester (12-HETE ester) and its geometric isomer, 12-hydroxy-5,8,10,14-*ZEEZ*-eicosatetraenoic acid methyl ester. Identification of *compounds* a_1 and a_2 was further confirmed as 12-KETE by GC/MS, using both chemical ionization and electron-impact mass spectrometry (10).

Stimulation of 12-KETE Production by Histamine

Application of histamine to [^{3}H]AA-labeled *Aplysia* neural tissue caused an ~10-fold increase in radioactivity associated with 12-KETE, compared with controls (10).

In contrast, formation of the aldehyde 12-oxododecatrienoic acid, which is formed when nervous tissue is incubated with AA, was not increased by this treatment. Thus application of histamine selectively raises 12-HETE and 12-KETE, whereas stimulation of L32 neurons releases 12-HETE and hepoxilin A_3. The reasons for the difference in metabolites generated by the two treatments remain to be determined.

Biological Actions of 12-KETE in Aplysia Neurons

Pharmacological experiments with the identified neuron L14 indicate that 12-KETE may participate in the intracellular signaling pathways used by histamine in this cell. Application of 12-KETE produced a membrane hyperpolarization that was often (62% of cases) followed by a slow hyperpolarization (Fig. 6). In contrast, applications of 12-HETE had no effect.

The pharmacological actions of 12-KETE that we have observed are in agreement with the idea that conversion of 12-HPETE to 12-KETE is necessary for at least some of the effects of the hydroperoxide. Voltage-clamp and patch-clamp studies will be necessary to determine whether these 12-lipoxygenase products affect the same ion channels modulated by histamine and the L32 transmitter.

In conclusion, the studies summarized here have shown the existence of a physiologically regulated 12-lipoxygenase pathway in neurons of *Aplysia*. Pharmacological and physiological stimulations lead to the formation of various compounds that are biologically active. These include hepoxilin A_3 and 12-KETE. Furthermore, there is experimental evidence suggesting that synthesis of these compounds may be under enzymatic control. These findings indicate that products of the 12-lipoxygenase pathway may act as intracellular second messengers in *Aplysia* neurons.

The author thanks Drs. F. Belardetti, J. H. Schwartz, E. Shapiro, S. J. Feinmark, S. Siegelbaum, A. Volterra, E. R. Kandel, and R. Zipkin, who collaborated at various stages of these studies.

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