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Role of Phospholipids in Calcium Accumulation in Brain Mitochondria from Adult Rat After Ischemic Anoxia and Hypoxic Hypoxia

by

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Summary. The accumulation of calcium ions by rat brain mitochondria isolated from the brain submitted to ischemic anoxia was decreased by about 35%. This process was also inhibited by about 18% after hypoxic hypoxia. At the same time the content of phospholipids decreased in ischemic and hypoxic brain mitochondria by about 27% and 16%, respectively. In particular, choline glycerophospholipids and ethanolamine glycerophospholipids were diminished. Simultaneously the content of free fatty acids increased. The addition of fatty acids, lysoglycerophospholipids, deoxycholate or sodium dodecyl sulfate, in low concentrations, inhibited calcium accumulation in the control mitochondria. In another experiment mitochondria were incubated with phospholipase A_2 (EC 3.1.1.4) or phospholipase C (EC 3.1.4.3) in the presence of albumin. After washing the mitochondria the rate of calcium accumulation increased considerably. These results suggest that the integrity of the membrane phospholipids is important for calcium transport. The decrease in ethanolamine glycerophospholipids may be a factor resposible for the inhibition of calcium accumulation in mitochondria after ischemic anoxia and hypoxic hypoxia. Another factor may be the uncoupling and detergent action of fatty acids and lysoglycerophospholipids.

The aim of the present study was to determine the role the phospholipids in the processes of calcium accumulation in the brain mitochondria from adult rats after ischemic anoxia and hypoxia.

Materials and methods

The experiments were performed on female Wistar rats weighing 180–200 g. Ischemic anoxia was realised by incubation of the decapitated animal heads at 37°C for 5 min. Hypoxic hypoxia was produced by keeping the animals for two h in a chamber of 12 l volume through which two liters of a gas mixture 7% of oxygen in nitrogen atmosphere was passed over per minute. The chamber was flushed with the gas mixture used 15 min before the experiments. The rectal temperature of animals was kept close to 37°C by means of heating pad.

Isolation of brain cortex mitochondria. The brain crude mitochondrial fraction was isolated according to Ozawa *et al.* [20]. The brain cortex was homogenized in the isolation medium containing 0.3 M mannitol 0.1 mM EDTA, 10 mM Tris-HCl pH 7.4 with a loose fitting glass teflon homogenizer with 10 up and down strokes of the pestle. The homogenate was centrifuged for six min at 900 *g*. The supernatant was carefully decanted and centrifuged at 10 000 *g* for 20 min.

The pellet (crude mitochondrial fraction) was suspended in a small volume of isolation medium for further subfractionation. The suspension of crude mitochondrial fraction (7.5 ml) was placed upon a discontinuous Ficoll mannitol gradient consisting of 3.5 ml of 6% (w/v) Ficoll in 0.3 mannitol with 0.1 mM EDTA, pH 7.4, 3.5 ml of 9% (w/v) Ficoll in mannitol medium, 3.5 ml of 12% and 5 ml 14% Ficoll in mannitol medium and centrifuged at 25 000 rpm for 45 min in an MSE Ultra Centrifuge (3×25 swing out rotor). After centrifugation the pellets (P) were suspended in the isolation medium and spun at 10 000 *g* for 20 min.

The purity of mitochondria was examined under an electron microscope and their integrity by measuring the oxidative phosphorylation by the polarographic method of Chance [7] and a Clark oxygen electrode. ADP/O and RCI coefficients of the normal mitochondrial fraction with 5 mM succinate as a respiratory substrate were about 1.3. and 2.0, respectively. These values diminished to 1.1 and 1.2, respectively, in mitochondria from animals submitted to 5 min of ischemic anoxia.

Determination of calcium accumulation by mitochondria. Brain cortex mitochondria (0.3 mg protein) were suspended in incubation medium consisting of 0.3 M sacharose, 5 mM Tris-HCl buffer (pH 7.4), 2 mM Na-phosphate (pH 7.4) 5 mM KCl, 5 mM $MgCl_2$, 5 mM Na-succinate, 2.5 μM rotenone, 3 mM Na_2ATP, 1 mM $^{45}CaCl_2$ (0.5 μCi/μmol) in final volume of 1.0 ml.

^{45}Ca accumulation was determined by measurement of the radioactivity remaining in the medium after 2.5-min incubation at 37°C. With subsequent chilling in ice bath and sedimentation of mitochondria at 10 000 g for 6 min [6] the values were corrected for passively bound ^{45}Ca at 0°C.

Extraction and analysis of phospholipids. Lipids were extracted according to Folch *et al.* [10]. Fractionation intact lipid classes was performed by spotting small sample of lipids resolved in choroform methanol 90:10 (v/v) on silica gel G layer, 0.5 mm in thickness. The lipids were separated by two-dimensional thin layer chromatography according to Horrocks [11]. Iodine vapour was used for detection of lipid spots which then were scraped from the silica gel for the phosphorus determination according to Bartlett [5]. Protein was estimated by the method of Lowry *et al.* [13].

Free fatty acids were extracted by the methods described by Dole and Meinertz [9] and determined by colorimetric method according to Duncomb as modified by Itaya and Ui [12] and by GLC.

Methyl esters of the acyl groups were formed by acid methanolysis and then were separated in a PYE Unicam S-104 and Varian 1400 gas chromatograph equipped with a flame ionization detector. The column was packed with 10% PEGA on chromosorb-W. The temperature of the column was 210°C. Argon flow was 40 ml/min. Methyl erucate was used as an internal standard. The peak area was measured by the peak height × 1/2 of its width.

Phospholipase A treatment. Purified phospholipase A (from *Vipera russelli*, Sigma) was used after elimination of possible contamination by the proteolytic enzymes by boiling in water bath for 5 min. The mitochondria were suspended in medium containing 0.32 M sucrose, 0.75 mM $CaCl_2$, 0.1 M triethanolamine at pH 8.0, 0.1% bovine serum albumine and phospholipase A_2 2.0 μg/mg protein in a final volume of 1.0 ml. Incubation was carried out at 37°C for 10 min. The reaction was stopped by the addition of 0.3 M mannitol with 1 mM EDTA and 1% serum albumin and centrifuged at 10 000 *g* for 10 min to remove phospholipase reaction products. The mitochondria were washed once more in 0.3 M monnitol with 1 mM EDTA, 0.01 M Tris-HCl pH 7.4 to remove albumine and resuspended for the assay of calcium accumulation.

The control mitochondria were treated in the same way except that no phospholipase A was added to the incubation medium.

Phospholipase C treatment. The mitochondria were suspended in a medium containing 0.32 M sucrose, 0.5 mM $CaCl_2$, 0.1 M KCl, 5 mM histidine buffer, pH 7.4. Phospholipase C from *Clostridium welchi*, Sigma, was added (0.0–2.0 mg). Mitochondrial protein concentration was about 1.0 mg. The incubation was carried out in a final volume 1.0 ml at 37°C for 10 min. Then the mitochondria were centrifuged at 10 000 *g* for 10 min and resuspended for the assay of calcium accumulation. The control mitochondria were treated in the same way but without phospholipase C addition to the incubation mixture.

Results

Effect of isochemic anoxia and hypoxic hypoxia on the phospholipids content in the brain mitochondria. After ischemic anoxia the content of phospholipids decreased by about 27%. In particular the content of choline glycerophospholipids was diminished by about 28% and the ethanolamine glycero phospholipids by about 30%.

The content of phospholipids after hypoxic hypoxia decreased by about 16%, cholineglycerophospholipids by about 15% and ethanolamine glycerophospholipids by about 21% (Table I).

TABLE I

The content of phospholipids in the mitochondria of rat brain submitted to ischemic anoxia and hypoxic hypoxia

Phospholipids	Normoxia	Ischemic anoxia	Hypoxic hypoxia
	μg Pi/mg protein		
Phosphatidylcholine and choline plasmalogen	7.73±0.24 [43.2]	5.56±0.26* [42.4]	6.56±0.31* [43.9]
Phosphatidylethanolamine and ethanolamine plasmalogen	6.84±0.27 [37.2]	4.80±0.07* [36.7]	5.41±0.12* [36.2]
Phosphatidyl serine and phosphatidylinositols	1.43±0.07 [8.0]	1.16±0.09 [8.9]	1.24±0.11 [8.3]
Sphingomyelin	1.27±0.12 [7.1]	1.00±0.07 [7.6]	1.14±0.03 [7.6]
Diphosphatidylglycerol	0.61±0.05 [3.4]	0.58±0.06 [4.4]	0.60±0.06 [4.0]
Total phospholipids	17.88±0.17	13.10±0.11	14.95±0.13

The values are the means from 3 experiments±SEM.
Values that are significantly different from controls by Student's test are marked* $p<0.05$.
Percentages of total phospholipids are shown in brackets

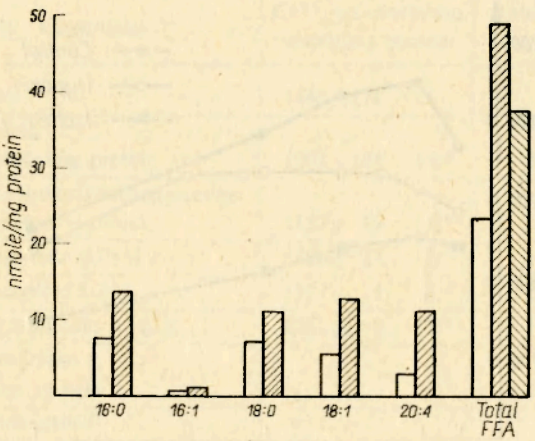

Fig. 1. The level of fatty acids in brain mitochondria after ischemic anoxia and hypoxia

16:0 palmitic acid, 16:1 palmiticoleic acid, 18:0 stearic acid, 18:1 oleic acid, 20:4 arachidonic acid. In Figs. 1, 3–5 each value is the mean of triplicate determinations
empty blocks—control; right-up hachure—ischemic anoxia; right-down hachure—hypoxia

Simultaneously the content of free fatty acids increased by about 110% after ischemic anoxia, mostly oleic and arachidonic acids were liberated.

After hypoxic hypoxia the level of free fatty acids increased by about 60% (Fig. 1).

TABLE II

Effect of ischemic anoxia and hypoxic hypoxia on Ca^{2+} accumulation in brain mitochondria

Conditions	Ca^{2+} accumulation nmole/mg protein	Inhibition %
Control	1437±153 (6)	
Ischemic anoxia	929± 48 (3)*	35
Hypoxic hypoxia	1184± 42 (4)*	18

Mitochondria 0.3 mg of protein/ml were incubated at 37°C for 2.5 min in 0.3 M sucrose, 5 mM Tris-HCl buffer pH 7.4, 2 mM Na phosphate pH 7.4 5 mM KCl, 5 mM MCl_2, 5 mM succinate, 2.5 μM rotenone, 3 mM ATP and 1 mM $^{45}CaCl_2$ (0.5 μCi/μmol).

Values represent means±SD.

Values that are significantly different from controls by Student's t test are marked* ($p<0.05$ at least).

The accumulation of calcium ions in brain mitochondria after ischemic-anoxia and hypoxic hypoxia diminished by about 35% and 18%, respectively, (Table II). The accumulation of calcium ions in mitochondria from normoxic, ischemic and hypoxic brain decreased evidently during 30 min of incubation (Fig. 2). All the other experiments were carried out during 2.5 min of incubation. Like other membrane-dependent processes, accumulation of calcium ions is dependent on temparature. The temperature dependence of calcium ions accumulation in brain mitochondria after ischemic anoxia and hypoxic hypoxia was demonstrated in Fig. 3.

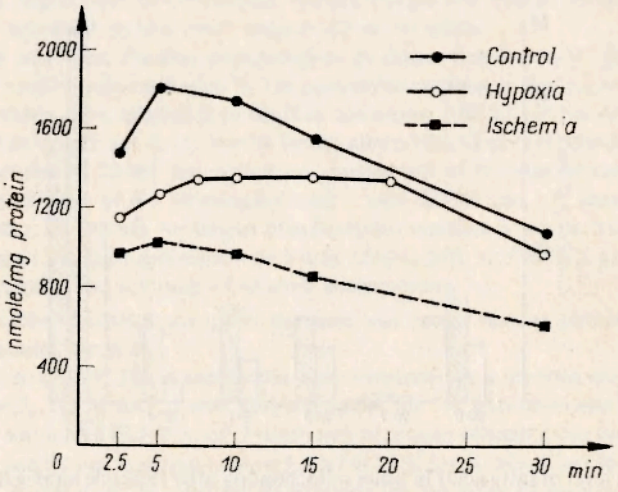

Fig. 2. Calcium accumulation in brain mitochondria

The mitochondria were incubated in the medium for massive Ca^{2+} uptake with succinate+rotenone as described in Table II. Data represent mean values obtained from at least 4 experiments

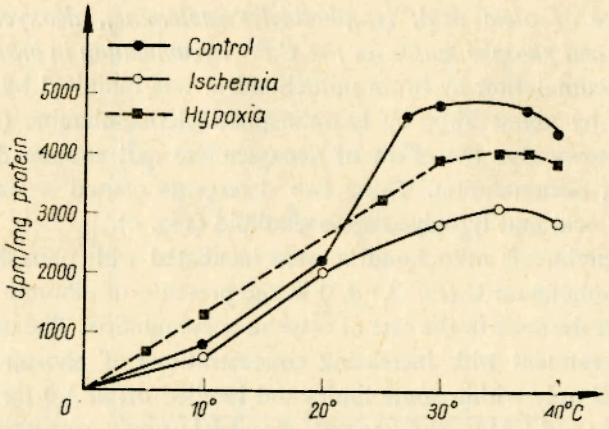

Fig. 3. Effect of temperature on Ca^{2+} uptake by rat brain mitochondria

The mitochondria (0.3 mg of protein per ml) were incubated in the medium for massive Ca^{2+} uptake as described in Table II

A sharp increase in calcium accumulation in brain mitochondria of untreated animals (control) was observed between 20°C and 38°C. The thermotropic transition was not observed in mitochondria isolated from the brain submitted to ischemic anoxia and hypoxic hypoxia. The higher accumulation of calcium ions was observed at 35°C–37°C.

TABLE III

The influence of oleic acid, lysophosphatidylethanolamine deoxycholate, dodecylsulphate and phospholipases on Ca^{2+} accumulation in brain mitochondria

Conditions	Ca^{2+} accumulation nmole/mg protein	Inhibition %
Control	1485±171 (4)	
Oleic acid 100 nmole/mg protein	1093±104 (4)*	26.4
Lysophosphatidylethanolamine 100 nmole/mg protein	1153± 49 (4)*	22.4
Deoxycholate 0.05%	1306± 22 (2)	12.1
Deoxycholate 0.1%	1132± 4 (2)*	23.8
Dodecylsulphate 0.125%	1211± 8 (2)*	18.5
Phospholipase A 2 μg/mg protein		47.0
Phospholipase C 0.5 mg/mg protein		43.7

The control values for the experiments with phospholipases. 967±93.6 (4) nmole/mg protein.

Other denotations as in Table II.

The influence of oleic acid, lysophatidylethanolamine,, deoxycholate, sodium dodecyl sulfate and phospholipases on the Ca^{2+} accumulation in brain mitochondria. The calcium accumulation by brain mitochondria was inhibited by about 26% by oleic acid and by about 22% by lysophosphatidylethanolamine (Table III).

Table III shows also the effect of deoxycholate and sodium dodecylsusphate on the calcium accumulation. These two detergents caused a similar inhibition to that of fatty acid and lysoglycerophospholipid (Fig. 4).

In other experiments mitochondria were incubated with phospholipase A (EC 3.1.1.4) or phospholipase C (EC 3.1.4.3) in the presence of albumin. After washing there was a large decrease in the rate of calcium accumulation. The inhibition corresponded to a treatment with increasing concentrations of phospholipase A, and phospholipase C only within some limits and levelled off at 1.0 μg PLA/mg mitochondrial protein and 1.5 mg PLC/mg mitochondrial protein, respectively (Fig. 4*a*, *b*).

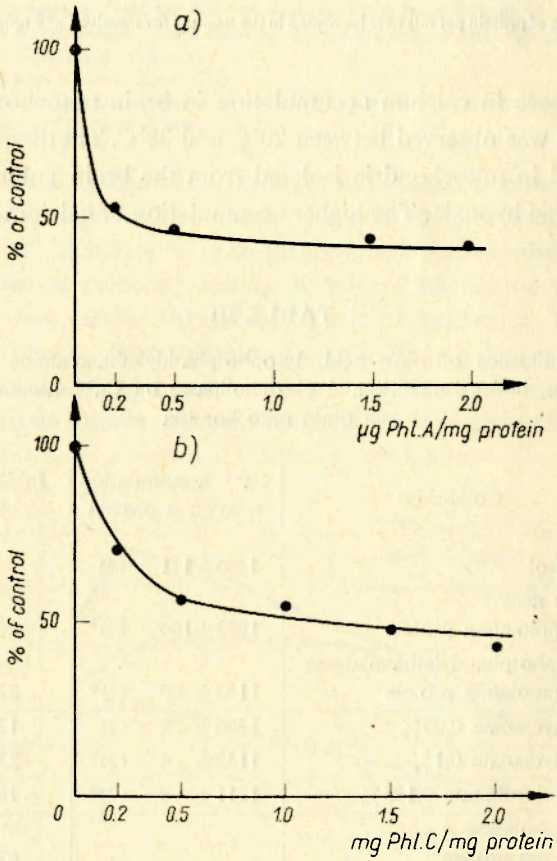

Fig. 4. Effect of phospholipase A (a) and phospholipase C (b) on Ca^{2+} accumulation by brain mitochondria

The mitochondria were incubated with phospholipase A_2 or phospholipase C in the presence of albumin. After washing mitochondria were incubated in the medium for massive Ca^{2+} uptake as described in Table II. Control mitochondria were treated in the same way except that no phospholipases were added to the incubation medium The massive uptake of Ca^{2+} for this control mitochondria was 967 ± 9.36 nmoles/mg protein. Values represent mean $\pm$SD for four experiments

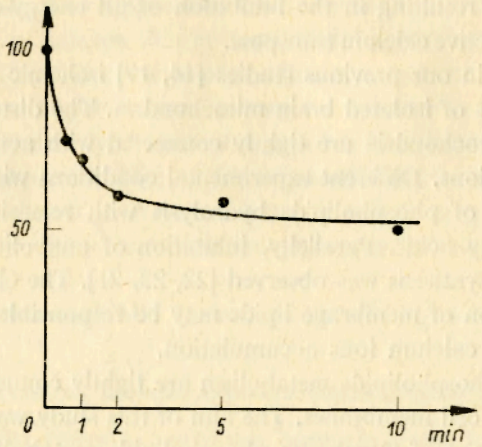

Fig. 5. Effect of the preincubation time with phospholipase A_2 on the Ca^{2+} accumulation by brain mitochondria

Mitochondria were incubated with 2 μg PLA/mg mitochondrial protein at 37°C for different time intervals

In other experiments mitochondria were incubated for different times with phospholipase A_2 in the presence of albumin. After washing the mitochondria were used for estimation of calcium ions accumulation. Large decrease in the rate of calcium accumulation was observed after 2-min incubation with phospholipase A_2. This inhibition of calcium accumulation was not increased substantially by increasing the time of incubation with phospholipase A (Fig. 5).

Discussion

Phospholipids are not only the backbone of mitochondrial membranes responsible for their barrier function but also they play a more activie role in such specific processes as oxidative phosphorylation and the active transport of ions and some metabolites [4, 24]. The content and composition of mitochondrial membrane phospholipids are regulated by endogenous enzymic activity. The presence of phospholipase A_1 and A_2 activity has been demonstrated in the mammalian brain [8, 25, 26]. Also plasmalogenase, the enzyme which hydrolyzes another type of ethanolamine phosphoglycerides, has been found in the central nervous system [2, 3].

In our previous studies [18, 19] the activation of phospholipid degradation in mitochondria isolated from brains submitted to ischemic anoxia with simultaneous increase in free fatty acids pool was described. We suggested that FFA may be responsible for such disfunctions of ischemic brain mitochondria as uncoupling of oxidative phosphorylation and inhibition of Ca^{2+} uptake [14, 15].

The ability for calcium accumulation characterises mitochondria of nearly all living cells including brain. Mitochondrial calcium uptake is an energy-dependent process coupled to respiration and ATP hydrolysis [6, 16]. Oxygen defficiency leads

to energy imbalance resulting in the inhibition of all energy-dependent processes of the cell including active calcium transport.

As was reported in our previous studies [14, 17] ischemic anoxia reduces also functional parameters of isolated brain mitochondria. The disturbances of functional behaviour of mitochondria are tightly connected with activation of phospholipids catabolic reactions. Different experimental conditions with oxygen deficiency produced activation of phospholipids hydrolysis with releasing the considerable amounts of free fatty acids. Parallelly, inhibition of anabolic reactions involved in phospholipids biosynthesis was observed [22, 23, 21]. The changes in the proper structural composition of membrane lipids may be responsible for the impairment of mitochondria for calcium ions accumulation.

The changes in phospholipids metabolism are tightly connected with the structure and function of cell membranes. The aim of this study was to answer to what degree the disturbances of membrane phospholipids metabolism may be responsible for decreasing the calcium accumulation in brain mitochondria after ischemic anoxia and hypoxic hypoxia. The free fatty acids which are liberated after ischemic and hypoxic episode exert the effect similar to that caused by the action of lysoglycerophospholipid and other detergents — deoxycholate and dodecylsulphate. The inhibition of calcium accumulation in mitochondria can be due to nonspecific action of fatty acids and lysoglycerophospholipids. Moreover, the liberation of unsaturated fatty acids from glycerophospholipids evidently changes the membrane properties, their physico-chemical state. Fatty acid chains in membrane are responsible for the transition of membrane from α-crystalline to liquid state. In the mitochondria from the brain of untreated animals the calcium accumulation increases sharply between 20°C–37°C. This thermotropic transition of mitochondria membranes from crystalline to liquid state facilitates the translocation of calcium ions across the membrane. In the mitochondria after ischemic anoxia and hypoxia the liberation of unsaturated fatty acids changes evidently the membrane thermotropic transition decreasing paralelly calcium ions accumulation. After ischemic anoxia mostly oleic and arachidonic acids are liberated. The arachidonic acid is located in the β position of glycerophospholipids. This glycerophospholipid is degraded by the action of phospholipases and plasmalogenase. The content of ethanolamine glycerophospholipids is remarkably diminished after ischemic anoxia and hypoxia by about 30, and 21%. It is possible that ethanolamine glycerophospholipids plays an important role in the transport of calcium ions. This hypothesis is supported by the results obtained with phospholipases, suggesting the existence of specific phospholipid pools responsible for calcium transport. The calcium accumulation was inhibited by about 50% after phospholipase A_2 and phospholipase C treatments. This effect was independent, within some limits of the amount of the added enzyme.

The results with phospholipase C suggested that the phosphoryl moiety of the membrane glycerophospholipids is also important for calcium transport. Possibly this groupis needed for the formation of Ca-ATP-phospholipid protein complex as it was suggested by Abood [1].

The present results lead to supposition that more than one mechanism is responsible for the inhibition of calcium ions accumulation by mitochondria after ischemic anoxia and hypoxia. The disturbances of the fine arrangement of membrane lipids by ischemic anoxia and hypoxic hypoxia may be responsible for the inactivation of mitochondrial membrane Ca^{2+} transport process. The decrease in ethanolamine glycerophospholipids is presumably a factor responsible for the inhibition of calcium accumulation in mitochondria. Another factor involved may be the uncoupling and detergent action of fatty acids and lysoglycerophospholipids.

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Й. Строшнайдер, **Роль фосфолипидов в накоплении кальция митохондриями мозга взрослых крыс после исхемической аноксии и гипоксической гипоксии.**

Содержание. Аккумуляция йонов кальция митохондриями, выделенными из мозга крыс после исхемической аноксии снижалась примерно на 35М. Снижение аккумуляции йонов кальция примерно на 18% наблюдалось также после гипоксической гипоксии. Содержание фосфолипидов было меньше на 27 и 16%, соответственно. Особенно понизилось количество холинглицеролипидов и этаноламиновых липидов. Одновременно увеличивалось содержание свободных жирных кислот. Низкие концентрации жирных кислот, лизоглицерофосфолипидов, дезоксихолата или додецилсульфата натрия тормозили накопление кальция в контрольных митохондриях. В ряде опытов митохондрии инкубировались с фосфолипазой A_2 (EC 3.1.1.4) или фосфолипазой С (EC 3.1.4.3) в присутствии альбумина. После промывки степень аккумуляции кальция этими митохондриями значительно понизилась. Полученные результаты указывают, что мембранные фосфолипиды являются важным звеном в транспорте кальция. Уменьшение содержания этаноламиновых липидов может оказаться фактором, ответственным за торможение аккумуляции кальция после исхемической аноксии и гипоксической гипоксии. Другим фактором можно считать распрягающее и детергентное воздействие жирных кислот и изоглицерофосфолипидов.