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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^+ M-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.
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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 A_4 (LTA_4). LTA_4 is short lived and may be rapidly transformed either into LTC_4, by LTC_4-synthase, or into LTB_4, by LTA_4-hydrolase (Samuelsson et al., 1987). Unlike other mammalian lipoxygenases, 5-LO requires multiple cofactors to become fully active, including Ca^{2+} and ATP (Ford-Hutchinson et al., 1994). Rises in intracellular Ca^{2+} 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, LTC_4 exerts multiple actions on neural cells, such as prolonged excitation of cerebellar Purkinje neurons (Palmer 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 noninactivating time- and voltage-dependent K^+ current (Brown and Adams, 1980) that, in

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Abbreviations used: FLAP, 5-lipoxygenase–activating protein; I_M, M-current, a noninactivating time- and voltage-dependent K^+ current; 5-LO, 5-lipoxygenase; LT, leukotriene; PCR, polymerase chain reaction; PLA_2, phospholipase A_2; RT, reverse transcriptase; SDS, sodium dodecyl sulfate; SSC, standard saline citrate.

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 LTB_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 to 459 of rat FLAP sequence (Dixon et al., 1990) and nucleotides 442–1,028 of rat 5-LO sequence (Balcarek et al., 1988). PCR conditions were as follows: 92°C, 56°C, and 72°C for 1 min each; amplification of FLAP cDNA was performed for 15 cycles and that of 5-LO cDNA for 20 cycles. We subjected the PCR products to agarose (1%) gel electrophoresis and transferred them onto nitrocellulose. Prehybridization was at 56°C for 2 h in 10× Denhardt's solution/20 mM Na_2HPO_4/NaH_2PO_4 buffer, pH 6.8/5× standard saline citrate (SSC)/1% sodium dodecyl sulfate (SDS)/denaturated salmon sperm DNA (0.1 mg/ml). We performed hybridization for 3 h at 56°C, in prehybridization solution containing 10% dextran sulfate and ^{32}P-labeled 46-mer oligonucleotide probes (5×10^6 dpm/ml) corresponding to nucleotides 86–132 (FLAP-1) and 285–331 (FLAP-2) of rat FLAP sequence (Dixon et al., 1990) and nucleotides 818–864 of rat 5-LO sequence (Balcarek et al., 1988). We washed blots twice with 2× SSC/0.1% SDS/10× Denhardt's solution/10 mM Na_2HPO_4/NaH_2PO_4 buffer, pH 6.8, at 56°C for 30 min.

In situ hybridization

We prepared and incubated brain sections essentially as described previously (Bouthenet et al., 1991). For FLAP, hybridizations were performed in the presence of 50% formamide and 10 mM dithiothreitol, using synthetic ^{35}S-labeled antisense oligonucleotides (base 89–109 for 5-LO and −6 to 14 for FLAP). We used as control a missense oligonucleotide (5′-ACA TAT CTG ACT TCG AAT GCC-3′). In some experiments, distribution of 5-LO mRNA was confirmed by using a riboprobe prepared with a Promega riboprobe kit from a *Sma*I–*Bam*HI DNA fragment corresponding to nucleotides 1,417–1,735 of human 5-LO sequence (Dixon et al., 1988), 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 a 1:500 dilution, rinsed, incubated with biotin-labeled anti-rabbit protein antiserum, 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 μm 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_2PO_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) and LTC_4 (Biomol Research Laboratories, Plymouth Meeting, PA, U.S.A.) in 0.05% dimethyl sulfoxide, which alone had no significant effect on I_M. We used sharp glass micropipettes filled with 3 M KCl (tip resistances, 55–88 mΩ) to penetrate CA1 pyramidal neurons for voltage clamp studies, using an Axoclamp preamplifier (Axon Instruments) in discontinuous single-electrode voltage-clamp mode. We added tetrodotoxin (0.5–1 μM) to block synaptic transmission and Na^+-dependent action potentials. We acquired current and voltage records by A/D sampling and acquisition software (Pclamp, Axon Instruments) and measured I_M amplitude with software (Clampfit, Axon Instruments) that fitted exponential curves of the I_M relaxation peak of the initial (quasi-instantaneous) current.

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

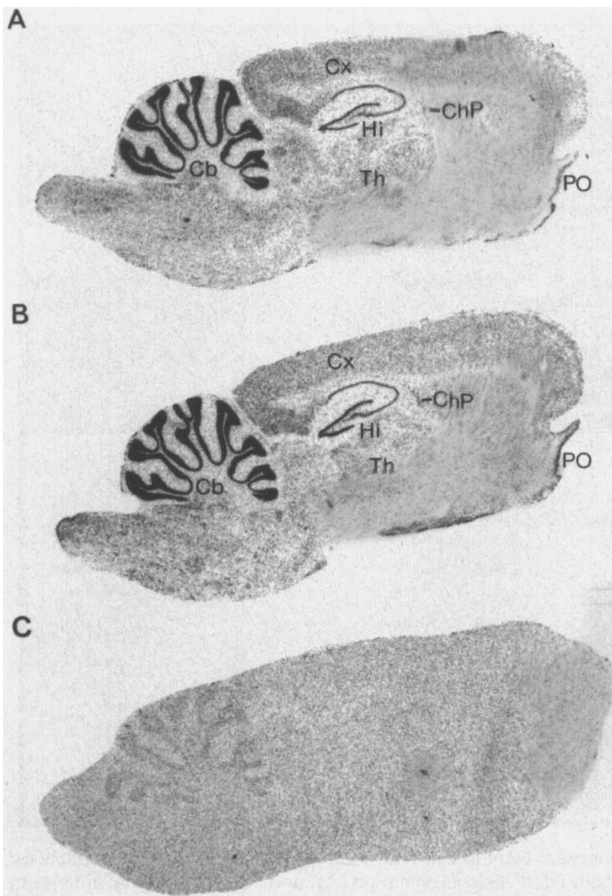

FIG. 1. Histologic localization of 5-LO and FLAP mRNAs in parasagittal sections of rat brain. Hybridization with a ^{35}S-labeled 5-LO oligoprobe (**A**), a ^{35}S-labeled FLAP oligoprobe (**B**), and a ^{35}S-labeled control missense probe (**C**) (for sequence, see Experimental Procedures). Highest labeling densities are observed in hippocampus (Hi) and cerebellum (Cb); moderate labeling is observed in neocortex (Cx), thalamus (Th), primary olfactory cortex (PO), and choroid plexus (ChP).

diffuse, nonspecific labeling was seen in adjacent sections treated with a control, ^{35}S-labeled missense probe (Fig. 1C). We obtained equivalent results using a ^{32}P-labeled 5-LO riboprobe (derived from human 5-LO cDNA, which displays 93% identity with its rat homologue) and two additional ^{35}S-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 ^{35}S-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). A 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 ^{35}S-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,

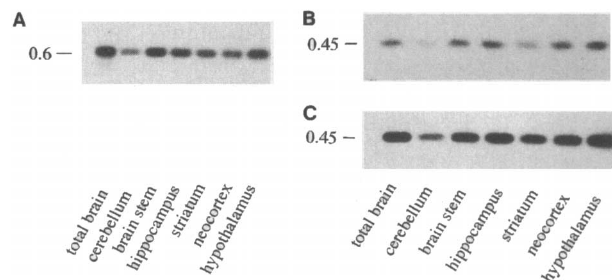

FIG. 2. Expression of 5-LO (**A**) and FLAP mRNAs (**B, C**) in various regions of the rat brain determined by RT-PCR followed by Southern blot analyses. Samples of poly(A)$^+$ RNA (1 μg) were subjected to RT-PCR, fractionated by agarose gel electrophoresis and hybridized with selective ^{32}P-labeled oligonucleotide probes. Two distinct probes, FLAP1 (B) and FLAP2 (C), were used to detect products of FLAP mRNA amplification (for sequences, see Experimental Procedures). Blots were exposed to x-ray films for 1 h at −80°C. Molecular sizes (in kb) of the PCR products obtained are indicated by the bars.

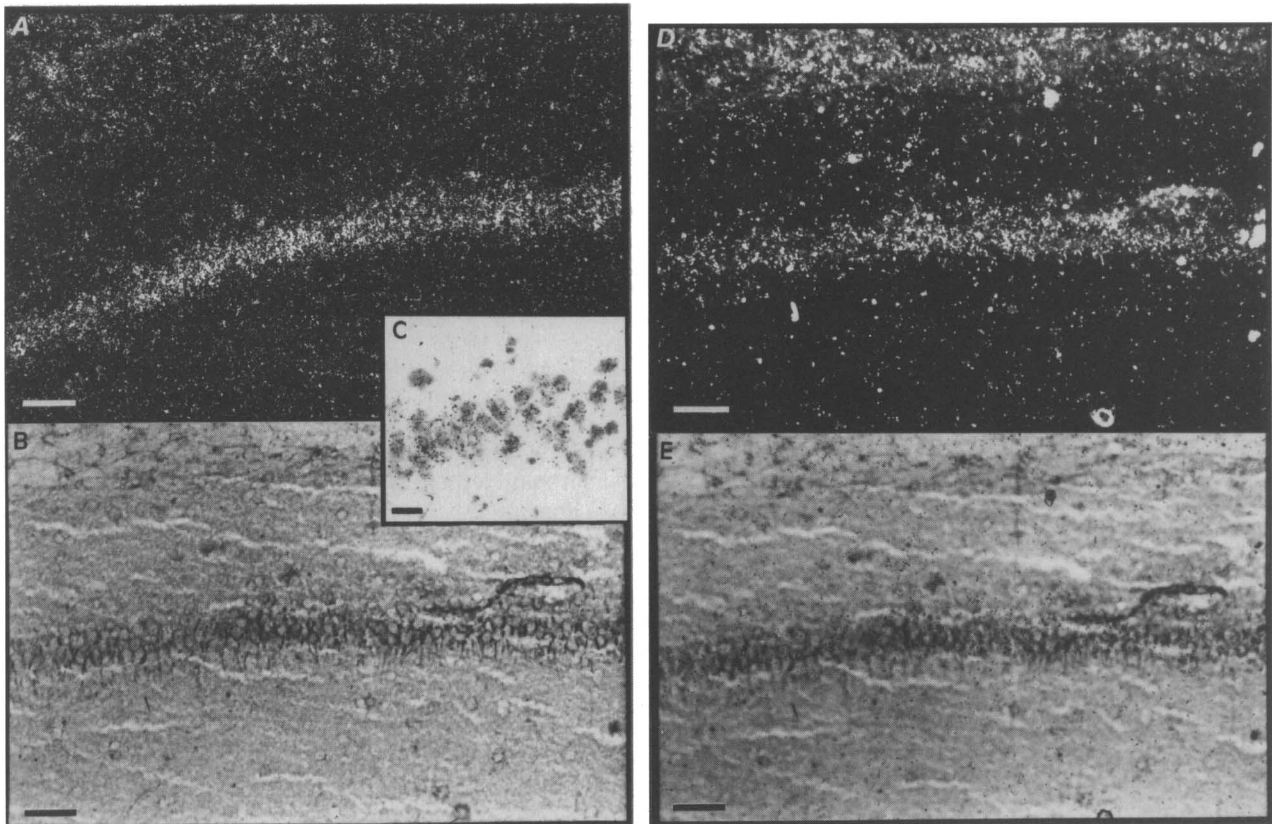

FIG. 3. Cellular localization of 5-LO and FLAP mRNAs and FLAP immunoreactivity in the hippocampal CA1 field. **A–C:** Localization of FLAP in sections exposed to ^{35}S-labeled FLAP oligoprobe, visualized using dark-field illumination (A), and FLAP polyclonal antiserum visualized using light-field illumination (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 ^{35}S-labeled 5-LO oligoprobe visualized using dark-field illumination. **E:** Colocalization of FLAP immunoreactivity and 5-LO mRNA. Note the silver grains (^{35}S-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 ± 0.6 mV (mean $\pm$ SEM) and action potential amplitude of 106 ± 1.3 mV. We used two drug administration protocols; in protocol 1, we first superfused somatostatin (1 μM, 2–4 min) to demonstrate an I_M increase, then washed out the peptide while superfusing MK-886 (0.25–1 μM, 18–25 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 $75 \pm 16\%$ 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 $14 \pm 10\%$ 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 $8 \pm 5\%$ increase overall; Fig. 4C). To rule out nonspecific effects of MK-886 on the M-channel, we also superfused LTC_4 in the presence of MK-886; LTC_4 (6–8 μM) still increased I_M (two cells; Fig. 4A), suggesting that MK-886 prevents the somatostatin response by inhibiting biosynthesis of LTC_4.

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

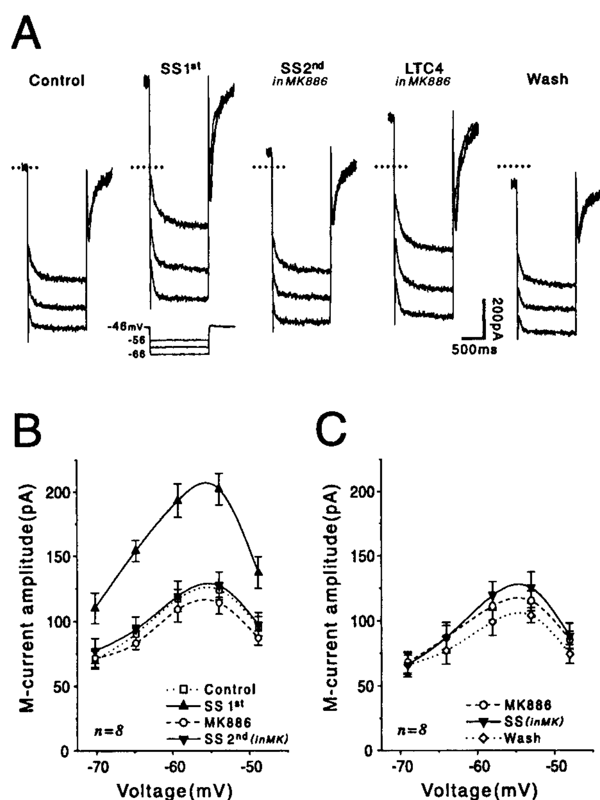

FIG. 4. Neutralization of the somatostatin effect on I_M by the FLAP inhibitor MK886. **A:** I_M recording of a CA1 neuron held at −46 mV (resting potential was −70 mV); I_M appears as a slow inward current "relaxation" after the instantaneous inward current drop. Somatostatin was applied at 1 μM and LTC_4 at 8 μM. **B:** Average of I_M amplitudes in eight neurons tested with protocol 1 [somatostatin first (▲); see text]. **C:** Average of I_M amplitudes in eight neurons tested with protocol 2, i.e., initial exposure to MK-886 (○) followed by somatostatin application (▼) 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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