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Reviews

A potentially critical role of phospholipases in central nervous system ischemic, traumatic, and neurodegenerative disorders

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Abstract

Phospholipases are a diverse group of enzymes whose activation may be responsible for the development of injury following insult to the brain. Amongst the numerous isoforms of phospholipase proteins expressed in mammals are 19 different phospholipase A_2's (PLA_2s), classified functionally as either secretory, calcium dependent, or calcium independent, 11 isozymes belonging to three structural groups of PLC, and 3 PLD gene products. Many of these phospholipases have been identified in selected brain regions. Under normal conditions, these enzymes regulate the turnover of free fatty acids (FFAs) in membrane phospholipids affecting membrane stability, fluidity, and transport processes. The measurement of free fatty acids thus provides a convenient method to follow phospholipase activity and their regulation. Phospholipase activity is also responsible for the generation of an extensive list of intracellular messengers including arachidonic acid metabolites. Phospholipases are regulated by many factors including selective phosphorylation, intracellular calcium and pH. However, under abnormal conditions, excessive phospholipase activation, along with a decreased ability to resynthesize membrane phospholipids, can lead to the generation of free radicals, excitotoxicity, mitochondrial dysfunction, and apoptosis/necrosis. This review evaluates the critical contribution of the various phospholipases to brain injury following ischemia and trauma and in neurodegenerative diseases.

Theme: Disorders of the Nervous System
Topic: Ischemia, Trauma

Keywords: Central nervous system; Free fatty acids; Phospholipase A; Phospholipase C; Phospholipase D; Arachidonic acid; Docosahexaenoic acid; Stroke; Trauma; Neurodegeneration

Contents

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1. Introduction

Phospholipases constitute a group of enzymes that catalyze the hydrolysis of phospholipids. This review will focus on the roles of phospholipases in the A, C, and D groups, which have a well-defined presence in the central nervous system (CNS). Although the presence of phospholipase B (PLB)-like activity in rabbit brain has been reported [119], less is known about the function of this enzyme. The primary substrates for these enzymes are the glycerophospholipids which account for 20–25% of the dry weight of adult brain, and which together with cholesterol and glycolipids represent approximately 50–60% of the total membrane mass with proteins accounting for the remainder [74]. The major glycerophospholipids in neural tissues are phosphatidylcholine (PtdCho), phosphatidylethanolamine (PtdEtn), phosphatidylserine (PtdSer) and phosphatidylinositol (PtdIns). Neural membranes also contain plasmalogens (PlsCho and PlsEtn), which are glycerophospholipids with vinyl ether linkages, and sphingomyelin, which contains ceramide linked to phosphocholine through its primary hydroxyl group. The glycerophospholipids, which affect membrane stability, fluidity and permeability, are required for the proper functioning of membrane ion channels and receptors.

In neural membranes, ethanolamine phospholipids and phosphatidylserine are concentrated in the inner layer of the plasma membrane bilayer, with choline glycerophospholipids and sphingomyelin occupying the outer layer [250]. Under normal conditions, glycerophospholipid homeostasis is maintained by a balance between catabolism, resynthesis by reacylation, and de novo synthesis [250]. In neural membranes, saturated free fatty acids (FFAs), palmitic or stearic, are usually esterified at the sn-1 position of the glycerol backbone whereas polyunsaturated free fatty acids (PUFAs), such as arachidonic and docosahexaenoic acids, are esterified at the sn-2 position.

Synthesis of glycerophospholipids occurs in the endoplasmic reticulum (ER). The newly created glycerophospholipids assemble into bimolecular layers, and then form into vesicles, which detach from the ER, and travel to other sites for transfer of their glycerophospholipids to membranes. This is achieved by both spontaneous interchange and transport of the glycerophospholipid molecules by phospholipid transfer proteins [4,74].

2. Phospholipases

Glycerophospholipids are hydrolyzed by groups of enzymes, the phospholipases. Phospholipase A_1, which catalyzes the hydrolysis of the ester bond at the sn-1 position forming a free fatty acid and a lysophospholipid, was identified in a soluble fraction of bovine brain [225]. This enzyme was present in cytosolic and particulate fractions enriched with microsomes and plasma membranes, but not in those enriched with mitochondria [226]. Phospholipase A_2's (PLA_2s, EC 3.1.1.4) act on the fatty acid ester bond at the sn-2 position liberating a free fatty acid and a lysophospholipid. PLB (EC 3.1.1.5), an enzyme that displays both PLA_2- and lysophospholipase-type activities has been tentatively identified in the rabbit brain [119]. PLB protein in the human epidermis had a molecular weight of 97 kDa and PLA_2-like activity [173]. However, little is known about its distribution and function in the brain. Phospholipase C (PLC, EC 3.1.4.3) hydrolyzes the phosphodiester bond at the sn-3 position forming 1,2-diacylglycerol (DAG) and a phosphobase. Phospholipase D (PLD, EC 3.1.4.4) hydrolyzes glycerophospholipids into phosphatidic acid (PA) and a free base. Phospholipases A_1, A_2, C, and D have been purified and characterized from brain tissue. All occur in brain in multiple forms, many of which have been cloned. Lysophospholipids generated by PLA_1 and PLA_2 are either hydrolyzed by lysophospholipases or resynthesized into phospholipids. The plasmalogens are hydrolyzed by plasmalogen-selective PLA_2 and lysoplasmalogenase [72]. The

expression and distribution of the various phospholipases in the CNS are described in the following sections.

2.1. Expression of PLA_2s in the CNS

At this time, 19 different groups of PLA_2 have been identified [12,15,297]. From a functional point of view, PLA_2s from mammalian tissues have been classified as: (a) secreted (groups I B, II A, II C, II D, II E, II F, III, V, X, and XII), which are typically small, extracellularly acting, $sPLA_2$s requiring in many instances millimolar levels of Ca^{2+} for activity; (b) higher molecular mass, micromolar Ca^{2+}-dependent, cytosolic ($cPLA_2$; groups IV A, IV B, and IV C) groups IV A, IV B, and IV C; and (c) Ca^{2+}-independent cytosolic PLA_2s ($iPLA_2$; groups VIA-1, VI A_2, and VI B) (groups VIA-11, VI A_2, and VI B). Other divisions of the PLA_2 family include groups VII A and VII B (the platelet-activating factor (PAF) acetylhydrolases, PAF-AH II) and group VIII (PAF-AH IB).

Molloy et al. [181] used the polymerase chain reaction (PCR) to determine the distribution of several cloned PLA_2 messenger RNAs (mRNAs) in the rat brain. Four low molecular weight forms of secretory PLA_2 (I B, II A, II C, and V) and a high molecular mass, calcium-independent, group VI ($iPLA_2$) were expressed. PLA_2s II A, II C, IV and VI mRNAs appeared to be ubiquitously expressed in the rat brain with relatively similar levels in all brain regions. $sPLA_2$ V mRNA was found at high levels in the hippocampus, and at lower levels in most other brain areas. Measurements of PLA_2 activity levels demonstrated that PLA_2 VI ($iPLA_2$) constitutes the dominant form of activity in adult rat brain, with the highest levels in the hippocampus and striatum [351,352].

2.1.1. Secretory phospholipases ($sPLA_2$ I B, II A, II C, and V)

The expression of mRNAs for $sPLA_2$ II A, II C, and V has been observed in the rat brain. Trace amounts of pancreatic $sPLA_2$ mRNA were also detected in some brains suggesting the possibility of a restricted expression of this mRNA [181]. $sPLA_2$ II A was observed at high levels in the brainstem and midbrain, with lowest levels in the cerebellum and corpus striatum. $sPLA_2$ II C mRNA expression was present in all brain regions with lower levels in the brainstem, thalamus and cerebellum. $sPLA_2$ V mRNA was present at low levels in most areas except the hippocampus, where it was expressed at high levels [181]. $sPLA_2$ II A and V activities were evenly distributed in all regions of the rat brain, with activity being higher for the $sPLA_2$ II A enzyme [351,352]. Group II A secreted $sPLA_2$ was primarily located in the particulate fraction. Total $sPLA_2$ activities in the cytosolic fraction did not change significantly during postnatal development, whereas activity in the particulate fractions peaked at 4 weeks after birth and then declined [351,352]. Rat brain synaptic vesicle fractions contain a PLA_2 activity which is completely neutralized by an antibody to rat type II $sPLA_2$. An $sPLA_2$ immunoreactive to this antibody was released, together with norepinephrine, from rat neural synaptosomes in response to K^+-induced depolarization in the presence of 2 mM Ca^{2+} [171]. Increases in the expression of $sPLA_2$ II A mRNA occur after a transient forebrain ischemia, with the most pronounced increases in selectively vulnerable areas, such as the hippocampus and neocortex [143]. Its expression was also enhanced after an endotoxic shock, with high levels in multiple brain areas [143]. Rodorf et al. [265] identified an $\approx$ 14 kDa secretory type PLA_2 associated with microsomal and mitochondrial fractions from the gerbil brain, the activity of which was enhanced by 50 μM Ca^{2+}. The specific activity of this enzyme was enhanced following ischemia/reperfusion.

An analysis of secreted PLA_2s in a human astrocytoma cell line failed to detect the expression of PLA_2s II A, II D, V, or X [12].

Of potential future interest is the identification of a novel $sPLA_2$ III enzyme in human tissues with homology to the group III bee venom enzyme[323]. This $sPLA_2$ III was abundant in heart, skeletal muscle, liver, and kidney, but had only weak expression in the brain.

2.1.2. Ca^{2+}-dependent phospholipase A_2 ($cPLA_2$)

The distribution of Ca^{2+}-dependent cytoplasmic PLA_2, an 85-kDa enzyme which hydrolyzes the sn-2 acyl ester bond of glycerophospholipids, in the CNS has been the subject of several investigations. Localization of $cPLA_2$ messenger mRNA in the rat brain by Northern blot analysis or in situ hybridization has revealed its presence in the gray matter of many regions, including the olfactory cortex, hippocampus, amygdala, thalamus, hypothalamus, and cerebellum. The most intense signals were predominantly from neurons, with fainter signals from glial cells [131]. Using rats and cytochemical techniques, Ong et al. [207,208] also observed a broad distribution of $cPLA_2$ activity with very dense immunostaining of neurons in hindbrain nuclei and lighter staining in the fore- and mid-brains. The rat spinal cord also had high levels of $cPLA_2$, with dense staining of motor neurons [207,208]. In another study, Sandhya et al. [275], using immunostaining, showed a weak expression of $cPLA_2$ in hippocampal neurons, but not in glial cells. Yang et al. [351,352] found $cPLA_2$ levels to be lower in both cytosolic and particulate fractions than were those of $iPLA_2$ and $sPLA_2$ groups II A and V. There were no significant changes in the level of $cPLA_2$ activity at all postnatal ages. A brain synaptic vesicle PLA_2, with properties somewhat comparable to those of $cPLA_2$, was characterized by Moskowitz et al. [186], who suggested that it might be involved in Ca^{2+}-evoked neurotransmitter release. Following a transient ischemic episode, $cPLA_2$ immunostaining has been observed in astrocytes and microglial cells of the rat brain [44,303,329] and an increased expression of the gene for $cPLA_2$ was apparent in neurons of the hippocampus [211]. Cultured rat brain microglia express $cPLA_2$ mRNA intensely [183].

$cPLA_2$ in mouse brain gray matter was reported to be confined to astrocytes at the pial surface and in the processes of fibrous astrocytes in white matter [144]. $cPLA_2$ mRNA and $cPLA_2$ immunoreactivity were not detected in neurons in this study. Information on $cPLA_2$ in the human brain is also limited. Immunohistochemical studies on human brain slices demonstrated that the enzyme is localized to astrocytes in gray matter [301]. Two newly identified human paralogs of PLA_2 (IV B and IV C) are widely expressed in human brain [240]. Increased intracellular Ca^{2+} concentrations (in the range of 100–500 nM) promote $cPLA_2$ translocation to intracellular membranes, including Golgi, endoplasmic reticulum and perinuclear membranes. Differential translocation to these structures is a function of Ca^{2+} amplitude and duration [66,280]. Expression of a human, Ca^{2+}-independent, group IV $cPLA_2$ isozyme ($cPLA_2$ gamma), a paralog of cytosolic $PLA_{2\alpha}$, in transfected CHO cells was apparent in the perinuclear membrane, endoplasmic reticulum and Golgi apparatus [11].

Translocation of $cPLA_2$ to the nuclear envelope, endoplasmic reticulum, Golgi apparatus, and possibly the plasma membrane occurs in response to increases in intracellular calcium mobilization [67,132,227,280].

2.1.3. Ca^{2+}-independent PLA_2

Yang et al. [351,352] purified an 80-kDa $iPLA_2$ from rat brain. The enzyme displayed a substrate preference for the fatty acid chain in the sn-2 position of phosphatidylcholine for linoleoyl>palmitoyl>oleoyl>arachidonyl, and a head group preference for choline ≥ ethanolamine ≫ inositol. Studies on the regional distribution and ontogeny of various phospholipase A_2 types indicated that the $iPLA_2$ is the dominant PLA_2 activity in the cytosolic fraction (whereas, as already stated, group II A secreted PLA_2 is the dominant activity in the particulate fraction). iPLA activity increased rapidly in the neonatal rat brain, peaking at 12 weeks of age, and then declining slightly over the following 48 weeks. The gene encoding a calcium-independent, high molecular weight isoform of $iPLA_2$ (PLA_2 VI), with pronounced expression in all rat brain regions, was identified by Molloy et al. [181]. A cytosolic $iPLA_2$ with somewhat different properties has been identified in the bovine brain [106] and a widely distributed form of the enzyme has been identified in the human brain [269]. There is also evidence for a human Ca^{2+}-independent PLA_2 gene which, with alternative splicing, can generate multiple $iPLA_2$ isoforms with distinct tissue distribution and localization [142].

Recent interest has focused on a newly identified Ca^{2+}-independent, $iPLA_2$ in rat liver and rabbit heart mitochondria [31,341]. The enzyme has pH optima in the pH 8.00–8.5 range and is inhibited by bromoenol lactone (BEL), a selective $iPLA_2$ inhibitor. It was suggested that this enzyme may play a critical role in initiating autolysis/autophagy of poorly functioning liver mitochondria [31] and in mitochondrial membrane catabolism in ischemic/reperfused hearts [341].

2.2. Phospholipase C

The PLC system is constituted by a family of isozymes, which have been identified by protein purification and molecular cloning. The 11 mammalian PLC isozymes, which have been identified to date, can be divided into three structural groups: β, γ, and δ [256,259]. All PLC isozymes are activated by Ca^{2+} and the different isozymes are linked to membrane receptors by distinct mechanisms [127,260]. The δ type isozymes are smaller (85,000 kDa) than the PLCβ and PLCγ isoforms (140,000–155,000 kDa). Phospholipases C hydrolyze a minor membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP_2), to produce two intracellular second messengers, diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3), which mediate the activation of protein kinase C (PKC) and intracellular Ca^{2+} release from the endoplasmic reticulum, respectively. A decrease in PIP_2 levels in the cell membrane is of itself an important signal because this phospholipid modulates the activities of several proteins. Diacylglycerol kinase converts DAG to phosphatidic acid, a newly emerging intracellular mediator of hormone action that targets a number of signaling proteins. The enzymatic activity of each of the PLC subtypes appears to be controlled by a distinct mechanism. PLCβ is activated by the G_q family of G-proteins, whereas PLCγ is a substrate for tyrosine kinase. Unlike the other PLC isoforms, neither protein phosphorylation nor heterotrimeric G-protein subunits significantly affect PLCδ activity. Evidence points to an atypical GTP-binding protein G_h, that appears to couple $PLC\delta_1$ to heptahelical receptors [189]. Even less is known about the regulation and function of $PLC\delta_{2-4}$ isoforms.

Mammalian PLCβ isoforms are differently distributed. $PLC\beta_1$ is most widely expressed with the highest concentrations found in specific regions of the rat brain [42,87]. High levels of $PLC\beta_1$ are present in the striatum and pyramidal cells of the hippocampus and to a lesser extent, in the granule cells of the dentate gyrus and in several thalamic nuclei. Rat $PLC\beta_1$ mRNA levels are highest in the cerebellar Purkinje and granule cells, frontal and pyriform cortex, hippocampus and dentate gyrus. Hindbrain structures have lower levels of this isoform [110,267]. $PLC\beta_3$ mRNA was detected weakly in rat brain cerebellar Purkinje and granule cells and $PLC\beta_4$ mRNA was expressed more intensely in olfactory mitral cells, thalamic nuclei, the medial habenula, caudate/putamen, cerebellar Purkinje granule cells. $PLC\gamma_2$ mRNA was localized only in Purkinje and granule cells in the vermal portions of lobules IX and X of the cerebellum [311]. $PLC\delta_1$ is concentrated in astroglial cells, whereas much lower levels are present in neurons [42,180]. PLCβ and γ were detected in rat oligodendrocytes [179].

Ruiz De Azua et al. [272] examined the protein levels of $PLC\beta_{1-4}$ in murine brains. Expression of $PLC\beta_1$ in the cerebral cortex and hippocampus was higher than in the cerebellum, whereas expression of $PLC\beta_3$ and $PLC\beta_4$ was

higher in the cerebellum. Protein levels of PLCβ_2 were very low in the murine brain. Western blot analysis revealed that mouse cerebellum expresses eight phospholipase C enzymes: β_1, β_2, β_3, β_4, γ_1, γ_2, δ_1, and δ_2. Further immunochemical analysis of cryosections showed distinct patterns of expression of each isoform. Purkinje cells had high levels of β_1, β_3, γ_2, and δ_2 isotypes. In the molecular layer, β_1 and γ_1 isozymes predominated, whereas in the granular layer, γ_1 and γ_2 isoforms were prevalent [168]. Sugiyama et al. [304] observed PLCβ_4 to be equally distributed in both rostral and caudal mouse cerebellum while PLCβ_3 was enriched in the caudal cerebellum.

The expression of PLCβ_{1-4} mRNAs in the mouse brain has been examined using in situ hybridization [178,333]. PLCβ_1 mRNA was expressed predominantly in the telencephalon, including the cerebral cortex, hippocampus, amygdala, lateral septum, and olfactory bulb, with little expression in most thalamic nuclei. PLCβ_2 mRNA was present in white matter, likely in oligodendrocytes. PLCβ_3 was specifically expressed in cerebellar Purkinje cells. High levels of PLCβ_4 were also expressed in Purkinje cells and in the thalamus and medial septum, with low levels in other telencephalic levels.

2.3. Phospholipase D

Phospholipase D is a widely distributed enzyme in the brain that catalyzes the hydrolysis of phospholipids at their terminal phosphodiester bond generating the signaling lipid PA and the polar head group. Phosphatidylcholine is the preferred substrate. Phosphatidic acid can be further metabolized to DAG and lysophosphatidic acid (LPA) by PA phosphohydrolase and phospholipase A_2, respectively [69]. PLD has been implicated in novel signaling pathways, membrane trafficking, secretion, and mitogenesis. There are currently three mammalian phospholipase D genes, whose products (PLD1a, PLD1b, PLD2) have distinct biochemical and regulatory properties [47,48,69,81]. PLDs require phosphatidyl-4,5-biphosphate (PIP_2) for activity. Phosphatidylinositol can substitute for PIP_2. PLD1 isozymes are regulated by PKC isozymes (PKCα_1, β_1, and γ). These interact with a domain in the N-terminus of PLD1. It is also regulated by the Rho and ARF subfamilies of the Ras superfamily. PLD2 shows no response to PKC isozymes or the Rho subfamily proteins and exhibits little or no stimulation with ARF [69,215].

The distribution of PLD1 in the rat brain and spinal cord has been studied by immunohistochemical localization [146,147]. Significant labeling was observed in many areas including the olfactory bulb, medial septum–diagonal branch complex, cerebral cortex, brainstem, cerebellum, and spinal cord. No significant labeling was found in the thalamus, epithalamus, and basal ganglia. Many of the stained cells were neurons and PLD1 was also expressed in glial cells. In another study, using in situ hybridization, PLD1 mRNA was detected mainly in presumptive rat oligodendrocytes and PLD2 mRNA in presumptive astrocytes [273]. Western blot analysis showed the presence of PLD1 protein in all the tissues studied with significantly higher levels in the brainstem and spinal cord [146,147]. PLD activity and PLD mRNA levels were determined in rat brains at ages ranging from embryonic day (E) 19 to postnatal day (P) 49. PLD activity increased between E19 and P24 and remained unaltered thereafter. A similar developmental pattern of mRNA levels of the PLD1 isoform was found by Northern blotting [360]. PLD2 mRNA distribution was examined in eight rat brain regions. In descending order of expression, these were: hypothalamus, hippocampus, striatum, frontal cortex, midbrain, cerebellum, brainstem, and spinal cord [223]. The levels of PLD2 mRNA in postnatal rats were lowest 1 day after birth and reached maximum levels in the adult brain. The intracellular location PLD1 and PLD2 are not well characterized. PLD1 is localized to Golgi apparatus, endoplasmic reticulum and late endosomes, whereas PLD2 appears to be associated with the plasma membrane and caveolae [69]. Kanfer and McCartney [116] evaluated the properties of a PLD in rat brain plasma membranes which was maximally active at pH 6.5, with a 60–80% reduction at pH 7.0. This suggests that it could be activated during an ischemic episode with concurrent tissue acidosis.

3. Physiological roles of PLA_2 in the nervous system

3.1. $sPLA_2$

Secretory phospholipases are subdivided into several groups based upon the position of cysteine residues. Type I $sPLA_2$ (so-called pancreatic type) and type II $sPLA_2$ (inflammatory type) are the best characterized members of this family. Pancreatic PLA_2 IB mRNA is not expressed in brain, whereas $sPLA_2$ IIA, IIC $sPLA_2$ IV, and $sPLA_2$ VI mRNAs are expressed in most areas of the rat brain. $sPLA_2$ V mRNA is present in the rat hippocampus at very high levels, but at low levels in most areas [181]. Amongst the various $sPLA_2$s, $sPLA_2$ IIE, $sPLA_2$ V, and $sPLA_2$ X are expressed in the human brain [306]. $sPLA_2$ IIA is not present in the normal brain but is elevated during the inflammatory state after ischemia [143]. A type II $sPLA_2$ is expressed in glial cells of the rat brain following endotoxin shock [82], and $sPLA_2$ II release from rat cerebral cortex mitochondria has been observed [78].

Several lines of evidence have suggested the participation of $sPLA_2$s in exocytosis in mast cells, chromaffin cells and neuroendocrine cells. PLA_2 inhibition depresses histamine release from mast cells or neurotransmitter release from neuroendocrine cells [28,174,185,186]. Matsuzawa et al. [171] demonstrated that synaptic vesicle fractions isolated from rat brain contain $sPLA_2$ type II, which is released by depolarization. When neuronally differentiated PC12 cells were stimulated with carbamycholine, $sPLA_2$

was released into the medium. Inhibitors specific to type II $sPLA_2$ suppressed catecholamine secretion by PC12 cells which had been stimulated by carbamylcholine or potassium, indicating that $sPLA_2$ released from neuronal cells may regulate the degranulation process leading to transmitter release [171].

Another aspect of the $sPLA_2$ family's function is that group IIA $sPLA_2$ is thought to be a key enzyme in the pathogenesis of inflammatory diseases, since its local and systemic levels are elevated in conditions such as septic shock, acute pancreatitis and rheumatoid arthritis [94,95,252]. However, some inbred mouse strains (199/SV; BALB/c) have a natural mutation in the $sPLA_2$ gene [125,165] and are susceptible to arthritis in the antigen-induced model in a comparable manner to $sPLA_2$ IIA-expressing mouse strains [27,347]. Transgenic mice expressing the human $sPLA_2$ IIA gene do not develop any overt inflammatory conditions [93]. These findings raise doubts about the role of $sPLA_2$ IIA in inflammatory diseases, suggesting that other $sPLA_2$ isoforms may be involved.

3.2. $cPLA_2$

Insights into the role of cytosolic PLA_2 has been gained from recent studies on knockout mice [26,277]. $cPLA_2$ (IV A) −/− 129/SV mice developed normally, gained weight at a rate equal to that of wild-type mice, and had life spans in excess of 1.5 years. Mean arterial blood pressures, baseline arterial pH, pCO_2 and rectal temperature were similar to those of the wild-type mice. However, the females had severe reproductive defects, producing small litters with poor survival of their offspring. Similar problems, observed in cyclooxygenase-2-deficient mice, have been attributed to the loss of unspecified prostaglandins necessary for the reproductive process [152,257].

Potential pathophysiological roles of $cPLA_2$ in the brain have been evaluated in a number of studies. PLA_2 and arachidonic acid metabolites have been implicated in ischemia/reperfusion injury [265]. Glutamate receptor excitation, which plays a role in ischemia/reperfusion-evoked neuronal death, causes $cPLA_2$ activation and arachidonic acid release [126,145,276]. PLA_2 activity can increase glutamate's action at non-NMDA receptors [169] and arachidonic acid enhances glutamate levels by blocking its reuptake [358]. $cPLA_2$ activity can also adversely affect neuronal viability by direct actions on plasma and mitochondrial membranes [326]. Reactive oxygen species (ROS) and lysophospholipids generated during reperfusion may also contribute to neuronal injury and death [89].

$cPLA_2$ −/− mice had a 34% reduction in brain infarct volume and suffered fewer neurological deficits following a 2-h period of middle cerebral artery occlusion with 3 days of reperfusion, in comparison with PLA_2 +/+ mice [26]. After injection of the neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), there was significantly less depletion of striatal dopamine in the $cPLA_2$ −/− mice compared to their normal littermates. The reduction in MPTP neurotoxicity could be a consequence of a decreased formation of free fatty acids and lysophospholipids, with a decrease in reactive oxygen metabolites, and reduced excitotoxicity and mitochondrial injury. As a caveat, it should be acknowledged that the 129/SV strain of mice used in the creation of this knockout have a naturally occurring disruption in the gene for group II A PLA_2 [125,165]. In these mice, group II A $sPLA_2$ mRNA was not detectable in the small intestine, whereas it was present at very high levels in BALB/c mouse small intestine. The $cPLA_2$ knockout mice would therefore have been deficient in both $cPLA_2$ and group II A PLA_2. However, as the ischemia/reperfusion and MPTP experiments compared wild-type 129/SV versus $cPLA_2$ −/− mice, the differences in the degree of histopathological and functional deficits between the two groups clearly demonstrate that $cPLA_2$ is a critical determinant of ischemia/reperfusion injury.

$cPLA_2$ −/− mice had a more rapid recovery from allergen-induced bronchoconstriction and no airway hyperresponsiveness. Peritoneal macrophages from $cPLA_2$ −/− mice failed to produce prostaglandins, leukotriene B_4 and cysteinyl leukotrienes after stimulation. Bone marrow-derived mast cells from these mice failed to produce eicosanoids. Thus, it was concluded that $cPLA_2$ has important roles in fertility, brain injuries, allergic responses, and the generation of eicosanoids from inflammatory cells that (in the absence of II A PLA_2) were not compensated for by other isoforms of PLA_2.

Compared with controls, $cPLA_2$ −/− mice demonstrated altered brain concentrations of several phospholipids, with reduced esterified linoleate, arachidonate and docosahexaenoate in choline glycerophospholipid, and reduced arachidonate in phosphatidylinositol. These mice had reduced rates of incorporation of arachidonate from the brain arachidonoyl-CoA pool into ethanolamine glycerophospholipids and choline glycerophospholipid, but elevated rates into phosphatidylinositol [266]. These differences would correspond to altered turnover and metabolic loss of esterified brain arachidonate, suggesting that $cPLA_2$ is necessary to maintain normal brain concentrations of phospholipids and their esterified polyunsaturated fatty acids.

Similar effects of targeted mutation of the $cPLA_2$ (IV A) gene, using virtually identical approaches were observed by Uozumi and Shimizu 320 and Uozumi et al. 321]. Renal, intestinal, and reproductive functions from these PLA_2 mice were impaired (effects on brain ischemia were not examined). Primary cultured cells from these mice produced significantly reduced amounts of prostaglandins and leukotrienes.

The physiological functions of $cPLA_2$s in the brain are just beginning to be understood in detail. Evidence suggests that these enzymes may be involved in neurotransmitter release, membrane remodeling, osmotically induced cell swelling, and repair or apoptosis [16,163,186,346]. A

well-defined action of $cPLA_2$ is its ability to catalyze the hydrolytic cleavage of membrane phospholipids at the sn-2 position, with the release of free fatty acids, preferentially liberating arachidonic and docosahexaenoic acids, together with lysophospholipids. To gain full catalytic activity, $cPLA_2$ requires phosphorylation at Ser^{505} by members of the mitogen-activated protein kinase family [14] and low micromolar Ca^{2+} levels in the cytoplasm. Arachidonic and docosahexaenoic acids can subsequently be metabolized by cyclooxygenases, lipoxygenases, and epoxygenases to form eicosanoids (prostaglandins, thromboxanes, leukotrienes, lipoxins, and epoxyeicosatrienoic acids) and docosanoids, respectively [19,73]. Many of these derivatives can subsequently function either as intracellular second messengers or as autocrine/paracrine agents. Lysophospholipids, the other product of $cPLA_2$-catalyzed reactions, have many effects on various systems. They can affect membrane fluidity and permeability and also serve as precursors for other pharmacologically active factors, including PAF and LPA [74]. Another pathway for arachidonic acid formation can be initiated by activation of PLC with the formation of DAG, which is subsequently broken down by mono- and diacylglycerol lipases to yield arachidonic acid and glycerol. Alternatively, PLA_1 can cleave membrane phospholipids at the sn-1 position, followed by lysophospholipase cleavage releasing arachidonic acid at the sn-2 position [224].

Arachidonic acid, itself, modulates ion channels and regulates the activity of many enzyme proteins, including protein kinase A, protein kinase C, NADPH oxidase, GTPase activating protein, diacylglycerol kinase, and Na^+/K^+ ATPase [73,123]. Amongst their many actions, arachidonic and docosahexaenoic acids may affect the release, uptake, and transport of several neurotransmitters [22,123,249]. Application of arachidonic acid to rat hippocampal slices induced a long-term potentiation (LTP) of synaptic transmission in most instances, with depression in other slices [56]. Both effects were antagonized by nordihydroguaiaretic acid (NDGA), suggesting the involvement of lipoxygenase metabolism. After induction of LTP in the hippocampal dentate gyrus there was a persistent increase in the level of free arachidonic acid in postsynaptic membrane preparations, which was initially attributable to elevated $cPLA_2$ activity and subsequently to that of PLC [45]. Docosahexaenoic acid has also been shown to induce LTP in rat hippocampal slices [83].

The signal cascade which is activated by osmotic cell swelling and which leads to the activation of osmolyte efflux from neurally derived and other cells has been reported to involve phospholipases, lipoxygenases and Ca^{2+} in the swelling-induced efflux of taurine [16,141]. Conversely, exogenous arachidonic acid depresses the efflux of taurine during hyposmotic conditions [217].

The cytosolic 85-kDa Ca^{2+}-dependent PLA_2 also exhibits significant lysophospholipase activity [332]. Unlike the action of $cPLA_2$ on glycerophospholipids, which requires the presence of low micromolar levels of Ca^{2+}, the lysoPLA activity of $cPLA_2$ is Ca^2-independent. By virtue of its $lysoPLA_2$ action, $cPLA_2$ may sequentially hydrolyze the sn-2, and then the sn-1 acyl chains of the phospholipid substrate, releasing a variety of free fatty acids [158].

3.3. $iPLA_2$

The $iPLA_2$s are the most recently identified of the PLA_2 superfamily. Although numerous Ca^{2+}-independent PLA_2 activities have been reported in various tissues, only one has been sequenced and characterized in detail. This $iPLA_2$ is the group VI enzyme [13]. It shares some characteristics with $sPLA_2$s and others with the $cPLA_2$. Like the $sPLA_2$s, $iPLA_2$ exhibits no apparent substrate specificity for arachidonic acid-containing phospholipids and appears not to be subjected to posttranslational covalent modifications. It shares the size, intracellular localization, and perhaps elements of the catalytic mechanisms with $cPLA_2$ [14]. $iPLA_2$ exhibits multiple activities including PLA_2, PLA_1, lysoPLA, transacylase, and PAF acetylhydrolase activities, with the relative activity of each dependent on substrate presentation [332]. A unique feature of the $iPLA_2$, in addition to the absence of a Ca^{2+} requirement, is that it contains eight ankyrin motifs at the N-terminal half of the molecule [162].

Of particular significance in the context of this review are two recent reports on the presence of a mitochondrial $iPLA_2$. Immunoanalysis of rat liver mitochondria and mitochondrial subfractions showed that a membrane bound protein is present that is recognized by antibody against authentic $iPLA_2$. The Ca^{2+}-independent activity displayed a broad pH optimum located between pH 8 and 8.5. It was strongly inhibited by bromoenol lactone, but not by $AACOCF_3$ [31]. These authors proposed that it is this enzyme, rather than the Ca^{2+}-dependent type II A PLA_2, which initiates the demise of poorly functioning mitochondria. Another study indicated that an $iPLA_2$ β is present in the rabbit heart mitochondria inner membrane, with no apparent translocation during ischemia [341]. Mitochondrion-associated $iPLA_2$ was catalytically competent and exhibited increases in activity following ischemia. Pretreatment of ischemic/reperfused hearts with bromoenol lactone prior to, but not post, ischemia significantly reduced infarct size. The authors concluded that mitochondrial-associated $iPLA_2$ is implicated in the signal transduction of myocardial ischemia/reperfusion injury.

3.4. Phospholipase C

The clearest indications of the roles of phospholipase C in the brain have come from studies with transgenic mice. The absence of $PLC\beta_1$ leads to sudden death due to epileptic-type seizures in mice [127,256]. The spontaneous seizures and hypersensitivity of these mice suggest that $PLC\beta_1$ is involved in the development and control of brain inhibitory pathways. In the temporal lobe, cerebellum, and hippocampus, PLC activation by muscarinic agonists is

substantially decreased, whereas the hippocampal response to metabotropic glutaminergic agonists is enhanced. The loss of muscarinic cholinergic tone may decrease the firing of inhibitory interneurons, which, coupled with unopposed glutaminergic stimulation, could account for the seizures.

Mice lacking PLCβ_2, which is highly expressed in the immune system, appear to show no outward behavioral differences from their wild-type littermates. Mice lacking PLCβ_4 are hypokinetic and exhibit a waddling gait that is not due to muscle weakness or bone deformities, suggesting a deficit in the cerebellum [127,256]. This would be consistent with the reduction in PLCβ stimulation by metabotropic glutaminergic and muscarinic agonists observed in cerebellar slices. In addition to ataxia, PLCβ_4 null mice have defective visual responses [115].

PLCδ isoforms are activated at Ca^{2+} concentrations in the range of 10^{-7} to 10^{-5} M, leading to the possibility that this enzyme functions as an amplifier, rather than initiator, of Ca^{2+} mobilizing signals. In this respect, the specific catalytic activity of PLCδ is 50–100-fold greater than the calcium-stimulated activities of the β and γ isoforms, measured in the absence of activating G-protein subunits, tyrosine protein kinases or PI(3,4,5) P_3. Measurements of the inositol triphosphate (IP_3R) and Ca^{2+} levels in intact cells support the idea that PLCδ_1 amplifies the PLCβ generated signal. In PC-12 cells, expression of PLCδ raises the level of IP_3, the increase in calcium, and secretion of norepinephrine stimulated by bradykinin [128]. This elevated calcium flux likely originates through store-operated channels. The situation may be comparable to that observed with PKCγ isoforms, which enhance PLCβ-generated Ca^{2+} signals by a similar mechanism [256].

Permeabilized adrenal medullary chromaffin cells have been used to study the role of PLC in stimulus-induced ^{3}H-norepinephrine release. Antibodies against PLCγ_1 or PLCβ_3 (but not PLCδ_1, δ_2, β_1, and β_2) inhibited ^{3}H-norepinephrine release evoked by Ca^{2+} and increased basal release, indicating that only specific PLC isozymes are involved in these actions. However, only PLCγ_I (but not PLCβ_3) antibodies inhibited the ability of Ca^{2+} to increase PLC activity in these cells [204]. Application of U73122, which would inhibit most, if not all, PLC isozymes, resulted in an increase in secretory activity in the absence of Ca^{2+}, together with a concentration-dependent decrease of Ca^{2+}-stimulated release. The authors suggested that U73122 enhancement of basal norepinephrine release may have been a consequence of an increase in membrane phospholipid levels following the inhibition of PLC.

3.5. Phospholipase D

PLD is thought to play a role in signal transduction in many cell types because it is activated by a wide variety of neurotransmitters, hormones, growth factors, cytokines, and other molecules that are involved in extracellular communication [68,69,156,261]. PLD may play key roles in regulation of vesicular transport, secretory events, cell motility, cytoskeletal reorganization and cell proliferation. PLD isoforms have reportedly been localized to the plasma membrane, Golgi apparatus, endoplasmic reticulum, mitochondria, cytoskeleton, cytosol, and secretory granules [156]. In mammals, the principal substrate is phosphatidylcholine and the generation of PA is itself of significant consequence in that PA can alter the activities of many enzymes and proteins. PA can also be metabolized to diacylglycerol and LPA by PA phosphohydrolase and phospholipase A_2, respectively. Both of these substances can function as active messengers in their own right. From the perspective of this review it is of interest that PLD has been implicated in cellular apoptosis [357]. The expression of mRNAs for PLD enzymes in C6 glial cells treated with C_2-ceramide, an apoptotic agent, was examined. Decreases in rPLD Ia and rPLD Ib mRNA expression were well correlated with decreases in GTPγs and Arf1-dependent PLD activity. The authors speculated that the inhibition of PLD activity was implicated in the C_2-ceramide-induced apoptosis. An increase in the expression of PLD1 protein, and in PLD activity associated with reactive astrocytes of the rat hippocampus was observed following cerebral ischemia [146,147].

Cannabinoids (marijuana and related drugs including the synthetic analog delta-9 tetrahydrocannabinol (THC)) have been proposed as treatments for a widening spectrum of medical disorders, including brain ischemia, brain trauma, and neurodegenerative diseases [29,96,97,190]. Central cannabinoid ($CB_{1\ and\ 2}$) receptors are coupled to several signal transduction pathways, including G-proteins that inhibit N-type voltage-gated calcium channels involved in the release of neurotransmitters [164], such as the excitatory transmitter L-glutamate, which has been implicated in stroke- and trauma-induced neuronal death.

Endocannabinoids are endogenous *N*-acyl-ethanolamine (NAE) ligands for the same cannabinoid receptors that mediate the effects of THC. Two such molecules, *N*-arachidonylethanolamine (anandamide) and sn-2-arachidonylglycerol (2-AG), have similar neuroprotective properties as the exogenous cannabinoids against ischemic and trauma-induced neuronal injury [214,296].

A *N*-acyl-ethanolamine phospholipid (NAPE)-hydrolyzing phospholipase D, which generates the formation of NAEs has undergone preliminary characterization in the rat brain. NAPEs, which are formed by a Ca^{2+}-stimulated *N*-acyltransferase from phosphatidylethanolamine, accumulate in the brain during excitotoxic episodes and ischemia. Neurons contain high levels of the *N*-acetyltransferase, whereas astrocytes have very low levels [100], suggesting that NAPE found in brain is mainly formed in neurons.

During neonatal development, *N*-acetyltransferase levels, which are initially high, decline, whereas the brain NAPE-PLD, which is low in neonates, increases 15-fold during development. Thus, in the adult brain, it may be NAPEs serving the function of being a precursor for NAE. In that

NAEs have cerebroprotective activity, the formation of these compounds in the stressed adult brain may constitute a valuable survival mechanism. At the level of injured cells, NAEs may be able to stabilize injured mitochondria, prevent Ca^{2+} leakage [63], and are reported to have antioxidant properties [100].

4. Ischemia-evoked activation of cerebral phospholipases

Free fatty acid levels in the brain begin to increase rapidly (within seconds) after the induction of cerebral ischemia in rat, mouse, and gerbil models involving vascular occlusion, cardiac arrest or decapitation [1,17,114,197,220,353,355]. However, Katsura et al. [124] failed to observe an increase in FFA acid levels until 60 s following cardiac arrest by a KCl injection into the rat jugular vein at which time there was a small increase in arachidonic acid levels. Depolarization of the brain cortex with calcium influx at circa 80 s was associated with massive increases in FFA levels. The formation of FFAs is attributed to the deacylation of membrane phospholipids by phospholipases combined with the failure of reacylation of membrane phospholipids as a consequence of ATP depletion. This breakdown of neural membrane phospholipids and the release and accumulation of FFAs following ischemia, or electroconvulsive shock has been referred to as the "Bazan effect" [111]. Two major pathways leading to free fatty acid generation have been proposed, namely their direct release from various phospholipids by PLA_2s, and alternatively by the catalysis of PIP_2 by PLC to form IP_3 and diacylglycerol [114]. DAG is then further metabolized to FFAs by lipases, including diacylglyceride lipase, which releases arachidonic acid. Based on studies of the time course of FFA formation and the use of a PLC inhibitor, Umemura et al. [318,319] proposed that release of FFAs during the initial 2 min of ischemia was mostly attributable to the action of PLC, which further influenced the activity of PLA_2, acting predominantly after the initial 2 min of ischemia. The degree of severity of ischemia has been correlated with the level of free fatty acid accumulation [2,121].

Analysis of the changes in brain FFAs during complete, or severe incomplete, 30-min ischemias demonstrated comparable increases in the brain levels of palmitic, stearic, oleic, arachidonic, and docosahexaenoic acids at 5 min, with further increases by 30 min, which were essentially reversed during a 30-min recirculation period [258]. However, in another study on FFA levels in rat brain mitochondrial membranes, the increases in arachidonic, docosahexaenoic, and other free fatty acids were sustained during 60 min of recirculation following a 30-min ischemia [305]. On a longer time scale, Nakano et al. [192] measured the changes in gerbil brain FFA levels following a 5-min cerebral ischemia. The levels of arachidonic, docosahexaenoic, palmitic, oleic, and stearic acids increased during ischemia and then returned towards control levels after 60 min of reperfusion. This was followed by a slow increase in brain levels of all 5 FFAs, which reached about seven times the control levels by 3 days, returning towards control levels after 6 days of recirculation. In another experiment, a 5-min ischemia/3-day reperfusion period, followed by a second 5-min ischemia evoked an extremely large release of stearic, oleic, and docosahexaenoic acids, with decreased levels of arachidonic acid, suggesting a non-specific activation of various lipases.

A prolonged increase in FFA levels following ischemia/reperfusion injury has been observed in other studies. In a rat model of permanent focal ischemia, an initial increase in arachidonic acid was observed within an hour of onset, and a delayed increase, especially of docosahexaenoic acid, occurred after 24 h [194]. Using magnetic resonance spectroscopy of rats following a 90-min focal ischemia, Gasparovic et al. [85] demonstrated increases in lipid signals for up to 5 days following insult.

Evidence that phospholipase activation makes a significant contribution to ischemic injury has been obtained in experiments which analyzed free fatty acid levels in the rat hippocampus [337]. Short periods of cerebral ischemia led to selective neuronal damage in the CA1, but not CA3 regions of the hippocampus [23]. Since both regions would be expected to experience similar ischemic insults, this suggests that there are important differences in the molecular mechanisms underlying the selective vulnerability. The Westerberg et al. experiments revealed that brief (8–10 min) ischemic episodes resulted in significantly higher levels of arachidonic acid in the CA1 region. There was also a tendency for CA1 levels of docosahexaenoic acid to increase to a greater extent than in the CA3 hippocampus.

Pharmacological evidence that phospholipases are involved in the ischemic injury process has come from several laboratories. Umemura et al. [318,319] demonstrated that the PLC inhibitor, phenylmethylsulfonyl fluoride, both inhibited increases in free fatty acid levels in the rat neocortex following cardiac arrest and protected rat CA1 hippocampal neurons against transient forebrain ischemia. A non-selective PLA_2 inhibitor, mepacrine, attenuated ischemia-induced increases in locomotor activity and hippocampal CA1 injury in stroked gerbils (5 min of ischemia, 5 days of reperfusion) [229] and reduced both infarct size and neurological deficits in rats after a 2-h middle cerebral artery occlusion with 7 days of reperfusion [64].

Inhibition of PLA_2s by bromophenacyl bromide and of $cPLA_2$ with the selective inhibitor $AACOCF_3$, which has a 500-fold greater inhibitory potency for $cPLA_2$ in comparison with $sPLA_2$ [308], prevented cell death in organotypic rat hippocampal slices subjected to oxygen/glucose deprivation [8,9]. An $iPLA_2$ inhibitor (bromoenol lactone) and the $sPLA_2$ inhibitor 3-(3-acetamide-1-benzyl-2-ethyl-indolyl-5-oxy) propane sulfonic acid (LY311727) were virtually ineffective in this in vitro ischemia model. However, the $sPLA_2$ inhibitor, indoxam, significantly reduced

cortical injury in a rat MCAO model [348,349]. The reduction in ischemia-evoked cerebral infarcts and neurological deficits in mice deficient in both $cPLA_2$ and group II $sPLA_2$ has already been referred to [26,277].

Several studies have demonstrated a marked increase in the levels of immunoreactive $cPLA_2$, and of PLA_2 enzyme activity during cerebral ischemia [44,59,265,274,303,329]. Oxygen/glucose deprivation activated hippocampal $cPLA_2$ in the Ammon's horn region (CA1 + CA3) but not in the dentate gyrus [8,9]. An increased expression of PLA_2 mRNA occurs following transient forebrain ischemia [211,143], and this may play a role in the reperfusion phase of an ischemic episode, but the rapid increases in activity during ischemia are more likely to be a consequence of the posttranslational modification of existing PLA_2 proteins.

$cPLA_2$ activity depends on Ca^{2+} levels and kinase-dependent phosphorylation [43,84,153,274]. $cPLA_2$ is a downstream target of ERK and p^{38} mitogen-activated (MAP) kinases[135,153]. ERK1 and 2 MAPKs are phosphorylated in response to cerebral ischemia [84,193]. Inhibition of tyrosine phosphorylation by genistein decreases ERK2 phosphorylation and prevents delayed neuronal death following ischemia [130]. Alessandrini et al. [5] have demonstrated that inhibition of MAP kinase/ERK kinase 1 (MEK1), the upstream activator of ERK1 and 2, with the agent PD098059 reduces infarct volume in a mouse transient cerebral ischemia model. Another MEK inhibitor, U0126, protected the mouse hippocampus against forebrain ischemia, and mouse primary cultured cortical neurons against oxygen deprivation for 9 h [193].

p^{38} MAP kinase is activated in neurons and microglia in response to various stress signals [101,330]. Phosphorylation and activation of a high molecular weight isoform of human $cPLA_2$ by p^{42} MAP kinase and protein kinase C results in an increase in catalytic $cPLA_2$-specific activity [196].

The expression of group II $sPLA_2$ is also stimulated in the rat brain during ischemia/reperfusion [143] and in cultured astrocytes by inflammatory mediators [206]. Mammalian group II A $sPLA_2$ generates arachidonic acid and triggers apoptosis in cultured cerebral cortical neurons [349]. $sPLA_2$-induced neuronal death was associated with a marked influx of Ca^{2+} into cortical neurons. A calcium chelator and a blocker of L-type voltage-sensitive Ca^{2+} channels (L-VSCC) protected neurons from $sPLA_2$ II A-induced death. Furthermore, the block of L-VSCC channels ameliorated PLA_2 II A-induced morphologic alterations and apoptotic features such as condensed chromation and fragmented DNA. Moreover, ROS were generated prior to apoptosis suggesting that eicosanoids and ROS generated during arachidonic acid oxidative metabolism were involved in $sPLA_2$ II A-induced apoptosis in cooperation with Ca^{2+} [348,350].

Activation of $cPLA_2$ can initiate activation of $sPLA_2$, which may occur at the level of regulation of enzyme activity itself (immediate response) or at the gene regulatory level (delayed response). Following its release into the interstitial space, the $sPLA_2$ enzyme appears to act on the outer cellular surface of adjacent cells where it hydrolyzes phospholipids. The enzyme may subsequently be reinternalized into the cell [188]. $cPLA_2$-derived arachidonic acid is oxidized by 12/15-lipoxygenase, the products of which not only augment the expression of $sPLA_2$, but also cause membrane perturbation, increasing cellular susceptibility to $sPLA_2$ [188].

5. Ischemia-evoked efflux of FFAs into the interstitial space of the brain

Brain cells in situ contain low concentrations of free polyunsaturated fatty acids (PUFAs), such as arachidonic acid, that are available for liberation into the interstitial space. During ischemia, the intracellular levels of these PUFAs increase rapidly and their extracellular concentrations rise. Arachidonic and docosahexaenoic acids are subject to oxidation by cyclo-, lip-, and epoxygenases to form eicosanoids and docosanoids, many of which may have deleterious actions, as well as oxygen-derived free radicals. Moreover, arachidonic acid itself can have effects independent of its metabolites, which may have deleterious consequences for a cell. Arachidonic acid may act as a detergent, thereby altering membrane fluidity and integrity [340]. It can directly enhance NMDA receptor currents [177] and may elevate extracellular glutamate concentrations by increasing its release [324,342] or blocking its reuptake [327,358]. These effects could potentiate excitotoxic injury to adjacent nerve cells. Unesterified arachidonic acid has been reported to inhibit mitochondrial respiration [309]. Given these potential causes for enhanced ischemic injury, it appears that inhibition of arachidonic acid formation and release should be a more effective protective strategy than prevention of its metabolism using inhibitors of the cyclo-, lip-, and epoxygenases.

The primary sources of arachidonic acid release during cerebral ischemia have been evaluated by simulating ischemic conditions with elevated extracellular K^+. The results of studies addressing this question have been discrepant. Rat cortical synaptosomes or isolated cerebellar glomeruli release ^{3}H-arachidonic acid when bathed in K^+-enriched media [28,55], but primary cultures of striatal or hippocampal neurons do not [57,58,276]. Taylor and Hewett [314] have recently evaluated the ability of high K^+ to elicit ^{3}H-arachidonic acid from a murine mixed cortical cell culture. Elevating K^+ induced a time-dependent increase in arachidonic release, which was diminished in Ca^{2+}-free perfusion solution and in the presence of tetanus toxin to block endogenous transmitter release. Interestingly, pharmacological antagonism of both ionotropic and metabotropic glutamate receptors completely prevented high K^+-evoked arachidonic acid release, indicating that presynaptic glutamate release, elicited by a K^+-evoked depolarization, with Ca^{2+} influx via voltage-gated Ca^{2+} channels, was actually responsible for the activation of PLA_2, and possibly diacylglycerol lipase, to yield

free arachidonic acid, rather than the direct activation of Ca^{2+}-dependent $cPLA_2$. Both astrocytes and neurons express various forms of ionotropic and metabotropic glutamate receptors, and either cell type is capable of releasing glutamate in a Ca^{2+}-dependent manner. The authors suggested that their use of the holotoxin form of tetanus toxin that cleaves neuronal but not astrocytic synaptobrevin-2, which effectively prevented the high K^+-evoked glutamate release, is indicative of a neuronal release of both glutamate and arachidonic acid. This conclusion was supported by evidence that arachidonic acid could not be evoked from pure astrocyte cultures, using either high K^+ or exogenous glutamate, which were effective in mixed cultures.

An alternative approach to studying changes in in vivo rat brain tissue free fatty acid levels during ischemia/reperfusion has been the cortical window technique, with repeated collections of cortical artificial cerebrospinal fluid (aCSF) [274]. FFA levels in 10-min aCSF collections were measured by high pressure liquid chromatography. Basal extracellular levels of FFAs in this study were: arachidonic acid, 76 ± 14 μg/l; myristic acid, 71 ± 9 μg/l; linoleic acid, 99 ± 17 μg/l; palmitic acid, 276 ± 61 μg/l; and oleic acid, 93 ± 17 μg/l [274]. Due to run-time limitations, stearic acid levels in cortical superfusates were not analyzed. Docosahexaenoic acid levels, measured in a subsequent study, were 113 ± 17 μg/l [246]. During a 20-min, four-vessel occlusion cerebral ischemia (2 × 10-min collections), FFA levels initially increased, with only that of arachidonic acid achieving significance, and then declined towards basal levels in the second ischemic collection. Reperfusion was marked by a rapid increase in superfusate levels of all FFAs, which then declined slowly during three subsequent 10-min collections. This pattern of efflux of FFAs varied somewhat between groups of animals, as well as with the investigator conducting the experiment, with some animals displaying more pronounced increases in FFA efflux during ischemia. This variation may have reflected the actual degree of reduction in cerebral blood flow, which involves the mean arterial blood pressure during ischemia and the extent to which collateral vascular beds supplied the cerebral cortex during the ische-

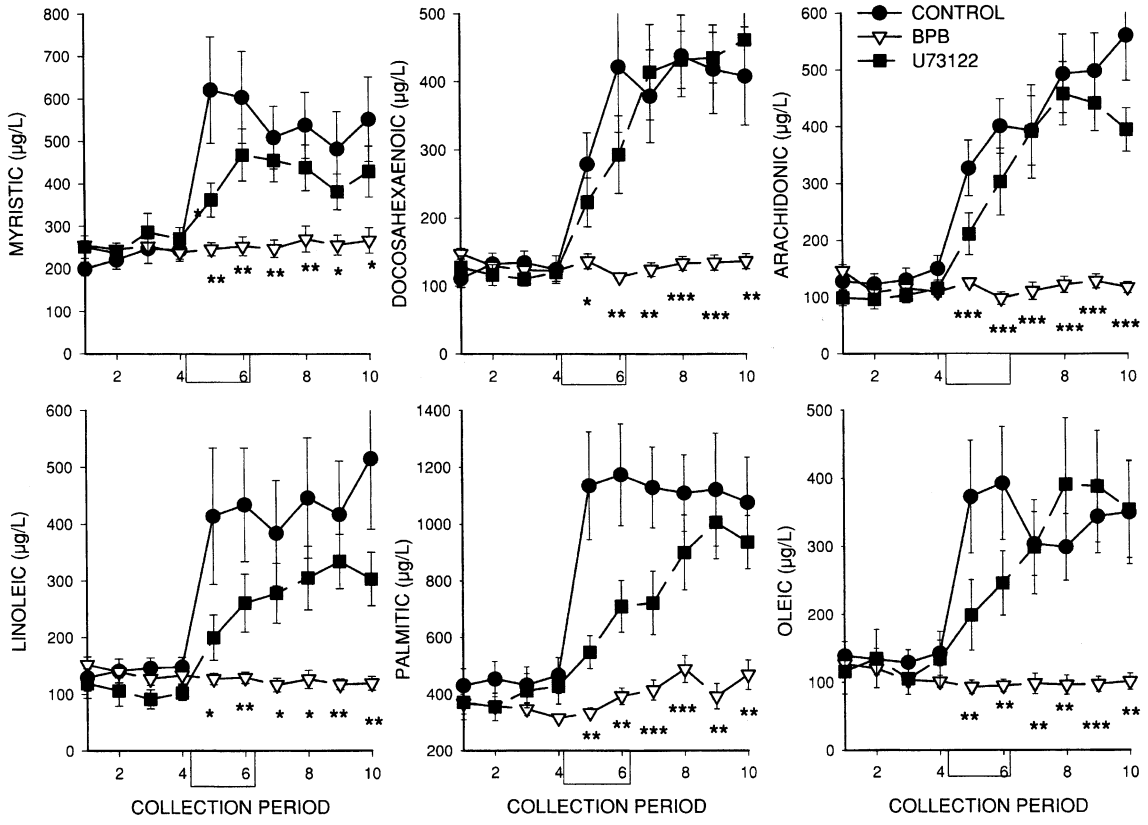

Fig. 1. Effects of non-selective PLA_2 inhibitor 4-bromophenylacyl bromide (BPB) (5 μM) and the PLC inhibitor U73122 (10 μM) in 0.05% DMSO aCSF on four-vessel occlusion ischemia/reperfusion-evoked efflux of free fatty acids into rat cerebral cortical superfusates. The line plots show the time course of changes in superfusate concentrations (μg/l) of FFA during 10-min basal collection periods (1 and 2) and following exposure to drug (3 and 4): line plot with open triangles for BPB, closed squares for U73122 or to control 0.5% DMSO (closed squares) in aCSF. Collections 5 and 6 represent ischemia, while collections 7–10 represent reperfusion periods. BPB and U73122 were initially applied 15 min prior to the onset of collection 3 and replaced at 5-min intervals. The drug-containing and DMSO-containing control solutions were present during all subsequent collections. Data represent mean ± S.E.M. Statistically significant differences between control and BPB data were determined by Student's *t* test. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. Reprinted from J.G. Pilitsis, F.G. Diaz, M.H. O'Regan, J.W. Phillis, Differential effects of phospholipase inhibitors on free fatty acid efflux in rat cerebral cortex during ischemia/reperfusion injury, Brain Res. 951 (2002) 96–106, with permission from Elsevier.

mic episode. (An isoelectric electrocorticogram was accepted as a valid indicator of cerebral ischemia.) Previous investigators have found that a reduced blood flow during cerebral ischemia may be more detrimental than complete cerebral ischemia [112,203]. Increases in extracellular cerebral cortical glutamate levels during ischemia were significantly greater in 4-vessel occlusion rats in comparison with 7- and 11-vessel occlusion animals, with greater reductions in cerebral blood flow [38,232]; a result that is consistent with PLA_2 activation being a contributor to glutamate release due to a smaller decrease in pH_i in the presence of a continuing blood supply.

In order to evaluate the contribution of individual groups of PLA_2s and PLC to the formation of FFAs, the rat cerebral cortex was superfused with phospholipases $sPLA_2$ and PLC, and specific PLA_2 or PLC inhibitors prior to and during ischemia/reperfusion. Melittin, a 26 amino acid polypeptide obtained from honey bee (*Apis mellifera*) venom was used to activate endogenous PLA_2s in the absence of ischemia/reperfusion. It induced a rapid, sustained, increase in FFA efflux into cortical superfusates [236]. The levels of arachidonic, linoleic, palmitic, and oleic acids were significantly elevated. (Docosahexanoic acid efflux was not monitored in this experiment.) Melittin also significantly enhanced the efflux of several amino acids, including glutamate, aspartate, taurine, GABA, phosphoethanolamine, glycine, glutamine, serine, and alanine from the non-ischemic rat cortex [236]. Topical applications of $sPLA_2$ (bee venom) and PLC (*Bacillus cereus*) also resulted in significant increases in FFA and amino acid efflux from the rat cerebral cortex [210,245].

Exposure of the rat cerebral cortex to the non-selective PLA_2 inhibitor, 4-bromophenylacyl bromide (BPB) significantly inhibited the efflux of arachidonic, docosahexaenoic, linoleic, palmitic, myristic, and oleic acids from the ischemic/reperfused rat cerebral cortex (Fig. 1) [245]. The $cPLA_2$ inhibitor, $AACOCF_3$, mirrored the effects of BPB, leading to pronounced reductions in all six FFA levels (Fig. 2). Exposure to the $sPLA_2$ inhibitor LY311727 and the $iPLA_2$ inhibitor BEL had only minimal effects on most FAA effluxes during ischemia/reperfusion, significantly reducing only myristic acid release. With the exception of myristic acid, the PLC inhibitor, U73122, did not significantly depress the increases in FFA levels during ischemia/reperfusion (Fig. 1). However, the initial rates of increase in the levels of linoleic, palmitic and oleic acids were reduced with the overall reduction in palmitic acid release during ischemia being significant ($p < 0.05$). This would be consistent with an earlier suggestion that PLC is the first phospholipase to be activated during ischemia [318,319].

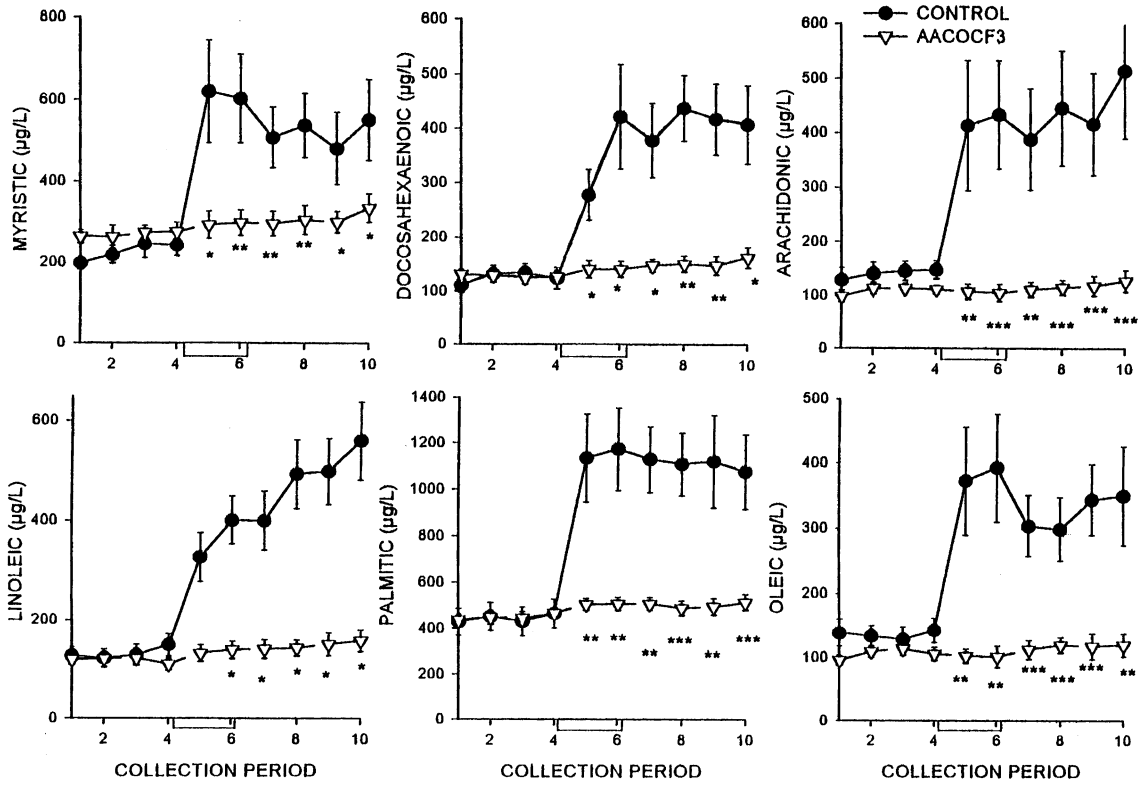

Fig. 2. Effects of the Ca^{2+}-dependent PLA_2 inhibitor $AACOCF_3$ (30 µM) in 0.05% DMSO aCSF on ischemia/reperfusion evoked efflux of FFA into rat cerebral cortical superfusates. See Fig. 1 for further details. Reprinted from J.G. Pilitsis, F.G. Diaz, M.H. O'Regan, J.W. Phillis, Differential effects of phospholipase inhibitors on free fatty acid efflux in rat cerebral cortex during ischemia/reperfusion injury, Brain Res. 951 (2002) 96–106, with permission from Elsevier.

In an earlier study, selective inhibitors of $sPLA_2$, $cPLA_2$, $iPLA_2$, and PLC were evaluated for their ability to depress ischemia/reperfusion evoked release of the excitotoxic amino acids glutamate and aspartate [230]. In this instance, inhibition of each of these phospholipases significantly reduced the amino acid efflux, suggesting that PLC and several PLA_2 isozymes may contribute to ischemia/reperfusion injury. Destabilization and deterioration of the plasma membrane, as a consequence of phospholipase activation, was proposed as a contributor to the amino acid leakage down concentration gradients into the extracellular fluids. Evidence in support of this concept was furnished by experiments demonstrating the efflux of lactic acid dehydrogenase (LDH) into rat cerebral cortical superfusates during a 30-min period of ischemia followed by reperfusion [233]. In that LDH has a molecular weight of circa 140,000, it is apparent that the ischemia must have inflicted considerable damage to at least some cortical cells. There is, indeed, ultrastructural evidence of early damage to CA1 hippocampal neurons following a 10-min ischemia with 20 min of reperfusion. This included postsynaptic cell membrane breaks [62] and evidence that horse radish peroxidase (MW 44,000) was able to gain access into pyramidal cell dendrites [54,62]. Tanaka et al. [312] subsequently described an early formation of membrane blebs in ischemic rat CA1 pyramidal cell membranes, with swelling and the formation of larger blebs 1.5–2 min after the onset of anoxic depolarization. Dye studies suggested that micropores in the blebs provided an access pathway for the diffusion of molecules with a molecular weight of <520 kDa.

Arai et al. [8,9] explored the relationship between PLA_2 activation and neuronal degeneration using organotypic hippocampal cultures. Exposure of the cultures to 35 min of oxygen/glucose deprivation resulted in a twofold increase in PLA_2 activity in the dissected pyramidal cell layer, demonstrated by measuring the amounts of radiolabeled fatty acids released from the substrate 1-palmitoyl-2-^{14}C arachidonoyl-sn-glycero-3-phosphoethanolamine. This enhancement lasted for at least 24 h, at which time neuronal death was evident in the CA1 field. OGD did not affect the level of PLA_2 activity in the dissected dentate gyrus, and no neuronal death was evident in this area. BPB and $AACOCF_3$ effectively prevented cell death in a concentration-dependent manner. Bromoenol lactone and LY311727 were virtually ineffective, confirming that it is $cPLA_2$, rather than $iPLA_2$ or $sPLA_2$, which plays a causative role in neuronal death following a simulated ischemic episode.

The pronounced increases in FFA levels in rat cerebral cortical superfusates at the onset of reperfusion following either 20- or 30-min periods of cerebral ischemia [237] suggested that phospholipase activity may have been inhibited during ischemia, with a rapid recovery at the onset of reperfusion. The explanation for this failure of phospholipase activation, proposed by Lemasters et al. [149], is that the reduction in pH that develops in ischemic tissues, due to ATP hydrolysis and lactate formation, can actually suppress the activation of hydrolytic enzymes such as phospholipases and proteases whose activity is inhibited by acidotic pH, would be applicable for $cPLA_2$ in the brain [73,356]. During reperfusion, the rapid recovery to normal pH releases this inhibition and hydrolytic activity ensures. Mild acidosis has been shown to protect hippocampal and cerebral cortical neurons from ischemia-like conditions [88,317]. Vornov et al. [328] subsequently explored the possibility that recovery of pH during reperfusion was actually responsible for the ischemic injury by lowering pH_e to 6.2 during the initial hour of recovery of neuronal cultures from metabolic inhibition, demonstrating profound protective effects. Slowing the rate of pH_i recovery by incubation with blockers of Na^+/H^+ exchange also had protective effects. Measurements of pH_i confirmed that the blockers slowed recovery from intracellular acidosis, and that a slower recovery was correlated with decreased injury as measured by LDH release.

6. Phospholipases and trauma-induced brain injury

Traumatic brain injury (TBI) is associated with glutamate release, elevated intracellular Ca^{2+}, phospholipase activation, and phospholipid degradation, leading to the generation of FFAs, including arachidonic and docosahexaenoic acids and their metabolites, formation of free radicals, and subsequent lipid peroxidation [39,108,120,200,213,284,293]. An increase in PLC activity in the cat brain after a fluid percussion injury [335], with the generation of diacylglycerol, was proposed as the source of arachidonic acid and the rise in brain prostaglandins that occur following brain trauma. Activation of brain PLA_2, with subsequent PGE_2 formation, following a closed head injury in the rat was subsequently demonstrated [291]. Identification of increased levels of PLCγ in cortical and hippocampal cytosols accounts for the liberation of stearic and arachidonic acids from PIP_2 [53]. Homayoun et al. [109] observed increases in cerebral cortical FFAs, which included arachidonic and docosahexaenoic acids, and diacylglycerols following cortical impact brain injuries in rats. The overall profiles of FFA formation suggested that phospholipases activated by TBI hydrolyzed primarily 20.4 phospholipids within minutes of the insult, followed by preferential hydrolysis of 22.6 phospholipids 24 h after the injury.

Inhibition of phospholipase activation as a treatment for brain trauma has received little attention to date. Using an in vivo rat brain fluid percussion model, Golding and Vink [91] were able to demonstrate that inhibition of PLC with neomycin improved both metabolic and neurological outcomes.

Weber et al. [334] used an in vitro traumatic injury mode to examine the effects of stretch (mechanical injury) on intracellular Ca^{2+} stress in cultured cortical neurons. Thapsigargin induced increases in Ca_i^{2+} were abolished 15 min

post-injury, indicating that endoplasmic reticulum stores of Ca^{2+} had been depleted. Pre-injury inhibition of PLC with neomycin maintained the Ca^{2+} stores 15 min post-injury, indicating that the initial injury-induced store depletion was due to PLC activation with IP_3 formation. Thapsigargin-stimulated increases in Ca_i^{2+} recovered over the next 3 h. Moreover, the Ca^{2+}-influx triggered by depletion of intracellular Ca^{2+} stores (capacitative Ca^{2+} influx) was enhanced 3 h post-injury, and this enhancement could be inhibited by inhibition of $cPLA_2$ with $AACOCF_3$ and of P450 epoxygenase with MS-PPOH or econazoles. The authors suggested that as intracellular Ca^{2+}-store-mediated signaling plays an important role in neuronal function, these changes, subsequent to ischemic injury, could contribute to the secondary late phase of dysfunction produced by traumatic brain injury. Inhibitors of PLC, $cPLA_2$, and P450 epoxygenase may have a valid role in the treatment of traumatic injuries to the central nervous system.

7. Phospholipase involvement in neurodegenerative disorders

Fatty acids are considered to be essential for normal cell membrane functioning as they affect such properties as permeability and fluidity. Polyunsaturated fatty acids can be released from membrane phospholipids by mechanisms involving the activation of phospholipase A_2 and phospholipase C/diacylglycerol lipase pathways, affecting the membrane phospholipid composition and properties such as permeability and rigidity. The arachidonic and docosahexaenoic acids thus released can subsequently be substrates for the generation of a variety of pro-inflammatory compounds including prostaglandins, thromboxanes, leukotrienes, platelet-activating factor, docosanoids, and reactive oxygen radicals. Excessive production of such pro-inflammatory mediators could potentially lead to disease states and neural injury [21,73,133,167,354].

7.1. Alzheimer's disease (AD)

The evidence for an involvement of phospholipases in a neurodegenerative condition is most convincing for Alzheimer's disease [71,161]. Nitsch et al. [201] demonstrated significant decreases in the initial phospholipid precursors, choline and ethanolamine, and increases in the phospholipid deacylation product glycerophosphocholine in Alzheimer brains. There was a near stoichiometric relationship between the decrease in phospholipids and increase in phospholipid catabolites. Similar abnormalities were not detected in brains of patients with Huntington's disease, Parkinson's disease (PD) or Down's syndrome, suggesting that the phospholipid abnormalities in AD brains were specific for the pathomechanism of Alzheimer's disease [201].

$cPLA_2$ levels in immunostained sections of human Alzheimer occipital cortex and cerebellum were elevated above those in neurologically normal brains. The staining was associated with astrocytes in both control and AD cases [302]. In the cerebral cortex, $cPLA_2$ immunoreactive astrocytes were detected in regions that contained numerous Aβ amyloid deposits. The finding of elevated levels of $cPLA_2$ in reactive astrocytes associated with the amyloid deposits supports the hypothesis that an active inflammatory response occurs in AD.

In a subsequent study, Stephenson et al. [303] confirmed the presence of cytosolic PLA_2 in reactive glial cells (both astrocytes and microglia) within the precise locations of neuronal loss in AD brains, suggesting that cytosolic PLA_2 may prove to be an attractive therapeutic target for neurodegenerative conditions, including Alzheimer's disease.

A rather different conclusion was reached by Gattaz et al. [86] who compared PLA_2 activity in postmortem brains from patients with AD and those from elderly, non-demented, controls. PLA_2 was significantly lower in the parietal, and to a lesser extent in the frontal cortices, of the AD brains. Lower PLA_2 activity correlated significantly with earlier onset of the disease, earlier age at death, and higher counts of neurofibrillary tangles and senile plaques.

Talbot et al. [310] examined the enzymes regulating membrane phospholipid composition in brains from patients with a frontal variant of Alzheimer's disease. In these brains, $cPLA_2$ levels in the dorsolateral prefrontal and lateral temporal cortices were normal. In contrast, there was a significant decrease (42%) in Ca^{2+}-independent PLA_2 in the dorsolateral prefrontal, but not in the lateral temporal, cortex. The dorsolateral prefrontal cortex also suffered a nonsignificant (42%) decrease in total free fatty acid content.

An involvement of PLA_2 in the production of ROS in Alzheimer's disease has been implicated in studies on rat brain synaptosomes exposed to amyloid β peptide fragment beta (25–35) [6]. This peptide fragment, but not the inverted peptide (35–25), stimulated production of reactive oxygen species in a concentration- and time-dependent manner. Reactive oxygen species formation was inhibited by the PLA_2 inhibitor 7,7-dimethyl-(5Z,8Z)-eicosadenoic acid suggesting that beta A (25–35) stimulated ROS production through a PLA_2-dependent pathway.

Shimohama et al. [287,288], using immunostaining, demonstrated an abnormal accumulation of PLCδ in the neurofibrillary tangles surrounding senile plaques and neuropil threads in AD brains. Further studies [289] showed that the concentration of PLCδ protein was significantly higher in the cytosolic fraction of AD brain cortical tissue. However, PLC activity was not enhanced indicating that the specific activity of the PLCδ is decreased in AD brains. A further analysis [290] established that $PLC\gamma_1$ activity in AD brains was significantly decreased and that $PLC\delta_1$ activity was significantly increased compared with controls, suggesting that PLC isozymes are differentially involved in Alzheimer's disease.

The activity of phospholipase D in homogenates of Alzheimer brain tissue was reduced by 63% in comparison

with controls [117]. As this enzyme releases choline directly from lecithin, its reduction could account for the decreased quantity of neurotransmitter acetylcholine in Alzheimer's brains. Kanfer et al. [118] attempted to clarify the relationship between the amyloid β peptide, which accumulates in AD brain tissue, by suggesting that the peptide is able to activate cellular phospholipases with the resultant production of a variety of potentially toxic second messengers.

Studies on CSFs from Alzheimer's disease and control subjects have failed to reveal any differences in total phosphatidylcholine (PC) levels [187,251]. Nor were there any differences in the concentrations of PC species containing linoleic acid or arachidonic acid [187]. However, lyso-PC concentrations tended to be lower, while the lyso-PC/PC ratio was significantly decreased, in the CSF of Alzheimer's disease patients compared to controls. A comparable decrease was found in the lyso-PC/PC ratios for PCs containing linoleic acid or arachidonic acid, respectively [187]. These lower lyso-PC/PC ratios may reflect alterations in the metabolism of choline-containing phospholipids in the brain in Alzheimer's disease, which could have an impact on the integrity of cell membranes.

7.2. Parkinson's disease

In order to determine whether increased oxidative stress in the substantia nigra of patients with idiopathic Parkinson's disease might be related to the decreased ability of nigral cells to detoxify oxidized membrane phospholipids, Ross et al. [268,269] measured the activity of PLA_2 in different brain regions. The levels of $cPLA_2$ and $iPLA_2$ in normal human brain, although relatively homogenous in their distribution, tended to be lowest in the substantia nigra. This coupled with low activity of phosphoethanolamine- and phosphocholine-cytidyltransferases, major regulatory enzymes of phospholipid synthesis in this brain region, suggested that the rate of phospholipid turnover was low in the substantia nigra, which might result in a reduced ability to repair oxidative membrane damage, as may occur in Parkinson's disease. In contrast, in Parkinson brains, although nigral levels of PLA_2 were normal, those of the biosynthetic enzymes were elevated, possibly representing a compensatory response to repair membrane lipids [270]. Enzyme activities in other brain areas were normal with the exception of an increase in $cPLA_2$ activity in the putamen.

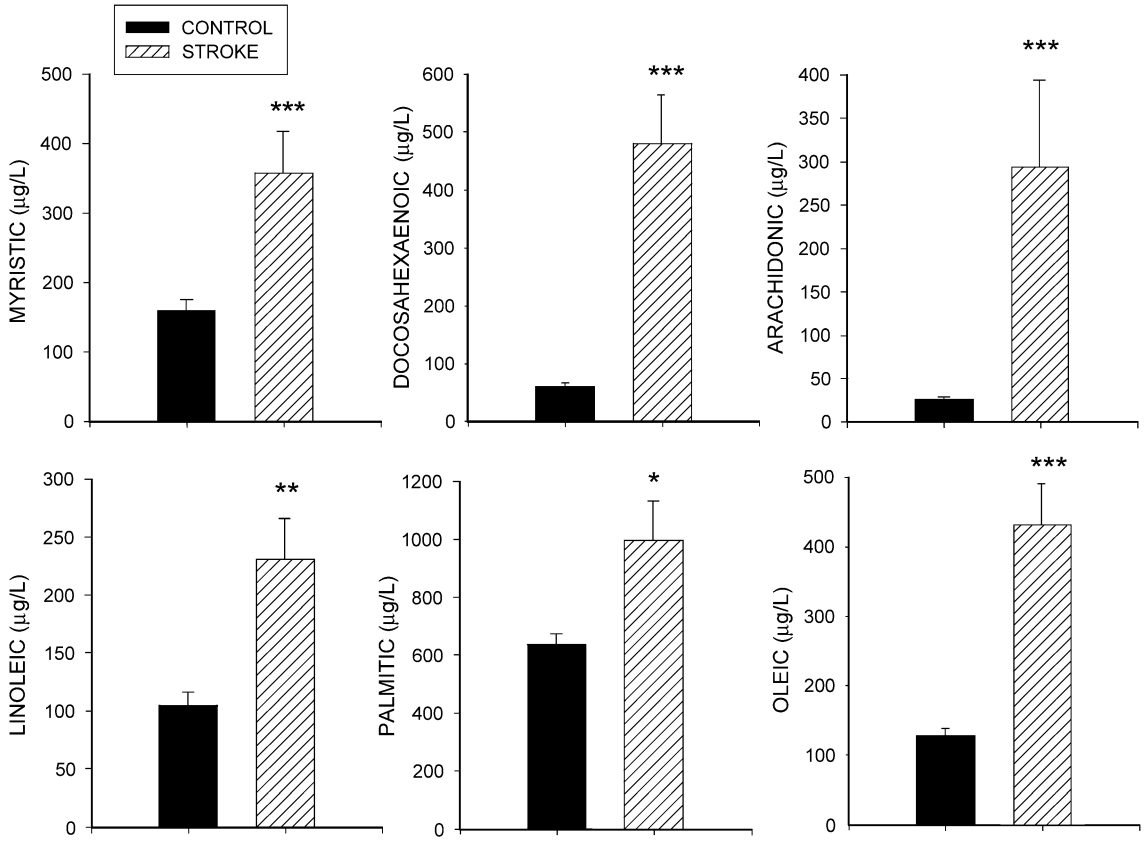

Fig. 3. CSF FFA concentrations from hospital patients taken within 48 h of ischemic or hemorrhagic stroke as compared to CSF FFA levels from control patients without evidence of acute neurological disease. This figure is reflective of the 15 stroke patients from whom samples were obtained within 48 h and 73 control patients. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Reprinted from J.G. Pilitsis, W.M. Coplin, M.H. O'Regan, J.M. Wellwood, F.G. Diaz, M.R. Fairfax, D.B. Michael, J.W. Phillis, Measurement of free fatty acids in cerebrospinal fluid from patients with hemorrhagic and ischemic stroke, Brain Res. (2003) (in press), with permission from Elsevier.

8. Free fatty acids in human CSF following brain injury

Pathologic processes initiated during and following cerebral injuries result in the significant increases in brain free fatty acid levels described in the earlier sections of this review. Bazan [18] appears to have conducted the initial studies on FFA levels in human CSFs, making the suggestion that there might be correlates between FFA levels and various neurological disorders, including ischemia, epilepsy and degenerative diseases. Studies conducted in the authors' laboratory have confirmed that examination of human CSF samples collected following strokes or cerebral trauma can be used to gain information about FFA levels in the human central nervous system, and yield potentially useful information regarding the likely clinical outcome of the insult. CSF free fatty acid concentrations in normal and stroked individuals were examined using high performance liquid chromatography. Concentrations of FFAs from ischemic and/or hemorrhagic stroke patients [248], obtained within 48 h of insult, were significantly greater than in the controls [243] ($p<0.001$, arachidonic, docosahexaenoic, linoleic and oleic acids; $p<0.01$, myristic acid) (Fig. 3). Higher concentrations of polyunsaturated fatty acids in CSF obtained within 48 h of insult were associated with lower admission Glasgow scores and worse outcomes at time of hospital discharge on the Glasgow Outcome Scale, suggesting that PUFA levels obtained within 48 h of insult may be a predictive marker for outcome [248].

Similar studies were conducted on patients admitted following subarachnoid hemorrhage (SAH), a condition that causes local vasospasm with disturbances in cerebral vascular autoregulation. All free fatty acid concentrations measured 24 h after SAH were significantly greater in comparison with control CSFs. FFA levels then declined, but had increased again at 8–10 days [244]. Samples of CSF collected within 48 h after SAH from patients in whom angiography and clinical examination confirmed the development of vasospasm after SAH were found to have significantly higher concentrations of arachidonic, linoleic and palmitic acids than samples collected from patients in whom vasospasm did not develop.

Concentrations of specific FFAs from TBI patients, obtained within 48 h of the insult were significantly greater than those in a control group ($p<0.001$, arachidonic, docosahexaenoic, myristic acids; $p<0.01$, oleic, palmitic acids; $p<0.05$, linoleic acid) (Fig. 4). Higher concentrations of total PUFAs ($p<0.0001$) and of arachidonic, myristic, and palmitic acids measured individually ($p<0.01$) in CSF

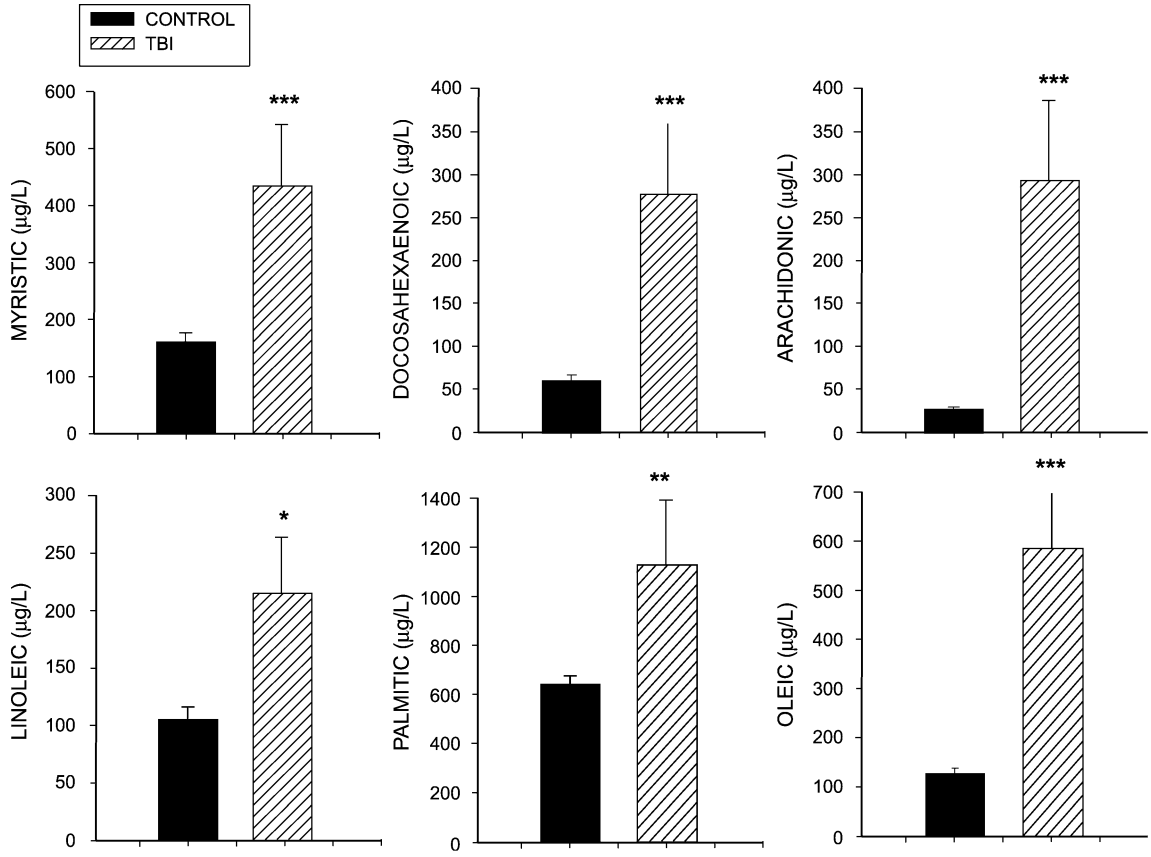

Fig. 4. CSF FFA concentrations obtained from 15 hospital patients within 48 h of traumatic brain injury as compared to values in samples from 73 control patients. *$p<0.05$; **$p<0.01$; ***$p<0.001$. Reprinted from J.G. Pilitsis, W.M. Choplin, M.H. O'Regan, J.M. Wellwood, F.G. Diaz, M.R. Fairfax, D.M. Michael, J.W. Phillis, Free fatty acids from patients with traumatic brain injury, Neurosci. Lett. 349 (2003) 136–138, with permission from Elsevier, [247].

obtained 1 week after the insult were associated with a worse outcome at the time of hospital discharge using the Glasgow Outcome Scale [247].

9. Excitotoxicity and phospholipase activation

The role of glutamate and *N*-methyl-D-aspartate (NMDA) in neural cell injury has been extensively investigated in order to gain a better understanding of the molecular processes underlying the phenomenon of excitotoxicity. Cell injury associated with exposure to these amino acids is accompanied by an accumulation of intracellular Ca^{2+}, degradation of membrane phospholipids, and accumulation of free fatty acids and lipid peroxides, implying the involvement of phospholipases [41,154,238,338]. Kainic acid-induced neurotoxicity also involves the activation of phospholipases with degradation of glycerophospholipids and the formation of free fatty acids and lipid peroxides [70,75]. An increase in $cPLA_2$ immunoreactivity in the rat hippocampus occurs following the intravenous administration of kainate [275], accompanied by an increase in $cPLA_2$ activity in neurons and astrocytes. This is associated with an increased lipid peroxidation as evidenced by accumulation of 4-hydroxynonenal modified proteins [159]. $cPLA_2$, but not $iPLA_2$, inhibitors attenuate the neural damage induced by kainate in hippocampal slices [160]. Significantly less 4-hydroxynonenal immunoreactivity was observed in slices pretreated with PLA_2 inhibitors than in those receiving kainate alone [209]. In that 4-hydroxynonenal is an especially neurotoxic endproduct of lipid peroxide decomposition [209], the reduction in its formation may be a significant aspect of the attenuation of PLA_2-induced cell death by phospholipase inhibitors. Inhibitors of PLC prevented glutamate neurotoxicity in primary cultures of cerebellar neurons [157].

Activation of glutamate receptors evokes the release of arachidonic acid from hippocampal slices [222] as well as from both neurons and astrocytes in culture [58,145,299]. Glutamate-evoked release from mouse cortical neurons in primary cultures was affected by changes in extra- and intracellular pH [300]. As pH was shifted from 7.2 to 7.8, the glutamate-evoked release of arachidonic acid was enhanced threefold. Arachidonic acid release evoked by NMDA and kainate was also enhanced by pH alkalinization. NMDA and kainate increases in intracellular Ca^{2+} were not affected by the change in pH. Membrane-bound phospholipase A_2 activity during glutamate exposure was stimulated by Ca^{2+} in a pH-dependent manner, increasing its activity threefold as pH was shifted from 7.2 to 7.8, which would be consistent with mediation by a pH-sensitive PLA_2.

Activation of NMDA receptors initiates an increase in cytosolic Ca^{2+}, activation of $cPLA_2$, with hydrolysis of 1-alkyl-2-arachidonyl glycero-3-phosphocholine at the sn-2 position, releasing free arachidonic acid and lyso-platelet-activating factor [3]. Lyso-PAF is then acetylated by acetyltransferases at the sn-2 position to yield PAF. PAF synthesis is enhanced during exposure to excitotoxic compounds [20]. When added to isolated rat brain mitochondrial preparations, PAF induced mitochondrial swelling, membrane permeability transition (MPT), and cytochrome *c* release; an effect that was blocked by the selective PAF antagonist BN50730 [216]. PAF may therefore make a significant contribution to cerebral injury and inflammation following exposure to excitotoxic agents [20].

The past decade has been characterized by the development of an intense interest in the role of metabotropic glutamate receptor activation in ischemic, traumatic and excitotoxic brain injury. Antagonists for groups 1, 3, and 5 metabotropic glutamate receptors have provided neuroprotection against such injuries to brain and spinal cord. mGlu receptor antagonists may also be beneficial in the treatment of chronic neurodegenerative diseases, such as amyotrophic lateral sclerosis, and Alzheimer's disease (see Ref. [32] for a recent review of this topic). In this context, it is of interest that metabotropic glutamate receptor subtypes 1 and 5 exhibit a high degree of sequence homology and that both are coupled to PLC and intracellular calcium mobilization [221], raising the possibility that activation of these receptors may be responsible for the postulated PLC-evoked early increase in FFA levels in the ischemic brain [319]. Glutamic acid may sequentially activate both phospholipases C and D via G-protein-coupled metabotropic glutamate receptors. In a study of primary cultures of astrocytes, Servitja et al. [285] observed activation by glutamate of both phospholipases with equal potency. PLD, but not PLC, activation by glutamate was dependent on Ca^{2+} mobilization, and fully blocked by both PKC inhibitors and PKC down-regulation. These results suggest that PLD activation by glutamate is secondary to PLC stimulation with PLC-induced Ca^{2+} mobilization via IP_3 receptors and protein kinase C activation. In addition, mGlu1 and mGlu5 can activate a variety of signaling cascades and modulate the activity of ion and ligand-gated channels through functional coupling with other transduction pathways such as adenylyl cyclase, PLA_2, tyrosine kinase, and mitogen-activated protein kinase [104,221,322].

10. Mitochondrial contributions to phospholipase activation and CNS injury

Neurons lose viability in response to ischemia and trauma. Cell death in these conditions can occur fairly rapidly with its onset within minutes of the ischemia/traumatic event or may take hours or days to become manifest. Cell death of rapid onset is distinguished as necrotic cell death and involves the rapid disruption of cellular metabolism, ATP depletion, ion dysregulation, mitochondrial and cellular swelling, activation of degradative enzymes, disruption of the plasma membrane, and cell lysis. Delayed cell death is

described as "apoptosis", which may take days or weeks to develop, and in which metabolism is not initially severely impaired and cells generally shrink rather than swell. Apoptosis is often accompanied by the expression of specific genes. Necrotic and apoptotic cell deaths share certain features in common and it has been suggested that the outcome depends in part on the extent to which mitochondria can sustain ATP levels. If ATP levels fall profoundly, plasma membrane permeability and rupture ensue. If ATP levels are partially maintained for some period of time early or late apoptosis will be the consequence [151]. A new term, "necrapoptosis" was proposed to describe such death processes, initiated by a common stress and culminating either in cell lysis (necrosis) or programmed cell resorption (apoptosis) depending on the extent to which ATP levels had been conserved [129,150,151].

Overstimulation of NMDA receptors by elevated levels of extracellular glutamic acid has been considered to be a major contributor to both cerebral ischemia/reperfusion and traumatic injury. NMDA receptor activation causes a rapid increase in intracellular Ca^{2+}, which can ultimately lead to a collapse of the mitochondrial membrane potential with energy failure and neuronal death via necrotic or apoptotic pathways [7,76,154,198,295]. The early increase in cell Ca^{2+} during ischemia, which is via NMDA receptor activated channels, subsequently triggers further release of Ca^{2+} from the endoplasmic reticulum by a process known as calcium-induced calcium release (CICR) [154]. CICR from the endoplasmic reticulum is likely to involve the IP_3R and ryanodine (RyR) receptor channels [218]. Activation of Ca^{2+} channels on the plasma membrane and on local intracellular storage sites, such as the endoplasmic reticulum, generates transient increases in mitochondrial Ca^{2+} concentrations which may be in the millimolar range [182]. This is a consequence of the close apposition of mitochondria to either Ca^{2+} release channels or plasma membrane Ca^{2+} channels [212,262,263]. In some cell types, including cerebellar Purkinje neurons

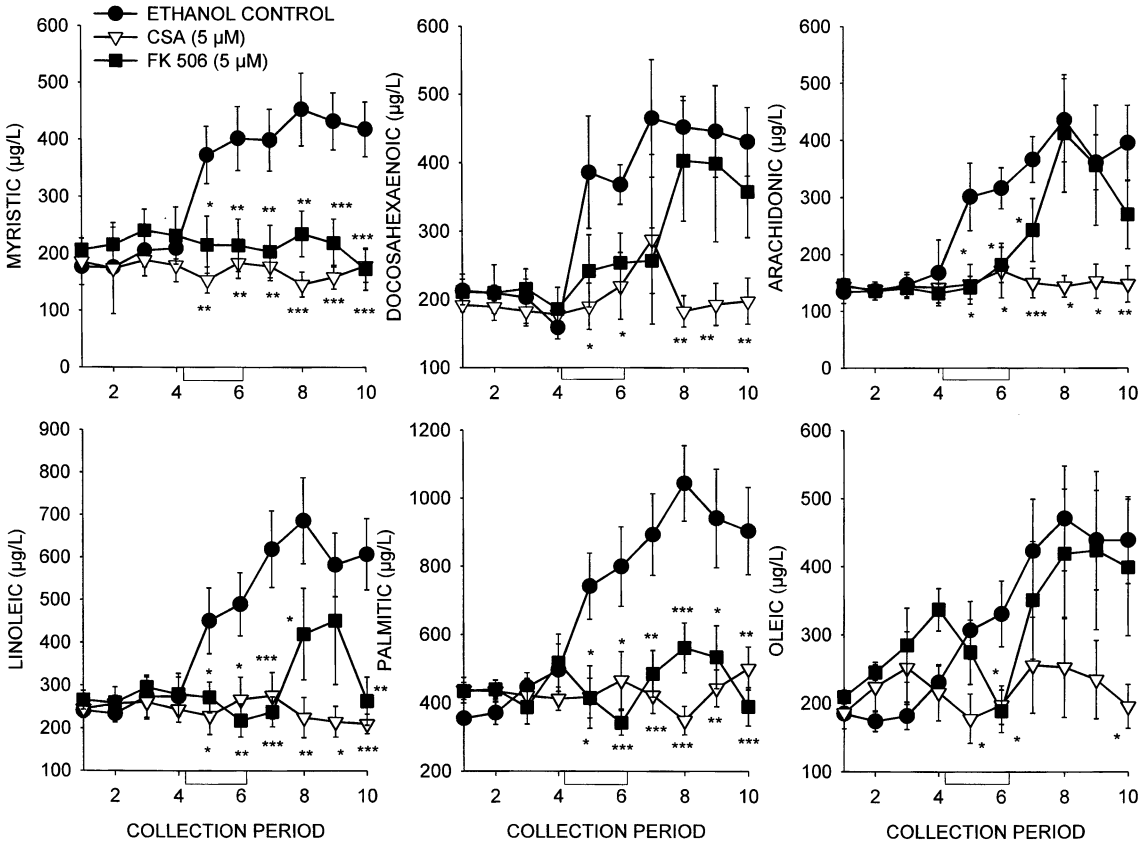

Fig. 5. Effects of the mitochondrial permeability transition (MPT) pore inhibitor cyclosporine A (CSA; 5 μM) and the calcineurin inhibitor FK506 (both in 0.05% ethanol aCSF)on 4 VO ischemia/reperfusion-evoked efflux of free fatty acids into rat cerebral cortical superfusates. The line plots show the time course of changes in superfusate concentrations of FFAs during 10 min basal collections (1 and 2) and following exposure to CSA (3 and 4, open triangles) FK506 (closed squares) or 0.05% ethanol (closed circles) in aCSF. Collections 5 and 6 represent ischemia, while collections 7–10 represent reperfusion periods. CSA, FK506 and 0.05% ethanol solutions were initially applied 15 min prior to the onset of collection 3 and replaced at 5 min intervals. The drug-containing and ethanol control solutions were present during all subsequent collections. Statistically significant differences between the control and CSA or FK506 data were determined by one-way ANOVA and contrasts to the appropriate control value for each collection period. *$p < 0.05$; **$p < 0.01$. Reprinted from J.W. Phillis, F.G. Diaz, M.H. O'Regan, J.G. Pilitsis, Effects of immunosuppressants, calcineurin inhibition, and blockade of endoplasmic reticulum calcium channels on free fatty acid efflux from the ischemic/reperfused rat cerebral cortex, Brain Res. 957 (2002) 12–24, with permission from Elsevier.

and cardiomyocytes, there is a close proximity of mitochondria to IP_3R-rich cisternae of the endoplasmic reticulum [255,279]. In Hela cells, close apposition of mitochondria to the endoplasmic reticulum (at distances of less than 100 nM) was a frequent finding and often involved a significant portion of the mitochondrial surface membrane [262]. There may therefore be a tight functional coupling between voltage-dependent Ca^{2+} channels in the plasma membrane, RyR, and IP_3R channels of the endoplasmic reticulum and intramitochondrial calcium levels, with Ca^{2+} cycling rapidly between the mitochondria and the endoplasmic reticulum [10].

Mitochondria appear to buffer large depolarization-induced increases in Ca^{2+}, using uptake of Ca^{2+} by the uniporter, with the mitochondrial Na^+/Ca^{2+} exchanger (mNCX) making a major contribution to its efflux and either uptake by the adjacent endoplasmic reticulum or extrusion back into the extracellular space by the plasma membrane Na^+/Ca^{2+} exchanger (NCX). An excessive accumulation of Ca^{2+} within the mitochondrion may, however, trigger a rapid loss of the mitochondrial membrane potential as a consequence of an inner mitochondrial MPT. During an MPT, a high conductance pore opens in the inner mitochondrial membrane. This precipitates collapse of the mitochondrial potential, an efflux of mitochondrial contents, including Ca^{2+}, and potentially mitochondrial demise [51,137,295]. MPT with cytochrome *c* release from the mitochondrial inner membrane are proposed as being the determining factors in the final step to necrosis and apoptosis. The mitochondrial membrane potential appears to control the permeability of the membrane, provide the appropriate conditions for oxidative phosphorylation with ATP generation, and regulate cytochrome *c* release. During ischemia, factors such as intracellular Ca^{2+} accumulation, long-chain FFA accumulation, and reactive oxygen species progressively increase mitochondrial susceptibility to MPT, increasing the likelihood that MPT will occur in reperfusion. Because neuronal recovery ultimately depends on mitochondrial recovery, protection must involve a reduction in these causative factors.

An approach to examine the role of free fatty acids in ischemia/reperfusion-evoked cerebral injury has involved measurements of free fatty acid efflux from the ischemic rat cerebral cortex. The magnitude of ischemia-evoked excitotoxic amino acid release from the brain has proven to be an effective prognosticator of the extent of ischemia/reperfusion-evoked injury in the brain [34,175,307]. It is likely that free fatty acid formation and efflux would serve

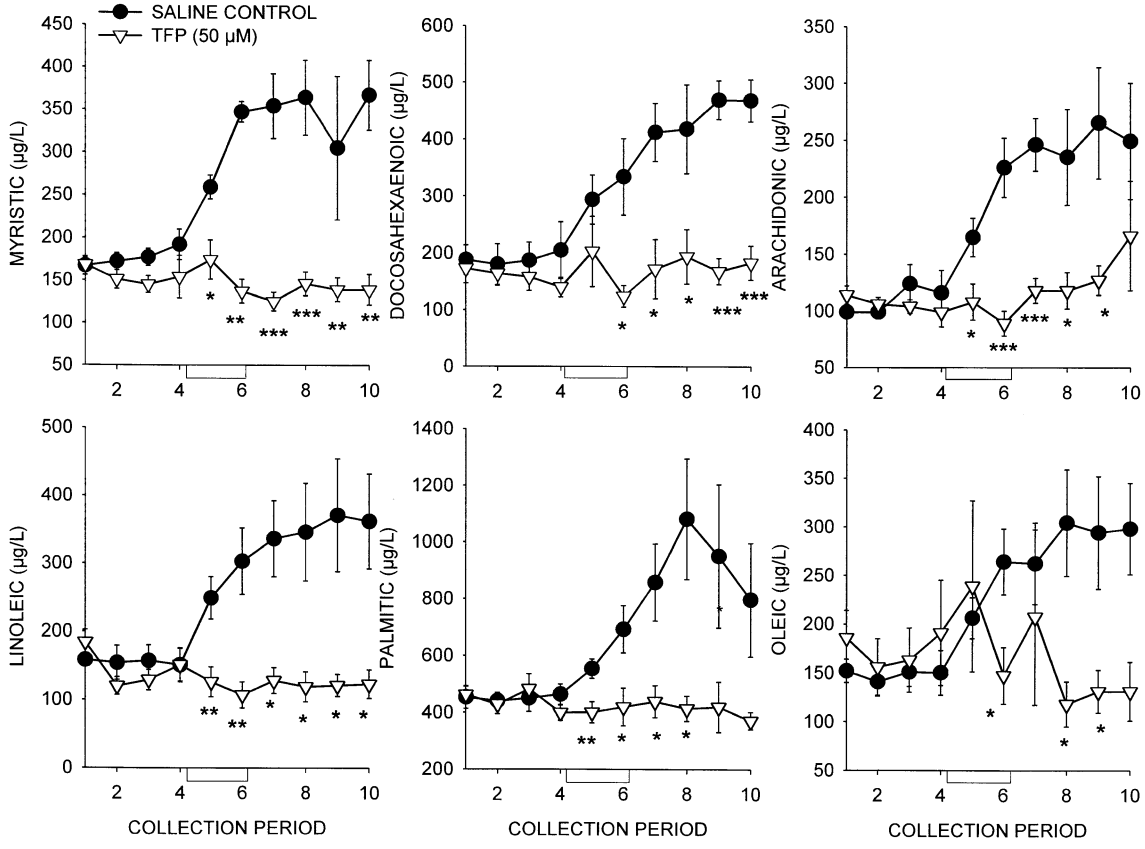

Fig. 6. Effect of the MPT pore inhibitor trifluoperazine (TFP, 50 μM) in aCSF on ischemia/reperfusion-evoked efflux of FFAs into rat cerebral cortical superfusates. See legend to Fig. 9 for further details. $*p<0.05$; $**p<0.01$; $***p<0.001$. Reprinted from J.W. Phillis, F.G. Diaz, M.H. O'Regan, J.G. Pilitsis, Effects of immunosuppressants, calcineurin inhibition, and blockade of endoplasmic reticulum calcium channels on free fatty acid efflux from the ischemic/reperfused rat cerebral cortex, Brain Res. 957 (2002) 12–24, with permission from Elsevier.

a comparable function, as phospholipase activation can occur very rapidly following the onset of cerebral ischemia [318,319], and FFAs readily diffuse across the plasma membrane. Free fatty acid efflux from the rat cerebral cortex was therefore used to monitor phospholipase activation during ischemia/reperfusion. Preliminary evidence for an involvement of phospholipases in the ischemia-evoked release of glutamate and aspartate was forthcoming in experiments demonstrating that the non-selective PLA_2 inhibitors mepacrine and 4-bromophenacyl bromide significantly depressed excitotoxic amino acid release from the ischemic rat cerebral cortex, whereas topical applications of PLA_2 and PLC to the non-ischemic cortex enhanced the extracellular levels of glutamate and aspartate [210]. A subsequent study revealed that selective inhibition of $sPLA_2$, $cPLA_2$, $iPLA_2$, and PLC significantly depressed ischemia-evoked excitotoxic amino acid release [230]. Cerebroprotective actions of the PLA_2 inhibitor, mepacrine, in gerbil forebrain ischemia, and rat middle cerebral artery occlusion, models were subsequently demonstrated [65,229].

Further evidence of a mitochondrial contribution to ischemia/reperfusion injury was sought after by evaluating the effects of two known inhibitors of the mitochondrial MPT pore, the calcineurin inhibitors cyclosporine A (CSA) and trifluoperazine (TFP), on the activation of phospholipases and generation of free fatty acids. Both compounds very effectively suppressed FFA efflux from the rat cerebral cortex during ischemia/reperfusion [239] (Figs. 5 and 6). FK506, which does not only directly affect the MPT but is also a calcineurin inhibitor, also suppressed the I/R-evoked efflux (Fig. 5), but less effectively than CSA and TFP. An interesting difference was that the effect of FK506 tended to fade during reperfusion in contrast to the sustained inhibition of FFA efflux observed during CSA and TFP application. This difference was especially pronounced for arachidonic, docosahexaenoic and oleic acids. Gossypol, a structurally unrelated inhibitor of calcineurin was also effective in depressing I/R-evoked FFA release, whereas rapamycin, a derivative of FK506, which does not inhibit calcineurin after binding to FKBP [184], and is not cerebroprotective [24], did not

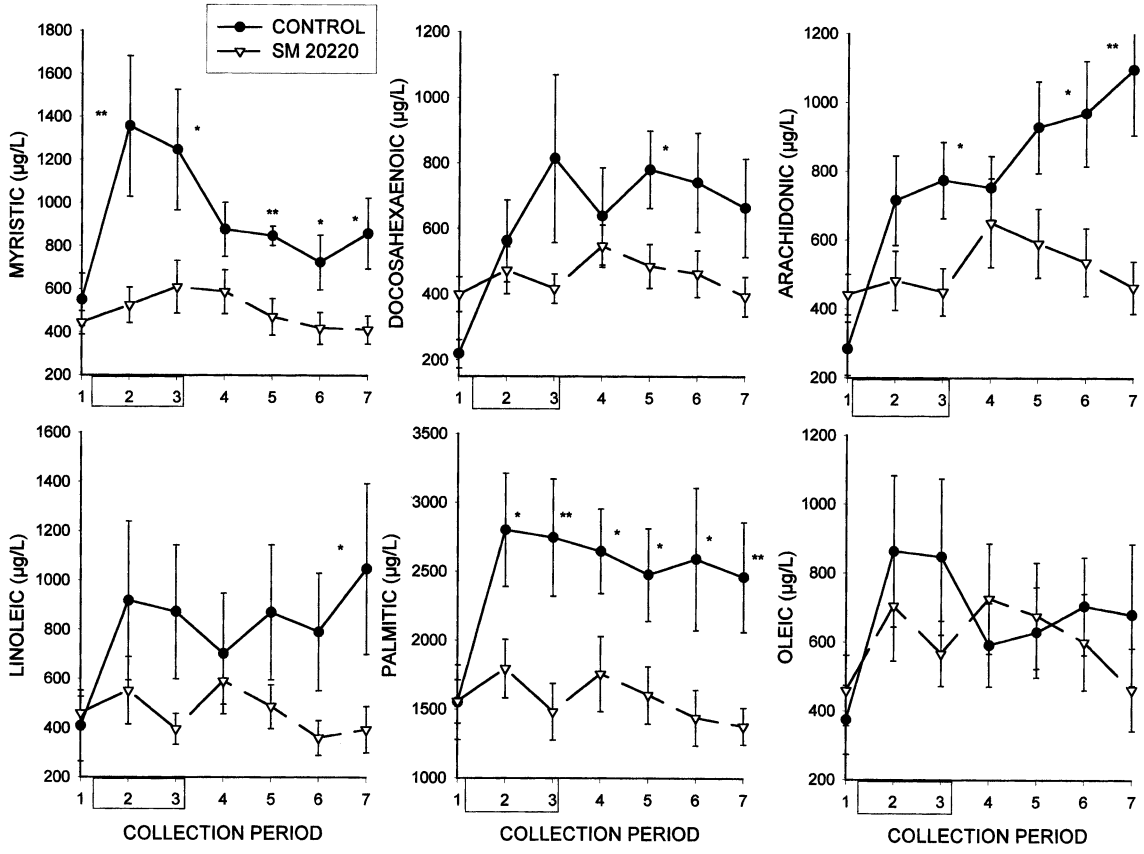

Fig. 7. Effects of the Na^+/H^+ exchange inhibitor SM 20220 on ischemia-evoked release of FFAs into rat cerebral cortical superfusates. The line plots show the time course of changes in superfusate concentrations prior to (collections 1, 2), during (collections 3, 4), and following reperfusion. SM 20220 (20 μM, open triangles, $n = 13$ cortices) was added to the aCSF 15 min prior to the start of collection 2 and replaced twice during this period. SM 20220 was thereafter present in all subsequent collections. Statistically significant differences between control ischemic (closed circles, $n = 11$) and SM 20220-treated animals were evaluated by the Student's t test. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Reprinted from J.G. Pilitis, F.G. Diaz, M.H. O'Regan, J.W. Phillis, Inhibition of Na^+/H^+ exchange attenuates free fatty acid efflux in rat cerebral cortex during ischemia/reperfusion injury, Brain Res. 913 (2001) 156–158, with permission from Elsevier.

suppress I/R-evoked FFA release [239], suggesting that mitochondrial calcineurin inhibition contributed to the actions of CSA, TFP and FK509.

10.1. Intracellular acidosis and phospholipase activity

Bond et al. [25] and Kim et al. [129] used the term pH paradox to account for the protective action of intracellular acidosis on cardiac myocytes exposed to simulated ischemia, proposing instead that the injury actually occurs during the return to normal pH. Delays in the recovery of intracellular pH with inhibitors of Na^+/H^+ exchangers, dimethylamiloride or HOE 694, slowed the return to normal pH during reperfusion with protection of the myocytes. Acceleration of pH_i recovery with monensin, a Na^+/H^+ ionophore, accelerated the increase in intracellular pH and caused more rapid cell death. Loss of cell viability and PLA_2 activity (measured by arachidonic acid release) in rat hepatocytes increased in parallel during chemical hypoxia in a pH 7.4 medium [102]. Acidosis (pH 6.5) or phospholipase inhibition with dibucaine and quinacrine delayed loss of cell viability and the release of arachidonic acid to a comparable extent. Staining of hypoxic rat hepatocytes with antibodies that recognize a pH-dependent group II phospholipase demonstrated a substantial increase in PLA_2 protein within 30 min of the initiation of hypoxic injury [331]. Treatment of hepatocytes with group II PLA_2-specific antisense DNA oligonucleotides abolished the hypoxia-evoked accumulation of PLA_2 protein, reduced arachidonic acid release, and significantly delayed cell death as indicated by propidium iodide uptake [331]. All of the above findings suggest that phospholipases play an important role in hypoxia/ischemia-evoked injury.

The pronounced increase in FFA levels in rat cerebral cortical superfusates at the onset of reperfusion following either 20- or 30-min periods of cerebral ischemia [237,242] suggested that phospholipase activity, which was largely suppressed during ischemia, developed rapidly with the onset of reperfusion and pH normalization. This concept has been tested by slowing the recovery of pH with two inhibitors of the Na^+/H^+ exchanger, EIPA and SM 20220 (Fig. 7). Both compounds significantly reduced FFA formation and release [237,242]. EIPA has previously been observed to protect gerbil CA1 hippocampal pyramidal

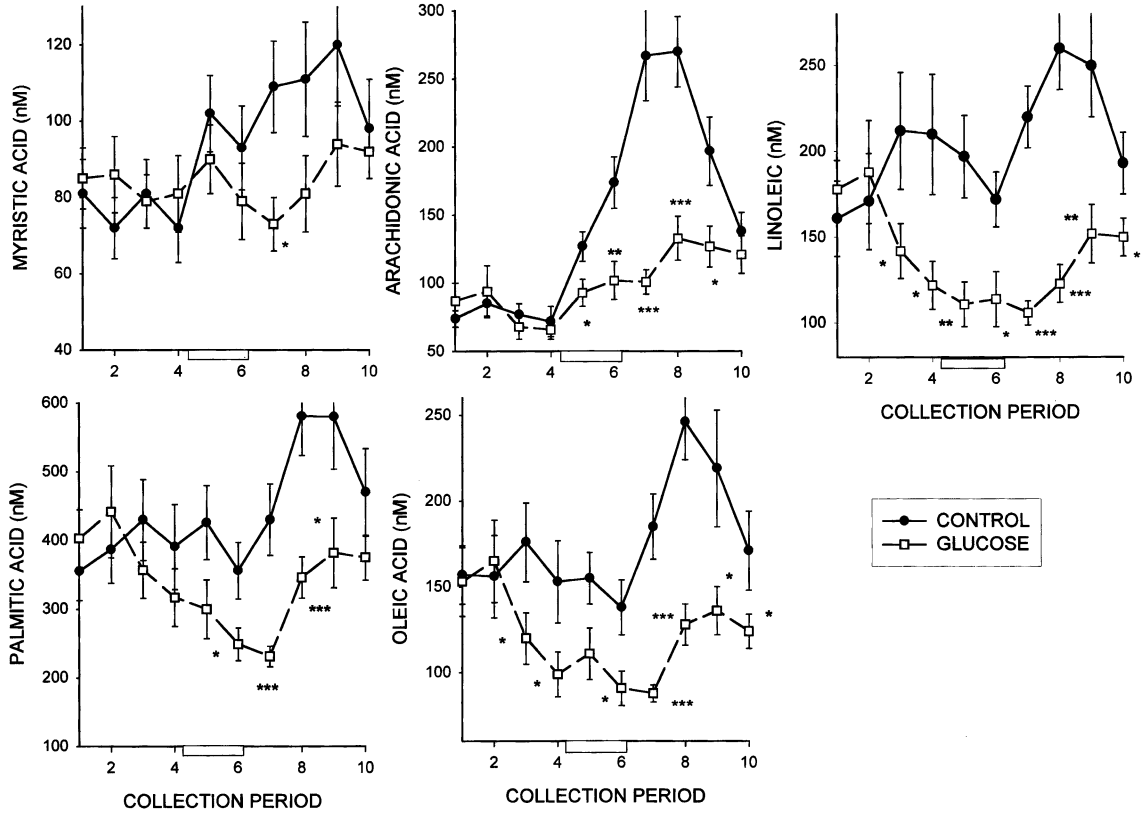

Fig. 8. Effects of hyperglycemia (pre-ischemic plasma glucose 603 ± 144 mg/dl) on ischemia/reperfusion evoked release of FFA into rat cerebral cortical superfusates. Line plots show time course of changes in superfusate concentrations of FFAs before, during, and after a 20-min period of four-vessel occlusion (4 VO) cerebral ischemia (collections 5 and 6; open box). D-Glucose (3.4 g/kg) was administered intravenously after collection of the initial two basal samples, and 10 min later, collection of sample 3 was initiated. The data (mean ± S.E.M.) are presented in comparison with those from saline injected, normoglycemic (73.5 ± 4.0 mg/dl) ischemic amounts. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Reprinted from J.W. Phillis, D. Song, M.H. O'Regan, Effect of hyperglycemia on extracellular levels of amino acids and free fatty acids in ischemic/reperfused rat cerebral cortex, Brain Res. 837 (1999) 177–183, with permission from Elsevier.

neurons from ischemic injury [234] and similar effects of SM 20220 have been reported in the gerbil hippocampal model [140] and in rat middle cerebral artery occlusion models [138,139].

10.2. Hyperglycemia: effect on cerebral cortical phospholipase activity

It has been evident for over two decades that pre-, but not post-, ischemic hyperglycemia can aggravate brain damage due to transient global or forebrain ischemia, as demonstrated by more extensive and rapidly developing neuronal necrosis and pannecrotic lesions involving glial cells [90,195,253,292], with failure of ATP recovery during reperfusion [122]. The mechanisms responsible for this effect have, however, remained unclear. A rise in lactate production with concomitant tissue acidosis have been proposed as an explanation for the hyperglycemia-evoked increase in brain damage [298].

A possible explanation for the actions of pre-ischemic hyperglycemia became apparent with the observation, cited above, that phospholipase activation, with free fatty acid formation, is depressed during the ischemic episode by the lowered pH, with recovery during reperfusion. The effects of hyperglycemia on ischemia-evoked free fatty acid efflux were studied in a four-vessel occlusion rat ischemia/reperfusion model [235]. Hyperglycemia (plasma glucose, 603 ± 144 mg/100 ml) resulted in significant reductions in arachidonic, linoleic, oleic, and palmitic acid levels in cerebral cortical superfusates in comparison with those observed in normoglycemic (73.5 ± 4.0 mg/100 ml) rats, and their levels continued to be significantly depressed during reperfusion, failing to recover to the levels recorded in the normoglycemic controls during the 40 min of reperfusion (Fig. 8). Conversely, in a separate study, hypoglycemia enhanced L-glutamate-evoked release of arachidonic acid from striatal neurons [343]. These effects of hyperglycemia were likely a consequence of an augmented reduction in pH, with inhibition of phospholipase A_2 activity, and a reduction in free fatty acid formation. The failure of free fatty acid levels to increase to those observed in the normoglycemic stroked rats implies that pH recovery was

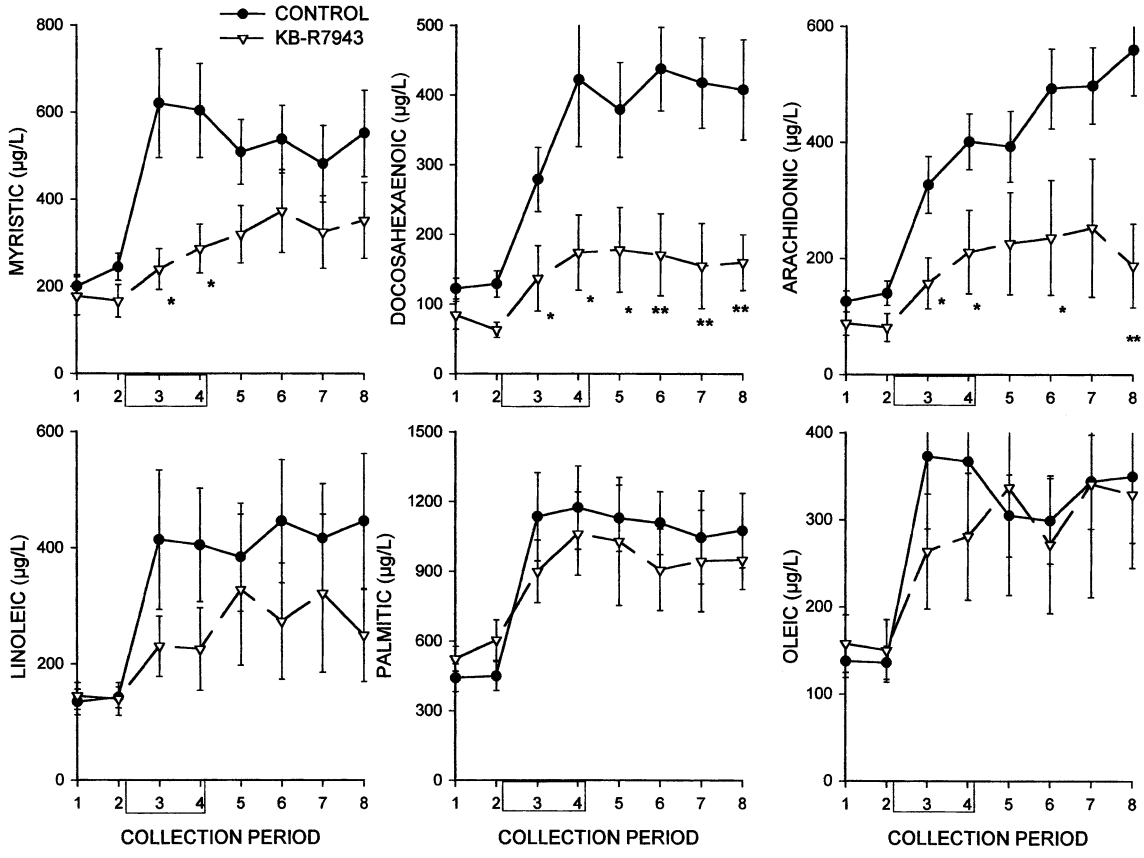

Fig. 9. Effects of KB-R7943 (50 µM), a Na^+/Ca^{2+} exchange inhibitor, on the release of free fatty acids into rat cerebral cortical superfusates during and following a 20-min, four-vessel occlusion ischemia. The line plots show the time course of changes in FFA levels before, during (open box) and after ischemia. Collection 1 represents the mean basal levels prior to exposure to KB-R7943, which was then added to the aCSF 15 min prior to the start of the superfusate 2 collection period and was present in all subsequent collections. The KB-R7943 data (12 cortices) was compared with those from eight control ischemic animals (15 cortices) (closed circles). $*p < 0.05$; $**p < 0.01$. Reported from J.G. Pilitsis, F.G. Diaz, M.H. O'Regan, J.W. Phillis, Inhibition of Na^+/Ca^{2+} exchange by KB-R7943, a novel selective antagonist, attenuates phosphoethanolamine and free fatty acid efflux in rat cerebral cortex during ischemia/reperfusion injury, Brain Res. 916 (2001) 192–198, with permission from Elsevier.

delayed in the hyperglycemic rats, possibly as a consequence of non-oxidative glycolytic energy production to fuel plasma membrane ion transport pumps (Na^+, H^+, and Ca^{2+}) [35,155,176,219,254,264,294]. A preference for ATP produced by glycolysis, rather than by mitochondrial oxidative phosphorylation, suggests that there may be advantages to such locally generated energy supplies for ion pumps, even though this may be associated with continuing H^+ production.

10.3. Plasma membrane and mitochondrial Na^+/Ca^{2+} exchange

During ischemia, ATP depletion leads to a loss of ion homeostasis, with increases in intracellular Na^+ and intracellular acidosis. Elevated levels of intracellular Na^+ can result in calcium entry via a reversed action of an NCX, which extrudes Na^+ ions in exchange for Ca^{2+} [60,286]. The calcium overload can lead to a variety of deleterious effects including: mitochondrial accumulation of Ca^{2+}, activation of phospholipases and lipolysis, activation of proteases, and free radical production. Inhibition of the NCX with KB-R7943 was neuroprotective in an in vitro model of ischemia/reperfusion injury [282]. When applied topically onto the rat cerebral cortex, KB-R7943 reduced the ischemia/reperfusion-evoked release of FFAs (Fig. 9) suggesting that it had decreased Ca^{2+} entry via the NCX with a reduction in activation of calcium-dependent phospholipases [241] (Fig. 9). KB-R7943 also reduced the ischemia/reperfusion-elicited release of phosphoethanolamine into cortical perfusates. The latter's presence is considered to be indicative of the degradation of the plasma membrane phospholipids [98]. Another NCX, SEA 0400 attenuated reperfusion injury in a rat MCAO cerebral ischemia model [170].

An increased intracellular Ca^{2+} concentration is a well-recognized contributor to neuronal death following ischemic injury [136]. In hippocampal slices, 50% of this increase in $[Ca^{2+}]_i$ has been attributed to calcium efflux via the mNCX [359]. The mNCX is activated as a result of

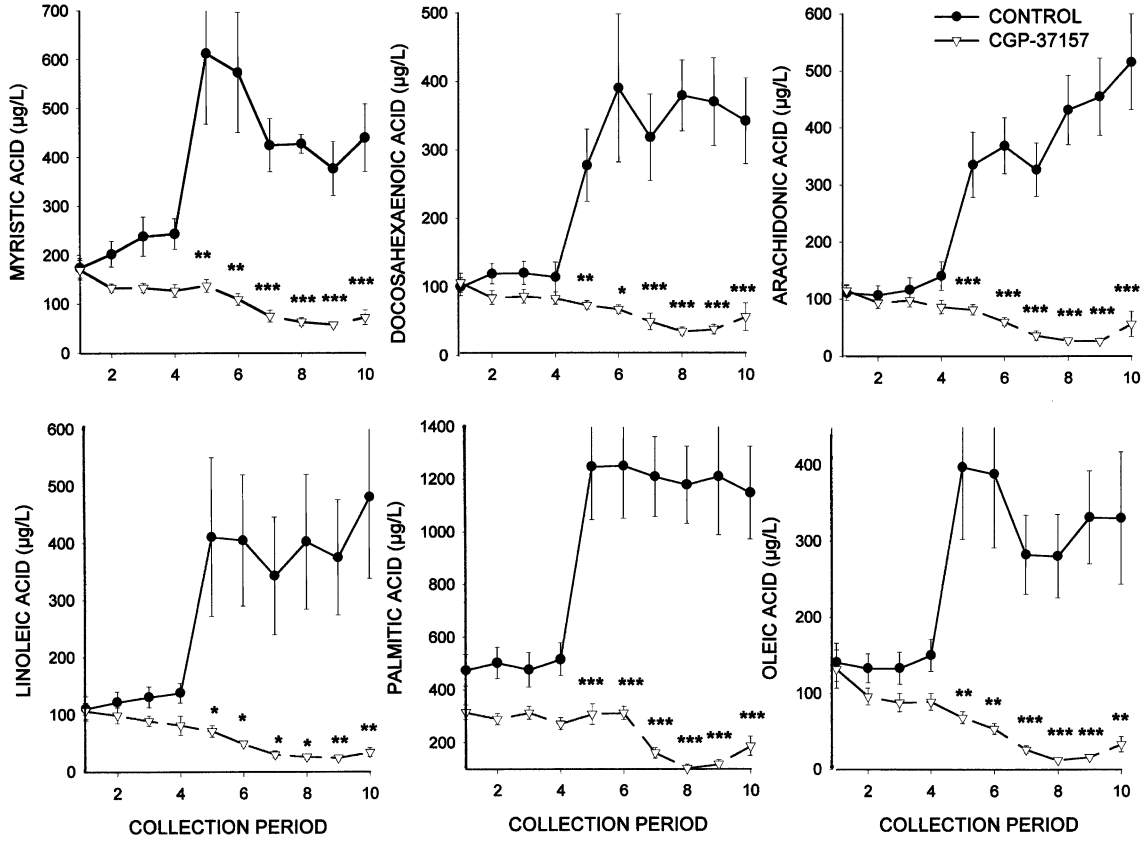

Fig. 10. Effects of CGP-37157 (50 µM), a mitochondrial Na^+/Ca^{2+} exchange inhibitor, on ischemia (4 VO) reperfusion-evoked release of FFAs from the rat cerebral cortex. The line plots show the time course of changes in superfusate concentrations of FFAs during basal collection periods (1 and 2) and following exposure to CGP-37157 (3 and 4; line plot with open triangles) or continued exposure to aCSF (filled circles). Collections 5 and 6 represent ischemia, while collections 7–10 represent reperfusion periods. CGP-37157 was added to this aCSF 15 min prior to collection 3 and was present during all subsequent collection periods. The control group (closed circles) was superfused with aCSF during all collection periods. Data represent mean ± S.E.M. *$p<0.05$; **$p<0.01$; ***$p<0.001$. Reprinted from J.G. Pilitsis, F.G. Diaz, M.H. O'Regan, J.W. Phillis, Inhibition of mitochondrial Na^+/Ca^{2+} exchange by 7-chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzothazepin-2(3*H*)-one attenuates free fatty acid efflux in rat cerebral cortex during ischemia/reperfusion injury, Neurosci. Lett. 321 (2002) 1–4, with permission from Elsevier.

increased levels of intracellular sodium, which causes an influx of Na^+ into mitochondria and mitochondrial Ca^{2+} release into the cytosol [49]. The exchanger is selectively inhibited by CGP-37157 [50]. CGP-37157 does not affect L-type voltage-dependent Ca^{2+} channels and does not inhibit Na^+ entry through the plasma membrane [359]. Inhibition of mNCX with CGP-37157 results in a rapid decline in elevated Ca^{2+} following glutamate stimulation of cultured neurons [339]. After removal of CGP-37157, this decrease in $[Ca^{2+}]_i$ is followed by an increase in $[Ca^{2+}]_i$, presumably as a result of the release of sequestered Ca^{2+} from the mitochondria. Following increases in $[Ca^{2+}]_i$, $cPLA_2$ is activated and free fatty acid accumulation occurs as production exceeds utilization [77,265]. After sustained increases in $[Ca^{2+}]_i$, the inducible secreted PLA_2 ($sPLA_2$) leads to the creation of a positive feedback loop, which amplifies the severity of cerebral ischemic damage [136]. Potentially, this could involve a cross-talk between $cPLA_2$ and $sPLA_2$, by which $sPLA_2$ regulates $cPLA_2$ activity and arachidonic acid release [99]. Application of CGP-37157 significantly inhibited FFA release from the rat cerebral cortex during ischemia/reperfusion [246] (Fig. 10), suggesting that inhibition of mitochondrial Na^+/Ca^{2+} exchange had prevented the activation of Ca^{2+}-dependent phospholipases during ischemia/reperfusion. Such a conclusion would firmly implicate the mitochondria as key contributors to phospholipase activation and the ischemia/reperfusion cycle of injury.

11. Contribution of the endoplasmic reticulum to phospholipase activation

Paschen and Doutheil [218] have proposed that disturbances of the functioning of the endoplasmic reticulum play an important role in neuronal cell injury. Their hypothesis is supported by observations that dantrolene, which blocks Ca^{2+} release via ryanodine-sensitive endoplasmic reticulum channels, has a cerebroprotective action [79,336]. Evidence for a role of the IP_3R channel in cerebral injury has been forthcoming in experiments with xestospongin C, which blocks Ca^{2+} release via this channel. Xestospongin protected cultured cortical neurons from glucose deprivation- and cyanide-induced injury [172].

Information was also sought on the role of the endoplasmic reticulum in I/R-evoked cerebral ischemia. Thapsigargin, which blocks the Ca^{2+}-ATPase uptake pump of the endoplasmic reticulum (SERCA) [315], allowing its stores of Ca^{2+} to dissipate by leakage through the RyR and IP_3P channels elicited significant elevations in the efflux of myristic, arachidonic, and linoleic acids in the absence of ischemia [239]. Dantrolene, which blocks the RyR channel of the endoplasmic reticulum, significantly inhibited the ischemia/reperfused-evoked release of arachidonic, docosahexaenoic, linoleic, and oleic acids [239]. Xestospongin C, an inhibitor of the IP_3P channel, failed to reduce FFA efflux during ischemia/reperfusion [239]. Collectively, the data favored an involvement of both endoplasmic reticulum and mitochondrial Ca^{2+} stores in the chain of events leading to phospholipase activation and free fatty acid release. The failure of FK506 to sustain a block of FFA formation, in comparison with CSA and TFP, appeared to indicate some difference in the activities of these compounds. It is possible that the primary action of FK506 is inhibition of Ca^{2+} release from the endoplasmic reticulum, whereas CSA and TFP likely affect both the endoplasmic reticulum and mitochondrial Ca^{2+} release, as well as inhibiting the MPT. It may be noteworthy that the mitochondrial Na^+/Ca^{2+} mNCX inhibitor CGP-37157 elicited a lasting inhibition of FFA formation and release in comparison with the fading action of FK506.

Another study examined the effect of selective inhibitors of various phospholipases on FFA efflux from the rat cerebral cortex. Pronounced inhibition of FFA efflux during ischemia (20 min)/reperfusion (40 min) was observed following the application of non-selective 4-bromophenylacyl bromide and the $cPLA_2$ inhibitor $AACOCF_3$. The PLC inhibitor U73122 significantly reduced the efflux of myristic acid and tended to attenuate the rate of formation of the other FFAs. The $iPLA_2$ inhibitor bromoenol lactone significantly depressed the efflux of myristic and linoleic acids and LY311727, an $sPLA_2$ inhibitor, was without effect [245]. These results suggest that the primary phospholipase activated during cerebral ischemia/reperfusion is $cPLA_2$, with lesser contributions from $iPLA_2$ and PLC, and apparently only a small input by $sPLA_2$.

12. Conclusions

The primary focus of this review has been to evaluate the potential contribution of phospholipases to cell death following cerebral ischemia and trauma, and in certain neurodegenerative diseases. Neuronal, and to a lesser extent, glial, death may exhibit features of necrosis, apoptosis, or of an intermediate state that displays characteristics of both forms. Necrotic cell death is associated with swelling, membrane disruption, the release of cellular contents, and inflammation, whereas apoptosis is characterized by cell shrinkage with distinctive nuclear fragmentation. It has recently been suggested that necrosis and apoptosis, rather than representing the consequences of two fundamentally different processes, are, in fact, initiated in an identical fashion, but with different outcomes [148,271]. Studies have indicated that apoptosis may be the earliest form of death in the ischemic myocardium with a delayed appearance of necrosis in some myocytes [92,325]. Tatsumi et al. [313] have proposed that although the apoptotic, non-inflammatory, elimination of cardiomyocytes would be biologically the preferred mechanism for cell elimination, its requirement for energy expenditure may trigger the onset of necrotic cell death as ATP is depleted during

severe ischemia. Consequently, the severity and duration of ischemia may dictate the degree to which dying cells demonstrate the characteristics of either apoptosis, necrosis or both [61,148,199,313]. In that mitochondria are the primary source of ATP, it might be supposed that it is the loss of the mitochondrial generation of this nucleotide that precipitates necrosis. This does not appear to be the case in some cell types as glycolytic ATP generation after reperfusion in the presence of adequate amounts of substrate can prevent necrotic killing of cardiomyocytes and hepatocytes [129,313]. It is uncertain if neurons also possess this ability, and in any case, it would not occur during ischemia in the absence of substrate.

Kim et al. [129] have argued that the cyclosporin A-sensitive mitochondrial permeability transition (MPT) is the key event leading to necrotic cell death of hepatocytes after ischemia/reperfusion. As a consequence of the MPT, mitochondria uncouple, depolarize, and swell. Outer membrane rupture after swelling allows the release of cytochrome *c* and other proapoptotic factors into the cytosol from the intermembrane space. An adequate supply of substrates for glycolytic ATP formation during reperfusion switches cell killing from MPT-dependent necrotic cell death to MPT and caspase-dependent apoptosis. According to this hypothesis, MPT would be a common mechanism responsible for both necrosis and apoptosis following ischemia/reperfusion or other forms of cerebral injury, with an adequate supply of ATP being the deciding factor.

The evidence presented in this review suggests that $cPLA_2$ plays a major role in ischemia/trauma-elicited brain injury. Cytoplasmic Ca^{2+} levels initially rise as a consequence of the opening of NMDA receptor-activated plasma membrane channels. This G-protein-coupled receptor also stimulates PLC to generate IP_3, which triggers further release of Ca^{2+} from the endopolasmic reticulum, a process known as calcium-induced calcium release, which can be blocked by the PLC inhibitor neomycin sulfate [334]. Intracellular Ca^{2+} concentrations rise, stimulating $cPLA_2$ with the release of arachidonic acid. sPLAs and $iPLA_2$ may play a more minor part in the arachidonic acid release. A role for the recently identified mitochondrial $iPLA_2$ remains to be established. Given the close proximity of mitochondria, endoplasmic reticulum and the plasma membrane, mitochondrial Ca^{2+} concentration may exceed tolerable levels, triggering a rapid loss of mitochondrial membrane potential, opening of the mitochondrial permeability pore, and the release of various apoptosis-inducing factors. It appears likely that elevated levels of arachidonic acid contribute to this event.

It is therefore significant that low concentrations of arachidonic acid cause swelling and the MPT in rat liver mitochondria, which is accompanied by cytochrome *c* release and cell death. All of these effects of arachidonic acid are prevented by cyclosporin A [283]. Furthermore, as MPT induction by tumor necrosis factor α (TNFα) is inhibited by the PLA_2 inhibitor aristolochic acid, which does not inhibit the permeability transition pore per se, it is likely that arachidonic acid released by PLA_2 participates in early apoptotic signaling to mitochondria in the TNFα pathway [283]. These findings have confirmed earlier reports that arachidonic acid is released by $cPLA_2$ activity in cells undergoing apoptosis induced by TNFα [103,107,316,345].

At this point in time, the mechanisms by which arachidonic acid induces the MPT, apoptosis, and cell death remain unresolved and include membrane depolarization and binding to the ADP/ATP carrier [281], inhibition of mitochondrial respiration activity [309], and effects on mitochondrial permeability, transport, and energy coupling processes [30,344]. Questions also remain as to whether arachidonic acid activates a distinct apoptotic pathway or is further metabolized by lipoxygenases and cyclooxygenases to injury-promoting eicosanoids and toxic oxygen free radicals. It has been suggested that a lipoxygenase product may function as the active second messenger in TNFα-induced apoptosis [40] or alternatively that oxygen free radicals, produced by lipoxygenation reactions, could be responsible for the apoptosis based on findings that lipoxygenase inhibitors can inhibit TNFα-induced apoptosis [40,166,205,228].

Evidence for an involvement of lipoxygenases in cerebral ischemic injury has been forthcoming in experiments on neurons in rat hippocampal slice cultures exposed to glucose/oxygen deprivation [9] but not in in vivo studies on forebrain ischemia in the gerbil [191].

Neuroprotection following deletion of the cyclooxygenase 2 gene in mice [113] and administration of cyclooxygenase inhibitors in rats and gerbils in cerebral ischemia has also been reported [33,36,37,46,202,231,278]. Attempts to use cyclooxygenase inhibitors to reduce the damaging effects of traumatic brain injury have yielded less promising results [52,134]. There is also evidence that a cyclooxygenase inhibitor (NS-398) attenuates NMDA-evoked cell death in primary cortical cell cultures [105]. Given the above findings, it appears likely that arachidonic acid metabolism plays a significant role in the apoptotic events initiated by this FFA. As stated above, whether or not arachidonic acid itself is the primary instigator of the injury is currently uncertain. It will be necessary to establish that the agents used to inhibit cyclooxygenases, lipoxygenases, and epoxygenases are selective for these enzymes and do not affect the activity of phospholipases (cf. Indomethacin [80]) and conversely that the various available, selective phospholipase inhibitors do not inhibit cyclooxygenases, lipoxygenases, and epoxygenases. Development and ready availability of knockout mice with single and multiple gene deletions of either phospholipases or arachidonic acid-oxygenases would contribute to a greater understanding of the contributions of these two groups of enzymes to ischemia/trauma-elicited apoptosis and cell death, and possibly to the part that they may play in various neurodegenerative disorders.

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