Lipoxygenase metabolites of arachidonic acid in neuronal transmembrane signalling

Daniele Piomelli and Paul Greengard

Studies of invertebrate and vertebrate nervous tissue have demonstrated that free arachidonic acid and its lipoxygenase metabolites are produced in a receptor-dependent fashion. The intracellular actions of these compounds include the regulation of activity of membrane ion channels and protein kinases. In this article Daniele Piomelli and Paul Greengard review the evidence that these lipophilic molecules constitute a novel class of intracellular second messenger, possibly involved in the modulation of neurotransmitter release.

Many neurotransmitters exert their actions on neurons by stimulating the formation of intracellular second messengers, which include such diverse molecules as cyclic nucleotides, Ca^{2+}, inositol 1,4,5-trisphosphate and 1,2-diacylglycerol. In most cases second messengers stimulate the activity of specific protein kinases, which phosphorylate and hence modulate the activity of a large number of enzymes, membrane ion channels and structural proteins (for review, see Ref. 1). However, second messengers can also activate phosphatases either directly (as in the case of the Ca^{2+}-calmodulin-dependent phosphatase calcineurin), or indirectly through phosphorylation of phosphatase inhibitors. In some cases, second messengers can act without the participation of enzyme intermediates, by binding to membrane ion channels (or to regulatory proteins closely associated with them) and changing their properties (for instance in the case of Ca^{2+}-activated K^+ channels; for review, see Ref. 3).

Recent work has suggested that the membrane polyunsaturated fatty acid arachidonic acid and its metabolites produced through the lipoxygenase pathways may constitute a novel class of neuronal second messengers. These lipids, which affect the activities of neuronal ion channels and protein kinases, might participate in the regulation of neurotransmitter release. Arachidonic acid is found in esterified form in cell membrane phospholipids from which it can be liberated through multiple enzymatic pathways (see Box 1). It can then diffuse out of the cell, be reincorporated into phospholipids, or undergo metabolism. Three pathways of arachidonic acid metabolism have been described in animal tissues: the cyclooxygenase pathway, the lipoxygenase pathway and the cytochrome P-450 or 'epoxysgenase' pathway (Box 1). In nervous tissue the major enzymatic route involved in the metabolism of arachidonic acid is the 12-lipoxygenase pathway (Box 1 and Fig. 1). There is also good evidence for the occurrence in nervous tissue of the 5-lipoxygenase pathway.

12-Lipoxygenase, first isolated from blood platelets and now purified to near homogeneity from leukocytes, is a cytosolic protein of 72 kDa (based on SDS-polyacrylamide gel electrophoresis) and has no apparent cofactor requirement. A cDNA encoding leukocyte 12-lipoxygenase has been isolated and sequenced. Like all other lipoxygenases, 12-lipoxygenase catalyses the introduction of molecular oxygen into a 1,4-(cis,cis)-pentadiene moiety, converting arachidonic acid into the hydroperoxide, (12s)-hydroperoxyeicosatetraenoic acid (12-HPETE) – a reaction that is both regiospecific and stereospecific. Other cis-polysaturated fatty acids, such as linoleic acid, linolenic acid and docosahexaenoic acid are also good substrates for the leukocyte 12-lipoxygenase.

Lipoxygenases in the brain

In a recent study the levels of 12-lipoxygenase in several areas of porcine brain were estimated by immunoassay, using a monoclonal antibody raised against leukocyte 12-lipoxygenase. Detectable levels of the enzyme were found in the cytosolic fractions of olfactory bulb, hypothalamus, cerebellum and pineal body; the highest levels were detected in parenchymal cells of the anterior pituitary lobe. Even though 12-HPETE is reduced very rapidly to the alcohol (12s)-hydroxyeicosatetraenoic acid (12-HETE), which has long been known to be a major metabolite of arachidonic acid in the brain, the metabolism of 12-HPETE is not limited to its reduction (Fig. 1). The fatty acid hydroperoxide can give rise to a group of biologically active hydroxyepoxides, the 'hepoxilins', two of which – hepoxilin A_3 and hepoxilin B_3 – have been identified in nervous tissue from both vertebrates and invertebrates. The 'hepoxilins' are short-lived compounds and undergo further metabolism by the action of a hepoxilin hydrolase, which has been purified from rat liver and shown to be present in brain. This enzyme catalyses the specific cleavage of the hepoxilin epoxide.
Liberation of arachidonic acid from membrane phospholipids mainly occurs by two pathways (Fig. 1). Hydrolytic cleavage at the sn-2 position of the glycerophospholipid backbone, catalysed by Ca²⁺-activated phospholipase A₂, yields free fatty acid and lysophospholipid. Alternatively, formation of free arachidonic acid can be initiated by the activation of a phospholipase C, which cleaves the phospholipid at the phosphate ester bond, producing 1,2-diacylglycerol. This intermediate is broken down by diacylglycerol- and monoaacetylglycerol lipases to yield free fatty acid and glycerol.

After liberation, free arachidonic acid has several fates. It can diffuse out of the cell. Alternatively, it can be either metabolized (see below) or converted to arachidonoyl-coenzyme A and reincorporated into phospholipids (Fig. 1). The two steps required for reincorporation—the reactions of arachidonoyl-CoA synthetase and arachidonoyl-lysophospholipid transferase—are thought to make up the major mechanism responsible for keeping the concentration of free arachidonic acid low within cells.

The three pathways of arachidonic acid metabolism described for other animal tissues—cyclooxygenase, lipooxygenase and cytochrome P-450 (Refs. 4–7)—have also been found in the brain (for review, see Ref. 5). The cyclooxygenase pathway leads to the formation of prostaglandins, prostaeycin (PGH₂) and thromboxane A₂ (TXA₂). Cyclooxygenase is inhibited by some non-steroidal anti-inflammatory drugs, such as indomethacin, by the antioxidant nordihydroguaiaretic acid and by the false substrate eicosatetraynoic acid.

Lipooxygenases, which are inhibited nonspecifically by nordihydroguaiaretic acid, eicosatetraynoic acid and baicalein, form hydroperoxyeicosatetraynoic acids (HPETE) as their primary products. HPETE can undergo a complex metabolism (see main text for details). 5-HPETE, formed by 5-lipoxygenase, is converted to the short-lived epoxide leukotriene, LTA₄, by a dehydrase activity intrinsic to 5-lipoxygenase. LTA₄ can be transformed either into LTB₄ by an LTA₄-hydrolase, or into LTC₄ by a glutathione-S-transferase (GSH-S-transferase). 5-Lipoxygenase activity is specifically inhibited by 5,6-methano- leukotriene A₄, methyl ester and 5,6-dehydroarachidonic acid. In addition, formation of 5-lipoxygenase products can be inhibited in intact cells by MK886, which prevents 5-lipoxygenase activation by binding to a newly described 5-lipoxygenase activating protein (FLAP). Metabolism of 15-HPETE shows considerable similarities to that of 12-HPETE (see Refs 10 and 11).

Cytochrome P-450 catalyses the conversion of arachidonic acid into an array of epoxyeicosatetraynoic acids (EET), which are hydrolysed to the corresponding diols by the action of an epoxide hydrolase (inhibited by trichloro(propene oxide). In addition, cytochrome P-450 has also been shown to produce hydroxyeicosatetraynoic acids (HETE)¹².

### Pathways of arachidonic

![Fig. 1. Pathways of arachidonic acid turnover. AA, arachidonic acid; PLₐ, phospholipase A₂; PLC, phospholipase C; DAG, 1,2-diacylglycerol; MAG, monoaacetylglycerol.](image)

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2. Lipooxygenases, which are inhibited nonspecifically by nordihydroguaiaretic acid, eicosatetraynoic acid and baicalein, form hydroperoxyeicosatetraynoic acids (HPETE) as their primary products. HPETE can undergo a complex metabolism (see main text for details). 5-HPETE, formed by 5-lipoxygenase, is converted to the short-lived epoxide leukotriene, LTA₄, by a dehydrase activity intrinsic to 5-lipoxygenase. LTA₄ can be transformed either into LTB₄ by an LTA₄-hydrolase, or into LTC₄ by a glutathione-S-transferase (GSH-S-transferase). 5-Lipoxygenase activity is specifically inhibited by 5,6-methano-leukotriene A₄, methyl ester and 5,6-dehydroarachidonic acid. In addition, formation of 5-lipoxygenase products can be inhibited in intact cells by MK886, which prevents 5-lipoxygenase activation by binding to a newly described 5-lipoxygenase activating protein (FLAP). Metabolism of 15-HPETE shows considerable similarities to that of 12-HPETE (see Refs 10 and 11).

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Receptor-dependent formation of lipoxygenase products

Neurotransmitters cause formation of 12-lipoxygenase metabolites in nervous tissue. NMDA receptor stimulation, apparently by increasing Ca\(^{2+}\) entry and activating phospholipase A\(_2\), stimulates liberation of arachidonic acid and the generation of 12-HETE in a variety of nervous tissue preparations\(^{20-22}\). The absolute configuration of the HETE formed (12s) gives definitive evidence of its origin from a 12-lipoxygenase-catalysed reaction\(^{21}\).

Norepinephrine, acting via \(\alpha\)-adrenoceptors, also elicits formation of 12-HETE in rat cortex\(^{21}\). In hippocampal neurons in culture, activation of 5-HT\(_2\) receptors stimulates arachidonic acid liberation, as a result of receptor-dependent activation of phospholipase A\(_2\) (Ref. 23).

In Aplysia, formation of 12-HETE by neural ganglia is stimulated by the application of histamine or the tetrapeptide FMRF amide\(^{24,25}\). These two modulatory substances can alter neuronal membrane potential by opening K\(^+\) channels, producing presynaptic inhibition of neurotransmitter release. It is interesting, therefore, that electrical stimulation of L32 neurons, an identified group of Aplysia cells, results in the generation of both 12-HETE and hepoxilin A\(_3\) as well as in the inhibition of neurotransmitter release from LI0 neurons\(^{8,24}\).

Modulation of K\(^+\) channels by lipoxygenase metabolites

The results obtained in Aplysia suggested that lipoxygenase metabolites of arachidonic acid may participate in the modulation of K\(^+\) channel activity and in presynaptic inhibition. This idea was substantiated by electrophysiological experiments. In two histaminergic neurons (L10 and L14), extracellular application of 12-HPETE produced changes in membrane potential that were similar to those produced by the neurotransmitter\(^{8}\). Similarly, in sensory cells, arachidonic acid and 12-HPETE mimicked the actions of FMRF amide, increasing the probability of opening of a subclass of K\(^+\) channels, the 5-HT-inactivated
IK$_{5-HT}$ (S-K$^+$) channels\textsuperscript{25}. By contrast, a 5-lipoxygenase-derived product, 5-HPETE, and the hydroxy acids, 12-HETE and 5-HETE, were inactive. In addition, the actions of histamine and FMRF amide on membrane potential could be prevented by treatment with the phospholipase A$_2$ inhibitor p-bromophenacylbromide, or with the lipoxygenase inhibitor nordihydroguaiaretic acid, but not with the cyclooxygenase inhibitor indometacin\textsuperscript{25,26}. (For a discussion of the drawbacks of the phospholipase A$_2$ inhibitors presently available, see Ref. 27.) On the basis of these findings it was proposed that a 12-lipoxygenase metabolite of arachidonic acid, possibly 12-HPETE, may act as an intracellular second messenger in the control of K$^+$ channel activity by histamine and FMRF amide in Aplysia neurons.

These findings raise several questions. Is 12-HPETE itself the active intracellular messenger, or does it require further metabolism? If it is metabolized, which of its products is the active one? Does this 12-lipoxygenase-derived messenger act directly on the IK$_{5-HT}$ channel, or does it act by modulating the activity of protein kinases or protein phosphatases? In cell-free patches of Aplysia sensory cell membranes, in the absence of cytosolic components, 12-HPETE could not affect the opening of IK$_{5-HT}$ channels\textsuperscript{15}. Its biological activity was restored, however, when the patches were bathed in a solution containing hematin (which catalyses the conversion of the lipid hydroperoxide to various products, including hepoxilins and 12-KETE). Thus biotransformation of 12-HPETE is necessary for biological activity. Since the experiments were carried out in the absence of ATP and GTP, they also indicate that K$^+$ channel modulation by 12-HPETE does not require the participation of a G protein or of a protein kinase; furthermore, it cannot be due to a phosphoprotein phosphatase, since it is reversible. It is still unclear what enzyme, if any, catalyses the metabolism of 12-HPETE and what metabolite is responsible for the biological response. Belardetti and colleagues found that the reversible cytochrome P-450 inhibitor proadifen (SKF525A) significantly reduced the response of sensory neurons to 12-HPETE, suggesting that metabolism is carried out by a cytochrome P-450-like enzyme\textsuperscript{15}.

The identity of the derivative of 12-HPETE that activates IK$_{5-HT}$ channels in Aplysia sensory cells is unknown. It is clear, however, that two of the candidates, hepoxilin A$_3$ and 12-KETE, have different electrophysiological actions on identified Aplysia neurons: hepoxilin A$_3$ hyperpolarizes the membrane of L14 cells, an effect that appears to result from increased conductance to K$^+$ ions\textsuperscript{8}; by contrast, 12-KETE produces a biphasic response (depolarization–hyperpolarization) in these neurons\textsuperscript{10}. Hepoxilin A$_3$ also hyperpolarizes neurons of the CA1 area in superfused rat hippocampal slices\textsuperscript{25}.

Recently, Buttner and co-workers reported that, although 12-HPETE is ineffective when applied to the intracellular side of the neuronal membrane, it can produce a large increase in IK$_{5-HT}$ channel activity when applied to the outside; 12-HPETE may therefore act as a local intercellular signalling molecule\textsuperscript{26}. While its tran-
sient chemical nature seems to argue against this possibility, there are other examples of extremely short-lived metabolites of arachidonic acid that appear outside the cell of origin and act on membrane receptors of neighboring cells. For example, the cyclooxygenase product thromboxane A2, a powerful platelet-aggregating and vasoconstricting autacoid, is released from blood platelets and acts both on the platelets themselves and on vascular smooth muscle (in both cases via a membrane receptor)\textsuperscript{32}. It is not understood how these short-lived metabolites leave cells. Perhaps an anion carrier of the type sensitive to the uricosuric agent probenecid may be involved.

Whatever the mechanism for the release of 12-HETE, its diffusion in a circumscribed area around the neuron of origin might bring about a local spreading of neuronal inhibition. This could represent a means of modulating or synchronizing the activity of a small ensemble of neighboring neurons involved in a single physiological function. Such a role would be analogous to the paracrine role played by the catecholamines in mammalian brain.

In rat hippocampal CA1 neurons, somatostatin, a putative transmitter in the hippocampus, augments a non-inactivating, voltage-gated K\textsuperscript{+} current, termed the M current (I\textsubscript{M}). Augmentation of I\textsubscript{M} results in a slow hyperpolarization of the membrane and decreased neuronal excitability\textsuperscript{30}. Lipoxigenase metabolites of arachidonic acid may be involved in the response to somatostatin. Schweitzer et al.\textsuperscript{31} found that phospholipase A\textsubscript{2} inhibitors (mepacrine and p-bromophenacylbromide) and specific 5-lipoxygenase inhibitors (5,6-methanoleukotriene A\textsubscript{4} methyl ester and 5,6-dehydroarachidonic acid) inhibit the response to somatostatin in CA1 cells. In addition, stimulation of I\textsubscript{M} by somatostatin can be mimicked by the application of arachidonic acid or LTC\textsubscript{4} (a 5-lipoxygenase metabolite)\textsuperscript{31}. These findings suggest that lipoxygenase-derived eicosanoids have a widespread role in reducing neuronal excitability through the regulation of K\textsuperscript{+} channel activity. There is also evidence that arachidonic acid regulates Ca\textsuperscript{2+} currents in hippocampal neurons\textsuperscript{32}, chick sympathetic\textsuperscript{33} and frog sympathetic neurons\textsuperscript{34}.

### Inhibition of neurotransmitter release

Phosphorylation of specific proteins in the presynaptic nerve terminal participates in modulating neurotransmitter release. The state of phosphorylation of the synaptic vesicle-associated protein, synapsin I, is thought to regulate the availability of synaptic vesicles for exocytosis (for review, see Ref. 35). In its dephosphorylated state, synapsin I may cross-link synaptic vesicles to the surrounding cytoskeletal lattice. According to this model, when synapsin I is phosphorylated on its 'tail'-region by Ca\textsuperscript{2+}-calmodulin-dependent protein kinase II, its interaction both with synaptic vesicles and with cytoskeletal elements is reduced, resulting in dissociation of the vesicles from the cytoskeleton. This would in turn increase the number of vesicles available for exocytosis. Therefore, reducing the state of phosphorylation of synapsin I may be a way to reduce

### Box 2

**Arachidonic acid in hippocampal long-term potentiation**

Long-term potentiation (LTP) is a mammalian model of synaptic plasticity and information storage, which can be produced in the hippocampus by high frequency stimulation of the perforant pathway (a group of fibers connecting the entorhinal cortex to the dentate gyrus)\textsuperscript{3,4}. LTP is generally believed to consist of two phases - induction and maintenance. Induction is initiated by the postsynaptic entry of Ca\textsuperscript{2+}, which occurs through cation channels associated with excitatory amino acid receptors of the NMDA type, and consequent activation of a Ca\textsuperscript{2+}-dependent protein kinase\textsuperscript{2-4}. Maintenance appears to be produced at least partly by presynaptic mechanisms\textsuperscript{5}.

Williams and collaborators\textsuperscript{6} reported that perfusion with arachidonic acid produced a transient inhibition of synaptic transmission in the dentate gyrus of the anesthetized rat. They also found, however, that by combining arachidonic acid perfusion with a weak stimulation of the perforant path (unable to produce LTP per se) a long-term enhancement in synaptic strength resulted, which was indistinguishable from LTP. This effect was not inhibited by the mixed lipoxygenase/cyclooxygenase blocker nordihydroguaiaretic acid indicating that it was produced by the free fatty acid, rather than by a metabolite. In agreement with this interpretation, oleic acid, a monounsaturated fatty acid which is not a substrate for metabolism by lipoxygenase or cyclooxygenase, was also able to produce LTP (Ref. 7). These findings suggest that free fatty acids, liberated by the action of a phospholipase, may play a role in the maintenance phase of LTP.

The cellular site of action of this lipid(s) is still unclear and both presynaptic\textsuperscript{6} and glial\textsuperscript{8} sites have been proposed. Likewise, little is known of their mechanism of action. An involvement of protein kinase C has been suggested\textsuperscript{7} on the grounds of the ability of unsaturated fatty acids to activate this protein kinase in a Ca\textsuperscript{2+}- and phospholipid-independent fashion\textsuperscript{6}.

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Thus FMRF amide, in addition to under these conditions, it still produced inhibition of neuronal secretory release. 12-HPETE and its metabolites may decrease Ca²⁺ influx during the action potential, also decreases the sensitivity of the secretory apparatus to an elevated concentration of intracellular Ca²⁺, possibly by affecting protein phosphorylation. Arachidonic acid and its metabolites may regulate neurotransmitter release partly by a direct action on neuronal K⁺ channels and partly by regulating Ca²⁺-dependent protein phosphorylation within the synaptic terminal. Lipoygenase-derived eicosanoids are potent and selective inhibitors of purified Ca²⁺-calmodulin-dependent protein kinase II (Ref. 37). 12-HPETE inhibited this enzyme with a half-maximal effect at a concentration of 0.7 μM. By contrast, it had no effect on the activities of protein kinase C, cAMP-dependent protein kinase, the Ca²⁺-calmodulin-dependent protein kinases I and III, or the Ca²⁺-calmodulin-activated phosphatase, calcineurin. Arachidonic acid was less potent than 12-HPETE to form 12-HPETE. A metabolite of 12-HPETE (possibly a hepoxilin) may act by modulating the activity of K⁺ channels, while 12-HPETE itself may inhibit Ca²⁺-calmodulin-dependent protein kinase II activity and reduce Ca²⁺-stimulated phosphorylation of synapsin I and other synaptic vesicle-associated proteins. These two independent actions might be synergistic in decreasing synaptic strength.

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Immunomodulatory activity of small peptides

Vassil St Georgiev

The activity of the immune system can be modulated by a wide variety of natural and synthetic peptides. Here, Vassil St Georgiev summarizes the actions of some of the immunostimulatory and immunosuppressant small peptides that have shown most promise as therapeutic agents. Some are already in use as vaccine adjuvants or to prevent graft rejection. There are now indications that these peptides may also be of benefit in conditions in which the immune system is compromised, in autoimmune disease and in cancer.

A large number of natural and synthetic peptides have been evaluated for their immunomodulatory effects. These derive from (or are modifications of) sources as diverse as bacterial cell wall peptidoglycans, microbial and fungal metabolites, peptide hormones, fragments of immunoglobulins and other plasma proteins and colostrum and milk proteins. Although in many cases little is known of the mechanism of action, the clinical potential of many of these peptides has clearly been demonstrated. Some of the less well-characterized peptides may also prove to be useful; their properties are summarized in Table I.

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Immunostimulatory peptides

Many peptides have been isolated that have nonspecific stimulatory effects on the immune system. Work is under way to develop these peptides as adjuvants in vaccines and as therapeutic agents for conditions including immune deficiencies and cancer.

- Muramyl peptides

Freund's complete adjuvant - killed mycobacterial cells emulsified in mineral oil - has long been known to be a powerful adjuvant of humoral and cellular mediated immune reactions. The smallest biologically active unit of bacterial cell wall peptidoglycan capable of replacing whole Mycobacteria in Freund's complete adjuvant is muramyl dipeptide (MDP). In addition to its immunomodulatory activity, manifested by a potent stimulation of the production of antibodies against antigen administered simultaneously, MDP stimulates nonspecific resistance against bacterial, viral and parasitic infections, and against tumors by stimulating cytotoxic macrophages and natural killer cells, and increases the duration of slow-wave sleep. However, it also has other, largely undesirable biological effects such as pyrogenicity, transient leukopenia, sensitization to endotoxin and induction of arthritis, granuloma and uveitis.

Other peptides with immunomodulatory activity include muramyl tripeptide (MDTP), muramyl tetrapeptide (MDTP), muramyl tetrapeptide (MDTP), muramyl pentapeptide (MDPP), muramyl hexapeptide (MDH), muramyl heptapeptide (MDHP), muramyl octapeptide (MDOP), muramyl nonapeptide (MDNP), muramyl decapeptide (MDDP), muramyl undecapeptide (MDUDP), muramyl dodecapeptide (MDDPD), muramyl tridecapeptide (MDTPD), muramyl tetradecapeptide (MDTDP), muramyl pentadecapeptide (MDTPD), muramyl hexadecapeptide (MDHTPD), muramyl heptadecapeptide (MDHTPD), muramyl octadecapeptide (MDOCTPD), muramyl nonadecapeptide (MDNCTPD), muramyl decadecapeptide (MDDCTPD), muramyl undecadecapeptide (MDUDCTPD), muramyl dodecadecapeptide (MDDDCTPD), muramyl tridecadecapeptide (MDTDDCTPD), muramyl tetradecadecapeptide (MDTDDCTPD), muramyl pentadecadecapeptide (MDTPDDCTPD), muramyl hexadecadecapeptide (MDHTPDDCTPD), muramyl heptadecadecapeptide (MDHHTPDDCTPD), muramyl octadecadecapeptide (MDOCTPDDCTPD), muramyl nonadecadecapeptide (MDNCTPDDCTPD), muramyl decadecadecapeptide (MDDCTPDDCTPD), muramyl undecadecadecapeptide (MDUDCTPDDCTPD), muramyl dodecadecadecapeptide (MDDDCTPDDCTPD), muramyl tridecadecadecapeptide (MDTDDCTPDDCTPD), muramyl tetradecadecadecapeptide (MDTDDCTPDDCTPD), and muramyl pentadecadecadecapeptide (MDTPDDCTPDDCTPD).