EFFECTS OF VARIOUS AGENTS ON SYNAPTOSOMAL ADENYLATE CYCLASE ACTIVITY IN THE ABSENCE AND PRESENCE OF THE BOILED SUPERNATANT

Hiroshi IZUMI and Hikaru OZAWA
Pharmaceutical Institute, Tohoku University, Aobayama, Sendai 980, Japan

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Abstract—A study was made of the effects of various agents on adenylate cyclase in synaptosomes in both the absence and presence of the boiled supernatant from rat cerebral cortex. The activity of adenylate cyclase in these preparations was inhibited by the sulfhydryl reactive agent p-chloromercuribenzoate. Sulfhydryl compounds such as cysteine, glutathione and Coenzyme A stimulated the enzymic activity in both the absence and presence of the boiled supernatant. The chelating agent 1,2-bis-(2-dicarboxymethylaminoethoxy)ethane caused a stimulation of the enzymic activity with and without the presence of the boiled supernatant. Adenine nucleotide (adenine, adenosine, AMP and ADP), GTP, Pi and carbamylcholine seemed to have little effect. The stimulatory substance in the boiled supernatant was estimated to have a molecular weight in the range of 1,000-1,300.

Recently certain properties of synaptosomal plasma membrane in rat brain were reported (1-3), and several studies have shown that synaptosomes have many of the properties of presynaptic nerve ending in intact nervous tissue (4-6).

We have demonstrated recently (7) that the 105,000 × g supernatant from the rat brain contains a heat-stable, dialyzable substance stimulating the cyclic AMP accumulation in synaptosomes and presumably this cyclic AMP accumulation in the presence of the boiled supernatant is due to increased synthesis. This suggested that the adenylate cyclase activity of synaptosomal membrane is regulated by endogenous substances in the brain. It was of interest therefore to investigate the synaptosomal adenylate cyclase in detail, and to characterize the stimulatory substance in the 105,000 × g supernatant.

MATERIALS AND METHODS

[3H]ATP (uniformly labeled) was obtained from New England Nuclear Corp. Crystalline disodium ATP, Tris, AMP, ADP and carbamylcholine were purchased from Sigma Chemical Co. Adenine, adenosine and GTP were purchased from Kohjin Co. Ltd. p-CMB was obtained from Nakarai Chemical Co. Ltd. All other chemicals were of analytical grade or the best commercially available.

Preparation of synaptosomes: Sprague-Dawley male rats (CLEA Japan, Inc.) weighing 250-300 g were used in these experiments. The animals were decapitated and the brains were isolated and chilled in ice-cold solution containing 0.32 M sucrose and 3.0 mM MgSO4. Subsequently, the cerebral cortex was isolated by the method of Glovinski and Iversen (8),
and tissues of the cerebral cortex were homogenized in 9 volumes of the same isotonic cold solution using a glass homogenizer with a loose Teflon pestle. Subcellular organelles were separated by the method of Whittaker (9).

Preparation of the boiled supernatant: The homogenates of rat cerebral cortex in the ice-cold solution containing 0.32 M sucrose and 3.0 mM MgSO₄ described above were centrifuged at 1,000 × g for 20 min, 10,000 × g for 20 min and 105,000 × g for 60 min. The 105,000 × g supernatant was heated in a boiling water bath for 5 min and then, after 10–20 min 0°C, centrifuged at 10,000 × g for 10 min at 4°C. This supernatant was used as the boiled supernatant in these experiments.

Assay of adenylate cyclase activity: Adenylate cyclase activities were measured as described previously (7). Unless otherwise described, the standard incubation medium (0.6 ml) consisted of 1.0 mM [3H]ATP (10 μCi), 3.3 mM MgSO₄, 10 mM NaF, 6.7 mM caffeine and 40 mM Tris-HCl buffer (pH 7.4). Test tubes (12 × 105 mm) containing all the ingredients for the assay of adenylate cyclase activity were preincubated for 5 min at 30°C prior to addition of substrate (ATP) which started the reaction. Incubation was run at 30°C for 15 min and terminated by the immersion of the tubes in a boiling water bath for 2 min.

Protein determination: The protein contents were determined by the procedure described by Lowry et al. (10) with crystalline bovine serum albumin as the standard.

RESULTS

Effects of the boiled supernatant concentration

The relationship between the rate of the cyclic AMP accumulation and the concentration of the boiled supernatant is given in Fig. 1. As the concentration of the boiled supernatant increased, the extent of the cyclic AMP accumulation was greater. In the assay of stimulatory activity described in the present work, preliminary experiments were routinely carried out to determine the amount of the boiled supernatant so that the linear part of the curve was used.

Estimation of molecular weight of the stimulatory substance

As can be seen from Fig. 2, when separation of the stimulatory substance in the boiled supernatant was carried out by column chromatography, the stimula-
FIG. 2. Chromatography of adenylate cyclase stimulatory substance on a Sephadex G-25 superfine. The boiled supernatant obtained as described in the Methods was freeze-dried and the resulting material dissolved in water. This material was fractionated by chromatography on a column (1.5 × 43 cm) of a Sephadex G-25 superfine equilibrated with 50 mM Tris-HCl buffer (pH 7.4). The column was eluted with the same buffer at 4 °C in 2.5 ml of fraction at a flow rate of 10 ml per hour. The stimulatory activity of an aliquot (100 μl) of each fraction was measured as described in the Methods.

( ), Absorbance at 280 μm (The boiled supernatant)

( ), Absorbance at 230 μm (Glucagon M.W. 3,500)

( ), Absorbance at 555 μm (Vitamin B12 M.W. 1,350)

A stimulation was found to be associated with a fraction of molecular weight less than 1,300.

To determine a more precise molecular weight of the stimulatory substance, an ultrafiltration method (11) was used (Table 1). The boiled supernatant was filtrated with a Ulvac ultrafiltration cell equipped with a G-01T membrane, and then an aliquot (100 μl) of the filtrate was assayed for its ability to stimulate adenylate cyclase activity. The results are shown in Table 1. This filtrate did not stimulate the enzymic activity at all, indicating that the stimulatory substance has a molecular weight of more than 1,000. From the above data, we estimated that the stimulatory substance has a molecular weight in the range of 1,000—1,300.

Role of sulfhydryl groups

The effects of p-chloromercuribenzoate (p-CMB), which reacts with sulfhydryl groups,
in various concentrations on synaptosomal adenylate cyclase in the absence and presence of the boiled supernatant are given in Fig. 3. Basal enzymic activity was not affected by the addition of p-CMB at $10^{-6}$ M concentration but was completely inhibited by the addition of more than $10^{-5}$ M p-CMB. On the other hand, adenylate cyclase activity was profoundly stimulated by the addition of the boiled supernatant as previously reported (7), however, this increased cyclic AMP formation was also gradually decreased by increasing concentration of p-CMB as can be seen from Fig. 3. The differences between the concentration of p-CMB required for the blockade of cyclic AMP formation in synaptosomes in the absence and presence of the boiled supernatant suggest that the boiled supernatant might have sulfhydryl groups, however, it is not clear at this time whether the stimulatory substance in the boiled supernatant does indeed have sulfhydryl groups, since purification of this stimulatory substance is still in progress.

To examine the more precise role of sulfhydryl groups of synaptosomal adenylate cyclase, the order of additions of synaptosomes (enzymes), p-CMB, the boiled supernatant and dithioerythritol, which maintains sulfhydryl groups, varied during preincubation and the

**Table 2. Effect of sulfhydryl reagents on the formation of cyclic AMP**

|              | A*            | B*          | Cyclic AMP formed (μmol/mg/min) |
|--------------|---------------|-------------|---------------------------------|
| Synaptosomes |               |             | 53.5 ± 4.2                      |
| + p-CMB (10^{-4}M) |               | 0           |
| + D.T.E. (10^{-5}M) |               | 345.3 ± 18.3 |
| + Boiled supernatant |               | 0           |
| Boiled sup.  | + Synaptosomes |             | 148.1 ± 9.3                     |
| Synaptosomes | + p-CMB       |             | 328.1 ± 5.0                     |
| + D.T.E.     | + Boiled sup. |             | 0                               |
| Boiled sup.  | + Synaptosomes| + D.T.E.    | 288 ± 18.8                      |

The boiled supernatant obtained as described in Methods, equivalent to 304 μg of protein, was added to the incubation medium. The incubation was performed as described in Methods. Each value represents the mean ± S.E. of 2 determinations on one preparation. Similar results were obtained on other preparations. *Materials indicated in each column were added to the flask; A: 60 sec B: 30 sec prior to the addition of substrate (3H-ATP).
results are shown in Table 2. As can be seen, when synaptosomes were added to the incubation medium after the addition of dithioerythritol (10^{-3} M), enzymic activity was not inhibited by p-CMB (10^{-4}). However, once sulfhydryl groups of synaptosomes had been occupied by p-CMB, enzymic activity was not restored by the addition of dithioerythritol.

As can be seen from Table 2, synaptosomal adenylate cyclase was sensitive to dithioerythritol and thus the following experiments were carried out. When sulfhydryl compounds such as cysteine glutathione and Coenzyme A were added to assay system, all three sulfhydryl compounds increased the enzymic activity in both the absence and presence of the boiled supernatant as can be seen in Fig. 4. Of the three compounds, cysteine was the most effective.

**Effects of adenine nucleotides**

The effects of adenine, adenosine and adenosine monophosphate (AMP) on synaptosomal adenylate cyclase in the absence and presence of the boiled supernatant are shown in Fig. 5. Higher concentrations of AMP (10^{-3} M) inhibited the basal enzymic activity. The increased cyclic AMP formation by the boiled supernatant was reduced by high concentrations of adenosine and AMP.

**Effect of EGTA (1,2-bis-(2-dicarboxymethylaminoethoxy)ethane)**

The effect of the inclusion of various concentrations of EGTA in the enzyme reaction mixture in the absence and presence of the boiled supernatant is shown in Fig. 6. As illustrated by the curve in Fig. 6, maximal stimulatory effects were obtained when the concentrations of EGTA were 10^{-4} M and 5 \times 10^{-5} M in the absence and presence of the boiled
FIG. 5. Effects of varying concentrations of adenine nucleotides on the formation of cyclic AMP in synaptosomes in the absence and presence of the boiled supernatant (0.25 mg of protein). Each value represents the means±S.E. of 3 determinations on one preparation. Similar results were obtained on two other preparations. The vertical bars represent the S.E.

Adenine. (••••••), Adenosine. (••••••), AMP.

FIG. 6. Effect of EGTA on the formation of cyclic AMP in synaptosomes in the absence and presence of the boiled supernatant (0.2 mg of protein). Varying concentrations of EGTA were included in the usual incubation mixture. Each value represents the mean±S.E. of 3 determinations on one preparation. Similar results were obtained on two other preparations. The vertical bars represent the S.E.

Effects of ADP and Pi

The effects of ADP (adenosine diphosphate) and Pi on synaptosomal adenylate cyclase are given in Table 3. In the absence of the boiled supernatant, ADP (10^{-4} M) and Pi (10^{-4} M) gave a slight stimulation, ADP (10^{-3} M) gave an appreciable inhibition but Pi (10^{-3} M) did not significantly affect the enzymic activity. Stimulation of cyclic AMP formation by the boiled supernatant was gradually decreased by increasing the concentration of either ADP or Pi.

Effect of GTP (guanosine triphosphate)

The effects of varying concentrations of GTP on synaptosomal adenylate cyclase in
TABLE 3. Effects of products of ATP breakdown on the formation of cyclic AMP in synaptosomes

| Additions                 | Cyclic AMP formed (µmoles/mg of protein/min) |
|--------------------------|---------------------------------------------|
| None                     | 62.2 ± 9.3                                   |
| Boiled supernatant       | 457.1 ± 15.3                                 |
| ADP (10^{-4}M)           | 73.6 ± 10.8                                  |
| Boiled sup. + ADP (10^{-4}M) | 321.8 ± 13.2                      |
| ADP (10^{-3}M)           | 26.4 ± 3.3                                   |
| Boiled sup. + ADP (10^{-3}M) | 180.6 ± 9.2                        |
| Pi (10^{-4}M)            | 73.3 ± 7.5                                   |
| Boiled sup. + Pi (10^{-4}M) | 309.2 ± 3.0                       |
| Pi (10^{-3}M)            | 61.8 ± 2.1                                   |
| Boiled sup. + Pi (10^{-3}M) | 256.7 ± 15.6                     |

The boiled supernatant obtained as described in Methods, equivalent to 304 µg of protein, was added to the incubation medium. The incubation was performed as described in the Methods. Each value represents the mean ± S.E. of 2 determinations on one preparation. Similar results were obtained on other preparations.

The effect of carbamylcholine on synaptosomal adenylate cyclase in the absence and presence of the boiled supernatant was investigated and the results are shown in Fig. 7. GTP seemed to have little effect on the basal and the boiled supernatant-stimulated cyclic AMP formation in synaptosomes as well as in the preparation of horse parathyroid gland (12).

**Effect of carbamylcholine**

The effect of carbamylcholine on synaptosomal adenylate cyclase in the absence and presence of the boiled supernatant is given in Fig. 7. As can be seen, carbamylcholine did not at all affect the basal and the boiled supernatant-stimulated cyclic AMP formation.

**DISCUSSION**

Recently, it has been reported that methylmercury is a potent inhibitor of adenylate
cyclase in rat liver plasma membranes (13). The results presented here that the synaptosomal adenylate cyclase from rat cerebral cortex was inhibited by the sulfhydryl reagents such as p-CMB (Fig. 3) suggest that organomercurials may react well with protein sulfhydryl groups of synaptosomal plasma membranes as liver plasma membranes. It has been reported, however, that sulfhydryl groups were required for adenylate cyclase of rat liver plasma membranes but were not required for the stimulation of this activity by sodium fluoride or ACTH (14). The cyclic AMP formation in the presence of both the boiled supernatant and 10^{-5} M p-CMB shown in Fig. 3 appears to be caused by the interaction with the remaining sulfhydryl groups of synaptosomes which had not reacted with p-CMB and a stimulatory substance in the boiled supernatant. Once these sulfhydryl groups of synaptosomes are lost as a result of reaction with p-CMB, enzymic activity is not restored by the addition of dithioerythritol as can be seen from Table 2. These results indicate that sulfhydryl groups are required for the basal enzymic activity and are required for the stimulation of this enzymic activity by the boiled supernatant. This is supported by the hypothesis that the sensitive sulfhydryl group(s) may be associated with the catalytic subunit of adenylate cyclase. It has been suggested by Storm and Dolginow that the reactivity of the sulfhydryl group(s) of liver plasma membrane is pronouncedly stimulated by glucagon and its binding might promote conformational changes within the adenylate cyclase system resulting in either increased or enhanced nucleophilicity of the crucial sulfhydryl group(s) (15).

Divalent cations play a crucial and to some extent not yet well understood role in regulation of adenylate cyclase activity. Sutherland and co-workers (16–19), discoverers of adenylate cyclase were first in pointing out that this enzyme has an absolute requirement for Mg^{2+}. Several inconclusive investigations concerning the effect of Ca^{2+} on adenylate cyclase have been reported (20–25). The result in Fig. 6 shows that EGTA, which is a specific chelator for Ca^{2+}, caused a stimulation of the enzymic activity with or without the presence of the boiled supernatant. This stimulatory effect has not been adequately explained. Ca^{2+} also inhibits adenylate cyclase in other tissues: fat, heart and adrenal tissues (26–28) but to a lesser extent. Birnbaumer et al. (26) suggested that calcium and magnesium compete for a putative allosteric site on the adenylate cyclase enzyme system in fat cell membrane. The data obtained in the present study that the increase of synaptosomal adenylate cyclase in response to EGTA in the presence of 3.3 mM Mg^{2+}, at which concentration the enzyme is approaching saturation with regard to Mg^{2+} suggest that in this tissue Ca^{2+} is exerting its effect by competing with Mg^{2+} for occupancy of an allosteric site. It therefore seems certain that Ca^{2+} plays a significant role in controlling the adenylate cyclase activity in synaptosomes.

Although the inhibitory effect of adenosine on adenylate cyclase of rat liver has been reported (29), we did not observe such an effect of adenosine on synaptosomal adenylate cyclase (Fig. 5). The inhibitory effects were seen by the addition of adenine nucleotide (AMP, ADP) at a higher concentration, however, the action does not seem to be physiological.

Purine nucleotide triphosphates were shown to be necessary for the expression of hormone stimulation, not only in the glucagon-sensitive system from liver parenchymal cell
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(30), but also in the glucagon-sensitive system from pancreatic cell (31), and in prostaglandin-sensitive system from human platelets (32). It is, therefore, of prime importance to test the hypothesis that the purine nucleotide-dependent step (notably GTP dependent) might be an invariant feature of the hormone-sensitive adenylate cyclase system, possibly acting at the step that couples hormone-receptor interaction to enzyme activation. We investigated the effect of GTP on synaptosomal adenylate cyclase in the absence and presence of the boiled supernatant in order to examine the interaction of GTP and the stimulatory substance in the boiled supernatant (Fig. 7). As can be seen, GTP, contrary to our expectation, did not significantly affect the enzymic activity in either the absence or presence of the boiled supernatant, suggesting that there is no interaction between the two. These results are in variance with those previously reported concerning GTP or Gpp(NH)p activation of basal adenylate cyclase preparations from rat liver or fat plasma membranes (33, 34) and mouse neuroblastoma × glioma hybrid cell (35). Such a variance may be due to the different assay condition of the incubation system or to the different enzyme preparation.

Further work is necessary to identify the stimulatory substance in the boiled supernatant and to determine whether it has a physiological role in the regulation of cyclic AMP formation.

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