RELATIONSHIP BETWEEN PEROXIDATION AND PHOSPHOLIPASE HYDROLYSIS OF LIPIDS IN SYNAPTOSOMES

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Undecomposed activation of lipid peroxidation (LPO) in nerve tissue has been shown to be an important stage in epileptogenesis [2, 5, 6, 8]. Meanwhile the initial causes of disturbance of the regulation of LPO in epilepsy are not yet clear. On the basis of our own results [1] and those of other investigators [13, 15], we have suggested [12] that one cause of LPO activation in epileptogenesis may be activation of endogenous phospholipase hydrolysis of lipids (PLH), found in the region of hyperactivity. As yet there has been no experimental verification of this hypothesis. The cause and effect relationships between LPO and PLH in general in biological membranes likewise remain unexplained.

In the investigation described below relations between LPO and PLH in synaptosomes were studied. The preliminary results were published previously [10].

EXPERIMENTAL METHOD

The fraction of unpurified synaptosomes (without separation in a sucrose density gradient) was isolated from the cerebral cortex of Wistar rats by the method in [16]. Synaptosomes were kept at 0°C for not more than 4-6 h before verification of their native state by the method of potential-sensitive fluorescence probes [9]. The same method is used to determine effective concentrations of α-tocopherol, mepacrine, and phospholipase A_2.

A suspension of synaptosomes (concentration 4 mg protein/ml) was incubated with constant mixing for 42 min (37°C), made up of 2 min of preincubation followed by 40 min of incubation in the presence of LPO and PLH activators or inhibitors. Samples were taken from the incubated suspension 5, 10, 15, 20, and 40 min after addition of the necessary additives for determination of their LPO and PLH activity. LPO and PLH processes in the samples were stopped by adding them to medium containing 1 mM EDTA. The results are given in Figs. 1-3 in the form of characteristic kinetic curves; each curve represents the results of 7-12 experiments.

LPO activity was estimated from the level of products reacting with 2-thiobarbituric acid (TBAP) in the synaptosomal suspension. To 100 μliters of synaptosomal suspension with 1 mM EDTA 1.5 ml of 25-30% solution of trichloroacetic acid and 1.5 ml of a 0.5% solution of 2-TBA were added. After incubation of the samples for 30 min at 60°C the optical density of the solutions was determined at 535 nm of a "Hitachi-320" spectrophotometer (Japan).

PLH activity was estimated as the concentration of free fatty acids (FFA), which was determined by using the reaction between diethyldithiocarbamate (DDTC), a reagent for copper, and an extract of copper salts of fatty acids, which proceeds with the formation of a colored complex. To 400 μliters of a suspension of synaptosomes containing 1 mM EDTA were added 600 μliters of the copper reagent (in mM): $Cu(NO_3)_2$ 50, triethanolamine 100, NaOH 167 (in a saturated solution of NaCl, pH 8.0, at 20°C) and 2 ml of extraction mixture (chloroform:heptane:methanol – 20:20:1). After vigorous shaking of the mixture for 5 min it was centrifuged for 15 min at 1000 rpm on an "OPN-8" centrifuge. After separation of the mixture

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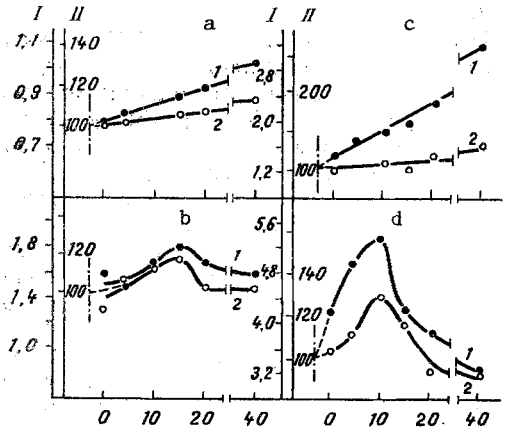

Fig. 1

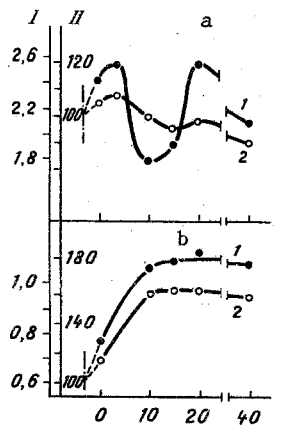

Fig. 2

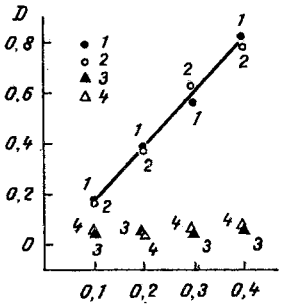

Fig. 3

Fig. 1. Effect of intensity of LPO (a, c) in synaptosomes on activity of their endogeneous phospholipases (b, d). a, b: 1) Native synaptosomes, 2) mepacrine. c, d: 19 Fe + ascorbate, 2) α-tocopherol + (Fe + ascorbate). Here and in Fig. 2, incubation medium of synaptosomes contained (in mM): NaCl 132, KCl 5, NaH_2PO_4 1.2, $MgCl_2$ 1.3, $CaCl_2$ 1.2, glucose 10, HEPES 20; pH 7.6 (20°C). Ordinate: a, c) TBAP content, b, d) FFA content. I) In relative units, II) in percent of initial level; time on abscissa given in minutes.

Fig. 2. Effect of PLH activity (a) in synaptosomes on intensity of LPO in them (b). a, b: 1) Phospholipase A_2, 2) mepacrine + phospholipase A_2. Ordinate: a) FFA content, b) TBAP content. I) In relative units, II) in percent of initial level; time along abscissa measured in minutes.

Fig. 3. Calibration curves for determining content of FFA with different lengths of their carbon chain (reaction with DDTC). 1-4) Arachidonic, stearic, butyric, and valeric acids, respectively. Ordinate, optical density at 550 nm; abscissa, FFA concentration (in mM).

into layers 1 ml of the top transparent phase was withdrawn and 100 μliters of a 0.2% solution of DDTC in ethanol was added to it. After 15 min the optical density of the sample was measured at 440 nm against the control sample containing the extraction mixture, and, in the corresponding cases, α-tocopherol, mepacrine, and phospolipase A_2 in the necessary concentrations.

The protein concentration in the suspension of synaptosomes was determined by the method of Lowry et al. The final concentrations of the additives were: $FeSO_4$ 10^{-5} M, ascorbic acid $2 \cdot 10^{-4}$ M, bee venom phospholipase A_2 0.55 mg/ml, α-tocopherol 10^{-4} M, and mepacrine $2 \cdot 10^{-3}$ M. All compounds were added in the form of aqueous solutions in incubation medium, with the exception of α-tocopherol, which was added in the form of an ethanol solution. The ethanol concentration in the suspension did not exceed 2.5%. Reagents were obtained from "Serva" (West Germany), salts from "Merck," (West Germany), and phospholipase A_2 and mepacrine from "Sigma."

EXPERIMENTAL RESULTS

The initial values of the TBAP and FFA levels in the experimental and control samples differed (Figs. 1 and 2). In samples with activators of LPO and PLH they were higher, and in samples with inhibitors – lower than the corresponding control samples. From the time of sampling until complete inhibition of LPO and PLH processes in them, a certain time evidently elapsed. The use of the method of extrapolation of the results enables this time to be determined (it was 3 min) and true initial values obtained for the levels of TBAP and FFA (Figs. 1 and 2). These values were sufficiently accurate for TBAP but less accurate for FFA, especially in the experimental with addition of phospholipase A_2.

Incubation of synaptosomes in medium at a temperature of 37°C for 40 min led to a steady and almost linear rise of the TBAP level in the suspension, on average by 20-40%, evidence of auto-oxidation of the synaptosomal lipids (Fig. 1a). Meanwhile the FFA level in the synaptosomes rose (Fig. 1b), due in all probability to temperature activation of endogenous phospholipases. The curve of dependence of the FFA level in the synaptosomes on their incubation time was more complicated than that for TBAP. A maximum was recorded in the region of incubation

for 25 min, when the increase in the FFA concentration amounted to 20-30% of the initial value. Later the FFA level fell toward the 20th minute to its initial value, at which it remained until the 40th minute of incubation.

Addition of LPO activators (a mixture of Fe^{2+} and ascorbate) to the incubation medium of the synaptosomes sharply increased the rate of oxidation of lipids, so that by the 40th minute of incubation the TBAP level was 2.5 times higher than initially (Fig. 1c). Changes in the FFA level in the synaptosomes under these circumstances were more marked than during auto-oxidation (Fig. 1b). At the maximum on the kinetic curve, which was shifted to the 10th minute of incubation, the rise of the FFA level amounted to 40-60% of the initial value. Addition of α-tocopherol to the synaptosomes inhibited the process of Fe-induced oxidation by 80% (Fig. 1c). The maximum for FFA on the kinetic curve was significantly higher in amplitude (Fig. 1d).

Addition of phospholipase A_2 to the suspension of synaptosomes greatly modified the shape of the kinetic curve for FFA. Two equally marked maxima were observed on the curve – in the regions of the 4th and 20th minutes of incubation (Fig. 2a). Meanwhile there was a significant increase in the rate of accumulation of TBAP in the synaptosomes. For instance, by the 40th minute of incubation the TBAP level was more than twice as high as that during auto-oxidation, amounting to about 80% of the initial value (Fig. 2b). The type A phospholipase inhibitor mepacrine smoothed the kinetic curves for FFA considerably both in the case of temperature activation of endogenous phospholipases of the synaptosomes (Fig. 1b) and for addition of the exogenous enzyme (Fig. 2a). Under these circumstances inhibition both of auto-oxidation of synaptosomal lipids (Fig. 1a) and peroxidation, stimulated by phospholipase A_2 (Fig. 2b) were inhibited. In the first case inhibition amounted to about 50%, and in the second case to about 25-30%.

Let us examine possible causes of the appearance of maxima on the kinetic curves for FFA. The presence of a phase (phases) of reduction of the FFA concentration in the synaptosomal suspension may be linked either with the formation of stable complexes of FFA or of their metabolic products with certain components of nerve endings or with FFA metabolism without complex formation, so that the FFA becomes "invisible" for the technique used to detect them. It must be pointed out that the sensitivity of the method used to determine FFA depends on the length of the carbon chain of the fatty acid. For instance, fatty acids containing fewer than 6 carbon atoms in their chain did not form a colored complex with DDTC (Fig. 3). During peroxidation, long carbon chains (20 carbon atoms) of unsaturated fatty acids are ruptured at their ω-double bond. As a result it can be expected that FFA with a carbon chain containing fewer than 8-9 carbon atoms will appear and, as a result, there will be an apparent fall of the FFA level in the synaptosomal suspension. Of two maxima marked on the kinetic curve for FFA in experiments with the addition of phospholipase A_2, the first was evidently connected with hydrolysis of phospholipids by exogenous phospholipase, the second with activation of endogenous phospholipases.

Evidence of an influence of intensity of LPO on PLH activity in synaptosomes is given chiefly by the high amplitude of the maximum on the kinetic curve for FFA during Fe-induced oxidation compared with the maximum during auto-oxidation. Experiments with the LPO inhibitor α-tocopherol are less informative in this direction, for reduction of the amplitude of the maximum for FFA against the background of α-tocopherol may be connected not with inhibition of LPO, but with the formation of FFA – α-tocopherol complexes, the possibility of which was demonstrated previously [3]. Other investigators have obtained results showing the stimulating effect of pro-oxidants on activity of type A phospholipases in mitochondria [17] and in microsomes of the liver [14]. The cause of this effect may be an increase in the concentration of Ca^{2+}, an activator of type A phospholipases, in "oxidizable" membranes, taking place as a result of an increase in the negative surface charge on the membranes because of accumulation of negatively charged peroxide groups. The role of intensification of PLH observed during activation of LPO in the membranes may be to repair membrane phospholipids whose fatty acids have undergone peroxidation, by a mechanism of monomolecular substitutions [4].

This activating effect of PLH on LPO which we observe in synaptosomes is in good agreement with the observations of Beckman and co-workers [14], who found a similar effect in liver microsomes. Activation of phospholipases, leading to accumulation of FFA and of lysoforms of phospholipids in membranes, which is accompanied by lowering of the viscosity of the lipid bilayer of synaptic membranes [11], evidently increases the accessibility of the oxidation substrate for initiators and pro-oxidants. Moreover unsaturated fatty acids found in the membrane in a free state are oxidized signifiantly faster than esterified fatty acids [7].

The results thus point to the existence of close direct interconnection between LPO and PLH processes in nerve endings.

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EFFECT OF ANTICOAGULANTS ON HUMAN PLASMA TRYPSIN-LIKE PROTEINASE ACTIVITY

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Changes in the proteolytic activity of human blood are nowadays an important parameter in clinical biochemistry in the diagnosis of several diseases, such as acute pancreatitis, shock of varied etiology, myocardial infarction, etc. [1, 2]. Reliable determination of proteolytic activity is therefore extremely important. Meanwhile anticoagulants (usually heparin, sodium citrate, or a combination of both) are widely used in clinical practice. The need for this is linked with the use of new methods of treatment, such as plasmapheresis, double and cascade ultrafiltration, and biospecific sorption on affinity sorbents [6, 7]. The aim of this investigation was to study the effect of anticoagulants on activity of human blood trypsin-like proteinases.

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