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Arachidonate 5-Lipoxygenase and Its Activating Protein: Prominent Hippocampal Expression and Role in Somatostatin Signaling


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Abstract: 5-Lipoxygenase—activating protein (FLAP) is an 18-kDa integral membrane protein required, in peripheral cells, for the activation of 5-lipoxygenase (5-LO) and for the resulting synthesis of leukotrienes from arachidonic acid. In the brain, the leukotrienes have been implicated in several pathophysiological events and in the electrophysiological effect of somatostatin, yet the cellular origin and role of these messenger molecules are still poorly understood. In the present study, we used reverse transcriptase—polymerase chain reaction, in situ hybridization, and immunohistochemistry to demonstrate that 5-LO and FLAP are expressed in various regions of the rat brain, including hippocampus, cerebellum, primary olfactory cortex, superficial neocortex, thalamus, hypothalamus, and brainstem. Highest levels of expression were observed in cerebellum and hippocampus. In the latter we demonstrate the colocalization of 5-LO and FLAP in CA1 pyramidal neurons. Moreover, electrophysiological experiments show that selective inhibition of FLAP with the compound MK-886 (0.25—1 μM) prevents the somatostatin-induced augmentation of the hippocampal K+ current. Our results provide necessary evidence for the presence and signaling role of 5-LO and FLAP in central neurons and strongly support their proposed participation in somatostatin-receptor transmembrane signaling.

Key Words: Arachidonic acid—Leukotrienes—Signal transduction—Hippocampus.


The leukotrienes, a family of biologically active metabolites of arachidonic acid, are synthesized through a sequence of enzymatic reactions initiated by 5-lipoxygenase (5-LO), a cytosolic dioxygenase that catalyzes both the initial oxidation of arachidonic acid to 5-hydroperoxy-6,8,11,14-eicosatetraenoic acid and its subsequent dehydration to leukotriene A4 (LTA4). LTA4 is short lived and may be rapidly transformed either into LTC4, by LTC4-synthase, or into LTD4, by LTA4-hydrolase (Samuelsson et al., 1987). Unlike other mammalian lipoxygenases, 5-LO requires multiple cofactors to become fully active, including Ca2+ and ATP (Ford-Hutchinson et al., 1994). Rises in intracellular Ca2+ cause the translocation of 5-LO from cytosol to membrane, where the enzyme interacts with an 18-kDa integral membrane protein, 5-lipoxygenase—activating protein (FLAP), required for leukotriene biosynthesis in peripheral cells (Dixon et al., 1990; Miller et al., 1990; Abramovitz et al., 1993). Arachidonic acid metabolism via the 5-LO pathway may play important roles in the CNS (for review, see Piomelli, 1994). In vitro, preparations of brain tissue convert exogenous and endogenous arachidonic acid to leukotrienes and to other 5-LO products (Lindgren et al., 1984; Adesuyi et al., 1985; Shimizu et al., 1987). In vivo, leukotriene biosynthesis is stimulated by the intracerebroventricular administration of platelet-activating factor and is associated with the induction of seizure activity, ischemia, and intracranial tumors (Simmet and Peskar, 1990; Hynes et al., 1991). Moreover, LTC4 exerts multiple actions on neural cells, such as prolonged excitation of cerebellar Purkinje neurons (Peer et al., 1981), stimulation of luteinizing hormone release from anterior pituitary cells (Kiesel et al., 1991), and enhancement of the M-current (I_M) in the hippocampal CA1 field (Schweitzer et al., 1990, 1993).

The I_M is a nonactivating time- and voltage-dependent K+ current (Brown and Adams, 1980) that, in
hippocampal pyramidal neurons, is reduced by muscarinic receptor agonists (Halliwell and Adams, 1982) and augmented by the neuropeptide somatostatin (Moore et al., 1988). Pharmacological evidence indicates that a 5-LO metabolite of arachidonic acid mediates the effect of somatostatin; $I_m$ enhancement by somatostatin is prevented by drugs that inhibit phospholipase $A_2$ (PLA$_2$) or 5-LO activities and mimicked by applications of exogenous arachidonic acid or LTC$_4$, but not by application of LT$\beta$$_4$ (Schweitzer et al., 1990, 1993).

Although these studies suggest a signaling role for leukotrienes in the CNS, it is not known whether the enzymatic machinery necessary for leukotriene biosynthesis is expressed in neurons and under what circumstances it may become activated. Here, we report that 5-LO and FLAP are coexpressed in rat brain neurons and enriched in discrete regions of the CNS including the hippocampus. To test whether FLAP is physiologically functional, and involved in somatostatin modulation of $I_m$, we used the compound MK-886, a potent and selective FLAP inhibitor (Dixon et al., 1990). We find that the effect of somatostatin on $I_m$ in the hippocampal slice preparation is abolished by MK-886. Thus, our results provide strong support to a requirement of 5-LO and FLAP in transmembrane signaling of somatostatin receptors in hippocampal neurons.

**EXPERIMENTAL PROCEDURES**

**Reverse transcriptase (RT)–polymerase chain reaction (PCR) analysis**

Poly(A)$^+$ mRNAs prepared from various regions of the Wistar rat brain served as templates for cDNA synthesis using avian myeloblastosis virus RT (25 units, Boehringer). We used the cDNAs obtained as templates to amplify, by PCR, DNA fragments corresponding to nucleotides 9–459 of rat FLAP sequence (Dixon et al., 1990) and nucleotides 1,417–1,735 of human 5-LO sequence (Dixon et al., 1990, 1998), obtained by excision of a recombinant 5-LO pUC 13 plasmid vector (generously provided by Dr. A. Ford-Hutchinson, Merck-Frosst, Canada) and subcloned in pGEM-4Z. We confirmed distribution of FLAP mRNA by using two $^{32}$P-labeled oligonucleotide probes, corresponding to nucleotides 86–132 and 285–331 of the rat FLAP sequence (Dixon et al., 1990).

**Immunohistochemistry**

After a 5-min fixation with 4% paraformaldehyde, sections were rinsed in phosphate-buffered saline solution, incubated for 12 h at 4°C with FLAP antiserum (a gift of Dr. A. Ford-Hutchinson) at 1:500 dilution, rinsed, incubated with biotin-labeled anti-rabbit protein antisera, treated with avidin/biotin/horseradish peroxidase complex and developed with diaminobenzidine for 5 min.

**Electrophysiology**

We performed intracellular recordings in rat hippocampal slices, as described previously (Schweitzer et al., 1993). In brief, we cut transverse hippocampal slices (350–400 $\mu$m thick) and superfused them (2.0–4.0 ml/min) with warm (30–31°C), gassed (95% O$_2$, 5% CO$_2$) artificial CSF of the following composition (mM): NaCl 130, KCl 3.5, NaH$_2$PO$_4$ 1.25, MgSO$_4$ 1.5, CaCl$_2$ 2.0, NaHCO$_3$ 24, glucose 10. We dissolved MK-886 (a gift of Dr. A. Ford-Hutchinson) at a 1:500 dilution, rinsed, incubated with avidin/biotin/horseradish peroxidase complex and developed with diaminobenzidine for 5 min.

**RESULTS**

We localized 5-LO mRNA in rat CNS by in situ hybridization, using a $^{35}$S-labeled oligonucleotide probe. We found the highest labeling densities in hippocampus and cerebellum and moderate labeling in primary olfactory cortex, superficial neocortex, thalamus, hypothalamus, and brainstem (Fig. 1A). In situ hybridization with a $^{35}$S-labeled FLAP probe revealed a distribution of transcripts that appeared to overlap with that of 5-LO, with highest hybridization signals in hippocampus and cerebellum. In addition, clear signals were visible in primary olfactory cortex, neocortex, brainstem, and thalamus (Fig. 1B). By contrast, only
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FIG. 1. Histologic localization of 5-LO and FLAP mRNAs in parasagittal sections of rat brain. Hybridization with a 35S-labeled 5-LO oligoprobe (A), a 35S-labeled FLAP oligoprobe (B), and a 35S-labeled control missense probe (C) (for sequence, see Experimental Procedures). Highest labeling densities are observed in hippocampus (Hi) and cerebellum (Cb); moderate labeling is observed in neocortex (Cx), thalamus (Th), primary olfactory cortex (P0), and choroid plexus (ChP). Diffuse, nonspecific labeling was seen in adjacent sections treated with a control, 35S-labeled missense probe (Fig. 1C). We obtained equivalent results using a 35P-labeled 5-LO riboprobe (derived from human 5-LO cDNA, which displays 93% identity with its rat homologue) and two additional 35S-labeled FLAP probes (data not shown).

RT-PCR analysis confirmed that 5-LO and FLAP mRNAs are expressed in rat brain. Southern blot analysis revealed PCR products of expected size when poly(A)\(^+\) RNA samples from various regions of the rat brain were subjected to RT-PCR using oligonucleotide primers selective for 5-LO or FLAP (Fig. 2). It is important that, although 5-LO and FLAP appear to be present throughout the brain, our RT-PCR conditions would not provide a quantitative assessment of their level of expression; i.e., more stringent quantitative PCR analyses must be performed to determine these levels.

To localize FLAP protein, we used a rabbit polyclonal antiserum selective for FLAP (Dixon et al., 1990). We assessed the selectivity of the immunostaining procedure by (1) showing that the antiserum stained a single band of ~18 kDa on western blots of rat brain particulate fractions, (2) replacing the primary antiserum with preimmune serum (not shown), and (3) demonstrating that FLAP antiserum and 35S-labeled FLAP probe labeled the same neuronal elements. Both FLAP mRNA and FLAP immunostaining were dense in hippocampus (Fig. 3A, B), where they were associated with pyramidal cells in CA1 (Fig. 3C) to CA3, and with granule cells in dentate gyrus (not shown).

We also determined the cellular distribution of 5-LO mRNA. Figure 3D and E show silver grains overlying neuronal elements within the hippocampal CA1 field. In agreement with the histologic localization, the number of silver grains associated with neurons was significantly higher in hippocampus than in neocortex or thalamus. We counted 16.2 ± 1.7 grains per neuron (mean ± SEM, n = 53) in the hippocampal CA1 field and 14.2 ± 1.3 grains per neuron in the dentate gyrus. By contrast, we measured only 6.5 ± 0.6 grains per neuron in the neocortex and 5.5 ± 0.9 grains per neuron in the thalamus (p < 0.05, ANOVA). Sense-oriented 5-LO probe revealed no specific labeling (1.6 ± 0.3 grains per neuron, n = 30 for all regions).

The extensive overlap between the cellular distribution of 5-LO and that of FLAP suggested that the two proteins may be coexpressed in CNS neurons. To examine this possibility, we stained sections of hippocampal tissue with FLAP antiserum and hybridized them with the 35S-labeled 5-LO probe. As shown in Fig. 3E, most neurons in the CA1 pyramidal cell layer showed both immunostaining and hybridization signals, confirming the colocalization of 5-LO and FLAP.

To determine if FLAP is functional in hippocampus,
FIG. 3. Cellular localization of 5-LO and FLAP mRNAs and FLAP immunoreactivity in the hippocampal CA1 field. A–C: Localization of FLAP in sections exposed to 35S-labeled FLAP oligoprobe, visualized using dark-field illumination (A), and FLAP polyclonal antiserum visualized using light-field illumination (B). C is a light-field detail of A with greater magnification, to show the cellular distribution of the FLAP transcript. D: Localization of 5-LO mRNA in a section exposed to 35S-labeled 5-LO oligoprobe visualized using dark-field illumination. E: Colocalization of FLAP immunoreactivity and 5-LO mRNA. Note the silver grains (35S-labeled 5-LO oligoprobe) overlying FLAP-immunoreactive neurons. Bars indicate 50 µm (except in C, where it indicates 20 µm).

we assessed the ability of the selective FLAP inhibitor MK-886 to affect the somatostatin augmentation of \( I_M \). We recorded intracellularly from 18 CA1 pyramidal neurons with an average resting membrane potential of \(-69 \pm 0.6 \text{ mV} \) (mean ± SEM) and action potential amplitude of \(106 \pm 1.3 \text{ mV} \). We used two drug administration protocols; in protocol 1, we first superfused somatostatin (1 \( \mu \text{M}, 2-4 \text{ min} \)) to demonstrate an \( I_M \) increase, then washed out the peptide while superfusing MK-886 (0.25–1 \( \mu \text{M}, 18-25 \text{ min} \)), and finally superfused somatostatin again (2–4 min) in the continued presence of MK-886. In protocol 2, to ensure that the somatostatin response was not reduced by desensitization, we superfused MK-886 first and then applied somatostatin in the presence of MK-886. Our results showed that MK-886 inhibited completely the effect of somatostatin on \( I_M \) using either protocol (Fig. 4). Superfusion of somatostatin alone (protocol 1) increased \( I_M \) amplitude by 45 ± 6 to 75 ± 16% in eight of 10 cells, with recovery to control levels after washout (15–25 min). In the eight responding cells, somatostatin showed very little effect when reapplied in the presence of MK-886 (8 ± 9 to 14 ± 10% increase; Fig. 4B ). When MK-886 was superfused first (protocol 2), seven of eight cells did not show any \( I_M \) increase with subsequent somatostatin addition (1 ± 7 to 8 ± 5% increase overall; Fig. 4C). To rule out nonspecific effects of MK-886 on the \( I_M \) channel, we also superfused LTC4 in the presence of MK-886; LTC4 (6–8 \( \mu \text{M} \)) still increased \( I_M \) (two cells; Fig. 4A), suggesting that MK-886 prevents the somatostatin response by inhibiting biosynthesis of LTC4.

DISCUSSION

In this report we show that 5-LO and FLAP, which play a pivotal role in the cellular biosynthesis of leukotrienes in peripheral tissue, are coexpressed in discrete neuronal populations of the CNS. By combining photographic emulsion and immunohistochemical techniques, we also demonstrate that 5-LO mRNA and FLAP protein are expressed in the same neurons, as suggested by their concerted role in leukotriene biosynthesis. Our findings, together with those of Nishiyama and coworkers (1993), establish that mammalian CNS...
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FIG. 4. Neutralization of the somatostatin effect on $I_M$ by the FLAP inhibitor MK886. A: $I_M$ recording of a CA1 neuron held at $-46 \text{ mV}$ (resting potential was $-70 \text{ mV}$); $I_M$ appears as a slow inward current “relaxation” after the instantaneous inward current drop. Somatostatin was applied at $1 \mu M$ and LTC4 at $8 \mu M$.

B: Average of $I_M$ amplitudes in eight neurons tested with protocol 1 (somatostatin first (A); see text). C: Average of $I_M$ amplitudes in eight neurons tested with protocol 2, i.e., initial exposure to MK-886 (O) followed by somatostatin application (V) in the continuous presence of MK-886.

neurons possess the enzymatic complement necessary to produce both 5- and 12-lipoxygenase metabolites. In further support of this conclusion, formation of leukotrienes was demonstrated in a preparation of purified pinched-off synaptic terminals (synaptosomes), which contain little or no astrocyte contamination (Yates et al., 1990). By contrast, embryonic neurons cultured in serum-free medium do not produce eicosanoids, a result that is likely explained by the culture conditions used in those experiments (Oomagari et al., 1991).

Arachidonic acid metabolites produced via the 12-lipoxygenase pathway have been proposed to act as intracellular second messengers in invertebrate and vertebrate neurons (Piomelli et al., 1987, 1989; Belardetti et al., 1989; Dorman et al., 1992). Several findings support the possibility that leukotrienes play a similar role. Here, we show that MK-886, a leukotriene biosynthesis inhibitor that acts by binding to FLAP, prevents somatostatin enhancement of the $K^+$ M-current in hippocampal pyramidal neurons. Previous studies have demonstrated that the $I_M$-augmenting effect of somatostatin in these neurons are prevented by inhibiting PLA$_2$ or 5-LO activities and mimicked by exogenous LTC$_4$ (Schweitzer et al., 1990, 1993). Thus, leukotrienes may serve, in CNS neurons, a signaling function similar to that proposed for 12-lipoxygenase metabolites in Aplysia.

Our results raise several questions. We do not know yet whether somatostatin stimulates formation of leukotrienes in hippocampal neurons, and, if so, through what mechanism. The discovery that a somatostatin receptor (SSTR4) highly expressed in hippocampus is linked to PLA$_2$ activation and release of arachidonic acid suggests the involvement of this transmembrane signaling pathway (Bito et al., 1994; Sakanaka et al., 1994). Further, the mechanism of action of LTC$_4$ in regulating M-channel function is still unknown. LTC$_4$ may act on intracellular targets (e.g., the M-channel), as shown for 12-lipoxygenase metabolites in Aplysia sensory neurons (Belardetti et al., 1989). Alternatively, LTC$_4$ may exit the cell of origin and act by binding to membrane receptors on adjacent neurons, a hypothesis supported by the occurrence of high-affinity binding sites for LTC$_4$ in neural tissue (Schalling et al., 1986). Interest in this question lies in the possibility that LTC$_4$ may provide a diffusible message, of the type postulated to operate in synaptic plasticity and development, linking intracellular to extracellular signaling (Harish and Poo, 1992). Finally, we have found other brain regions, besides hippocampus, that express relatively high levels of 5-LO and FLAP mRNAs. Further experiments are necessary to demonstrate the cellular localization of these transcripts in such regions, for instance, in the cerebellum, and to determine whether 5-LO metabolites serve signaling functions similar to that proposed here for hippocampal CA1 neurons.

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