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Activities of Enzymes Metabolizing Phospholipids in Rat Cerebral Ischemia

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ABSTRACT

Ischemic rat brains were prepared by decapitation followed by incubation in an artificial cerebrospinal fluid at various times at 37°C, and the levels of phospholipids, free fatty acids, and enzymes involved in their metabolism were studied.

Activities of phospholipase A, phospholipase C, and di- and monoglyceride lipase, assayed with optimal concentrations of Ca^{2+} and lysophospholipase, did not significantly change by 60 min of ischemia, whereas acylation enzymes of lysophospholipid decreased in activity to an extent of 70% of control at 15 min after the ischemic treatment. The maximal activities were found at $8 \times 10^{-3}M$, $1 \times 10^{-3}M$, and $2 \times 10^{-2}M$ Ca^{2+} for phospholipase A, phospholipase C, and di- and monoglyceride lipases, respectively in microsomal fractions of both control and ischemic brain.

Furthermore, the sensitivity of microsomal enzymes to endogenous Ca^{2+} was estimated in control and ischemic brain. The sensitivity of phospholipase C was found to be increased after 1 min of ischemic treatment, but those of phospholipase A and di- and monoglyceride lipase were not increased.

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Index Entries: Free fatty acid; phospholipid; phospholipase A; phospholipase C; lysophospholipase; monoglyceride lipase; diglyceride lipase; acylCoA synthetase; acylCoA:lysophospholipid acyltransferase; brain ischemia; calcium.

Abbreviations: Gro*P*Cho, *sn*-glycero-3-phosphocholine; Gro*P*Ins, *sn*-glycero-3-phosphoinositol; ChoGpl, choline glycerophospholipids; EtnGpl, ethanolamine glycerophospholipids; SerGpl, serine glycerophospholipids; InsGpl, inositol glycerophospholipids; PtdCho, 1,2-diacyl-Gro*P*Cho; PtdEtn, 1,2-diacyl-Gro*P*Etn; PtdIns, 1,2-diacyl-Gro*P*Ins; PlsEtn, 1-alk-1′-enyl-2-acyl-Gro*P*Etn.

INTRODUCTION

Phospholipids are the major constituents of the membranous structure of cells. They not only constitute the backbone of the biomembrane, but also provide the membrane with a suitable environment, fluidity, and permeability. The metabolism of phospholipids is altered in ischemia. A significant decrease in various phospholipids has been reported to occur in ischemic brain (DeMedio et al., 1980; Yoshida et al., 1980; Abe et al., 1987). The earliest biochemical event that occurs during ischemia is the release of free fatty acids from various phospholipids (Bazan, 1970; Yoshida et al., 1980; Rehncrona et al., 1982) and accumulation of lysophospholipids (Sun and Foudin, 1984) and diacylglycerols (Abe et al., 1987). A stimulation of phospholipases A_1 and A_2 activities has been reported by Edgar et al. in acetone-dried powder of gerbil ischemic brain (Edgar et al., 1982). Recently, Abe et al., (1987) speculated that the action of phospholipase C and diglyceride lipase on polyphosphoinositides may be responsible for the release of free fatty acids in the early ischemic stage. Another pathway liberates fatty acids by utilizing a lysophospholipase, preceded by phospholipase A_1 (van den Bosch, 1982). Plasmalogenase, coupled with lysophospholipase, may also liberate free fatty acids from plasmalogens (Horrocks et al., 1984). Although the relative contribution of these pathways to the release of free fatty acids during ischemia is unknown, the importance of direct action of phospholipase A_2 and diglyceride lipase, preceded by phospholipase C, has been suggested (Abe et al., 1987).

In the present study, we have examined the activities of enzymes relating to the metabolism of phospholipids using the ischemic brain of the rat, prepared by decapitation followed by incubation in an artificial cerebrospinal fluid, with respect to the action of intracellular Ca^{2+} ions.

EXPERIMENTAL PROCEDURES

Materials

Male Wistar LWH rats (Toyama Laboratory Animals Co., Toyama, Japan), weighing about 250 g, were used for experiments. The following

were purchased from Funai Pharmaceutical Co., Tokyo, Japan: 1-stearoyl-2-arachidonyl-Gro*P*Cho, 1,2-dipalmitoyl-Gro*P*Cho, 1-palmitoyl-2-lysoGro*P*Cho, 1,2-diacyl-Gro*P*Ins, and arachidonic acid. The radioactive reagents were products of Amersham International Ltd., Amersham, UK. Phospholipase C from *Clostridium perfringens* was purchased from Sigma Chemical Co., St. Louis, MO. Silica gel plates were purchased from Schleicher and Schuell, Dassel, West Germany. Other chemicals and solvents in this study were of reagent grade.

Preparation of Ischemic Brain

The animals were killed by decapitation. The heads were immersed in the artificial cerebrospinal fluid prepared by the method of Ban (Ban et al., 1978) and incubated in the fluid at 37°C for 1, 5, 15, 30, or 60 min. The brains were removed from the heads, transferred into liquid nitrogen, and stored at −80°C until use. A brain specimen obtained without incubation at 37°C was used as the control brain.

Analysis of Phospholipids

Brain tissue was weighed and homogenized with 3 vol of 0.25*M* sucrose. Total lipids were extracted from the homogenate by the method of Bligh and Dyer (Bligh and Dyer, 1959). The lipid extracts were subjected to quantitative thin layer chromatography (TLC). TLC plates were developed with the solvent system composed of chloroform/methanol/acetic acid/water (25/15/4/2, by vol) and lipids were located with iodine vapor. The areas corresponding to EtnGpl, ChoGpl, SerGpl, and InsGpl fractions were scraped off, and the phospholipid contents were determined by the method of Rouser et al. (1966). The amount of total phospholipids was calculated by adding the amounts of the individual lipids. SerGpl and InsGpl were separated by rechromatography with a solvent composed of chloroform/methanol/acetic acid/water (81/10/45/5, by vol).

Analysis of Free Fatty Acids

Free fatty acids were obtained from the lipid extracts by TLC with the solvent system composed of *n*-hexane/diethyl ether/acetic acid (80/20/1, by vol). Conversion into methyl esters, after extraction and evaporation, was via diazomethane derivatization. The methylated fatty acids were analyzed by gas-liquid chromatography on a 15% DEGS column (0.3 × 200 cm) at 200°C with nitrogen as carrier gas. Fatty acids were located by comparison of their retention times with those of authentic fatty acids. The amounts of the individual free fatty acids were determined by comparing the areas recorded with specific amounts of nonadecanoic acid as an internal standard.

Subcellular Fraction of Brain

Subcellular fractionation was carried out essentially by the method of Shum et al. (1979). The brains were homogenized with 9 vol of ice-cold 0.25*M* sucrose. The homogenate was centrifuged at 750 × *g* for 10 min. The pellet containing nuclei was discarded. Mitochondrial fraction was obtained by centrifugation of the supernatant at 12,000 × *g* for 10 min. The postmitochondrial supernatant was again centrifuged at 106,000 × *g* for 60 min to obtain the microsomal fraction. Protein was determined by the method of Lowry et al. (1951).

Preparation of 1,2-di[1-^{14}C]palmitoyl-sn-glycerol

The substrate for di- and monoglyceride lipase activities was prepared from 1,2-di[1-^{14}C]palmitoyl-Gro*P*Cho (2 Ci/mol) by the reaction of phospholipase C from *Clostridium perfringens*. The reaction mixture consisted of 100 m*M* Tris-HCl buffer, pH 7.0, lipid (25 μmol), phospholipase C (50 mg), 0.1% sodium deoxycholate, 100 m*M* $CaCl_2$ in a total vol of 10 mL. After incubation for 10 h at 37°C, the reaction was stopped by the addition of chloroform:methanol (2:1, by vol). Using the solvent system composed of petroleum ether/diethyl ether/acetic acid (60/40/1, by vol), 1,2-di[1-^{14}C]palmitoy-*sn*-glycerol was purified with TLC.

Enzyme Assays

For the phospholipase A assay, the mixture consisted of 20 m*M* Tris HCl buffer (pH 7.0), 0.2% Triton X-100, an appropriate concentration of $CaCl_2$ or 1 m*M* EGTA, 1 m*M* 1-stearoyl-2-[1-^{14}C]arachido nyl-Gro*P*Cho (6 Ci/mol), and enzyme preparation in a total vol of 100 μL. The tubes were incubated for 2 h at 37°C, and the reaction was stopped by the addition of 400 μL chloroform:methanol (2:1, by vol).

Lysophospholipase was assayed in a final volume of 60 μL. The reaction mixtures contained 34 m*M* Tris HCl buffer (pH 7.0) and 1.7 m*M* 1-[1-^{14}C]palmitoyl-Gro*P*Cho (2 Ci/mol) and enzyme preparation. After incubation for 15 min at 37°C, the reaction was stopped by the addition of 240 μL chloroform:methanol (2:1, by vol).

Phospholipase C activity was determined by the method of Lapetina and Michell (1973). The assay was carried out in a final volume of 125 μL. The reaction mixture contained 20 m*M* Tris HCl buffer (pH 7.0), 0.002% sodium deoxycholate, an appropriate concentration of $CaCl_2$ or 1 m*M* EGTA, 0.8 m*M* 1,2 diacylGro*P*[U-^{14}C]Ins (0.5 Ci/mol), and enzyme preparation. The tubes were incubated for 30 min at 37°C, and the reaction was stopped by the addition of 500 μL chloroform:methanol (2:1, by vol).

For the determination of the sum of di- and monoglyceride lipase activities, the reaction mixture consisted of 20 m*M* Tris HCl buffer (pH 7.0), an appropriate concentration of $CaCl_2$ or 1 m*M* EGTA, 0.5 m*M* 1,2-di[1-

^{14}C]palmitoyl-*sn*-glycerol, and enzyme preparation in a total volume of 125 μL. The tubes were incubated for 30 min at 37°C, and the reaction was stopped by the addition of 500 μL chloroform:methanol (2:1, by vol).

Acylation enzymes of lysophospholipid activity were determined by the method of Webster and Alpern (1964). The reaction mixture consisted of 50 m*M* Tris HCl buffer (pH 7.0), 1 m*M* 1-palmitoyl-Gro-*P*Cho, 0.1 m*M* CoA, 2.5 m*M* ATP, 10 m*M* $MgCl_2$, 150 m*M* NaF, 1 m*M* [1-^{14}C]arachidonic acid (2 Ci/mol), and enzyme preparation in a total of 100 μL. The tubes were incubated for 2 h at 37°C, and the reaction was stopped by the addition of 400 μL chloroform:methanol (2:1, by vol).

All the reactions were initiated by the addition of the enzyme preparation (0.3–0.8 mg protein). After terminating the reactions, the radioactive products, [1-^{14}C]palmitate and [1-^{14}C]arachidonate were separated by TLC. We used the two solvent systems, chloroform:methanol:acetic acid:water (25:15:4:2, by vol) for phospholipase A, lysophospholipase, and acylation enzymes of lysophospholipid assays and petroleum ether: diethyl ether:acetic acid (60:4:1, by vol) for di- and monoglyceride lipase assay. The distribution of radioactivity was monitored by the radio thin-layer scanner (Thin Layer Scanner II, Berthold) and the radioactivities of [1-^{14}C]palmitic acid and [1-^{14}C]arachidonic acid were measured by the liquid scintillation counter (LSC-671, Aloka). D-myo-[U-^{14}C]inositol monophosphates, such as D-myo-[U-^{14}C]inositol-1-phosphate and D-myo-[U-^{14}C]inositol 1:2-cyclic phosphate were recovered from an aqueous phase by the extraction. We also confirmed that the radioactive compound in the organic phase is only 1,2-diacyl-Gro*P*[U-^{14}C]Ins. The radioactivities of aqueous and organic phases were measured for the determination of phospholipase C activity after evaporation of both phases by azeotropic procedures and nitrogen.

Determination of the Amount of Calcium

The amount of calcium in the subcellular fraction, such as free calcium ion and binding calcium, was measured by the method of inductively coupled plasma atomic emission spectrometry (Scott et al., 1974).

Statistical Analysis

Numerical values were statistically evaluated by Dunnett's test (Dunnett, 1955) and $p < 0.05$ was defined to be a statistically significant difference.

RESULT

A comparison of phospholipid and free fatty acid content between control and ischemic rat brain at various time intervals is shown in Fig. 1.

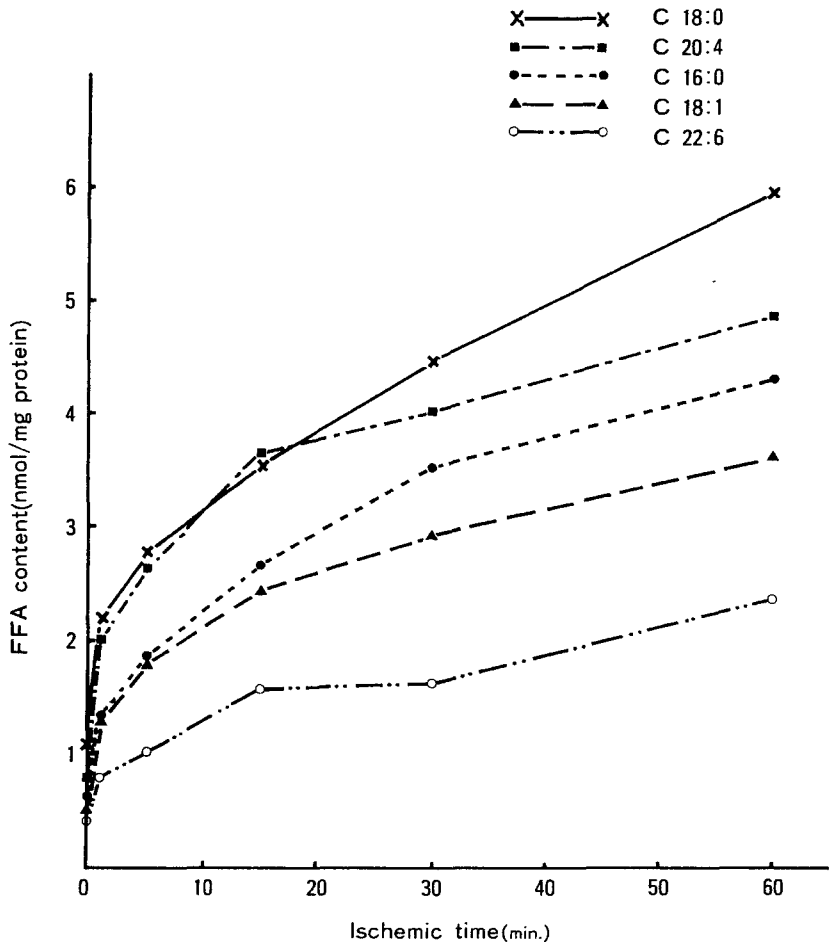

Fig. 1. Effect of different times of ischemia on the major components of the free fatty acid pool. Periods of ischemia were 0 (control), 1, 5, 15, 30, and 60 min. The mean values of five animals are presented at each time period. The amount of stearate and arachidonate showed a significant increase at 1 min of ischemia ($p < 0.05$). The amounts of all free fatty acids showed a significant increase after 5 min of ischemia ($p < 0.01$). C 16:0, palmitate; C 18:0, stearate; C 18:1, oleate; C 20:4, arachidonate; C 22:6, docosahexanoate.

The amounts of palmitic acid (C 16:0), stearic acid (C 18:0), oleic acid (C 18:1), arachidonic acid (C 20:4), and docosahexaenoic acid (C 22:6) were 0.62 ± 0.43, 1.08 ± 0.25, 0.54 ± 0.52, 0.78 ± 0.21, and 0.41 ± 0.38, respectively, on the basis of nmol/mg protein (mean ±SD) in the control brains ($n = 5$). The free fatty acids significantly increased in the brain during the ischemia caused by decapitation and followed by incubation in the artificial cerebrospinal fluid. The amounts of free fatty acids accumulated in the 60-min ischemic brain were about six to seven times higher compared to the control brains. The rates of free fatty acid accu-

mulation seemed to be more rapid during the early period of ischemia than in the late period. However, after even 60 min decapitation, the release of the fatty acid was not complete. Furthermore, the accumulation of stearic and arachidonic acids was higher than other fatty acids. Accumulation of stearic and arachidonic acids seemed to precede the increase of other free fatty acids. The amounts of stearic and arachidonic acids showed significant increases ($p < 0.05$), but others did not show statistically significant changes at 1 min of ischemia. After 5 min of ischemia, all free fatty acids showed significant increases ($p < 0.01$). Since the amount of total phospholipids was estimated to be about 600 nmol/mg protein under our experimental conditions, the amounts of free fatty acids released did not exceed 2% of the total content of acyl moiety of phospholipids in the brain. As would be expected, no significant changes in the amounts of the individual phospholipids could be detected during the entire course of ischemia tested (data not shown).

Phospholipase C in the microsomal fraction was strongly stimulated by calcium ions. The concentration of Ca^{2+} needed for half of the maximum stimulation was $4 \times 10^{-4}M$. Phospholipase C of mithochondrial and cytosolic fractions also showed similar stimulation with respect to Ca^{2+} (data not shown). Phospholipase A and di- and monoglyceride lipases are also stimulated by Ca^{2+}. However, the degree of activation was lower than phospholipase C. Maximal stimulation was observed at $8 \times 10^{-3}M$ (for phospholipase A) and $2 \times 10^{-2}M$ (for di- and monoglyceride lipase). Furthermore, prolonged ischemic treatment did not result in any additional changes in the enzymes' dependency on Ca^{2+} (data not shown). The effect of Ca^{2+} ions on activities of lysophospholipase and acylation enzymes of lysophospholipid is also shown in Fig. 2. They were not Ca^{2+} dependent.

We also determined the activities of these enzymes in microsomal fractions prepared from brains treated under ischemic conditions at various time intervals and compared them with control brains under identical conditions. The activities of the enzymes tested with optimal Ca^{2+} concentrations were found to show variations at the initial period of ischemia indicated. However, as a consequence of evaluation by the method of Dunnett (1955), we could not give the apparent variations of the enzyme activities. We have also determined the activity of acylation enzymes of lysophospholipid, enzymes responsible for the resynthesis of membrane phospholipids. This activity of the enzymes is decreased (30%) after 15 min ischemia compared to control. The enzyme preparation from ischemic brain contains a higher amount of free fatty acids than control brain. However, the dilution effect of RI-labeled arachidonic acid of 60 min ischemic brain was negligible (Table 1).

In Fig. 2, we examined the sensitivity of microsomal enzymes for exogenous Ca^{2+}. Furthermore, the sensitivity of enzymes to endogenous Ca^{2+} was also estimated before and after ischemia. The enzymic activity determined in the presence of EGTA was defined as the basal activity.

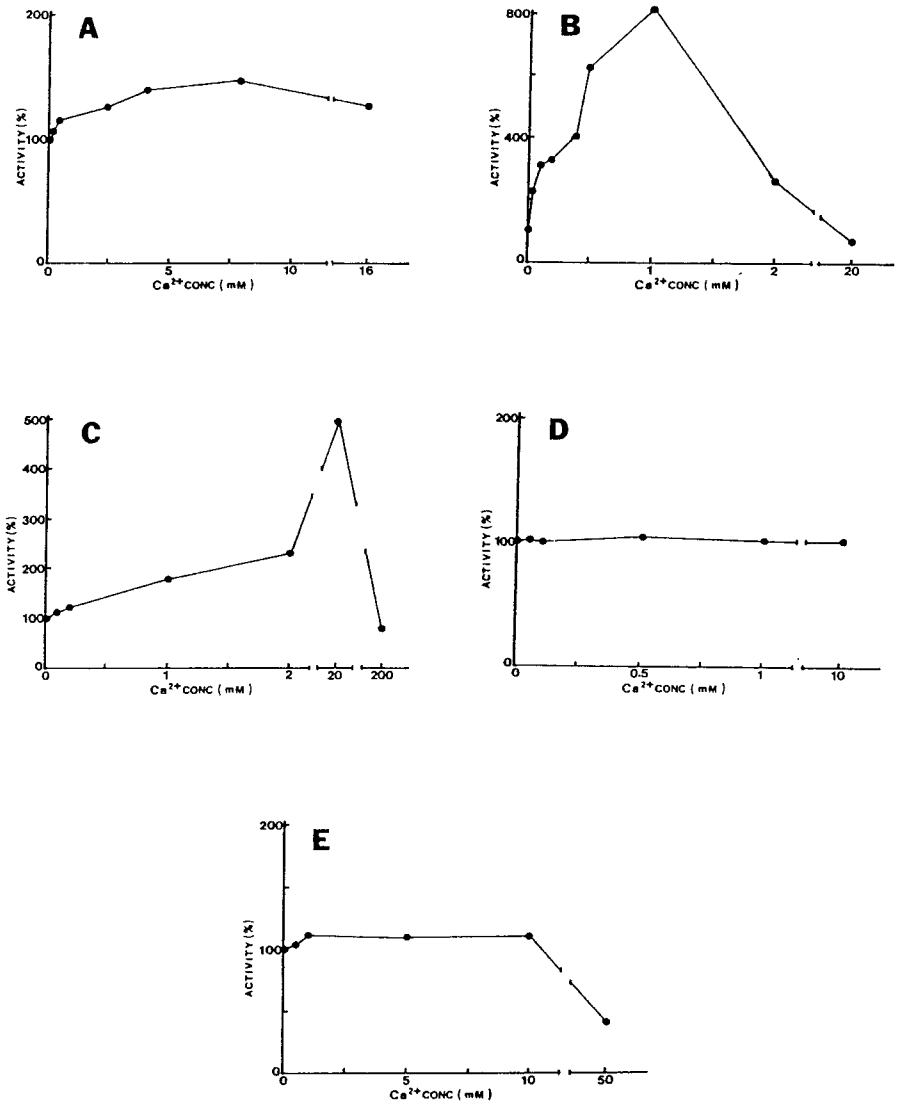

Fig. 2. Effect of Ca^{2+} concentration on the activities of microsomal phospholipase A, phospholipase C, di- and monoglyceride lipase, lysophospholipase, and acylation enzymes of lysophospholipid. Experimental details are shown in the text. Values are expressed as percent of control activities. A, phospholipase A; B, phospholipase C; C, di- and monoglyceride lipases; D, lysophospholipase; E, acylation enzymes of lysophospholipid.

The enzymic activity determined in the absence of EGTA and exogenous Ca^{2+} was defined as the native activity. Native phospholipase C activity in the microsomal fraction from the control brain was slightly higher than the basal activity. In contrast, the enzymic activity in the ischemic brains was significantly elevated. Since the basal activity seems to be the same in control and ischemic brains, the difference between basal and native

Table 1
Effect of Ischemia on the Activities of Rat Brain of Microsomal Enzymes[a]

Enzyme[b]	Duration of ischemia, min					
	0	1	5	15	30	60
PLA	0.79 ± 0.19 (n = 10)	1.06 ± 0.67 (n = 10)	1.16 ± 0.18 (n = 6)	1.34 ± 0.22 (n = 6)	1.15 ± 0.42 (n = 10)	1.17 ± 0.31 (n = 6)
LPL	641 ± 125 (n = 6)	708 ± 296 (n = 6)	503 ± 164 (n = 6)	552 ± 84 (n = 6)	553 ± 86 (n = 6)	464 ± 110 (n = 6)
PLC	83.1 ± 8.40 (n = 6)	83.0 ± 11.2 (n = 6)	88.9 ± 4.61 (n = 6)	87.2 ± 5.08 (n = 6)	73.2 ± 9.12 (n = 6)	73.0 ± 5.62 (n = 6)
DG + MG Lipase	6.18 ± 2.42 (n = 10)	8.66 ± 3.92 (n = 10)	9.14 ± 3.56 (n = 6)	9.80 ± 3.34 (n = 6)	7.74 ± 1.86 (n = 10)	5.56 ± 1.40 (n = 6)
Acylation Enzymes	3.66 ± 0.25 (n = 6)	4.26 ± 1.28 (n = 6)	2.75 ± 1.12 (n = 6)	2.56 ± 0.76[c] (n = 6)	2.50 ± 0.5[d] (n = 6)	2.31 ± 0.3[d] (n = 6)

[a]Enzyme assays are shown in the text. Concentrations of $CaCl_2$ were optimal for phospholipase A, phospholipase C, and di- and monoglyceride lipases, respectively. Values are means ± SD, expressed as nmol/h/mg protein; n indicates sample size.

[b]PLA, phospholipase A; LPL, lysophospholipase; PLC, phospholipase C; DG + MG lipase, di- and monoglyceride lipase; Acylation enzymes, acylation enzymes of lysophospholipid.

[c]Significance at $p < 0.05$ compared with control.

[d]Significance at $p < 0.01$ compared with control.

Table 2
Effect of Ischemia on the Difference Between Native and Basal Activities of Microsomal Enzymes[a]

Enzyme[b]	Duration of ischemia, min	
	0 (n = 4)	1 (n = 4)
PLC	3.85 ± 2.75	16.1 ± 9.47[c]
PLA	0.30 ± 0.26	0.15 ± 0.15
DG + MG lipase	0.32 ± 0.27	0.88 ± 1.60
Ca content, nmol/mg protein	12.7 ± 1.75	8.98 ± 1.38

[a]Experimental details are given in the text. The native activity was assayed without addition of both EGTA and Ca^{2+}; the basal activity was assayed with 1 m*M* EGTA. Values represent the difference between the native and basal activities as mean ±SD nmol/30 min/mg protein for PLC and DG + MG lipase and nmol/2 h/mg protein for PLA.

[b]PLC, phospholipase C; PLA, phospholipase A; DG + MG lipase, di- and monoglyceride lipase.

[c]Denotes significance at $p < 0.05$ compared with control; $n = 4$ indicates assays of microsomes obtained from four animals.

phospholipase C activities was found to increase after 1 min of ischemic treatment ($p < 0.05$) (Table 2). Maximal stimulation of phospholipase C activity in microsomal fraction at a concn. of $1 \times 10^{-3} M$ in control and ischemic brain were obtained. To investigate the factor capable of affecting phospholipase C, we determined the amount of total calcium in microsomal fractions. The amount of calcium in the microsomal fractions seemed to be in practically the same range (Table 2, the lowest line). Thus, the level of total calcium added into the assay mixture with enzyme can be calculated to be 54 or 76 μ*M* at the most. These values were considerably lower than necessary for expression of half the maximal activity of phospholipase C in the microsomal fraction. Phospholipase A and di- and monoglyceride lipases in microsomal fraction were also examined for their native activities. These did not show any distinguishable stimulation of the native activities. In these assays, the calculated concentrations of Ca^{2+} were around 100 μ*M*.

DISCUSSION

We did not observe significant changes in phospholipid contents of rat brain during 1–60 min ischemia. Similar results were observed by Rehncrona et al. (1982) and Bhakoo et al. (1984). However other investigators (DeMedio et al., 1980; Yoshida et al., 1980; Abe et al., 1987) reported significant decreases in various phospholipids after ischemia. These authors found differences of phospholipid content between ischemic and control brain, such as PlsEtn, PtdCho, PtdIns, PtdEtn, and

polyphosphoinositides. Abe et al. (1987) showed substantial reductions specific to polyphosphoinositides in early ischemia.

Stimulation of phospholipase A_1 and A_2 activities was observed by Edgar et al. (1982) in acetone-dried powder of gerbil ischemic brain. In the present study, however, the activities of phospholipase A, lysophospholipase, phospholipase C, and di- and monoglyceride lipase did not change significantly during ischemia. Although there is a marked difference between preparation of these enzymes, the reasons for the discrepancy are not clear.

Activities of phospholipases and lipases are regulated by Ca^{2+} ions (van den Bosch, 1982; Bell et al., 1979; Farooqui et al., 1985). Our data on the effect of calcium on phospholipase A and C and di- and monoglyceride lipases in vitro also indicated that these enzymes are stimulated by Ca^{2+} ions in microsomal fraction. The determination of total calcium content in microsomal fractions obtained from control and ischemic brain indicated that there was no difference in total calcium concentrations. However, based on the effect of Ca^{2+}, there was a marked difference between basal and native phospholipase C activity of ischemic brain compared to control brain. One possibility is that in the very early stage of ischemia, activation of phospholipase C may occur initially at lower Ca^{2+} concentration by an unknown mechanism. Prolonged ischemia may also increase intracellular Ca^{2+} concentration by many mechanisms (Siesjo, 1984). One of these mechanisms is mobilization of Ca^{2+} from intracellular storage. Phospholipase C activation may contribute to this mechanism. An increase in Ca^{2+} concentration will activate other phospholipases and lipases. Besides this, several other studies have indicated that activities of phospholipases and lipases are stimulated by neuropeptides (β-endorphin and bradykinin) (Richter et al., 1983; Gecse et al., 1987; Farooqui et al., 1988). It has also been reported that neuropeptide metabolism is related to arachidonic acid metabolism and phospholipase A_2 activity (Knepel and Meyen, 1986). Thus, it may be possible that alterations in levels of neuropeptide are also responsible for increased activities of phospholipases and lipases in ischemia.

The alteration in activity of acylation enzymes of lysophospholipids also may be important for the pool of free fatty acid in brain. Earlier studies on acylation of lysophospholipid in guinea-pig brain (Fisher and Rowe, 1980) have indicated that P_3 (microsomes) has the highest specific activity among subcellular fractions. Therefore, the decrease of acylation activity in the microsomal fraction may significantly contribute to the accumulation of free fatty acids in the ischemic brain. Lysophosphatidylchloline was shown to inhibit the action of acylCoA:lysophospholipid acyltransferase (Wittels and Hurlbert, 1977; Weltzien et al., 1979). Although we did not measure lysophospholipid levels in this study, accumulation of lysophosphatidylcholine was reported in ischemic brain (Sun and Foudin, 1984). Therefore, it is possible that lysophosphati-

dylcholine may inhibit this enzyme in ischemia. Fisher and Rowe (1980) have indicated that the acylation process results from the action of two enzymes, acylCoA synthetase and acylCoA:lysophospholipid acyltransferase. AcylCoA synthetase is ATP dependent and our results suggest that both ATP exhaustion and inactivation of acylation enzymes of the lysophospholipid may be involved in the decrease of acylation of the lysophospholipid in ischemic brain.

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