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THE EFFECT OF LIPOXIDATION ON SYNAPTOSOMAL ($Na^+ + K^+$)-ATPase ISOLATED FROM THE CEREBRAL CORTEX OF SQUIRREL MONKEY*

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SUMMARY

1. A system comprised of lipoxygenase (EC 1.99.2.1) and H_2O_2 ($8.8 \cdot 10^{-6}$ M) was employed to induce lipid peroxidation on polyunsaturated fatty acids of synaptosomal membranes.

2. Treatment of synaptosomal membranes with lipoxygenase–H_2O_2 led to a marked inactivation of the ($Na^+ + K^+$)-ATPase (ATP phosphohydrolase EC 2.6.1.3) activity; whereas similar treatment did not affect the acetylcholinesterase (acetylcholine acyl hydrolase EC 3.1.1.8) activity.

3. Kinetic studies revealed that lipoxygenase–H_2O_2 treatment increased the K^+ concentration required for half maximal rates; changes also in both K_m and V of enzyme for Na^+ were observed after such treatment. Since the inactivation of enzyme after lipoxygenase–H_2O_2 treatment is of the competitive type with respect to K^+, it may be concluded that the K^+ site is affected by lipoxidation.

4. Lipid analysis showed that the percentages of 20:4, 22:4 and 22:6 acyl groups in the synaptic membranes were lowered after lipoxygenase–H_2O_2 treatment.

5. Preincubation of membranes with peroxidized linolenic acid did not affect the ($Na^+ + K^+$)-ATPase activity. The loss in activity of ($Na^+ + K^+$)-ATPase after lipoxygenase–H_2O_2 treatment is probably due to a change in the microenvironment of the K^+ channels in the membranes.

INTRODUCTION

Enzymic activity of the membrane-bound synaptosomal ($Na^+ + K^+$)-ATPase (ATP phosphohydrolase EC 3.6.1.3) has been shown to depend on the structural integrity of the membrane[1–4]. Results from our laboratory[2,3] as well as others[5–7] have also demonstrated that the phospholipids of synaptosomal membranes are essential for the activity of ($Na^+ + K^+$)-ATPase. It was found that phospholipid-depleted enzyme could not be reactivated by adding synthetic lecithin or ethanolamine obtained from commercial sources. However, partial or full reactivation has been achieved by adding phospholipids prepared from brain[7]. These results suggest that enzyme activation may be related to the hydrophillic character as well as the degree

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of unsaturation of the membrane lipids. Like other metabolically active membrane systems, the synaptosomal membranes contain phospholipids with large proportions of polyunsaturated fatty acids[8] (A. Y. Sun and G. Y. Sun, unpublished data). Since these double bonds are important in providing the molecular configuration and hydrophillic character of the membrane, they may also play a critical role in determining the functional activity of $(Na^+ + K^+)$-ATPase.

Peroxidation of unsaturated lipids in mitochondrial and microsomal preparations may alter the structure of membranes and impair some of the membrane-dependent enzymes[9–13]. It has also been demonstrated that oxidative damage by ionizing radiation[14–16] and other agents such as alcohol[17] and carbon tetrachloride[18] are probably due to the formation of lipid peroxides. More recently, Bishayee and Balasubramanian[19] have reported that lipid peroxides are present in subcellular fractions of the brain. The synaptosomal membranes may be highly susceptible to oxidative damage due to their high content of polyunsaturated fatty acids and the continuous diffusion of oxygen through the membranes.

A homogeneous synaptosomal membrane fraction rich in junctional components and $(Na^+ + K^+)$-ATPase and acetylcholinesterase (acetylcholine acyl hydrolase EC 3.1.1.8) has been isolated from cerebral cortex of the squirrel monkeys[2]. The isolated membrane fraction has provided a useful biochemical system for studying the molecular processes related to synaptic transmission and for elucidating the relationship between the structure and function of membranes. Since lipoxygenase (EC 1.99.2.1) is a dioxygenase capable of stereospecific attack on selected fatty acid substrates[20], this enzyme system has been used to induce lipid peroxidation in the synaptosomal membranes. The present investigation was undertaken to examine the effect of lipid peroxidation on $(Na^+ + K^+)$-ATPase and acetylcholinesterase of synaptosomal membranes isolated from cerebral cortex of the squirrel monkey and to present further information on the functional relationship between changes in microenvironment and biochemical properties of synaptosomal membranes in the central nervous system.

METHODS AND MATERIALS

Preparation of synaptic membranes

Synaptic membranes were prepared from cerebral cortex of squirrel monkey according to the method described previously[2].

Enzyme assays

The synaptic membranes were suspended in an incubation medium containing 50 mM Tris buffer (pH 7.4), 5 mM $MgCl_2$, 0.2 mg of lipoxygenase and 0.2 mg of membrane protein in a total volume of 1.0 ml. Lipoxygenase was added to the mixture and was incubated for various time periods at 37 °C. In later experiments, $8.8 \cdot 10^{-6}$ M H_2O_2 was routinely added to the preincubation mixture (see Table I). The control membranes were treated the same way except that no lipoxygenase and H_2O_2 were added to the preincubation system. At the end of preincubation, the reactions were stopped by addition of 1.0 ml of 0.03 M EDTA and the membranes were isolated by centrifugation at $43000 \times g$ for 20 min. The membranes were then resuspended in cold doubly distilled water. Both the control and lipoxygenase–H_2O_2 treated

membranes were analyzed for enzymic activities of $(Na^+ + K^+)$-ATPase and acetylcholinesterase[2]. Protein was determined by the method of Lowry *et al.*[21].

Assay for lipid peroxide

Lipid peroxide was measured by the thiobarbituric acid color reaction as described by Wilbur *et al.*[22]. A 1-ml aliquot of the incubation mixture was added to 1 ml of 10% trichloroacetic acid. A color reaction was developed immediately after treating the solution with thiobarbituric acid reagent. The thiobarbituric acid color was read at 530 nm after centrifuging the protein precipitate. The amount of malonaldehyde formed in the color reaction was determined by using $\varepsilon_{530\ nm} = 1.56 \cdot 10^5$ cm/mmole[23].

Analysis of fatty acid composition

Portions of the control and lipoxygenase–H_2O_2 treated membranes were added to 20 vol. of chloroform–methanol (2:1, v/v) for extraction of total lipids. The ester-linked fatty acids were transmethylated with 0.5 M NaOH–methanol and the methyl esters were analyzed by gas–liquid chromatography[24].

Reagents

Soybean lipoxygenase was obtained from Sigma Chemical Co. (St. Louis, Mo.). Tris–ATP was prepared from Na_2–ATP according to the method of Järnefelt[25]. Other chemicals were of the highest grade commercially available.

RESULTS

The effect of H_2O_2 on lipid peroxide formation

Table I illustrated that there was no apparent reduction of $(Na^+ + K^+)$-ATPase activity when the membrane preparation was incubated with a low concentration of H_2O_2 for 30 min at 37 °C. In the presence of lipoxygenase, about 25% of the enzymic activity was inactivated. However, when lipoxygenase together with H_2O_2 ($8.8 \cdot 10^{-6}$ M) was added to the preincubation system, the $(Na^+ + K^+)$-ATPase

TABLE I

EFFECT OF H_2O_2 AND LIPOXYGENASE ON $(Na^+ + K^+)$-ATPase

The control membranes (0.2 mg protein) were preincubated in a medium containing 50 mM Tris buffer (pH 7.4), 5 mM $MgCl_2$ making a total volume of 1.0 ml. After 30 min of preincubation at 37 °C, 1.0 ml of 0.03 M EDTA was added. The membrane mixture was centrifuged at 43000 × *g* for 20 min, suspended in doubly distilled water and assayed for $(Na^+ + K^+)$-ATPase activity. Data were the means from two triplicate experiments.

Addition to preincubation medium	*$(Na^+ + K^+)$-ATPase activity (μmoles P_i/10 min per mg membrane protein)*	*% of control*
None (control)	8.72	100.0
Plus H_2O_2 ($8.8 \cdot 10^{-6}$ M)	8.59	98.5
Plus Lipoxygenase (0.2 mg/ml)	6.59	75.6
Plus H_2O_2 and lipoxygenase	3.62	41.6
Plus H_2O_2 and lipoxygenase and EDTA (7.0 mM)	9.07	104.0

activity was greatly inhibited. EDTA inhibited the action of lipoxygenase and thus protected $(Na^+ + K^+)$-ATPase from inactivation.

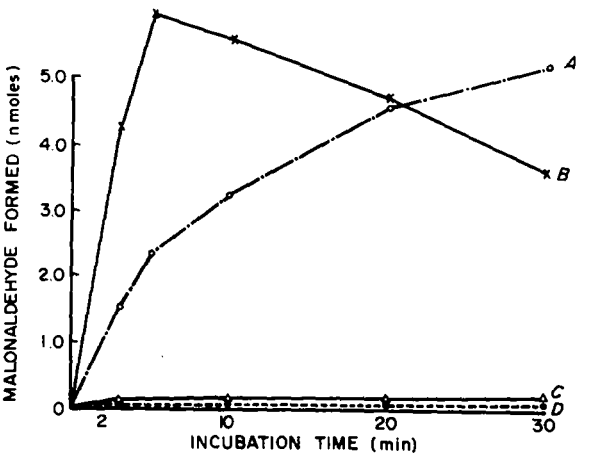


Fig. 1. The time course for lipoxidation using linolenic acid as substrate. The reaction medium consisted of 50 mM Tris buffer (pH 7.4), 6 mM $MgCl_2$, $8.8 \cdot 10^{-6}$ M H_2O_2, 0.2 mg lipoxygenase, 0.3 mg linolenic acid in a final volume of 1.0 ml. The reaction was stopped at the specified intervals by adding 1.0 ml trichloroacetic acid and analyzed for malonaldehyde formation as described in the Methods and Materials. Curve B (×—×), complete system; Curve A (○–·–○), H_2O_2 was omitted from the system; Curve C (△—△) 15 mM of EDTA was present in the incubation mixture; Curve D (●---●) lipoxygenase was omitted from the system. Each point is the mean of three experiments.

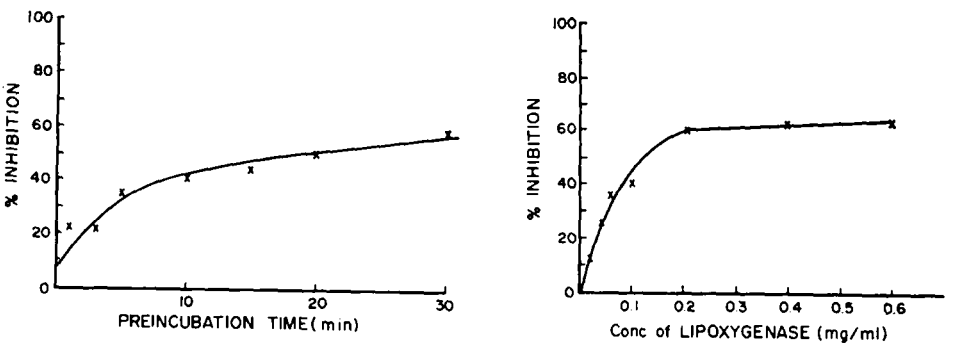


Fig. 2. The effect of lipoxidation on $(Na^+ + K^+)$-ATPase in synaptosomal membranes. Synaptosomal membranes (0.2 mg protein) were incubated for a specific period at 37 °C in a medium containing 50 mM Tris buffer (pH 7.4), 6 mM $MgCl_2$, $8.8 \cdot 10^{-6}$ M H_2O_2, 0.2 mg lipoxygenase in a final volume of 1.0 ml. At the end of each incubation interval, the reaction was stopped by adding 1.0 ml of 0.03 M EDTA after which the membranes were sedimented at 43000 × *g* for 20 min. The membranes were subsequently resuspended in cold doubly distilled water and assayed for $(Na^+ + K^+)$-ATPase activity. Control membranes were treated similarly except no lipoxygenase–H_2O_2 was added. The results are expressed as a percentage of inactivation in relation to values obtained for control membranes.

Fig. 3. The effect of lipoxygenase on membrane-bound $(Na^+ + K^+)$-ATPase activity. The synaptosomal membranes (0.2 mg protein) were treated with lipoxygenase (0–0.6 mg) and H_2O_2 ($8.8 \cdot 10^{-6}$ M) at 37 °C for 30 min in the preincubation system described in the legend of Fig. 2. Control membranes were treated in the same way except that no lipoxygenase and H_2O_2 were added to the preincubation system. The activities of $(Na^+ + K^+)$-ATPase remaining after treatment are compared with values obtained for the control membranes.

The effect of H_2O_2 on lipid peroxide formation was further examined in a system containing pure linolenic acid as substrate (Fig. 1). The addition of $8.8 \cdot 10^{-6}$ M H_2O_2 to the incubation system did not cause peroxidation of the linolenic acid (Curve D). The addition of lipoxygenase resulted in lipid peroxidation which increased with time up to 30 min (Curve A). However, in the presence of both H_2O_2 and lipoxygenase, the lipid peroxidation was greatly enhanced at the initial stages of incubation but subsequently decreased (Curve B). Results also indicated that lipoxidation induced by lipoxygenase and H_2O_2 was completely inhibited by 15 mM EDTA (Curve C).

The effect of lipoxygenase on the activity of synaptosomal ($Na^+ + K^+$)-ATPase and acetylcholinesterase

The enzymic activities of ($Na^+ + K^+$)-ATPase and acetylcholinesterase were tested after treating the membrane system with lipoxygenase. The degree of inactivation of ($Na^+ + K^+$)-ATPase in synaptosomal membranes after lipoxygenase–H_2O_2 treatment at various time periods of preincubation is shown in Fig. 2. The rate of inactivation became slower after 10 min of preincubation. Fig. 3 shows that the activity of ($Na^+ + K^+$)-ATPase is increasingly inactivated by increasing concentrations of lipoxygenase. However, no further inactivation could be achieved when the lipoxygenase reached a concentration of 0.2 mg/ml. Treatment of the synaptic membranes with lipoxygenase–H_2O_2 did not show any significant alteration of the acetylcholinesterase activity (Fig. 4).

A kinetic study on the ($Na^+ + K^+$)-ATPase reaction was performed with respect to the concentration of Na^+ and K^+. At a fixed concentration of Na^+ (100 mM) and ATP (2.5 mM), results indicated that the activation of ($Na^+ + K^+$)-ATPase by K^+ followed the Michaelis–Menten form (Fig. 5). The concentration of K^+ required for half maximal rate of ATP hydrolysis was $3.45 \cdot 10^{-3}$ M. However, a higher K^+ concentration was required for the half maximal rate of action after lipoxygenase–H_2O_2 treatment. At a fixed concentration of K^+ (20 mM) and ATP (2.5 mM), the activation of ($Na^+ + K^+$)-ATPase by Na^+ gave an apparently linear relationship which was maintained both before and after lipoxygenase–H_2O_2 treatment (Fig. 6).

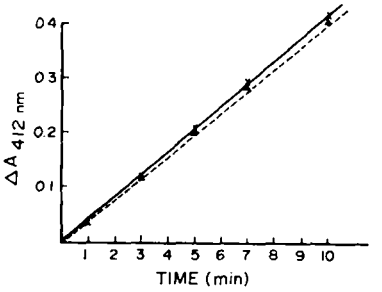


Fig. 4. The time course for acetylcholinesterase before and after lipoxygenase–H_2O_2 treatment. Synaptosomal membranes (0.2 mg protein) were treated with lipoxygenase (0.2 mg) and H_2O_2 ($8.8 \cdot 10^{-6}$ M) at 37 °C for 30 min in the preincubation system as described in the legend of Fig. 2. Control membranes were treated the same way except no lipoxygenase and H_2O_2 were added to the preincubation system. Both the lipoxygenase–H_2O_2 treated and control membranes were assayed for acetylcholinesterase activity according to Ellman *et al.*[41]. The amount of acetylthiocholine ester hydrolyzed is expressed as the increment of absorbance at 412 nm. ×—×, control; ▲---▲, lipoxygenase–H_2O_2 treated.

Both K_m and V of the enzyme for Na^+, were altered after lipoxygenase–H_2O_2 treatment (Fig. 6).

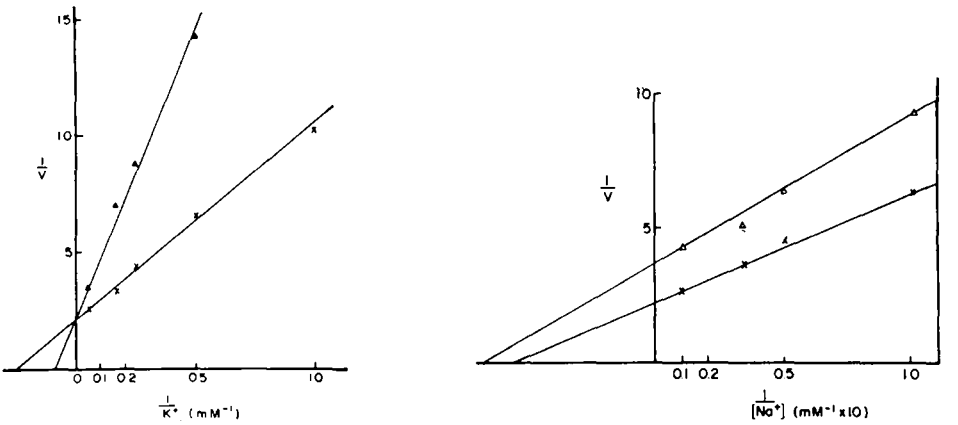


Fig. 5. The effect of lipoxygenase–H_2O_2 treatment on the affinity of $(Na^+ + K^+)$-ATPase for K^+. The control and lipoxygenase–H_2O_2 treated membranes were obtained as described in the text. except that the whole system was increased 10-fold to obtain more pretreated membrane materials. Thus, the membranes containing 2.0 mg protein were treated with or without lipoxygenase (2.0 mg)–H_2O_2 at 37 °C for 30 min in the preincubation system. The membranes were assayed for $(Na^+ + K^+)$-ATPase at various concentrations of K^+ in the incubating medium with a fixed concentration of Na^+ and ATP at 100 mM and 2.5 mM, respectively. The rates (v) are expressed in terms of relative amount of P_i hydrolyzed from ATP/min per mg protein. ×—×, control; ▲—▲, lipoxygenase–H_2O_2 treated.

Fig. 6. The effect of lipoxygenase–H_2O_2 treatment on the affinity of $(Na^+ + K^+)$-ATPase for Na^+. The control and lipoxygenase–H_2O_2 treated membranes were obtained as described in the legend of Fig. 5. The membranes were assayed for $(Na^+ + K^+)$-ATPase at various concentrations of Na^+ in the incubation medium with a fixed concentration of K^+ and ATP at 20 mM and 2.5 mM respectively. The rates (v) are expressed in terms of the relative amount of P_i hydrolyzed from ATP/min per mg protein. ×—×, control; △—△, lipoxygenase–H_2O_2 treated.

The effect of peroxidized fatty acids on $(Na^+ + K^+)$-ATPase

Upon treatment of the membrane with lipoxygenase–H_2O_2, 55 % of the $(Na^+ + K^+)$-ATPase activity was lost and this corresponded to 3.85 nmoles of malonaldehyde formed after preincubation (Table II). To further study the possible effects of the peroxidized lipids on $(Na^+ + K^+)$-ATPase activity, linolenic and oleic acids were emulsified in bovine serum albumin and incubated with the lipoxygenase–H_2O_2 system. After the incubation, 13.3 nmoles of malonaldehyde was formed with linolenic acid as substrate and no malonaldehyde was detected when oleic acid was used as substrate. The peroxidized lipids were added to the membrane suspension, incubated for 30 min at 37 °C and assayed for $(Na^+ + K^+)$-ATPase activity (Table II). Results showed that the addition of peroxidized linolenic or oleic acids to the system did not significantly affect the $(Na^+ + K^+)$-ATPase activity in the membrane preparation. Similar results were also obtained when untreated linolenic or oleic acids were added to the system.

Changes in fatty acid composition of synaptic membrane after lipoxygenase–H_2O_2 treatment

A study of the chemical composition of the synaptic membrane revealed that

TABLE II

EFFECT OF PEROXIDIZED FATTY ACIDS ON $(Na^+ + K^+)$-ATPase

The conditions for preincubation were the same as described in Table I. After 30 min of preincubation at 37 °C, half of the membrane suspension (containing 0.1 mg protein) was taken for the thiobarbituric acid test as described under Methods and Materials. The remaining membrane suspension was treated with EDTA (15 mM), centrifuged, resuspended and assayed for $(Na^+ + K^+)$-ATPase activity. The "peroxidized" fatty acids were generated by treating the acid (0.3 mg) with lipoxygenase–H_2O_2 system as described in the legend of Fig. 1 except that the lipoxidation reaction was stopped by EDTA (15 mM). "Native" fatty acids were treated the same way in the absence of lipoxygenase and H_2O_2. The "oxidized" or "native" fatty acid was added to the preincubation medium containing membrane preparation (0.2 mg protein). After 30 min of preincubation at 37 °C, the membrane suspension was then centrifuged, resuspended and assayed for $(Na^+ + K^+)$-ATPase activity.

Expt No.	*Treatment*	*Malonaldehyde formed in preincubation medium (nmoles)*	*$(Na^+ + K^+)$-ATPase activity (% of control)*
1	Control membranes	0.00	100
2	Lipoxygenase–H_2O_2 treated membranes	3.85	45.3
3	Membranes treated with "peroxidized" linolenic acid (0.3 mg)	13.30	94.1
4	Membranes treated with "native" linolenic acid (0.3 mg)	4.10	95.3
5	Membranes treated with "peroxidized" oleic acid (0.3 mg)	0.00	97.5
6	Membranes treated with "native" oleic acid (0.3 mg)	0.00	97.1

TABLE III

CHANGES IN FATTY ACID COMPOSITION AFTER LIPOXYGENASE TREATMENT

Membranes containing 2.0 mg of protein were treated with lipoxygenase (2.0 mg)–H_2O_2 at 37 °C for 30 min as described in Methods and Materials. Lipoxidation reaction was stopped by EDTA and the membranes were obtained by centrifuging at 43000 × *g* for 20 min. Membrane lipids were extracted by chloroform–methanol (2:1, v/v). Analysis of the fatty acid composition was described in Methods and Materials.

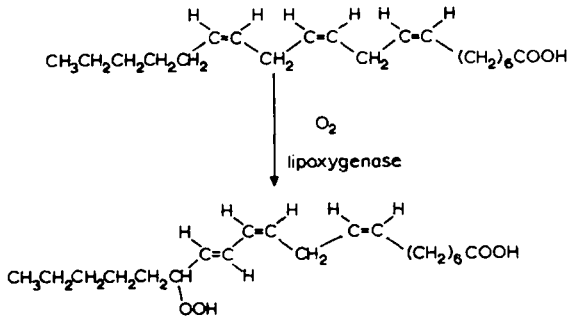
Fatty acid	*Percent of the total fatty acids*	
	Control ($n = 4$)	*Lipoxygenase–H_2O_2-treated ($n = 2$)*
16:0	18.62 ± 1.40	24.25 ± 0.66
18:0	25.23 ± 1.01	24.71 ± 1.09
18:1	20.53 ± 0.29	21.90 ± 0.21
20:4	6.79 ± 0.26	5.23 ± 0.12
22:4	6.16 ± 0.30	5.44 ± 0.12
22:6	22.57 ± 0.58	18.32 ± 0.28

the membrane fraction has a lipid:protein ratio of 1.19. The lipids were mainly phospholipids and cholesterol. The phospholipids were rich in unsaturated fatty acids, especially the long-chain polyunsaturated fatty acids such as 22:6(*n*-3) (A. Y. Sun and G. Y. Sun, unpublished data). Analysis of the fatty acid composition of synaptic membranes by gas–liquid chromatography before and after lipoxygenase–H_2O_2 treatment showed that a small proportion of the polyunsaturated fatty acids

in the membrane was affected by lipoxygenase treatment (Table III). The percentage of 20:4, 22:4 and 22:6 acyl groups in lipoxygenase–H_2O_2 treated membranes was lower than in control preparations indicating that some of the polyunsaturated fatty acids originally present in the synaptic membranes could be converted to lipid peroxides and other oxidative products during the lipoxygenase–H_2O_2 treatment.

DISCUSSION

Lipoxygenase is a dioxygenase capable of stereospecific attack on selected fatty acid residues[21]. The primary products after peroxidation are optically active *cis,trans* diene hydroperoxides as shown below:



According to Tappel[26], this reaction sequence may include a highly reactive free radical intermediate. Addition of a low concentration of H_2O_2 may induce the formation of the intermediate and thus accelerates lipid peroxidation. H_2O_2 itself is too stable to generate free radicals. In the presence of the olefinic groups or transition metals such as heme iron, free radicals can be produced at a much faster rate through molecular-induced homolysis or one-electron redox reaction[27]. In most cases, a variety of carbonyl-containing residues are produced in this type of multiple chain cleavage. However, some of these reaction products do not form malonaldehyde. Thus, a decrease in malonaldehyde formation may occur after prolonged incubation of linolenic acid with the lipoxygenase–H_2O_2 system (Fig. 1). A similar pattern has been demonstrated by Tam and McCay[13] in a microsomal enzyme system.

Although lipoxidative products have been shown to inhibit glycolysis and respiration[25], the resulting inactivation of $(Na^+ + K^+)$-ATPase by lipoxygenase–H_2O_2 cannot be explained as due to the toxic effects of the lipoxidative products. The present study showed that more peroxides were formed from the incubation of linolenic acid with lipoxygenase–H_2O_2 than from the membrane system *per se* as indicated in the thiobarbituric acid test (Table II). However, addition of the peroxidized linolenic acid to the membrane system did not result in an appreciable loss of $(Na^+ + K^+)$-ATPase activity. Since long-chain fatty acids have been demonstrated to inhibit $(Na^+ + K^+)$-ATPase of rat brain[28], the small degree of inactivation shown in Table II may be due to the residual effect of free linolenic and oleic acids adhered to the membranes.

Results from previous experiments have indicated that the activity of $(Na^+ + K^+)$-ATPase is dependent on the structural integrity of the membrane. Inhibitions

of the enzyme with alcohols[1,29] as well as by phospholipase treatment[2,3] have provided support for a close relationship between the arrangement of lipid molecules and membrane function. The present results indicate also that the active transport system is further dependent on the degree of unsaturation of the hydrophobic side chains. It has been reported that the permeability of membranes depends on the degree of unsaturation of the membrane lipids[30–33]. Furthermore, it is probable that there is a high requirement of polyunsaturated fatty acids for the synaptosomal membranes in order to maintain a liquid crystalline state which facilitates translocation of small molecules across the membranes[32]. Analysis of fatty acid composition of the membrane preparation did in fact indicate that the membrane contained very high proportions of polyunsaturated fatty acids (A. Y. Sun and G. Y. Sun, unpublished data). However, treatment with lipoxygenase–H_2O_2 which caused a 60% inhibition of the ($Na^+ + K^+$)-ATPase activity, resulted only in a small decrease in 20:4, 22:4 and 22:6 acyl groups of the membrane. Evidently, not all of the polyunsaturated fatty acids of the synaptic membrane are involved in active ion transport.

We have previously reported that phospholipase A preferentially attacks the fatty acids linked to the phospholipid molecules at the β-glycerol position causing a complete inactivation of synaptosomal ($Na^+ + K^+$)-ATPase[3]. Structural analysis of phospholipid molecules by several investigators have shown that most polyunsaturated fatty acids are linked to the β position of the glycerol moiety[35,36]. The present results are in good agreement with the reported inhibitory action of phospholipase A on ($Na^+ + K^+$)-ATPase. The polyunsaturated fatty acids at the β position of phospholipids may play an important role in the mechanism of active transport. However, increased concentration of the lipoxygenase did not produce further inhibition of the enzymic system. Perhaps some of the polyunsaturated fatty acids associated with the active sites are not readily accessible to lipoxidation.

Peroxidation of polyunsaturated fatty acids at the active sites of ion transport may affect either the binding affinity of substrates or the pore size through which Na^+ or K^+ are being transported. Results of the kinetic studies have suggested that the primary action of lipoxygenase is on the K^+ site; whereas changes in both apparent K_m and V of the enzyme with respect to Na^+ after lipoxygenase–H_2O_2 treatment is due to an allosteric effect on the enzyme system. Since the activation of ($Na^+ + K^+$)-ATPase by K^+ follows an apparent Michaelis–Menten formulation (Fig. 5), it is apparent that ($Na^+ + K^+$)-ATPase may operate through a carrier mechanism with ($Na^+ + K^+$)-ATPase itself functioning as a carrier protein[34]. Thus, lipoxygenase–H_2O_2 treatment may cause either (1) a change in the conformation of the carrier protein or (2) a change in the microenvironment of the K^+ channel. In view of the fact that ($Na^+ + K^+$)-ATPase is a membrane-bound protein, the first possibility appears reasonable because a change in the enzymic conformation due to an alteration of the hydrophobic bonds between the protein and phospholipid of the synaptic membrane may render the protein less efficient in carrying K^+ across the membrane. However, the second possibility seems more likely based on the relationship between the action of enzyme and ion transport. A cyclic conformational change of the membrane-bound ($Na^+ + K^+$)-ATPase has been suggested during the active state of enzymic action[4,37,38]. They have suggested also that ($Na^+ + K^+$)-ATPase is bound across the cell membrane with inward affinity for Na^+ and outward affinity for K^+. The steric conversion caused by the hydrolysis of ATP may be accom-

panied by the eversion of pore openings in the membrane[39]. The polyunsaturated fatty acids may be located in a position which may facilitate the eversion of a pore containing the K^+–ligand complex. More experiments are currently in progress in an attempt to isolate the enzyme in a pure form so that physicochemical studies may be conducted.

In view of the high proportion of polyunsaturated fatty acids in synaptosomal membrane and the presence of NADPH dependent microsomal oxidase system in living tissues[13], it is suggested that the peroxidation of membrane may occur as a consequence of irradiation or some other conditions which may accelerate free radical formation. Other subcellular membranes which contain high proportions of unsaturated fatty acid moieties are also subject to free radical attack. Consequently, the synaptic membrane system has provided a unique model for the study of the mechanism of peroxidative damage to membranes in brain.

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