STUDIES ON ARACHIDONIC ACID RELEASE AND METABOLISM BY THE 12-LIPOXYGENASE PATHWAY IN RAT BRAIN SLICES

by
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"The optimism of biochemists that even the highest order neural functions, i.e. reasoning, rationality and emotion, can be analyzed and understood finally at the molecular level rests on the conviction that there exist phylogenetically old and universal mechanisms of signal transduction and signal amplification of the kind that I have discussed which have been retained in the course of evolution. If that assumption should turn out to be correct, then the detailed study of receptor-mediated signal transduction mechanism whereby external signals of various kinds are received, amplified and translated into biological functions should bear rich fruits in the future and should prove to be of great heuristic value for studies of the biology of learning and behaviour. Certainly an a priori pessimism seems unwarranted. As exemplified by the work with *Aplysia*, the merger of biochemistry, genetics and neurobiology gives every hope that a satisfying, in itself coherent and precise description of mental processes on a molecular level is feasible and that this goal is achievable perhaps at a time not as long off as one was inclined to believe only a few years ago."

Ernst J.M. Helmreich

Discussions in neurosciences
vol. I, no. 3, p.38, 1984

Abstract

The present work was aimed at studying the release of arachidonic acid and formation of lipoxygenase metabolites in rat brain slices maintained *in vitro*, as well as exploring possible physiological roles for them in the mammalian central nervous system. A particularly active 12-(S)-lipoxygenase activity was found, which could be stimulated by various stimuli including the neurotransmitters norepinephrine and glutamate. Activation of α-adrenergic and N-methyl-D-aspartate (NMDA) receptor subtypes appear responsible for the effect observed in each case. Arachidonic acid on the other hand was found to have profound effects on synaptic transmission, inducing a long-lasting potentiation which appears dependent on the formation of lipoxygenase metabolites. In return, pharmacological conditions which can potentially lead to long-term potentiation (LTP) of synaptic transmission and for most of them activate NMDA receptors also induced arachidonic acid release. As these observations suggest, it is proposed that arachidonic acid and its lipoxygenase metabolites belong to a new group of messengers in the nervous system possibly acting as modulator of synaptic transmission both intra- and transcellularly. This new class of messengers constitutes an essential component of the molecular machinery involved in synaptic plasticity.

Résumé

Les travaux réunis dans cette thèse avaient pour but d'étudier le relâchement de l'acide arachidonique et la formation de ses dérivés synthétisés par les lipoxygénases dans les tranches de cerveau de rat maintenues *in vitro*, ainsi que de tenter de leur attribuer un rôle physiologique particulier dans le système nerveux central des mammifères. Une activité enzymatique importante associée à une 12-(S)-lipoxygénase fut mise à jour et il fut démontré qu'elle pouvait être stimulée par certains neurotransmetteurs comme la norépinéphrine et le glutamate. L'activation des récepteurs α-adrénergiques et au N-méthyl-D-aspartate (NMDA) semble responsable de l'effet observé dans chaque cas. D'autre part it fut démontré que l'acide arachidonique induit de profonds changements au niveau de la transmission synaptique. En effet, une potentiation de longue durée qui semble requérir la formation des dérivés synthétisés par les lipoxygénases a été observée. De plus, il fut démontré que l'utilisation de différentes conditions pharmacologiques qui sont en mesure d'induire une potentiation à long terme de la transmission synaptique, et qui pour la plupart activent les récepteurs au NMDA, conduisent aussi à une libération de l'acide arachidonique. Ces observations nous ont amené à proposer que l'acide arachidonique ainsi que ses dérivés synthétisés par les lipoxygénases appartiennent à une nouvelle classe de messagers au niveau du système nerveux qui peuvent agir comme modulateur de la transmission synaptique. Cette action pourrait s'accomplir aussi bien intracellulairement au niveau de la cellule d'origine que de façon transcellulaire. Cette nouvelle classe de messagers constituerait alors un élément essentiel des mécanismes moléculaires nécessaires à la plasticité synaptique.

Acknowledgements

Many years of sometimes arduous work, of which this thesis represents the culminating point, have been spent in this enterprise. It has been an occasion for extraordinary intellectual and personal evolution. But because of the highly demanding personal involvement required, it would have been impossible to successfully carry out this project without the participation of a certain number of individuals. I would like to take the time here to acknowledge their contribution.

First my supervisor Dr. L.S. Wolfe deserves my full appreciation. His infectious excitement has given me the necessary drive to pursue even my wildest ideas and has allowed me to go through the gloomiest moments. His wisdom has guided me all along the tortuous path that one has to follow to pursue knowledge. Overall, interacting with him has been an extremely rewarding learning experience.

I have appreciated the numerous conversations I had with Dr. H.M. Pappius. Sharing our common fascination for the brain and the many molecular mechanisms regulating it has made my day-to-day work easier to carry on.

I am particularly in debt to a number of people in the Department of Neurophysiology. First I should acknowledge the contribution of Christian Drapeau in carrying out the experiments presented in chapter 3. Paul Perreault and Granger G.C.

Hwa collaborated in the electrophysiological experiments presented in chapter 4. They have attempted to make a physiologically knowledgeable biochemist out of me, but even if they came short of that goal, the fact that they made me aware of the physiology of the brain can be already considered a significant advance. Moreover, the numerous epistemological and philosophical discussions I had with them will certainly have a long lasting influence on my personal endeavor. Finally the supervision and contact I had with Dr. M. Avoli were appreciated.

A number of people have contributed to make my life easier in the lab. A very special thanks is due to Klara Rostworowski for her dedication in providing expert technical assistance. Naila Chughtai provided technical assistance in the last part of the project. Sylvie Gauthier and Marjorie Laurent have been helpful at various times. Violaine Bégin, a graduate student in the laboratory, patiently accepted and coped with the chaotic state of matter and mind that surrounded me. It was also a pleasure to interact with James Fabian, a summer student. Being in contact with all these people has been a gratifying human experience. Mr E. Puodziunas and Mr. J. Roy from the neuroelectronic department made the perfusion bath and the temperature controller, and have provided expert technical assistance in numerous occasions. I wish to thank Dr O. Mamer and the people at the Mass Spectrometry Unit for their help in obtaining the mass spectra.

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My parents, Yvon and Cécile deserve special thanks for providing the computer on which this thesis was laboriously elaborated as well as sincere encouragements. Finally, I would like to express my deep affection and admiration for my wife Sylvie. She kindly accepted all the sacrifices I have imposed over the six years. But more importantly, she made me discover through our son Raphaël that a family life can also bring its enlightening moments .

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Preface

This thesis is presented in accordance with the indications provided for the presentation of a doctoral thesis in the Guidelines Concerning Thesis Preparation prepared by the Faculty of Graduate Studies and Research of McGill University. It is constituted of an abstract, in both english and french, claims of original research, a general introduction, three chapters of results and a general conclusion. Each chapter of results has its own introduction, discussion and references. They do not correspond as such however to published articles, although some of the data presented have been used in publications. For chapter 2, part of the data from figure 3, 4 and table 1 as well as figure 6 and 9 have been published in Journal of Neural Transmission ([Suppl] 29:29-37, 1990). Figure 4, 5 and 6 of chapter 3 have been published in Neuroscience Letters (115:286-292, 1990). Finally, figure 2, 6, 7 and 8 of chapter 4 are part of a manuscript that has been accepted for publication in Neurochemical Research (16:983-989, 1991).

Claims to original research

The work presented in this thesis studied the release of arachidonic acid and the formation of its lipoxygenase metabolites in rat brain, and explored the possibility that they could actively participate in and modulate synaptic transmission. Different approaches requiring a combination of biochemical and electrophysiogical techniques were used and a particular effort was made in order to preserve the physiological condition of the tissue while performing the biochemical experiments. The major contributions to knowledge brought by this work are listed below.

1. Although previous reports had indicated the presence of a 12-lipoxygenase in the mammalian central nervous system, it was shown for the first time to be stimulated by specific neurotransmitters. Both norepinephrine and glutamate were found to stimulate 12-HETE formation. It was also shown that this effect involves an α-adrenergic and an N-methyl-D-aspartate type of receptor, respectively. This situation appears analogous to what has been described in the mollusk *Aplysia californica* and suggests that 12-lipoxygenase metabolites might also serve as second messengers in the mammalian central nervous system.

2. For the first time, the chirality of an arachidonic acid metabolite from the 12-lipoxygenase pathway in rat brain was determined. Only the S enantiomer was found thus clearly establishing that a 12-S-lipoxygenase is present in rat brain cerebral cortex.

3. It was demonstrated for the first time in *in vitro* hippocampal slice preparations that both arachidonic acid and 12-lipoxygenase metabolites can modulate synaptic transmission. A long-lasting potentiation was induced by arachidonic acid which appears to depend on the formation of lipoxygenase metabolites. This is the first evidence obtained *in vitro* in the CA1 subfield of the hippocampus that arachidonic acid and its lipoxygenase metabolites could act as retrograde messengers in the phenomenon of long-term potentiation (LTP).

4. In some cases arachidonic acid, as well as 12-HPETE elicited a rather long-lasting depression of synaptic transmission in hippocampal slices maintained *in vitro*. The dual effect of arachidonic acid (potentiation and depression) could depend on different lipoxygenase metabolites, a situation analogous to what has been previously demonstrated in *Aplysia*.

5. Melittin, a 26-amino acid peptide obtained from bee venom was shown to induce a long-lasting potentiation in hippocampal slices maintained *in vitro*. This was correlated with the ability of melittin to elicit arachidonic acid release, possibly via a phospholipase A_2-based mechanism.

6. A new type of perfusion bath and incubation chamber system was developed for maintaining brain slices in a physiologically active state while allowing easy pharmacological and biochemical manipulations. It was further demonstrated that it could

be used successfully to study the release of unlabelled, endogenous fatty acids in this preparation.

7. Using the hippocampal slice preparation, it was clearly demonstrated that conditions which are able to elicit a long lasting potentiation induce the release of arachidonic acid. The approach was truly original since it involved the measurement of the mass of fatty acids (endogenous, unlabelled) from brain slices maintained physiologically responsive. Surprisingly release of stearic acid also accompanied the release of arachidonic acid. This provides evidence that phosphatidylinositol might serve as a source pool. The effect also appears critically dependent on the activation of the N-methyl-D-aspartate receptors since it could be prevented by the non-competitive NMDA receptor antagonist MK-801. In addition, it was observed that the effect is already apparent after a short period of time (2 min). These observations strengthen the hypothesis that arachidonic and/or lipoxygenase metabolites participate in events related to synaptic plasticity and particularly long-term potentiation (LTP).

8. An easy and rapid photochemical method to synthesize hydroxy- and hydroperoxyeicosatetraenoic acids was developed. It avoids the problem of previous methods which had to use high intensity lamps and a cooling system by employing an optic fiber lamp.

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Chapter 2

Formation of lipoxygenase metabolites of arachidonic acid in rat cerebral cortex slices

"Experience does not ever err, it is only your judgement that errs in promising itself results which are not caused by your experiments"

Leonardo da Vinci

1510

Chapter 1

GENERAL INTRODUCTION

The study of eicosanoids, a term introduced by Corey et al. (1980) which includes all the oxidized derivatives of the carbon-20 unsaturated fatty acid arachidonic acid, has developed into an extremely vast and complex field in the recent years. It would be unrealistic to pretend to be able in a short introduction to give an accurate and complete summary of all the various aspects of this large research area. For this reason, only a general overview of the relevant aspects will be presented here, and the reader is referred to numerous reviews and references therein for a more exhaustive or detailed account of the different subjects pertaining to the eicosanoids (Wolfe, 1982; Samuelsson, 1983; Borgeat et al., 1985; Sirois, 1985; Needleman et al., 1986; Samuelsson et al., 1987; Spector et al., 1988; Fitzpatrick and Murphy, 1989; Pace-Asciak and Asotra, 1989; Shimizu and Wolfe, 1990; Simmet and Peskar, 1990; Schaad et al., 1991).

BRIEF HISTORICAL ACCOUNT OF THE DISCOVERY OF THE MAJOR EICOSANOIDS

Von Euler and Goldblatt independently discovered a smooth muscle stimulating factor that also exhibited vasodepressor properties obtained from lipid extracts of seminal vesicles and fluid, which was termed 'prostaglandin' by von Euler (von Euler, 1935; Goldblatt, 1935; von Euler, 1936). Almost thirty years later had to pass before the chemical identity of these compounds was determined, and the metabolic connection between arachidonic acid and the prostaglandins was established (Bergström, S., 1949;

Bergström et al., 1960; Bergström et al., 1964; Van Dorp et al., 1964; Bergström et al., 1968). The elucidation of the structure of the first prostaglandins (PGE_2, $PGF_{2\alpha}$) was soon to be followed by the isolation and characterization of a number of other members of the same pathway, which is controlled by the enzyme fatty acid cyclooxygenase. These includes the prostaglandin endoperoxides PGG_2 and PGH_2 (Hamberg and Samuelsson, 1973; Hamberg et al., 1974), precursors of all the other metabolites belonging to this pathway, thromboxane A_2 (TxA_2) and its hydrolysis product thromboxane B_2 (TxB_2) (Hamberg et al., 1975) as well as prostacyclin (PGI_2) and its stable hydrolysis product 6-keto $PGF_{1\alpha}$ (Moncada et al., 1976; Gryglewski et al., 1976; Johnson et al., 1976; Pace-Asciak, 1976). Also worth mentioning was the discovery by Vane et al. (1971) of the mode of action of non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin and indomethacin which inhibit the fatty acid cyclooxygenase.

A new pathway was discovered with the isolation and characterization from platelets of 12-(S)-5,8,14-*Z*,10-*E*-eicosatetraenoic acid (12-HETE) (Hamberg and Samuelsson, 1974; Nugteren, 1975). Its formation from arachidonic acid, which was not inhibited by aspirin-like drugs, depended on a 12-lipoxygenase. Studies on leukocytes extended the number of lipoxygenase-derived metabolites to include the 5-lipoxygenase products, the leukotrienes, which include LTB_4, LTC_4, LTD_4 and LTE_4 (Borgeat and Samuelsson, 1979a,b; Murphy et al., 1979; Morris et al., 1980). Of great interest is the elucidation at the same time of the nature of the slow reacting substances of anaphylaxis (SRS-A), powerful mediators of immediate hypersensitivity reactions which consisted of

a mixture of LTC_4, D_4 and E_4 (Morris and Taylor, 1978; Morris et al., 1980). Subsequently, a host of new metabolites were described, including hepoxilins, derived from the 12-lipoxygenase product 12-hydroperoxyeicosatetraenoic acid (12-HPETE) (Pace-Asciak, 1984), lipoxins, obtained by double oxygenation of arachidonic acid by the 15- and the 5-lipoxygenases (Serhan et al., 1984; reviewed by Samuelsson et al., 1987), and more recently epoxyeicosatrienoic acids, the synthesis of which is catalyzed by cytochrome P-450 (Capdevila et al., 1981; Morrison and Pascoe, 1981; Oliw et al., 1982; reviewed by Fitzpatrick and Murphy, 1989).

In the nervous system, the first arachidonic acid metabolite to be reported was $PGF_{2\alpha}$ (Samuelsson, 1964). It was followed by the other cyclooxygenase products PGE_2, PGD_2, TxA_2 (detected as TxB_2) and PGI_2 (detected as 6-keto-$PGF_{1\alpha}$) (reviewed by Wolfe, 1982). The first lipoxygenase metabolite to be discovered was 12-HETE (Sautebin et al., 1978; Spagnuolo et al., 1979). The leukotrienes LTC_4,D_4 and E_4 were more recently detected in rat brain (Lindgren et al., 1984).

DISTRIBUTION OF THE PRECURSOR FATTY ACID

Arachidonic acid, or all *Z*-5,8,11,14-eicosatetraenoic acid is a fatty acid preferentially found esterified in phospholipids. The brain is particularly rich in arachidonate-containing phospholipids. The unique distribution of arachidonic acid, as well as other fatty acids among the various phospholipid species in the nervous system

has been quite extensively reviewed by Sastry (1985). In synaptosomes, arachidonic acid constitutes the following percentage of total fatty acids found for each type of phospholipid: 6% in phosphatidylcholines (PC), 14.2% in phosphatidylethanolamines (PE), 18.6% in ethanolamine plasmalogens (PEpl), 2.3% in phosphatidylserines (PS) and 31.5% in phosphatidylinositols (PI) (Sun and Su, 1979). A particular predominance of stearoyl-arachidonoyl species in PI has been noted (Baker and Thompson, 1972). However, due to the fact that the total mass of PC and PE is more important the contribution of each phospholipid to the esterified pool of arachidonic acid is as follow: PE 36.5%, PC 31.5%, PEpl 13%, PI 8.2% and PS 4.7% (Sun and Su, 1979). A peculiar observation is that arachidonic acid is found almost exclusively esterified at carbon 2 of the glycerol backbone in all phospholipid species (Baker and Thompson, 1972; Baker, 1979). Finally, the concentration of free versus esterified arachidonic acid was estimated to be smaller by a factor of one thousand in brain (Bazan et al., 1986), an observation that suggests an important regulation of the level of free arachidonic acid in brain as well as in other tissues.

LIBERATION AND REACYLATION OF ARACHIDONIC ACID

Since the concentration of free arachidonic acid is very low in the basal state, its release from phospholipids is generally thought to be the rate-limiting step in the formation of eicosanoids. A wide range of stimuli have been shown to induce the release of arachidonic acid from the various phospholipids in the nervous system including

neurotransmitters, peptides, growth factors, seizures, trauma and ischemia, among others (Wolfe, 1988). The receptor-mediated generation of arachidonic acid is thought to occur through either a G protein or calcium-dependent activation of phospholipases (Axelrod et al., 1988). Two main pathways have been identified, one controlled by a phospholipase A_2 (PLA_2), the other involving a phosphoinositide-specific phospholipase C (PLC) and a diacylglycerol lipase (DAG lipase) (see Figure 1). Both pathways and their key enzymes have been the subject of numerous reviews (Van den Bosch, 1980; Irvine, 1982; Downes, 1983; Nishizuka, 1984; Fisher and Agranoff, 1987; Waite, 1987; Van den Bosch, 1990). It can be added however that while the PLC-DAG lipase pathway uses the phosphoinositides as a source of arachidonic acid, the PLA_2 pathway appears to use predominantly phosphatidylcholine and phosphatidylethanolamine, although a phosphatidylinositol-specific PLA_2 associated with the formation of eicosanoids has also been described (Hong and Deykin, 1981). It was also shown that the release of arachidonic acid originates predominantly from a PLA_2-based mechanism than a PLC-DAG lipase, at least in thrombin-stimulated platelets (Broekman, 1986). Recently other pathways have been discovered such as one controlled by a phosphatidylcholine-specific phospholipase C (Exton, 1990), although the importance of this is not yet clear (Burgoyne and Morgan, 1990). In the nervous system, release of arachidonic acid via both PLA_2 and PLC-DAG lipase mechanisms have been described (Farooqui et al., 1989; Sun and MacQuarrie, 1989) but their importance as well as the relative contribution of their associated phospholipid pool to free arachidonic acid, particularly in physiological situation is still not clearly established.

Figure 1: Arachidonic acid (AA) deacylation-reacylation mechanisms. There are two major routes for receptor-mediated release of esterified arachidonic acid from phospholipids (hatched arrows). The first one is controlled by a phospholipase A_2, which can be activated either by an increase in intracellular Ca^{2+} concentration or particular subunits of a G-protein. The second pathway involves the sequential action of a phospholipase C, which forms diacylglycerol (DAG) from phospholipids, and a DAG lipase which finally release arachidonic acid from DAG. The reacylation pathway (open arrows) involves two steps. First the coenzyme A derivative of arachidonic acid (AACoA) is formed by the acylCoA synthetase or ligase. Then a lysophospholipid acyltransferase (LAT) combines lysophospholipids and arachidonylCoA to form phospholipids.

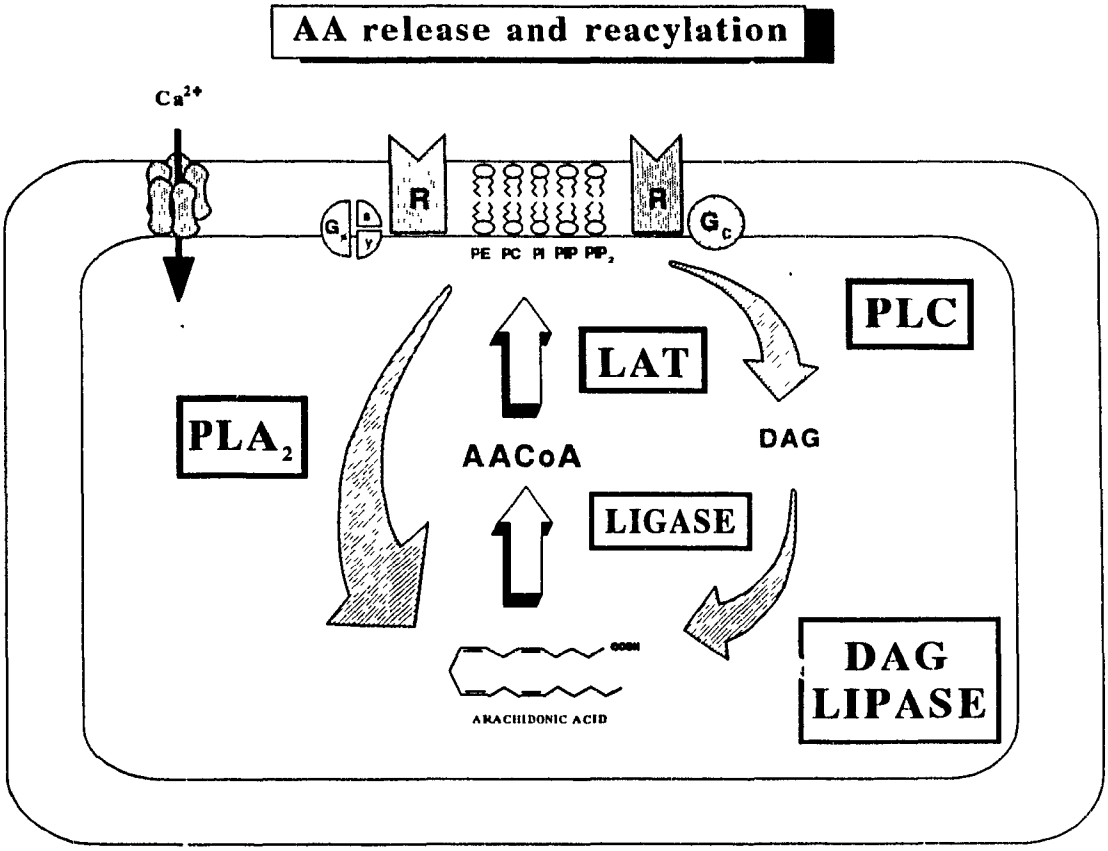
AA release and reacylation
Ca^{2+}
R
R
G_q
G_c
PE PC PI PIP PIP_2
PLC
LAT
PLA_2
DAG
AACoA
LIGASE
DAG
LIPASE
ARACHIDONIC ACID

Figure 1

Although the liberation of arachidonic acid represents a critical step for the synthesis of eicosanoids, reacylation might also represent an important regulatory mechanism. Two enzymes are involved in this process (see figure 1). First an acyl-CoA synthetase forms the coenzyme A ester derivative of arachidonic acid. Then a lysophospholipid acyl transferase (LAT) is responsible for reacylating arachidonic acid back into phospholipids. This pathway, initially described by Lands (1960) constitutes with release mechanisms a deacylation-reacylation cycle known as the Lands cycle. In a particular macrophage-derived cell line, it was demonstrated that reacylation was in fact more important than release in regulating the formation of eicosanoids (Kaever et al., 1988). The importance of the reacylation mechanism in the nervous system has not been clearly established yet, but it is definitely present and particularly active at least in synaptosomes (Sun and MacQuarrie, 1989).

METABOLISM OF ARACHIDONIC ACID INTO THE VARIOUS EICOSANOIDS

Arachidonic acid metabolism in mammals occurs through three major routes. The first one involved the enzyme PGH synthase (also known as fatty acid cyclooxygenase) which catalyzes the formation of the prostaglandin endoperoxides PGG_2 and PGH_2. These will be subsequently metabolized by different enzymes to give rise to various prostaglandins and thromboxanes. The second route depends on cytochrome P-450 which is responsible for the formation of various epoxyeicosatrienoic acids (EETs) as well as hydroxyeicosatetraenoic acids (HETEs). Finally three lipoxygenases, the 5-, 12- and 15-

lipoxygenase form hydroperoxyeicosatetraenoic acids (HPETEs) which become the precursors of a whole new series of metabolites (see Figure 2). The first two routes will not be further described here and the reader is referred to reviews for more extensive description (Wolfe, 1982; Needleman et al., 1986; Fitzpatrick and Murphy, 1989; Shimizu and Wolfe, 1990). Lipoxygenase pathways, particularly those involving the 5- and 12-lipoxygenases will be considered in more detail.

A) **The 5-lipoxygenase pathway**

The 5-lipoxygenase enzyme catalyzes the conversion of arachidonic acid into 5-(S)-hydroperoxy-6-*E*-8,11,14-*Z*-eicosatetraenoic acid (5-HPETE). The enzyme has been purified, its cDNA cloned and sequenced and it shows homologies with the soybean lipoxygenase (Ueda et al., 1986; Balcarek et al., 1988; Dixon et al., 1988; Matsumoto et al., 1988). Both the homogenate activity and the purified enzyme are dependent on Ca^{2+} and ATP as well as other low molecular weight factors including hydroperoxides for expressing full activity (Rouzer and Samuelsson, 1985; Shimizu et al., 1987). It is primarily a soluble enzyme, but upon stimulation it translocates to the membrane to become associated with a five lipoxygenase activating protein or FLAP (Dixon et al., 1990; Miller et al., 1990). Associated with the 5-lipoxygenase is a LTA_4 synthase activity which converts 5-HPETE into 5,6-oxido-7,9-*E*,11,14-*Z*-eicosatetraenoic acid (leukotriene A_4, LTA_4) (Rouzer et al., 1986). Leukotriene A_4 can then be metabolized into 5(S),12(R)-dihydroxy-6,14-*Z*-eicosatetraenoic acid (leukotriene B_4, LTB_4) via a

Figure 2: The arachidonic acid cascade. Arachidonic acid is metabolized via three major routes. The enzyme PGH synthase (cyclooxygenase) is involved in the formation of the endoperoxides PGG_2 and PGH_2, which are subsequently transformed enzymatically to give the various prostaglandins and thromboxanes. Cytochrome P-450 on the other hand catalyzes the formation of various epoxyeicosatrienoic acids (EETs) which can be further metabolized into dihydroxyeicosatrienoic acids (diHETEs) and monohydroxyeicosatetraenoic acids (HETEs). Finally the last route is composed of three major pathways, each one involving a specific lipoxygenase enzyme (LOX). Each lipoxygenase catalyzes the formation of a different hydroperoxyeicosatetraenoic acid (HPETE). The HPETEs are then metabolized differently into a number of products such as lipoxins (Lx), leukotrienes (LT) and hepoxilins (Hx), depending on the particular pathway.

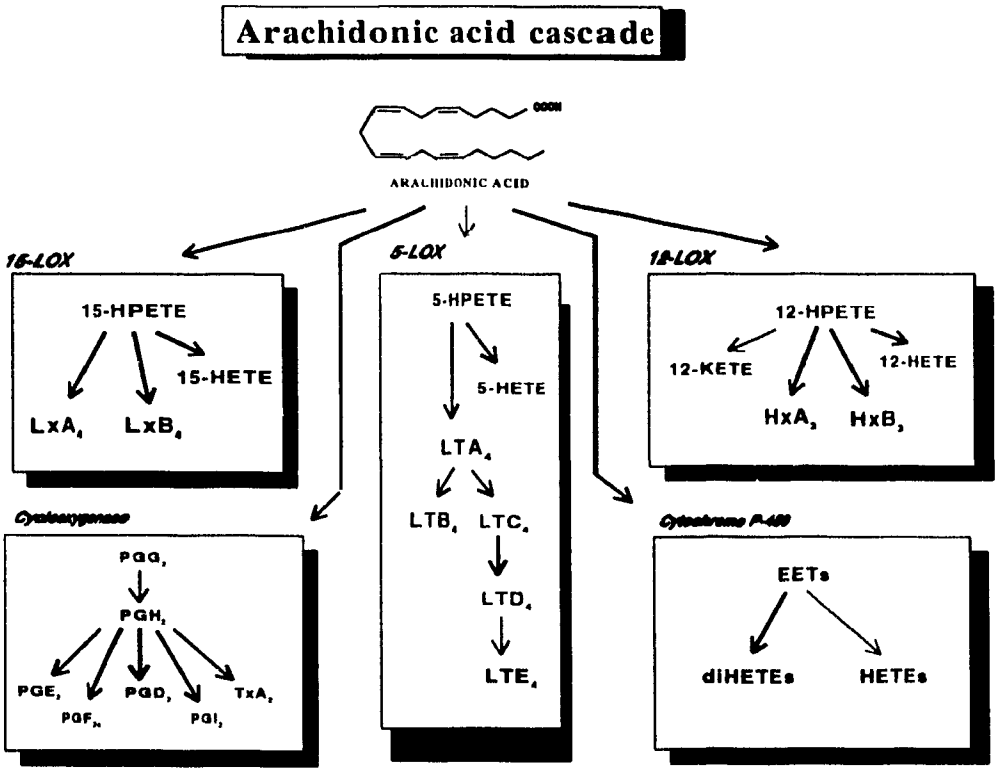
Arachidonic acid cascade
ARACHIDONIC ACID
15-LOX
15-HPETE
15-HETE
LxA4
LxB4
5-LOX
5-HPETE
5-HETE
LTA4
LTB4
LTC4
LTD4
LTE4
12-LOX
12-HPETE
12-KETE
12-HETE
HxA3
HxB3
Cyclooxygenase
PGG2
PGH2
PGE2
PGF2α
PGD2
PGI2
TxA2
Cytochrome P-450
EETs
diHETEs
HETEs

Figure 2

LTA_4 hydrolase (Rådmark et al.,1984; Evans et al., 1985). Leukotriene A_4 may also be conjugated with a glutathione molecule, catalyzed by either a LTC_4 synthase or a specific isozyme of glutathione-S-transferase. The product is 5(S)-hydroxy-6(R)-sulfido-glutathionyl-7,9-*E*,11,14-*Z*-eicosatetraenoic acid (leukotriene C_4, LTC_4) (Yoshimoto et al., 1985; Tsuchida et al., 1987; Izumi et al., 1988) . Leukotriene C_4 can be successively transformed by a γ-glutamyl transpeptidase and an aminopeptidase into leukotriene D_4 (LTD_4) and leukotriene E_4 (LTE_4) respectively, by cleavage of aminoacids from the glutathionyl moiety. Finally 5-HPETE can be reduced by an hydroperoxidase to 5-(S)-hydroxyeicosatetraenoic acid (5-HETE). The entire pathway is illustrated in Figure 3.

Interaction between the 5-lipoxygenase and 15-lipoxygenase products also leads to a whole new series of metabolites. Conversion of 15-(S)-hydroperoxyeicosatetraenoic acid (15-HPETE) by the 5-lipoxygenase to 5,15-dihydroperoxyeicosatetraenoic acid is subsequently followed by a series of transformations giving rise to trihydroxyl, conjugated tetraene derivatives collectively known as lipoxins. Whether these arise enzymatically or not is not clear however. Lipoxins formation has been reviewed more extensively elsewhere (Samuelsson et al., 1987; Rokach and Fitzsimmons, 1988).

B) The 12-lipoxygenase pathway

The 12-lipoxygenase is responsible for the conversion of arachidonic acid into

Figure 3: The arachidonate 5-lipoxygenase pathway. The 5-lipoxygenase transforms arachidonic acid into 5-hydroperoxyeicosatetraenoic acid (5-HPETE). This compound can be either reduced by an hydroperoxidase to form 5-hydroxyeicosatetraenoic acid (5-HETE) or it can be further metabolized by the 5-lipoxygenase into leukotriene A_4 (LTA_4). Leukotriene A_4 can be subsequently metabolized via two routes. In the first case LTA_4 hydrolase will catalyze the formation of LTB_4. The second one involves a glutathione-S-transferase which adds a glutathionyl moiety onto the leukotriene A_4 molecule, forming the leukotriene C_4 (LTC_4). Finally, the successive cleavage of the glutamate and glycine residue of the LTC_4 glutathionyl moiety, by the action of a glutamyl transpeptidase and an amino peptidase will form the leukotriene D_4 (LTD_4) and E_4 (LTE_4), respectively. Modified from Samuelsson (1983).

Arachidonate 5-lipoxygenase pathway

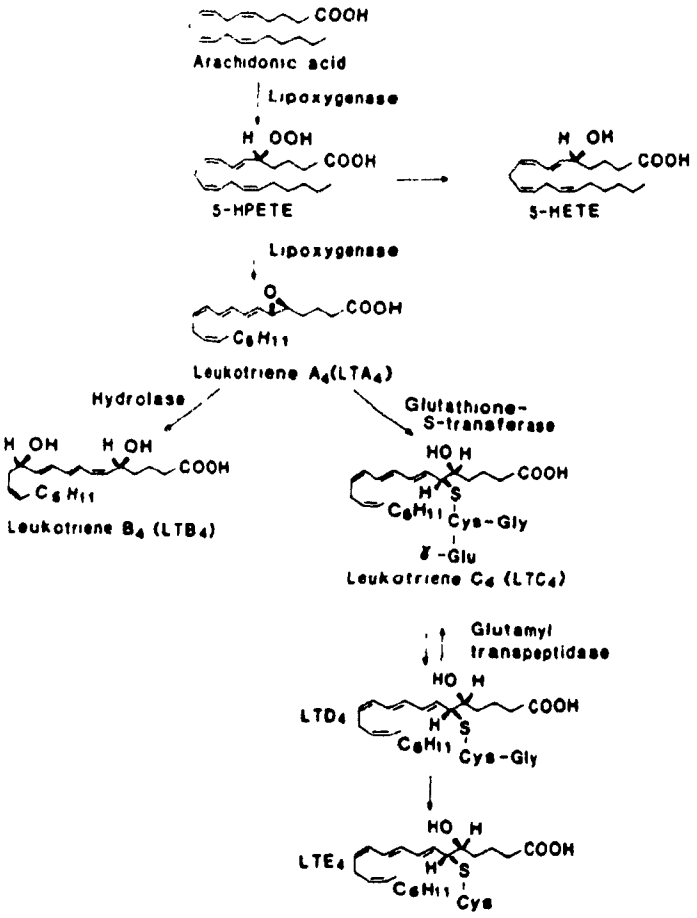

Figure 3

12-(S)-hydroperoxy-5,8,14-*Z*,10-*E*-eicosatetraenoic acid (12-HPETE). The enzyme appears in both cytosolic and membrane fractions of platelets (Nugteren, 1982). The purified enzyme exhibits a kinetic lag period and is activated by hydroperoxides (Hwang, 1982). It does not appear to have cofactor requirements or need addition of Ca^{2+} to be fully active although a rat basophilic leukemia cell 12-lipoxygenase was stimulated by elevation of Ca^{2+} levels (Hamasaki and Tai, 1984). The enzyme was shown to translocate however from cytosol to membrane upon exposure to high concentration of Ca^{2+} (Baba et al., 1989). An other form of 12-lipoxygenase was purified from porcine leukocytes and shown to be distinctly different from the platelet enzyme (Yokoyama et al., 1986). Recently the cDNA for both forms of 12-lipoxygenase was cloned and sequenced (Yoshimoto et al., 1990a,b; Funk et al., 1990). It showed homologies with both the 5- and the 15-lipoxygenases but appeared more closely related to the 15-lipoxygenase.

The first 12-lipoxygenase product, 12-HPETE, can be converted via a number of different routes. A hematin-catalyzed intramolecular rearrangement leads to the formation of epoxy-alcohol compounds, the hepoxilin A_3 and B_3, as well as their trihydroxyl derivatives trioxilin A_3 and B_3, whose formation is catalyzed by an epoxide hydrolase (Pace-Asciak, 1984; Pace-Asciak and Lee, 1989). Alternatively, 12-HPETE can be converted either to 12-oxo-5,8,14-*Z*-10-*E*-eicosatetraenoic acid (12-KETE), or to 11,12-LTA_4 and 11,12-diHETE, or finally to the short-chain aldehyde 12-ketododecatrienoic acid (Piomelli et al., 1988; Kitamura et al., 1988; Miki et al., 1989; Glasgow and Brash, 1987). 12-HPETE can also be reduced by a glutathione peroxidase to 12-(S)-hydroxy-

Figure 4: The arachidonate 12-lipoxygenase pathway. The first step in this pathway involves the conversion of arachidonic acid by the 12-lipoxygenase into 12-hydroperoxyeicosatetraenoic acid (12-HPETE). Then several possibilities can occur. It can be reduced by a glutathione peroxidase to form 12-hydroxyeicosatetraenoic acid (12-HETE). It can also be nonenzymatically transformed into the epoxy-alcohols hepoxilin A_3 and hepoxilin B_3, or the epoxide 11,12-leukotriene A_4 (11,12-LTA_4). An epoxide hydrolase can then catalyze the conversion of hepoxilin A_3 and B_3 into the trihydroxy derivatives trioxilin A_3 and B_3, or converts 11,12-LTA_4 into 11,12-dihydroxyeicosatetraenoic acid (11,12-diHETE). Finally 12-HPETE can give rise to 12-ketoeicosatetraenoic acid (12-KETE) or the short-chain aldehyde 12-ketododecatrienoic acid. Solid lines indicate an enzymatic process, whereas broken lines indicate non-enzymatic process or unknown enzyme or process. Taken from Shimizu and Wolfe (1990).

Arachidonate 12-lipoxygenase pathway

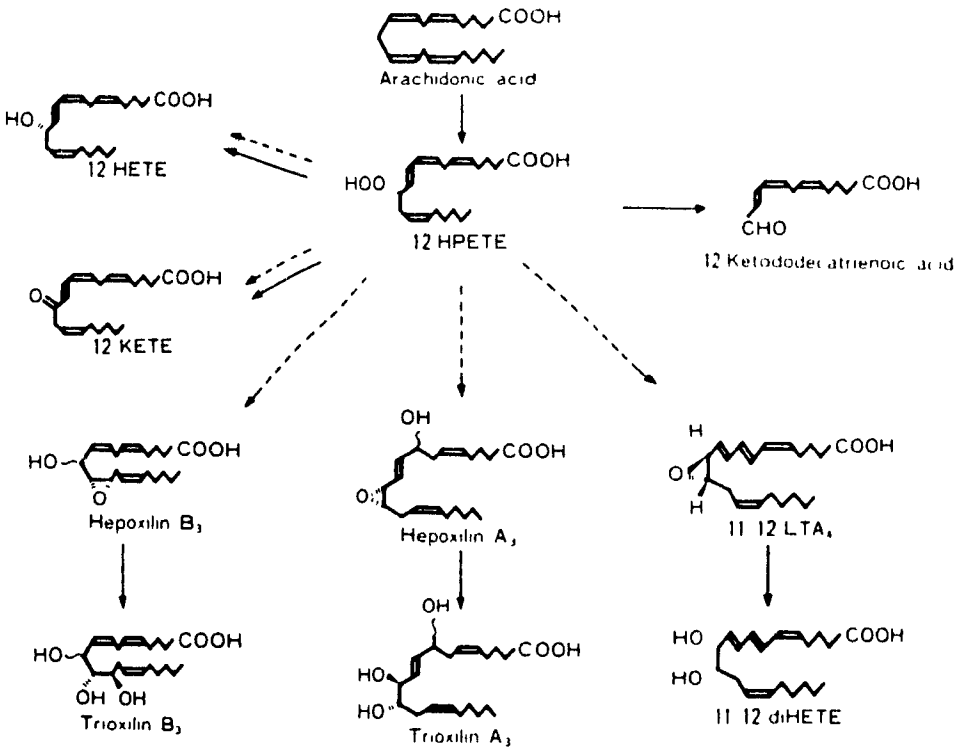

Figure 4

eicosatetraenoic acid (12-HETE) (Bryant et al., 1982; Chang et al., 1982; Bryant et al., 1983). The multiple routes characterizing the 12-lipoxygenase pathway are illustrated in Figure 4.

SCOPE OF THE THESIS

Our knowledge about arachidonic acid and its lipoxygenase metabolites in the mammalian central nervous system has been rather limited. Information has usually been obtained through preparations exhibiting pathological features (ischemia, seizures) or very reduced preparations (homogenates, synaptosomes). In these circumstances it is rather difficult to infer possible physiological roles that might be played by arachidonic acid and its lipoxygenase metabolites. The aim of the work presented in this thesis was to gather clues which might help clarifying the role of these compounds in the nervous system. In chapter 2, an evaluation of the capacity of rat cortical slices to synthesize lipoxygenase metabolites of arachidonic under various conditions, including stimulation by various neurotransmitters was performed. In chapter 3 the possible involvement of arachidonic acid and its lipoxygenase metabolites in the modulation of synaptic transmission was investigated in hippocampal slices maintained *in vitro*. Finally chapter 4 presents a study of arachidonic acid release performed on hippocampal slices maintained physiologically responsive *in vitro* and exposed to pharmacological conditions involving activation of a subset of glutamate receptors which can induce an enhancement of synaptic transmission.

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Chapter 2

FORMATION OF LIPOXYGENASE METABOLITES OF ARACHIDONIC ACID IN RAT CEREBRAL CORTEX SLICES

INTRODUCTION

Arachidonic acid metabolites or eicosanoids are known to be formed and released in most mammalian tissues, where they are thought to act as "local hormones" and for this reason were called autacoids (see Shimizu and Wolfe, 1990 for a recent review). However, recent work in the nervous system of the marine mollusc *Aplysia californica* has revealed that some lipoxygenase metabolites of arachidonic acid act as second messengers, and it was suggested that they could also participate in cell-cell communication (Piomelli et al., 1987a; Piomelli et al., 1987b). In the sensory neurons of this organism, serotonin and the molluscan tetrapeptide FMRFamide (Phe-Met-Arg-Phe amide) close and open respectively a specialized potassium channel known as the S-K^+ channel (Belardetti and Siegelbaum, 1988). It has been shown that the effect of FMRFamide on the S-K^+ channel can be mimicked by the 12-lipoxygenase metabolite 12-hydroperoxy-eicosatetraenoic acid (12-HPETE) which is formed in these neurons (Buttner et al., 1989). 12-HPETE was reported in fact to exert a dual action in sensory neurons, causing first a fast depolarization followed by a slow hyperpolarization (Piomelli et al., 1989a). The slow hyperpolarization, caused by the opening of the S-K^+ channel, appear to be mediated by a metabolite of 12-HPETE, most likely 8-hydroxy-11,12-epoxy-5,9,14-eicosatrienoic acid (hepoxilin A_3) (Piomelli et al., 1989a; Belardetti et al., 1989) although 12-HPETE itself might play a role (Buttner et al., 1989). It has been suggested that the fast depolarization is mediated by another 12-HPETE metabolite formed in *Aplysia* neurons, namely 12-keto-5,8,10,14-eicosatetraenoic acid or 12-KETE (Piomelli et al., 1988).

Much less is known about lipoxygenase metabolites in the central nervous system of vertebrates. The presence of various 5- and 12- lipoxygenase metabolites has been reported in the brain of various species including man (Sautebin et al., 1978; Lindgren et al., 1984; Adesuyi et al., 1985; Hambrecht et al., 1987; Miyamoto et al., 1987a; Shimizu et al., 1988; Simmet et al., 1988a; Pace-Asciak, 1988; reviewed recently by Simmet and Peskar, 1990 and Schaad et al., 1991). The formation of 12-hydroxyeicosatetraenoic acid (12-HETE) appears to be particularly important in a number of structures associated with the nervous system including the rat pineal gland (Yoshimoto et al., 1985), the rat anterior pituitary (Pilote et al., 1982), the rat hypothalamus (Gerozissis et al., 1984), the rat retina (Birkle and Bazan, 1984) and the bovine cornea (Hurst et al., 1991). In addition, 12-HETE was reported to be formed by primary cultures of mouse striatal neurons (Dumuis et al., 1988) and by rat brain subcellular fractions, together with other lipoxygenase products (Birkle and Bazan, 1987). These observations suggest the presence of an efficient 12-lipoxygenase in the central nervous system of vertebrates.

None of these studies however has directly addressed the question of a possible physiological role for lipoxygenase metabolites in the central nervous system. Moreover, an important number of studies have used ischemic or injured brain preparations (Moskowitz et al., 1984; Dempsey et al., 1986; Simmet et al., 1987) or broken cell preparations (Birkle and Bazan, 1987). In the planning of experiments presented in this chapter a number of goals were set. (1) Identification of lipoxygenase metabolites formed

in metabolically active rat brain cerebral cortex slices; (2) demonstration of induced formation of specific lipoxygenase metabolites by relevant stimuli (calcium ionophore, neurotransmitters and agonists); (3) specific chemical and stereochemical characterization of the lipoxygenase metabolites formed under these conditions and (4) identification of an intact neuronal cell preparation suitable for the study of receptor-mediated formation of lipoxygenase metabolites in the central nervous system.

MATERIAL AND METHODS

A) **Preparation and incubation of rat cerebral cortex slices**

Male Sprague-Dawley rats weighing 250-300 g (Charles River, St-Constant, Québec) were decapitated with a guillotine without anesthesia. The brain was rapidly taken out of the skull and washed with chilled Dulbecco's phosphate buffer saline (DPBS) without Ca^{2+} and Mg^{2+} and supplemented with glucose. The exact composition of the DPBS buffer was as follows in mM: KCl 2.68, KH_2PO_4 1.47, NaCl 136.9, Na_2HPO_4 15.2, glucose 5.6. The cortex was excised with scissors and was placed on a petri dish containing a small amount ($\approx$ 1ml) of chilled modified DPBS. Cortical slabs were sliced by hand with a scalpel and slices were transferred to a scintillation vial with a broken pasteur pipette. Fresh chilled modified DPBS was added to a final volume of 3 ml. $CaCl_2$ was added to a final concentration of 2.0 mM and the slices (cortex from 2 hemispheres/vial) were preincubated for 5 minutes at 37°C. In the case of the calcium

ionophore A23187, it was added first and $CaCl_2$ was added after the preincubation period (Murphy and Mathews, 1982).

The various agonists or stimulatory compounds were added either in solution in modified DPBS, in ethanol or dimethylsulfoxide (DMSO). The final concentration of the solvents was 0.1%. Incubations proceeded at 37°C for the various periods of time tested and were stopped by addition of 1 volume of methanol at -80°C. Vials were kept at -80°C until extraction was started. All reagents were from Sigma, St-Louis, MO, with the exception of A23187 that was from Calbiochem, CA, and arachidonic acid from Nu-chek-prep, Elysian, MN. The stock solution of phorbol myristate acetate (PMA) was kept frozen in DMSO. Aliquots were thawed and used only once. Arachidonic acid was kept as a stock solution in isooctane at a concentration of 1 mg/ml. The solution was stored at -20°C protected from light and blanketed with nitrogen after each opening to prevent oxidation. Common chemicals were from Fisher scientific, St-Laurent, Quebec.

B) Extraction of lipoxygenase metabolites

The content of incubation vials were thawed and transferred to centrifuge tubes. Radiolabelled leukotriene B_4 ([^{3}H]-LTB_4 $\approx$ 12000 dpm, from Amersham, Oakville, Ontario) was added to each tube and the tubes were then centrifuged at 1000 g for 20 min. Supernatants were collected and the pellets were set aside for protein assay (see other procedures below). The volume of supernatants was adjusted with HPLC grade H_2O

so that the final methanol content was 10%. This is crucial for optimal recovery of all lipoxygenase metabolites (Powell, 1987). An octadecylsilylsilica cartridge (Sep-pak C18, Waters, Mississauga, Ontario) was first prewashed with the following sequence: 10 ml methanol, 2 ml H_2O, 2 ml 0.1% EDTA solution, 2 ml H_2O (modified from Verhagen et al., 1986). The pH of the supernatants was adjusted to 8.0 with NaOH and they were loaded on the cartridge at a flow rate of 2-4 ml/min. After rinsing with 2 ml H_2O, lipoxygenase metabolites were eluted with 5 ml of methanol. Methanol (from Anachemia, Lachine, Quebec) was HPLC grade. Water was purified by a Millipore Q filtration system (Millipore, Mississauga, Ontario) or obtained from Anachemia (HPLC grade) and was passed through an octadecylsilylsilica cartridge prior to use.

C) Separation and quantitation of lipoxygenase metabolites by RP-HPLC

The HPLC system originally consisted of a model M6000A pump, a U6K injector and a model 450 variable wavelength UV detector, all from Waters. The detector was connected to a Berthold LB510 chromatography data system that was used for integration. A chart recorder and a dot matrix printer were also attached to the system. Quantitation was based on 15-HETE used as external standard. During the course of this work, the system was upgraded. An eluent stabilization system (Waters) using helium as the inert gas has been added to help stabilize the baseline and improve sensitivity. The pump was replaced by a model 510 pump while the more sensitive lambda max 481 variable wavelength UV detector was used. It was connected to a 760 series intelligent interface

from Nelson Analytical Inc. (Cupertino, CA) as part of the 3000 series chromatography data system. Integration was performed using the Nelson Analytical 2600 software (v. 4.1) installed on an IBM PC computer. A standard line for each compound monitored was constructed inside the program using pure standards and was later used for quantitation. A chart recorder and an inkjet printer were also used.

The methanol fraction obtained after extraction of each sample was evaporated to dryness under a stream of nitrogen and the products were rapidly redissolved in 25 μl of methanol:H_2O (65:35 by vol.). Two different columns and solvent systems were used to separate the various lipoxygenase metabolites. Separation of the leukotrienes (LTB_4,C_4,D_4 and E_4) was achieved by injecting samples on a 4.6 mm x 25 cm, 5 μm particles Nucleosil C18 column from Alltech (Deerfield, IL). A 4.6 mm x 2.3 cm guard column packed with Nucleosil C18, 15-25 μm particles (Macherey-Nagel, Düren, Germany) was used to protect the column. The solvent system was methanol:H_2O:acetic acid (69:31:0.06 by vol.) to which oxalic acid was added to 0.5 mM concentration and then adjusted to pH 6.0 with ammonium hydroxide (Muller and Sorrell, 1985). Flow rate was 1.0 ml/min and elution of leukotrienes was monitored at wavelength 280 nm (System I). Separation of leukotriene B_4 and its isomers 12S,6-*trans* and 12R,6-*trans* LTB_4 as well as the different hydroxyeicosatetraenoic acids (HETEs) was accomplished by injecting samples on a 4.6 mm x 25 cm, Spherisorb ODS II column with 5 μm particles from Jones Chromatography (Llanbradach, Mid Glamorgan, UK). A 3.2 mm x 1.5 cm RP-18 MPLC New Guard cartridge from Pierce (Rockford, IL) was used as guard column. The solvent

Figure 1: Separation of various lipoxygenase metabolites by reversed-phase HPLC. Chromatogram showing the elution profile of authentic standards after injection on a Spherisorb ODS II 5 μM particles 4.6 x 250 mm column. Solvent system was methanol:H_2O:acetic acid 75:25:0.01 by volume and flow rate was 1 ml/min (System II). Elution of 12R-6trans-LTB_4 (6 ng) and LTB_4 (50 ng) was monitored at 280 nm while 15,11,12 and 5-HETE (50 ng each) were monitored at 235 nm. Recorder was set at 0.01 AUFS.

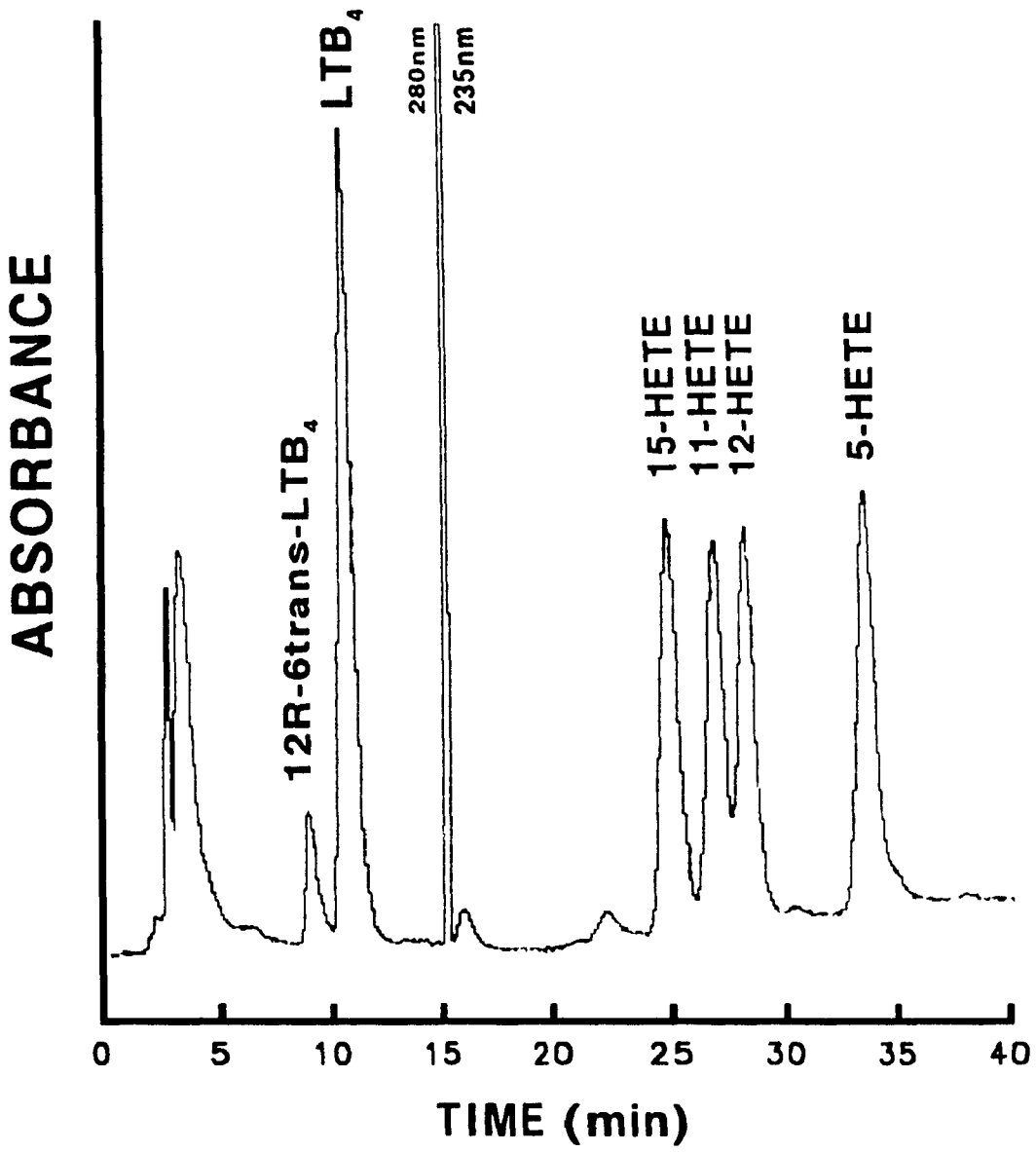

ABSORBANCE
12R-6trans-LTB4
LTB4
280nm
235nm
15-HETE
11-HETE
12-HETE
5-HETE
0
5
10
15
20
25
30
35
40
TIME (min)

Figure 1

system was methanol:H_2O:acetic acid (75:25:0.01 by vol.) and the flow rate 1.0 ml/min (Borgeat and Samuelsson, 1979). Elution of LTB_4 and its isomers was monitored at 280 nm between 0-15 minutes while elution of HETEs was followed at 235 nm between 15-40 minutes (System II). An example of the separation achieved is shown on Figure 1.

Recovery was estimated by the following procedure. After collecting the peak corresponding to LTB_4 for each sample into separate scintillation vials and adding 15 ml of liquid scintillation cocktail (Scintiverse LC, Fisher Scientific, Montreal, Quebec), they were all counted with a 1219 RackBeta liquid scintillation counter (LKB, Bromma, Sweden). The recovery was calculated then with the following equation:

$$Recovery(\%) = \frac{[LTB_{4 samplecounts} - Bkgd]}{[LTB_{4 stockcounts} - Bkgd]} \times 100$$

The amount of each lipoxygenase metabolite (LM) was calculated using the following equation:

$$Amount_{(LM)} = \frac{HPLC\ Amount_{(LM)} \times 100}{Recovery \times Tissue\ protein\ or\ weight}$$

All calculations were performed by an application program developed with Lotus

1-2-3 software v. 2.01 (Lotus Development Corp., Cambridge, MA). Standards were either purchased from Seragen, Boston, MA (monoHETEs) or Cayman Chemical, Ann Arbor, MI (mono and diHETEs) or were a generous gift from Dr J. Rokach, Merck Frosst, Pointe-Claire, Quebec (leukotrienes and isomers).

D) Derivatization procedures and GC-MS analysis of HETEs

Methyl esters of HETEs were prepared with diazomethane. To a 50 ml pear-shaped flask containing 3.5 ml of 2-(2-ethoxyethoxy)-ethanol and 1 ml of 60% aqueous potassium hydroxide, a solution of 20 ml diethylether and 2.15 g of N-methyl-N-nitroso-p-toluenesulfonamide (Sigma, St-Louis, MO) was gradually added. The flask was fitted with a condenser for distillation. An adapter at the end of the condenser dipped into a graduated cylinder cooled by ice-water. The reaction flask was warmed on a water bath at 70°C and the ethereal diazomethane solution collected in a 15 ml cylinder (adapted from Arndt, 1943). After the reaction had been completed, the glass-capped cylinder was covered with foil and kept in a refrigerator. The diazomethane solution lasted one week. For methylation, samples were evaporated to dryness and approximatively 20 μl of methanol was added followed by 0.5 ml of ethereal diazomethane solution. They were left at room temperature in total darkness for 20 minutes and then solvent was evaporated with a stream of nitrogen followed by resuspension in appropriate solvent. Trimethylsilyl ethers were prepared by adding 10 ul of BSA and 10 μl of Trisil/BSA (Pierce, Rockford, IL) to the dry methyl esters and heating at 60°C for 5 minutes.

Hydrogenation of HETEs were carried out with platinum oxide as a catalyst. Approximately 1 mg of PtO_2 (EM Science, Cherry Hill, New Jersey) was placed in 1 ml ethyl acetate. The solution was bubbled with hydrogen gas until the catalyst formed clumps ($\approx$ 1 min). Then the supernatant was removed and the catalyst resuspended in 1 ml ethyl acetate. 0.1 ml of the suspension was taken up and added to the dry sample which was then bubbled with hydrogen gas for 1 min. The sample was finally filtered through glass wool to remove the catalyst.

Gas chromatography-mass spectrometry of trimethylsilyl ether methyl ester derivatives of HETEs was carried out on a HP5890 gas chromatograph (Hewlett-Packard, Kirkland, Quebec) connected to a VG-ZAB-HS mass spectrometer operated in the electron impact mode at 70 eV. The source temperature was 240°C. The GC inlet was connected to a J & W 30 meter DB1 capillary column (Chromatographic Specialties, Brockville, Ontario). The temperature program started at 170°C and increased at a rate of 10°C/minute until 285°C.

E) Photochemical synthesis of HETEs

This procedure was adapted from Camp et al. (1983). Twenty mg of arachidonic acid (Nu-Chek) was dissolved in 2 ml of methanol containing 0.1% methylene blue. Photooxidation was carried out by exposing the solution to the light provided by an optic fiber lamp (Fiber-lite high intensity illuminator series 180, Dolan-Jensen Industries Inc.,

USA) for 1 hr while oxygen was gently bubbled through it. The solution was evaporated to near dryness with a stream of nitrogen and 2 ml of H_2O added. After acidification to pH 3.0 with 1 N HCl, the hydroperoxyeicosatetraenoic acids (HPETEs) were extracted twice with 4 ml ethyl acetate. The ethyl acetate fractions were evaporated to near dryness and 1 ml of methanol added. The HPETEs were reduced to HETEs by gradual addition of 10 mg of sodium borohydride powder. The solution was maintained at 4°C and after 60 minutes 2 ml of H_2O were added, the solution was acidified to pH 3.0 and HETEs extracted twice with 4 ml ethyl acetate. The HETEs in the ethyl acetate fractions, after evaporation and addition of methanol were separated on the same column and solvent system used before (Spherisorb C18, methanol:H_2O:acetic acid 75:25:0.01). Each HETE was collected individually and stored at -80°C.

F) Stereochemical of analysis of 12-HETE by chiral phase column HPLC

Stereochemical analysis of 12-HETE as the methyl ester was carried out according to the method of Hawkins et al. (1988). Briefly, samples were injected on a 4.6 mm x 25 cm (R)-N-(3,5-dinitrobenzoyl)phenylglycine (DNBPG) [ionic] chiral column (J.T.Baker, Phillipsburg, NJ) with 5 μm particles. The solvent system was hexane:isopropanol (100:0.5 by vol.) at a flow rate of 1.2 ml/minute. Elution of the stereoisomers was monitored at 235 nm.

G) Neuronal cell preparations

Two neuronal cell lines, NG-108-15 and NCB-20 (gift from M. Nirenberg) were grown in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum (Gibco Canada Inc., Burlington, Ontario), penicillin (100 U/ml), streptomycin (100 μg/ml) and l-glutamine (0.3 g/l). Cells were plated on Nunc 100 mm tissue culture plates (Gibco Canada Inc.) and incubated at 37°C in a 95/5% mixture of air and CO_2. To induce differentiation, cells were cultured for three days in presence of either dibutyryl cAMP (1 mM) or phorbol myristate acetate (100 ng/ml).

Primary cultures from mouse brain were prepared as described by Keller et al. (1985). The cephalic region of 13 days mouse embryos was minced finely and incubated at 37°C for 30 minutes in DISGH solution [5% Colorado serum(NaCl 8 g/liter, KCl 0.4 g/liter, $Na_2HPO_4 \cdot 7H_2O$ 0.45 g/liter, KH_2PO_4 0.03 g/liter), 0.1% glucose, 2% sucrose and 9.86 mM HEPES, pH 7.4] containing 0.25% trypsin. The trypsin was neutralized by adding minimum essential medium (MEM) with 10% horse serum and 10% fetal calf serum (Gibco Canada Inc.). The tissue was dissociated mechanically by trituration. Cells were plated at a density of 2 x 10^6 per dish in 100 mm dishes coated with collagen (Calbiochem, San Diego, CA, USA). For the first three days, cells were cultured in MEM containing 10% fetal calf serum. Then the medium was changed to N3 medium (Romijn et al., 1982) plus 5% horse serum. At day five, 20 μM cytosine-ß-D-arabinofuranoside (Calbiochem) was added for three days to prevent overgrowth of non-neuronal cells (Durham, 1988).

Prior to stimulation of the cultures, the culture medium was removed and cells were washed twice with DPBS containing 0.1% glucose without Ca^{2+} and Mg^{2+} at pH 7.4 at 37°C. Cells were then allowed to equilibrate in 2 ml of DPBS in an incubator at 37°C in an atmosphere of 95/5% air/CO_2 with agitation on a rotative plate. Substances to be tested were added either in DPBS (e.g. Melittin, Ca^{2+}), in ethanol (arachidonic acid, A23187) or dimethylsulfoxide (PMA) with a final concentration of less than 0.25% for the solvents. Cells were incubated for 20 minutes under the same conditions as for the equilibration period and the reaction was stopped by addition of 2 ml of methanol at -80°C. It is important to note that when A23187 was tested, it was added before the equilibration period and the stimulation begins with the addition of Ca^{2+}. This was suggested to produce a more robust stimulation (Murphy and Mathews, 1982). Finally, the plates were scraped with a rubber policemen and suspensions were transferred to centrifuge tubes. Tubes were placed at -80°C until extraction started, as described above. The same HPLC system was also used for separation and quantitation of lipoxygenase metabolites.

H) Other procedures

The pellets obtained after extraction and centrifugation of cortical slices were resuspended in 1N NaOH. They were then analyzed for their protein content by the method of Lowry et al. (1951). Calculations for the protein assays were performed using a program developed with the Lotus 1-2-3 software (Pellerin, 1990). For statistical

analysis, analysis of variance (ANOVA) followed by a Bonferroni's *t* test was used where appropriate (Wallenstein et al., 1980) and was performed with InStat statistical software package.

RESULTS

A) Formation of lipoxygenase metabolites in presence of exogenous arachidonic acid

The capacity of rat cerebral cortex slices to synthesize lipoxygenase metabolites was evaluated by incubating them with arachidonic acid (75 μM). In these conditions the slices were found to produce five compounds that could be separated by reverse phase HPLC (Figure 2A). Four of the five peaks co-eluted with authentic standards and were identified as 15-hydroxyeicosatetraenoic acid (15-HETE), 11-hydroxyeicosatetraenoic acid (11-HETE), 12-hydroxyeicosatetraenoic acid (12-HETE) and 5-hydroxyeicosatetraenoic acid (5-HETE). The remaining peak was tentatively identified as 9-hydroxyeicosatetraenoic acid (9-HETE) on the basis of its relative retention time. None of the compounds was formed in incubations without tissue or when tissue was denatured with methanol prior to incubation. Similar findings have been reported by Shimizu et al. (1987), and more recently by Kim et al. (1991) using rat brain homogenates. LTB_4 and its isomers 12R,6-*trans* and 12S,6-*trans* LTB_4, as well as LTC_4, LTD_4 and LTE_4 were not detected under the incubation conditions used here.

Figure 2: Lipoxygenase metabolites formed by rat cerebral cortex slices incubated with exogenous arachidonic acid (75 μM). **A:** HPLC chromatogram of lipoxygenase metabolites formed after 10 min incubation and separated by reversed-phase HPLC according to System II (see Material and Methods section). Retention times for authentic standards are indicated. An asterisk indicates peak tentatively identified as 9-HETE. **B:** Time course of synthesis of 12-HETE (□), 15-HETE (●), 5-HETE (◇), 11-HETE (▾) and 9-HETE (△) by rat cerebral cortex slices incubated with arachidonic acid (75 μM).

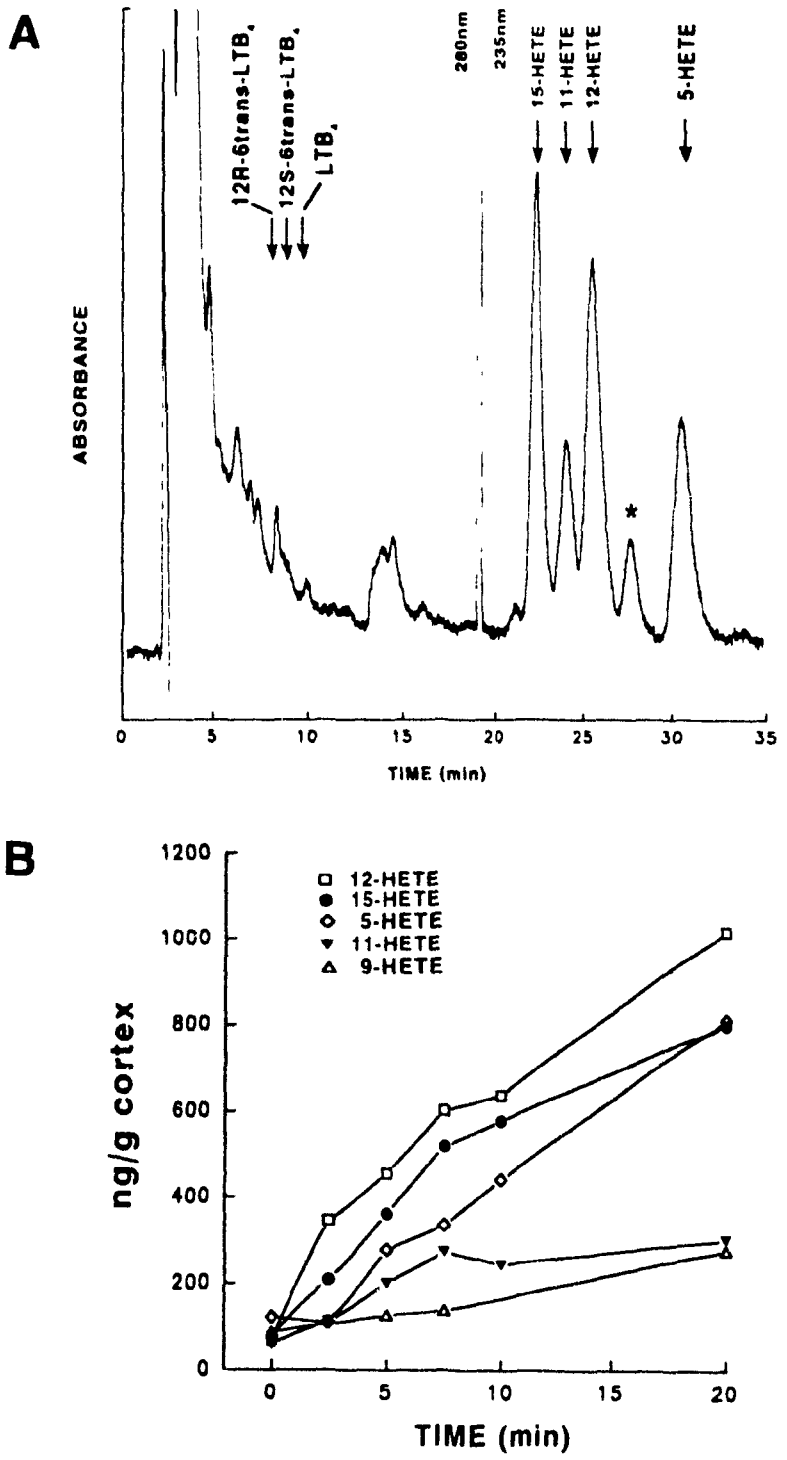

A
ABSORBANCE
12R-6trans-LTB4
12S-6trans-LTB4
LTB4
280nm
235nm
15-HETE
11-HETE
12-HETE
5-HETE
*
0
5
10
15
20
25
30
35
TIME (min)
B
12-HETE
15-HETE
5-HETE
11-HETE
9-HETE
ng/g cortex
1200
1000
800
600
400
200
0
0
5
10
15
20
TIME (min)

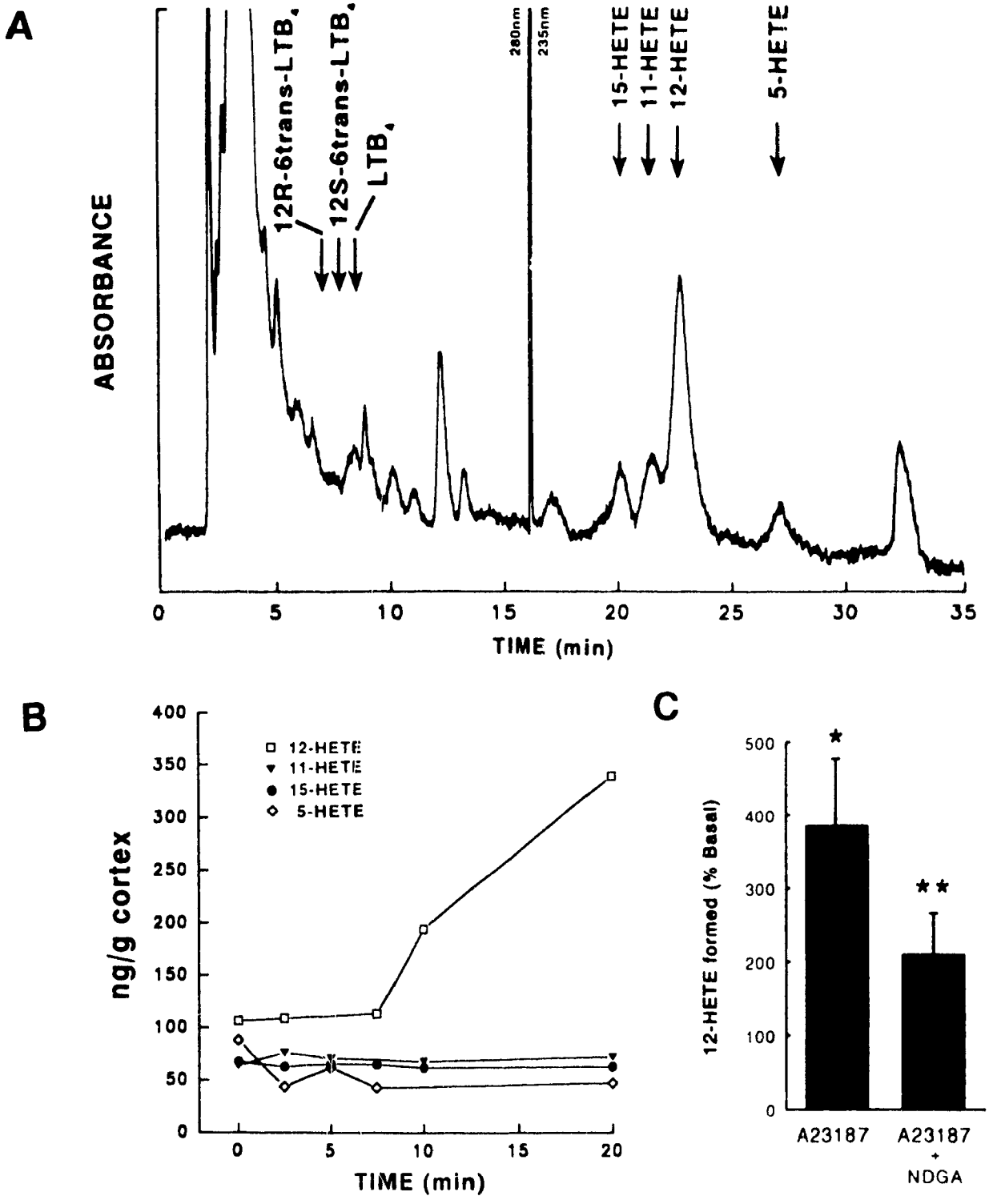
Figure 2

The formation of all HETEs appeared to increase more or less linearly with time of incubation although at different rates, with 12-HETE being quantitatively the major product (Figure 2B). 11-HETE is generally considered to be a side product of the cyclooxygenase reaction since its synthesis can be inhibited by indomethacin (Miyamoto et al., 1987a). Its rate of formation here differs significantly from the three known lipoxygenase products, 15,12 and 5-HETE. A 9-lipoxygenase has not been identified so far in mammalian systems that can explain the presence of 9-HETE, although it has been suggested by Miyamoto et al. (1987a) who also observed the formation of 9-HETE in brain tissue. The rate of formation of 9-HETE is also low compared to the other HETEs.

B) Effect of A23187 on the formation of lipoxygenase metabolites

The calcium ionophore A23187 has been used in the past to stimulate lipoxygenase metabolite formation in various preparations (Borgeat and Samuelsson, 1979; Saad et al., 1983; Fructeau de Laclos et al., 1984; Tripp et al., 1985; Hartung and Toyka, 1987; see Spector et al., 1988 for a more exhaustive list) . Following application of the ionophore on rat cerebral cortex slices the major lipoxygenase metabolite found was 12-HETE (Figure 3A). Small amounts of 15, 11 and 5-HETE were also seen but not 9-HETE. These findings confirm observations originally made in this laboratory (Wolfe et al., 1985) that have been further confirmed subsequently by others (Miyamoto et al., 1987a; Miyamoto et al., 1987b). Surprisingly however, no leukotrienes could be detected under

Figure 3: Lipoxygenase metabolites formed by rat cerebral cortex slices stimulated with the calcium ionophore A23187 (10 μM). **A:** HPLC chromatogram of lipoxygenase metabolites formed after 20 min incubation and separated by reversed-phase HPLC according to System II (see Material and Methods section). Retention times for authentic standards are indicated. **B:** Time course of synthesis of 12-HETE (□), 11-HETE (▾), 15-HETE (●) and 5-HETE (◇) by rat cerebral cortex slices stimulated with A23187 (10 μM). **C:** Calcium ionophore (10 μM) induced a significant increase of 12-HETE over basal level after 20 min incubation (* $p<0.001$ vs basal, $n=4$). Nordihydroguaiaretic acid (10 μM) partially inhibited the effect of A23187 (** $p<0.05$ vs A23187, $n=3$). Basal level was set at 100%.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12R-6trans-LTB4
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15-HETE
11-HETE
12-HETE
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TIME (min)
B
ng/g cortex
12-HETE
11-HETE
15-HETE
5-HETE
400
350
300
250
200
150
100
50
0
0
5
10
15
20
TIME (min)
C
12-HETE formed (% Basal)
500
400
300
200
100
0
*
**
A23187
A23187
+
NDGA

Figure 3

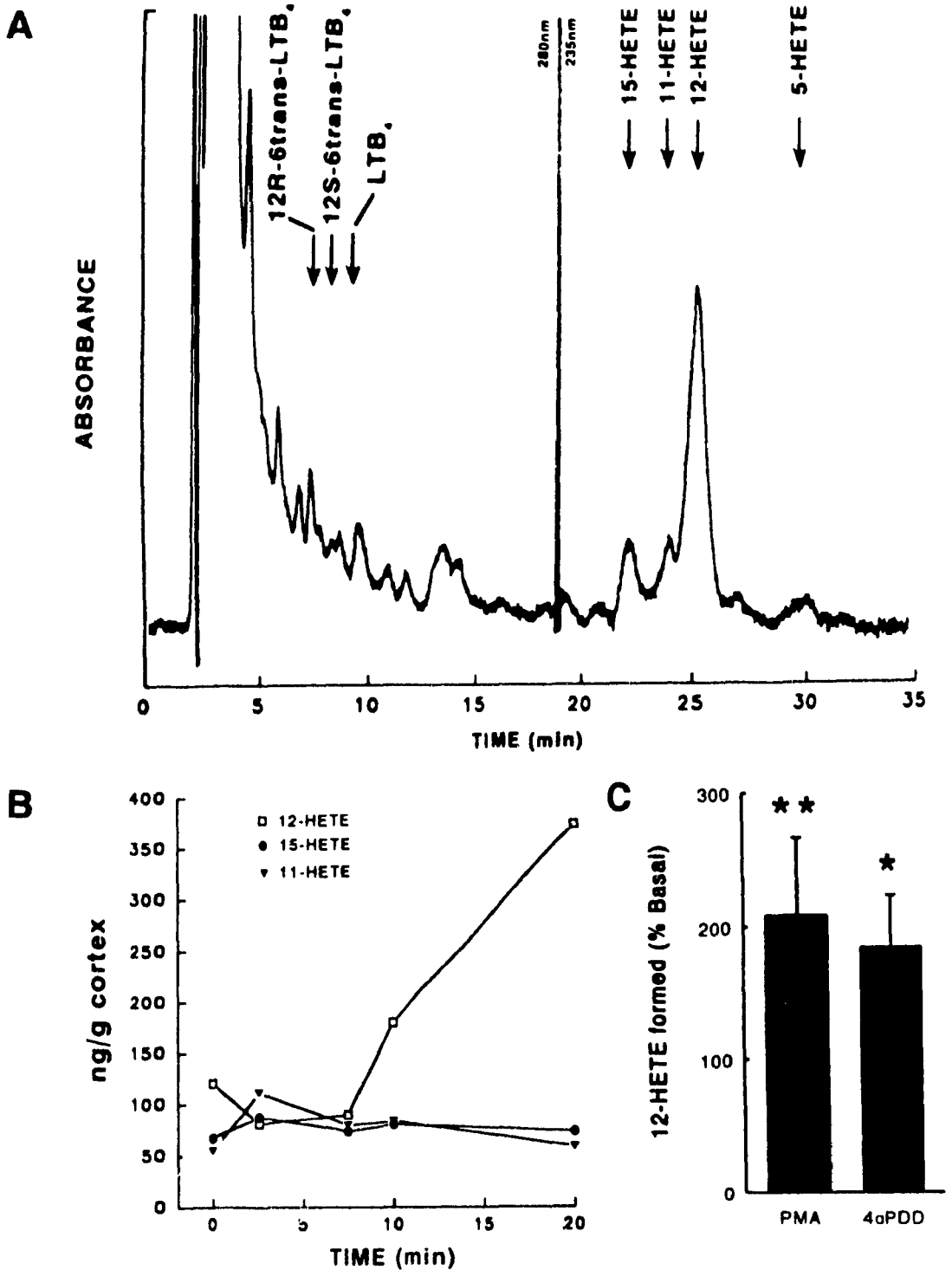
our conditions. The time course reveals that only 12-HETE formation was increased over time by the ionophore (Figure 3B), in contrast with Miyamoto et al. (1987b) who found that production of all HETEs was independent of A23187. An interesting lag phase was also observed in the synthesis of 12-HETE. A similar lag phase was reported for the formation of PGE_2 in Madin-Darby Canine Kidney cells (Slivka and Insel, 1987). Quantitatively, A23187 caused on average a 285% increase in 12-HETE level following a 20 minutes incubation period (Figure 3C). This effect was partially blocked (65% inhibition) by the lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA).

C) **Effect of phorbol myristate acetate on the formation of lipoxygenase metabolites**

Phorbol esters, and particularly phorbol myristate acetate (PMA) are known activators of protein kinase C (PKC) (Nishizuka 1986; Baraban, 1987; Rando, 1988). They have also been shown to induce arachidonic acid release and eicosanoid formation in a variety of preparations (Emilsson and Sundler, 1986; Jeremy et al., 1987; Jeremy and Dandona, 1987; Parker et al., 1987; Portilla et al., 1988; recently reviewed by Kiss, 1990) . Rat cerebral cortex slices synthesized 12-HETE after exposure to PMA and it constituted the major lipoxygenase metabolite formed (Figure 4A). 15, 11 and 5-HETE were also present but in small amounts. Again no appreciable amount of leukotrienes were detected.

When the formation of lipoxygenase metabolites was followed with time, only

Figure 4: Lipoxygenase metabolites formed by rat cerebral cortex slices stimulated by phorbol esters (100 ng/ml). **A:** HPLC chromatogram of lipoxygenase metabolites formed after 20 min incubation and separated by reversed-phase HPLC according to System II (see Material and Methods section). Retention times for authentic standards are indicated. **B:** Time course of synthesis of 12-HETE (□), 15-HETE (●) and 11-HETE (▼) by rat cerebral cortex slices stimulated with phorbol myristate acetate (100 ng/ml). Amount of 5-HETE was below limit of sensitivity. **C:** Phorbol myristate acetate (PMA;100 ng/ml) induced a significant increase in 12-HETE level over basal after 20 min incubation (** $p<0.01$ vs basal,$n=7$). 4α-phorbol didecanoate (4αPDD;100 ng/ml) was also found to stimulate 12-HETE synthesis (* $p<0.05$ vs basal,$n=5$). Basal level was set at 100%.


A
ABSORBANCE
12R-6trans-LTB4
12S-6trans-LTB4
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15-HETE
11-HETE
12-HETE
5-HETE
0
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10
15
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25
30
35
TIME (min)
B
ng/g cortex
12-HETE
15-HETE
11-HETE
400
350
300
250
200
150
100
50
0
0
5
10
15
20
TIME (min)
C
12-HETE formed (% Basal)
300
200
100
0
**
*
PMA
4αPDD

Figure 4

12-HETE synthesis was found to increase after a short lag phase, reminescent of the effect of A23187 (Figure 4B). When the effect of PMA was evaluated quantitatively, it was found to cause a 107% increase in 12-HETE level over control following a 20 minutes incubation period (Figure 4C). 4α-phorbol didecanoate (4αPDD), an inactive phorbol ester toward PKC (Castagna et al., 1982) was found however to mimic the effect of PMA on 12-HETE with an average increase of its synthesis of 86%.

D) **Effect of neurotransmitters on the formation of lipoxygenase metabolites**

In order to determine if lipoxygenase metabolite formation could be induced by a receptor-mediated event, the effect of seven different neurotransmitters or their agonists were tested. Two of them, L-glutamate and norepinephrine significantly increased the level of 12-HETE over basal following a 20 minute incubation period (Table 1). The others (serotonin, cholinergic agonist carbachol, gamma-aminobutyric acid, histamine and dopamine) appeared to have only a small inhibitory effect.

To further characterize the effect of norepinephrine (NE), the effect of two α-adrenergic antagonist on NE-induced 12-HETE synthesis was evaluated. Prazosin, an α_1 antagonist (Subbarao and Hertz, 1990) completely prevented the effect of norepinephrine on 12-HETE synthesis (Figure 5). Surprisingly yohimbine, an α_2 antagonist (Subbarao and Hertz, 1990) was found to be almost as effective as prazosin (90% inhibition). This effect might be explained at least in part by the fact that both antagonists can also

Table 1 Effects of neurotransmitters on 12-HETE synthesis by rat cerebral cortex slices

Condition	12-HETE (% Basal)[a]	(n)
NE	199.2±38.9[b]	(6)
NE+PRA	100.3±2.3[c]	(3)
NE+YO	111.8±3.6[d]	(3)
Glutamate	158.7±17.3[e]	(6)
NMDA	223.3±40.3[f]	(3)
Kainate	89.6,110.0	(2)
Serotonin	111.5,46.6	(2)
Carbachol	74.6,78.0	(2)
GABA	71.4,81.8	(2)
Dopamine	52.3,46.9	(2)
Histamine	74.9,55.9	(2)

[a]The basal level was 2321.0±417.2 pg/mg prot./20 min and was set at 100%. Determinations were average±STD unless otherwise indicated.
[b]Significant at $p<0.001$ vs basal
[c]Significant at $p<0.01$ vs NE
[d]Significant at $p<0.05$ vs NE
[e]Significant at $p<0.05$ vs basal
[f]Significant at $p<0.01$ vs basal

Figure 5: HPLC chromatograms of lipoxygenase metabolites formed by rat cerebral cortex slices stimulated by some neurotransmitters or agonists. NE is norepinephrine (100 μM), Pra is prazosin (1 μM), Yo is yohimbine (1 μM), Glu is L-glutamate (100 μM), NMDA is N-methyl-D-aspartate (100 μM) and Kai is kainate (100 μM). Slices were preincubated 5 minutes in presence of antagonist (Pra or Yo) prior to the addition of NE. Incubation in presence of neurotransmitter or agonist lasted 20 minutes. Lipoxygenase metabolites were separated according to System II (see Material and methods section).

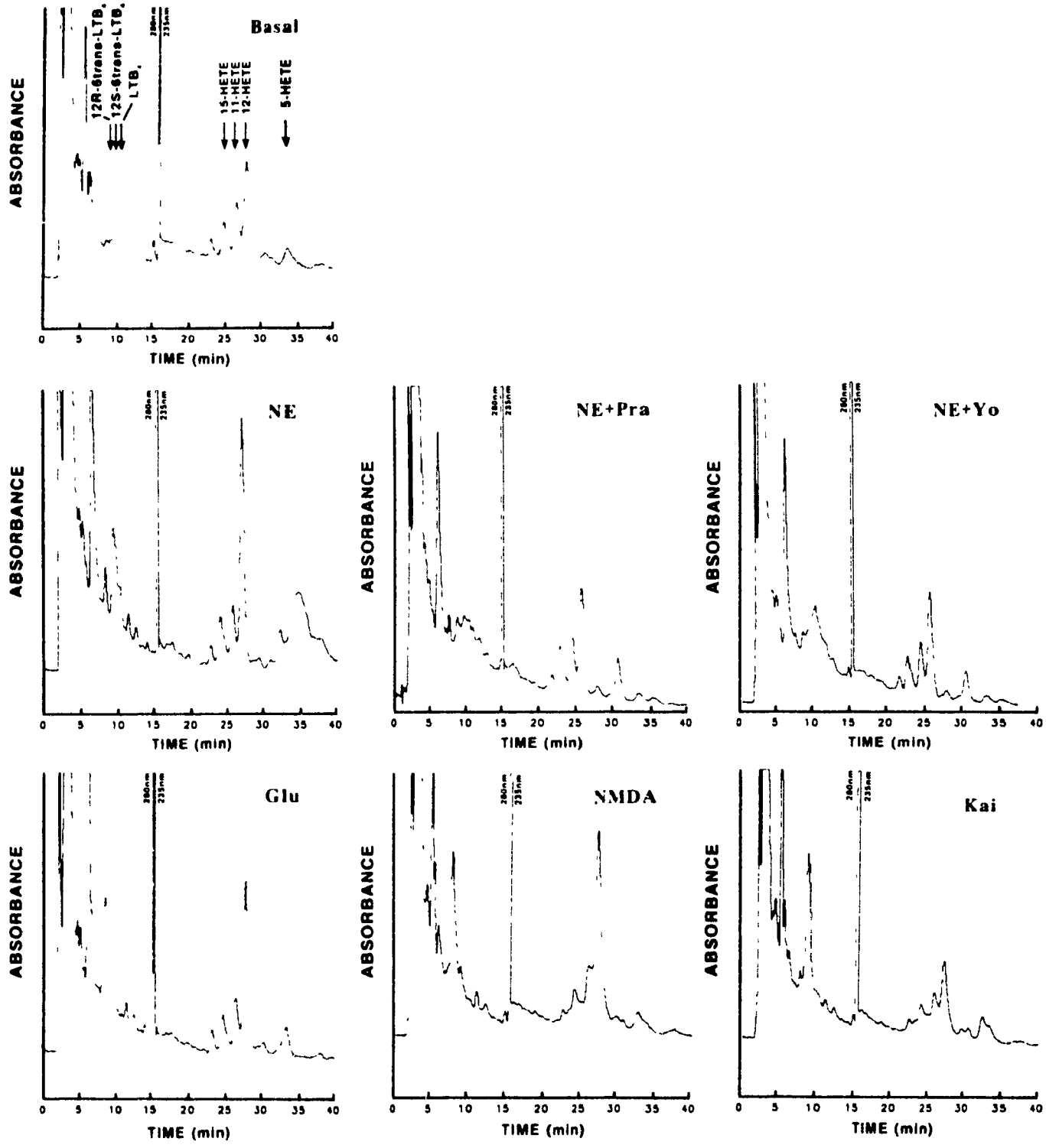
Basal
12R-6trans-LTB4
12S-6trans-LTB4
LTB4
15-HETE
11-HETE
12-HETE
5-HETE
NE
NE+Pra
NE+Yo
Glu
NMDA
Kai
ABSORBANCE
TIME (min)
0 5 10 15 20 25 30 35 40

Figure 5

partially antagonize responses mediated by the other type of α-adrenergic receptor (Lees, 1981). Nevertheless it suggests that the effect of norepinephrine on 12-HETE involves an α-adrenergic receptor subtype.

It was also of interest to look more closely at the effect of glutamate on 12-HETE synthesis for several reasons. L-glutamate represents the major excitatory neurotransmitter in the central nervous system including the cerebral cortex. Its involvement in various physiological as well as pathophysiological processes is now well established and specific glutamate receptor subtypes have been identified (for review see Collingridge and Lester, 1989). Although the situation appears somewhat more complex now, one can still loosely divide glutamate receptor subtypes in two classes, the NMDA (or N-methyl-D-aspartate) and the non-NMDA class. While kainate, an agonist of the non-NMDA receptor class was found to be without effect on 12-HETE synthesis, N-methyl-D-aspartate (NMDA) caused more than 100% increase over basal level (Figure 5 and Table 1). NMDA appears in fact more potent than glutamate (123% vs 59% stimulation). Activation of NMDA receptors thus appears solely responsible for the effect of glutamate on 12-HETE synthesis.

E) **Establishment of the chemical identity of glutamate-induced 12-HETE**

It was essential at this point to confirm unequivocally the chemical identity of the compound that was shown to be synthesized following stimulation by glutamate of

Figure 6: Electron impact mass spectra of the methyl ester, trimethylsilyl ether derivatives of the compound synthesized by rat cerebral cortex slices and corresponding to 12-HETE on reverse phase HPLC. **A:** native compound. **B:** saturated compound obtained by catalytic hydrogenation with activated platinum oxide.

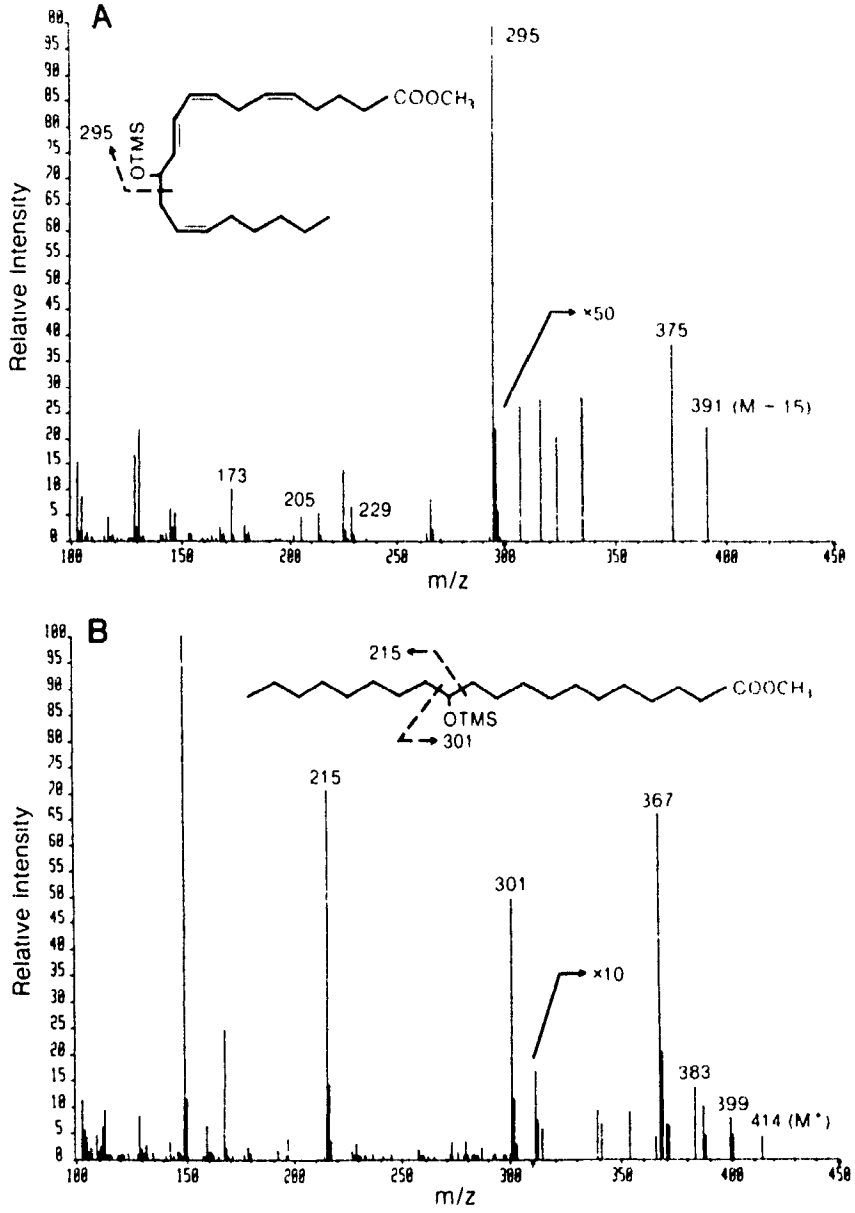
A
295
COOCH3
295
OTMS
×50
375
391 (M − 15)
173
205
229
Relative Intensity
m/z
B
215
COOCH3
OTMS
301
215
367
301
×10
383
399
414 (M+)
Relative Intensity
m/z

Figure 6

NMDA receptors. For this purpose, the peak on reverse phase HPLC corresponding to 12-HETE was collected. A first aliquot was derivatized to form the trimethylsilyl ether methyl ester derivative of the suspected hydroxyeicosatetraenoic acid. It was then submitted to electron impact negative ion mass spectrometry. The fragmentation pattern obtained (Figure 6A) was found to be similar to spectra published in the literature for this 12-HETE derivative (Boeynaems et al., 1980; Pace-Asciak, 1989). Although the molecular ion at m/z 406 was not observed, the base peak at m/z 295 is very characteristic of this compound.

A second aliquot was hydrogenated using PtO_2 as a catalyst, then derivatized to form the trimethylsilyl ether methyl ester derivative prior to being submitted to mass spectrometry. Again the mass spectrum obtained (Figure 6B) was almost identical to published spectra for the hydrogenated derivative of 12-HETE (Boeynaems et al., 1980]; Pace-Asciak, 1989). Together with the molecular ion at m/z 414, fragments at m/z 215 and 367 confirm the identification of the glutamate-induced compound as 12-hydroxyeicosatetraenoic acid.

Despite the fact that their synthesis was not increased by glutamate or any other stimulus tested so far, the peaks corresponding to 15, 11 and 5-HETE were also collected. After appropriate derivatization, they were analyzed by gas chromatography-mass spectrometry. Both 11 and 5-HETE were positively identified (Figure 7A and 7B) on the basis of mass spectra from their hydrogenated derivatives. Fragments at m/z 229

Figure 7: Electron impact mass spectra of the saturated methyl ester, trimethylsilyl ether derivatives of compounds formed by rat cerebral cortex slices and corresponding to **A:** 11-HETE and **B:** 5-HETE on reverse phase HPLC. The two fragments in A marked by an asterisk belong to 12-HETE. This contamination is due to incomplete resolution of the two peaks by reversed-phase HPLC.

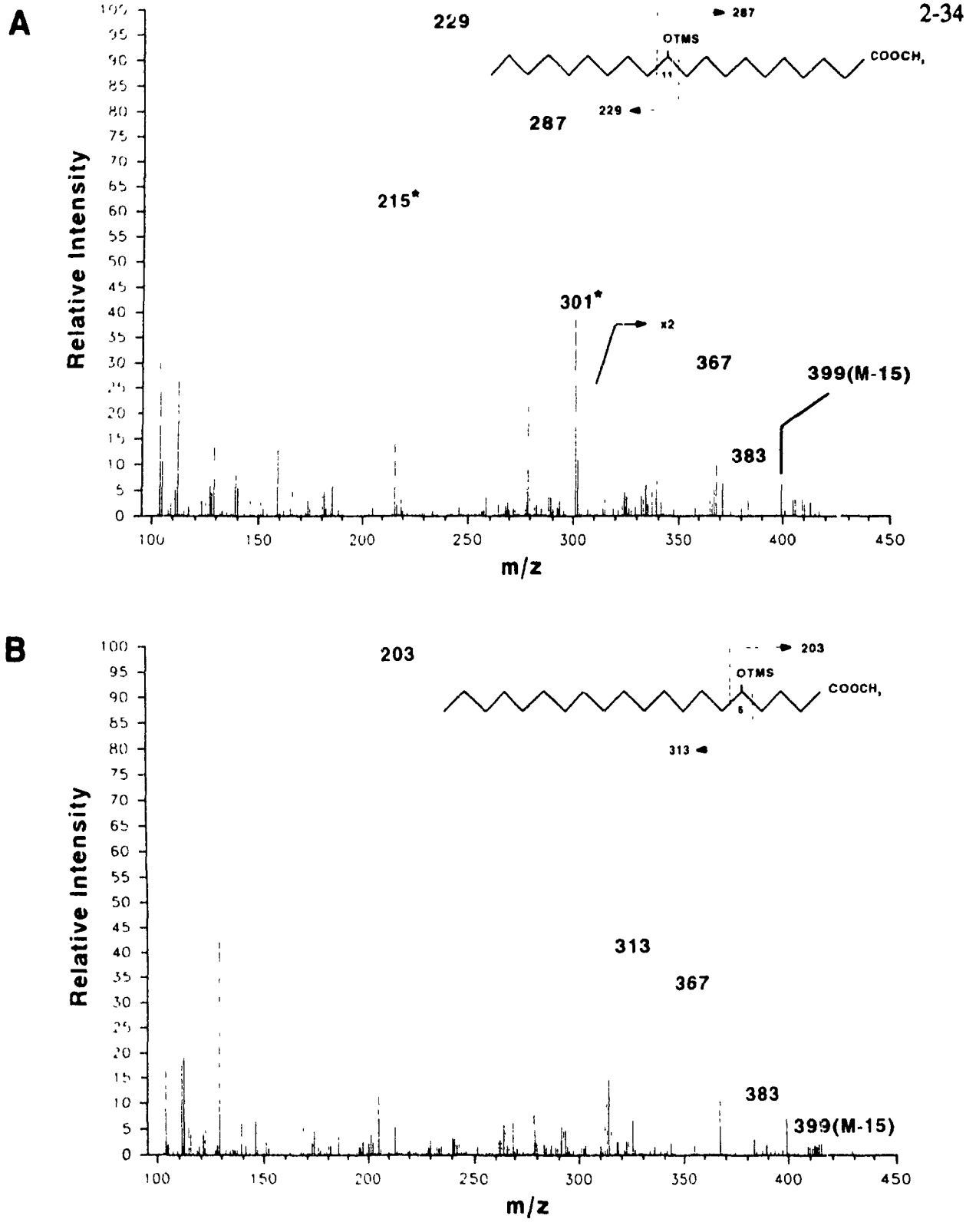
A
229
287
OTMS
COOCH,
229
287
215*
301*
x2
367
399(M-15)
383
Relative Intensity
m/z
B
203
203
OTMS
COOCH,
313
313
367
383
399(M-15)
Relative Intensity
m/z

Figure 7

and 287 are characteristic of 11-HETE while those at m/z 203 and 313 belong to 5-HETE (Boeynaems et al., 1980; Pace-Asciak, 1989). The fragments at m/z 215 and 301 in the mass spectrum of 11-HETE (Figure 7A) are due to a cross-contamination by 12-HETE since those two peaks were not totally resolved by reversed-phase HPLC. Satisfactory mass spectra from the non-hydrogenated derivatives could not be obtained. Acceptable spectra for both derivatives of 15-HETE could not be obtained. These failures are likely related to an insufficient amount of material available.

Finally, one question left open was the stereochemistry of 12-HETE. Although the original study in platelets had shown that the 12-lipoxygenase forms the S isomer (Hamberg and Samuelsson, 1974), recent work has demonstrated that the R form constitutes the major isomer found in some tissues such as the skin (Woollard, 1986). In addition it has been shown recently that the R isomer can arise from a pathway that does not involve a lipoxygenase but rather cytochrome P-450 (see Fitzpatrick and Murphy, 1989 for review). Determining the stereochemistry of the brain 12-HETE could then potentially indicate the route used for its formation. Three pathways can possibly account for 12-HETE formation in rat cerebral cortex and each predicts a different outcome regarding the stereochemistry (Figure 8). While a 12-lipoxygenase would only form the S enantiomer, a pathway involving the cytochrome P-450 would generate the R form as the major product (approximately 80% R and 20% S according to Capdevila et al., 1986). A third possibility would be autooxidation of arachidonic acid by free radicals and in this case an equimolar amount of each isomer should be present.

Figure 8: Diagram representing the possible pathways by which 12-HETE could arise in rat cerebral cortex. Information on the cytochrome P-450 mediated pathway was taken from Fitzpatrick and Murphy (1989).

Possible pathways for 12-HETE formation

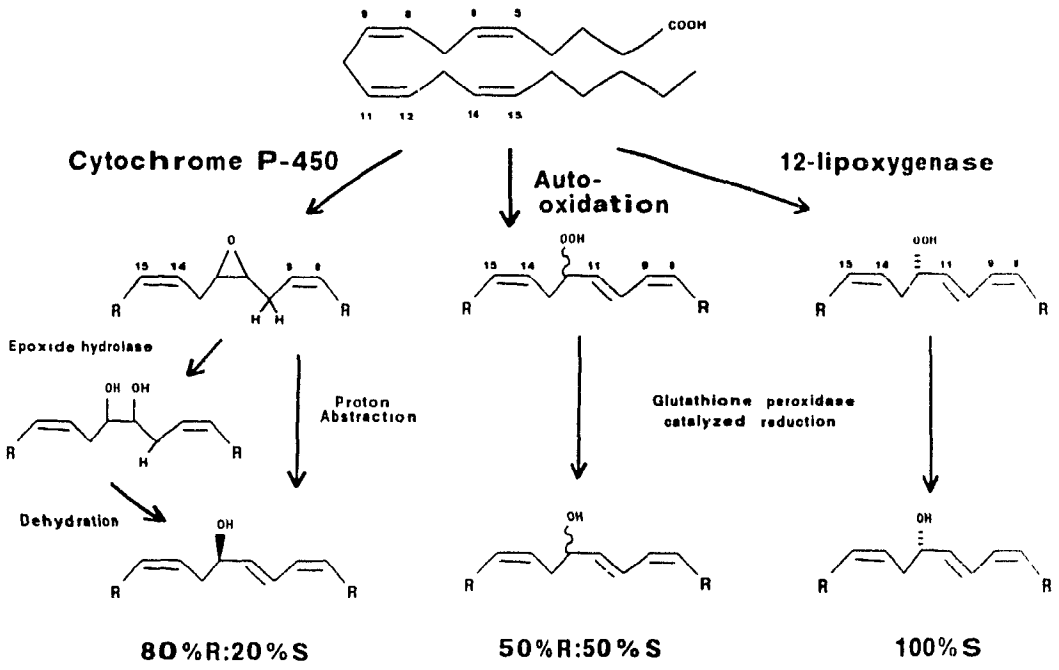

Figure 8

To answer this question an equimolar mixture of the two enantiomeric forms of 12-HETE was synthesized by a photochemical method. Using a method based on chiral phase column HPLC, the separation of the two enantiomers as methyl ester derivatives was realized, with retention time of 16.15 and 16.60 minutes for the S and R forms, respectively (Figure 9A). In order to validate the method a sample of 12-(S)-HETE isolated from neutrophils was obtained from Dr. William S. Powell of the Royal Victoria Hospital, Montreal, Canada. When injected alone on the chiral phase column, a single peak was apparent (Figure 9B). When an aliquot of this 12-(S)-HETE was added to an amount of the chemically synthesized 12-(S,R)-HETE mix and the new mixture injected on the chiral phase column, the peak corresponding to the S isomer increased in size as expected (Figure 9C). Now when 12-HETE isolated from rat cerebral cortex slices was analyzed by chiral phase HPLC after forming its methyl ester derivative, a single peak was observed when injected alone (Figure 9D). Finally when an aliquot was added to the photochemically synthesized mixture and the whole injected, the peak corresponding to the S isomer increased over the peak for the R form (Figure 9E). This clearly established that the compound formed in rat cerebral cortex after glutamate and NMDA stimulation correspond to 12-(S)-HETE. In addition the most likely pathway by which it is formed would involve a specific 12-(S)-lipoxygenase. It was recently demonstrated that in *Aplysia*, 12-lipoxygenase products formed by neurons are also of S configuration (Feinmark et al., 1990).

Figure 9: Stereochemical characterization of 12-HETE formed by rat cerebral cortex slices. **A:** Equimolar mixture of 12-HETE enantiomers photochemically synthesized and resolved by chiral phase column HPLC (retention time S=16.15 R=16.60 min) **B:** 12-(S)-HETE from neutrophils. **C:** Mix of 12-(S)-HETE from neutrophils and photochemically synthesized 12-HETE, showing the correct assignment of configuration. **D:** 12-HETE from rat cerebral cortex slices. **E:** Mix of 12-HETE from brain and 12-HETE photochemically synthesized, clearly showing the S configuration of the brain 12-HETE.

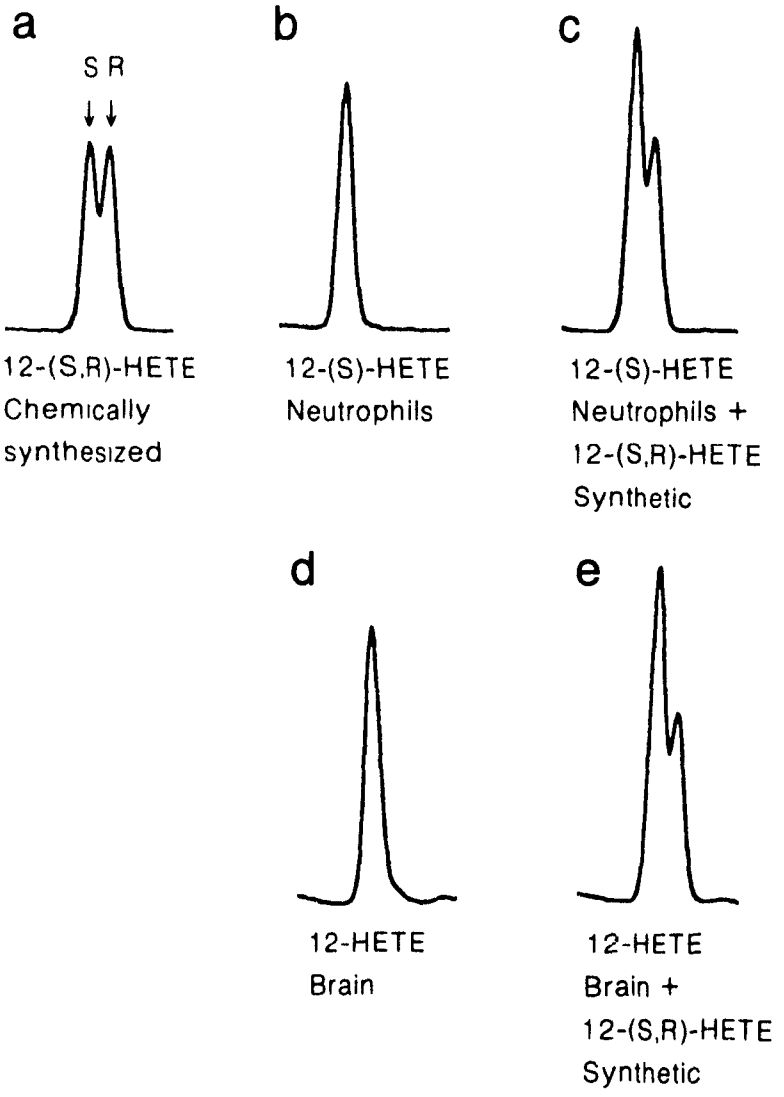
a
S R
12-(S,R)-HETE
Chemically
synthesized
b
12-(S)-HETE
Neutrophils
c
12-(S)-HETE
Neutrophils +
12-(S,R)-HETE
Synthetic
d
12-HETE
Brain
e
12-HETE
Brain +
12-(S,R)-HETE
Synthetic

Figure 9

F) Lipoxygenase metabolite formation in neuronal cell cultures

A more careful biochemical study of the various molecular components involved in receptor-mediated formation of lipoxygenase metabolites would require a model system constituted of a single homogenous cell population. This is obviously far from being the case in cerebral cortex slices. This is the reason why it was attempted to establish such a system by looking at two neuronal cell preparations. Two cell lines, NG108-15 and NCB-20 were selected for that purpose. NG108-15 is an hybrid between a neuroblastoma and a glioma cell line and was selected on the basis that it expresses α-adrenergic receptors (Nirenberg et al., 1983). NCB-20 is an hybrid between the same neuroblastoma cell line as for NG108-15 and fetal chinese hamster brain cells (Nirenberg et al., 1983) and was selected because it was the only cell line known to express NMDA receptors (Lerma et al., 1989). Both cell lines have been used extensively for the study of receptor-mediated stimulation of second messengers (Nirenberg et al., 1983; Cubitt et al., 1987; Zhu and Chuang, 1988). In addition, a primary culture of brain cells prepared from 13 days mouse embryos was also tested.

The two cell lines were cultured under different conditions, including culture in presence of dibutyryl cAMP (dbcAMP) or phorbol myristate acetate (PMA) for three days to induce differentiation. Usually between 10^6 and 10^7 cells were used per incubation in all cases. The primary cultures were assayed at two stages: at 14 days, when they are considered mature and at 21 days when they are considered senescent. The

Figure 10: HPLC chromatograms obtained following stimulation of three neuronal cell culture systems by melittin (10 μg/ml). NCB and NG108 are two neuronal cell lines while PCN represents primary cultures of brain cells from 13 days mouse embryos (for more details see Material and methods section). No significant differences were observed between control (unstimulated) and melittin-stimulated preparations.

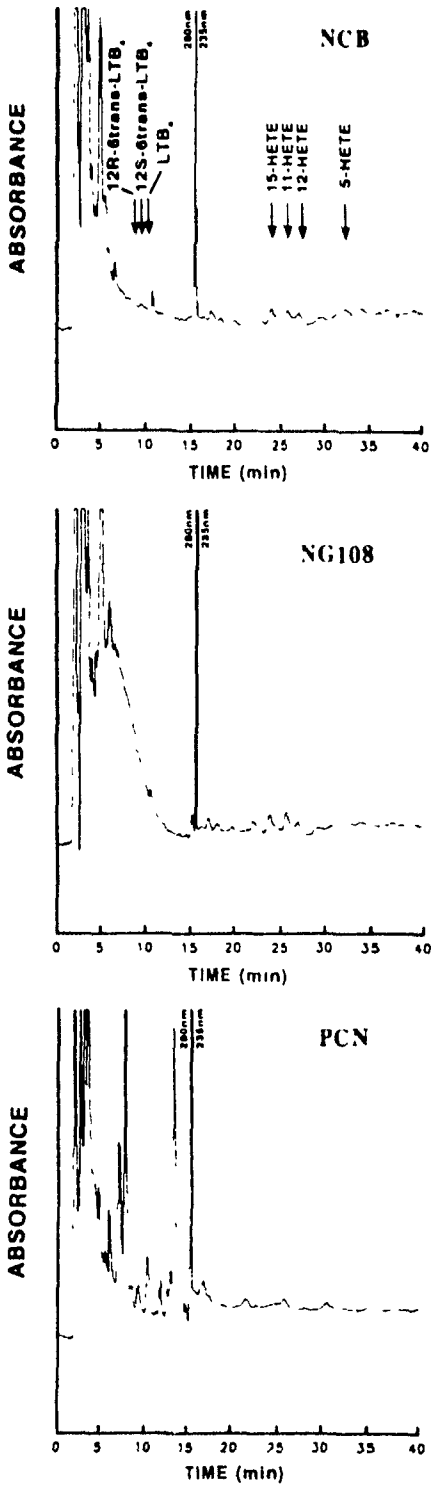
NCB
ABSORBANCE
12R-6trans-LTB4
12S-6trans-LTB4
LTB4
15-HETE
11-HETE
12-HETE
5-HETE
280nm
235nm
0 5 10 15 20 25 30 35 40
TIME (min)
NG108
ABSORBANCE
280nm
235nm
0 5 10 15 20 25 30 35 40
TIME (min)
PCN
ABSORBANCE
280nm
235nm
0 5 10 15 20 25 30 35 40
TIME (min)

Figure 10

Table 2 Conditions of culture and stimulation used to evaluate the synthesis of lipoxygenase metabolites of arachidonic acid by the neuronal cell lines NCB-20 and NG108-15

CULTURE	STIMULUS
normal	none
normal	A23187 (2μM)
normal	A23187 (2μM) + AA (10μM)
normal	A23187 (2μM) + AA (30μM)
normal	Melittin (10μg/ml)
+dbcAMP	none
+dbcAMP	A23187 (2μM)
+dbcAMP	A23187 (2μM) + AA (10μM)
+dbcAMP	A23187 (2μM) + AA (30μM)
+PMA	A23187 (2μM) + AA (30μM)
+PMA	Melittin (10μg/ml)

Table 3 Conditions of culture and stimulation used to evaluate the synthesis of lipoxygenase metabolites of arachidonic acid by primary cultures of brain cells

CULTURE	STIMULUS
14 days	none
14 days	A23187 (5μM)
14 days	AA (10μM)
14 days	A23187 (5μM) + AA (10μM)
14 days	Melittin (5μg/ml)
14 days	PMA (100ng/ml)
21 days	none
21 days	A23187 (5μM)
21 days	AA (10μM)
21 days	A23187 (5μM) + AA (10μM)
21 days	Melittin (5μg/ml)

results can be resumed as follows: very little, if any lipoxygenase metabolites including the HETEs were observed, even when cells were stimulated with the phospholipase A_2 activator melittin (see Figure 10). Exposure of the neuronal cell preparations to various culture conditions and treatments to stimulate lipoxygenase product formation as presented in Table 2 and 3 did not produce any significant effect on the formation of the lipoxygenase metabolites monitored.

DISCUSSION

The experiments reported here were aimed at verifying the capacity of rat brain cortex to synthesize lipoxygenase metabolites of arachidonic acid, as well as investigating receptor-mediated stimulation of their formation. Cerebral cortex slices were chosen since these have been a successful model for the study of receptor-mediated generation of second messengers (Brown et al., 1984; Kendall, 1986; Schaad et al., 1989) as well as prostaglandins (Wolfe et al., 1976; Reichman et al., 1987) in the nervous system. The results further confirmed the presence of an active 12-lipoxygenase pathway in the rat central nervous system. Moreover, this particular pathway appears responsive to stimulation by some neurotransmitters.

A) Capacity of rat brain cortex to form lipoxygenase metabolites

In the presence of exogenous arachidonic acid, rat cerebral cortical slices

synthesized five hydroxyeicosatetraenoic acids (HETEs). Three of them, 15,12 and 5-HETE are possible lipoxygenase products. Shimizu et al. (1987), obtaining similar results with guinea pig brain homogenates concluded that 15, 12 and 5-lipoxygenases were present in nervous tissue. Kim et al. (1991) have recently challenged however these allegations. Despite the fact that they do observe the formation of all five HETEs in rat brain homogenates and could prevent their formation with lipoxygenase inhibitors or by either boiling or denaturing the tissue, stereochemical analysis of the products lead them to conclude that they arise most likely by autooxidation. They hypothesized that free radicals generated by other endogenous enzymatic reactions might react with arachidonic acid and be responsible for HETEs formation. This is certainly a possibility that can not be dismissed for the moment and it could explain at least in part the present results, particularly the formation of 9-HETE. The observation however that both A23187 and PMA selectively induce the formation of 12-HETE argues in favor of a specific enzymatic origin in this case. Moreover the stereochemical analysis of 12-HETE obtained not only indicates it is formed enzymatically but clearly suggest the involvement of a 12-(S)-lipoxygenase over a cytochrome P-450-dependent mechanism. This leads to the conclusion that an active 12-lipoxygenase is present in the nervous tissue of vertebrates.

Cellular localization of lipoxygenase metabolism represents a more difficult problem since slices are quite heterogeneous, containing various cell types including endothelial cells, glial cells and of course neurons. It has been reported previously that the amount of 12-HETE found in brain slices is reduced by half when the brain of the

animal is perfused with saline prior to decapitation (Wolfe et al., 1985; Adesuyi et al., 1985). This points out to a certain contribution of residual blood elements to 12-HETE level found in brain tissue, despite the relatively small amount of blood present. In addition, endothelial cells and brain microvessels (Hambrecht et al., 1987; Spector et al., 1988) as well as glial cells (Ishizaki et al., 1989; Petroni et al., 1990) have been found to synthesize 12-HETE under various conditions. As mentioned previously however, 12-HETE formation has also been described in a neuronal cell preparation (Dumuis et al.,1988). We have observed that N-methyl-D-aspartate (NMDA), a selective agonist of a subclass of glutamate receptors known as NMDA receptors, causes a selective increase of 12-HETE formation in rat cerebral cortex slices. Since NMDA receptors have not been described in any cell types other than neurons, this observation argues in favor of a 12-lipoxygenase present in neurons as well. Recently, a 12-lipoxygenase was purified from dog cerebral cortex and shown to synthesize 12-(S)-HPETE (Nishiyama et al., 1990; S. Yamamoto, personal communication). Using an antibody raised against this enzyme, it was demonstrated immunohistochemically that it was located mostly in pyramidal neurons both in the cortex and the hippocampus, and to a lesser extent in some glial cells. It thus appears likely that neurons can be also a source of 12-lipoxygenase metabolites including 12-HETE. It is still possible however that release of arachidonic acid occurs in neurons, but it is metabolized in non-neuronal cells. Many such examples of transcellular metabolism have been reported previously for eicosanoids (Feinmark et al., 1986; Bigby and Meslier, 1989). Whether it is the case here will have to be looked at more closely in culture systems.

Leukotrienes have been reported by several investigators to be formed in the central nervous system of vertebrates (Lindgren et al., 1984; Shimizu et al., 1987; Simmet et al., 1988b; reviewed by Simmet and Peskar, 1990). Stimulation by the calcium ionophore A23187 appears essential in order to observe the formation of these compounds (Miyamoto et al., 1987b). Some factors, such as adenosine triphosphate (ATP), Ca^{2+} and some hydroperoxides have been found to be also required (Shimizu et al., 1987). Failure to observe formation of leukotrienes even when A23187 was used as a stimulus is reported here. One of the reason for this failure might be the amount of tissue used. While incubations were routinely performed with approximately 500 mg of cortex wet weight, Miyamoto et al. (1987a) have reported to use up to 10 g of tissue per incubation. In addition, the lower sensitivity of conventional UV detection might have prevented the detection of the small amounts of leukotrienes formed in the nervous tissue, as reported by others using more sensitive radioimmunoassay methods (Hartung and Toyka, 1987; Simmet et al., 1987; Kiwak et al., 1985; Dempsey et al., 1986; Black et al., 1986).

B) Stimulation of lipoxygenase metabolism by neurotransmitters

An important step toward the recognition of lipoxygenase metabolites of arachidonic acid as second messengers in the central nervous system is the demonstration that their synthesis can be stimulated by endogenous substances acting through specific receptors. It is then of great interest that a selective stimulation of 12-HETE was

observed following the application of two neurotransmitters, norepinephrine and glutamate.

Because of its extensive and diffuse distribution across the entire cerebral cortex, norepinephrine is considered to play a modulatory role on synaptic transmission (Descarries et al., 1988). It can act through a series of receptor subtypes that have been reasonably well characterized in term of their signal transduction mechanisms (Strange, 1988). Typically, ß-adrenoceptors have been associated with a stimulation of adenylate cyclase through a G-protein (G_s) and increase cAMP, while α_2-adrenoceptors are considered to be negatively coupled to adenylate cyclase through the G-protein G_i and thus decrease cAMP. On the other hand α_1-adrenoceptors have been shown to activate phosphoinositide turnover.

Activation of either α_1 or α_2 receptors has been reported to induce arachidonic acid release in various preparations including thyroid cells, platelets, adipocytes and ovarian cells transfected with the gene encoding the receptor (Burch et al., 1986a; Sweatt et al., 1986; Slivka and Insel, 1987; Schimmel, 1988; Jones et al., 1991). Although it has been previously reported that NE can stimulate the formation of cyclooxygenase products such as $PGF_{2\alpha}$ (Wolfe et al., 1976; Birkle et al., 1981), nobody has previously demonstrated that NE can induce 12-HETE synthesis in rat cortex. Despite the fact that our results do not allow us to distinguish between the involvement of α_1- or α_2-adrenoceptors, an interesting question that comes to mind is whether arachidonic acid

release and 12-HETE synthesis could be linked to the phosphoinositide pathway. Following the activation of a phospholipase C and generation of inositol phosphates and diacylglycerol, arachidonic acid can be released from diacylglycerol by a diacylglycerol lipase as was shown in platelets following activation of α_2-adrenoceptors (Sweatt et al., 1986). Alternatively mobilization of calcium by inositol trisphosphate and activation of protein kinase C by diacylglycerol could subsequently induce arachidonic release via a phospholipase A_2-based mechanism, as demonstrated in the case α_1-adrenoceptors activation in Madin-Darby Canine Kidney cells (Weiss and Insel, 1991). On the other hand it has been also demonstrated that arachidonic acid release via PLA_2 activation and the phosphoinositide pathway can be activated independently from each other by α_1-adrenoceptors in spinal cord neurons (Kanterman et al., 1990) and moreover via separate G-proteins in FRTL5 thyroid cells (Burch et al., 1986b). Finally it is possible that norepinephrine might not directly lead to arachidonic acid release but simply potentiate the effect of an other endogenously released neurotransmitter like glutamate, as recent experiments in cerebral cortex have suggested (Mouradian et al., 1991).

The results obtained with the calcium ionophore A23187 and the phorbol ester PMA could be interpreted as favoring the possibility that Ca^{2+} mobilization and activation of PKC would be involved in NE-induced arachidonic acid release and 12-HETE synthesis. Ho and Klein (1987), looking at arachidonic acid release in the pineal gland have obtained similar results and have concluded that norepinephrine acting via α_1-adrenoceptors activates a PLA_2 and this action involves Ca^{2+} and PKC. The effect of

4αPDD however apparently argues against a role for PKC, although similar effects of active and inactive phorbol esters on arachidonic acid release, operating via different mechanisms have been described (Fischer et al., 1991). In addition, the possibility remains that NE could stimulate the non-enzymatic reduction of 12-HPETE to 12-HETE via its anti-oxidant properties, as was suggested for the stimulation of $PGF_{2\alpha}$ formation (Wolfe et al., 1976). So despite the interesting possibility suggested by our data, the exact mechanism remains to be more firmly established.

Glutamate (together with aspartate) is considered the major excitatory neurotransmitter of the mammalian central nervous system. Its effects are mediated by three main types of receptors named after their more specific agonists, N-methyl-D-aspartate (NMDA), kainate (K) and quisqualate (Q) (for review see Cotman et al., 1987; Collingridge and Lester, 1989). Glutamatergic synapses operate usually with a system involving two types of receptor. K or Q receptors (also referred to as non-NMDA receptors) are normally responsible for carrying out fast synaptic transmission. Their activation increases permeability of the postsynaptic membrane to sodium which leads to fast depolarization. NMDA receptors on the other hand are not normally activated at normal membrane resting potential. Because of a Mg^{2+} blockade their activation is voltage dependent. Only when depolarization (usually provided by K or Q receptors activation) reach a certain threshold and alleviate the Mg^{2+} blockade that NMDA receptors can be activated. In addition to Na^{+} they also allow Ca^{2+} to enter the cell, which is thought to mediate most of the effects attributed to NMDA receptor including

activation of different second messenger systems. These unique properties make this receptor ideally suited for mediating slower types of synaptic transmission, like activity-dependent processes which often involve long-term modification of cell responses such as synaptic plasticity.

The observation that 12-HETE synthesis in rat cerebral cortex is stimulated by glutamate, most likely through activation of NMDA receptors certainly represents an important finding. A similar observation has been made in striatal neurons in cultures (Dumuis et al., 1988). In addition it was shown in cerebellar granule cells that NMDA induces arachidonic acid release (Lazarewicz et al., 1988) and this might be linked to cGMP formation via 12-lipoxygenase metabolites (Wroblewska et al., 1990). Calcium was shown to be essential in inducing these effects on arachidonic acid release and metabolism. The stimulation of 12-HETE synthesis observed with the calcium ionophore A23187 appears compatible with this view. Dumuis et al. (1988) have similarly observed stimulation of arachidonic acid release by the calcium ionophore ionomycin in striatal neurons. Whether PKC is involved in the activation of arachidonic acid release and metabolism following NMDA receptor stimulation has not been tested in those two neuronal cell preparations. It has been shown however that PKC is activated following NMDA receptor stimulation (Vaccarino et al., 1987).

C) Suitability of the slice preparation for studying lipoxygenase metabolism and the necessity to develop a cell culture model

The use of a well-characterized, intact and homogenous preparation has allowed rapid progress in the study of arachidonic acid metabolism through lipoxygenases and their potential role as neuromodulators in the nervous system of the invertebrate *Aplysia californica*. Unfortunately the situation is not the same in the mammalian nervous system. Brain slices have been a rather popular and surprisingly successful model for the study of receptor-mediated formation of second messengers as mentioned earlier. However the use of slices for the study of arachidonic acid metabolites formation turned out to be more difficult than anticipated. Several reasons might explain the difficulties encountered. One should not forget that a slice is a damaged piece of tissue and numerous alterations in the physiology and biochemistry of the tissue might occur (Elliott, 1969; Andersen, 1981; Reid et al., 1988). It is also highly susceptible to environmental manipulation such as oxygen level, pH variation, temperature, bathing medium composition, etc (nicely reviewed by Reid et al., 1988). In addition the brain is extremely heterogenous in term of cell types it contains and it can express considerable regional variability for a number of parameters. Finally contamination from blood elements could also contribute to the variability observed.

This prompted a search for alternative preparations. The retina has already been proposed as a model to study lipoxygenase metabolism in the nervous system (Birkle and

Bazan, 1984). However, a more accessible, easy to use and better characterized preparation would be a cell culture system, which appears more suitable for studying receptor-mediated induction of lipoxygenase metabolism. Unfortunately no satisfactory one has been developed so far. Two preparations, a neuronal cell line called Neuro2A and striatal neurons in culture have been reported to synthesize the lipoxygenase metabolite 12-HETE (Birkle and Ellis, 1983; Dumuis et al., 1988). The evidence however remains incomplete since no mass spectrometric data were obtained to confirm the claim. In an other neuronal cell line named N1E 115, lipoxygenase metabolites were suggested to participate, on the basis of inhibitor studies, in guanylate cyclase activation after stimulation of a muscarinic receptor (Snider et al., 1984). Lipoxygenase metabolite formation was never demonstrated however. Experiments reported here with the two neuronal cell lines NG-108-15 and NCB-20 as well as a mouse primary culture of brain cells were essentially negative. This situation is not necessarily unusual since neuronal cell lines do not display all the characteristics of adult neurons and often have a phenotype closer to embryonic neurons. Similar difficulties for finding a suitable model system have been encountered in the case of Ca^{2+}/calmodulin kinase II, an enzyme most likely activated following NMDA receptor stimulation (Molloy and Kennedy, 1991). These authors have successfully used organotypic cultures of slices of rat hippocampus in their study of Ca^{2+}/camodulin kinase II, which might represent an interesting alternative to acutely prepared slices (see Gähwiler, 1988 for a review). Another promising possibility is related to the recent development of techniques to immortalize brain cells (see Lendahl and McKay, 1990 for review). With the recent identification by

immunohistochemistry of pyramidal neurons containing a 12-lipoxygenase in canine cerebral cortex (Nishiyama et al., 1990, S. Yamamoto, personal communication), it might be possible to produce a neuronal cell line that would conserve both its capacity to form lipoxygenase metabolites as well as most of its adult neuron characteristics. Development of such preparations appears the next essential step in order to study more closely at the biochemical level receptor-mediated induction of lipoxygenase metabolism in nerve cells of vertebrates.

D) Potential roles for lipoxygenase metabolites of arachidonic acid in the nervous system

A number of recent studies have suggested roles for arachidonic acid metabolites, and particularly lipoxygenase metabolites in the nervous system (see Schaad et al., 1991 for review). Magistretti and coworkers for example have shown that lipoxygenase metabolites play a role in the release of the neuropeptide VIP (vasoactive intestinal peptide) and modify its action in the neocortex of mice (Martin and Magistretti, 1989; Schaad et al., 1989). In rat hippocampus the enhancing effect of somatostatin on the neuronal M-current was shown to be mediated by a 5-lipoxygenase metabolite (Schweitzer et al., 1990). In rat brain synaptosomes, 12-HPETE and 12-HETE have been shown to inhibit Ca^{2+}/calmodulin kinase II, an enzyme involved in the regulation of neurotransmitter release, suggesting a role for these arachidonic acid metabolites in presynaptic modulation of neurotransmitter release (Piomelli et al., 1989b). Finally the

well known work of Piomelli and co-workers (1987a) in the mollusc *Aplysia californica* has revealed the involvement of certain 12-lipoxygenase metabolites as second messengers in a form of synaptic plasticity called habituation . Their effects in this case appear to arise from interaction with specific ion channels (Belardetti and Siegelbaum, 1988). The results presented here provide some support for the idea that 12-lipoxygenase metabolites could be second messengers in the vertebrate central nervous system since they reveal the occurrence of receptor-mediated formation for these arachidonic acid metabolites. Evidences that lipoxygenase metabolites of arachidonic acid participate in neuronal transmembrane signalling in both vertebrate and invertebrates have been reviewed lately (Piomelli and Greengard, 1990).

Several authors have suggested that arachidonic acid and its lipoxygenase metabolites, and particularly 12-lipoxygenase metabolites might play a role in some forms of synaptic plasticity in the mammalian central nervous system (Piomelli et al.,1987a; Williams and Bliss, 1988; Dumuis et al., 1988; Lazarewicz et al., 1988; Collingridge and Davies, 1989; Lynch, 1989). The results presented here also provide some evidence in favor of such an hypothesis. NMDA receptors are known to participate in various forms of synaptic plasticity such as the phenomenon of long-term potentiation (Collingridge and Singer, 1990). Induction of arachidonic acid release and metabolism by the 12-lipoxygenase pathway following activation of NMDA receptors might provide some of the necessary messengers to instruct the cells how to bring about the specific changes occurring in some forms of synaptic plasticity. A more direct testing of this idea using the *in vitro* hippocampal slice preparation will be pursued in the following chapters.

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Chapter 3

EFFECTS OF ARACHIDONIC ACID AND ITS LIPOXYGENASE METABOLITES ON SYNAPTIC TRANSMISSION IN RAT HIPPOCAMPAL SLICES

INTRODUCTION

Since its description by Bliss and Lømo in 1973, long-term potentiation (LTP) of synaptic transmission in the hippocampus has been used as a model for the study of learning and memory. Over the years, it has been the subject of an impressive number of investigations and several reviews on this topic have appeared (Gustafsson and Wigström, 1988; Nicoll et al., 1988; Brown et al., 1988; Kennedy, 1989; Kuba and Kumamoto, 1990; Lynch et al., 1990; Madison et al., 1991). In brief, long-term potentiation can be defined as a persistent enhancement of synaptic efficacy usually triggered by brief, high frequency tetanic activation of excitatory pathways.

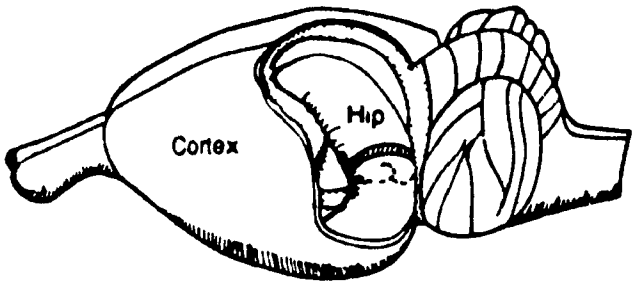
Evidence was provided recently for the involvement of arachidonic acid and/or its lipoxygenase metabolites in LTP as occurring in the hippocampus. Okada et al. (1989) have shown that p-bromophenacylbromide, an inhibitor of PLA_2 and thus of arachidonic acid release, can prevent tetanically-induced LTP in hippocampal slices. In addition, Bliss and his coworkers have shown that (1) calcium-induced LTP in dentate gyrus and area CA1 of the hippocampal slices is prevented by nordihydroguaiaretic acid (NDGA), an inhibitor of lipoxygenases (Williams and Bliss, 1988) (2) tetanus-induced LTP in area CA1 is also blocked by NDGA (Williams and Bliss, 1989) (3) NDGA blocks tetanus-induced LTP and the increased glutamate release *in vivo* in the dentate gyrus of the hippocampus (Lynch et al., 1989) (4) finally, arachidonic acid itself was shown to induce LTP *in vivo* in the same area (Williams et al., 1989).

The hippocampal slice preparation provides an ideal system to study the modulation of synaptic transmission as occurring in the phenomenon of LTP *in vitro*. Its lamellar organization with densely packed neuronal cell bodies allows easy extracellular and intracellular electrophysiological recordings. Three major excitatory synapses are usually distinguished (see Figure 1): a first one in the dentate area between the granule cells and the perforant path arising from cells in the entorhinal cortex (1); a second one in the CA3 area between pyramidal cells and the mossy fibers coming from the dentate (2); finally the last one in the CA1 area between pyramidal cells and the Schaffer collateral pathway originating from the CA3 area (3). Glutamate has been recognized as the neurotransmitter acting at all three synapses. All three types of glutamate receptors, the N-methyl-D-aspartate (NMDA), kainate and quisqualate types have been described, although their distribution varies from one area to the other (see Cotman et al., 1987). The area CA1, which is particularly rich in NMDA receptors has been the most extensively used for the study of LTP. For a more complete description of the hippocampal anatomy and physiology, as well as the properties and use of hippocampal slices, the reader is referred to books and reviews that have appeared on these topics (see Andersen et al., 1971; Isaacson and Pribram, 1975; O'Keefe and Nadel, 1978; Dingledine, 1984).

Taking advantage of the *in vitro* hippocampal slice preparation, investigation of the following points was planned: (1) observation of the effects on extracellular components of synaptic transmission (population spike and extracellularly recorded excitatory postsynaptic potential) in area CA1 induced by arachidonic acid when

Figure 1: The hippocampal slice preparation. **A:** Diagram showing the position of the hippocampus under the cortex in the rat brain. Slices, as illustrated, are prepared by cutting the hippocampus transversely. **B:** Schematic representation of the hippocampal slice illustrating its trisynaptic cicuitry. D, dentate gyrus;CA3, field CA3 of the hippocampus;CA1, field CA1 of the hippocampus;S, subicular complex;p.p., perforant path;m.f., mossy fiber pathway;s.c.p., Schaffer collateral pathway;f., fimbria. Adapted from Kennedy (1989).

A



B

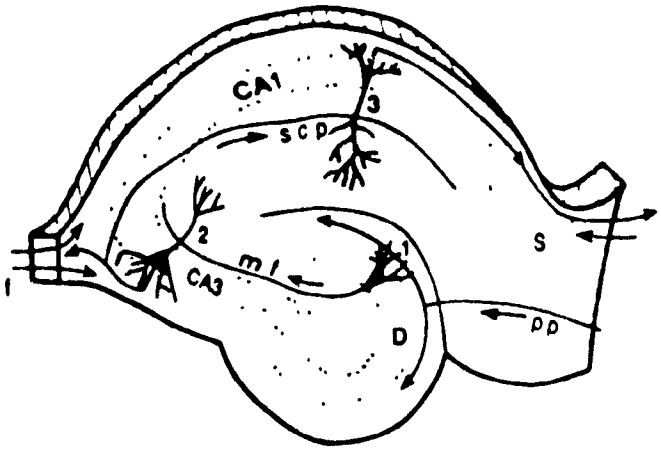


Figure 1

exogenously applied on hippocampal slices, (2) determination of lipoxygenase metabolite involvement in the effects observed, if any, using the lipoxygenase inhibitor NDGA, (3) demonstration that endogenously released arachidonic acid, using the phospholipase A_2 activator melittin, has similar effects and (4) investigation of possible modulation of synaptic transmission by 12-lipoxygenase metabolites using the 12-lipoxygenase inhibitor baicalein and 12-hydroperoxyeicosatetraenoic acid (12-HPETE), a 12-lipoxygenase metabolite.

MATERIAL AND METHODS

A) **Electrophysiological measurements**

Hippocampal slices (450 μm thick) were obtained from male Sprague-Dawley rats (250-300 g) and were maintained *in vitro* in a tissue chamber (see Perreault and Avoli (1989) for further details). Slices were perfused with oxygenated (O_2 95%, CO_2 5%) artificial cerebrospinal fluid (ACSF) whose composition was in mM: NaCl, 124; KCl, 2; KH_2PO_4, 1.25; $MgSO_4$, 2; $NaHCO_3$, 26; $CaCl_2$, 2; glucose 10. Extracellular recordings were obtained from the pyramidal and the dendritic layers of the CA1 subfield using glass microelectrodes (resistance: 5-20 MΩ) filled with 3 M NaCl (see Figure 2). Low frequency (0.083 Hz) stimulation was provided by a bipolar electrode placed in the stratum radiatum of the CA2 area. Tetanic stimulation in order to elicit LTP consisted of five bursts of 1 second at 100 Hz applied at 1 second interbursts intervals. Signals

Figure 2: Experimental set-up to investigate modulation of synaptic transmission in the CA1 subfield of the hippocampus. The top diagram represents the hippocampal slice preparation. A stimulating electrode (SE) was positioned in the stratum radiatum of the CA2 area to activate the Schaffer collaterals. A first recording electrode (RE1) was positioned in the cell body layer while a second one (RE2) was placed in the dendritic layer. The diagram at the bottom left shows the various layer (stratum) of the hippocampus: *alv*, alveus;*or*, oriens;*pyr*, pyramidale;*rad*, radiatum;*mol*, moleculare. Tracings at the bottom right show the responses obtained following stimulation of the Schaffer collaterals. Top tracing display a population spike as recorded by the first electrode in the pyramidal layer. Bottom tracing shows an EPSP (excitatory postsynaptic potential) as recorded by the second electrode in the dendritic layer.

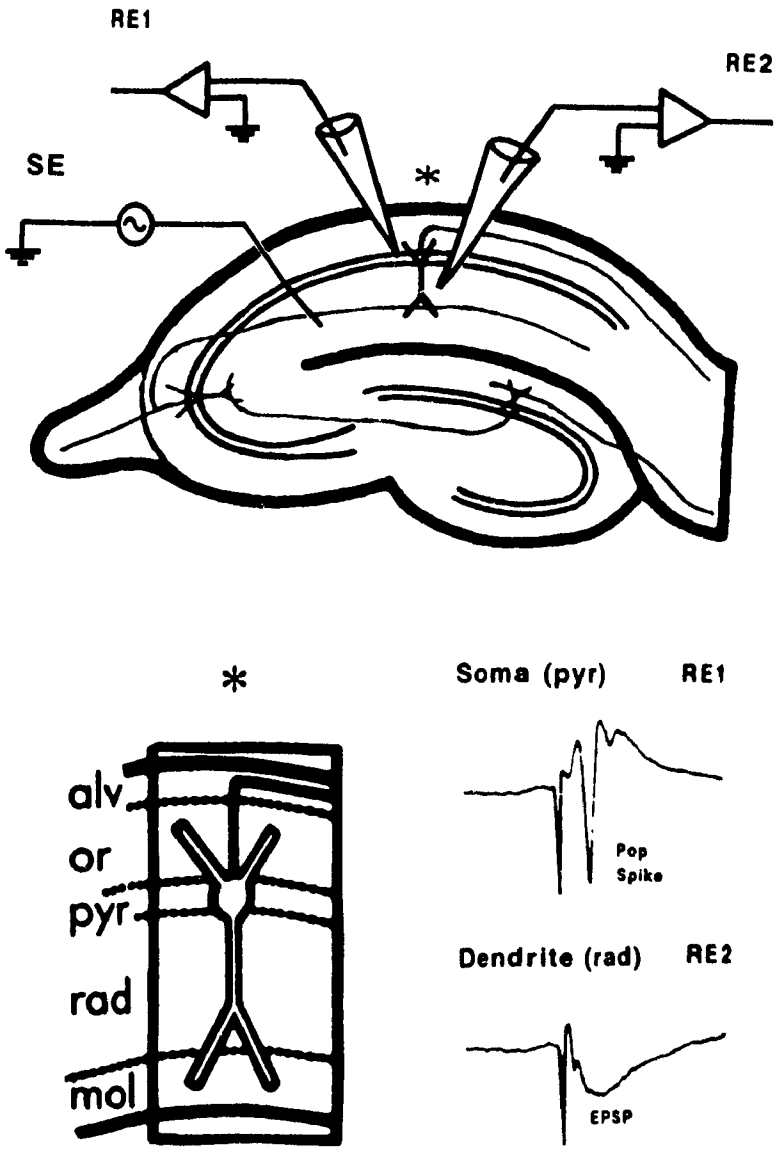

RE1
RE2
SE
*
*
alv
or
pyr
rad
mol
Soma (pyr)
RE1
Pop
Spike
Dendrite (rad)
RE2
EPSP

Figure 2

were fed to a negative capacitance, DC amplifier and displayed on paper or stored on video-tape for later analysis.

Arachidonic acid was purchased from Nu-Chek Prep, Elysian, MN. Its purity was assessed by thin layer chromatography and by gas chromatography and was found to be over 99%. It was kept in isooctane in the dark at -20°C and was prepared fresh on the day of the experiment by resolubilizing it in ethanol. Arachidonic acid (50 μM) was always added to the ACSF in presence of indomethacin (50 μM; obtained from Sigma, St-Louis, MO) in order to prevent its conversion into cyclooxygenase products. In some experiments NDGA (obtained from Sigma) was dissolved in ethanol and applied at a 50 μM concentration. Baicalein (obtained from Biomol, Plymouth Meeting,PA) was dissolved in ethanol and applied at a 3 μM concentration. The final concentration of ethanol in ACSF was always less than 0.2 %. Melittin (obtained from Sigma) was directly dissolved in ACSF at a concentration of 5 μg/ml.

B) Preparation and quantitation of 12-HPETE

12-HPETE (as an equimolar mixture of S and R isomers) was obtained from arachidonic acid by the same photochemical synthesis method described in the Materials and methods section of chapter 2 with some modifications. Following the photochemical reaction and after acidification of the reaction mixture with HCl, HPETEs were extracted twice with 4 ml ethyl ether. After evaporation of ethyl ether fractions to near dryness,

HPETEs were dissolved in approximately 1 ml of hexane.

Aliquots were injected on 4.6 x 250 mm Spherisorb HPLC column (Jones Chromatography, Mid Glamorgan, UK) with 5 μM particle size. Solvent system was hexane:isopropanol:acetic acid 100:1.5:0.1 at a flow rate of 1 ml/min. Elution was monitored at 235 nm. This system allows the resolution of 12,15,11,9,8 and 5-HPETE (see Figure 3). The first peak, corresponding to 12-HPETE was collected and stored at -80°C.

Just before performing electrophysiological experiments, an aliquot of 12-HPETE was taken and the compound redissolved in methanol. The amount of 12-HPETE was quantitated using the method of Boeynaems et al. (1980), which is based on the oxidation of triphenylphosphine by HPETEs at an equimolar ratio to form triphenylphosphine oxide. In brief, approximatively 2 nmole of 12-HPETE was allowed to react with 5 nmole of triphenylphosphine at room temperature in methanol. After solvent evaporation the dry residue was dissolved in methanol and analyzed by gas chromatography. The separation of triphenylphosphine and triphenylphosphine oxide was performed on a HP5890A instrument equipped with flame ionization detector and on-column injector. The column was a Hewlett Packard ultra1 cross-linked methyl silicone capillary column 50 m x 0.32 mm with a film thickness of 0.17 μm. Oven temperature was initially set at 230°C and increased at a rate of 10°C/min up to 285°C. The amount of 12-HPETE was given by reporting the amount of triphenylphosphine oxide on a

Figure 3: Separation by straight phase HPLC of the various hydroperoxyeicosatetraenoic acids (HPETEs) formed by photooxidation of arachidonic acid. Conditions were as described in the Material and methods section.

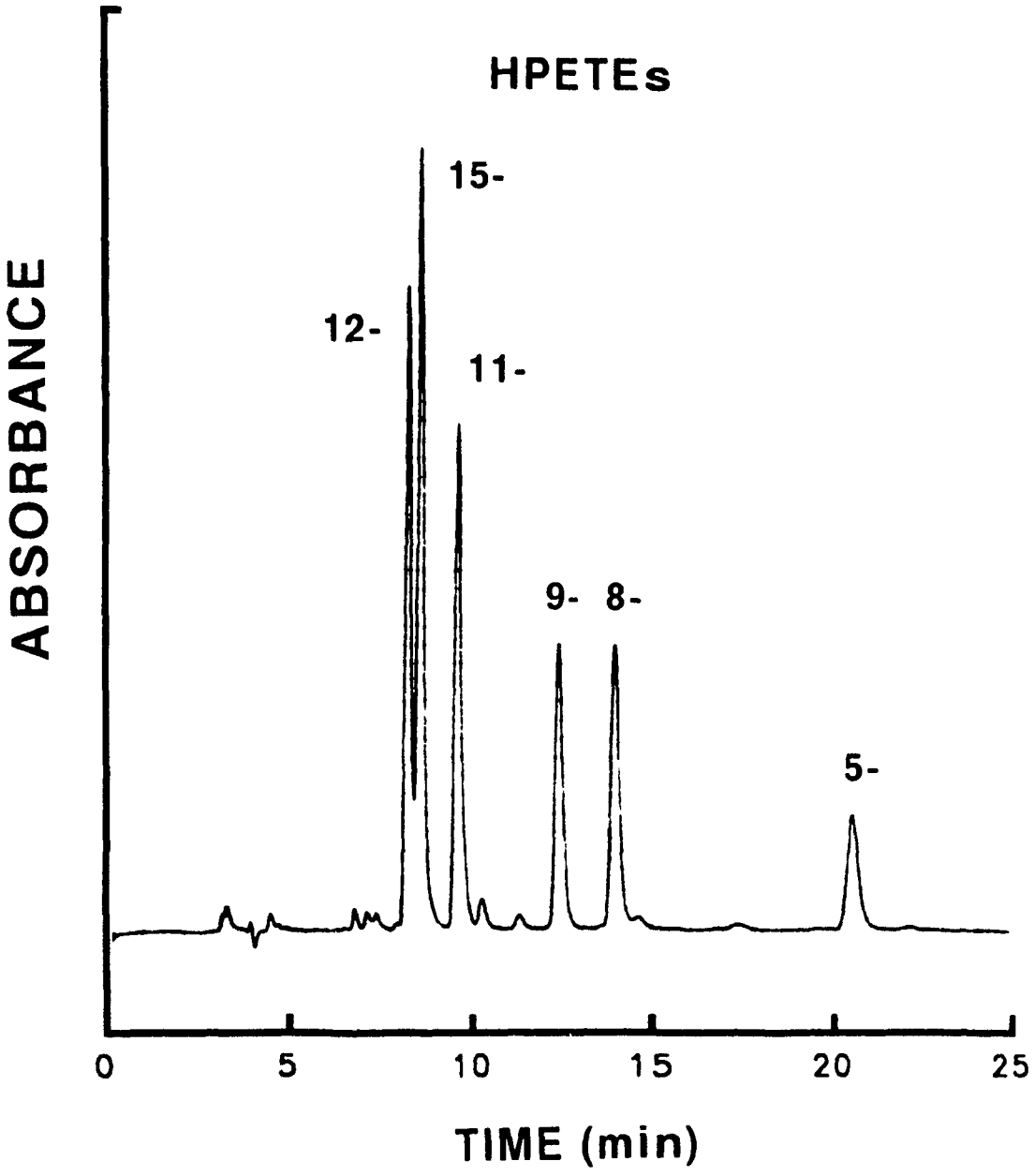


Figure 3

calibration curve made with hydrogen peroxide. Finally, the appropriate amount of 12-HPETE from the stock solution was redissolved in ethanol and applied at a 3 μM concentration on the slices.

RESULTS

A) **Effects of arachidonic acid on synaptic transmission**

The population spike response as well as the extracellularly recorded excitatory postsynaptic potential (EPSP) following a test stimulus were recorded. While the population spike amplitude is a reflection of the number of cells that synchronously fires an action potential, the slope and the amplitude of the EPSP give a measure of the postsynaptic depolarization induced by the stimulus. Both measures can be used as an index of synaptic efficacy (Bliss and Lynch, 1988). Perfusion of hippocampal slices with arachidonic acid for 30 minutes induced in 68.4% of the experiments an increase in the amplitude of both the extracellular EPSP recorded in stratum radiatum and in the population spike recorded in stratum pyramidale (Figure 4A). In the dendritic layer (s. radiatum), the effects induced by arachidonic acid were also expressed as an increase in the slope of the rising phase of the EPSP (Figure 4B) and were not accompanied by any changes in the properties of the presynaptic volley which could be detected in some experiments.

Figure 4: Long-lasting potentiation of synaptic transmission in CA1 hippocampal slice induced by a brief perfusion with arachidonic acid and indomethacin (50 μM). **A:** and **B:** Input-output curves obtained in the same experiment from the somatic and dendritic layers, respectively, in control condition (open circle, dashed line), at 30 minutes of perfusion with arachidonic acid and indomethacin (filled circle, filled line), and at 60 minutes of washout of the drugs (triangle, dotted line). **C:** Arachidonic acid was applied on the hippocampal slice for 30 minutes (black bar; AA). Stimulation was provided every 12 seconds and the amplitude of the population spike was constantly monitored. The stimulus intensity was set to obtain one third of the maximal amplitude. Traces **1**, **2** and **3** represent recordings from the pyramidal (top) and from the dendritic (bottom) layers, in control, during perfusion with AA and after 60 min of washing. The data are from one representative experiment out of thirteen with similar results.

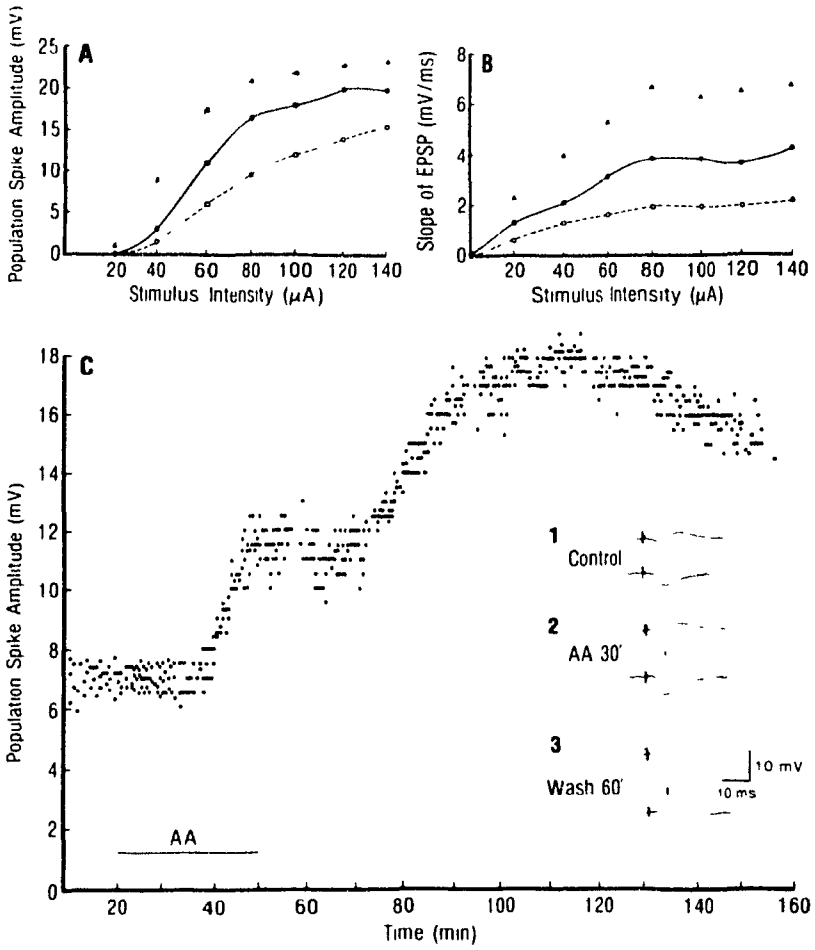

A
Population Spike Amplitude (mV)
Stimulus Intensity (μA)
B
Slope of EPSP (mV/ms)
Stimulus Intensity (μA)
C
Population Spike Amplitude (mV)
Time (min)
AA
1
Control
2
AA 30'
3
Wash 60'
10 mV
10 ms

Figure 4

The increase in excitability overlasted the perfusion of arachidonic acid (Figure 4C) and the potentiation even further increased after washout of arachidonic acid. In most of the experiments the response started to decrease 90 minutes after the removal of arachidonic acid and in one instance, the synaptic transmission was still potentiated relative to control 160 minutes after washing arachidonic acid. In all experiments the increase in synaptic transmission efficacy was best noted at low intensity of stimulation, although the response evoked by high intensity stimulation also increased. The average increase in population spike amplitude at low intensity of stimulation, defined as the stimulus strength able to elicit a population spike amplitude 20-30% of the maximal amplitude, was $232 \pm 126\%$ after washing arachidonic acid for 60 min.

In 26.3% of the experiments, perfusion with ACSF containing arachidonic acid for 30 minutes induced a decrease in the amplitude of the population spike (Figure 5). This effect was also associated with a decrease in the amplitude and the slope of the rising phase of the extracellular EPSP recorded in stratum radiatum. The depression of synaptic transmission developed during the perfusion of arachidonic acid and lasted between 20-60 minutes after its washing. The depression did not further develop after removal of arachidonic acid from the perfusate. The maintenance of this relatively long lasting depression of synaptic transmission was shorter in duration compared to the potentiation. In one case where extracellular recordings were performed simultaneously in both CA3 and CA1 subfields, arachidonic acid induced a long lasting potentiation of synaptic transmission in CA1 but a short lasting depression in CA3. In one experiment

Figure 5: Input-output curves of the response obtained in the cell body layer showing a long-lasting depression of synaptic transmission in CA1 induced by perfusion of the slice with arachidonic acid and indomethacin (50 μM). The response had been stable for more than 30 minutes (open circle, dashed line) before the application of arachidonic acid for 30 minutes (filled circle, filled line). Near maximal effect was already reached after 20 minutes of perfusion. The depression lasted 30 minutes and slowly recovered to control after 40 minutes of perfusion with ACSF (triangle, dotted line). The data presented are from one representative experiment out of five with similar results.

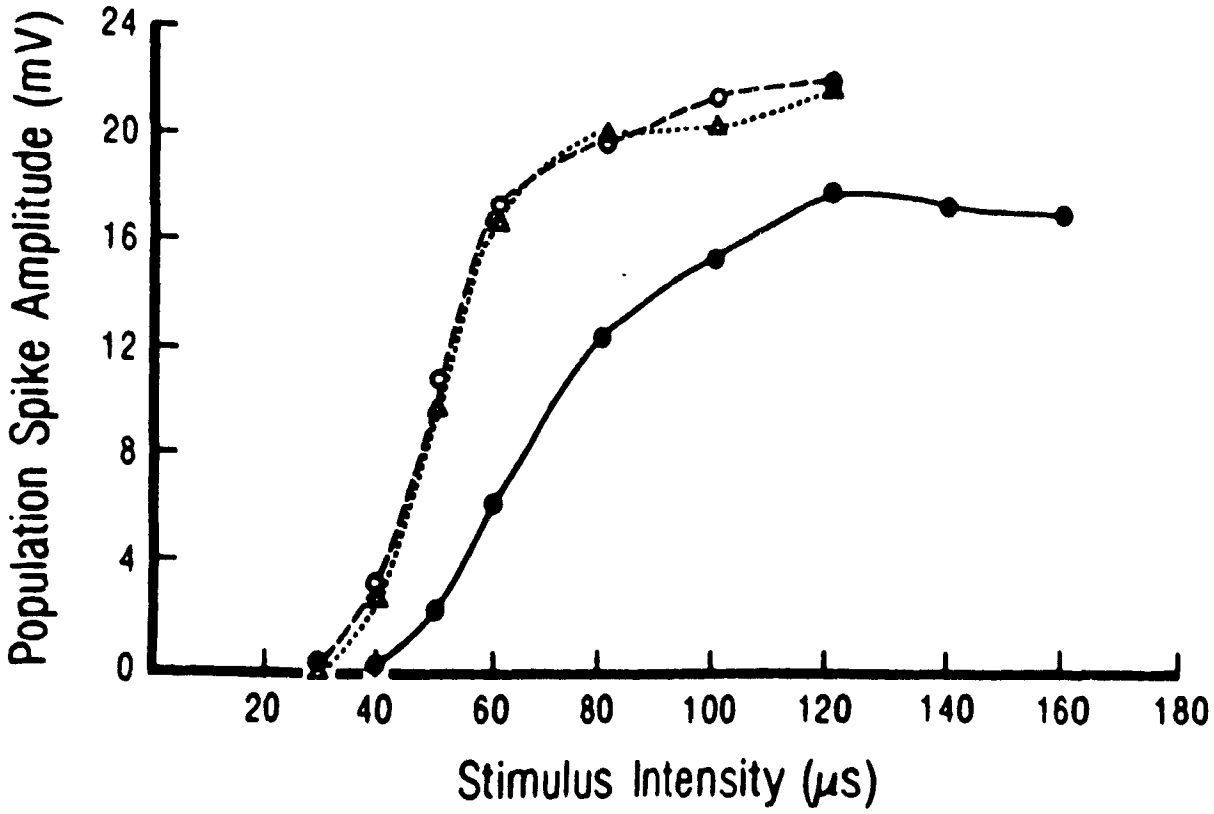

Population Spike Amplitude (mV)
24
20
16
12
8
4
0
20
40
60
80
100
120
140
160
180
Stimulus Intensity (μs)

Figure 5

arachidonic acid induced no effect.

B) Effect of nordihydroguaiaretic acid on arachidonic acid-induced potentiation

NDGA was used to determine whether the effects induced by arachidonic acid were caused by arachidonic acid itself or by one, or even several of its lipoxygenase metabolites. In these experiments NDGA was applied 15-30 minutes before the application of arachidonic acid and was continued during and after the perfusion of arachidonic acid. In 33.3% of the experiments, NDGA alone induced within 15-20 minutes a significant and reversible decrease of the amplitude of the stratum radiatum-induced response recorded in the pyramidal (population spike) and the dendritic layer (EPSP), while in the remaining experiments NDGA had no effect. In 77.8% of the experiments, perfusion of arachidonic acid in presence of NDGA (50 μM) did not affect the amplitude of both the population spike and the extracellular EPSP (Figure 6). After washout of NDGA, reperfusion of arachidonic acid alone induced a reversible depression of synaptic transmission. We have not been able to induce a long lasting potentiation of synaptic transmission in any slice that had been previously exposed to NDGA. When NDGA was applied on an established, arachidonic acid-induced potentiation however it did not affect the maintenance of the potentiation (Figure 7).

Figure 6: Sensitivity of the arachidonic acid-induced changes in excitability to the lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA). Stratum radiatum induced responses were recorded in stratum pyramidale. Arachidonic acid (AA) and indomethacin (50 μM) were applied in presence of NDGA (50 μM) which inhibited the action of arachidonic acid. After the washout of NDGA, arachidonic acid induced in the same slice a reversible depression of synaptic transmission. This represents the data from a typical experiment out of seven with similar results.

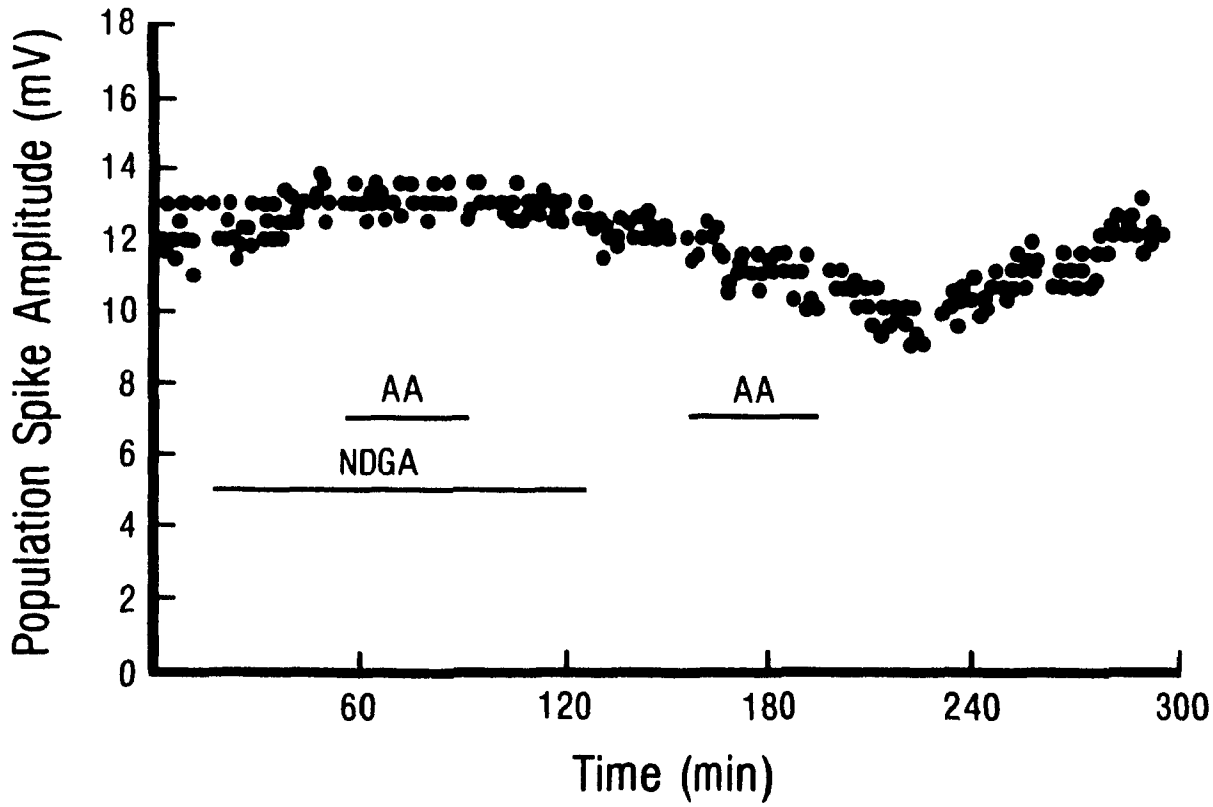

Population Spike Amplitude (mV)
18
16
14
12
10
8
6
4
2
0
AA
AA
NDGA
60
120
180
240
300
Time (min)

Figure 6

Figure 7: Effect of nordihydroguaiaretic acid (NDGA) on established potentiation. Application of arachidonic acid (AA) for 20 minutes induced a long lasting potentiation of synaptic transmission as measured by the amplitude of the population spike. After the response had reached a plateau, application of NDGA (50 μM) did not affect it. This represents the data from one out of two experiments with similar results.

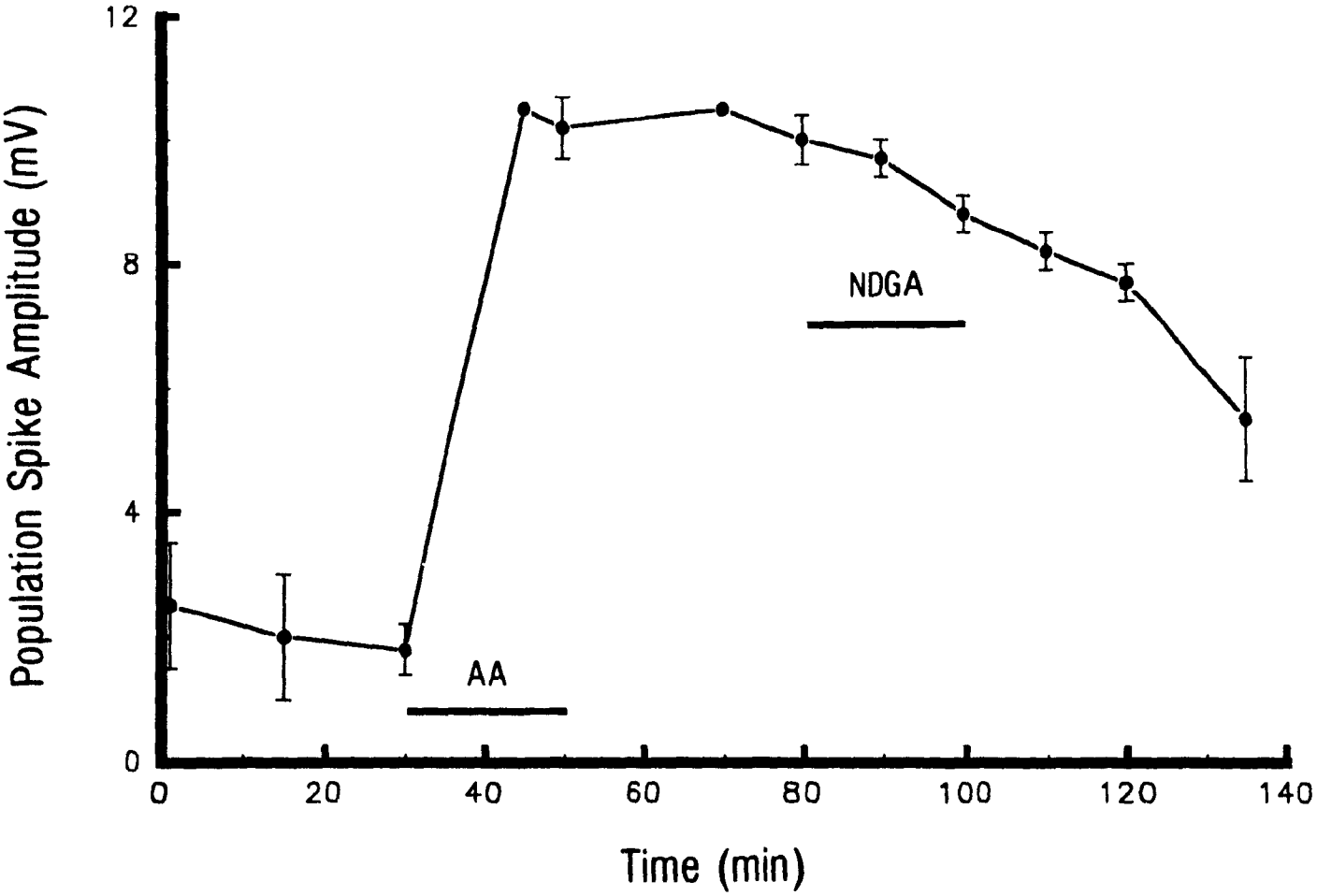

12
8
4
0
Population Spike Amplitude (mV)
AA
NDGA
0
20
40
60
80
100
120
140
Time (min)

Figure 7

C) Effect of melittin on synaptic transmission

Melittin, a 26 amino acid peptide isolated from bee venom has been frequently used as a stimulator of arachidonic acid release, via phospholipase A_2 activation, and metabolism (Shier, 1979; Salari et al., 1985). It was used here to determine if endogenously generated arachidonic acid could mimic the effect observed with exogenously applied arachidonic acid on synaptic transmission. Melittin, when perfused at a concentration of 5 μg/ml induced a dramatic and rather long lasting increase in the amplitude of the population spike (Figure 8). Following return of the population spike amplitude to close to the basal level, reapplication of melittin in presence of NDGA (50 μM) did not produce a similar increase in population spike amplitude.

D) The 12-lipoxygenase pathway and synaptic transmission

Baicalein has been described as a more specific inhibitor of the 12-lipoxygenase (Sekiya and Okuda, 1982). It has been used here in order to assess the possibility that 12-lipoxygenase metabolites might be involved in the mechanism giving rise to long-term potentiation. Baicalein, at a concentration of 3 μM, was found to prevent tetanically-induced LTP (Figure 9).

12-hydroperoxyeicosatetraenoic acid (12-HPETE) is the product of the reaction catalyzed by the 12-lipoxygenase with arachidonic acid as substrate. It is also the

Figure 8: Long lasting potentiation of synaptic transmission in the CA1 area of the hippocampal slice induced by a brief perfusion with melittin (5 μg/ml) and indomethacin (50 μM). The effect was observed as an increase in the population spike amplitude. After it had declined to almost basal level, application of melittin at the same concentration in presence of nordihydroguaiaretic acid (NDGA) at a concentration of 50 μM did not produced a similar response. This represents the data from one out of two experiments with similar results.

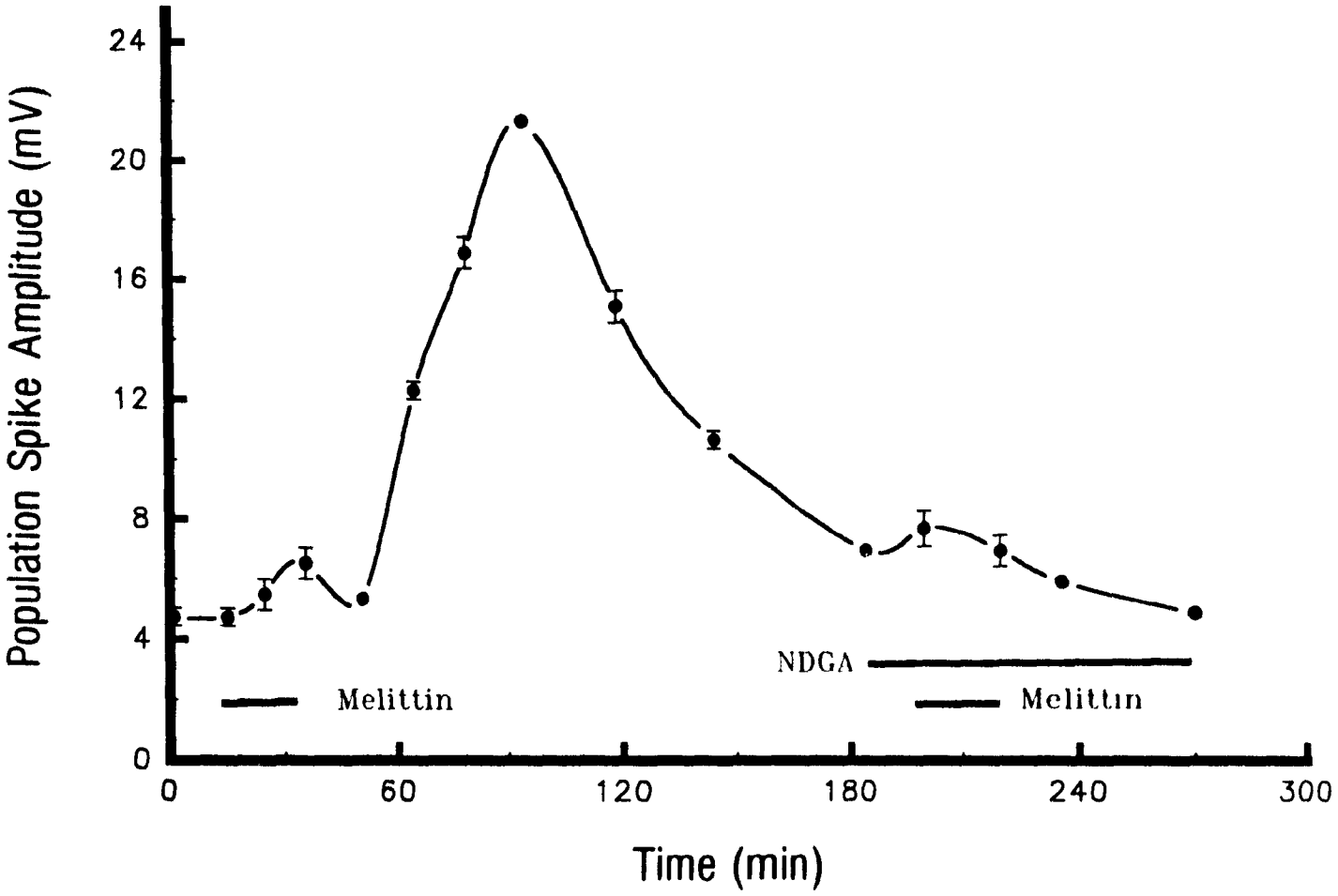

Population Spike Amplitude (mV)
24
20
16
12
8
4
0
0
60
120
180
240
300
Time (min)
Melittin
NDGA
Melittin

Figure 8

Figure 9: Effect of baicalein on tetanically-induced LTP. The graph shows the population spike amplitude during perfusion of baicalein (3 μM) prior to the tetanus (circle, filled line), 10 minutes after the tetanus (open triangle, dotted line) and 40 minutes after the tetanus (filled triangle, dashed line). Potentiation was completely prevented by the drug. This represents the data from one out of two experiments with similar results.

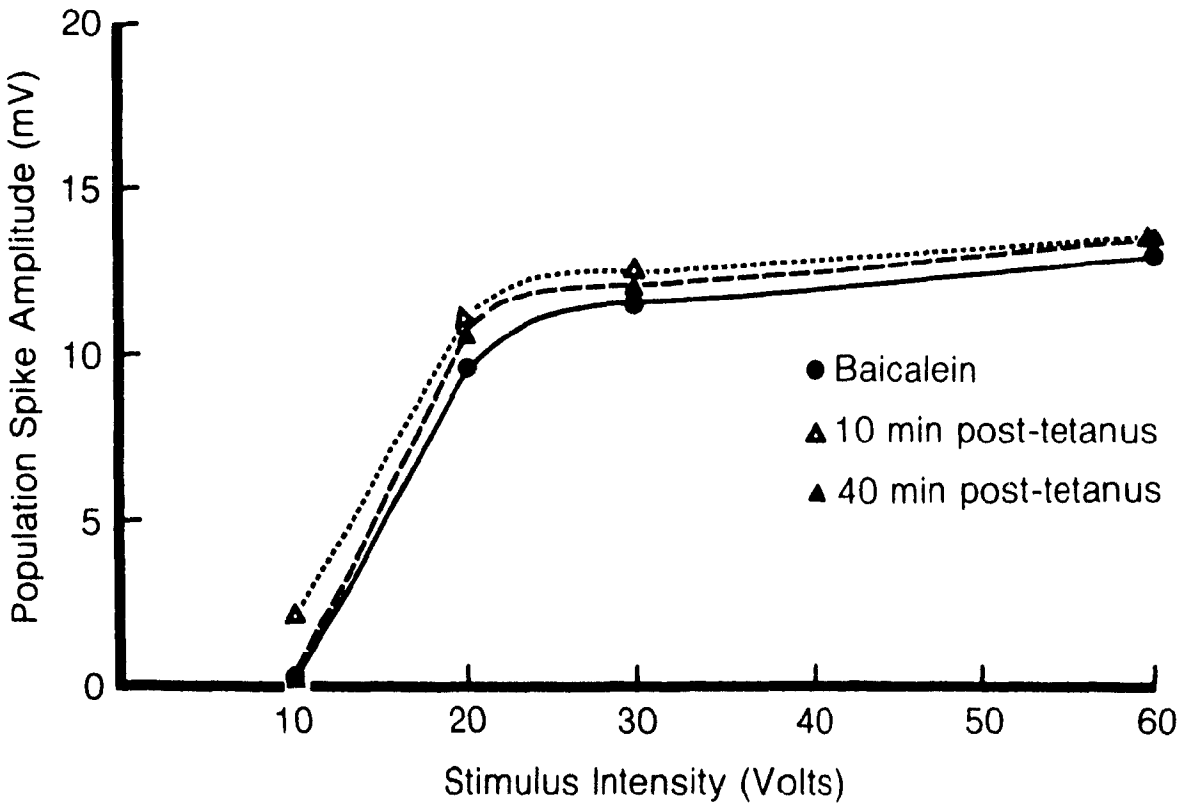

Population Spike Amplitude (mV)
20
15
10
5
0
10
20
30
40
50
60
Stimulus Intensity (Volts)
● Baicalein
▲ 10 min post-tetanus
▲ 40 min post-tetanus

Figure 9

Figure 10: Long lasting depression of synaptic transmission in area CA1 of the hippocampus induced by a brief perfusion with 12-hydroperoxyeicosatetraenoic acid (12-HPETE). Traces at the top represent population spikes obtained at three selected stimulus intensities before 12-HPETE application (control), after applying 12-HPETE for 20 minutes and after 60 minutes washout. The graph at the bottom shows the population spike amplitude after obtaining a stable response (Control; open circle, dotted line), after 20 minutes of perfusion with 12-HPETE (closed circle, filled line) and after washing the compound for 60 minutes (triangle, dashed line). The data shown constitute one representative experiment of three with similar results.

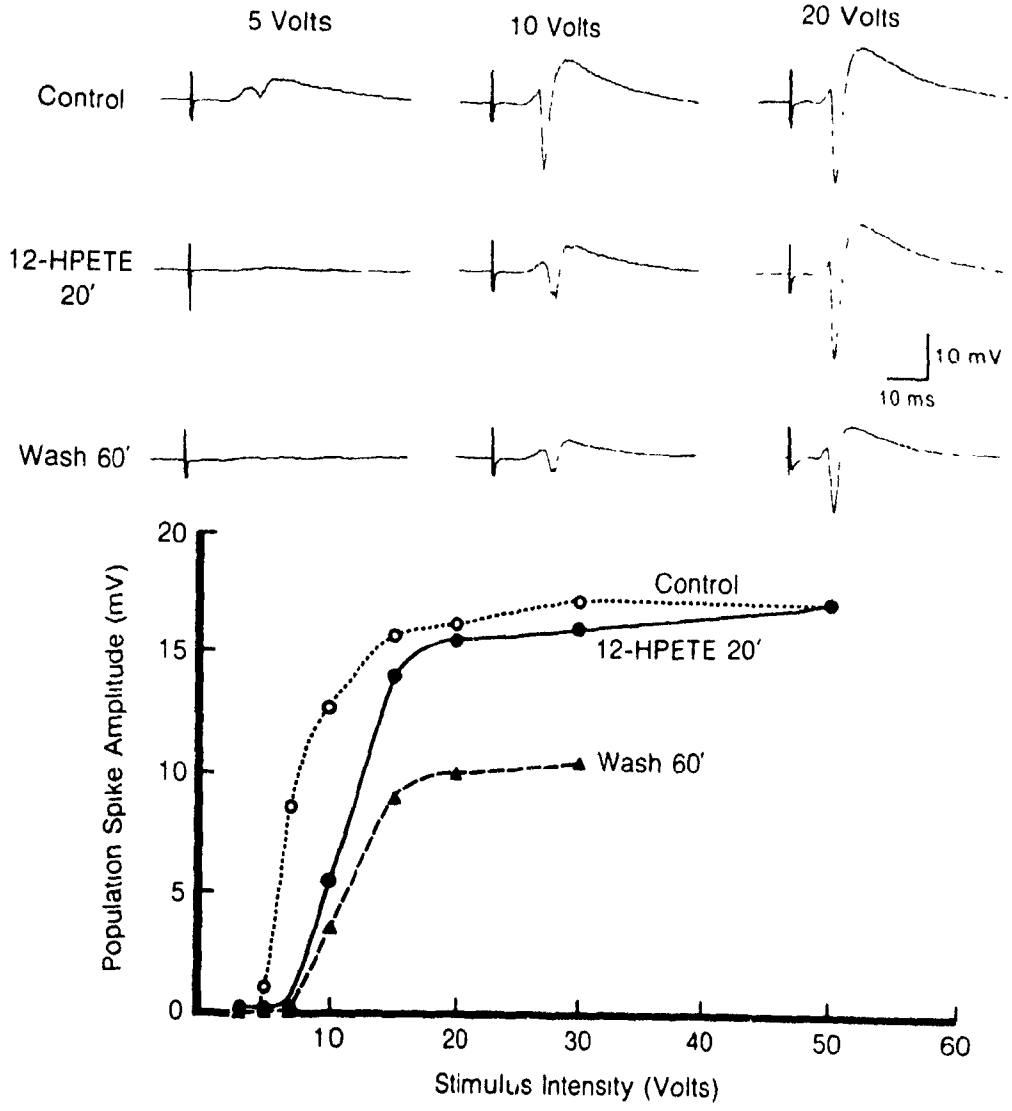

5 Volts
10 Volts
20 Volts
Control
12-HPETE
20'
Wash 60'
10 mV
10 ms
Population Spike Amplitude (mV)
20
15
10
5
0
Control
12-HPETE 20'
Wash 60'
10
20
30
40
50
60
Stimulus Intensity (Volts)

Figure 10

precursor of all the metabolites belonging to the 12-lipoxygenase pathway including 12-hydroxyeicosatetraenoic acid (12-HETE) and the hepoxilins (see Shimizu and Wolfe, 1990). 12-HPETE was synthesized by photooxidation of arachidonic acid and its effect on synaptic transmission in the CA1 subfield of the hippocampal slice was evaluated. Application of 12-HPETE at a concentration of 3 μM induced a long lasting decrease in the amplitude of the population spike (Fig. 10). The effect was originally more evident at low intensity of stimulation but further developed upon washing and was then evident at all stimulus intensities.

DISCUSSION

A) Modulation of synaptic transmission by arachidonic acid

The results reported here demonstrate that arachidonic has a major effect on synaptic transmission. In a majority of slices studied (68.4%), arachidonic acid induced a long lasting potentiation of synaptic transmission as measured by an increase in both the population spike amplitude and the slope of the extracellular EPSP in the CA1 subfield of the hippocampus. These results are consistent with those obtained *in vivo* in the dentate area of the hippocampus by Williams et al. (1989). A few points however deserve some comments.

Williams et al. observed a potentiation of the extracellular EPSP when they paired

arachidonic acid application with the delivery of weak tetani that were subthreshold for the induction of long-term potentiation. When arachidonic acid was applied alone (only in presence of the regular test stimulus), a reversible depression was observed. In the experiments reported here, a majority of slices exhibited long-lasting potentiation after arachidonic acid application without having to use any other stimulus than the normal test shock. A small proportion of slices (26.3%) displayed however a depression. A possible explanation for these discrepancies might lie in the frequency used for the test shocks. While the experiments here were performed with a test stimulus frequency of 0.083 Hz, Williams et al. reported using a lower frequency (0.033 Hz). Moreover they have also observed that increasing the frequency of test shocks from 0.033 to 0.25 Hz during arachidonic acid application allowed them to see potentiations without having to use weak tetani. What can be concluded from these observations is that arachidonic acid seems to require at least another concomitant event, here activated by test shocks, in order to elicit a long lasting potentiation. This other event, which is activated by electrical stimulation, displays a frequency-sensitive threshold above which potentiation can be induced when paired with arachidonic acid application. Below this threshold, a depression would be elicited by arachidonic acid. It can be added that at least the potentiation appears specific to arachidonic acid since no other fatty acid including unsaturated ones were reported to have the same effect (Bliss et al., 1991).

What might be the nature of this accompanying event is at this point speculative. It is possible that the test stimulus allows the build up of a certain level of Ca^{2+} in the

presynaptic terminal. Coupled with arachidonic acid, this might be sufficient to activate a kinase, such as the protein kinase C which could in turn, through phosphorylation of key proteins, favor neurotransmitter release (see Linden and Routtenberg, 1987). Such an increase in neurotransmitter release is one mechanism proposed to be responsible for the enhancement of synaptic transmission observed in LTP (Lynch, 1989). Arachidonic acid has already been reported to activate protein kinase C and enhance glutamate release in presence of small amounts of Ca^{2+} (Shearman et al., 1989; Shearman et al., 1991; Shinomura et al., 1991; Lynch and Voss, 1990; Freeman et al., 1990). This does not explain however how sometimes a depression results. More work will be needed to determine the exact mechanism that explains this dual effect of arachidonic acid.

Melittin on the other hand provided the opportunity to verify whether endogenously released arachidonic acid can elicit the same changes in synaptic transmission and thus possibly be a component of LTP. It was based on the assumption that melittin can effectively induce arachidonic acid release in hippocampal slices as it does in other preparations (Shier, 1979; Salari et al., 1985). Melittin was found to induce a long lasting potentiation of synaptic transmission which resembles the effect obtained with exogenously applied arachidonic acid. In addition, it was later confirmed that melittin elicits arachidonic acid release in hippocampal slices (Pellerin and Wolfe, 1991; see also chapter 4). One distinctive feature of the effect observed with those two treatments is the decremental aspect of the potentiation once it has reached its maximum. On the contrary tetanically-induced LTP can remain stable for hours in slices (see Kuba

and Kumamoto, 1990 for a review). This might be taken as an indication that other processes, either in parallel or in association with arachidonic acid release, are necessary to lead to a persistent potentiation.

B) Modulation of synaptic transmission by lipoxygenase metabolites of arachidonic acid

The lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA) was used in order to evaluate the possible involvement of lipoxygenase metabolites in the effects obtained on synaptic transmission with arachidonic acid. It has been previously shown that NDGA can either block or reduce both calcium-induced and tetanically-induced LTP (Williams and Bliss, 1988; Williams and Bliss, 1989; Lynch et al., 1989). It was reported however that NDGA was unable to prevent arachidonic acid-induced potentiation (Williams et al., 1989). Previous results obtained with NDGA were then explained on the basis that it can also inhibit the phospholipase A_2 at high concentration, thus preventing arachidonic acid release. It was argued that arachidonic acid itself and not its lipoxygenase metabolites is responsible for the potentiating effect observed. This is in contrast with results reported here. NDGA was found to prevent the effect of both arachidonic acid and melittin on synaptic transmission. Since in the case of melittin the effect of NDGA was tested after obtaining a first potentiation, caution should be exerted until it is demonstrated that melittin can elicit two consecutive potentiations in the same slice. In addition, NDGA does not appear to affect the pre-established potentiation, an observation which would

restrict the involvement of lipoxygenase metabolites to the induction phase of the potentiation. The discrepancy noted above emphasized the need to use in the future more specific inhibitors of lipoxygenases or different approaches in order to investigate further the possible involvement of lipoxygenase metabolites in modulating synaptic transmission.

Lynch and Voss (1990) have recently shown that both 12-hydroxyeicosatetraenoic acid (12-HETE) and 12-hydroperoxyeicosatetraenoic acid (12-HPETE) can increase glutamate release from synaptosomes prepared from the dentate area of the hippocampus. On the contrary, Freeman et al. (1991) have observed an attenuation of glutamate release evoked by depolarization following exposure of synaptosomes prepared from mossy fiber nerve endings (area CA3) to 12-lipoxygenase products. Interestingly in one of the experiments reported here where dual recording from area CA1 and CA3 was obtained, arachidonic acid was found to cause simultaneously a potentiation in area CA1 and a depression in area CA3. Whether 12-lipoxygenase metabolites could be responsible for this dual effect of arachidonic acid as suggested by the aforementioned studies remains to be seen but this is certainly an attractive possibility.

To further investigate the possibility that 12-lipoxygenase metabolites could modulate synaptic transmission or even participate in long-term potentiation, two types of experiments were performed. First the 12-lipoxygenase inhibitor baicalein was found to prevent tetanically-induced LTP. Although this could argue in favor of a participation of 12-lipoxygenase products in LTP, it is not known whether baicalein can inhibit

phospholipase A_2 and thus also prevent arachidonic acid release. Moreover, baicalein was reported to inhibit leukocyte 5-lipoxygenase (Kimura et al., 1987). Caution should then be exerted in interpreting these results until a more detailed study is performed. Finally application of 12-HPETE, the first product of the 12-lipoxygenase pathway, induced a rather long-lasting depression of synaptic transmission. This effect appears more compatible with the results obtained by Freeman et al. (1991) in mossy nerve endings than those of Lynch et al. (1990) in synaptosomes from the dentate area as presented before. Since the number of experiments was small (n=3), it might be necessary to carry out more experiments to make sure that a potentiating effect could not also be observed. The situation may well be analogous to what has been seen in the mollusk *Aplysia californica*. In this organism, it was observed that 12-HPETE has different effects at two distinct sites (hyperpolarization in cell L10, depolarization-hyperpolarization in cell L14, see Feinmark et al., 1990). The dual effect obtained in one cell was shown to be mediated by different metabolites. The one responsible for the hyperpolarization is hepoxilin A_3 (HxA_3), which has been shown to be formed in the rat hippocampus (Pace-Asciak, 1988). HxA_3 was also shown to prevent 4-aminopyridine-induced neurotransmitter release in hippocampal slices (Pace-Asciak et al., 1990) as well as having effects compatible with a depression intracellularly (Carlen et al., 1989). So this compound could be responsible for the depression that was observed with both arachidonic acid and 12-HPETE. It could then be interesting to investigate whether a metabolite such as 12-ketoeicosatetraenoic acid (12-KETE), which mediate the depolarizing effect of 12-HPETE in *Aplysia* (Piomelli et al., 1988), could be formed in

the rat hippocampus and could affect synaptic transmission.

In summary, evidence has been obtained that both arachidonic acid and 12-lipoxygenase metabolites modulate synaptic transmission. It was suggested that one (or both) may participate in the phenomenon of long-term potentiation. A more complete demonstration would require however that arachidonic acid release and/or formation of 12-lipoxygenase metabolites be shown to occur selectively following conditions which are known to lead to the synaptic modification that underly long-term potentiation. In the previous chapter, it was demonstrated that activation of NMDA receptors, which represents a necessary step for the induction of LTP, causes a selective increase in the formation of 12-HETE in the cerebral cortex. Recently Bliss and his coworkers have observed an increase in the level of free arachidonic acid, 12-HETE and 12-HPETE following the induction of LTP in the dentate area of the hippocampus *in vivo* (Lynch et al., 1991; Bliss et al., 1991). Using the *in vitro* hippocampal slice preparation, the question of whether arachidonic acid release occurs following conditions which can potentially induce LTP will be more specifically addressed in the next chapter.

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Chapter 4

PHARMACOLOGICAL INDUCTION OF A LONG LASTING POTENTIATION OF SYNAPTIC TRANSMISSION IN RAT HIPPOCAMPAL SLICES: EFFECT ON ARACHIDONIC ACID RELEASE

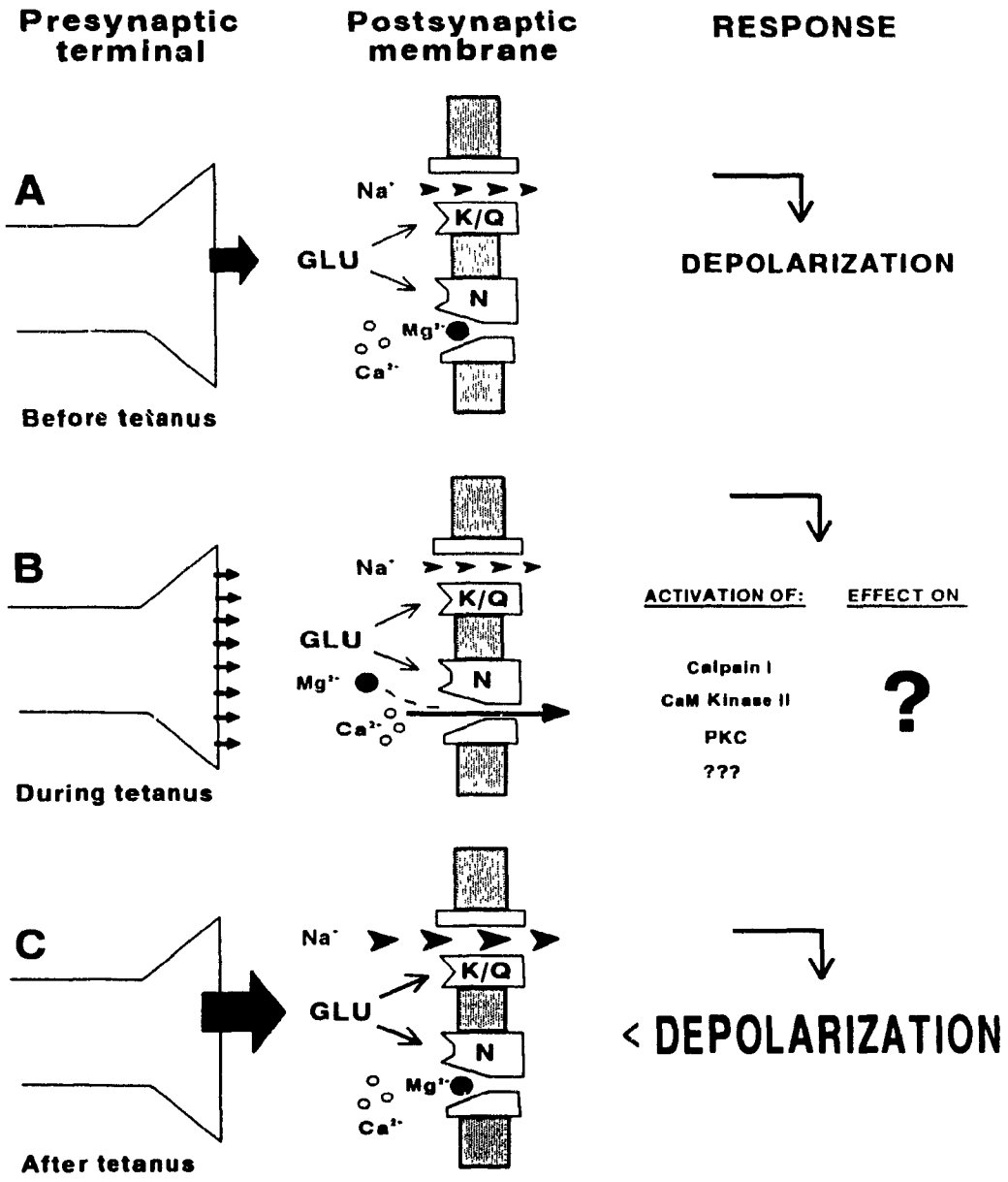
INTRODUCTION

In the past few years, the molecular steps leading to induction of long-term potentiation (LTP) in the hippocampus have been elucidated (for review, see Collingridge and Bliss, 1987). Following a high frequency tetanic stimulation of afferent fibers, glutamate is released in large amounts from the presynaptic terminals and binds to its postsynaptic receptors. Activation of glutamate receptors of the kainate/quisqualate class causes sufficient depolarization of the postsynaptic cell membrane to relieve the Mg^{2+} blockade on N-methyl-D-aspartate (NMDA) receptors. Calcium then enters the cell via the ion channel associated with the NMDA receptor and activates a series of Ca^{2+}-dependent processes which initiate a cascade of unknown events leading at the end to a persistent enhancement of synaptic transmission (see Figure 1). So far, activation of the protease calpain I (Lynch and Baudry, 1984) as well as the Ca^{2+}/calmodulin-dependent kinase and protein kinase C (Akers et al., 1986; Malinow et al.. 1989) have been reported but other molecular mechanisms are likely to be involved.

The mechanism responsible for maintenance of LTP has remained on the other hand rather poorly understood. Over the years, models have mainly focused around two possibilities: (1) an increase in presynaptic glutamate release (Lynch et al., 1989; see Lynch, 1989 for review) or (2) a change in sensitivity or in the number of postsynaptic glutamate receptors (Kauer et al., 1988a; see Lynch et al., 1990 for review). The presynaptic hypothesis has recently received some support from quantal analysis studies

Figure 1: The three steps leading to long-term potentiation (LTP). **A:** During normal transmission that precedes induction of LTP, stimulation of afferent fibers leads to depolarization of the postsynaptic membrane, mostly due to the activation of kainate/quisqualate type of receptors by the excitatory neurotransmitter glutamate. **B:** A series of repetitive, high-frequency stimulation or tetanus applied to the afferent fibers will cause massive release of glutamate in the synaptic cleft which will in turn induce an important and sustained depolarization of the postsynaptic membrane. This allows removal of the Mg^{2+} that normally prevents activation of the NMDA receptor. As a consequence, binding of glutamate to the receptor now leads to Ca^{2+} influx through the channel associated with the receptor. A cascade of Ca^{2+}-dependent processes is then initiated, a number of these still not characterized, with effects on unknown targets which also remain to be determined. **C:** Finally upon normal afferent fiber stimulation some time after the tetanus, a greater depolarization of the postsynaptic membrane is obtained. A major feature of this phenomenon is that this potentiating effect persists for hours, days and even weeks *in vivo* after its induction. Whether the maintenance mechanism involves an increase in presynaptic glutamate release, an increase in sensitivity or number of postsynaptic glutamate receptors, or a combination of both is still debated.

Long-term potentiation



modified from Collingridge and Bliss,TINS,7 288,1987

Figure 1

(Voronin, 1988; Malinow and Tsien, 1990; Bekkers and Stevens, 1990 but see also Foster and McNaughton, 1991 for a different view). Since the induction of LTP depends on a postsynaptic event (activation of NMDA receptors), it becomes necessary for a presynaptic model to postulate the existence of a retrograde messenger. Bliss and his coworkers (Williams and Bliss, 1989; Williams et al., 1989) have recently proposed that arachidonic acid might play such a role. Conversely, a number of reports have argued in favor of a postsynaptic mechanism involving an increase in the sensitivity or in the number of postsynaptic glutamate receptors of the kainate/quisqualate type. An increase in sensitivity, detected as an increase in binding of a specific agonist for glutamate receptors of the kainate/quisqualate class, was shown to be dependent on the activation of phospholipase A_2 (Massicotte et al., 1991). This suggests that perhaps arachidonic acid and/or its metabolites might also have a role to play in a postsynaptic aspect of the mechanism responsible for the maintenance of long-term potentiation.

It remains to be determined whether arachidonic acid is actually released following induction of LTP. It has been reported that activation of NMDA receptors induces arachidonic acid release and/or 12-HETE formation in striatal neurons (Dumuis et al., 1988), cerebellar granule cells (Lazarewicz et al., 1988; Lazarewicz et al., 1990), cortical slices (Wolfe et al., 1990) and hippocampal neurons (Sanfeliu et al., 1990; Patel et al., 1990). It appears to be restricted to neurons since it was not observed in astrocytes (Sanfeliu et al., 1990). In addition it has been reported that induction of LTP in the dentate gyrus of the rat hippocampus *in vivo* leads to a sustained increase in the

concentration of free arachidonic acid as well as 12-HPETE and 12-HETE, as measured by the push-pull cannula technique (Lynch et al., 1989; Lynch et al., 1991; Bliss et al., 1991).

In order to investigate this question further in vitro, the following steps were taken: (1) development of a perfusion bath and incubation chamber system that maintains hippocampal slices viable and potentially able to express LTP while being convenient enough for biochemical experiments, (2) demonstration that arachidonic acid release can be obtained in this preparation using melittin, a phospholipase A_2 activator, as a positive control and finally, (3) observation of the effect on arachidonic acid release of pharmacological manipulations, involving excitatory amino acids and their receptors, which have been shown to induce LTP electrophysiologically.

MATERIAL AND METHODS

A) Tissue preparation and incubation

Adult male (300-325g; 90 days old) or young (100-125g; 17 days old) Sprague-Dawley rats were decapitated without anesthesia. Their brain were rapidly removed from the skull and put in ice-cold oxygenated artificial cerebrospinal fluid (ACSF). The composition of ACSF was as follows (in mM): NaCl 124, KCl 2, $CaCl_2$ 2, $MgSO_4$ 2, KH_2PO_4 1.25, $NaHCO_3$ 26, glucose 10 (pH 7.4). The two hemispheres were separated

and the hippocampus from each hemisphere was dissected free. Each hippocampus was then cut transversely into 500 μm sections using a McIlwain tissue chopper (see Teyler, 1980 or Dingledine, 1984 for further details on the technique). Slices were placed in a modified Haas-type perfusion chamber and were kept at the interface between oxygenated ACSF and a humidified atmosphere made of 95% O_2 and 5% CO_2. The slices were perfused continuously at a rate of 0.5-1.0 ml/min with oxygenated ACSF warmed at a temperature of 34.0$\pm$0.5°C for at least one hour prior to their use for experimental testing. This period of time has been shown to be essential for electrophysiological and metabolic recovery (Whittingham et al., 1984). Both the perfusion bath and the temperature controller were made by the Department of Neuroelectronics, Montreal Neurological Institute, Montreal, Canada. The temperature probe (YSI model 403) was purchased from VWR Scientific, Weston, Ontario, Canada.

For incubations, slices were transferred into incubation chambers consisting of 20 ml borosilicate vials containing 1 ml of oxygenated ACSF (4 slices/vial). Chambers were maintained in a water bath kept at 36.0$\pm$1.0°C, gently shaken and supplied with a warmed and humidified gas mixture of 95% O_2 and 5% CO_2 at a flow rate of 1 L/min. Melittin and parachloromercuribenzoate (pCMB) were added to the incubation medium at a final concentration of 5 μg/ml and 100 μM respectively. L-glutamate and NMDA were added at a final concentration of 100 μM. For the first conditioning solution (CS), ACSF was replaced by a low Mg^{2+}-high K^+ solution, which consists of ACSF without added Mg^{2+} and 13.75 mM KCl (final $[K^+]$=15 mM). Then glutamate at a final

concentration of 100 μM was added. For the second conditioning solution (LTP), ACSF was replaced by a low Mg^{2+} solution consisting of ACSF without added Mg^{2+}. Then an NMDA-glycine-spermine mixture was added to obtain final concentrations of 100 μM, 10 μM and 850 μM respectively. MK-801 ((+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine maleate), a non-competitive NMDA receptor antagonist, was added to the perfusion medium at a final concentration of 10 μM. Slices were perfused with this medium for 2 hrs (following 1 hr in normal medium) prior to incubations. MK-801 was also added to the incubation medium at the same concentration. AMPA (α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid) was used at a concentration of 30 μM. All chemicals were from Sigma (St-Louis, Mo) except MK-801 that was a gift from Dr. M. Avoli, Neurophysiology Dept., Montreal Neurological Institute, Montreal, Canada and AMPA which was obtained from Tocris Neuramin, Bristol, UK. Incubation was stopped by dipping the incubation chambers in liquid nitrogen and storing them in a deep freezer at -80°C until the extraction was started.

B) Lipid extraction and purification of free fatty acids

Lipid extraction from hippocampal slices was performed according to the method of Bligh and Dyer as described by Christie (1982). Briefly, individual samples were taken out of the freezer and 5 μg of heptadecanoic acid ($C_{17\,0}$) was added to each to serve as internal standard. Five ml of chloroform:methanol 1:2 was then added and each sample was homogenized with a "Polytron" (Brinkmann Instruments, Rexdale, Ont,

Canada). An amount of 1.25 ml of chloroform was added and the resulting mix was transferred to a test tube, followed by the addition of 1.25 ml of a solution of 0.88 % KCl. The samples were then centrifuged in a clinical centrifuge at top speed for ~20 min. The lower phase of each sample was removed with a pasteur pipette, evaporated to dryness with a stream of nitrogen and resuspended in about 50 μl of hexane. The upper phase was thrown out and the protein interface was kept for protein determination.

Whatman silica gel TLC plates, 20 x 20 cm, 250 μm thickness (Chromatographic specialties, Brockville, Ont, Canada) were first washed with (1) chloroform:methanol:acetic acid:water 52:20:7:3 and (2) hexane:diethylether:acetic acid 90:10:1 prior to activation at 100°C for 1 hr. After the samples and arachidonic acid (as a free fatty acid marker) were applied on TLC plates, these were developed in hexane:diethylether:acetic acid 120:30:3. The lanes containing the marker were sprayed with a solution of phosphomolybdic acid (10% in ethanol) and the position of the compound revealed by blowing hot air over it to form a blue spot. The corresponding region for each sample was carefully scraped off and the free fatty acids eluted from the silica gel with chloroform:methanol 2:1.

C) Quantitation of free fatty acids by gas chromatography

The free fatty acids were first converted to their methyl ester derivatives using ethereal diazomethane (procedure described in chapter 2). Separation of the various

compounds was obtained with an HP5890A gas chromatograph equipped with on column injection port and flame ionization detector (Hewlett-Packard,Canada). An Ultra1 cross-linked methyl silicone capillary column (length 50 m; film thickness 0.52 μm; int. diam. 0.31 mm ; obtained from Hewlett-Packard) was used with a temperature gradient from 100 to 285°C and comprising multiple ramps to optimize separation and minimize length of analysis. Integration was performed by an HP3390A integrator connected to the gas chromatograph. All fatty acids used as standards including the internal standard were obtained from Nu-Chek-Prep, Elysian, MN.

D) Electrophysiological measurements

Evaluation of hippocampal slice viability was carried out directly on the slices as they were sitting in the modified perfusion bath, or in the case of the incubation chambers after the slices had been transferred into a conventional Haas-type tissue chamber. They were kept at the interface between oxygenated ACSF and humidified atmosphere gassed with 95% O_2 and 5% CO_2, and were perfused with oxygenated ACSF at a rate of 0.3-0.5 ml/min. A recovery period of at least 15 minutes was allowed prior to any testing when slices had to be transferred. Extracellular recordings were obtained from the pyramidal layer of the CA1 subfield using glass microelectrodes (resistance: 40-60 MΩ) filled with 3 M NaCl. The Schaffer collaterals were stimulated via a bipolar electrode placed in the stratum radiatum of area CA2. Signals were fed to a negative capacitance, DC amplifier and displayed on an oscilloscope and a Gould pen writer.

Amplitude of the population spike was measured by hand and input/output curve was plotted from these measurements.

E) Other procedures

Protein pellets were dissolved in 1 N NaOH and assayed using the method of Lowry et al. (1951). Calculations for the protein assays were performed using a program developed with Lotus 1-2-3 software (Pellerin, 1990). For statistical analysis, analysis of variance (ANOVA) followed by a Bonferroni's *t* test was used where appropriate (Wallenstein et al., 1980) and was performed with the InStat statistical software package from Graphpad, San Diego, CA. SlideWriteplus v.4 software package from Advanced Graphics Software inc., Sunnyvale, CA, and Sigmaplot v.4.0 software package from Jandel Scientific, Corte Madera, CA, were used to realize some of the diagrams and graphics presented.

RESULTS

A) Design of a perfusion bath and incubation system suitable for studying fatty acid release in tissue slices

In order to investigate the possible relationship between arachidonic acid release and long-term potentiation in the rat hippocampus, it was necessary to develop a system

which would ensure the viability of the tissue slices and at the same time remain convenient enough for biochemical investigations. A number of designs for maintaining tissue slices *in vitro* have been developed over the years, mostly by electrophysiologists (Kelly, 1982; Dingledine, 1984). However they have shortcomings that render them not very useful for biochemical analyses. The two major ones are the limited amount of tissue that can be handled at once and the difficulty to obtain an environment that can be used for biochemical manipulations and determinations. The solution to these problems was to design separate systems for maintaining the tissue *in vitro* and then performing the incubation experiments.

The perfusion bath design was based on the Haas-type interface tissue chamber (Dingledine, 1984). It was made of 0.6 cm thick clear plexiglass (except the top plate which is 0.9 cm thick) and has the following dimensions: 14.5 cm long, 12 cm wide and 8.5 cm high (see Figure 2 for diagram). The major difference pertains to the size of the tissue holding platforms, which are 4.7 cm long by 4 cm wide. They were grooved in the top plate about 4-5mm deep, slightly slanted to favor liquid flow. A fine mesh nylon net covers the bottom of the platform. Slices sit on lens paper strips directly lying on the nylon net. They are maintained at the interface between oxygenated ACSF and an atmosphere made of a humidified gas mixture of 95% O_2 and 5% CO_2. The oxygenated ACSF is warmed to the appropriate temperature by circulating inside a narrow bore teflon tubing which is immersed in the water contained in the compartment under the platforms. A gas mixture made of 95% O_2 and 5% CO_2 bubbles through the water of that

Figure 2: Diagram of the perfusion bath for maintaining hippocampal slices *in vitro*. 1- Warming water tank 2- Oxygenated ACSF inlet 3- 95% O_2:5% CO_2 inlet 4- Temperature probe 5- Heating filament 6- Gas diffuser stone 7- Waste buffer suction device 8- Nylon mesh. The heating filament and the temperature probe are connected to a bath temperature controller. Slices are placed in perfusion areas on lens paper strips that sit on the nylon mesh. Perfusion areas are covered with plexiglass plates such that warmed, humidified 95% O_2:5% CO_2 gas coming from the water tank flows over the slices which are maintained at the interface between the gas and the perfusing ACSF (see Results section for more details).

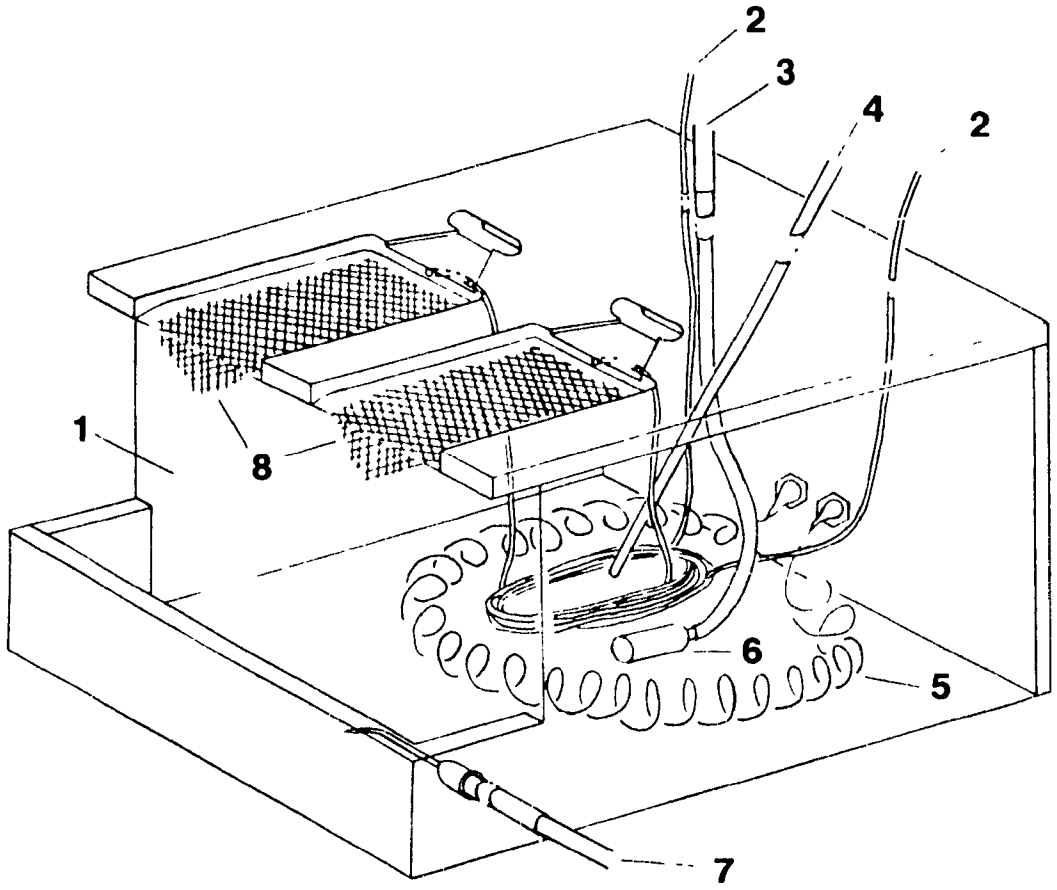


Figure 2

Figure 3a: Perfusion bath to maintain brain slices *in vitro*.

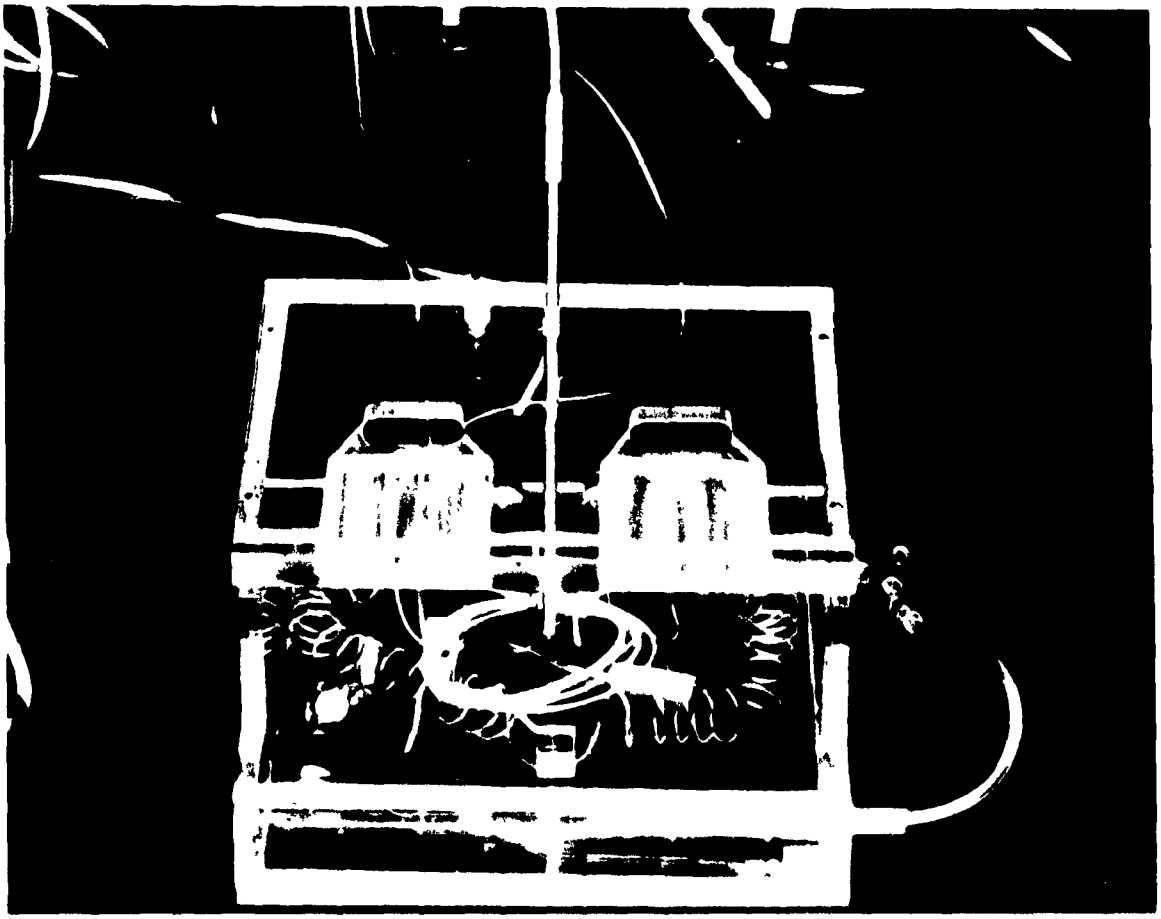
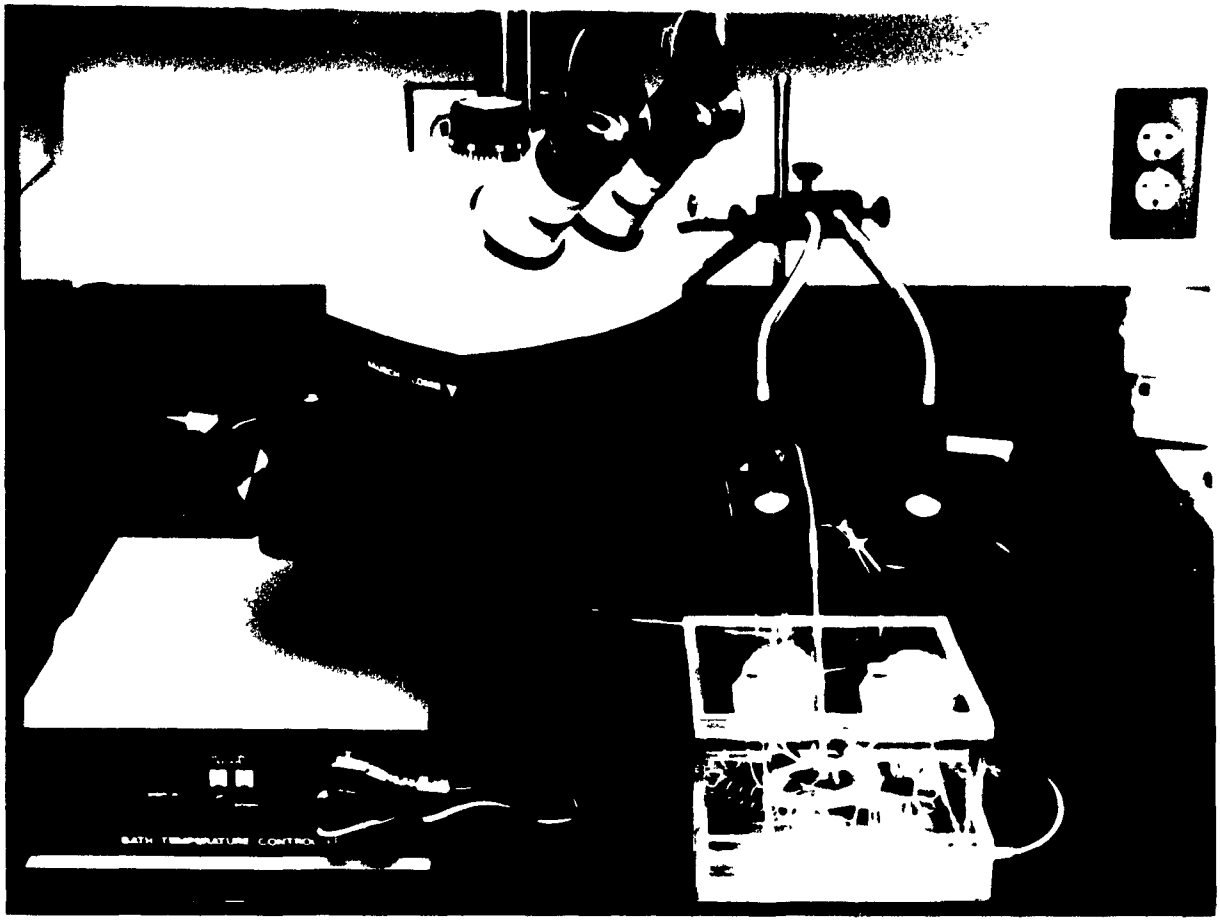


Figure 3b: Experimental setup used to maintain brain slices *in vitro*. It includes the perfusion bath, a temperature controller connected to the bath and a stereomicroscope with a fiber optic lamp to visually inspect the slices.



same compartment and reaches the slices through holes in the top plate communicating with the platforms. Small plates made of thin clear plexiglass cover the platforms in order to preserve the humidified atmosphere and prevent the slices from drying. An electronic module ensures proper control of the temperature. A probe connected to the module reads at regular intervals the temperature of the inside compartment water. Depending on the reading, a heating filament immersed in the water is activated to maintain the temperature at the desired value (see Figure 3 for pictures of the chamber and of the whole setup).

The condition of the slices maintained *in vitro* with this perfusion bath were evaluated electrophysiologically. Slices perfused for 2.5 hrs displayed characteristic responses of healthy hippocampal slices. Population spike responses were obtained with no epileptic signs (usually characterized by the appearance of multiple population spikes following single low intensity stimulation) reaching sometimes more than 20 mV in amplitude at high intensity stimulation (Figure 4a). The input/output curve, which represents the increase in population spike amplitude with increasing stimulus intensity displays a normal shape (Figure 4b). The peak latency was also found to decrease as stimulus intensity increases, as expected from healthy slices (see Kelly, 1982 for a discussion of these points). Routinely 24 slices were placed on each platform, although it could be possible to use twice that number.

For biochemical experiments, slices were transferred into incubation chambers.

Figure 4: Viability of hippocampal slices maintained in a modified Haas-type tissue chamber. **A:** Population spike elicited in the CA1 subfield by a 250 μA stimulus delivered through the Schaffer collaterals. Note the absence of a second population spike despite the near maximal stimulus intensity. Arrow indicates when the stimulus was delivered. **B:** Input/output curve from the same slice. Both population spike and input/output curve were obtained after 2.5 hrs of perfusion. Same results were obtained in three slices tested consecutively.

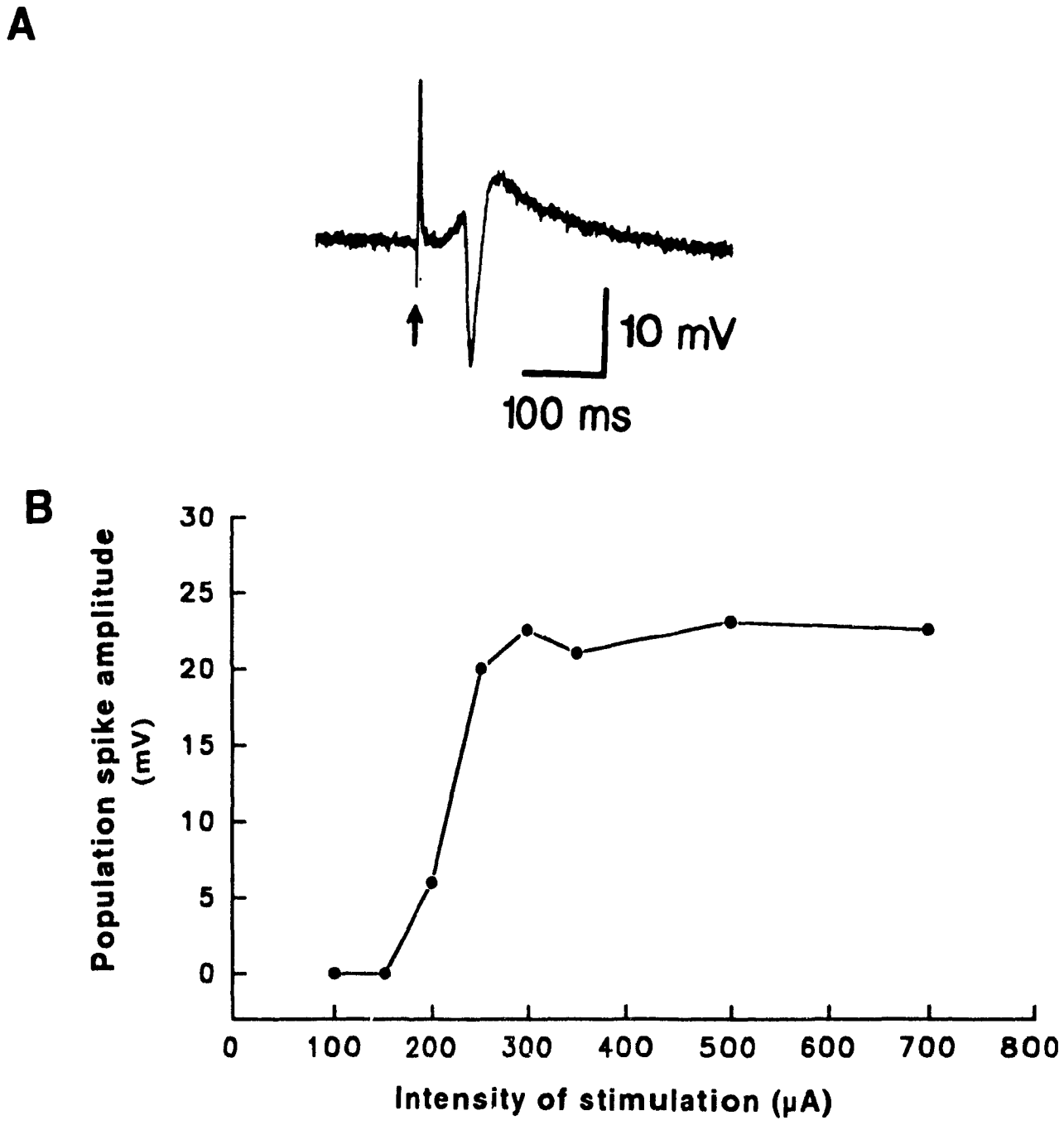

A
10 mV
100 ms
B
Population spike amplitude (mV)
30
25
20
15
10
5
0
0
100
200
300
400
500
600
700
800
Intensity of stimulation (μA)

Figure 4

This can be easily done by taking one end of the lens paper strip with a pair of tweezers and lifting it. Slices will stick to the lens paper. It is then inserted in the incubation chamber already containing oxygenated ACSF. While in contact with ACSF, gentle agitation made normally the slices come off the paper but it was sometimes necessary to use a fine bristle paintbrush to gently remove them. The lens paper was finally removed. The incubation chamber, which consists of a 20 ml borosilicate scintillation vial, was capped and connected to a source of warm, humidified gas mixture of 95% O_2 and 5% CO_2. The cap was modified so that gas can be delivered easily to the solution through a 22-gauge needle (see Figure 5). The particular conditions used for oxygenation (1 L/min; humidified gas mixture blown close to the liquid surface; gentle agitation; low volume of ACSF) were arrived at by trial and error. They minimize damage to the slices caused by too violent stirring of the solution while providing maximal oxygenation, since submerged slices are known to require more oxygen than slices maintained in interface conditions (Kelly, 1982). Since incubation times planned to be used were relatively short (a few minutes), it was assumed that these conditions might be sufficient to maintain the slice viability. If longer periods of incubation were to be used, it would be necessary to assess slice viability before adopting these conditions. In a modified version of the chamber where the gas mixture was directly delivered into the solution, the viability of hippocampal slices was tested. It was found that even after 2.5 hrs of incubation they exhibited normal electrophysiological responses as illustrated by the population spike response (Figure 6a) and input/output curve (Figure 6b) obtained from one such slice. In addition, two short-term forms of synaptic plasticity, paired-pulse inhibition and

Figure 5: Incubation chamber for conducting biochemical experiments with hippocampal slices. 1- I.V. set connector and tubing 2- Spinal needles gauge 18 and 22 3- Scintillation vial 4- ACSF 5- Hippocampal slices 6- Water 7- Gas dispersion tube 8- Hot plate. A warmed, humidified mixture of 95% O_2:5% CO_2 is delivered to the incubation chamber which is maintained half-immersed in a shaking water bath kept at 37°C. The various elements are not necessarily drawn proportionally to each other.

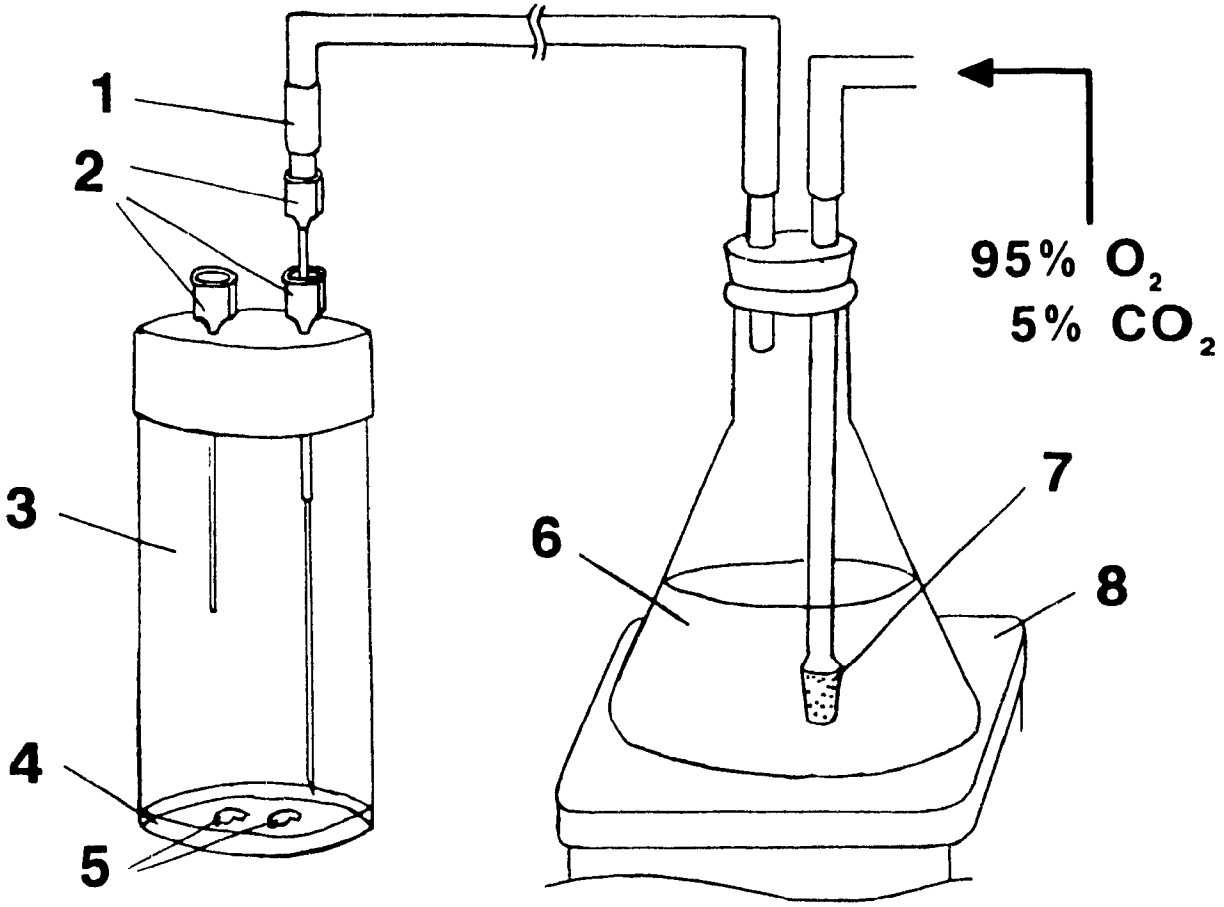


Figure 5

Figure 6: Viability of hippocampal slices maintained in an incubation chamber designed for biochemical experiments. **A:** Population spike elited in the CA1 subfield by a 90 μA stimulus delivered through the Schaffer collaterals. Note the absence of a second population spike. **B:** Input/output curve from the same slice. **C:** Paired-pulse inhibition (1-2) and facilitation (4-5) demonstrated in a different slice. Intervals between stimuli (70 μA each): 1. 10 2. 20 3. 30 4. 40 5. 50 msec. Same results were obtained from 11/11 slices tested after 1.5 hr of incubation on average.

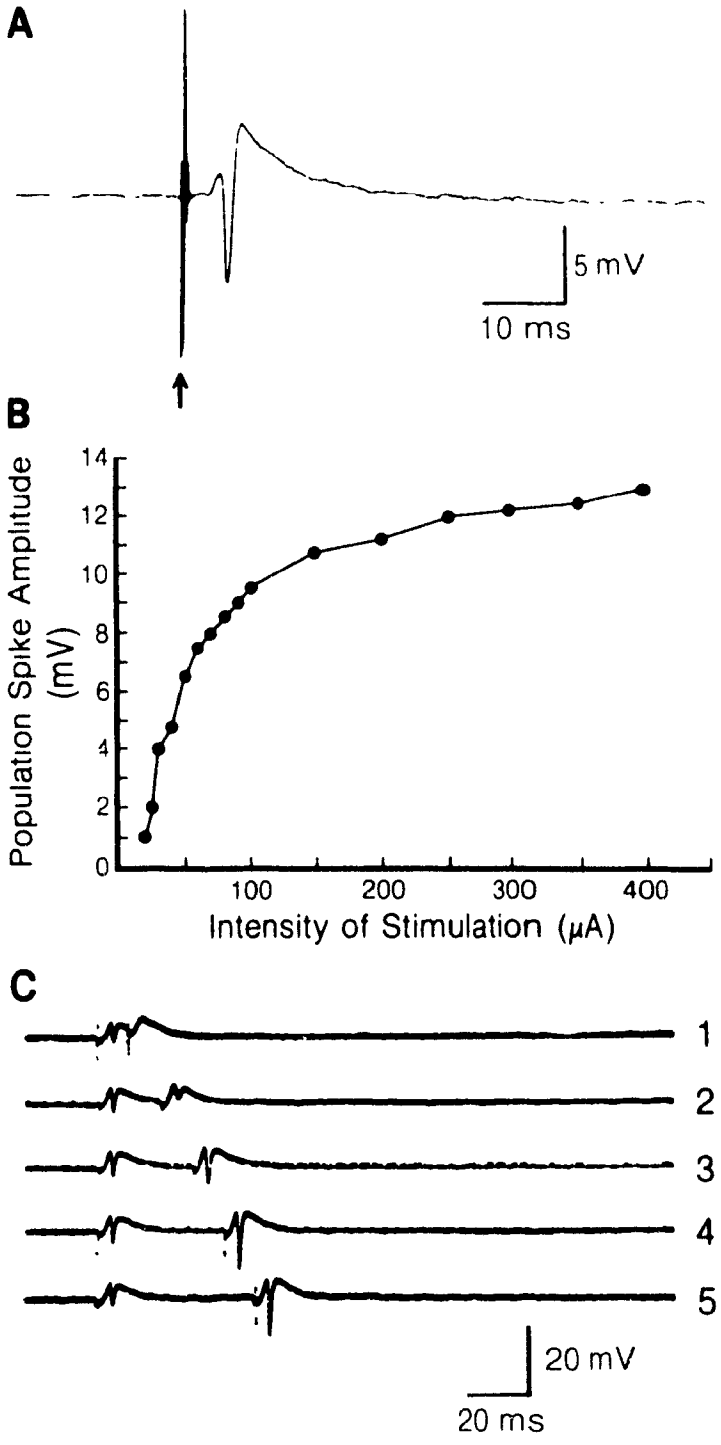

A
5 mV
10 ms
B
Population Spike Amplitude (mV)
14
12
10
8
6
4
2
0
100
200
300
400
Intensity of Stimulation (μA)
C
1
2
3
4
5
20 mV
20 ms

Figure 6

paired-pulse facilitation could be elicited (Figure 6c). The condition of the slices was such that good intracellular recordings could be obtained in them with preservation of inhibition which is normally quite sensitive to damage or anoxia. In this modified version of the chamber, slices were maintained between two tight nylon nets to avoid too much agitation.

B) Stimulation of fatty acid release from rat hippocampal slices by melittin, a PLA_2 activator

Melittin, a small peptide isolated from bee venom has been recognized as an activator of the enzyme phospholipase A_2 (Shier, 1979), although its mechanism of action is still unclear (see for example Dempsey, 1990 for a review on the actions of melittin on membranes). It was observed previously that it can have some long-term effects on synaptic transmission in rat hippocampal slices maintained *in vitro* (see chapter 3). It was now of interest to try to correlate the effect of melittin on synaptic transmission with its effect on fatty acid release in the same preparation. If this is so, it could be used as a positive control for comparison with the effect of other pharmacological manipulations on fatty acid release.

When hippocampal slices were incubated for 10 minutes with melittin, an increase in the level of oleate ($C_{18:1}$), arachidonate ($C_{20:4}$) and docosahexaenoate ($C_{22:6}$) was observed (Figure 7). The effect appeared restricted to the unsaturated fatty acids since

Figure 7: Effect of melittin on fatty acid release in rat hippocampal slices maintained *in vitro*. Melittin (5 μg/ml) induced the release of all three unsaturated fatty acids but not the two saturated ones ($^{*}p<0.01$ vs basal, $^{**}p<0.001$ vs basal). Parachloromercuribenzoate (pCMB; 100 μM), a blocker of reacylation caused only a small but not significant increase in the release of arachidonate when used with melittin (5 μg/ml) as compared to melittin alone. The values are the average$\pm$STD of at least three determinations.

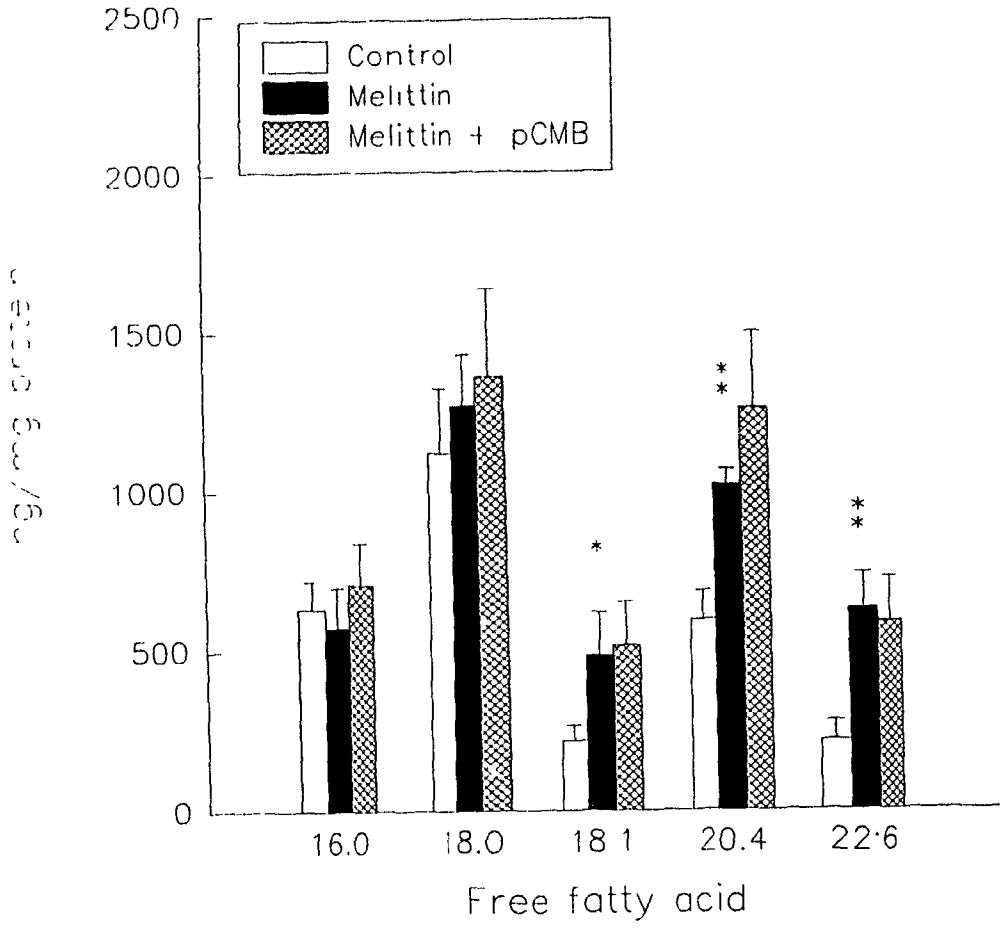

2500
2000
1500
1000
500
0
Control
Melittin
Melittin + pCMB
*
**
**
16.0
18.0
18 1
20.4
22·6
Free fatty acid

Figure 7

both palmitate ($C_{16\,0}$) and stearate ($C_{18\,0}$) were not affected. When the slices were first preincubated with parachloromercuribenzoate (pCMB), an inhibitor of the fatty acid reacylation pathway (Hunter et al., 1984) and then stimulated with melittin, only a small but not significant increase in the level of arachidonate was noticed with no effect on the other fatty acids.

C) Stimulation of fatty acid release in rat hippocampal slices by pharmacological manipulations involving the excitatory ar.ino acid glutamate and its receptors

Since it was previously shown that arachidonic acid can induce a form of long-lasting potentiation in rat hippocampal slices (Williams et al., 1989; Drapeau et al., 1990; see also chapter 3), it became of interest to investigate whether an increase in arachidonic acid release could be observed following induction of LTP. Conventional induction of LTP requires brief electrical high-frequency stimulation (tetanus) usually delivered through a bipolar electrode to afferent fibers. This however posed technical difficulties for subsequent biochemical analysis. Fortunately, pharmacological manipulations which have been shown to induce some forms of long-term facilitation which resemble classical LTP in rat hippocampal slices have been described (Collingridge et al., 1983; Izumi et al., 1987; Thibault et al., 1989). Taking advantage of this, the effect of those pharmacological treatments on fatty acid release in rat hippocampal slices was measured.

Figure 8: Effect of various treatments involving the activation of receptors for the excitatory neurotransmitter glutamate on fatty acid release in hippocampal slices from adult rats maintained *in vitro*. Both glutamate (100 μM) and N-methyl-D-aspartate (100 μM) induced a significant release of arachidonate. A conditioning solution made of a low Mg^{2+}, high K^+ buffer and glutamate (100 μM), which favors activation of NMDA receptors, also induced the release of arachidonate but in addition caused a small increase in the release of stearate and oleate ($^{*}p<0.05$ vs basal, $^{**}p<0.01$ vs basal and $^{***}p<0.001$ vs basal). The values are the average$\pm$STD of at least three determinations.

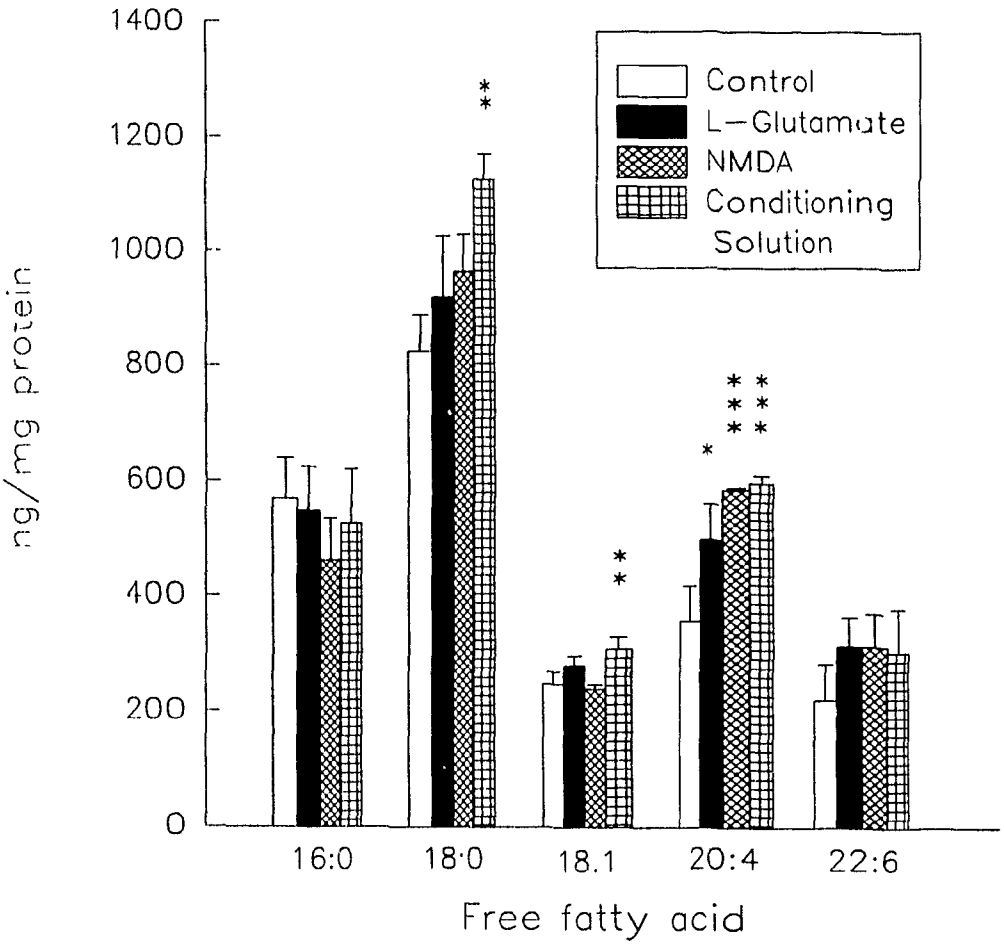

1400
1200
1000
800
600
400
200
0
ng/mg protein
Control
L—Glutamate
NMDA
Conditioning Solution
16:0
18:0
18.1
20:4
22:6
Free fatty acid

Figure 8

The effect of either glutamate alone, N-methyl-D-aspartate (a specific glutamate receptor subtype agonist) alone or a conditioning solution made of glutamate and a low Mg^{2+}, high K^+ buffer on fatty acid release from hippocampal slices of adult rats was studied. Glutamate alone was found to cause a small but significant increase in arachidonic acid ($C_{20\,4}$) release with no effect on other fatty acids (Figure 8). A more robust increase in arachidonic acid was obtained with NMDA and still no significant effect on the release of other fatty acids was observed. Finally, the conditioning solution (CS) induced an increase in arachidonic acid of similar magnitude when compared to NMDA, but in this case a small increase in both stearate ($C_{18\,0}$) and oleate ($C_{18\,1}$) was also observed.

It has been reported that hippocampal slices taken from young rats (around 15 days of age) exhibit LTP with a magnitude which is over twice that seen in the adult rat (Teyler, 1989). This preparation might then represent a more sensitive system to monitor the release of fatty acids following induction of LTP. First a combination of NMDA and AMPA (a specific agonist at ionotropic quisqualate receptors) was found to increase the level of arachidonate and stearate (Figure 9). The same combination had been found previously to induce arachidonate release in primary culture of neurons (Sanfeliu et al., 1990). Then the effect of a solution containing NMDA, glycine and spermine (two allosteric regulators at NMDA receptors) in combination with a low Mg^{2+} buffer was tested. This treatment was termed LTP since the same conditions had previously been shown to be a reliable method to induce LTP by a bath-applied method in hippocampal

Figure 9: Effect of various treatments involving the activation of receptors for the excitatory neurotransmitter glutamate on fatty acid release in hippocampal slices from 17 days old rats maintained *in vitro*. A combination of NMDA and AMPA caused an increase in both stearate and arachidonate. A combination of NMDA (100 μM), glycine (30 μM) and spermine (850 μM) in presence of a low Mg^{2+} buffer, called LTP, induced a robust increase in the release of arachidonate and stearate ($^{*}p<0.05$ vs basal). MK801, a specific non-competitive NMDA receptor antagonist completely prevented the effect of the LTP condition on both arachidonate and stearate ($^{+}p<0.05$ vs LTP). The values are the average$\pm$STD of at least three determinations except control which represent the average of two.

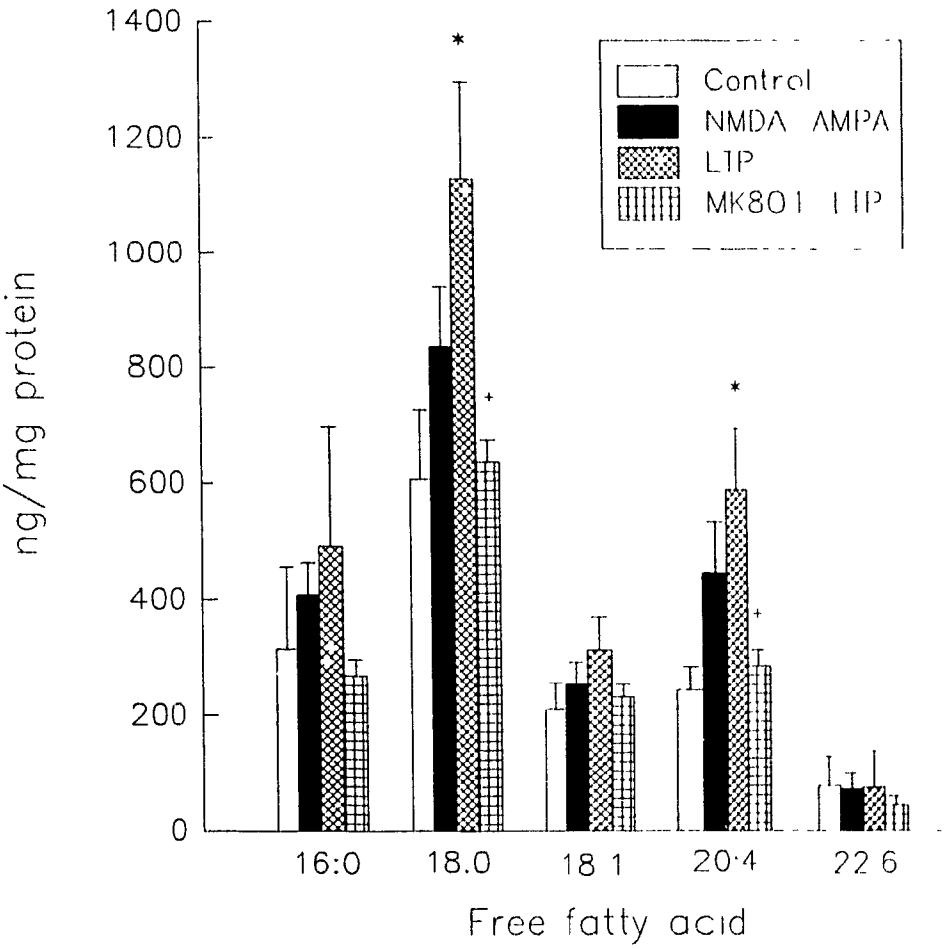

1400
1200
1000
800
600
400
200
0
ng/mg protein
Control
NMDA AMPA
LTP
MK801 LTP
*
*
+
+
16:0
18.0
18 1
20·4
22 6
Free fatty acid

Figure 9

slices of adult rats (Thibault et al., 1989). With these conditions a robust increase in both arachidonate and stearate was found (Figure 9). Finally MK-801, a non-competitive NMDA receptor antagonist (Wong et al., 1986; Huettner and Bean, 1988) completely prevented the effect of the LTP treatment on both fatty acids.

DISCUSSION

A) Use of brain slices for the study of fatty acid release

Brain slices have been used in the past to study a variety of biochemical processes such as oxygen consumption, metabolism, neurotransmitter release or second messenger systems. The study of fatty acid release on the other hand has been carried out mainly in cell culture systems using radiolabeled products or on the whole brain *in vivo* or immediately following the death of the animal. In this last case it was shown that free fatty acids rapidly accumulate in the tissue (Bazan et al., 1971; Hirashima et al., 1989). The same was shown to occur in slices rapidly prepared after the death of the animal (Marion, 1979). No special efforts were made however to preserve physiological responsiveness of slices, to confirm their viability and study the release of fatty acids by physiologically relevant stimuli.

It was intended to use rat hippocampal slices for the study of fatty acid release and particularly arachidonic acid following specific pharmacological manipulations. In

order to do so, a tissue chamber system was developed with the aim of preserving slices viability while allowing easy access for biochemical determinations. The system described here was shown to fulfill these requirements. In addition, contrary to cell culture systems, the use of radiolabelled compounds was not found to be necessary. This is particularly significant since a number of problems with prelabelling of phospholipid pools in isolated cells have been revealed recently. In fact, major discrepancies have been noted between results obtained with radiolabels and those by mass spectrometry which uses only endogenous pools (Chilton and Connell, 1988; Ramesha and Taylor, 1991). These reports stress the importance of looking at the total mass of fatty acids which reveals in a better way the extent of the fatty acid release as well as reflects more accurately the various phospholipid species that serve as pools.

In order to demonstrate the validity of this approach for the study of fatty acid release, rat hippocampal slices maintained *in vitro* in this tissue chamber system were stimulated with melittin. The results, which show a clear increase in unsaturated fatty acids with no change in saturated ones, do not appear to reflect the composition of a particular phospholipid class. Considering now that phospholipids preferentially contain unsaturated fatty acids at their *sn*-2 position (Sastry, 1985), it can be postulated that activation of a PLA_2 species might have produced the results observed. Whether one or more phospholipid pools has been used as a source cannot be deduced however and would require a more specific investigation. Finally the small effect observed with parachloromercuribenzoate (pCMB), an inhibitor of fatty acid reacylation, argues against

an important activation of reacylating mechanisms, at least during the period of time investigated. Overall it can be concluded that the preparation appears suited for studies on free fatty acid release *in vitro*.

B) Induction of long-term potentiation and fatty acid release

The present results confirm in hippocampal slices the effect of both glutamate and N-methyl-D-aspartate (NMDA) on arachidonic acid release as described in striatal, cerebellar and hippocampal neurons in cultures (Dumuis et al., 1988; Lazarewicz et al., 1990; Sanfeliu et al., 1990; Patel et al., 1990). In slices however the effect of NMDA appeared slightly more robust than the effect of glutamate. Interestingly, bath application of NMDA has been reported to induce a decremental form of LTP (Kauer et al., 1988b; Collingridge and Singer, 1990). To investigate further the relationship between arachidonic acid and LTP, pharmacological conditions that were previously shown to induce LTP were selected (Izumi et al., 1987) and found to give arachidonic acid release similar to NMDA. In addition a small increase in both stearate and oleate was also obtained in this case. Whether these two fatty acids have a specific role to play remains to be seen but oleate was previously reported to activate protein kinase C and to prolong the time course of a decremental form of long-term potentiation in hippocampal slices (Linden et al., 1987). From the data reported here however arachidonic acid, which has been reported also to activate protein kinase C (Shearman et al., 1989; Shearman et al., 1991; Shinomura et al., 1991) as well as to induce a form of long-lasting potentiation

(Williams et al., 1989; Drapeau et al., 1990), appears more likely to be the fatty acid involved in the NMDA-dependent enhancement of synaptic transmission as seen in LTP.

The profile of fatty acids released seems to match the fatty acid content of phosphatidylinositol (PI) and its derivatives phosphatidylinositol phosphate (PIP) and phosphatidylinositol bisphosphate (PIP_2) (Sastry, 1985) suggesting that this phospholipid might serve as the source pool. We have not obtained specific information however on the mechanism responsible for the release. Two major pathways that can be responsible for arachidonic acid release have been described (see Irvine, 1982 for review). The first one involves a phospholipase A_2 which can act usually on both phosphatidylcholine and phosphatidylinositol. The second one involves the sequential action of a particular phospholipase C species, which has a preference for the phosphoinositides followed by a diacylglycerol lipase. In all three cell lines where NMDA-induced arachidonic acid release was demonstrated, this process was shown to be due to phospholipase A_2 activation on the basis of inhibition with mepacrine, a specific inhibitor which apparently does not interfere with the PLC-DAG lipase pathway (Lazarewicz et al., 1990; Dumuis et al., 1988; Sanfeliu et al., 1990) or at least does not appreciably inhibits the DAG lipase (Majerus and Prescott, 1982). It is of interest that establishment of a stable tetanically-induced LTP could be prevented by mepacrine (Linden et al., 1987) as well as by parabromophenacylbromide (Okada et al., 1989; Massicotte et al., 1990), another PLA_2 inhibitor. A phospholipase A_2 species which has a preference for phosphatidylinositol in the nervous system has also been described recently (Chock et al.,

1991). Our results however imply that another enzyme such as a lysophospholipase should be activated to release stearate which constitutes the major fatty acid found in position 1 of the glycerol backbone of phosphatidylinositol. A phospholipase A_2 which possesses also a lysophospholipase activity was discovered very recently (Leslie, 1991) and could account for the present results. The author explained this activity as necessary for the elimination of rapidly accumulating lysophospholipids following PLA_2 activation which are toxic for the cells. It is important to stress however that the results presented here do not exclude the possiblity that a PLC-DAG lipase pathway might be responsible for or at least be involved partially in the fatty acid release observed. Further experiments will be required to clarify this point.

Developmental studies have shown that LTP first appears at postnatal day 5, increases in magnitude to peak around day 15 and gradually decreases to reach adult level as measured at 60 days (Teyler, 1989). This pattern is paralleled by a somewhat similar pattern in the number of NMDA receptors expressed (Insel et al., 1990) which is thought to explain the effect on LTP magnitude. It was thought that this feature could be advantageously used to further investigate the effect of NMDA receptor activation on fatty acid release. Another set of conditions selected for their ability to activate the NMDA receptors were tested for their capacity to induce fatty acid release in hippocampal slices taken from 17 days old rats. The results obtained with this preparation have further confirmed our previous results on slices from adult rats which clearly link the activation of NMDA receptors with the release of both arachidonic and stearic acids.

Moreover the effect appears quantitatively more important. For arachidonic acid it amounts to roughly twice as much as what is seen in slices from adult rats (140% vs 67% over basal level), which agrees with the increase in the number of NMDA receptors at this age, although the stimuli were not exactly the same between young and adult. The fact that the condition termed LTP, which was shown to reproducibly induced a long-term potentiation in hippocampal slices (Thibault et al., 1989), elicited a robust release of arachidonic acid is very significant and further increases the likelihood of a role of this fatty acid in long-term potentiation. Quite important also is the observation that MK-801, a non-competitive NMDA receptor antagonist completely prevented the release of the two three fatty acids normally observed after the LTP-inducing treatment. This definitively establishes the specific involvement of NMDA receptors in stimulating the release of fatty acids in rat hippocampal slices. MK-801 was also found to prevent tetanically-induced LTP in hippocampal slices (Coan et al., 1987). Finally it is interesting to point out that the release of fatty acids including arachidonate seems to take place quite rapidly after activation of the NMDA receptors (changes detectable two minutes after stimulation in both preparations although shorter periods of time were not tested). This fulfills another of the requirements expected for a potential retrograde messenger (Malinow and Tsien, 1990).

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Chapter 5

GENERAL CONCLUSION AND PERSPECTIVE

The work presented in this thesis constitutes a study of arachidonic release and metabolism by lipoxygenases, and also represents an attempt at understanding the physiological role of arachidonic acid and its lipoxygenase metabolites in the mammalian central nervous system. It was originally based on the hypothesis that arachidonic acid, but more specifically its lipoxygenase metabolites could act as second messengers in nerve cells, as has been demonstrated in the case of the mollusk *Aplysia californica* (Piomelli et al., 1987).

A particularly active 12-S-lipoxygenase activity was found to be present in rat cerebral cortex and was responsive to stimulation by an increase in intracellular calcium. In addition, the lipoxygenase was stimulated by specific receptor-mediated mechanisms. This is the first essential feature to be considered for a role as second messenger. In the hippocampal slice preparation, arachidonic acid was found to induce long lasting changes in synaptic transmission which depended at least in part on lipoxygenase metabolite formation. Activation of NMDA receptors, a particular subset of glutamate receptors which are known to be essential for induction of long-term potentiation of synaptic transmission, lead to arachidonic acid release and formation of 12-lipoxygenase metabolites. These observations suggest a role for arachidonic acid and/or its lipoxygenase metabolites in this particular form of synaptic plasticity. Because of the nature of the mechanism responsible for the induction and maintenance of LTP and the role as a retrograde messenger suggested for arachidonic acid or one of its metabolites, this raised the question of whether arachidonic acid and its lipoxygenase metabolites can

be considered strictly as second messengers in the nervous system. It is proposed here instead that they belong, along with nitric oxide (NO; see review by Collier and Vallance, 1990), to a new class of diffusible messengers which can act both inside the cell of origin as well as affect neighbouring cells by acting on intracellular targets. This new mode of communication could be used to synchronize a large population of neurons within discrete and localized areas, a concept which is not too remote from chemotaxis in the immune system where lipoxygenase metabolites of arachidonic acid are known to be involved (see for example Spector et al., 1988 for a review of chemotaxic activity of HETEs and particularly 12-HETE). Based on this idea, a model to account for synaptic plasticity and activity-dependent changes in the nervous system has been proposed by Edelman and his group (Gally et al., 1990). In addition, as outlined by Schwartz (1991), the arachidonic acid cascade with its numerous products offers unique opportunities for diverse modulatory effects on synaptic transmission that are only beginning to be explored.

Many aspects still remain to be worked out and clarified. For example, the possible targets of arachidonic and its lipoxygenase metabolites will need to be more clearly determined. Already some targets have been suggested such as protein kinase C (Shearman et al., 1989; Shearman et al., 1991; Shinomura et al., 1991), neurotransmitter uptake systems (Barbour et al., 1989; Chan et al., 1983; Saltarelli et al., 1990; Yu et al., 1986) and ion channels (Belardetti et al., 1989; Buttner et al., 1989; Fluri et al., 1990; Hwang et al., 1990; Kim and Clapham, 1989; Kurachi et al., 1989; Ordway et al., 1989;

Premkumar et al., 1990; Vacher et al., 1989; see Ordway et al., 1991 for a review). Since some of the effects of arachidonic acid and its lipoxygenase products are rather long-lasting, it is possible also that gene expression and protein synthesis might be involved. It has been shown in some preparations that either arachidonic acid or some of its lipoxygenase metabolites participate in the induction of specific genes such as the immediate early gene *c-fos* (Kacich et al., 1988; Haliday et al., 1991; Sellmayer et al., 1991;), the protooncogene *c-fms* (Stone et al., 1990) and the gene encoding for tumor necrosis factor (Horiguchi et al., 1989; Spriggs et al., 1990). This possibility will need to be investigated directly in the nervous system. In addition the exact phospholipid pool acting as a source of arachidonic acid, as well as the precise release mechanism will need to be more firmly established. Determination of all the lipoxygenase metabolites formed following stimulation also will be required. The participation of cell types other than neurons, such as astrocytes needs to be assessed, both as a source and a target for arachidonic acid and its lipoxygenase metabolites. Finally it is unlikely that such a signalling mechanism would work in isolation. So in order to fully appreciate its importance it will be necessary eventually to pay attention to the cross talk with other signalling systems. Only by studying these interactions can one hope to achieve a better understanding at the molecular level of the complex information processing which takes place in the nervous system.

"...We are now standing on an exciting threshold of scientific history, where we can hope that at least some aspects of how the human mind works can be understood, not necessarily in the traditional terms of biophysics and biochemistry-because such a hope would betray a too naively reductionist point of view-but in terms of mechanisms that involve organizational levels of which biophysical and biochemical processes are the indispensable building blocks."

Pierre Gloor

In: Neurotransmitters and cortical function: from molecules to mind
book edited by M. Avoli et al., New York: Plenum, 1988, p.11.

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