RECIPROCAL REGULATION OF FATTY ACID RELEASE IN THE BRAIN BY GABA AND GLUTAMATE

Dale L. Birkle

Department of Pharmacology and Toxicology
West Virginia University
Health Science Center North
Morgantown, WV

INTRODUCTION

Free fatty acids (FFA) and their metabolites have many effects on neurochemical processes, including altering receptor-effector coupling, modulating the activity of protein kinase C, and changing ion channel conductance in the cell membrane. However, the neurotransmitters and other factors that control the release of FFA in neurons in normal or pathological states are not well defined. The following studies investigate the regulation of FFA release in intact brain, synaptosomes, and isolated neurons in culture in response to drugs that interact at γ-aminobutyric acid (GABA) and glutamate receptors. The results suggest that neuronal excitation via stimulation of glutamate receptors or blockade of GABA receptors causes the activation of FFA release, and that phosphatidylcholine (PC) is a major source of FFA. Conversely, inhibition of neuronal activity reduces FFA release. FFA release occurs via activation of phospholipase A_2 and possibly via activation of a PC-specific phospholipase C, followed by diacylglycerol (DG) lipase. The common pathway for these effects may be alterations in intracellular calcium.

FFA are normally maintained at extremely low levels in nervous tissue (Bazan et al., 1986). Accumulation can occur by activating phospholipase A_2, which removes the fatty acid esterified at the 2-position of phospholipids (Lands and Crawford, 1976). FFA are re-esterified into lipids by the energy-requiring conversion to acyl-CoA derivatives and subsequent incorporation into lysophospholipids or mono- and diacylglycerols (via acyltransferases). DG accumulate via the activation of phospholipase C, which removes the phosphoryl-base group of phospholipids. DG are converted to phosphatidic acid (PA) via DG kinase, and to other phospholipids via cytidylphosphotransferases. Alternatively, DG can be catabolized by DG lipase to produce FFA (see Horrocks, this volume).

Calcium appears to be an important regulator of phospholipase A_2 (Ho and Klein, 1987; Moskowitz et al., 1984; Felder et al., 1990; Sanfeliu et al., 1990; Dumuis et al., 1988; Lazarewicz et al., 1990). Calcium binds to the active site of phospholipase A_2 and enhances the substrate-enzyme interaction. Intracellular calcium also regulates the translocation of phospholipase A_2 from cytosol to membrane, a step that may be critical

Neurobiology of Essential Fatty Acids
Edited by N.G. Bazan *et al.*, Plenum Press, New York, 1992

to activation (Channon and Leslie, 1990). Activation of arachidonic acid (20:4) release in cultured neurons in response to N-methyl-D-aspartate (NMDA) (Sanfeliu et al., 1990; Dumuis et al., 1988; Dumuis et al., 1990; Lazarewicz et al., 1990), serotonin ($5HT_2$) agonists (Felder et al., 1990), and quisqualate/AMPA (Dumuis et al., 1990) is dependent on receptor-gated calcium influx. Voltage-gated calcium influx via K^+ depolarization is not sufficient in some preparations (Sanfeliu et al., 1990; Dumuis et al., 1988) but, in the retina (Birkle and Bazan, 1984) and in synaptosomes (Birkle and Bazan, 1987; Dorman, 1991), 20:4 release is stimulated by K^+ depolarization. Obviously the regulation of phospholipase A_2 by calcium is complex and may be influenced by compartmentalization within the intracellular space.

The studies described below characterize the activation of phospholipase A_2, the loss of fatty acids from particular glycerolipid classes, and the release and accumulation of FFA and DG in neurons. Studies have been conducted in the intact brain and in various model systems, including synaptosomes, primary neuronal cultures, and cultures of a neuroblastoma cell line. The advantages and disadvantages of the various experimental paradigms are discussed. The focus of the work is on the effects of inhibition and stimulation of the GABA and glutamate systems, to determine the possible reciprocal regulation of phospholipase A_2 via these neurotransmitters.

FFA AND DG ACCUMULATION IN THE RAT BRAIN *IN VIVO*

In these types of studies, animals are treated with a drug and killed by head- focused microwave irradiation. This method of sacrifice heats the brain tissue very rapidly, reaching 90°C in less than 3 seconds. Therefore enzymes are denatured rapidly, and postmortem activation of phospholipase A_2 and other lipases does not occur. The measurements of FFA and DG represent levels at a single time point, and reflect the balance between FFA release and reacylation and DG production and conversion to phospholipids, triacylglycerols (TG), or monoacylglycerols (MG), and FFA.

Increased neuronal activity causes rapid (within seconds) changes in membrane lipids in brain. A striking example of this phenomenon is the accumulation of FFA during the earliest periods of seizures (Bazan, 1970). Seizures induced by the GABA antagonist, bicuculline, cause a prompt and dramatic increase in FFA (Rodriguez de Turco et al., 1983). This occurs in whole brain and in hippocampus and cerebral cortex (Siesjo et al., 1982), and can be measured in synaptosomes isolated from bicuculline-treated rats (Birkle and Bazan, 1987). Whether these effects are mediated via direct interaction with the GABA receptor complex or via other neurotransmitter systems is not known.

There is substantial evidence that ischemia-induced elevation in FFA is due in large part to the activation of phospholipase A_2, while elevation in DG is due in large part to activation of phosphoinositide (PI)-specific phospholipase C (see Sun, this volume). Drugs that change FFA levels may be affecting phospholipase A_2 directly through their receptors or via a drug-induced alteration in another neurotransmitter system. Isolation of particular subcellular fractions from microwave-fixed brains is not possible, but specificity in the responses to pharmacological treatments can be assessed to a limited degree by studying discrete brain regions. Changes in response to a generalized stimulus from one brain region to another reflect the neurotransmitters and receptors that are present in that region.

Effects of Diazepam on FFA in the Rat Brain

Diazepam acts at the $GABA_A$ receptor complex to increase the affinity of GABA binding sites for GABA, thus enhancing inhibitory tone in the brain. These experiments

(Flynn et al., 1986) were done to test the hypothesis that positive modulation of the GABA system would affect fatty acid metabolism in a manner opposite to negative modulation, i.e., since $GABA_A$ blockade by convulsants (bicuculline, picrotoxin, pentylenetetrazol) increases FFA in brain (Bazan et al., 1986), then increasing $GABA_A$ activity may decrease FFA levels.

Rats were treated with diazepam (2 or 4 mg/kg, i.p.) or drug vehicle and were killed 1 hr later by head-focused microwave irradiation. Whole brain less cerebellum and brain stem was removed and homogenized in hexane:2-propanol (3:2) to extract lipids. FFA were isolated by thin layer chromatography (TLC) (Birkle et al., 1988b), derivatized to form methyl esters (Morrison and Smith, 1964), and quantified by gas-liquid chromatography (GLC) using an internal standard method (Birkle et al., 1988b). There was no significant effect of diazepam on the levels of saturated fatty acids (palmitic, 16:0 or stearic, 18:0) and no effect on oleic (18:1) or linoleic (18:2) acids (Fig. 1). However, levels of free 20:4 and docosahexaenoic acid (22:6) were reduced about 60%. One explanation of these results is that the basal levels of

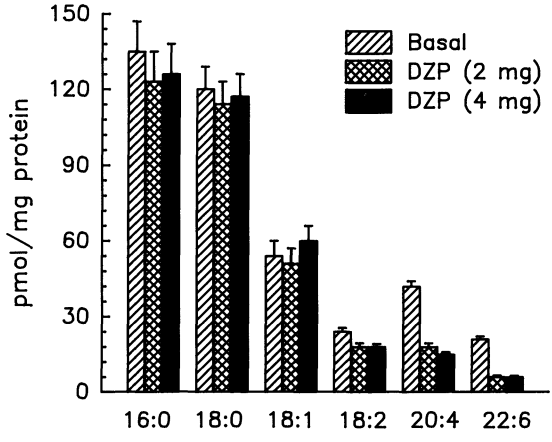

Figure 1. Diazepam (DZP) reduces the basal levels of 20:4 and 22:6 in the whole rat brain. Rats were treated with drug vehicle (basal) or 2 or 4 mg/kg diazepam (DZP), i.p., and sacrificed by head-focused microwave irradiation 1 hr after drug treatment. Values are means ± SE of 6 animals per group.

unsaturated FFA in brain can be reduced by enhancing the activity of GABA at $GABA_A$ receptors. Another possible explanation is that so-called basal levels of FFA in microwave-fixed rat brain are truly above normal because of the stress caused by a novel environment and by the 5 to 10 sec restraint in the microwave holder. Control animals were not acclimated to the microwave holder, so FFA levels in control rats may have been higher than true basal levels. Because diazepam is an anxiolytic drug, treated rats would not experience the stress, mild as it may be, and thus the FFA levels in treated rats would be reduced compared to the "stressed" controls.

Seizure-Induced Alterations in Lipid Metabolism in Specific Brain Regions

Most of the previous work on the effects of neuronal stimulation on brain lipids has been done in whole brain (Bazan et al., 1986). The following experiments examined the changes in FFA and DG in specific brain regions and found a remarkable heterogeneity in these responses.

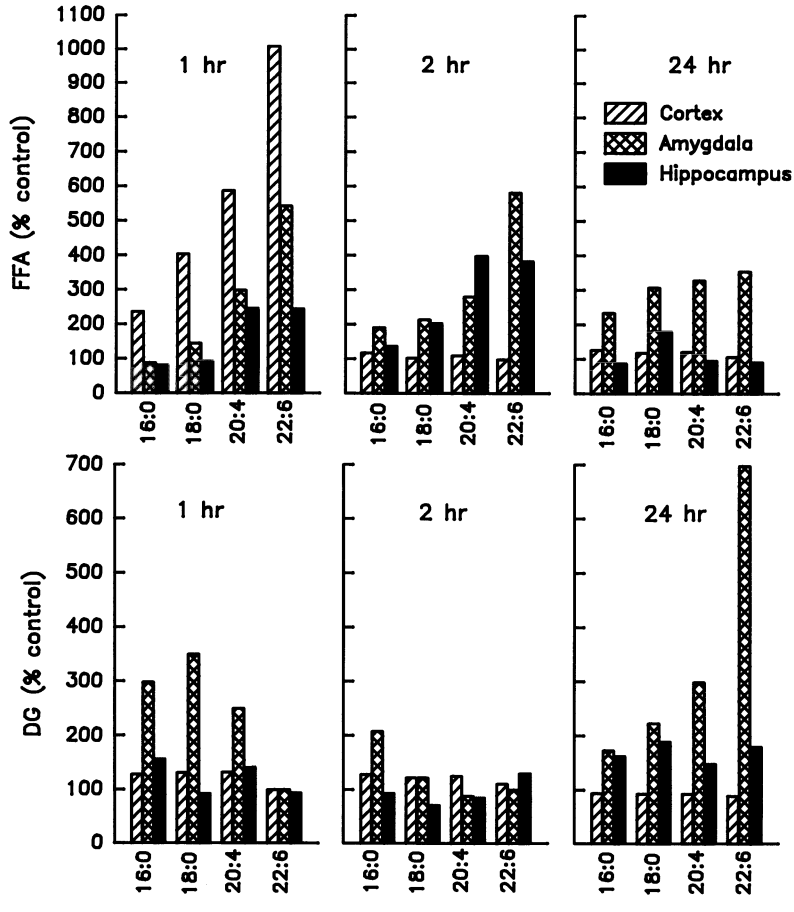

Figure 2. Alterations in FFA and DG in selected brain regions 1, 2, or 24 hr after kainic acid treatment *in vivo* (10 mg/kg, s.c.). Values are means of % of control (experimental/control), N = 6 to 8. Values greater than 120% of control were statistically significant (ANOVA, Tukey's protected t-test).

Effects of Kainic Acid

Kainic acid acts at the kainic acid subtype of glutamate receptors, induces limbic status epilepticus, and produces characteristic pathology in the brain (Sperk et al., 1983). Rats were treated with kainic acid (5 to 15 mg/kg, s.c.) or 0.9% NaCl. Behavioral responses were rated (no response to full limbic status epilepticus) over time, and rats were killed by head-focused microwave irradiation at 1, 2, or 24 hr after drug treatment. Brains were dissected to yield frontal cortex, hippocampus, and amygdala/pyriform cortex, areas rich in glutamate receptors (London and Coyle, 1979). FFA and DG were isolated from lipid extracts by TLC and quantified by capillary GLC. In all brain areas, an increase in FFA, particularly 20:4 and 22:6, was evident at 1 hr (Fig. 2, top panel), 20 min prior to any behavioral manifestations of seizure activity. FFA accumulation was most pronounced in the frontal cortex. By 2 hr, FFA levels in cortex had returned to normal values, but in the hippocampus and amygdala, FFA continued to increase. This time point was taken during the period of most intense seizure activity, which occurred

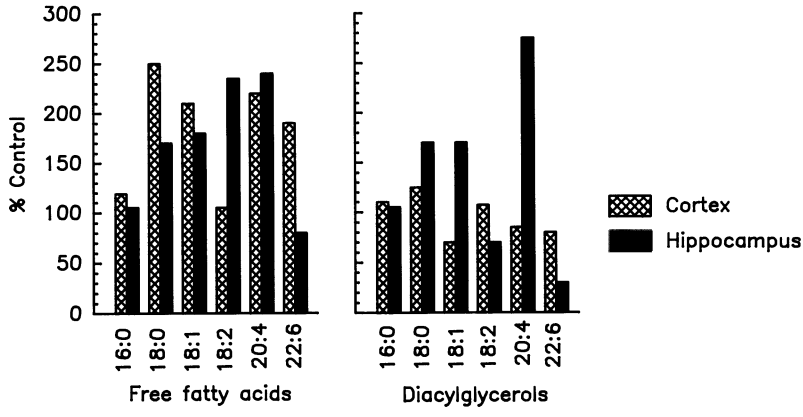

Figure 3. Effect of a single electroconvulsive shock on the accumulation of FFA and DG in the rat hippocampus and cortex. Values are % of control (experimental/control) and are means of 6 samples.

between 80 and 200 min post drug. Twenty-four hours later, when histological evidence of neuronal damage is apparent in the amygdala (data not shown), FFA levels had normalized in hippocampus, but remained elevated in amygdala.

DG levels were increased at 1 hr in amygdala, but there was a delayed increase in DG in the hippocampus, occurring at 24 hr (Fig. 2, bottom panel). DG did not change at any time point in cortex. DG in the amygdala remained elevated at 24 hr. The DG pool was not specifically enriched in 18:0/20:4; however, increases in 16:0 and 22:6 in the DG pool were observed. This suggests that the PI cycle was not the sole source of this lipid.

These data show a heterogeneity in the changes in lipid mediators in response to kainic acid. This suggests that at least some of the changes in lipids are not due directly to an action of kainic acid at its receptor, but rather to the release of other neurotransmitters induced by kainic acid. The neurotransmitters vary with the brain area, and so the lipid responses also vary.

Effects of Electroconvulsive Shock

Electroconvulsive shock causes a reversible increase in FFA and DG and a loss of phosphatidylinositol-4,5-bis-phosphate (PIP_2) in whole brain (Reddy and Bazan, 1987). These experiments investigated brain lipid metabolism in specific brain regions important in the generation and propagation of generalized seizures, the hippocampus and cerebral cortex (Birkle et al., 1988a), in response to a single tonic-clonic seizure induced by maximal electroconvulsive shock. Rats were subjected to a single electroconvulsive shock, then killed by microwave irradiation 30 sec later (during the clonic phase of the seizure). Hippocampus and cerebral cortex were dissected and the lipids extracted. FFA and DG were isolated by TLC and quantified by capillary GLC.

The data show that although FFA levels increase in both brain regions, DG levels increased only in the hippocampus (Fig. 3). This could indicate that seizure-induced stimulation of the PI cycle (the main pathway for the production of DG in this model) occurs mainly in the hippocampus, whereas activation of phospholipase A_2, leading to FFA release, occurs in both brain regions. Alternatively, DG lipase may be selectively

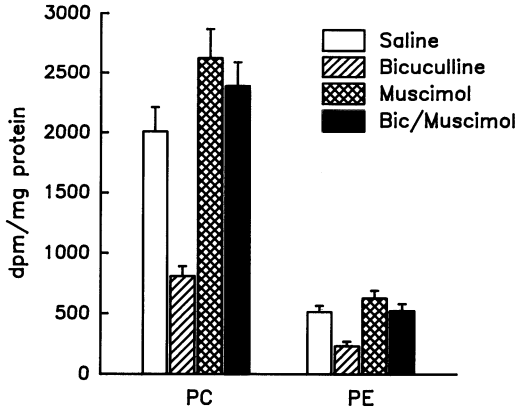

Figure 4. Loss of ^{14}C-22:6 from phosphatidylcholine (PC) and phosphatidylethanolamine (PE) in rat brain synaptosomes incubated 30 min with 1 μM bicuculline methiodide with or without 10 μM muscimol. Values are means ± SE of 6 samples.

activated in the cortex, so that DG are catabolized to produce FFA, and thus do not accumulate.

FFA RELEASE IN RAT BRAIN SYNAPTOSOMES

The following experiments were conducted in preparations of synaptosomes from the whole rat cerebrum. This model system consists largely of synaptic boutons and the biochemical events represent, for the most part, actions of drugs at presynaptic receptors. Synaptosomal preparations are highly purified in neuronal elements compared to preparations of the intact brain. Intact circuitry is absent from a synaptosomal preparation. However, depolarization of synaptosomes does cause the release of neurotransmitters (de Belleroche and Bradford, 1972), which must be considered in the interpretation of results in terms of direct vs. indirect effects of the stimuli. Synaptosomes are also a "damaged" preparation, and subject to postmortem alterations in lipids that occur during the isolation procedures. For this reason, high levels of endogenous FFA and DG are present (Birkle and Bazan, 1987; Birkle and Bazan, 1988). Therefore, to study FFA release mechanisms, radiotracer techniques must be used. Radiotracer studies do have the advantage of allowing measurement of the changes in phospholipid pools, which can be used to infer possible sources of released FFA.

Bicuculline-induced status epilepticus causes the release and accumulation of FFA in the brain *in vivo* (Rodriguez de Turco et al., 1983; Siesjo et al., 1982). Previous experiments have not targeted any particular neurotransmitter system as a putative link to FFA release. Bicuculline is a $GABA_A$ antagonist, so its effect *in vivo* to release FFA may be due to interaction at $GABA_A$ receptors. In the intact animal, however, the contribution of disinhibition of other neurotransmitter systems must be considered. These experiments were designed to investigate the direct effects of bicuculline on membrane lipid metabolism and to investigate the phospholipid sources of the released FFA.

Synaptosomes were isolated from whole cerebrum of naive rats (Birkle and Bazan, 1987). The membrane lipids were labeled with ^{14}C-22:6 by incubation at 37°C for 1 hr in a Tris buffer containing CoASH, ATP, Mg^{2+}, DTT, EGTA, and 1-^{14}C-22:6. The labeled membranes were washed in Tris buffer containing BSA to remove excess

unincorporated ^{14}C-22:6, then re-incubated (pulse chased) in Tris buffer with 1 μM Ca^{2+}, 2 μM unlabeled 22:6, and varying concentrations of bicuculline methiodide (0.1, 1, 10 μM) for 5, 10, and 30 min. The incubations were stopped by addition of 2 vol chloroform:methanol (1:1), the lipids were extracted, and the radioactivity in the FFA pool and various glycerolipids was assessed chromatographically and by liquid scintillation counting.

Bicuculline methiodide caused a loss of ^{14}C-22:6 from PC and phosphatidylethanolamine (PE) (Fig. 4). There was a corresponding increase in the amount of free ^{14}C-22:6 (data not shown). The effect of bicuculline was both concentration and time dependent (data not shown). When synaptosomes were incubated with bicuculline (1 μM) after a 10 min pre-incubation in the presence of the $GABA_A$ agonist, muscimol (10 μM), the bicuculline-induced loss of radioactivity from PC and PE was inhibited (Fig. 4).

These experiments demonstrate that a $GABA_A$ antagonist can stimulate FFA release in a subcellular preparation devoid of intact neuronal circuitry. Synaptosomes contain substantial amounts of GABA, which exerts a tonic hyperpolarizing effect on the membrane potential. It has been shown previously that bicuculline causes a voltage-dependent influx of calcium into synaptosomes, presumably by antagonizing this tonic hyperpolarization by GABA (Straub et al., 1990; de Belleroche and Bradford, 1972). This depolarization-induced calcium influx may be responsible for activating FFA release mechanisms. When synaptosomes were exposed to the potent $GABA_A$ agonist, muscimol, the effects of bicuculline were blocked. This observation lends further support to the hypothesis that the effects of bicuculline are mediated via the $GABA_A$ receptor. Another possible (or additional) mechanism for bicuculline's effect is the depolarization-induced release of other neurotransmitters, which could then activate their receptors to induce FFA release. FFA release in these experiments is likely to be the result of the activation of phospholipase A_2, because the target lipids were PC and PE. Activation of the PI-specific phospholipase C would result in a loss of phosphoinositides, which was not observed (data not shown).

FATTY ACID RELEASE IN NEUROBLASTOMA CELLS IN CULTURE

Synaptosomes are somewhat limited in their usefulness for studies of FFA release because they are a damaged preparation that is leaky to ions. Furthermore, synaptosomes represent presynaptic membranes mainly, so postsynaptic effects cannot be observed. Neurons in cell cultures form synapses with each other, so both pre- and postsynaptic elements are present. Use of these cell cultures allows pharmacological manipulation of receptors with simultaneous measurement of phospholipid metabolism, without causing any damage to the cells and, therefore, with less artifactual activation of lipases. Moreover, tumor cell lines are easily maintained and quickly yield the large numbers of cells needed for biochemical studies.

Previous studies investigated the basal and norepinephrine-stimulated metabolism of 20:4 in Neuro-2A cells and found reasonable similarity in responses, as compared to the brain homogenates (Birkle and Ellis, 1983). Incubation of confluent, differentiated cultures of Neuro-2A with radiolabeled 20:4 resulted in labeling of various glycerolipid pools and the synthesis of cyclooxygenase and lipoxygenase products. Phospholipid labeling patterns were similar to the distribution of endogenous 20:4 in brain lipids. In a short term incubation (20 min), about 40% of the radiolabel was converted to prostaglandins and hydroxyeicosatetraenoic acids (HETE), a rate of conversion much greater than that usually observed in brain using exogenous substrates. Over a 24-hr period, about 75% of the fatty acid was incorporated in phospholipids; the remainder was in the form of free (10%) or oxygenated (15%) metabolites. When prelabeled cells were incubated in fresh medium, about 20% of the fatty acid was released from

glycerolipids, mainly from PE. The loss of label from PE was stimulated by norepinephrine (5 μM), and the effect of norepinephrine was blocked by the ß-antagonist, propranolol (10 μM). These data, coupled with the report that Neuro-2A cells have binding sites for GABA and benzodiazepines that are similar in density and affinity to those found in the brain (Baraldi et al., 1979), led to the studies on the use of these cells as a model system for studying the interaction of GABA and phospholipase A_2.

Effects of Bicuculline and Calcium Ionophore (A23187) on Fatty Acid Metabolism in Neuro-2A Cells

These experiments examined the release of FFA from Neuro-2A cells after stimulation with bicuculline or A23187 (Birkle and Wiley, 1991). The objectives were to characterize the system for use as a model to study the regulation of phospholipase A_2, and to further investigate the phospholipid sources of released FFA. Confluent monolayer cultures of Neuro-2A cells in 35-mm wells were prelabeled in growth medium by addition of 0.1 or 0.5 μCi 1-^{14}C-22:6 or 1-^{14}C-20:4. After 30 min, 60 min, or 24 hrs of labeling, cells were washed to remove serum-containing growth medium and unesterified fatty acid. Cells were then re-incubated for various times in fresh buffer with 10 μM A23187, 1 or 10 μM bicuculline methiodide, or drug vehicles (0.01% DMSO or normal saline). At the end of the incubation time, ice-cold methanol (2 vols) was added and the lipid extracts were prepared. Major glycerolipids and FFA were separated by TLC and quantified by liquid scintillation counting or capillary GLC.

Endogenous FFA were released to the medium during re-incubation of Neuro-2A cells for 10 or 30 min (data not shown). Treatment with either 10 μM A23187 or 1 μM bicuculline stimulated this release. The FFA pool was specifically enriched in 18:1 and 20:4 after the 10 min treatment with A23187 or bicuculline; at 30 min, drug treatment caused an enrichment of 20:4 only. This enrichment in unsaturated fatty acids in the free pool is an indication of the specific activation of phospholipase A_2.

FFA measured at 10 min represented about 2% of the total fatty acids esterified in phospholipids; therefore the source of released FFA could not be determined by measuring loss of particular glycerolipids. However, prelabeling cells with 1-^{14}C-22:6 or 1-^{14}C-20:4 provided an indication of the source of the released fatty acid. A time-dependent release of free 1-^{14}C-22:6 or 1-^{14}C-20:4 occurred during re-incubation, similar to the release of endogenous FFA (data not shown). Release was markedly stimulated by 1 μM bicuculline or 10 μM A23187. Loss of 1-^{14}C-22:6 from PC was observed, while 1-^{14}C-20:4 was lost from both PC and PE. Other glycerolipids were not significantly altered over time, with the exception of TG. Labeling of TG increased during the re-incubation period, possibly indicating that some of the FFA was rapidly re-esterified.

The results of this study suggest that FFA release induced by A23187 or bicuculline is mediated by phospholipase A_2. This mechanism is supported by the specific enrichment of unsaturated fatty acids (18:1, 18:2, and 20:4) in the free pool and labeling studies, which demonstrate a loss of 1-^{14}C-20:4 and 1-^{14}C-22:6 from PC and PE. In addition, no accumulation of DG or PA and no loss of radiolabel from PI were detected. These latter two observations argue against release of FFA via the phospholipase C/DG lipase pathway. The results of this study suggest that the Neuro-2A cell line, with its advantages of homogeneous cell type and easily controllable milieu, is a potentially useful system for determining the molecular mechanisms that control the normal and pathological regulation of phospholipase A_2 in neurons. However, this model is limited by the types of receptors that are present in this cell line and potentially by derangements in receptor-effector coupling related to the loss of control of cell division that characterizes immortal cell lines.

There are some valid concerns about the use of a clonal cell line for studies of receptor-mediated changes in lipase activity. While a clonal cell line has advantages in terms of availability of the cells and the studies described above suggest it may be a useful system, the question of what types of receptors are present and if the receptors change as a function of the degree of differentiation is a crucial consideration.

Primary cultures of hippocampal neurons have been well characterized electrophysiologically and pharmacologically, in terms of receptors for excitatory (Murphy and Miller, 1988; Furuya et al., 1989) and inhibitory amino acids (Zorumski and Yank, 1988). Recently some studies on the regulation of 20:4 release in hippocampal neurons have been reported. Release of 20:4 is stimulated by NMDA (Sanfeliu et al., 1990), muscarinic (M1) agonists (Kanterman et al., 1990), and agonists at $5HT_2$ receptors (Felder et al., 1990). In all cases the effects of the agonist were blocked by removing calcium from the incubation medium. The effects of other neurotransmitters have not been reported. The sources of released 20:4 have not been identified, but a dissociation from stimulation of the PI cycle has been well established (Kanterman et al., 1990; Felder et al., 1990; Sanfeliu et al., 1990).

Uptake of Radiolabeled Fatty Acids into Hippocampal Neurons

The purpose of these experiments was to determine the ability of hippocampal neuron cultures to incorporate radiolabeled fatty acid and to determine the pattern of labeling of glycerolipids. Hippocampal cultures in 35-mm, 6-well plates were prepared from rat embryos at E16 or E17 (Shahar et al., 1989; Conn, 1990). Cells were seeded at a density of 1 to 2 million cells per well. On the 10th day of culture, 1-^{14}C-20:4 was added to the growth medium (0.2 to 0.5 μCi per well). Twenty-four hours later the cells were rinsed and the lipids were extracted to assess the uptake of the fatty acid into cellular glycerolipids. About 70% of the added fatty acid was incorporated into the cells. About 80% of the incorporated label was in the phospholipid fraction, 3% in DG, 11% in TG, and 6% in FFA. In the phospholipid fraction, the radiolabel was distributed as follows: phosphatidylserine (PS), 1.3%; PA, 1.8%; PI, 42.5%; PC, 49.7%; and PE, 4.7%. This pattern of distribution differs from Neuro-2A in that in the primary cells, there is more labeling of PI and DG and less labeling of PE, suggesting a more active PI cycle in the hippocampal cells.

Release of 20:4 in Response to Bicuculline

Cells prelabeled for 24 hrs were washed and re-incubated with 1 μM bicuculline. At 5, 10, 15, or 30 min, incubations were stopped by addition of methanol and lipid extracts prepared and analyzed. Bicuculline caused significant increases in radioactivity in the incubation medium, representing an increase in FFA released from the cells over time (Fig. 5). In untreated cells, FFA release increased rapidly over the first 10 min, then reached a plateau. In the bicuculline-treated cells, there was an enhanced release of FFA during the first 10 min, followed by a slower increase over the 10 to 30 min period. The release of FFA was accompanied by a decline in PC and a rapid increase in DG during the first 10 min. DG began to decrease during the 5 to 30 min period, perhaps indicating an activation of DG lipase. The level of label in PC increased slightly during the 10 to 30 min period, indicating a reacylation of this phospholipid. At no time were any alterations observed in PI (Fig. 5) or in any other glycerolipid (data not shown).

These results demonstrate several points. First, the response of hippocampal cells to bicuculline is similar to that of Neuro-2A in terms of accumulation of FFA and loss

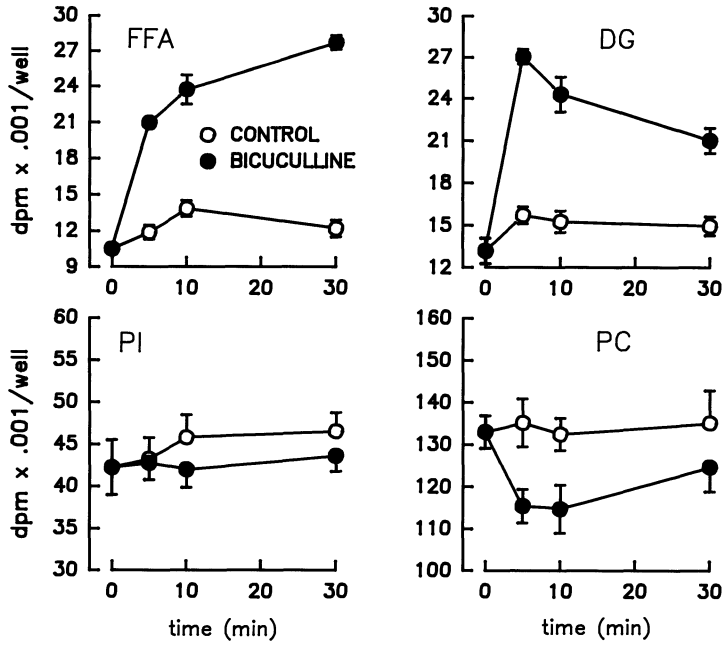

Figure 5. Activation of FFA release, DG accumulation, and loss of radiolabel from PC in hippocampal neurons in primary culture incubated with 1 μM bicuculline methiodide. Each well was seeded at a density of 1.5 x 10^6 cells per well and data were normalized to a total radioactivity of 200,000 dpm/well. Values are means ± SE of 6 wells.

of PC. However, no accumulation of DG was observed in the Neuro-2A cells. Second, bicuculline's effects are most prominent during the early periods of incubation. In both Neuro-2A cells and hippocampal neurons, the effects of bicuculline appear to be on the release of FFA from PC via phospholipase A_2. In hippocampal cells phospholipase C may also be involved, since an accumulation of DG was observed. However, since no loss of PI was measured, phospholipase C may be acting on PC to produce DG.

Release of 20:4 in Response to Kainic Acid

In these experiments, cells were prelabeled for 24 hrs with ^{14}C-20:4, washed, then re-incubated in fresh buffer. At 10 min, 100 μM kainic acid was added, and the incubations were continued an additional 20 min. Aliquots of the incubation medium were taken every 2 min (250 μl out of 2 ml) and the medium replaced to maintain the volume and concentration of drug. Aliquots were placed in scintillation vials and counted to obtain cumulative counts present in the medium. At the end of the incubation, methanol was added to extract the lipids of the cells and the amounts of radioactivity present in the various lipid fractions were determined.

In untreated cells, an increase in FFA occurred over the first 10 min, and then reached a plateau (Fig. 6). This probably reflects a nonspecific stimulation of FFA release during the washing steps, as this rapid release was also observed in the experiments with bicuculline (see Fig. 5). When kainic acid was added, the release of FFA continued to increase over the remaining 20 min of incubation, instead of reaching a plateau (Fig. 6, top panel). In kainic acid-treated cells there was a substantial loss of label from PC, a minor loss of label from PS, and an increase in DG (Fig. 6, bottom

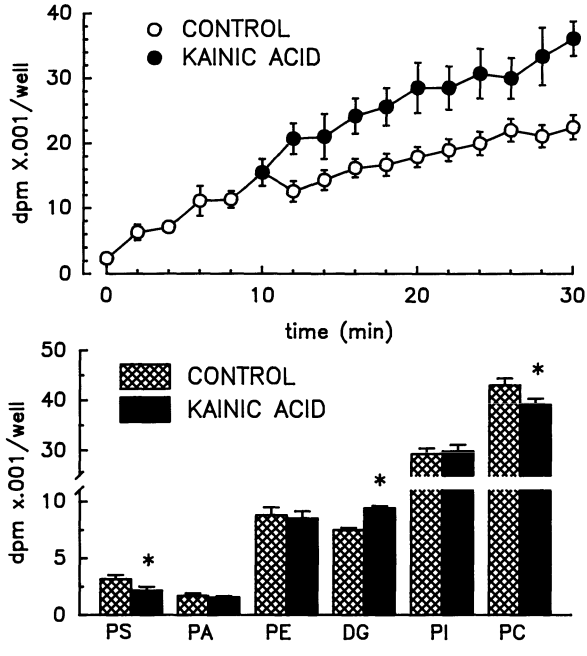

Figure 6. Effects of 100 μM kainic acid on FFA release and loss of label from phospholipids in hippocampal neurons in primary culture. Each well was seeded at a density of 2.5 x 10^6 cells per well. Values are means $\pm$ SE of 12 samples.

panel). There was no change in other phospholipids, including PI and PA.

These results, like those of the experiments with bicuculline, suggest that depolarization of hippocampal neurons by the glutamate agonist, kainic acid, causes an activation of phospholipase A_2 and a PC-specific phospholipase C.

SUMMARY AND CONCLUSIONS

Several model systems have been used to test the hypothesis that the release of FFA in the brain is regulated by depolarization of neurons. This FFA release is likely the result of the activation of phospholipase A_2. The increased neuronal activity that occurs due to synchronous depolarization during seizures causes activation of phospholipase A_2. Decreasing neuronal activity by administering the anxiolytic, diazepam, appears to decrease the activity of phospholipase A_2. The GABA antagonist, bicuculline, which causes depolarization by negating the hyperpolarizing tone imposed on neurons by GABA, causes FFA release in synaptosomes and in neurons in tissue culture. Likewise, the glutamate agonist, kainic acid, which depolarizes neurons by opening sodium channels, increases the activity of phospholipase A_2. PC-specific phospholipase C, another enzyme important in the generation of the second messenger, DG, is also activated by depolarization.

Several important questions remain to be answered. The site of FFA release, in terms of the pre- vs. postsynaptic membrane, is not clear, although the experiments with synaptosomes support the hypothesis that activation of phospholipase A_2 may be an important regulator of presynaptic events. This idea has also been suggested by studies on the phenomenon of long-term potentiation, where free 20:4 or its metabolites may

be involved in presynaptic facilitation of neurotransmitter release (Freeman et al., 1990; Massicotte et al., 1990; Williams et al., 1989; also see Dorman, this volume). The activation of the PI cycle and subsequent stimulation of protein kinase C may be a postsynaptic event important in the integration of inputs at the dendrite and soma or a presynaptic event involved in the modulation of neurotransmitter release (Taniyama et al., 1990; El-Fakahany et al., 1990; also see Nishizuka, this volume). Therefore the stimulation of a PC-specific phospholipase C, which is capable of generating large amounts of DG over a prolonged period of time (Exton, 1990; Martinson et al., 1990; Diaz-Laviada et al., 1990), could occur at either site.

Another important question is the role of FFA and DG in affecting cell-cell signaling events, particularly with regard to ion fluxes. Modulation of an acetylcholine-linked K^+ channel in the heart by FFA and their oxygenation products has been reported (Kim and Clapham, 1989). The cardiac muscarinic receptor is linked to a hyperpolarizing K^+ channel via a G protein. Receptor-activated dissociation of the G protein releases $\beta\gamma$ subunits, which stimulate phospholipase A_2, resulting in the release of 20:4 and subsequent conversion to lipoxygenase products; these metabolites increase the activity of the K^+ channel (Kim et al., 1989). Lipoxygenase products also modulate responses of *Aplysia* sensory neurons to FRMF-amide (Piomelli et al., 1987; Volterra and Siegelbaum, 1988; also see Volterra, this volume) via changes in a K^+ channel. In the heart and in smooth muscle cells, there are also other K^+ channels that are activated by FFA directly (Kim and Duff, 1990). In hippocampus, 20:4 and its metabolites cause hyperpolarization and increased inhibitory postsynaptic potentials (IPSPs), which may be related to changes in K^+ conductance (Carlen et al., 1989). In the rat brain, $GABA_B$ and $5HT_{1a}$ receptors are linked via a G protein to a hyperpolarizing K^+ channel (Andrade et al., 1986; Innis et al., 1988), which may be modulated by phospholipase A_2 activation (Duman et al., 1986). These observations underscore the importance of determining the regulation of FFA release in the brain.

It is now clear that FFA and DG are important lipid-derived messengers in neurons, and that their metabolism is highly regulated. The techniques and model systems now available should provide the means for discerning regulatory factors, contributions to the normal function of neurons, and mechanisms of pathological alterations in diseases of the central nervous system.

ACKNOWLEDGMENTS

Supported by grants from Epilepsy Foundation of America, Pharmaceutical Manufacturers Association Foundation, NSF #BNS-8919943, and NIH-BMRS #2S07-RR05433-28. The technical assistance of B.J. Victor and K.S. Wiley is appreciated.

REFERENCES

Andrade R, Malenka RC, Nicoll RA (1986) A G protein couples serotonin and $GABA_B$ receptors to the same channels in hippocampus. Science 234:1261-1265.

Baraldi M, Guidotti A, Schwartz JP, Costa E (1979) GABA receptors in clonal cell lines: A model for study of benzodiazepine action at molecular level. Science 205: 821-823.

Bazan NG (1970) Effects of ischemia and electroconvulsive shock on free fatty acid pool in the brain. Biochim Biophys Acta 218:1-10.

Bazan NG, Birkle DL, Tang W, Reddy TS (1986) The accumulation of free arachidonic acid, diacylglycerols, prostaglandins and lipoxygenase reaction products in the brain during experimental epilepsy. In: Advances in Neurology, Vol 44

(Delgado-Escueta AV, Ward AA, Woodbury DM, Porter RJ, eds) pp 879-902. New York: Raven Press.
Birkle DL and Bazan NG (1984) Effect of K^+ depolarization on the synthesis of prostaglandins, hydroxyeicosatetraenoic acid (HETE) and other eicosanoids in the rat retina: Evidence for esterification of 12-HETE in lipids. Biochim Biophys Acta 795:564-573.
Birkle DL and Bazan NG (1987) Effect of bicuculline-induced status epilepticus on prostaglandins and hydroxyeicosatetraenoic acids in rat brain subcellular fractions. J Neurochem 48:1768-1778.
Birkle DL and Bazan NG (1988) Cerebral perfusion of metabolic inactivators: A new method for rapid fixation of labile lipid pools in brain. Neurochem Res 13: 849-852.
Birkle DL and Ellis EF (1983) Conversion of arachidonic acid to cyclooxygenase and lipoxygenase reaction products and incorporation into phospholipids in the mouse neuroblastoma clone, Neuro-2A. Neurochem Res 8:319-332.
Birkle DL and Wiley KS (1991) Bicuculline induces free fatty acid release from phospholipids in Neuro-2A cells in culture. Neurochem Res 16:1285-1293.
Birkle DL, Kurian P, Bazan NG (1988a) Seizure-induced alterations in lipid metabolism in hippocampus and cerebral cortex. Soc Neurosci Abstr 14:574.
Birkle DL, Kurian P, Braquet P, Bazan NG (1988b) The platelet activating factor antagonist, BN52021, decreases accumulation of free polyunsaturated fatty acids in mouse brain during ischemia and electroconvulsive shock. J Neurochem 51:1900-1905.
Carlen PL, Gurevich N, Wu PH, Su WG, Corey EJ, Pace-Asciak CR (1989) Actions of arachidonic acid and hepoxilin A3 on mammalian hippocampal CA1 neurons. Brain Res 497:171-176.
Channon JY and Leslie CC (1990) A calcium-dependent mechanism for associating a soluble arachidonoyl-hydrolyzing phospholipase A_2 with membrane in the macrophage cell line RAW 264.7. J Biol Chem 265, 5409-5413.
Conn PM (1990) Methods in neuroscience, Vol 2: Cell culture. San Diego: Academic Press Inc.
de Belleroche JS and Bradford HF (1972) Metabolism of beds of mammalian cortical synaptosomes: Response to depolarizing influences. J Neurochem 19:585-602.
Diaz-Laviada I, Larrodera P, Diaz-Meco MT (1990) Evidence for a role of phosphatidylcholine-hydrolysing phospholipase C in the regulation of protein kinase C by *ras* and *src* oncogenes. EMBO J 9:3907-3912.
Dorman RV (1991) $PGF_{2\alpha}$ synthesis in isolated cerebellar glomeruli: Effects of membrane depolarization, calcium availability and phospholipase activity. Prostaglandins Leukot Essent Fatty Acids 42:233-240.
Duman RS, Karbon EW, Harrington C, Enna SJ (1986) An examination of the involvement of phospholipases A_2 and C in the alpha-adrenergic and GABA receptor modulation of cAMP accumulation. J Neurochem 47:800-810.
Dumuis A, Pin JP, Oomagari K, Sebben M, Bockaert J (1990) Arachidonic acid released from striatal neurons by joint stimulation of ionotropic and metabotropic quisqualate receptors. Nature 347, 182-184.
Dumuis A, Sebben M, Haynes L, Pin J-P, Bockaert J (1988) NMDA receptors activate the arachidonic acid cascade system in striatal neurons. Nature 336:68-70.
El-Fakahany EE, Alger BE, Lai WS, Pitler TA, Worley PF, Baraban JM (1990) Neuronal muscarinic responses: Role of protein kinase C. FASEB J 2:2575-2583.
Exton JH (1990) Hormonal regulation of phosphatidylcholine breakdown. Adv Second Messenger Phosphoprotein Res 24:152-157.

Felder CC, Kanterman RY, Ma AL, Axelrod J (1990) Serotonin stimulates phospholipase A_2 and the release of arachidonic acid in hippocampal neurons by a type 2 serotonin receptor that is independent of inositolphospholipid hydrolysis. Proc Natl Acad Sci USA 87:2187-2191.

Flynn CJ, Birkle DL, Wecker L (1986) Diazepam prevents seizure-induced increases in free fatty acids and choline in rat cerebrum. Soc Neurosci Abstr 12:454-454.

Freeman EJ, Terrian DM, Dorman RV (1990) Presynaptic facilitation of glutamate release from isolated hippocampal mossy fiber nerve endings by arachidonic acid. Neurochem Res 15:743-750.

Furuya S, Ohmori H, Shigemoto T, Sugiyama H (1989) Intracellular calcium mobilization triggered by a glutamate receptor in rat cultured hippocampal cells. J Physiol 414:539-548.

Ho AK and Klein DC (1987) Activation of $alpha_1$-adrenoceptors, protein kinase C, or treatment with intracellular free Ca^{2+} elevating agents increases pineal phospholipase A_2 activity. J Biol Chem 262:11764-11770.

Innis RB, Nestler EJ, Aghajanian GK (1988) Evidence for G protein mediation of serotonin- and $GABA_B$-induced hyperpolarization of rat dorsal raphe neurons. Brain Res 459:27-36.

Kanterman RY, Ma AL, Briley EM, Axelrod J, Felder CC (1990) Muscarinic receptors mediate the release of arachidonic acid from spinal cord and hippocampal neurons in primary culture. Neurosci Lett 118:235-237.

Kim D and Clapham, DE (1989) Potassium channels in cardiac cells activated by arachidonic acid and phospholipids. Science 244:1174-1176.

Kim D and Duff RA (1990) Regulation of K^+ channels in cardiac myocytes by free fatty acids. Circ Res 67:1040-1046.

Kim D, Lewis DL, Graziadei L, Neer EJ, Bar-Sagi D, Clapham DE (1989) G-protein beta-gamma subunits activate the cardiac muscarinic K^+-channel via phospholipase A_2. Nature 337:557-560.

Lands W and Crawford C (1976) Enzymes of membrane phospholipid metabolism in animals. In: Enzymes of biological membranes (Martonosi A, eds) pp 3-85. New York: Plenum Press.

Lazarewicz JW, Wroblewski JT, Costa E (1990) *N*-methyl-D-aspartate-sensitive glutamate receptors induce calcium-mediated arachidonic acid release in primary cultures of cerebellar granule cells. J Neurochem 55:1875-1881.

London ED and Coyle JT (1979) Specific binding of [^{3}H]kainic acid to receptor sites in rat brain. Mol Pharmacol 15:492-505.

Martinson EA, Goldstein D, Heller Brown J (1990) Muscarinic receptor activation of phosphatidylcholine hydrolysis: Relationship to phosphoinositide hydrolysis and diacylglycerol metabolism. J Biol Chem 264:14748-14754.

Massicotte G, Oliver MW, Lynch G, Baudry M (1990) Effect of bromophenacyl bromide, a phospholipase A_2 inhibitor, on the induction and maintenance of LTP in hippocampal slices. Brain Res 537:49-53.

Morrison WR and Smith LM (1964) Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron-trifluoride-methanol. J Lipid Res 5:600-608.

Moskowitz N, Schook W, Puszkin S (1984) Regulation of endogenous calcium-dependent synaptic membrane phospholipase A_2. Brain Res 290:273-280.

Murphy SN and Miller RJ (1988) A glutamate receptor regulates Ca^{2+} mobilization in hippocampal neurons. Proc Natl Acad Sci USA 85:8737-8741.

Piomelli D, Shapiro E, Feinmark SJ, Schwartz JH (1987) Metabolites of arachidonic acid in the nervous system of *Aplysia*: Possible mediators of synaptic modulation. J Neurosci 7:3675-3686.

Reddy TS and Bazan NG (1987) Arachidonic acid, stearic acid and diacylglycerol accumulation correlates with the loss of phosphatidylinositol 4,5-bisphosphate in cerebrum two seconds after electroconvulsive shock: Complete reversion of changes 5 minutes after stimulation. J Neurosci Res 18:449-455.

Rodriguez de Turco EB, Morelli de Liberti S, Bazan NG (1983) Stimulation of free fatty acid and diacylglycerol accumulation in cerebrum and cerebellum during bicuculline-induced status epilepticus: Effect of pre-treatment with alpha-methyl-*p*-tyrosine and *p*-chlorophenylalanine. J Neurochem 40:252-259.

Sanfeliu C, Hunt A, Patel AJ (1990) Exposure to N-methyl-D-aspartate increases release of arachidonic acid in primary cultures of rat hippocampal neurons and not in astrocytes. Brain Res 526:241-248.

Shahar A, de Vellis J, Vernadakis A, Haber B (1989) A dissection and tissue culture manual of the nervous system. New York: Alan R. Liss, Inc.

Siesjo BK, Ingvar M, Westerberg E (1982) The influence of bicuculline-induced seizures on free fatty acid concentrations in cerebral cortex, hippocampus and cerebellum. J Neurochem 39:796-802.

Sperk G, Lassman H, Baran H, Kish SJ, Seitelberger F, Hornykiewicz O (1983) Kainic acid induced seizures: Neurochemical and histopathological changes. Neuroscience 10:1301-1315.

Straub H, Speckmann EJ, Bingmann D, Walden J (1990) Paroxysmal depolarization shifts induced by bicuculline in CA3 neurons of hippocampal slices: Suppression by the organic calcium antagonist verapamil. Neurosci Lett 111:99-101.

Taniyama K, Saito N, Kose A, Matsuyama S, Nakayama S, Tanaka C (1990) Involvement of the gamma subtype of protein kinase C in GABA release from the cerebellum. Adv Second Messenger Phosphoprotein Res 24:399-404.

Volterra A and Siegelbaum SA (1988) Role of two different genuine nucleotide binding proteins in the antagonistic modulation of the S-type K^+ channel by cAMP and arachidonic acid metabolites in *Aplysia* sensory neurons. Proc Natl Acad Sci USA 85:7810-7814.

Williams JH, Errington ML, Lynch MA, Bliss TVP (1989) Arachidonic acid induces a long-term activity-dependent enhancement of synaptic transmission in the hippocampus. Nature 341:739742.

Zorumski CF and Yank J (1988) Non-competitive inhibition of GABA currents by phenothiazines in cultured chick spinal cord and rat hippocampal neurons. Neurosci Lett 92:86-91.