A glutathione conjugate of hepoxilin A₃: Formation and action in the rat central nervous system

(hippocampus/CA1 neurons/hyperpolarization/post-spike train afterhyperpolarization/inhibitory postsynaptic potential)

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ABSTRACT Incubation of (8R)- and (8S)-[1-^{14}C]hepoxilin A₃ [where hepoxilin A₃ is 8-hydroxy-11,12-epoxyeicosa-(5Z,9E,14Z)-trienoic acid] and glutathione with homogenates of rat brain hippocampus resulted in a product that was identified as the (8R) and (8S) diastereomers of 11-glutathionyl hepoxilin A₃ by reversed-phase high performance liquid chromatographic comparison with the authentic standard made by total synthesis. Identity was further confirmed by cleavage of the isolated product with γ-glutamyltranspeptidase to yield the corresponding cysteinylglycinyl conjugate that was identical by reversed-phase high performance liquid chromatographic analysis with the enzymic cleavage product derived from the synthetic glutathionyl conjugate. The glutathionyl and cysteinylglycinyl conjugate are referred to as hepoxilin A₃-C and hepoxilin A₃-D, respectively, by analogy with the established leukotriene nomenclature. Formation of hepoxilin A₃-C was greatly enhanced with a concomitant decrease in formation of the epoxide hydrolase product, trioxilin A₃, when the epoxide hydrolase inhibitor trichloropropene oxide was added to the incubation mixture demonstrating the presence of a dual metabolic pathway in this tissue involving hepoxilin epoxide hydrolase and glutathione S-transferase processes. Hepoxilin A₃-C was tested using intracellular electrophysiological techniques on hippocampal CA1 neurons and found to be active at concentrations as low as 16 nM in causing membrane hyperpolarization, enhanced amplitude and duration of the post-spike train afterhyperpolarization, a marked increase in the inhibitory postsynaptic potential, and a decrease in the spike threshold. These findings suggest that these products in the hepoxilin pathway of arachidonic acid metabolism formed by the rat brain may function as neuromodulators.

Hepoxilins are biologically active epoxy alcohols formed from arachidonic acid by initial 12-lipoxygenation and subsequent intramolecular rearrangement of (12S)-hydroperoxy-eicosatetraenoic acid (1–3). This transformation is facilitated by the ferric ion protoporphyrin subunit, which is present in hematin, hemoglobin (2, 3), and other hemeproteins (unpublished observations). Two position-isomeric hepoxilins have been isolated, hepoxilin A₃ [mixture of (8R) and (8S) diastereomers of 8-hydroxy-11,12-epoxyeicosa-(5Z,9E,14Z)-trienoic acid (hepoxilin A₃)] and hepoxilin B₃ (hydroxyl at C-10) (1). Both products have been shown to enhance the release of insulin from rat pancreatic islets (4). Hepoxilin A₃ is capable of modulating synaptic neurotransmission and neuronal excitability (5), facilitating the transport of calcium across membranes (6), and raising cytosolic calcium concentrations in human neutrophils with intracellular acidification (7). Hepoxilin A₃ has also been shown to be formed by

Aplysia neurons, on which it produces slow hyperpolarization (8). These findings suggest a potential second messenger role for these products in the cell.

The formation of prostaglandins and the glutathione-containing leukotrienes are catalyzed by specific isozymes of glutathione S-transferases. Thus, prostaglandins E₂ and F₂α appear to be formed through the reaction of the prostaglandin endoperoxide, prostaglandin H₂, with isozymes containing the Yₐ subunit (9), while leukotriene C₄ is catalyzed by isozymes containing the Yᵦ subunit (10, 11). Hepoxilin A₃ is metabolized to a glutathione conjugate, 8-hydroxy-(11R)-glutathionyl-(12S)-hydroxyeicosa-(5Z,9E,14Z)-trienoic acid (hepoxilin A₃-C), by an affinity-purified mixture of glutathione S-transferases (12), and enzymatic glutathione conjugation with hepoxilin A₃ occurs at C-11 rather than at the allylic C-9 position (12).

This report describes the formation of the glutathione C-11 conjugate termed hepoxilin A₃-C by the rat brain and the demonstration of its biological activity on hippocampal CA1 neurons. In addition we provide evidence of the conversion of hepoxilin A₃-C into another product, 8-hydroxy-(11R)-cysteinylglycinyl-(12S)-hydroxyeicosa-(5Z,9E,14Z)-trienoic acid (hepoxilin A₃-D), by reaction with γ-glutamyltranspeptidase.

MATERIALS AND METHODS

Materials. [1-^{14}C]Hepoxilin A₃ was prepared as described from [1-^{14}C]arachidonic acid (Amersham; specific activity, 59 mCi/mmol; 1 Ci = 37 GBq), using rat platelets as the source of 12-lipoxygenase and (12S)-hydroperoxyeicosatetraenoic acid, and hematin (Sigma) to effect the rearrangement of (12S)-hydroperoxyeicosatetraenoic acid to hepoxilin A₃ (2). The product was purified to radiochemical homogeneity using straight-phase high performance liquid chromatography (HPLC) and consisted of a mixture of two isomers racemic at C-8. Trichloropropene oxide (TCPO) was purchased from Sigma. Two synthetic hepoxilins A₃ were formed (13), which were termed more polar and less polar isomers, with the configuration at C-8 now assigned as 8R and 8S, respectively (E.J.C. and W.-G.S., unpublished method). Chemical coupling of each synthetic hepoxilin A₃ with glutathione was effected by the following sequence: (i) reaction of 2.0 equivalents of N-trifluoroacetylglutathione trimethyl

Abbreviations: hepoxilin A₃, 8-hydroxy-11,12-epoxyeicosa-(5Z,9E,14Z)-trienoic acid; hepoxilin A₃-C, 8-hydroxy-(11R)-glutathionyl-(12S)-hydroxyeicosa-(5Z,9E,14Z)-trienoic acid; hepoxilin A₃-D, 8-hydroxy-(11R)-cysteinylglycinyl-(12S)-hydroxyeicosa-(5Z,9E,14Z)-trienoic acid; trioxilin A₃, 8,11,12-trihydroxyeicosa-(5Z,9E,14Z)-trienoic acid; TCPO, trichloropropene oxide; ACSF, artificial cerebrospinal fluid; EPSP, excitatory postsynaptic potential; IPSP, inhibitory postsynaptic potential; AHP, afterhyperpolarization.

ester with the methyl ester of hepoxilin A$_3$ in 4:1 (vol/vol) methanol/triethylamine at 80°C for 24 hr under an atmosphere of argon to give a mixture of the C-11 (*R*) thioether conjugate (a single isomer) and two (*R* and *S*) C-9 thioether conjugates in a ratio of 1:1.5, respectively; (*ii*) chromatographic separation of the C-11 and C-9 thioether conjugates using preparative thin layer chromatography on silica gel plates with 45:45:10 (vol/vol) ethyl acetate/hexane/methanol for development (*R$_f$* values 0.37 and 0.44 for the C-11 and C-9 conjugates, respectively); and (*iii*) saponification with 0.15 M potassium carbonate in 3:1 (vol/vol) water/methanol at 23°C for 24 hr under argon in the dark, separately for the C-11 and C-9 thioether conjugates. In this way the pure C-11 (*R*) thioether conjugate of glutathione and hepoxilin A$_3$ (hepoxilin A$_3$-C) was obtained in both 8*R* and 8*S* forms. The thioether conjugate of glutathione with hepoxilin A$_3$ at C-9 was obtained separately as a mixture of (9*R*) and (9*S*) diastereoisomers. The determination of the structure of these conjugates was accomplished by 500 MHz ^{1}H NMR analysis with spin decoupling using both 8-deuterated and undeuterated synthetic samples.

Brain Tissue. Male Wistar rats (200–250 g, pathogen free; Charles River Breeding Laboratories) were anaesthetized with halothane and decapitated, and the brain was dissected and placed in ice-cold oxygenated (95% O$_2$/5% CO$_2$) artificial cerebrospinal fluid [pH 7.4, ACSF(5)]. Coronal sections of hippocampus were prepared using a Vibratome (400 μm thick) and were allowed to equilibrate at 30°C for 60 min in oxygenated ACSF. The hippocampal slices were then pooled and homogenized in 1 ml of ACSF by using a Polytron tissue homogenizer. Each experiment made use of a homogenate resulting from five hippocampal slices.

Incubations. The hippocampal homogenates were incubated with 50,000 cpm of [1-^{14}C]hepoxilin A$_3$ in the presence of reduced glutathione (0, 1, and 10 mM) and in the presence of TCPO (0–3 mM). Incubations were routinely carried out at 37°C for 60 min. Incubations were terminated by the addition of 5 M formic acid (50 μl), and the mixture was centrifuged quickly at 3000 rpm in a laboratory centrifuge (Hettich EBA35).

In experiments where metabolism of hepoxilin A$_3$-C into hepoxilin A$_3$-D was investigated, hepoxilin A$_3$-C was first isolated by reversed-phase liquid chromatography (see below) and the purified material was treated with commercial γ-glutamyltranspeptidase (50 μg) in 0.1 M Tris·HCl (pH 8.0) for 60 min at 37°C. The sample was then acidified with 5 M formic acid (50 μl), desalted, and concentrated by passing it on a Sep-Pak C$_{18}$ cartridge (Waters; see below).

Extraction and Analysis. Supernatants from acidified incubations were passed through Sep-Pak C$_{18}$ cartridges. The cartridges were then washed with water until the eluate was neutral, and the hepoxilin products were eluted with pure methanol. Recovery of added radioactivity to this stage was better than 90%. The methanol solution was evaporated to near dryness *in vacuo*, and the concentrated solution was diluted with methanol/water/acetic acid, 55:45:0.01 (vol/vol), and analyzed by HPLC on a μBondapak C$_{18}$ column (Waters) equilibrated with the same solvent. The eluent from the chromatograph was analyzed by passage through an on-line ultraviolet spectrophotometer (Waters) operated at 215 nm and then through an on-line radioactivity monitor (LB5026, Berthold) using PCS scintillation fluid (Amersham) admixed with the chromatograph eluent at a ratio of 1:2, respectively.

Electrophysiological Studies. Electrophysiological intracellular recordings were carried out in an interface-type chamber at 34°C with glass micropipettes filled with 3 M KCl or 3 M potassium acetate (resistances, 60–150 MΩ). Data were stored on tape and recorded on chart paper. Orthodromic stimulation for excitatory postsynaptic potentials (EPSPs)

and inhibitory postsynaptic potentials (IPSPs) was done by mono- or bipolar tungsten electrodes. Post-spike train afterhyperpolarizations (AHPs) were generated by 100-ms constant current depolarizing pulses. During drug perfusion, the pulse was adjusted if necessary to obtain the same number of spikes as required to generate the pre-drug AHP. All measurements of the AHP, postsynaptic potentials, and the input resistance (*R$_{in}$*) were done at the same membrane potential as the control pre-drug membrane potential, which variably required constant current injection. Measurements included the maximum amplitude of the EPSP, IPSP, and AHP from the prestimulus baseline. The duration of the AHP was measured after the offset of the 100-ms depolarizing pulse until the AHP reached the level of the prestimulus baseline. Stock solution of hepoxilin A$_3$-C methyl ester [(11*R*)-glutathionyl derived from the more polar (8*R*)-hepoxilin A$_3$ isomer] or the methyl ester of the C-9 glutathionyl isomer [derived from the less polar (8*S*)-hepoxilin A$_3$ isomer] were dissolved in dimethyl sulfoxide (1 μg/μl) and diluted to the appropriate concentrations (3 μM–16 nM) with ACSF. The drug was applied by perfusion to nine neurons in total.

RESULTS

Formation of Hepoxilin A$_3$-C in Hippocampal Homogenates—Dependence on Exogenous Reduced Glutathione. Incubations of [1-^{14}C]hepoxilin A$_3$ [mixture of (8*R*) and (8*S*) diastereomers] with fresh homogenates of rat hippocampus in the absence of glutathione led to the appearance of only trioxilins A$_3$ [8,11,12-trihydroxyeicosa-(5*Z*,9*E*,14*Z*)-trienoic acid], resulting from the enzymatic hydrolysis of the substrate by hepoxilin epoxide hydrolase (14) (Fig. 1*A*). When glutathione was included in the incubation, more polar products were detected on reversed-phase HPLC whose formation was dependent on the concentration of glutathione used (Fig. 1 *B* and *C*). Formation of these polar products was enhanced when TCPO, the hepoxilin epoxide hydrolase inhibitor, was used (Fig. 1*D*). That these more polar products were formed enzymatically was shown by their lack of formation when heat-inactivated enzyme was used (Fig. 1*E*). The polar products were shown to consist of two C-8 diastereomers that migrated chromatographically with authentic standards of (8*R*) and (8*S*)-hepoxilin A$_3$-C, the 11-glutathione conjugate of hepoxilin A$_3$ derived from reaction of glutathione *S*-transferase with the more polar (8*R*) and less polar (8*S*) isomers of hepoxilin A$_3$ (Fig. 1*F*) (12) and compared with authentic chemically derived products. [Retention times were: hepoxilin A$_3$-C: more polar isomer = 15.5 min; less polar isomer = 19.6 min. 9-Glutathione conjugate of hepoxilin A$_3$: more polar isomer = 13.5, 18.0 min; less polar isomer = 3.5, 13.5 min; product derived from brain = 15.5 and 19.6 min. Solvent: methanol/water/acetic acid, 55:45:0.01 (vol/vol). Column: μBondapak C$_{18}$, at a flow rate of 1.5 ml/min.]

Effect of TCPO on the Formation of Hepoxilin A$_3$-C. We found that TCPO inhibits a purified preparation of hepoxilin epoxide hydrolase from rat liver (unpublished results) as well as the rat brain preparation used in this study (Fig. 1*D*). Addition of TCPO at various concentrations from 0.3 to 3 mM caused progressive inhibition of trioxilin A$_3$ formation by the hippocampus preparation with a concomitant dose-dependent increase in formation of hepoxilin A$_3$-C (Fig. 2).

Formation of Hepoxilin A$_3$-D. Brain-derived hepoxilin A$_3$-C [mixture of (8*R*) and (8*S*) isomers] formed in the above incubations was isolated by reversed-phase HPLC and treated with γ-glutamyltranspeptidase to investigate whether the biological product indeed had a conjugated glutathione. Analysis of the reaction mixture indicated the appearance of a less-polar metabolite (two isomers) as expected of a cysteinylglycinyl conjugate (hepoxilin A$_3$-D) (Fig. 3*B*). The same products were formed when glutathione *S*-transferase-

derived hepoxilin A_3-C was treated with γ-glutamyltrans-peptidase (Fig. 3D).

Electrophysiological Effects of Hepoxilin A_3-C [(8R) form] on Hippocampal CA1 Neurons. Hepoxilin A_3-C [(8R) form] caused hyperpolarization in eight out of nine hippocampal CA1 neurons that took 5–15 min to develop (Fig. 4A). Hepoxilin A_3-C caused an increase in the duration and amplitude of the AHP (Fig. 4B). There was no appreciable effect of hepoxilin A_3-C on the EPSP but both the early chloride-dependent and the later potassium-dependent phases of the IPSP were increased by hepoxilin A_3-C (Fig. 4C). All effects persisted for the duration of the perfusion, up to 30 min. Washout with ACSF for up to 30 min did not reverse any of the effects. When the membrane was depolarized to the control resting membrane potential, spontaneous spiking was often noted, which did not occur at that potential prior to drug application. At 16 nM concentration,

all the above-described effects to (8R)-hepoxilin A_3-C were observed.

DISCUSSION

The present report reveals the presence in rat brain of two competing pathways for the metabolism of hepoxilin A_3 [(8R) and (8S) forms]—i.e., an epoxide hydrolase pathway and a glutathione conjugating pathway (Fig. 5). Through the use of TCPO, an epoxide hydrolase inhibitor that also inhibits hepoxilin epoxide hydrolase, the molecular flux through the glutathione-conjugating pathway could be increased to favor the formation of hepoxilin A_3-C [(8R) and (8S) forms] in significant amounts. Rat brain is capable of forming hepoxilin A_3 (15). This product has also been shown to exert synaptic and neuromodulatory effects on hippocampal CA1 neurons at low nanomolar concentrations with hyperpolarization of the membrane potential, augmentation of the post-spike train AHP and an increase in the amplitude of the IPSP and a decrease in the spike threshold (5). We have also shown that glutathione S-transferase catalyzes the conjugation of hepoxilin A_3 into the glutathione conjugate hepoxilin A_3-C (12).
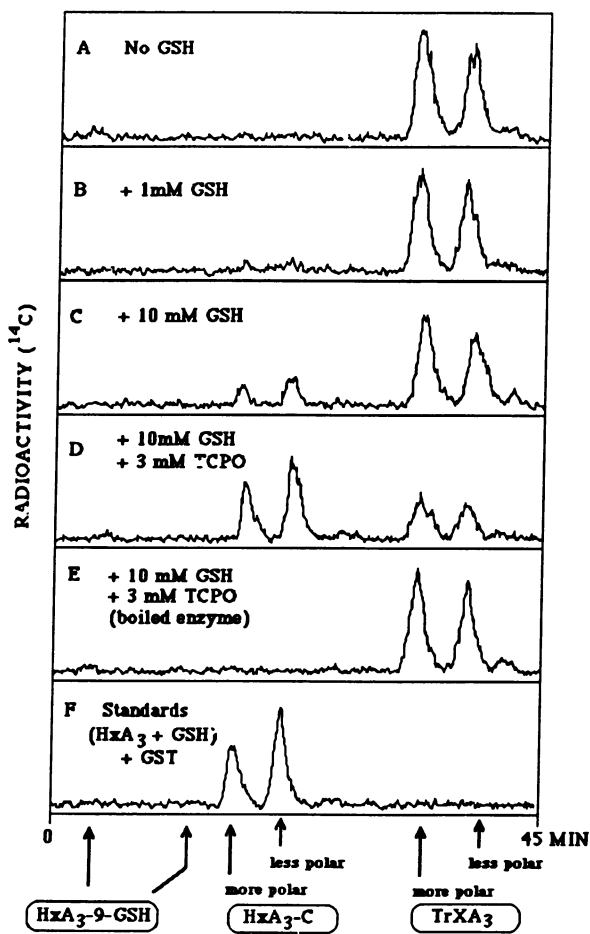

FIG. 1. Reversed-phase high performance liquid radiochromatograms showing the glutathione dependence of the conversion of [1-^{14}C]hepoxilin A_3 [mixture of (8R) and (8S) isomers] into hepoxilin A_3-C (the 11-glutathione conjugate of hepoxilin A_3) by homogenates of rat brain hippocampus in the absence of glutathione (A), in the presence of 1 and 10 mM glutathione (B and C, respectively), same as C but with TCPO (an inhibitor of hepoxilin epoxide hydrolase) (D), same as D but with heat-inactivated enzyme (E). (F) Migration of the two isomers of hepoxilin A_3-C generated by treatment of the two isomers of hepoxilin A_3 (racemic at C-8) with glutathione S-transferase (12). Homogenate was equivalent to five hippocampal slices (400 μm thick) prepared using a Vibratome. GSH, glutathione; GST, glutathione S-transferase; HxA$_3$, hepoxilin A_3; HxA$_3$-C, hepoxilin A_3-C; TrXA$_3$, trioxilin A_3 [a product resulting from the enzymatic (hepoxilin epoxide hydrolase) and nonenzymatic (acid workup) conversion of hepoxilin A_3]. Solvent: methanol/water/acetic acid, 55:45:0.01 (vol/vol). Column: C_{18} μBondapak. Flow rate: 1.5 ml/min.
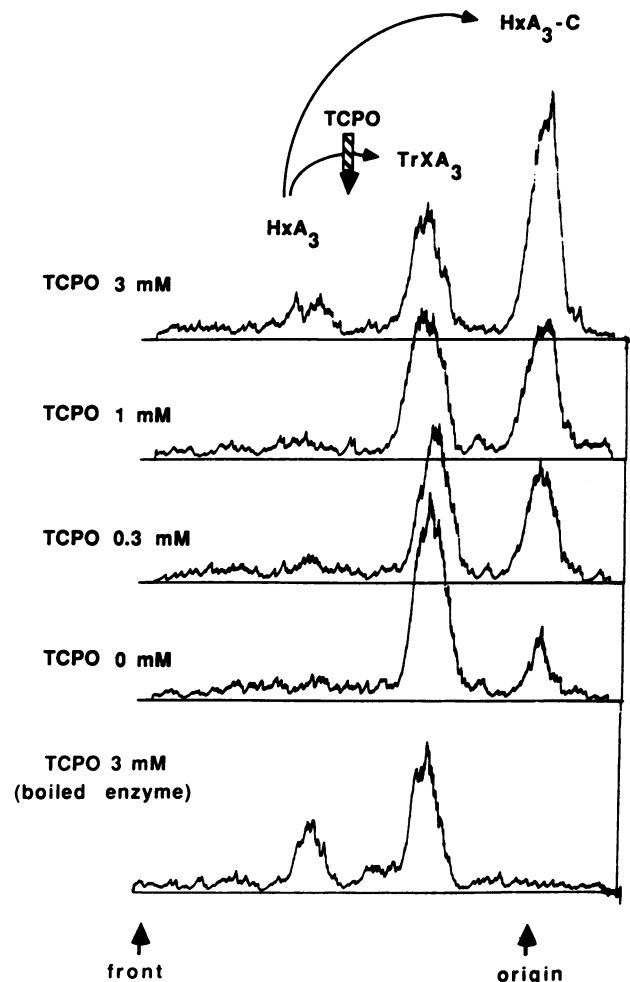

FIG. 2. Thin layer radiochromatograms showing the effects of TCPO at various concentrations on blocking hepoxilin epoxide hydrolase activity [trioxilin A_3 (TrXA$_3$) formation] and augmenting the synthesis of hepoxilin A_3-C (HxA$_3$-C) (through glutathione S-transferase) by homogenates of rat brain hippocampus. The 3 mM TCPO (boiled enzyme) sample demonstrates that the positive effects of 3 mM TCPO on hepoxilin A_3-C formation are abolished by heat denaturation of the brain enzyme. In the boiled enzyme reaction mixture, trioxilin is nonenzymatically generated during the acidic workup.
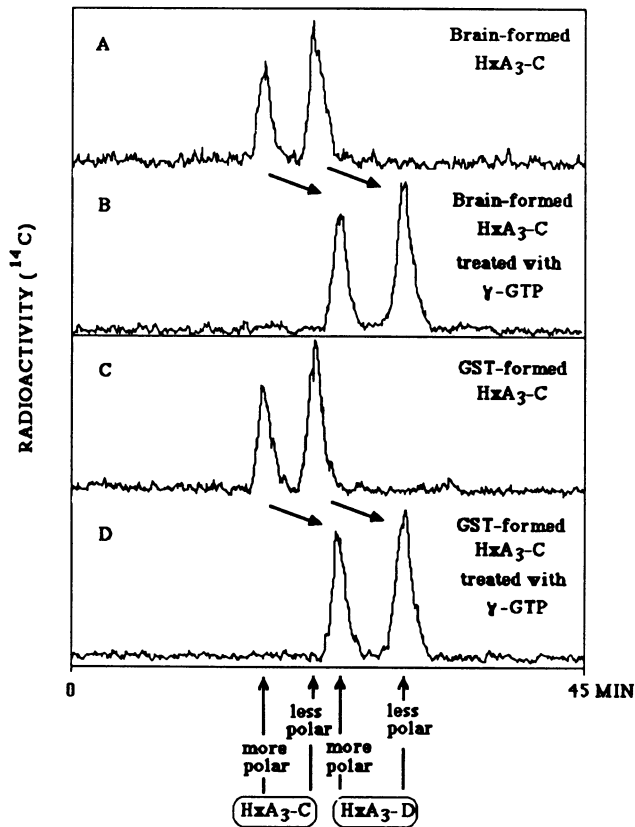
RADIOACTIVITY (^{14}C)

A Brain-formed
HxA$_3$-C

B Brain-formed
HxA$_3$-C
treated with
γ-GTP

C GST-formed
HxA$_3$-C

D GST-formed
HxA$_3$-C
treated with
γ-GTP

0   45 MIN

less   less
polar   polar
more   more
polar   polar

(HxA$_3$-C) (HxA$_3$-D)

FIG. 3. Reversed-phase HPLC showing that hepoxilin A$_3$-C (HxA$_3$-C) from brain contained glutathione by its reaction with γ-glutamyltranspeptidase (γ-GTP). Hepoxilin A$_3$-C was isolated by HPLC from brain incubations (A) as well as from a standard prepared by reaction of hepoxilin A$_3$ with glutathione S-transferase (GST) (C) and treated with γ-glutamyltranspeptidase (B and D). Results show that both substrates are converted into a less-polar product [mixture of (8R) and (8S) diastereomers] identified as hepoxilin A$_3$-D (HxA$_3$-D), a cysteinylglycinyl-containing product.

The present results demonstrate the formation of hepoxilin A$_3$-C by the rat brain and that this compound also has

synaptic and neuromodulatory effects not unlike those of its precursor hepoxilin A$_3$.

The formation of the hepoxilins infers the presence of a 12-lipoxygenase in the hippocampus, since these products are formed from 12-hydroperoxyeicosatetraenoic acid (2, 3). Indeed, 12-hydroxyeicosatetraenoic acid has been isolated from brain tissue and formation of this product is stimulated by certain neurotransmitters (16–21). Brain tissue also contains a variety of other enzymes that metabolize arachidonic acid into biologically active products. Thus, 5-lipoxygenase activity has been demonstrated through the isolation of 5-hydroxyeicosatetraenoic acid and the glutathione conjugate leukotriene C$_4$ (18), the latter being found in most brain regions but especially in the median eminence and hypothalamus. Leukotriene C$_4$ has further been localized in nerve fibers in the external layer of the median eminence associated with luteinizing hormone releasing hormone (18). Other metabolites of arachidonic acid have been shown to occur in mammalian brain. Although prostaglandin F$_{2\alpha}$ was first shown to occur in brain more than two decades ago (22), no specific function for this prostanoid has been proposed to date. The occurrence of prostaglandins E$_2$ and D$_2$ and thromboxane B$_2$ in brain has been demonstrated (23), and roles for prostaglandins E$_2$ and D$_2$ have been proposed in pyrogen-altered body temperature (24, 25) and in sleep pattern (26), respectively. Hence, the diversity of the arachidonic acid cascade is well expressed in the mammalian central nervous system offering a variety of products that may be critically involved with synaptic transmission and neuronal function.

The formation of hepoxilin A$_3$-C in the present study is analogous to the formation of leukotriene C$_4$ in that both products are produced by a glutathione-conjugating system, likely a form of glutathione S-transferase (10–12). It is interesting to note that a synthetic isomer of hepoxilin A$_3$-C in which the glutathionyl residue is located at the 9-carbon position instead of the 11-carbon position as in hepoxilin A$_3$-C is completely inactive on hippocampal CA1 neurons (n = 5 cells) within the concentration range used for hepoxilin A$_3$-C (unpublished observations). The 9-glutathionyl analog is not formed enzymatically when either glutathione S-transferase or hippocampal homogenate is incubated with hepoxilin A$_3$.
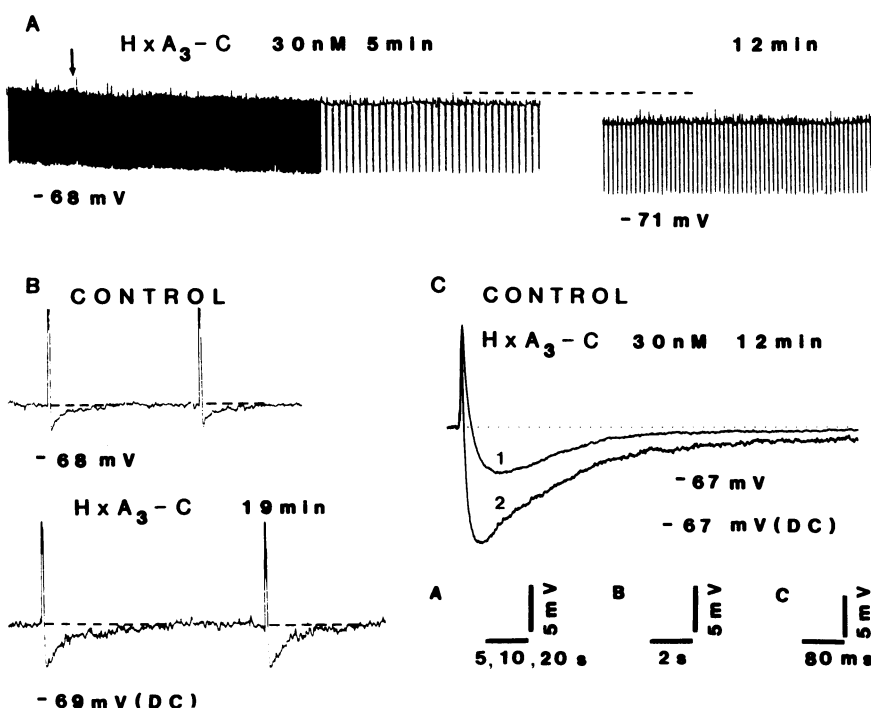
A H x A$_3$-C 30 nM 5 min

12 min

-68 m V

-71 m V

B CONTROL

-68 m V

H x A$_3$-C 19 min

-69 m V (D C)

C CONTROL

H x A$_3$-C 30 nM 12 min

1
2

-67 m V

-67 m V (D C)

A   B   C
5,10,20 s   2 s   80 ms

FIG. 4. Electrophysiological responses of hippocampal CA1 neurons to perfusion of hepoxilin A$_3$-C [(8R) isomer]. (A) Hepoxilin A$_3$-C (HxA$_3$-C) starts hyperpolarizing the cell within 5 min of onset of perfusion. The control resting membrane potential (−68 mV) is indicated by the dashed line. Input resistance (R_{in}), as measured by 0.2 nA 100 ms hyperpolarizing pulses (downward deflections), was unchanged. The hyperpolarization (−3 mV) lasted for 30 min during the perfusion of the drug and persisted for another 25 min of washout with ACSF. (B) The AHPs, which followed a train of six spikes, were increased in both depth and duration by hepoxilin A$_3$-C. Repolarization of the membrane potential to near the control resting membrane potential made the increase of the AHP more apparent. Electrode: 3 M KCl. (C) In another CA1 neuron, the orthodromic EPSP amplitude was not changed by hepoxilin A$_3$-C, but the IPSP amplitude and duration were significantly enhanced (compare curve 1 = control and curve 2 = hepoxilin A$_3$-C perfusion). The resting membrane potential was repolarized to the control level (dashed line). Each trace is the average of eight sweeps. Electrode: 3 M KCl.
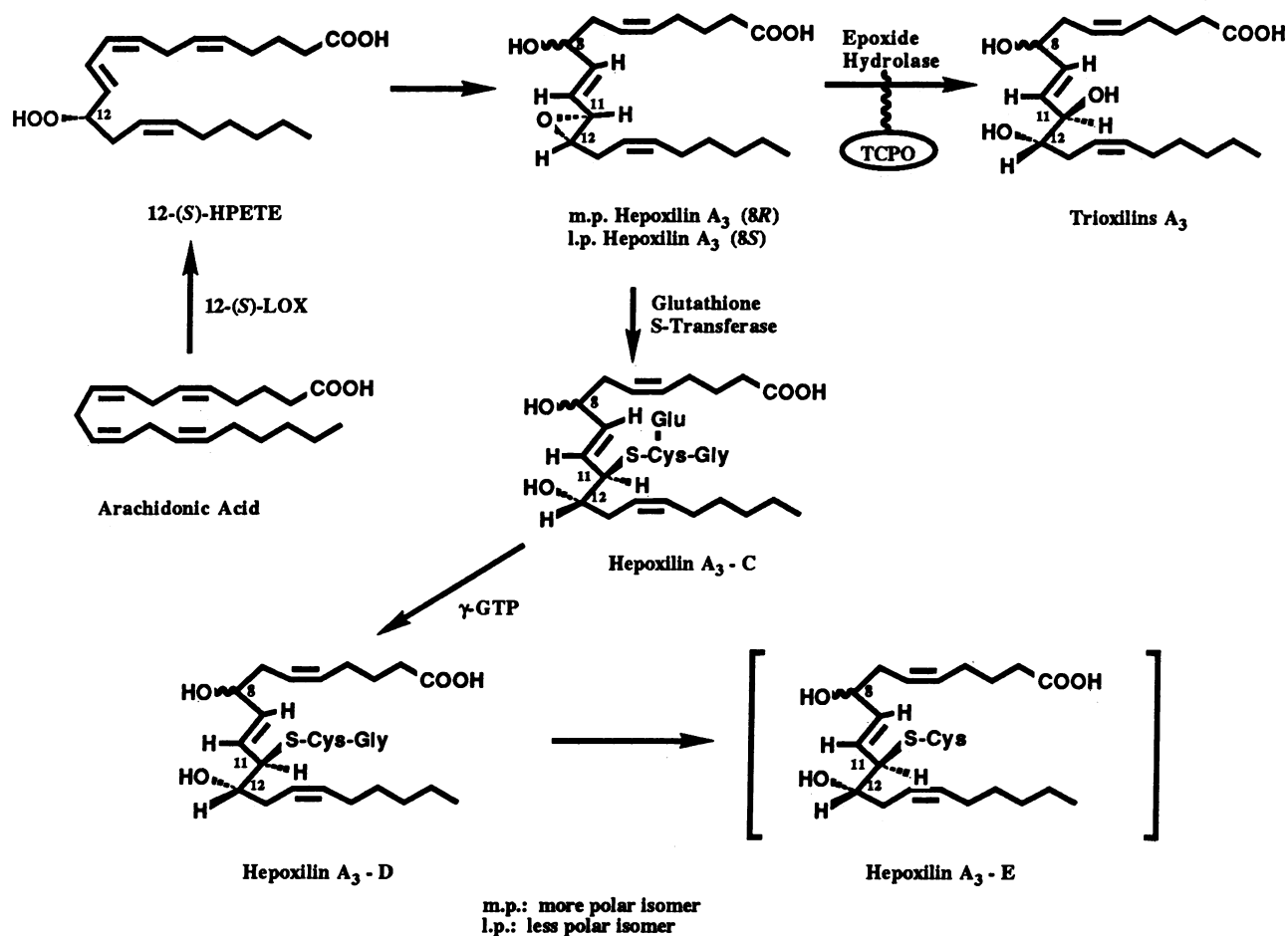
FIG. 5.　Scheme describing the two pathways of metabolism of hepoxilin A₃ by the hepoxilin epoxide hydrolase pathway to form trioxilin A₃ and by the glutathione *S*-transferase pathway to form the glutathionyl-containing hepoxilin, hepoxilin A₃-C. The latter product is transformed into a cysteinylglycinyl-containing product named hepoxilin A₃-D, by analogy with the leukotriene nomenclature. Occurrence of hepoxilin A₃-E in parentheses is inferred by analogy with the leukotrienes and has not yet been isolated. 12-(*S*)-LOX, 12-lipoxygenase.

These findings further confirm the biological relevance of hepoxilin A₃-C in the central nervous system.

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