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Release of Arachidonic Acid by NMDA-Receptor Activation in the Rat Hippocampus*

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In hippocampal slices arachidonic acid released after NMDA post-synaptic receptor activation is thought to act as a retrograde trans-synaptic messenger which facilitates the pre-synaptic release of L-glutamate to be involved in the expression of long-term synaptic potentiation (LTP). We measured the mass amount of arachidonic acid released from hippocampal slices incubated under conditions which maintain the electrophysiological responsiveness of the slice. Melittin released arachidonic, oleic and docosahexaenoic acids by phospholipase A_2 activation but not palmitic or stearic acids. Of greater interest L-glutamate, N-methyl-D-aspartate and incubation conditions known to induce LTP selectively and rapidly increased the release of archidonic acid in amounts over basal levels of 200–300 ng/mg protein. This is the first direct determination of the mass amount of arachidonic acid released following NMDA receptor activation in the hippocampus

KEY WORDS: Arachidonic acid; hippocampal slices; long-term potentiation; L-glutamate, N-methyl-D-aspartate, melittin

INTRODUCTION

The dietary essentiality of polyunsaturated fatty acids of the n-6 series particularly linoleic and arachidonic acid has been known since the pioneering studies of George and Mildred Burr in 1929 (1). Five years latter von Euler in Sweden, and Goldblatt in England, reported a smooth muscle stimulating principle in lipid extracts from human seminal fluid and seminal vesicles of sheep and goats which was called by von Euler prostaglandin (2–4). Thirty years were to pass before the metabolic connection between arachidonic acid and prostaglandins was made by Bergström, Samuelsson and co-workers in Sweden (5). In the almost thirty years since these pivotal discoveries, a multitude of potent biologically important arachidonic metabolites have been discovered; prostaglandins, thromboxanes, leukotrienes, lipoxins, hydroxy- and epoxy octadeca- and eicosenoic acids, and hepoxilins all derived by enzymatic transformations of essential fatty acids. The list grows each year (6,7). However, it is only recently that the reason for the essentiality of all *cis* polyunsaturated fatty acids is becoming clear. It lies not only in the selectivity of their acylation in membrane phospholipids, but also in their release after many specific receptor activations and the versatile transformations which they undergo in all tissues of the body to a fascinating array of potent paracrine substances. It was known for some time also that dietary essential fatty acids were required for normal brain growth and development, synaptic function, myelination and response to autoimmune challenge (8). Yet it is less than ten years that the important involvement of arachidonic acid metabolites in synaptic receptor signal transduction pathways, ion channel activities and neurotransmitter release has been discovered.

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The invertebrate and mammalian nervous systems have an active 12-(S)-lipoxygenase which initiates the synthesis through the unstable 12-hydroperoxy intermediate of a variety of arachidonic acid metabolites (7). The marine mollusk *Aplysia californica* has been the subject of intensive neurobiological investigations in recent years. In the mechanosensory neurons which control the gill-withdrawal reflex serotonin closes the K_s^+ ion channel through a cyclic AMP-dependent phosphorylation. On the other hand, histamine and the molluscan tetrapeptide FMRFamide evoke the release of arachidonic acid from membrane phospholipids and through the 12-(S)-lipoxygenase pathway the 12-hydroperoxyeicosatetraenoic (12-HPETE) intermediate is formed. Arachidonic acid and 12-HPETE mimic the dual response (rapid depolarization followed by a longer hyperpolarization) caused by histamine which is blocked by nordihydroguaiaretic acid (NDGA) (9–14). The rapid depolarization has been convincingly shown to be due to 12-HPETE constitutively formed. However, the slow hyperpolarization dependent on the opening of a K_s^+ channel which inhibits neurotransmitter release appears to be due to the metabolite of 12-HPETE, 8-hydroxy-11,12-epoxyeicosatrienoic acid (hepoxilin A_3). In inside-out patch-clamp studies of *Aplysia* sensory neurons 12-HPETE itself fails to modulate the K_s^+ channel, but in the presence of hematin which catalyses the metabolism of 12-HPETE to hepoxilin A_3 by a cytochrome P450 mechanism, the K_s channel is opened (15,16). There are other examples of modulation of potassium currents by arachidonic acid lipoxygenase metabolites. In atrial cardiac myocytes, lipoxygenase metabolites activate the G-protein gated muscarinic K^+-channel. In both heart muscles patches and smooth muscle cells arachidonic acid directly activates potassium channels (17,18).

At the time the *Aplysia* results were reported, Williams and Bliss showed that arachidonic acid and lipoxygenase metabolites were likely involved in the induction and expression of tetanic stimulus induced long-term potentiation (LTP) in area CA1 of the rat hippocampus (19,20). Wolfe and Pellerin have also shown that the formation of 12-(S)-hydroxyeicosatetraenoic acid (12-HETE) by cerebral cortical slices was markedly stimulated by glutamate through NMDA but not kainate receptors (21,22). It has also been shown that following the induction of LTP there was a small but sustained release of arachidonic acid which did not occur when the induction was blocked by NDGA (23). Because NDGA can inhibit phospholipase A_2 as well as lipoxygenases it has been difficult to specifically implicate 12-lipoxygenase metabolites in LTP induction. Dumuis and co-workers found that glutamate and NMDA stimulated the release of arachidonic acid and 12-HETE in primary cultures of striatal neurones (24). A current hypothesis is that arachidonic acid and its lipoxygenase metabolites are released from post-synaptic membranes during the induction of LTP and act as retrograde trans-synpatic messengers facilitating the pre-synaptic release of glutamate thus enhancing synaptic efficiency (25). In our recent studies, on perfusion of hippocampal slices, arachidonic acid induced the LTP response but in some experiments a depression was observed and NDGA antagonized both responses (26). However, we have not been able to reverse the NDGA inhibition. The phospholipase A_2 activator melittin had a similar effect to arachidonic acid. It is also of considerable interest that Carlen, Pace-Asciak and coworkers reported that hepoxilin A_3 hyperpolarizes rat hippocampal CA1 neurones by increasing K^+ conductance as also found for *Aplysia* sensory neurones (27). The evidence is thus becoming more convincing that arachidonic acid and/or 12-lipoxygenase metabolites are involved in the complex synaptic events which produce LTP.

Most investigations on the neurotransmitter and agonist-stimulated release of arachidonic acid in neurones in culture or brain slices have used preparations prelabelled with [^{3}H] or [^{14}C] arachidonic acid. However, it is known that arachidonic acid release generated after the exogenous incorporation of labelled arachidonic acid may not reflect a neurotransmitter sensitive pool (28,29). In the experiments reported here we have measured directly the mass of endogenous arachidonic acid released from phospholipids in physiologically responsive rat hippocampal slices after NMDA-receptor activation and in conditions that generate LTP. An important technical difficulty in the preparation of brain slices to study arachidonic acid release and metabolism is the damage response after cutting the slices which results in the release of K^+ and glutamine with consequent development of spreading depression. With specially designed platforms on which humidified oxygenated gas mixtures flow over the perfused slices it was possible after 1 h of preincubation for the neurons in the slice preparation to return to an electrophysiological active condition. In such preparations glutamate and NMDA and conditions which induce LTP were found to significantly increase the amount of arachidonic acid released over the basal level.

EXPERIMENTAL PROCEDURES

Preparation of hippocampal slices. Male Sprague-Dawley rats (300–325g) were decapitated with a guillotine, the brain rapidly excised and placed in chilled 10°C oxygenated Ringer solution. Each

hippocampus was quickly dissected and slices 450μm thick prepared with a McIlwain tissue chopper. The slices were then placed in a specially designed modified Haas-type perfusion bath (Figure 1) (30). Each of the two perfusion platforms was designed to hold 24 hippocampal slices. The slices were perfused at 33–35°C for at least one hour and oxygenated with (95% O_2, 5% CO_2) in artificial cerebrospinal fluid of composition in mM: NaCl 124, KCl 2, KH_2PO_4 1.25, $MgSO_4$ 2, $NaHCO_3$ 2, $CaCl_2$ 2, and glucose 10. The slices were then transferred into borosilicate scintillation vials, four slices to each chamber containing 1ml of prewarmed oxygenated perfusion solution. The vials were then placed in a water bath at 37°C with continued oxygenation with warmed humidified 95% O_2, 5% CO_2. Additions of the various compounds under study were made at this stage and incubations continued for 2–5 min. The reaction was stopped by dipping the chamber into liquid nitrogen for 1 min and the vials stored in a −80°C freezer until the start of the fatty acid extractions. The electrophysiological responsiveness of slices after 1 h in the perfusion chamber before the incubation period was tested with stimulating electrodes positioned in the Schaffer collaterals of the CA1 region while recording electrodes were placed in the stratum pyramidale.

Extraction of Free Fatty Acids. To each of the frozen incubated samples 4.5ml of chloroform-methanol (1:2 by vol) was added containing 5μg of heptadecanoic acid internal standard and then rapidly homogenized in a Polytron homogenizer. Chloroform (1.25 ml) and 1.25 ml of 0.88% KCl were added, agitated and then the mixture transferred into a test tube and centrifuged. The upper phase and interface were kept for Lowry protein assay. The lower phase was removed, evaporated to dryness and dissolved in 50 μl of hexane. The lipid extract was then spotted on twice washed thin-layer plates of silica gel 150A (Whatman) activated for 1 h at 100°C. Arachidonic acid was spotted as a marker on each side of the plates with the samples between. Separation was done in hexane-diethylether-acetic acid (40:10:1). The band corresponding to free fatty acids was scraped off and eluted with 2:1 chloroform-methanol. The samples were evaporated to dryness, methylated with diazomethane and resuspended in 200 μl of isooctane. The free fatty acids were quantitated by capillary gas chromatography (50 m Ultra 1 methyl silicone column, Hewlett Packard, Mississuaga, Ontario, Canada) on an HP5890A gas chromatograph connected to an HP 3390A integrator. A dual rate temperature program was used from 100°C to 285°C.

Materials. Fatty acid standards were obtained from Nu-Chek, Elysian, MN. L-glutamate, N-methyl-D-aspartate and mellitin were from Sigma, St. Louis, MO. CPP (3-((-)-2-carboxypiperazine-4-yl)propyl phosphonate was from Tocris Neuramin, Essex, UK, and MK-801 (+)5-methyl-10,11,dihydro-5H-dibenzo[a,d]cyclohep-ten-5,10-imine hydrogen maleate was from Merck Frosst, Pointe-Claire, Quebec.

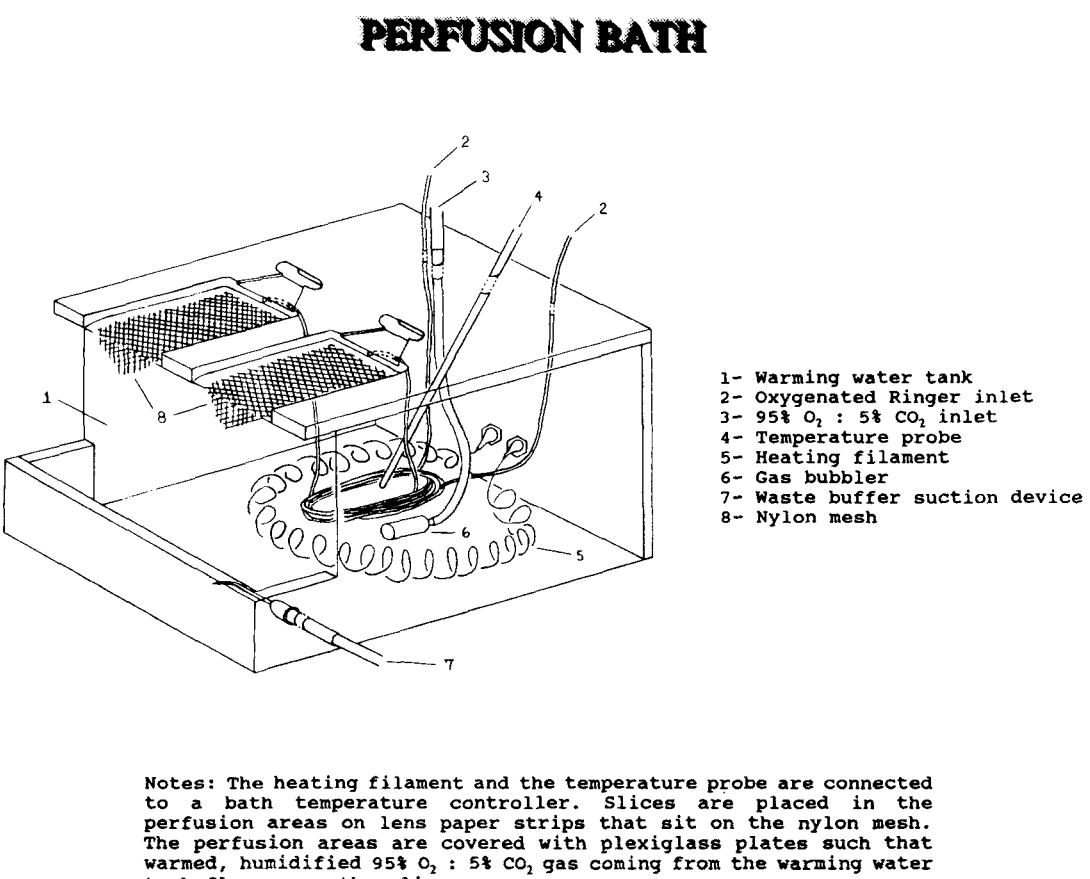

Fig. 1. Design of perfusion bath for the recovery and maintenance of rat hippocampal slices for electrophysiological and biochemical studies. Each of the nylon mesh perfusion platforms is capable of maintaining 24 slices.

RESULTS

The physiological responsiveness to electrical stimulation of the hippocampal slices which were used for investigation of the agonist-stimulated release of endogenous arachidonic acid is shown in Figure 2. With such slices it was found that arachidonic acid and the bee venom peptide, melittin, were capable of inducing long-term potentiation (26, and Drapeau, Pellerin, Avoli & Wolfe, unpublished observations). The addition of 7 μg of melittin to these hippocampal slices and then incubation for a further 2 min in the oxygenation set-up as described in the methods section, significantly increased the release of arachidonic acid and to a lesser extent oleic and docosahexaenoic acids. Palmitic and stearic acid release was unaffected (Figure 3). Inhibition of reacylation with p-chloromercuribenzoate (p-CMB) caused a further small increase in arachidonic acid release. In these experiments the hippocampal slices had been preincubated for one hour in the holding chambers (Figure 1) and several slices tested for their responsiveness to electrical stimulation of the Schaffer collaterals with recording of the population spikes in the stratum pyramidale. It was clear that the neurons and synaptic circuitry had recovered from the initial slicing injury. We were never able to reduce the basal lavel of free arachidonic acid in the slices below 200 ng/mg protein.

Under the slice conditions already described we tested the effect of L-glutamate, N-methyl-D-aspartate and a conditioning solution without magnesium but containing 15 mM potassium and 100μM glutamate, which has been

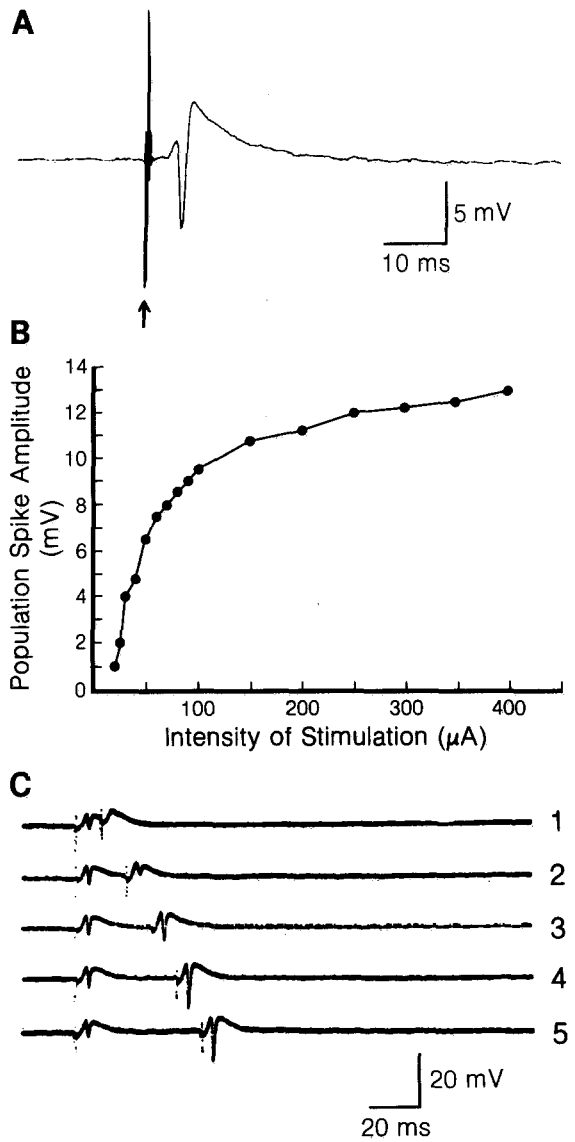

Fig. 2. Electrophyiological properties of the hippocamal slices maintained for 1 h in the perfusion bath. (A) Population spike elicited by a 200μ Amp stimulus in the Schaffer collaterals and recorded in the stratum pyramidale. (B) Input/output response in the CA1 region. (C) Paired pulses demonstrating inhibition (1,2) and facilitation (4–5). Intervals between each 70μ Amp stimulus were 1.1, 2.2, 3.3, 4.4, and 5.5, msec.

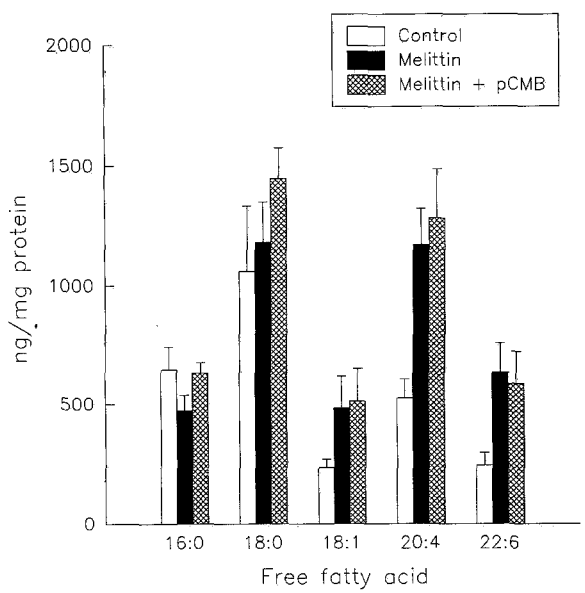

Fig. 3. Release of free fatty acids in hippocampal slices after stimulation for 2 min by 7μg/ml of melittin without and with the addition of p-chloromercuribenzoate (pCMB). The effect of saturated fatty acids was not statistically significant compared to the level control slices. However, the increase in oleic, arachidonic and docosahexaenoic acid is significant at $p = 0.01$, $p < 0.001$, $p < 0.1$ levels respectively. The results are shown as means ± SD for at least four separate incubations.

shown by Izumi and co-workers to induce LTP (31). In Figure 4 it is seen that after 2 min L-glutamate increased the release of arachidonic acid significantly ($p = 0.01$). NMDA and the LTP condition solution markedly and at a highly significant level ($p < 0.001$) increased arachidonic acid release to over 100 percent of the control condition. It is of interest to note that only arachidonic acid release is affected, the release of oleic and docosahexaenoic acids was not significant or at borderline levels. Thus, the activation of NMDA receptors in the slices likely affects only a specific pool of esterified arachidonic acid. The competitive NMDA-receptor antagonist CPP at 10μM concentration reduced the arachidonic acid release but not to the control levels. The non-competitive NMDA-receptor antagonist, MK-801, at 10μM concentration completely blocked the NMDA-induced release of arachidonic acid but only if the slices were preincubated with the inhibitor for 2 h before the addition of NMDA (data not shown). In a series of experiments in which the release of arachidonic acid was measured after exposure to NMDA for periods longer than 2 min a significant increase over basal levels was not found. Thus, the NMDA-induced release of arachidonic acid in hippocampal slices under the conditions studied here is a very rapid event.

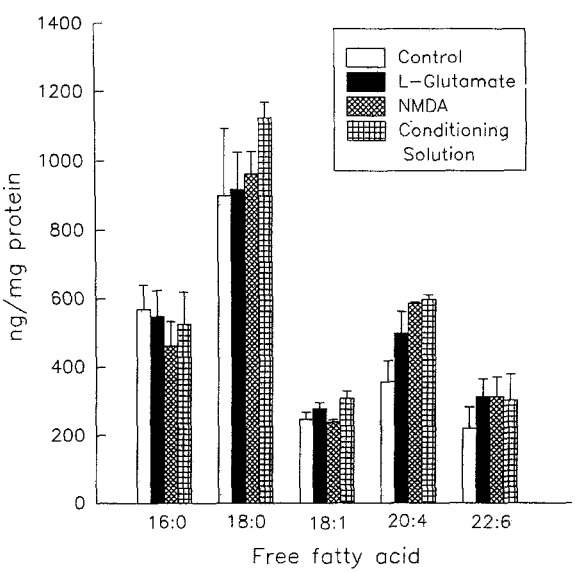

Fig. 4. Release of fatty acids from control slices and after the addition and incubation for 2 min in the presence of 100μM L-glutamate, 100μM NMDA and a low Mg^{2+}-15mM K^+ - 100μM L-glutamate conditioning solution for induction of LTP. L-glutamate stimulated arachidonic acid release (20:4) significantly at $p = 0.01$. With NMDA and the LTP conditioning solution $p < 0.01$. Results for the other fatty acids was not significant. Results shown as means ± SD for at least four separate incubations.

DISCUSSION

For the first time in a preparation used to study LTP, a model of synaptic plasticity, we have shown directly that NMDA receptor activation can induce release of arachidonic acid. This release appears selective for arachidonic acid and may indeed come from a restricted pool of membrane phospholipids closely associated with the L-glutamate receptors. In most previous studies of arachidonic acid release following agonist stimulation the arachidonic acid release was measured from prelabelled phospholipids. Since our studies measured the mass increase of arachidonic acid release, it is more likely to reflect the specific changes related to the receptor activation. A weakness of studies on radioactively prelabelled preparations is that the radiolabel may not enter pools that are sensitive to hormonal or neurotransmitter stimulation and even if it does it might not reflect quantitatively the changes induced by receptor stimulation (see 28).

Recently there has been much discussion on the mediation of arachidonic acid and its lipoxygenase metabolites in the generation of LTP. Evidence is clear that the induction of LTP depends on NMDA receptor activation. What is less clear is how post-synaptic and pre-synaptic events participate in the expression and maintenance of the synaptic enhancement. Recent research indicates that an increase in presynaptic neurotransmitter release does occur and can explain in part the enhancement in synaptic efficiency (32,33). Because LTP induction is post-synaptic but its expression is pre-synaptic, a retrograde messenger has been postulated (34). Arachidonic acid itself or one of the 12-lipoxygenase metabolites is a likely candidate and the results in this paper provide further evidence for this possibility. The pre-synaptic events involved in the expression of LTP probably take place over a short time interval of about 10 to 30 min starting 2–3 min after induction, then post-synaptic events become more important in its maintenance (34). The short time window when pre-synaptic events are operative in LTP would require a rather fast signal following post-synaptic induction (33). The fast release of arachidonic acid observed in our experiments is in agreement with this view. Indeed, arachidonic acid has been shown to induce a release of L-glutamate in synaptosomes (35,36). It is still uncertain if arachidonic acid itself or a lipoxygenase metabolite is the active mole-

cule. A logical next step in our biochemical studies is to determine directly the time frame of lipoxygenase metabolite formation in relation to arachidonic acid release in hippocampal slices during the induction and maintenance of LTP.

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