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# SOLUBILIZATION OF ADENYLATE CYCLASE OF BRAIN MEMBRANES BY LIPID PEROXIDATION

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**Adenylate cyclase in the membrane fractions of bovine and rat brains, but not in rat liver plasma membranes, was solubilized by treatment with $Fe^{2+}$ (10 $\mu$M) plus dithiothreitol (5 mM). Solubilization of the enzyme by these agents was completely prevented by simultaneous addition of *N,N'*-diphenyl-*p*-phenylenediamine (DPPD), an inhibitor of lipid peroxidation. Ascorbic acid also solubilized the enzyme from the brain membranes. Lipid peroxidation of the brain membranes was characterized by a selective loss of phosphatidylethanolamine. Solubilization of membrane-bound enzymes by $Fe^{2+}$ plus dithiothreitol was not specific for adenylate cyclase, because phosphodiesterase, thiaminediphosphatase and many other proteins were also solubilized. Solubilized adenylate cyclase had a high specific activity and was not activated by either NaF, 5'-guanylyl imidodiphosphate (Gpp[NH]p) or calmodulin. These results suggested that lipid peroxidation of the brain membranes significantly solubilized adenylate cyclase of high specific activity.**

## Introduction

Lipid peroxidation of the membrane is thought to be associated with damage to the membrane structure of subcellular particles and inactivation of membrane-bound enzymes [1,2]. However, several membrane-bound enzymes are known to be activated by lipid peroxidation, e.g., phospholipase $A_2$ [3] and glucuronyltransferase [4] in liver membranes and thiaminediphosphatase in rat brain microsomes [5].

In previous study, we found that lipid peroxidation of the membranes caused marked stimulation of adenylate cyclase in the synaptic membranes, but not in the peripheral membranes, with concomitant decrease in turbidity of the synaptic membrane suspension [6,7]. These findings led us to examine the possibility of adenylate cyclase being solubilized by lipid peroxidation. Solubilization of adenylate cyclase, an integral membrane protein, can be achieved by non-ionic detergents such as Triton X-100 and Lubrol-PX, and thereby some characteristics of the solubilized enzyme were studied [8–10]. However, there has been no way to solubilize adenylate cyclase in the absence of the detergent. In the present study, we solubilized adenylate cyclase from the membrane fractions of bovine and rat brains by lipid peroxidation, and studied some characteristics of the solubilized enzyme.

## Materials and methods

*Materials.* [G-$^3$H]cyclic AMP (36.4 Ci/mmol) was purchased from New England Nuclear; di-

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Abbreviations: DPPD, *N,N'*-diphenyl-*p*-phenylenediamine; Gpp[NH]p, 5'-guanylyl imidodiphosphate.

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thiothreitol was from Boehringer Mannheim; DPPD was from Tokyo Kasei; Membrane filter (0.45 $\mu$m pore) was from Millipore Co. All other reagents were of the highest analytical grade available.

*Preparation of brain membranes.* Bovine cerebral cortex was homogenized with 5 vol. of 10 mM Tris-HCl (pH 7.4) containing 2 mM EGTA. The homogenate was centrifuged 20000 $\times$ *g* for 30 min at 2°C. The supernatant was discarded, and the pellet was suspended in 10 mM Tris-HCl (pH 7.4). The centrifugation step was repeated twice, and the final pellet was suspended in the same buffer for the assay. Synaptic membranes and liver plasma membranes of rat were also prepared as described previously [7].

*Solubilization of brain membranes.* The membrane preparations were incubated with 80 mM Tris-maleate (pH 7.4), 8 mM $MgSO_4$, 10 $\mu$M $Fe^{2+}$ and 5 mM dithiothreitol. The concentration of $Fe^{2+}$ and dithiothreitol was optimal to solubilize adenylate cyclase. Unless otherwise indicated, the reaction mixture was incubated at 30°C for 20 min. Then, the mixture was immediately centrifuged at 104000 $\times$ *g* for 60 min. Enzyme activities in the resulting supernatant were determined and were referred to as solubilized enzymes, because the enzyme activities in this fraction were not sedimented by further centrifugation (104000 $\times$ *g* for 2 to 3 h). Bovine brain membranes were also solubilized by Lubrol-PX by the method of Toscano et al. [11]. Calmodulin was purified from bovine brain by the method of Wang and Desai [12]. Membranes and solubilized proteins were analysed on SDS-polyacrylamide slab gel electrophoresis [13] with slight modifications [14].

*Assays.* The activities of adenylate cyclase, 5'-nucleotidase, phosphodiesterase, acetylcholinesterase and ATPase were determined as described previously [7]. Protein was measured by the method of Bennet [15]. Lipid peroxidation was measured by thiobarbituric acid reaction as described previously [7]. The lipids were extracted from the membranes by the method of Bligh and Dyer [16]. Phospholipids were separated by thin-layer chromatography [17], then the content of lipid phosphorus was determined [18].

## Results

Fig. 1 shows that the treatment of $Fe^{2+}$ plus dithiothreitol solubilizes adenylate cyclase from the membranes of bovine brain. Solubilization ratio of the enzyme depends on the protein concentration of the membranes; 70% of the total enzyme activity and the highest specific activity in the supernatant fraction were observed at 200 $\mu$g protein of membrane per ml. Both solubilization of the enzyme and the membrane proteins were decreased at protein concentration of more than 200 $\mu$g/ml of the reaction mixture (Fig. 1b).

Solubilization of adenylate cyclase and the membrane proteins by $Fe^{2+}$ plus dithiothreitol was dependent on incubation time (Fig. 2). Even during 5 min of incubation, a significant enzyme activity and proteins were appeared in the supernatant fraction. A maximum solubilization of the enzyme was seen at 15 to 20 min of incubation. Lipid peroxidation by $Fe^{2+}$ plus dithiothreitol of the membranes, which was determined by the formation of malondialdehyde, preceded the solubilization of adenylate cyclase and membrane proteins (Fig. 2). DPPD, an inhibitor of lipid peroxidation, blocked solubilization of the enzyme by $Fe^{2+}$ plus dithiothreitol. In the absence of these

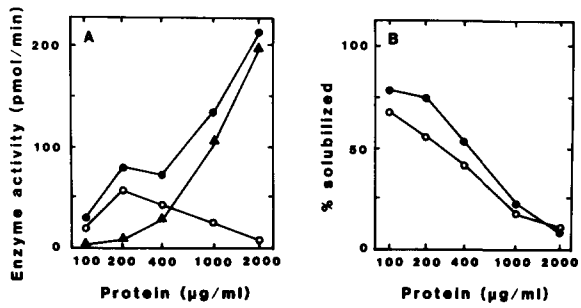


Fig. 1. Solubilization of bovine brain adenylate cyclase by $Fe^{2+}$ plus dithiothreitol. Membrane pellets of various protein concentrations from bovine brain were treated with 10 $\mu$M $Fe^{2+}$ plus 5 mM dithiothreitol as described in the Methods. After the incubation was stopped, the suspension was centrifuged, and the resulting pellet and the supernatant was used for adenylate cyclase assay as described in Methods. (A) Total enzyme activity (pmol/min). ●, Pellet + supernatant; ○, supernatant; ▲, pellet. (B) Percent solubilization. ●, Adenylate cyclase activity; ○, protein.

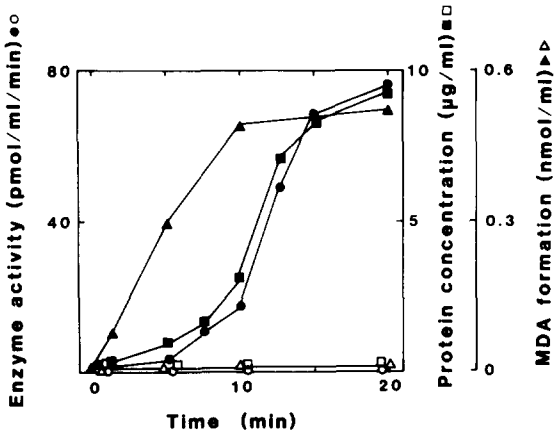


Fig. 2. Time course of solubilization of adenylate cyclase and of malondialdehyde formation. Bovine brain membranes were incubated with 10 μM $Fe^{2+}$ plus 5 mM dithiothreitol in the presence and absence of DPPD (0.1 μg/ml), an inhibitor of lipid peroxidation. After the incubation was stopped, the suspension was centrifuged. The resulting supernatant was used for assays of adenylate cyclase (●,○), malondialdehyde (▲,△) and protein (■,□) as described in the Methods. ●,▲,■; −DPPD, ○,△,□; +DPPD.

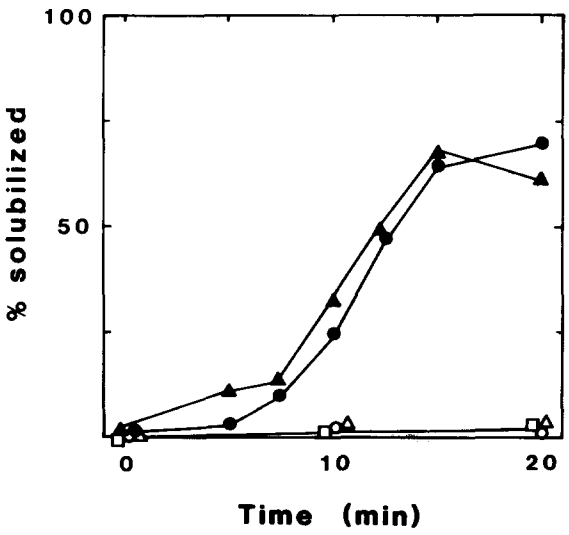


Fig. 3. Solubilization of thiaminediphosphatase and phosphodiesterase by $Fe^{2+}$ plus dithiothreitol from bovine brain membranes. Experiments were done at the same condition as that for Fig. 2. ▲, Thiaminediphosphatase; ●, phosphodiesterase; △, acetylcholinesterase; ○, 5′-nucleotidase; □, ATPase.

agents, the enzyme and proteins were not significantly solubilized.

Solubilization of the enzyme from bovine brain membranes by $Fe^{2+}$ plus dithiothreitol was not specific for adenylate cyclase, because phosphodiesterase and thiaminediphosphatase activities were also detected in the supernatant. The time-course of the solubilization of these enzyme was similar to that of adenylate cyclase (Figs. 2 and 3). However, other enzyme activities such as 5′-nucleotidase, acetylcholinesterase and ATPase were not significantly solubilized (Fig. 3). Moreover, $Fe^{2+}$ plus dithiothreitol also solubilized adenylate cyclase from crude synaptic membranes of rat cerebral cortex, but not from the liver plasma membranes (Table I). Total enzyme activity (pellet + supernatant) increased by about 7-fold after the treatment of $Fe^{2+}$ plus dithiothreitol. Specific activity of solubilized adenylate cyclase was 9-times as high as that of intact membranes. Ascorbic acid (0.1 mM), an inducer of lipid peroxidation, also solubilized the enzyme from crude synaptic mem-

TABLE I

SOLUBILIZATION OF PROTEINS AND ADENYLATE CYCLASE FROM THE MEMBRANES OF RAT BRAIN AND LIVER

Brain $M_1$ fraction (3.5 mg) and liver plasma membranes (3.9 mg) were incubated as described in the Methods in a total volume of 20 ml with $Fe^{2+}$ plus dithiothreitol (DTT) and ascorbic acid (0.1 mM) for 10 and 20 min, respectively. (a) Total activity (pmol/min). (b) Specific activity (pmol/mg protein/min). P, pellet; S, supernatant. * Not detected (less than 0.5 pmol/min).

| | | Protein (mg) | Adenylate cyclase | |
|---|---|---|---|---|
| | | | (a) | (b) |
| Brain | | | | |
| Control | P | 3.09 | 235.8 | 76.3 |
| | S | 0.43 | 4.1 | 9.5 |
| $Fe^{2+}$ + DTT | P | 1.62 | 368.1 | 227.2 |
| | S | 1.75 | 1247.2 | 712.7 |
| Ascorbate | P | 2.69 | 462.4 | 171.9 |
| | S | 0.79 | 41.3 | 52.3 |
| Liver | | | | |
| Control | P | 3.41 | 30.0 | 8.8 |
| | S | 0.51 | n.d. * | – |
| $Fe^{2+}$ + DTT | P | 3.41 | 27.5 | 8.1 |
| | S | 0.51 | n.d. * | – |

TABLE II

EFFECTS OF VARIOUS STIMULANTS ON THE ACTIVITIES OF ADENYLATE CYCLASE SOLUBILIZED FROM BOVINE BRAIN MEMBRANES

DTT, dithiothreitol.

| | Adenylate cyclase (pmol/mg protein per min) | | |
|---|---|---|---|
| | Intact membranes | Lubrol-PX-solubilized | $Fe^{2+}$ + DTT-solubilized |
| Control | 35.2 | 158.5 | 128.3 |
| NaF (10 mM) | 85.6 | 445.4 | 127.0 |
| Gpp[NH]p (0.1 mM) | 63.7 | 590.0 | 130.7 |
| Calmodulin (50 μg/ml)+$Ca^{2+}$ | 52.7 | 222.3 | 113.6 |

branes, although its effect was much less than that of $Fe^{2+}$ plus dithiothreitol.

It is well known that adenylate cyclase activity is stimulated by NaF, Gpp[NH]p and calmodulin. As shown in Table II, these stimulants increased the enzyme activities in intact membranes and Lubrol-PX-solubilized fraction from bovine brain. In contrast, the solubilized enzyme by $Fe^{2+}$ plus dithiothreitol was not activated by these stimulants (Table II).

TABLE III

EFFECT OF $Fe^{2+}$ PLUS DITHIOTHREITOL (DTT) ON PHOSPHOLIPID COMPOSITIONS OF RAT CEREBRAL CORTEX $M_1$ FRACTION

Values are means ± S.E. of four separate experiments.

| Phospholipids | μg/mg protein | |
|---|---|---|
| | Control | $Fe^{2+}$ + DTT |
| Lysophosphatidylcholine | 0.5 [a] | 1.0±0.1 |
| Sphingomyelin | 2.3±0.9 | 3.4±0.4 |
| Phosphatidylcholine | 25.5±3.2 | 26.5±2.2 |
| Phosphatidylinositol | 2.7±0.5 | 1.7±0.5 |
| Phosphatidylserine | 5.3±1.1 | 4.5±0.3 |
| Phosphatidylethanolamine | 27.9±1.0 | 16.4±0.6 [b] |
| Phosphatidylglycerol | 0.8±0.4 | 0.5±0.1 |
| Phosphatidic acid | 1.3±0.6 | 0.3 [a] |
| Cardiolipin | 1.5±0.5 | 2.2 [a] |
| Unknown | 2.2±0.2 | 4.1±0.4 |
| Total phospholipids | 68.7 | 57.6 |
| Cholesterol/protein | 0.11 | 0.18 |
| Phospholipids/cholesterol | 0.79 | 0.53 |

[a] Means of two determinations.
[b] $P < 0.01$.

To obtain the information on the state of the lipid environment of rat brain membranes after the treatment of $Fe^{2+}$ plus dithiothreitol, phospholipid compositions of the membranes were determined (Table III). Lipid peroxidation of the brain membranes resulted in a specific loss of the total phosphatidylethanolamine. In the liver plasma membranes, lipid peroxidation was accompanied by significant decrease in phosphatidylethanolamine and phosphatidylcholine (data not shown). Solubilized proteins from the brain membranes were then analyzed by SDS-polyacrylamide slab gel electrophoresis. Results showed that many of the membrane proteins were solubilized (data not shown).

## Discussion

In the present study, we found that adenylate cyclase was considerably solubilized from the membrane fractions of bovine and rat brains by $Fe^{2+}$ plus dithiothreitol which induced lipid peroxidation of synaptic membranes and the following activation of adenylate cyclase [7]. Solubilization of the enzyme by $Fe^{2+}$ plus dithiothreitol was dependent on the incubation time and the protein concentration of the reaction mixture. $Fe^{2+}$ plus dithiothreitol did not solubilize the enzyme when DPPD, an inhibitor of lipid peroxidation, was present in the reaction mixture. Ascorbic acid, an other inducer of lipid peroxidation, also solubilized the enzyme, though it was less effective than $Fe^{2+}$ plus dithiothreitol. These results indicate that lipid peroxidation of the brain membranes is

followed by solubilization of adenylate cyclase.

It should be pointed out that solubilization of membrane-bound enzymes by lipid peroxidation was not restricted to adenylate cyclase; thiamine-diphosphatase, phosphodiesterase and many other membrane proteins were also released into the supernatant. There was no previous study showing that lipid peroxidation of the membranes resulted in solubilization of membrane-bound enzymes. Tappel [19] described some of the damaging biochemical reactions in the membranes induced by lipid peroxidation; propagation of the free radical chain reaction through the polyunsaturated fatty acids of phospholipid in the membranes was followed by polymerization, cross-linking of enzymes and production of structural abnormalities in the membranes. Degradation of brain membrane structure by lipid peroxidation was previously detected by a decrease in the turbidity of the membrane suspension [7]. Thus, it is speculated that changes in lipid-protein interaction of nerve membranes induced by lipid peroxidation release adenylate cyclase and other membrane proteins. Specific reduction in phosphatidylethanolamine content in the brain membranes might reflect its interaction to adenylate cyclase in the brain membranes (Table III). This result was in good accordance with the recent study of showing that $Fe^{2+}$ and ascorbate-induced lipid peroxidation was accompanied by specific degradation of phosphatidylethanolamine [20]. Lipid peroxidation of the liver plasma membranes did not solubilized adenylatye cyclase, though it significantly reduced phosphatidylethanolamine and phosphatidylcholine contents in the liver plasma membranes. Thus, it is difficult to correlate the solubilization of adenylate cyclase to the changes in the membrane lipid composition.

It has been demonstrated that adenylate cyclase was solubilized by non-ionic detergents from rat brain. In general, adenylate cyclase solubilized by Lubrol-PX as well that of intact membranes is activated by NaF, Gpp[NH]p and calmodulin [10,22]. As shown in Table II, similar results were obtained in the present study. Nevertheless, these stimulants did not activate the solubilized adenylate cyclase obtained by lipid peroxidation. In the previous study, we found that adenylate cyclase in $Fe^{2+}$ plus dithiothreitol-treated membrane suspension (pellet + supernatant) was activated by NaF, Gpp[NH]p and calmodulin [7]. Further experiments are required to characterize the solubilized adenylate cyclase by lipid peroxidation.

It should be notable that adenylate cyclase was solubilized without detergents. Usually, aggregation of solubilized adenylate cyclase was accompanied by inactivation of the enzyme [8]. Although the solubilized adenylate cyclase by lipid peroxidation had a high specific activity the enzyme might be aggregated, because the solubilized enzyme activity was retained on Sepharose 6B column only when 20% ethyleneglycol was present in the elution medium (Baba, A., unpublished data).

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