The relationship between adenylate cyclase activity in the synaptic membrane fraction (M1) of rat brain and lipid peroxidation of these membranes was examined. In the presence of 5 mM dithiothreitol (DTT), 1 to 10 mM Fe2+ activated adenylate cyclase 2- to 4-fold. Of several metal ions, Fe2+ was the most effective. Other enzymes in M1, such as Mg2+-ATPase, (Na+—K+)-ATPase, 5'-nucleotidase, acetylcholinesterase, and phosphodiesterase, were not activated by Fe2+ plus DTT. Activation of adenylate cyclase by Fe2+ plus DTT was accompanied by production of malondialdehyde, a product of lipid peroxidation. Formation of malondialdehyde was completely parallel with enzyme activation. Ascorbic acid or a NADPH system also stimulated enzyme activity and caused lipid peroxidation. Activation of the enzyme and lipid peroxidation induced by Fe2+ plus DTT, ascorbic acid, or NADPH was completely prevented by simultaneous addition of NaF, N,N'-diphenyl-p-phenylenediamine, an inhibitor of lipid peroxidation. This inhibitor also prevented the decrease in turbidity of the enzyme preparation induced by Fe2+ plus DTT. The stimulatory effects of NaF, guananyl-5'-imidodiphosphate and calmodulin, respectively, and that of Fe2+ plus DTT on the enzyme activity were additive. Activation of adenylate cyclase by Fe2+ plus DTT was only observed in brain synaptic membranes, not in erythrocyte ghosts, liver plasma membranes, or cardiac sarcolemma. These results indicate that lipid peroxidation of synaptic membranes was accompanied by specific stimulation of adenylate cyclase activity.

It is well established that the activity of adenylate cyclase is stimulated by hormones (1, 2), neurotransmitters (3) interacting with their receptors, and calmodulin (4, 5). Adenylate cyclase activity is also affected by changes of the membrane lipid environment. Increasing the level of cholesterol (6) or of certain phospholipids (7) in membranes was accompanied by an increase of basal adenylate cyclase activity, while depleting membranes of essential fatty acids (8) caused a decrease in the basal activity. Phospholipids may also be involved in the receptor-mediated activation of adenylate cyclase by glucagon (9, 10) and norepinephrine (11). Moreover, adenylate cyclase activity can be influenced by the ordering of membrane lipids (12). These observations suggest that the organization of membrane lipids may play an important role in regulation of the adenylate cyclase system.

Lipid peroxidation in the membrane is thought to be associated with damage to the membrane structure of subcellular particles and inactivation of membrane-bound enzymes (13, 14). (Na+—K+)-ATPase in neural membranes is also inhibited by lipid peroxidation (15). Previously, we found that Cu2+ activated adenylate cyclase of rat brain in the presence of dithiothreitol and caused lipid peroxidation. In subsequent experiments, we found that Fe2+ was more effective than Cu2+ both in stimulation of the enzyme activity and in lipid peroxidation. Here, we report that lipid peroxidation of synaptic membranes is accompanied by stimulation of adenylate cyclase and that this effect is not observed in plasma membranes of peripheral tissues.

**EXPERIMENTAL PROCEDURES**

Materials—[G-T-H]cAMP (34.4 Ci/mmol) and [2,8-3H]ATP (34.8 Ci/mmole) were purchased from New England Nuclear; 3-isobutyl-1-methylxanthine, glucagon, isoproterenol, Gpp(NH)p, and NADPH were from Sigma; DTT was from Boehringer Mannheim; DPPD was from Tokyo Kasei. All other reagents were of the highest analytical grade available.

Preparation of Enzyme—Male Sprague-Dawley rats, weighing about 150 to 250 g, were killed by decapitation. The cerebral cortex was immediately removed and homogenized with 9 volumes of 0.32 m sucrose (containing 2 mM Tris, 2 mM maleic acid, and 2 mM EGTA, pH 7.4). The crude synaptic membrane fraction (M1) was prepared by the method of De Hobertis et al. (16). Excess EGTA was removed by suspending the M1 pellet in 0.032 m sucrose (containing 2 mM Tris-maleate buffer, pH 7.4) and centrifuging it. This washing procedure was repeated 3 times. The final pellet was suspended in EGTA-free buffer and used as an enzyme source. In some experiments, the M1 fraction was layered on a sucrose gradient (16) to obtain synaptic membranes, M0.9. For comparison, liver plasma membranes (17), cardiac sarcolemma (18), and erythrocyte ghosts (19) were also prepared from rats for assay of adenylate cyclase activity.

Assay of Enzymes—The standard assay medium for measurement of adenylate cyclase activity consisted of 80 mM Tris-maleate buffer (pH 7.4), 0.5 mM ATP, 8 mM MgSO4, 1 mM 3-isobutyl-1-methylxanthine, and the M1 fraction (about 100 pg of protein) in a total volume of 0.5 ml. Unless otherwise indicated, the reaction mixture, without ATP, was preincubated at 30°C for 10 min. The reaction was started by adding ATP and carried out at 30°C for 2.5 min. Metal ions and other compounds were added at zero time of preincubation. The reaction was stopped by placing the tube in boiling water for 3 min. The amount of cAMP formed in each tube was measured by the protein binding method (20) as described previously (21). Preliminary experiments showed that Fe2+ and the other agents used in the present study had no significant effect on values in the protein-binding method. In the absence of an ATP-regenerating system, less than 20% of ATP, added in the reaction mixture, was hydrolyzed during 2.5 min of incubation under standard conditions. Addition of 10 mM Fe2+ plus 5 mM DTT reduced the hydrolysis of ATP to about 10%. In some experiments, adenylate cyclase activity was also determined in the presence of an ATP-regenerating system using [3H]ATP as described by Lynch et al. (22) and [14C]cAMP was assayed by the method of Krishna et al. (23).

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1. A. Baba, T. Kihara, E. Lee, and H. Iwata, unpublished observation.
2. The abbreviations used are: Gpp(NH)p, guananyl-5'-imidodiphosphate; DTT, dithiothreitol; DPPD, N,N'-diphenyl-p-phenylenediamine; EGTA, ethylene glycol bis(b-aminomethyl ether)N,N',N'-tetraacetic acid.
The activities of (Na⁺-K⁺)-ATPase (24), 5'-nucleotidase (25), phosphodiesterase (26), and acetylcholinesterase (27) were also determined. Protein was measured by the method of Lowry et al. (28). Calmodulin was purified from bovine brain by the method of Wang and Desai (29).

**Assay of Lipid Peroxidation**—Lipid peroxidation was measured by the thiobarbituric acid reaction (30). The reaction mixture without ATP for assay of adenylate cyclase was mixed with 0.5 ml of 10% trichloroacetic acid after an appropriate time. It was then centrifuged and 0.5 ml of the resulting supernatant was mixed with 0.5 ml of 0.67% thiobarbituric acid and placed in boiling water for 10 min. Then the absorbance of the supernatant fluid was read at 530 nm. An extinction coefficient of A₄₃₀ = 1.56 × 10⁴ was used in calculating the amount of malondialdehyde formed (31).

**Preparation of Malondialdehyde**—Malondialdehyde, a reaction product of lipid peroxidation, was prepared (32), and its purity was determined to be 99%.

**RESULTS**

The effect of substrate concentration on the adenylate cyclase of M₁ (in the absence of an ATP-regenerating system) is shown in Fig. 1. At concentrations of more than 0.2 mM, ATP was saturating for the enzyme (Kₘ for ATP, 50 µM). Addition of 10 µM Fe²⁺ and 5 mM DTT caused a marked increase in adenylate cyclase activity which was not dependent on ATP concentration. Fe²⁺ plus DTT increased Vₘₜₐₖ of the enzyme without affecting Kₘ for ATP.

Figure 2 shows that activation of the adenylate cyclase by 10 µM Fe²⁺ plus 5 mM DTT was dependent on the preincubation time. Fe²⁺ plus DTT caused a 2-fold increase in enzyme activity without preincubation, and a 4- to 5-fold increase with preincubation for 10 to 20 min. Fe²⁺ plus DTT also caused lipid peroxidation (formation of malondialdehyde) of the enzyme preparation in a time-dependent manner (Fig. 2). Maximal lipid peroxidation was observed after incubation for 10 to 20 min. The stimulatory effects of Fe²⁺ plus DTT on the enzyme activity and on lipid peroxidation were concentration-dependent (Figs. 3 and 4). In the presence of 5 mM DTT, 10 µM Fe²⁺ elicited maximal stimulation of the enzyme activity and lipid peroxidation, and even 1 µM Fe²⁺ caused both significant activation of the enzyme and lipid peroxidation. Adenylate cyclase was not activated by Fe²⁺ or DTT alone. Both enzyme activation and lipid peroxidation were decreased at Fe²⁺ concentrations of more than 10 µM. The dose-response curves of Fe²⁺ and DTT for activation of the enzyme were parallel with those for lipid peroxidation (Figs. 3 and 4). Effect of 10 µM Fe²⁺ plus 5 mM DTT on adenylate cyclase activity was also examined with preincubation time of 10 min in the presence of an ATP-regenerating system, showing that Fe²⁺ plus DTT markedly stimulated the enzyme activity (control, 133 pmol/mg of protein/min; Fe²⁺ plus DTT, 805 pmol/mg of protein/min).

Among the metals tested, Fe²⁺ caused the most stimulation of the enzyme activity and lipid peroxidation in the presence of DTT (Fig. 5). Fe²⁺ also activated the enzyme and caused lipid peroxidation in the presence of DTT. At concentrations of 10 µM, Zn²⁺ and Cu²⁺ strongly inhibited the enzyme activity. On the contrary, Cu²⁺ stimulated the enzyme activity in the presence of DTT. Other metal ions at concentrations of 10 µM had little effect on the enzyme activity or lipid peroxidation either in the absence or presence of DTT.

Fig. 6 shows the effects of sulfur-containing agents on the enzyme activity and lipid peroxidation in the absence and presence of 10 µM Fe²⁺. DTT caused the most stimulation of the enzyme and lipid peroxidation, but GSH, cysteine, and 2-
As reported previously, disulfide compounds such as mercaptoethanol and cystamine inhibited the enzyme activity in the absence of an ATP-regenerating system and lipid peroxidation were examined as described under "Experimental Procedures." MDA, malondialdehyde.

**TABLE I**

<table>
<thead>
<tr>
<th>Experiment A</th>
<th>pmol/mg protein/min</th>
<th>MDA formation (nmol/mg/10 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>77.4</td>
<td>1.6</td>
</tr>
<tr>
<td>DPPD (0.1 μg/ml)</td>
<td>67.4</td>
<td>0.8</td>
</tr>
<tr>
<td>Fe²⁺ (10 μM) + DTT (5 mM)</td>
<td>268</td>
<td>59.1</td>
</tr>
<tr>
<td>Fe²⁺ + DTT + DPPD</td>
<td>56.7</td>
<td>3.4</td>
</tr>
<tr>
<td>Ascorbic acid (0.1 mM)</td>
<td>166</td>
<td>35.4</td>
</tr>
<tr>
<td>Ascorbic acid + DPPD</td>
<td>70.7</td>
<td>1.2</td>
</tr>
</tbody>
</table>

**Experiment B**

| None         | 74.3                | 1.4                             |
| DPPD (0.1 μg/ml) | 79.7          | 0.9                             |
| NADPH (0.1 mM) + EDTA-Fe²⁺ | 170          | 20.4                            |
| NADPH + EDTA-Fe²⁺ + DPPD | 80.9          | 0.7                             |

**TABLE II**

<table>
<thead>
<tr>
<th>Experiment A</th>
<th>pmol/mg protein/min</th>
<th>Increase over basal activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>71.3</td>
<td></td>
</tr>
<tr>
<td>Fe²⁺ (10 μM) + DTT (5 mM)</td>
<td>290</td>
<td>219</td>
</tr>
<tr>
<td>NaF (10 mM)</td>
<td>370</td>
<td>299</td>
</tr>
<tr>
<td>NaF + Fe²⁺ + DTT</td>
<td>641</td>
<td>570</td>
</tr>
<tr>
<td>Gpp(NH)p (100 μM)</td>
<td>125</td>
<td>53.6</td>
</tr>
<tr>
<td>Gpp(NH)p + Fe²⁺ + DTT</td>
<td>391</td>
<td>320</td>
</tr>
</tbody>
</table>

**Effect of Fe²⁺ plus dithiothreitol on NaF-, Gpp(NH)p-, and calmodulin-stimulated adenylate cyclase activity of the M₁ fraction of rat cerebral cortex**

Adenylate cyclase activity was determined in the absence of an ATP-regenerating system as described under "Experimental Procedures." MDA, malondialdehyde.

**FIG. 4.** Effect of the concentration of DTT on adenylate cyclase activity and lipid peroxidation in the M₁ fraction. Adenylate cyclase activity without an ATP-regenerating system (○, ○) and lipid peroxidation (●, ●) were examined as described under "Experimental Procedures" in the absence (○, ●) or presence (●, ●) of 10 μM Fe²⁺. MDA, malondialdehyde.

**FIG. 5.** Effects of metal ions on adenylate cyclase activity and lipid peroxidation in the M₁ fraction. Metal ions (10 μM) were added at 0 time of preincubation with (□) or without (□) 5 mM DTT. Adenylate cyclase activity in the absence of an ATP-regenerating system and lipid peroxidation were examined as described under "Experimental Procedures." MDA, malondialdehyde.

**FIG. 6.** Effects of sulfur-containing agents on adenylate cyclase activity and lipid peroxidation in the M₁ fraction. Sulfur-containing compounds (5 mM) were added at 0 time of preincubation with (□) or without (□) 10 μM Fe²⁺. Adenylate cyclase activity in the absence of an ATP-regenerating system and lipid peroxidation were examined as described under "Experimental Procedures." MDA, malondialdehyde.
tive when these stimulants were added with Fe^{2+} plus DTT at 0 time of preincubation. The activation of the enzyme by Fe^{2+} plus DTT is irreversible (Table III). Even when the membrane fraction was pretreated with Fe^{2+} plus DTT and then washed 3 times to remove excess Fe^{2+} and DTT, the enzyme activity still remained stimulated. Moreover, addition of chelating agents for Fe^{2+} had no effect on the stimulated activity (data not shown). As shown in Table III, Fe^{2+} plus DTT inhibited the other membrane-bound enzymes, Mg^{2+}-ATPase, (Na^+K^+)-ATPase, and 5'-nucleotidase, and had no influence on phosphodiesterase or acetylcholinesterase. The stimulatory effects of Fe^{2+} plus DTT on adenylate cyclase in the membrane fractions of other tissues were also examined. Fig. 7 shows that Fe^{2+} plus DTT activated only the enzyme in brain synaptic membranes, M,0.9. The enzyme activities in liver plasma membranes and cardiac sarcolemma were stimulated by glucagon and isoproterenol, respectively, and NaF markedly stimulated the adenylate cyclase in all preparations. But, Fe^{2+} plus DTT did not stimulate the enzyme activity in liver plasma membranes, cardiac sarcolemma, or erythrocyte ghosts. Fe^{2+} plus DTT did not affect the enzyme activity in these membranes even in the higher concentration of ATP (1.0 mM). In addition, Fe^{2+} plus DTT had no effect on glucagon- or NaF-stimulated adenylate cyclase activity in liver plasma membranes (data not shown). Moreover, various concentrations of Fe^{2+} (1 to 100 μM) in the presence of 5 mM DTT had no effect on adenylate cyclase activity in liver plasma membranes.

**TABLE III**

**Effect of Fe^{2+} plus dithiothreitol on enzyme activities in the M fraction of rat cerebral cortex.**

<table>
<thead>
<tr>
<th>Enzyme activity</th>
<th>Control</th>
<th>Fe^{2+} + DTT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenylate cyclase</td>
<td>75.5</td>
<td>234</td>
</tr>
<tr>
<td>Mg^{2+}-ATPase</td>
<td>0.43</td>
<td>0.34</td>
</tr>
<tr>
<td>(Na^+K^+)-ATPase</td>
<td>0.65</td>
<td>0.37</td>
</tr>
<tr>
<td>5'-nucleotidase</td>
<td>1.10</td>
<td>0.38</td>
</tr>
<tr>
<td>Acetylcholinesterase</td>
<td>9.92</td>
<td>8.81</td>
</tr>
<tr>
<td>Phosphodiesterase</td>
<td>67.7</td>
<td>58.1</td>
</tr>
</tbody>
</table>

**DISCUSSION**

The present study demonstrated that Fe^{2+} activated adenylate cyclase in the membrane fraction of rat brain in the presence of DTT. Other membrane-bound enzymes, such as (Na^+K^+)-ATPase, 5'-nucleotidase, phosphodiesterase, and acetylcholinesterase, were not activated by Fe^{2+} plus DTT. Tryptophan 5-monoxygenase is known to be affected by a combination of DTT and Fe^{2+}: the velocity of activation in the presence of Fe^{2+} was markedly accelerated by Fe^{2+} (35, 36). Maximal activation of tryptophan 5-monoxygenase required preincubation with Fe^{2+} plus DTT under anaerobic conditions (37). In the present experiments, adenylate cyclase activity in the M fraction of rat brain was not stimulated by DTT alone (Fig. 3) or by Fe^{2+} plus DTT under anaerobic conditions. Therefore, the mechanism of activation of adenylate cyclase by Fe^{2+} plus DTT is different from that of tryptophan 5-monoxygenase.

Fe^{2+} plus DTT might increase the enzyme activity by...
preserving ATP since Fe^{2+} plus DTT inhibited ATPase activity of the membrane fraction (Table III) and prevented ATP hydrolysis in the assay medium for adenylate cyclase. However, this possibility can be excluded by the following findings: 1) as it appeared in ATP concentration curves (Fig. 1), ATP hydrolysis (less than 20%) during the incubation had no actual effect on the adenylate cyclase activity; 2) the apparent K_m for ATP was not changed in the presence of Fe^{2+} plus DTT; 3) as described in this text, activation of the enzyme was also observed when the enzyme activity was determined in the presence of an ATP-regenerating system. Thus, the results from the assays both in the presence and absence of an ATP regenerating system were comparable, except for the difference in the specific activity.

Iron plus a reducing agent constitutes one of the most powerful catalytic systems for lipid peroxidation of liver microsomes. In the present study, Fe^{2+} evoked lipid peroxidation of the enzyme preparation in the presence of DTT. The dose-response curves of Fe^{2+} and DTT for stimulation of adenylate cyclase were parallel with those for lipid peroxidation (Figs. 3 and 4). Moreover, these stimulatory effects of Fe^{2+} plus DTT on the enzyme activity and lipid peroxidation of the membranes were completely blocked by DPPD and reduced under anaerobic conditions. Ascorbic acid or a NADPH system also caused lipid peroxidation (33) and stimulated adenylate cyclase activity. In addition, the stimulation of enzyme by ascorbic acid or a NADPH system was inhibited by DPPD (Table I). Thus, lipid peroxidation of the membranes seems to stimulate adenylate cyclase. Lipid peroxidation of the membranes is associated with inhibition of several membrane-bound enzymes (14). The only enzymes known to be activated by lipid peroxidation are microsomal thiamine diphosphatase in rat brain (38) and glucurononitransferase in liver microsomes (39).

Malondialdehyde, a reaction product of lipid peroxidation, had no effect on adenylate cyclase at concentrations of 1 to 100 μM (data not shown). Polyunsaturated fatty acid peroxides, other reaction products of peroxidation, might possibly activate the enzyme.

Proteins within the membranes are highly mobile (40), but their activity and function may be affected by both the lipid microenvironment and the membrane fluidity (41, 42). Several lines of evidence indicate that adenylate cyclase is dependent upon membrane lipid for basal and hormone-stimulated activities (7-11). Moreover, it was demonstrated that increase of membrane fluidity causes a linear increase in the bimolecular process of cyclase activation by hormone-bound β-receptor (43, 44) and that increase in membrane ordering enhances the basal activity of adenylate cyclase (12). The mechanism of activation of adenylate cyclase by lipid peroxidation is not known, but three hypothetical explanations may be considered. 1) Dobretsov et al. (45) reported lowering of the membrane fluidity after lipid peroxidation. Adenylate cyclase activity is enhanced by increase in membrane ordering (12) or in the cholesterol level (6). Thus, lipid peroxidation may increase adenylate cyclase by increasing the rigidity of the membranes. 2) The change of membrane structure may be related to activation of the enzyme since peroxidation causes degradation of the membrane structure (13). The turbidity of the membrane suspension was decreased by treatment with Fe^{2+} plus DTT, and DPPD completely inhibited this decrease in turbidity induced by Fe^{2+} plus DTT (Fig. 8). 3) A possible inhibitor of adenylate cyclase may be freed from intact membranes or inactivated during lipid peroxidation. Lipid peroxidation attacks unsaturated fatty acids in the β-position of phospholipids and is accompanied by the disappearance of some polyunsaturated fatty acids from the phospholipids (46).

Anderson and Jaworski (47) reported that unsaturated fatty acids inhibited adenylate cyclase activity of fibroblasts. In preliminary experiments, we found that some unsaturated fatty acids, such as oleic, linoleic, and arachidonic acids, inhibit adenylate cyclase of rat brain. These fatty acids possibly modulate the enzyme activity in the neural membrane.

It should be noted that the activation of adenylate cyclase by Fe^{2+} plus DTT was specific for the brain enzyme. Moreover, when ascorbic acid or a NADPH system was used as an inducer of lipid peroxidation, only the enzyme in the membrane fraction of the brain was stimulated. In the present study, the responses of adenylate cyclase in liver plasma membranes, cardiac sarcolemma, and erythrocyte ghosts to NaF, glucagon, and isoproterenol were similar to those reported previously (17, 48, 49), showing that the integrities of these membranes were maintained. As shown in Fig. 8, Fe^{2+} plus DTT also caused lipid peroxidation and consequent decrease in the turbidity of the liver plasma membrane fraction, although the change was less than that of the brain fraction. We propose that the specificity of activation of adenylate cyclase in neural membranes by lipid peroxidation may depend on a specific difference in the composition of its membrane phospholipids (50) or of the membrane organization of its cyclase.

REFERENCES


2 A. Baba, E. Lee, and H. Iwata, unpublished observation.
Activation of Adenylate Cyclase by Lipid Peroxidation