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Lipoxygenase metabolites of arachidonic acid as second messengers for presynaptic inhibition of *Aplysia* sensory cells

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Biochemical and biophysical studies on Aplysia *sensory neurons indicate that inhibitory responses to the molluscan peptide FMRFamide are mediated by lipoxygenase metabolites of arachidonic acid. These compounds are a new class of second messengers in neurons.*

In many of the synaptic actions of neurotransmitters, the binding of transmitter to a cell membrane receptor alters the activity of ion channels indirectly through a second messenger. The number of substances that have been recognized as second messengers in neurons continues to increase since Sutherland's discovery of cyclic AMP in 1957[1] and now includes, in addition to cAMP[2-4], cGMP[5], Ca^{2+} (ref. 6) and the phospholipid metabolites, diacylglycerol[7-10] and inositol phosphates[9-11]. In addition to these diffusible second messengers, recent evidence indicates that GTP-binding proteins[12] can also modulate ion channels[13-16]. Are there other kinds of second messengers yet to be identified?

A promising mechanism for generating second messenger molecules for neuronal signalling is the receptor-mediated release of arachidonic acid from membrane phospholipids. Release of arachidonic acid has been well characterized in non-neural cells, where free arachidonate is rapidly metabolized to a family of bioactive products (the eicosanoids) that are thought to be both intracellular and intercellular messengers (see ref. 17 for review). Although the metabolism of arachidonic acid in the brain has been studied for more than 20 years[18-23], the complexity of the mammalian central nervous system has made it difficult to assign specific functions to the eicosanoids. We have taken advantage of the simple nervous system of the marine mollusc *Aplysia*, where it is possible to examine identified nerve cells of known behavioural function both in intact ganglia and in dissociated cells in culture. Our results indicate that inhibitory synaptic actions of the neuroactive peptide FMRFamide, which produces presynaptic inhibition of transmitter release in sensory neurons of *Aplysia*, are mediated by lipoxygenase metabolites of arachidonic acid.

Previous studies of *Aplysia* sensory neurons have characterized an excitatory effect of serotonin (5-hydroxytryptamine or 5-HT) that involves a prolonged all-or-none closure of a specific class of K^+ channels (the S channels) through cAMP-dependent protein phosphorylation[24-27]. Closure of these channels by 5-HT produces slow depolarization, broadens the action potential in the cell body and increases transmitter release from sensory neuron terminals (presynaptic facilitation). Recently, application of FMRFamide (a tetrapeptide denoted by FMRF in single-letter code) to these sensory neurons was found to hyperpolarize the membrane, decrease the duration of the action potential[28], increase an outward K^+ current[29,30] and inhibit synaptic transmission (presynaptic inhibition)[28]; actions that are opposite in effect to those of 5-HT. In other molluscan neurons, FMRFamide has also been shown to decrease the inward calcium current[30,31] and to activate an inward cationic current[32,33]. Using single-channel recording, Belardetti *et al.*[34] showed that FMRFamide increases the net outward K^+ current in sensory neurons by increasing the probability that S channels are in the open state (see also ref. 30). FMRFamide can also reverse the closure of S channels produced by 5-HT or cAMP[34]. Thus, the actions of FMRFamide and 5-HT are antagonistic both at the cellular level and at the level of the single channel.

Several independent lines of evidence suggested that the effects of FMRFamide are produced by a second messenger other than cAMP[29,30,34]. We have examined the arachidonic acid cascade because its metabolites are formed in *Aplysia* neural tissue in response to application of histamine (ref. 35), a neuromodulator capable of producing presynaptic inhibition in other neurons of *Aplysia*[36].

Aplysia eicosanoid pathways

Arachidonate is a prominent component of *Aplysia* neural phospholipids (~10% of total fatty-acid content) and can be metabolized in *Aplysia* neurons through both the 12-lipoxygenase and the 5-lipoxygenase pathways, as well as through the cyclooxygenase pathway[35]. These pathways are shown in Fig. 1*a*, with the sites of action of the various drugs used in this study. Two stable lipoxygenase products, the hydroxy acids 12-hydroxyeicosatetraenoic acid (HETE) and 5-HETE, were previously identified in neural tissue from *Aplysia*[35]. In mammals, these hydroxy acids are formed by enzymatic reduction of the corresponding short-lived hydroperoxides (HPETE)[23]. The presence of hydroperoxy acids is only inferred in *Aplysia*, because we have not yet isolated these unstable precursors. Both 12-HPETE and 5-HPETE may be metabolized to products other than the hydroxy acids[17]. Finally, the cyclooxygenase pathway leads to the synthesis of various prostaglandins, which in *Aplysia* nervous tissue include PGE_2 and $PGF_{2\alpha}$ (ref. 35).

Arachidonate mimics FMRFamide

To investigate the possible physiological function of arachidonic acid metabolites, we first examined whether exogenously applied arachidonic acid can simulate the actions of FMRFamide on the sensory neurons of the abdominal and pleural ganglia (Fig. 2). When applied extracellularly by pressure ejection from a large pipette[37], arachidonic acid (4.5–50 μM) simulated the macroscopic actions of FMRFamide, producing a slow hyperpolarization of the membrane (Fig. 2*a*) associated with an increase in membrane conductance (not shown), decreasing the duration of the action potential (Fig. 2*b*), and inhibiting synaptic transmission between the sensory neuron and motor neuron (Fig. 2*c*). These effects were seen both in intact ganglia (Fig. 2*b* and *c*) and in isolated neurons in culture[38] (Fig. 2*a*). Eicosa-11-monoenoic acid, a 20-carbon chain fatty acid with one double

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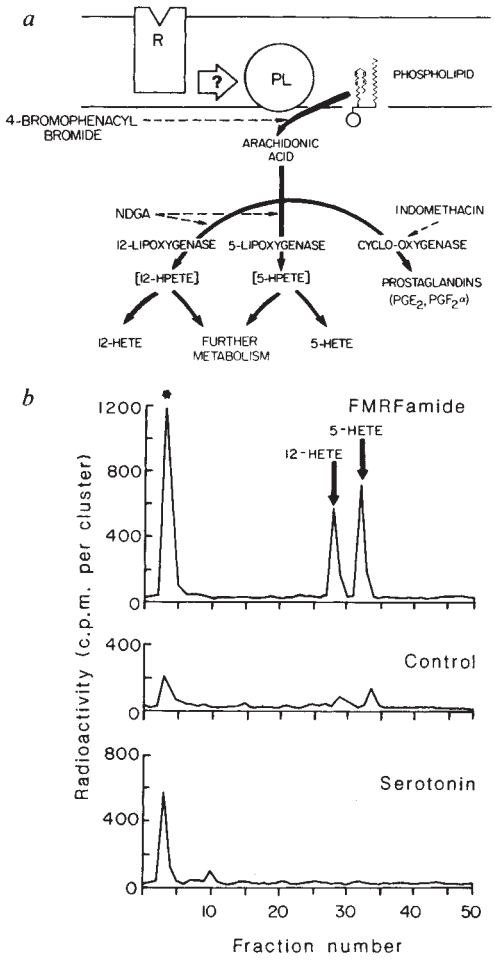

Fig. 1 *a*, Pathways of arachidonic acid metabolism in neural tissue of *Aplysia californica*. In mammals, arachidonic acid release is catalysed by a phospholipase A_2 or by the combined action of a phospholipase C and a diacylglycerol-lipase[39,40] (PL). Both enzymes can be stimulated in a receptor-mediated (R) fashion and are irreversibly inhibited by 4-bromophenacyl bromide[42]. In *Aplysia*, esterified arachidonate accounts, on average, for 10% of total fatty acids in neural phospholipids[35]. Free arachidonic acid can be metabolized in *Aplysia* neural tissue through the 12-lipoxygenase, the 5-lipoxygenase and the cyclooxygenase pathways[35]. Two lipoxygenase products, 12-hydroxyeicosatetraenoic acid (12-HETE) and 5-HETE have been identified[35]. These stable end-products are thought to be derived from the corresponding unstable hydroperoxy acids (HPETE). The lipoxygenase reaction is selectively inhibited by low concentrations of nordihydroguaiaretic acid (NDGA). *Aplysia* neural tissue also has cyclooxygenase activity and forms several prostaglandins, of which PGE_2 and $PGF_{2\alpha}$ have been identified[35]. Prostaglandin formation is inhibited by indomethacin. *b*, FMRFamide stimulates the release of lipoxygenase products from sensory clusters of *Aplysia*. Representative reversed-phase HPLC fractionation of samples from the incubation of one sensory cluster (≈200 cells) with FMRFamide (10 μM for 1 min, top trace), without FMRFamide (middle) or with 5-HT (10 μM for 1 min, lower trace). Arrows, retention volumes of authentic 12-HETE and 5-HETE standards. The radioactive material associated with the solvent front (*) was consistently increased in the presence of FMRFamide (control: 536 ± 136 c.p.m. per 100 cells; FMRFamide: 1,701 ± 377 c.p.m. per 100 cells, mean ± s.e.m.; $n = 4$, $P < 0.001$. Student's *t*-test). This component includes polar metabolites of arachidonic acid that are not yet identified.

bond, which constitutes 4.3% of *Aplysia* neural fatty acids[35], had little effect on resting potentials, action potentials, or synaptic potentials (Table 1).

The similarity between the physiological effects of FMRFamide and arachidonic acid results from their sharing common ionic mechanisms. Voltage-clamp experiments on

Table 1 Comparison of fatty acid metabolites and FMRFamide

Resting potential analysis*

Compound	Concentration (μM)	ΔV_m (mV)	Number responding	*n*
Cell culture				
FMRFamide	2	−6.4 ± 4.9	6	9
Arachidonic acid	4.5–45	−5.8 ± 7.3	4	5
12-HPETE	1.5	−4.6 ± 2.9	3	4
5-HPETE	1.5	0	0	4
12-HETE	1.5	−0.6 ± 0.8	2	5
5-HETE	1.5	0	0	4
Intact ganglia				
Arachidonic acid	10–50	−4.6 ± 5.2	12	15
Eicosa-11-monoenoic acid	50	+0.2 ± 0.7	2	9

Action potential analysis†

Compound	Concentration (μM)	APD (%)	Recovery (%)	*n*
FMRFamide	5	−18.2 ± 5.7	91.5 ± 7.8	5
Arachidonic acid	50	−17.2 ± 5.4	95.0 ± 3.5	5
Eicosa-11-monoenoic acid	50	−4.5(−10, +1)	95.0	2
12-HPETE	60	−27.4 ± 12.4	94.4 ± 9.7	5
5-HPETE	60	−11.8 ± 5.2	96.7 ± 3.9	5
12-HETE	60	−5.7 ± 2.2	98.3 ± 1.6	6
5-HETE	60	−4.3 ± 2.5	98.3 + 1.9	6

Synaptic potential analysis‡

Compound	Concentration (μM)	PSP Size (%)	Recovery (%)	*n*
FMRFamide	5–10	−46.8 ± 7.2	85.0 ± 13.9	4
Arachidonic acid	50	−45.0 ± 14.7	84.0 ± 19.0	5
Eicosa-11-monoenoic acid	50	−7.7 ± 1.7	95.7 ± 2.1	3

Single-channel analysis§

Compound	Concentration (μM)	*I*(%)	*i*(%)	*n*
FMRFamide	2–10	+442.2 ± 210.4	−0.06 ± 0.08	5
Arachidonic acid	50	+269.0 ± 110.0	−8.1 ± 5.1	5
Eicosa-11-monoenoic acid	50	+1.2	−9.1	1

* Changes in membrane potential (ΔV_m) as peak response to test compound. Data from sensory neurons in cell culture and intact ganglia as marked. Numbers given are mean ± s.e.m.; number responding indicates number of cells showing a detectable response (≥1 mV). Mean values include responders and non-responders.

† Action potential duration in sensory neurons in intact abdominal ganglia in response to brief suprathreshold current pulses. %APD, maximum per cent decrease in action potential duration from control value in response to test compound; % recovery, per cent return of APD to control value after washing out compound for 5–10 min. Action potential duration measured at time when membrane had repolarized by 80% from peak amplitude.

‡ Postsynaptic potential amplitudes measured in motor neurons from intact pleural ganglia in response to firing the sensory neuron. Maximal % decrease in motor neuron fast EPSP and % recovery are plotted.

§ Single-channel analysis in sensory neurons in cell culture. Mean single S channel current (*I*) measured by integrating 1-min long stretches of data before and after addition of compound to the bath. Maximal % change in *I* and in open channel current amplitudes (*i*) are listed.

sensory neurons in culture show that arachidonic acid and FMRFamide produce similar effects on the membrane current–voltage relation (Fig. 3*a*). The currents in response to a series of voltage steps, before and shortly after application of FMRFamide, are compared in Fig. 3*a*I. Although the time course of these current traces is complicated by overlapping capacitative-, time- and voltage-dependent ionic currents, FMRFamide clearly shifts the current in the outward direction over most of the voltage range.

The FMRFamide-sensitive current can be obtained by subtracting the trace recorded in the absence of FMRFamide from that recorded in its presence (Fig. 3*a*II). This FMRFamide-sensitive difference current is likely to contain contributions from several different ionic current[30–34]. At voltages below −30 mV, the difference current displays the characteristic outward rectification typical for the S current[25,26]. Arachidonic acid produces a similar outward shift in the unsubtracted current record (Fig. 3*b*I) which yields a similar difference current (Fig. 3*b*II).

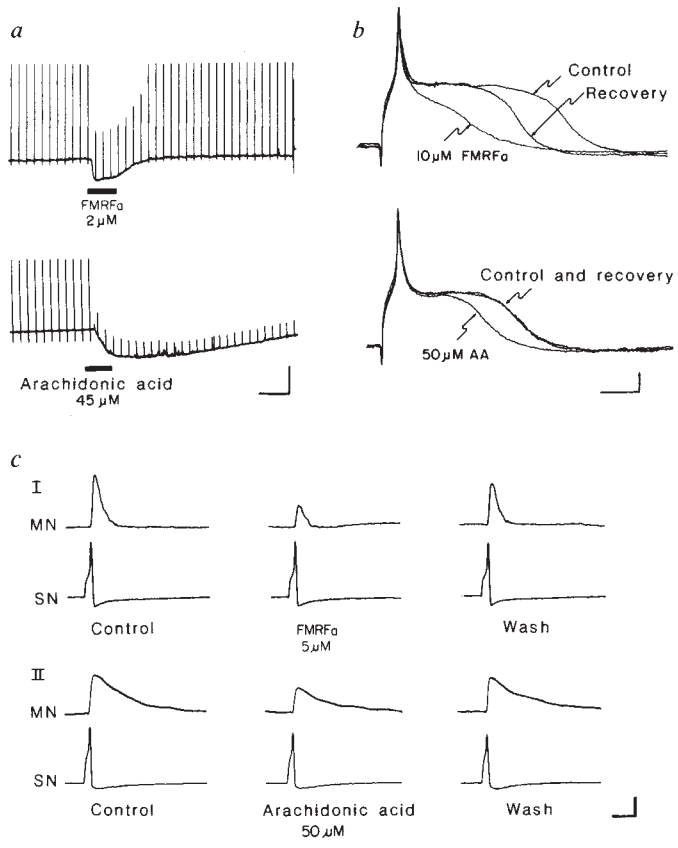

Fig. 2 Arachidonic acid simulates the action of FMRFamide on whole cell potential responses. *a*, Changes in membrane potential and spike firing of sensory neurons in culture in response to FMRFamide (2 μM, top trace) or arachidonic acid (45 μM, lower trace). Solid bar, period of application. Intracellular records are from the same cell. Initial resting potential was −41 mV (top) or −35 mV (below). Scale bar: 20 mV vertical, 40 s horizontal. Upward spikes, action potentials elicited once every 10 s by brief depolarizing (constant) current stimuli. The smaller spikes reflect action potential inhibition and are the passive depolarizing responses to the current step. The slower time course of the arachidonate-induced hyperpolarization most probably reflects the time required for diffusion (or transport) across the plasma membrane and metabolism. *b*, FMRFamide and arachidonate (AA) cause a reversible decrease in action potential duration. Action potentials recorded from sensory neuron in an intact abdominal ganglion in presence of 50 mM tetraethyl-ammonium (TEA) (control). In absence of TEA, decrease in action potential duration is also observed with FMRFamide and arachidonic acid; the brief duration of the control action potential makes shortening difficult to measure accurately, however. Resting potential in the top trace was −43 mV before FMRFamide and −48 mV in presence of peptide. In the lower trace, resting potential was −43 mV before arachidonate and −44.5 mV in presence of the fatty acid. Scale bar: 20 mV vertical, 10 ms horizontal. In the intact ganglia, response to arachidonic acid is somewhat slower than in the culture, probably owing to the restricted access and diffusion of the fatty acid. *c*, Reduction in fast excitatory synaptic potential in follower motor neuron (MN traces) in response to firing an action potential in sensory neuron (SN traces). Records I and II are from two different pairs of cells in intact abdominal ganglia. Scale bar: vertical 8 mV for MN, 32 mV for SN traces; horizontal 50 ms.

Methods. Membrane potentials were recorded using conventional intracellular 3 M KCl microelectrodes (10–20 MΩ). Action potentials were elicited by passing brief depolarizing currents through the recording electrode using a bridge circuit. Two preparations were studied: the gill and siphon mechanoreceptor sensory neurons in intact abdominal ganglia and identified tail mechanoreceptor sensory neurons from the pleural ganglia plated in dissociated cell culture[38]. Test compounds were applied to sensory neurons by pressure ejection from a wide mouthed pipette[37]. Concentrations given here were the concentrations of compounds in the pipette. FMRFamide obtained from Peninsula Laboratories. Arachidonic acid obtained from Nu-Check-Prep (Elysian, MN). The artificial sea water (ASW) contained (in mM): 460 NaCl, 10 KCl, 11 $CaCl_2$, 55 $MgCl_2$, 10 HEPES, *p*H 7.4. Arachidonic acid in toluene was dried in the dark under a gentle stream of nitrogen immediately before use, ASW added and the mixture sonicated for 15 s. Preparations containing oily droplets, as seen under the microscope, were not used. Arachidonic acid purity was 99%, as measured by thin-layer chromatography (using hexane/ethylether/acetic acid/methanol, 85:20:4:2, by volume). Because of the poor solubility of arachidonic acid in water, the free concentration in solution is likely to be lower than the nominal value.

To obtain more direct evidence that FMRFamide and arachidonic acid share a common mode of action, we studied single S-channel currents in cell-attached membrane patches (Fig. 4). S-channels were identified by their characteristic elementary current size, outwardly rectifying current-voltage relation and weak voltage dependence of gating[26].

Within 1–2 min of applying either FMRFamide or arachidonic acid in the bath, we observed a large and consistent increase in S-channel opening. The effects on S-channel activity were quantified by measuring the mean (time-averaged) current in the patch (I) and the single-channel current amplitude (i). In the experiment in Fig. 4, FMRFamide increased the mean S-channel current up to sixfold over a range of membrane voltages (Fig. 4*b*, top) with no significant effect on the single-channel current amplitude (Fig. 4*c*, top). Arachidonic acid increased mean channel current up to fourfold (Fig. 4*b*, bottom) with no significant effect on single-channel amplitude (Fig. 4*c*, bottom). This effect of arachidonic acid was seen in five out of five experiments, whereas eicosa-11-monoenoic acid was ineffective (Table 1).

The mean current through a patch, I, depends on the product $N_f \times i \times p$, where N_f is the number of functional channels in the patch, i the single-channel current amplitude, and p the probability that a channel is open. Previously Belardetti *et al.*[34] found that FMRFamide increases I by increasing p, with no effect on N or i. In experiments using a binomial analysis, we were able to confirm that the mode of action of arachidonic acid is similar to that of FMRFamide because it increases p with no marked effect on N or i. Thus, by all the criteria we have tested, the actions of arachidonic acid and FMRFamide are identical.

Although these experiments show that arachidonic acid simulates the action of FMRFamide, they do not prove that metabolites of endogenous arachidonate mediate the response to the peptide. Arachidonic acid could be acting on the K^+ channel through an independent parallel mechanism. We have obtained evidence for an obligatory involvement of arachidonic acid using two separate approaches, one pharmacological and the other biochemical.

Effects of inhibitors

The first step in the receptor-mediated generation of eicosanoids is the release of free arachidonate from membrane phospholipids, a reaction that in mammals is most commonly catalysed by a phospholipase A_2, or by the combined action of phospholipase C and diacylglycerol lipase[39,40]. Although selective inhibitors of either phospholipase are not yet available, both enzymes can be effectively blocked by 4-bromophenacyl bromide[41]. This inhibitor, at a concentration of 10 μM, completely suppresses the hyperpolarization produced by FMRFamide in cultured neurons ($n=4$) (Fig. 5*b*). The depolarizing response to 5-HT is not affected in the same cells, indicating that the drug does not interfere with S-channel function.

The experiments with the phospholipase inhibitor suggest that the release of arachidonic acid is a necessary step in the response

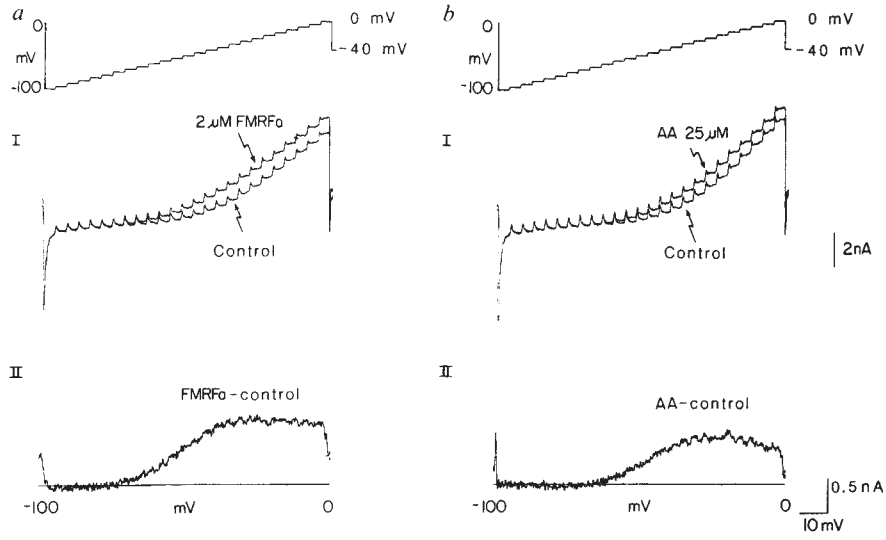

Fig. 3 Comparison of the actions of FMRFamide and arachidonic acid (AA) on the total ionic current in voltage-clamped sensory neurons. *a*, Effect of FMRFamide (2 μM); *b*, effect of arachidonic acid (25 μM). Membrane potential held at −40 mV. Once every 30 s, a 500-ms staircase voltage command composed of 20-ms depolarizing steps of 4-mV increments, from −100 mV to 0 mV, was applied (upper traces in *a* and *b*). FMRFamide and arachidonic acid solutions prepared and applied as described in Fig. 2 legend. *a*, Trace I, superimposed records of total membrane current before (lower trace, average of 4 records, control) and at peak action of the peptide (upper trace, average of 2 records, FMRFa). Trace II, the control trace subtracted from the FMRFa trace to yield the FMRFamide-sensitive current. *b*, Current records from the same cell, after prolonged washing with normal sea water. Trace I, superimposed averaged records of total current before (lower trace, average of 5 records, control) and during (upper trace, average of 2 records, AA 25 μM) application of 25 μM arachidonic acid. Trace II, arachidonic acid-sensitive current, obtained by subtraction of the records in trace I. Below −30 mV, it seems that the difference current largely reflects the outwardly rectifying S current. Positive to −30 mV contributions from other FMRFamide-sensitive currents (including a decreased calcium and calcium-activated K current) overlap with the S current[30–33].

Methods. Sensory neurons in culture were voltage-clamped using a single microelectrode discontinuous voltage-clamp (Axoclamp-2A). Microelectrodes were filled with 2.5 M KCl and had resistances of 10 MΩ. In some experiments, electrodes were coated with a silicone polymer (Sylgard, Dow Corning) to reduce capacitance. Switching frequencies of 5–7 kHz were employed. Current records low pass filtered at 300 Hz using an 8-pole Bessel filter (Frequency Devices), digitized at 1.0 kHz and stored on a hard disk using a DEC 11/73 computer and an INDEC laboratory interface. The computer also applied the voltage clamp command stimuli. Stored current records were averaged and subtracted by computer.

to FMRFamide. To determine which metabolic pathway is responsible for producing the active eicosanoids, we tested the actions of indomethacin (indometacin), which blocks cyclooxygenase[42] and nordihydroguaiaretic acid (NDGA), which blocks lipoxygenases[43]. Indomethacin, which we find inhibits formation of prostaglandins in *Aplysia* ganglia with a half-maximal inhibitory concentration of 0.5 μM without affecting lipoxygenase activity ($IC_{50} > 10$ μM), has no effect on the hyperpolarization induced by FMRFamide (Fig. 5*c*). In five separate experiments, the response to FMRFamide in the presence of 5 μM indomethacin was 84 ± 15% of the control response. In contrast NDGA, which inhibits lipoxygenase activity in *Aplysia* nervous tissue with an IC_{50} of 3 μM, suppresses the hyperpolarizing response of sensory cells in culture to FMRFamide (Fig. 5*d*). On average, the hyperpolarization produced by FMRFamide (2 μM) after treatment with NDGA (5 μM; $n = 3$) was only 9.3 ± 7.4% of the response in the absence of the drug. The depolarization produced by 5-HT was not affected. These experiments suggest that a lipoxygenase rather than cyclooxygenase is involved in the intracellular signalling process.

Release of hydroxy acids

To determine whether lipoxygenase metabolites of arachidonic acid were actually released in response to FMRFamide, we studied clusters of sensory neurons dissected from pleural ganglia and labelled by incubation with [^{3}H]arachidonic acid (60 Ci mmol^{-1}). FMRFamide stimulates the formation of radioactive material that migrated on reversed-phase HPLC with authentic 12-HETE and 5-HETE. In four experiments, after a 1-min exposure to FMRFamide (10 μM), 448 ± 66 c.p.m. per cluster ($\bar{x} \pm$ s.e.m.) were recovered in HPLC fractions corresponding to 12-HETE and 1,622 ± 628 c.p.m. per cluster in 5-HETE after reversed-phase HPLC purification. A representative chromatogram is shown in Fig. 1*b* (top trace). In all experiments, the radioactive material at the solvent front also increased in the presence of FMRFamide (control: 1,072 ± 272 c.p.m. per cluster; FMRFamide: 3,402 ± 754 c.p.m. per cluster, $n = 4$, $P < 0.001$, Student's *t*-test). This component includes more polar metabolites of arachidonic acid (possibly prostanoids) that are not yet identified. In control incubations (Fig. 1*b* middle) or incubations with artificial sea water containing 5-HT (10 μM) (Fig. 1*b*, bottom), only background radioactivity was found in the 12-HETE and 5-HETE fractions (20 ± 3 c.p.m., $n = 8$).

12-HPETE mimics FMRFamide

Independent support for the involvement of lipoxygenase metabolites comes from experiments where we directly tested the action of exogenously applied metabolites on the sensory neurons. Lipoxygenases convert arachidonate into hydroperoxy acids; in mammals these are either reduced to the corresponding hydroxy acids or transformed into metabolites that are distinctive for each of the lipoxygenase pathways[17]. We tested the actions of both 12- and 5- hydroperoxy and hydroxy acids on the resting and action potentials of sensory neurons (Fig. 6). Application of 12-HPETE (1.5 μM) simulates the slow hyperpolarization of the resting potential and the decrease in excitability seen with FMRFamide (Fig. 6*a*). 5-HPETE, 5-HETE and 12-HETE have little effect on resting potential (Fig. 6*a* and Table 1). Both 12-HPETE and 5-HPETE simulate the decrease in action potential duration observed with FMRFamide (Fig. 6*b*). The average decrease in action potential duration with 12-HPETE, however, was two- to threefold greater than the decrease in response to 5-HPETE. Again, 12-HETE and 5-HETE had little effect on the duration of the action potential (Fig. 6*b* and Table 1). These results confirm the biochemical and pharmacological experiments that implicate lipoxygenase metabolites in the actions of FMRFamide; they further suggest that the active second messenger derives from a hydroperoxy acid, possibly 12-HPETE, rather than from a hydroxy acid.

Discussion

We find that the receptor-stimulated release of arachidonic acid and its metabolism through a lipoxygenase pathway mediate the inhibitory synaptic response to the tetrapeptide FMRFamide in *Aplysia* sensory neurons. Our results provide strong evidence that lipoxygenase products act as second messengers in nerve

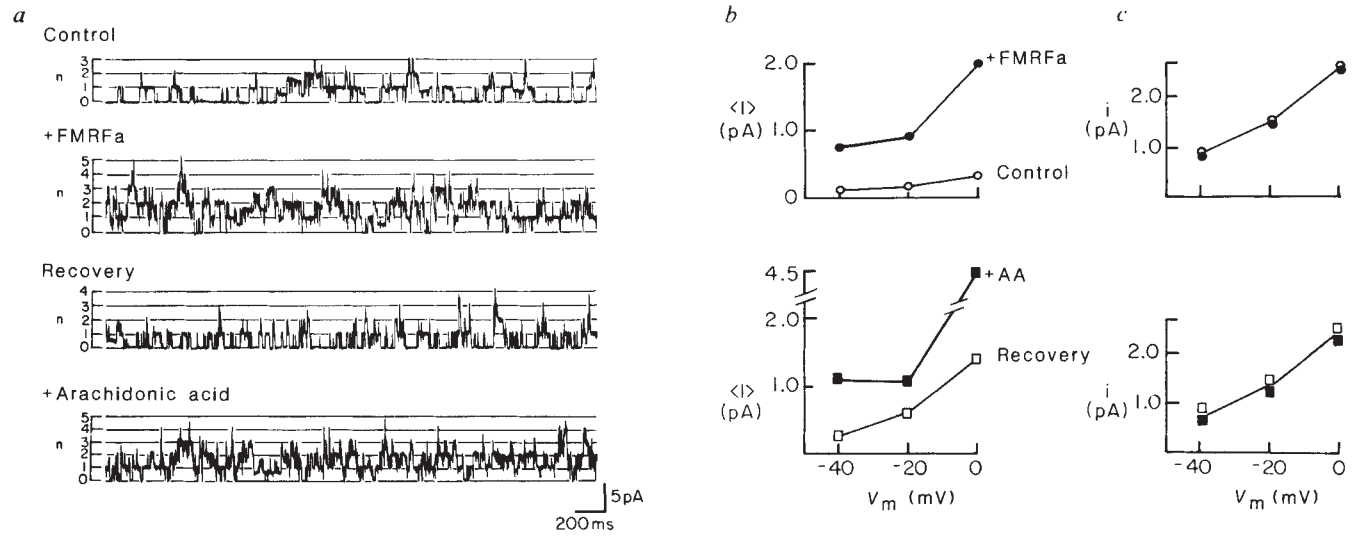

Fig. 4 Comparison of the effects of FMRFamide and arachidonic acid (AA) on single S-channel currents. *a*, Single-channel recordings from a cell-attached patch on a pleural sensory neuron in culture. Control record: before application of any compounds; +FMRFa: 7 min after application of 5 μM FMRFamide to the bath; recovery: 20 min after washing the peptide out of the bath; +arachidonic acid: 6 min after application of 50 μM arachidonic acid. Binomial fit to data yields estimate of $n = 6$ for the number of active S channels under all conditions. Records obtained at a patch potential depolarized by +80 mV above the resting potential. *b*, Effect of compounds on mean (time-averaged) current through patch. Mean current ($\langle I \rangle$) measured from the time integral of patch current above baseline for 30 s stretches of records at three membrane potentials (V_m). Membrane potential was altered from rest by changing voltage in the patch pipette. Voltages on abscissa are approximate transmembrane potentials of the patch, assuming a resting potential of −60 mV. Current records filtered at 500 Hz. *c*, Effects of compounds on single-channel current amplitude, *i*. Analysis in *b* and *c* is for the experiment shown in *a*, and symbols are as follows in both *b* and *c*: ●, +FMRFamide; ○, control; ■, +arachidonic acid; □, recovery.

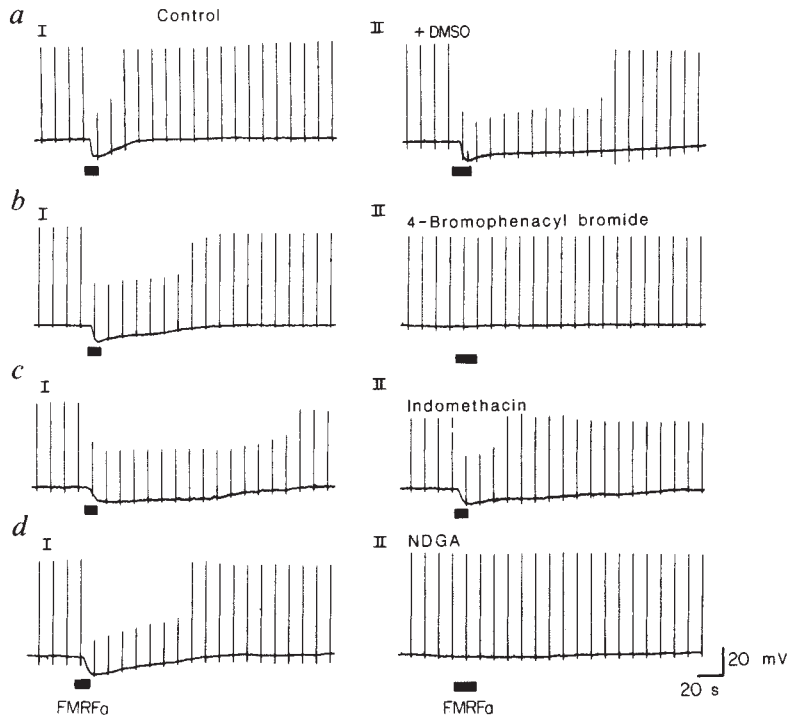

Fig. 5 Actions of inhibitors of arachidonic acid metabolism on the response to FMRFamide. Chart records of membrane potential under current clamp from sensory cells in culture. Large brief upward deflections are action potentials elicited by depolarizing current pulses. Smaller upward deflections are the passive depolarizations following action potential blockade. Bars, application of 2 μM FMRFamide onto the cell body. *a–d*, Representative tracings from four different experiments. Left (traces I), control responses to the peptide recorded during continuous superfusion with artificial sea water (resting potential ranged between −38 and −57 mV). Right (traces II), responses to a second application of FMRFamide on the same cells after superfusion of a test drug. *a*, After superfusion with dimethylsulphoxide (DMSO) in artificial sea water (1:10,000 dilution). *b–d*, After superfusion of the sea water/DMSO mixture, containing in addition: in *b*, the phospholipase inhibitor 4-bromophenacyl bromide (10 μM, Sigma); in *c*, the cyclooxygenase blocker indomethacin (5 μM, Sigma); in *d*, the lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA, 5 μM, Biomol). **Methods.** At the beginning of each day, a concentrated stock solution of the blocker in DMSO was prepared, and kept at 0 °C in the dark. Portions of this solution were dissolved in artificial sea water to reach the final concentration, vortexed and superfused in the culture dish with a peristaltic pump (at ~7 ml min^{-1}). In some of the experiments, indomethacin was dissolved in an ethanol/sea water mixture (1:1,000-1:20,000) or a 100 mM Tris *p*H 8.4/sea water mixture (1:400-1:1,000).

cells and meet the criteria originally proposed for second messenger systems[44]. These are: (1) synthesis—the enzymatic machinery for producing lipoxygenase metabolites is present in neurons; (2) receptor mediation—formation of lipoxygenase metabolites is stimulated by applying the neuropeptide FMRFamide; (3) simulation—arachidonic acid and its lipoxygenase metabolites simulate the actions of FMRFamide; (4) selective blockade—agents that block arachidonic acid release and metabolism effectively inhibit the physiological response to FMRFamide; (5) termination—two mechanisms for terminating the action of the putative second messengers could be reduction to the inactive hydroxy acids and re-incorporation into membrane phospholipids[17,35].

This function for the arachidonic acid cascade is probably not unique to *Aplysia* sensory cells or to FMRFamide, because other experiments show that the presynaptic inhibition produced by stimulating the putative histaminergic L32 cells on the L10-RB connection[45] operates through the same second messenger mechanism[35]. It will be of interest to see whether arachidonic acid participates in presynaptic inhibition in vertebrates, such as that produced in dorsal root ganglion cells by noradrenaline[46] and enkephalin [47].

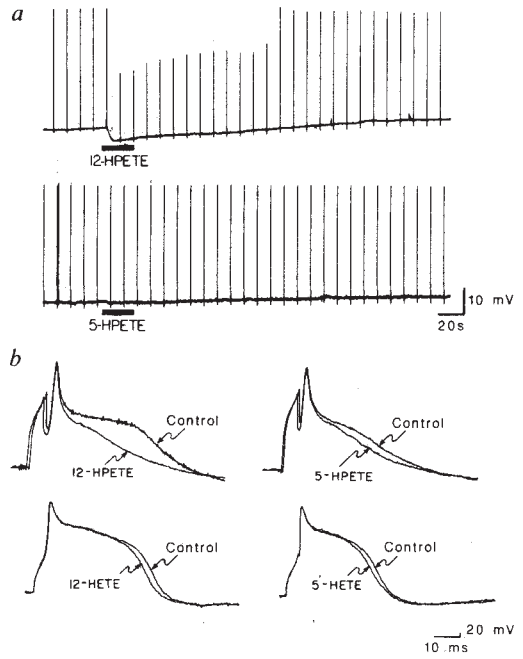

Fig. 6 Effect of lipoxygenase metabolites on membrane and action potentials. *a*, Effect of 1.5 μM 12-HPETE (top trace) and 1.5 μM 5-HPETE (bottom trace) on resting potentials and stimulated action potentials (brief upward spikes) in pleural sensory neurons in culture. 12-HPETE inhibited spike firing for about 2 min (during this period the smaller spikes are subthreshold). Initial resting potential was −43 mV for top trace and −39 mV for bottom trace. *b*, Effects of metabolites on action potential duration recorded in 50 mM TEA. Action potentials recorded from sensory neuron in intact abdominal ganglia. 12-HPETE and 5-HPETE (60 μM) were applied to one cell (upper traces); 12-HETE and 5-HETE (60 μM) were applied to a second cell (lower traces). Action potentials elicited by brief depolarizing stimuli. In the two upper traces the neuron fired a spike after termination of the brief current pulse. Action potentials have been aligned to superimpose the rising phase.

Methods. Lipoxygenase metabolites obtained from Biomol. The hydroperoxy acids were purified by normal phase HPLC using an Alltech silica 4.6 mm × 25 cm column, eluted with hexane/isopropanol/acetic acid, (98:2:0.01) at 1 ml min^{-1} and stored at −70 °C before use. Purity was checked regularly. Immediately before an experiment, a sample of the stock solution was dried under nitrogen, artificial sea water was added and the mixture sonicated.

Eicosanoids differ from other intracellular second messengers in one important way. Unlike other messengers, the eicosanoids are able to leave the cell in which they are generated and act as first messengers on neighbouring cells[48]. For example, a metabolite of arachidonic acid might provide a diffusible extracellular signal from a postsynaptic cell to a presynaptic one. This type of signal could explain the operation of a Hebbian type of synapse of the kind recently postulated to be important for long-term potentiation of pyramidal cells in the CA1 region of the hippocampus, where depolarization of the postsynaptic cell leads to enhanced release from the presynaptic region[49,50].

Although our results implicate arachidonic acid metabolites as second messengers for FMRFamide action in the sensory neurons, several questions remain to be answered. First, the inhibitory action of FMRFamide results from a dual ionic mechanism, an increase in outward S current and a decrease in the inward Ca^{2+} current. Our analysis here has concentrated on the increase in S current and we do not know whether the decrease in Ca^{2+} current is also mediated by arachidonic acid metabolites, although the marked decrease in action potential duration with 12-HPETE and arachidonic acid does suggest a concomitant inhibition of inward calcium current. Second, we have not identified the active metabolite (or metabolites) ultimately responsible for S-channel modulation, although our results indicate that the active molecule is closely related to a hydroperoxy acid. Finally, we do not know either the mechanism coupling transmitter binding to the production of arachidonic acid metabolites or how the active metabolite modulates the channel.

Whatever the answers to these questions, the inhibitory effect of FMRFamide acting through the eicosanoids is functionally antagonistic to the facilitatory action produced by 5-HT through cAMP. Because 5-HT and FMRFamide converge on the same K^+ channel, 5-HT to decrease and the peptide to increase channel activity, our results show that an individual channel can serve as a final common effector for different second messenger systems.

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