THE STIMULATORY EFFECT OF THE BOILED SUPERNATANT ON CYCLIC AMP FORMATION IN SYNAPTOSOMES FROM RAT CEREBRAL CORTEX

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

Abstract—The effect of the boiled supernatant on synaptosomal adenyl cyclase activity of rat cerebral cortex was investigated. The boiled supernatant markedly increased the accumulation of cyclic AMP in synaptosomes when added to adenyl cyclase system by a mechanism presumably unrelated to inhibition of cyclic AMP phosphodiesterase or adenosine triphosphatase. Magnesium ion was required for synaptosomal adenyl cyclase activity and its stimulation by the boiled supernatant. The result discerned by double reciprocal plots showed an increase in Vmax value of adenyl cyclase by addition of the boiled supernatant without significantly altering the affinity for substrate. The enzyme activity was not stimulated by dopamine, histamine and serotonin in either the absence and presence of the boiled supernatant.

Adenosine 3',5'-monophosphate (cyclic AMP) mediates the action of variety of different hormones. Its tissue level is critically related to the intensity and duration of hormone action (1). Since cyclic AMP levels in cells have been found to be regulated by its formation, catalyzed by adenyl cyclase, and its hydrolysis to AMP which is catalyzed by a specific nucleotide phosphodiesterase, a study of the factors affecting the levels would be deemed worthy. Investigations concerning the soluble activator of cyclic AMP phosphodiesterase were made by various investigators (2, 3, 4) but studies of substance(s), except hormone, which affect adenyl cyclase are few.

Previous work from this laboratory showed that post-microsomal supernatant (105,000 x g supernatant) increased the accumulation of cyclic AMP in synaptosomes from rat cerebral cortex when added to adenyl cyclase system (5, 6). Although the properties of this stimulatory substance(s) in the 105,000 x g supernatant have yet to be clarified, the site of action of the stimulatory substance(s) on cyclic AMP formation was investigated by measuring the activity of enzymes which may affect the level of cyclic AMP in synaptosomes.

MATERIALS AND METHODS

[3H]ATP (uniformly labelled) was obtained from New England Nuclear Corp. [8-14C]cyclic AMP was obtained from Schwarz Bio-Research. Crystalline disodium ATP and Tris were purchased from Sigma Chemical Co.

Preparation of synaptosomes: Sprague-Dawley male rats (CLEA Japan, Inc.) weighing 250-300 g were used. After these animals were decapitated, the brain was 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 (7), 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 (8).

**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 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 at 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 adenyl cyclase activity:** Adenyl cyclase activity was determined on the rate of conversion of [3H]ATP to [3H]cyclic AMP by means of a modified method of Krishna et al. (9). 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 adenyl 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. One-tenth ml [8-14C] cyclic AMP (about 5,000 dpm) as an internal standard for calculation of percent conversion of [3H]ATP to [3H]cyclic AMP and 0.1 ml each of ZnSO₄ (0.17 M) and Ba(OH)₂ (0.15 M) were added to each test tube and, after agitation, centrifuged at 1,000 × g for 10 min. The resulting supernatant was applied to a 0.3 × 8 cm column of Dowex 50 W-X4 in the hydrogen form, and then cyclic AMP was eluted with water. Every 1 ml of the eluate was continuously collected and transferred into a counting vial with 10 ml of scintillating fluid (4 g of PPO and 100 mg of dimethyl POPOP in 1,500 ml of triton X-100-toluene mixture (1:2)). The radioactivity was then determined by a liquid scintillation spectrometer (Packard Tri-Carb Model 3380) and corrected for quenching using an external standardization system.

**Assay of cyclic AMP phosphodiesterase activity:** Cyclic AMP phosphodiesterase assay was done by the method of Pösch (10) with a cyclic AMP concentration of 3.6 × 10⁻⁴ M. Enzymatic activity was measured as the rate of hydrolyzing cyclic AMP in a standard reaction medium (final volume 500 μl) containing [8-14C] cyclic AMP (0.05 μCi), 3.0 mM Mg-acetate, 2.0 mM 5'-AMP, 100 mM Tris-HCl buffer, pH 7.4 and enzyme. Incubations were run at 30°C for 15 min.

**Assay of ATPase activity:** Reaction mixtures (1.0 ml) contained 5.0 mM MgCl₂, 14 mM KCl, 140 mM NaCl, 50 mM Tris-HCl buffer, pH 7.4 and tissue suspension. The mixtures were shaken at 30°C for 5 min and the reaction was initiated by the addition of Tris-ATP (0.1 ml) to give a final concentration of 3.0 mM. Shaking was continued for a further 15 min, and 2.0 ml of 10% (W/V) trichloroacetic acid solution then added. Protein precipitate was spun down, and inorganic phosphate was measured in samples from
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...the supernatant by the method of Fiske and Subbarow (11).

Protein determination: The protein contents were determined by the procedure described by Lowry et al. (12) with crystalline bovine serum albumin as standard.

RESULTS

Time course of adenyl cyclase reaction

Fig. 1 shows the rate of the accumulation of $^3$H-labelled cyclic AMP formed from $[^3$H] ATP in synaptosomes from rat cerebral cortex in the absence and presence of the boiled supernatant. The cyclic nucleotide accumulated linearly for the first 20 min period incubation, both in the absence and presence of the boiled supernatant. The rate of formation of cyclic AMP was increased approximately five-fold by addition of the boiled supernatant.

Effect of the native and boiled supernatant on ATPase activity

In order to determine whether the boiled supernatant-induced enhancement of cyclic AMP accumulation was due to increased availability of substrate (ATP)

for adenyl cyclase, we investigated the effects of the native and boiled supernatant on ATPase activity in synaptosomes (Table 1). As can be seen in Table 1, the native and boiled supernatant inhibited ATPase activity by about 20%.

Effects of sodium fluoride on adenyl cyclase activity and ATPase activity

In order to compare the concentration-response relationships of sodium fluoride with inhibition of ATPase and the accumulation of cyclic AMP, the following experiments were carried out. As can be seen from Fig. 2, as the concentration of sodium fluoride increased, the extent of inhibition of synaptosomal ATPase activity was proportionally greater, on the other hand, activation of synaptosomal adenyl cyclase by sodium fluoride was maximal at a concentration of about 5 mM; higher concentrations of sodium fluoride reduced enzy-
mic stimulation, indicating that there was no correlation between the two effects.

Effect of the boiled supernatant on cyclic AMP phosphodiesterase activity

To determine whether the boiled supernatant-induced stimulation of cyclic AMP accumulation occurred at the cyclic AMP phosphodiesterase step, the effects of the boiled supernatant on cyclic AMP phosphodiesterase activity in synaptosomes were investigated and the results shown in Table 2. As can be seen, synaptosomal cyclic AMP phosphodiesterase activity was also stimulated by the addition of the boiled supernatant about 1.5-fold, indicating that the stimulatory effect of the boiled supernatant on cyclic AMP formation was not the result of the inhibition of cyclic AMP phosphodiesterase activity.

| Preparations          | Addition          | Cyclic AMP hydrolyzed (nmoles/15 min) |
|-----------------------|-------------------|---------------------------------------|
| Synaptosomes (100 µg protein) | boiled supernatant | 40.4 ± 7.8                            |
| Synaptosomes (200 µg protein) | boiled supernatant | 65.1 ± 1.1*                           |

*p<0.001 compared with each control.

The boiled supernatant obtained as described in Materials and Methods, equivalent 300 µg of protein, was added to each incubation medium. Data are averages ± S.D. of 3 separate analyses.

Effect of Mg** concentration on adenyl cyclase activity

Effect of Mg** concentration on adenyl cyclase activity in synaptosomes in the absence and presence of the boiled supernatant is given in Fig. 3. When ATP was fixed at 1 mM, the enzyme activity was not detected in the absence and presence of the boiled supernatant whereas an increase in Mg** concentration caused a progressive increase in the activity of adenyl cyclase with or without the presence of the boiled supernatant.

Effect of substrate concentration on adenyl cyclase activity

Assays of adenyl cyclase in synaptosomes were conducted by various ATP concentrations (Fig. 4). The calculation on the basis of the data obtained in this experiment revealed that Km values were $0.40 \times 10^{-3}$ M and $0.41 \times 10^{-3}$ M in the absence and presence of the boiled supernatant, respectively and Vmax values were 80 nmoles/min per mg pro-
FIG. 3. Effects of Mg^{2+} concentrations on adenyl cyclase activity in synaptosomes in the absence (open symbols) and presence (closed symbols) of the boiled supernatant (0.3 mg of protein).

FIG. 4. Mechanism of the stimulatory effect of adenyl cyclase by the boiled supernatant (0.3 mg of protein). The effect of the boiled supernatant was studied by the double reciprocal plots. (inset)

Effect of dopamine, histamine and serotonin on adenyl cyclase activity

The effects of neurotransmitters such as dopamine, histamine and serotonin on enzyme activity are shown in Fig. 5. The basal enzyme activity was not affected by addition of such neurotransmitters but the boiled supernatant-stimulated enzyme activity was reduced by a high concentration of dopamine and serotonin.

DISCUSSION

We have previously found that the addition of the 105,000 x g supernatant from rat brain to adenyl cyclase system increased the accumulation of cyclic AMP in synaptosomes.
significantly and that this stimulatory substance(s) in the 105,000 x g supernatant was heat-stable (100°C, 5 min), dialyzable (24 hr) and had a molecular weight of about 1,000-1,300 (5, 6). Although the purification of the stimulatory substance(s) was in the preliminary stage, studies were undertaken in the present report to determine the sites of action of the stimulatory substance(s) by using the boiled supernatant as a stimulator.

As shown in Fig. 1, the boiled supernatant increased the accumulation of cyclic AMP about five-fold when added to adenyl cyclase system in synaptosomes from rat cerebral cortex. Adenyl cyclase from most tissues is not readily purified, primarily because it is membrane bound and is relatively unstable. In the present investigation, we utilized synaptosomes as enzyme preparation. In this preparation, ATPase and cyclic AMP phosphodiesterase activities often are significant contaminants and their presence must be considered. Therefore, the ability of the boiled supernatant to stimulate cyclic AMP accumulation in synaptosomes may be due to the following three reasons: 1) direct stimulation of synaptosomal adenyl cyclase activity, 2) increase of available substrate (ATP) by inhibition of synaptosomal ATPase activity, 3) inhibition of synaptosomal cyclic AMP phosphodiesterase activity. As can be seen from Fig. 2, a study of the concentration response relationships of sodium fluoride on inhibition of ATPase and stimulation of the accumulation of cyclic AMP revealed no correlation between the two effects, indicating that ATPase inhibition and cyclic AMP accumulation are not causally related. This result suggests that the slight inhibition of ATPase activity by the boiled supernatant might not induce an increase in the stimulation of cyclic AMP accumulation. The presence of the soluble inhibitory substance(s) on ATPase of rat brain has been previously reported by Schaefer et al. (12). On the other hand, synaptosomal cyclic AMP phosphodiesterase was not inhibited but was rather stimulated by the addition of the boiled supernatant as can be seen in Table 2. This result indicates that the stimulatory effect of the boiled supernatant is apparently unrelated to cyclic AMP phosphodiesterase activity. Although we cannot exclude completely at this time other possibilities, it is conceivable from the above results that the mechanism responsible for the stimulation of accumulation of cyclic AMP by the stimulatory substance(s) might be due to an activation of adenyl cyclase. However, proof for a mechanism of this stimulatory effect on cyclic AMP formation in synaptosomes must await purifications of both the enzyme and the stimulatory substance(s).

As can be seen from Fig. 3, adenyl cyclase requires Mg++ for its activity and its stimulation by the boiled supernatant. The basal activity of the enzyme was increased and then reached a plateau at Mg++ concentration of 4 mM and in the presence of the boiled supernatant the activity peaked at the same Mg++ concentration of 4 mM.

We conclude that while the stimulatory effect of the boiled supernatant is due to an increase in Vmax, there is no significant effect on the apparent affinity for substrate (Fig. 4). Our results suggest that this stimulation is similar kinetically to fluoride (13) and hormone stimulation (14). Although we found the optimum concentration of ATP to be 0.8 mM, the significance of this value is difficult to assess since the isolated synaptosomes contain other enzymes which act on ATP, ADP and AMP. These enzymes are ATPase, myokinase,
ATP pyrophosphohydrolase and 5'-nucleotidase. Hence the actual concentration of ATP added to the synaptosomes is very rapidly lowered by the active ATPase and partially regenerated by myokinase. Birnbaumer and Rodbell (15) attempted to circumvent this problem by adding ATP regenerating enzyme. However, unless the ATP levels are actually measured at different time intervals, the true concentration of ATP remains unknown.

It has been previously reported that homogenates from brain tissues were not stimulated by catecholamines (16) whereas slice preparations of brain readily responded to the hormone (17, 18). Recently Harwood and Rodbell reported that the loss of hormone sensitivity in the preparation of fat cell ghost was probably due to fluoride ion (19). In the present experiments, since enzyme activity was not detected in the absence of fluoride, we added sodium fluoride to the incubation medium. As can be seen from Fig. 5, no stimulatory effects by catecholamines such as dopamine, histamine and serotonin were observed in the absence and presence of the boiled supernatant. These results suggest that the process of cell disruption may cause a dissociation of the functional relationship of receptor and catalytic component of brain adenyl cyclase.

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