CHEMICAL & PHARMACEUTICAL BULLETIN

Vol. 24, No. 4 April 1976

Regular Articles

[Chem. Pharm. Bull. 24(4) 555—559 (1976)]

UDC 577.159.04 : 612.82-06

The Stimulation of Synaptosomal Adenyl Cyclase Activity by the Supernatant from Rat Brain Cerebral Cortex[1]

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(Received February 27, 1975)

Effects of the 105000 ***g*** supernatant on adenyl cyclase of rat cerebral cortex have been investigated and following results were obtained.

1) The 105000 ***g*** supernatant significantly stimulated the activity of the synaptosomal adenyl cyclase in rat cerebral cortex. As the concentration of the supernatant increased, the progressive stimulation was observed with a plateau at a concentration of about 200 μg of protein.

2) This stimulatory effect of the supernatant was completely abolished by a dialysis of the supernatant for 24 hours at 4°.

3) When the enzyme system was incubated with combination of the supernatant and norepinephrine (100 μM), no activation by norepinephrine was observed.

4) Studies on the combined effects of the supernatant and sodium fluoride (NaF) showed that the supernatant additively enhanced NaF stimulated adenyl cyclase activity.

5) Gel filtration indicated that this stimulatory substance (s) in the supernatant had a molecular weight of 1000—1300.

It is well established that adenyl cyclase is stimulated by various neurotransmitters such as norepinephrine, 5-hydroxytryptamine and histamine in brain slice[3] and by direct administration of monoamine into the central system.[4] However, failure to obtain stimulation of brain adenyl cyclase activity by hormones and putative neurotransmitters in cell-free systems have been reported.[5] Similar loss of hormone sensitivity was observed in other tissue homogenates.[6] As these losses of hormone sensitivity were caused only in broken cell preparations, it seems likely that some essential substance(s) for the stimulation of the enzyme by such neurotransmitters is removed from the membrane during homogenization and fractionation of the tissue.

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1) A part of this work was presented at the 93th Annual Meeting of Pharmaceutical Society of Japan, Tokyo, April 1973.
2) Location: *Aobayama, Sendai.*
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The purpose of the present work is to investigate the effect of the 105000 ***g*** supernatant and the effects of combination of the 105000 ***g*** supernatant and either norepinephrine or sodium fluoride on the adenyl cyclase activity of rat cerebral cortex.

Materials and Methods

Materials——[^{3}H]ATP was obtained from New England Nuclear Corp. Crystalline adenosine 5′-triphosphate disodium salt (ATP), tris(hydroxymethyl)aminomethane and D,L-norepinephrine were purchased from Sigma Chemical Co. All other chemicals were reagent grade preparations obtained from various commercial sources.

Preparation of Subcellular Fractions——Sprague-Dawley male rats (CLEA Japan, Inc.), weighing 250—300 g, were used in the experiments. After animals were guillotined, brains were isolated and chilled in the ice-cold solution containing 0.32 M sucrose and 3.0 mM $MgSO_4$. Subsequently, the cerebral cortex was isolated by the method of Glowinski and Iversen[7] and was homogenized in 9 volumes of the same isotonic cold solution using a glass homogenizer with a loose Teflon pestle. The homogenates were centrifuged at 4° for 20 min at 1000 ***g***. The precipitate was washed twice by brief homogenization in the sucrose medium and centrifuged to discard the nuclei fraction. The supernatant fluid (1000 ***g*** supernatant) was pooled and centrifuged at 10000 ***g*** for 20 min at 4°. The precipitate was diluted with the same sucrose medium and designated as the crude mitochondrial fraction (10000 ***g*** sediment). The supernatant was centrifuged at 105000 ***g*** for 60 min at 4°. The precipitate was designated as the microsomal fraction (105000 ***g*** sediment) and the supernatant fraction as the supernatant (105000 ***g*** supernatant).

Assay of Adenyl Cyclase——Adenyl cyclase activity was measured using the method originally devised by Krishna, *et al.*[8] Unless otherwise described, the standard incubation medium (0.6 ml) consisted of 1.0 mM [^{3}H]ATP (10 μCi), 3.3 mM $MgSO_4$, 10 mM NaF, 6.7 mM caffeine, 40 mM Tris-HCl buffer (pH 7.4) and enzymes. Reaction mixtures were preincubated for 5 min and the reaction was initiated by the addition of substrate. After 15 min of incubation, the reaction was terminated by the immersion of the test tubes in a boiling water bath for 2 min. After the addition of 0.1 ml of [^{14}C] cyclic adenosine monophosphoric acid (cyclic AMP) (about 5000 dpm) as an internal standard for calculation of conversions of [^{3}H] ATP to [^{3}H] cyclic AMP, 0.1 ml of each of $ZnSO_4$ (0.17 M) and $Ba(OH)_2$ (0.15 M) solutions was added to each test tube. The mixture was agitated and centrifuged at 1000 ***g*** for 10 min. The resulting supernatant was applied to a column of Dowex 50W-X4 in the hydrogen form (0.3×8 cm) and then cyclic AMP was eluted with water. Every 1 ml of the eluate was collected and transferred to a counting vial with 10 ml of scintillating fluid which contained 4 g 2,5-diphenyloxazde (PPO) 100 mg 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)] benzene (dimethyl POPOP) and 500 ***g*** triton X-100 in 1 liter of toluene. The radioactivity was determined by a liquid scintillation spectrometer (Packard Tri-Carb Model 3380) and corrected for quenching using an external standardization system. The recovery of cyclic AMP was 75—80%. In the assay of adenyl cyclase activity, amounts of protein of the supernatant added as stimulator was not taken into account in calculation of the specific activity of adenyl cyclase.

Protein Determination——The protein contents were determined by the procedure described by Lowry, *et al.*[9] with crystalline bovine serum albumin as standard.

Results and Discussion

The effects of the supernatant on adenyl cyclase activity of subcellular fractions of rat cerebral cortex homogenates were examined. The data in Table I show that the enzyme activity in crude mitochondrial fraction was stimulated by the addition of the supernatant. Previously, Weiss and Costa[10] have reported the effects of the soluble supernatant on adenyl cyclase of sebcellular fractions of rat cerebellum. In agreement with our results, they reported that the enzyme activity of the 11000 ***g*** (mitochondrial) fraction was stimulated by the soluble supernatant material. Furthermore, we fractionated the crude mitochondrial fraction to three particulate fractions, *i.e.*, myelin, synaptosomes and mitochondria by a sucrose density gradient ultracentrifugation method[11] and then determined each enzyme activity in the absence or presence of the supernatant. As shown in Table II, three particulate fractions

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Table I. Effects of the Supernatant on Adenyl Cyclase Activity in Subcellular Fractions from Rat Cerebral Cortex

Fractions	Adenyl cyclase activity (pmoles cyclic AMP/min/mg protein)	
	Without supernatant	With supernatant
1000 *g* supernatant (whole homogenate)	71.7± 1.3	
10000 *g* sediment (crude mitochondria)	61.8± 4.0	150.0±4.6
105000 *g* sediment	92.0±22.4	102.0±5.1
105000 *g* supernatant	0	0

The subcellular fraction of rat cerebral cortex was obtained as described in Methods. The supernatant obtained after centrifugation at 105000 *g*, equivalent to 180 μg protein, was added to each of other fractions. Each value represents the mean of 3 experiments±S.E.

Table II. Effects of Supernatant on Adenyl Cyclase Activity in Submitochondrial Fractions

Fractions	Adenyl cyclase activity (pmoles cyclic AMP/min/mg protein)	
	Without supernatant	With supernatant
Mitochondria	21.3± 3.4	49.3± 2.8
Synaptosomes	38.8± 5.6	117.7± 1.3
Myelin	34.5±12.1	63.1±10.3

Fractions from rat brain homogenates (1000 *g*—10000 *g*) were subfractionated by a discontinuous sucrose gradient centrifugation. The supernatant obtained as described in Methods, equivalent to 180 μg protein, was added to each incubation medium. Each value represents the mean of 3 expreiments ±S.E.

showed about the same activities in the absence of the supernatant, while upon addition of the supernatant the enzyme activity in synaptosomes was activated more markedly than in other two fractions, indicating that the stimulation of adenyl cyclase activity in the crude mitochondrial fraction by the addition of the supernatant may be due largely to the stimulation of the synaptosomal enzyme activity. These results suggest that adenyl cyclase of synaptosomes may be different from the enzyme present in other particulate fractions of rat cerebral cortex.

The relationship between the degree of the stimulation and the concentration of the supernatant is depicted in Fig. 1. As the concentration of the supernatant increased, the accumulation of cyclic AMP increased progressively, reaching a plateau at a concentration of about 200 μg of protein. In the assay of the stimulatory activity described in the present work, preliminary experiments were usually carried out to determine the amount of protein of the supernatant so that the linear part of the curve was used.

The effect of dialysis of the supernatant has been examined (Table III). The supernatant was dialyzed for 6 hours at 4° (2×500 volumes). Dialysis of the supernatant caused a marked loss of the stimulatory activity. Longer dialysis (24 hours, 4×500 volumes) of the supernatant resulted in a complete loss of the stimulatory activity.

The stimulatory substance(s) was purified using chromatography on Sephadex G-25 Superfine. Fig. 2 depicts the elution profile of the lyophilized supernatant from a Sephadex G-25 Superfine. As shown in Fig. 2, the active fraction eluted at 53 ml. Calibration of this column with Glucagon (mol. wt. 3350) and Vitamin B_{12} (mol. wt. 1350) as markers indicated that this elution volume corresponded to a molecular weight of about 1000—1300.

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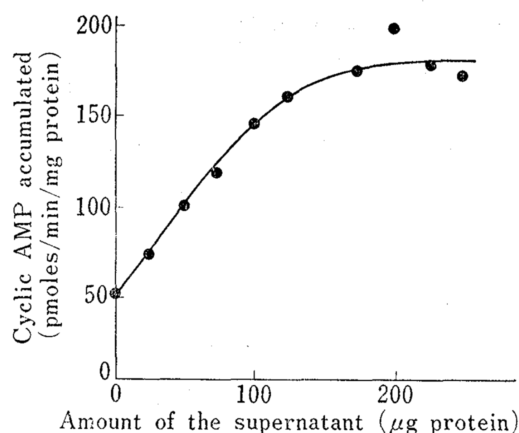

Fig. 1. Effects of the Supernatant Concentration on Adenyl Cyclase Activity in Synaptosomes from Rat Cerebral Cortex

The supernatant was obtained as described in Methods. The amounts of the supernatant varied from 25 to 250 μg of protein. Adenyl cyclase activity was determined as described in Methods.

Table III. Effect of Dialysis of Supernatant on Synaptosomal Adenyl Cyclase Activity

Additions	Adenyl cyclase activity (pmoles cyclic AMP/min/mg protein)
None-control	39.3±10.9
Supernatant	126.3± 8.7
Supernatant (dialyzed for 6 hr)	61.5±11.4
Supernatant (dialyzed for 12 hr)	40.5± 3.8

The supernatant (130 μg protein) was obtained as described in methods and then dialyzed for 6 hours (2×500 volumes) and 24 hours (4×500 volumes) *versus* 50 mM Tris-buffer (pH 7.4). Adenyl cyclase activity was determined as described in Methods. Data were averages ±S.D. of 3 separate analyses.

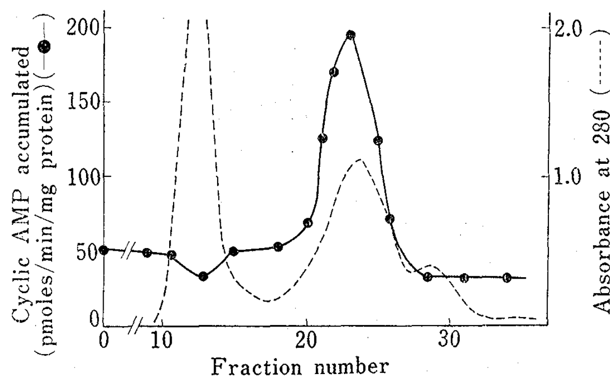

Fig. 2. Chromatography of Adenyl Cyclase Stimulatory Substance (s) on Sephadex G-25 Superfine

Rat brain cerebral cortex was homogenized in five volumes of ice-cold 10 mM Tris-buffer (pH 7.4). The homogenate was centrifuged: at 1000 *g* for 20 min, at 10000 *g* for 20 min and at 105000 *g* for 60 min. The resulting supernatant was lyophilized and applied to a column of Sephadex G-25 Superfine (1.5×43 cm) equilibrated previously in 50 mM Tris-buffer (pH 7.4). The column was eluted at 4° with the same buffer in 2.5 ml fractions at a flow rate of 10 ml per hour. An aliquot (100 μl) of each fraction was assayed for its ability to activate the synaptosomal adenyl cyclase activity. The void volume of the column was 23 ml and the stimulatory activity was eluted at 53 ml.

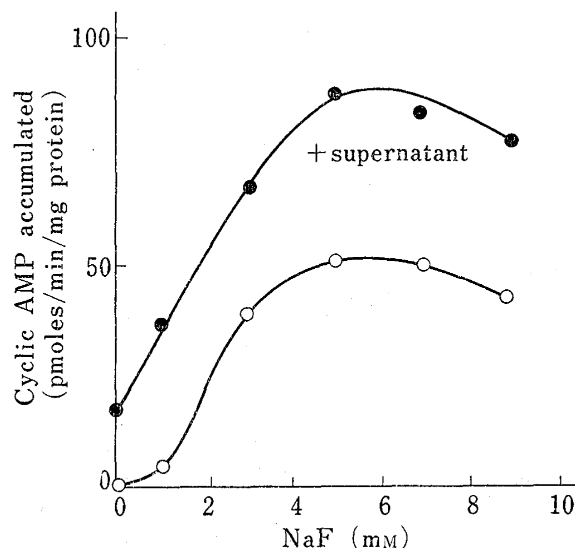

Fig. 3. Effects of the Supernatant on the Synaptosomal Adenyl Cyclase Activity in the Presence of Various Concentrations of Sodium fluoride (NaF)

Sodium fluoride was included the assay mixture at the concentrations indicated. The supernatant was obtained as described in Methods, equivalent 180 μg of protein, was added to incubation medium. Adenyl cyclase activity was determined as described in Methods.

Since it has been reported that homogenate from brain tissue was not stimulated by catecholamine,[6,12] we have investigated the effect of norepinephrine on synaptosomal adenyl cyclase activity in the absence or presence of the supernatant. As shown in Table IV, in contrast to the stimulatory effect of the supernatant, norepinephrine (100 μM) did not influence the basal enzyme activity significantly. On the other hand, the stimulatory effect of the supernatant was markedly reduced by the addition of norepinephrine. The reason for this inhibitory effect of norepinephrine is not clear.

12) K.V. Hungen and S. Roberts, *Nature New Biology*, 242, 58 (1973).

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Table IV. Effects of Norepinephrine and Supernatant on Adenyl Cyclase Activity in Synaptosomes

Additions	Adenyl cyclase activity (pmoles cyclic AMP/min/mg protein)
None	38.1±2.4
Supernatant	104.7±3.3
Norepinephrine (100 μM)	33.4±4.7
Supernatant+Norepinephrine	54.7±5.0

The supernatant obtained as described in Methods, equivalent to 140 μg of protein, was added to each incubation medium. Norepinephrine (100 μM) was added. Data are mean ±S.D. from 3 separate determinations.

Sodium fluoride (NaF) is a well-known potent activator of adenyl cyclase in various tissue homogenates.[13)] In order to determine whether the sites of action of the stimulatory substance(s) in the supernatant and NaF on the cyclic AMP accumulation are the same or not, the effect of the supernatant on the adenyl cyclase activity in the absence or presence of NaF was investigated. As shown in Fig. 3, the stimulatory substance(s) induced a further increase in the rate of the formation of cyclic AMP in the presence of NaF, indicating that the stimulatory substance(s) and NaF act at distinctly different sites on the adenyl cyclase system of rat brain synaptosomes.

Recently, it has been reported that synaptosomes have many of the properties of presynaptic nerve ending in intact nervous system.[14)] Although it is not clear whether the stimulatory substance(s) have any physiological function, the finding that adenyl cyclase in synaptosomes is very sensitive to the stimulatory substance(s) suggests that alternation of concentration of the stimulatory substance(s) may regulate the level of cyclic AMP in synaptosomes.

Adenyl cyclase is a multicomponent macromolecular system existing as integral parts of mammalian plasma membranes. Two models[15)] have been proposed which have in common the basic concept that adenyl cyclase is composed of a receptor-regulatory component(s) and a catalytic component(s). It has been reported by Perkin and Moore[13a)] that NaF activation is the result of the dissociation of the normal inhibition imposed on the catalytic component. There is at least two possibilities for the activation of synaptosomal adenyl cyclase by the stimulatory substance(s) in the supernatant. One possibility is that the stimulatory substance(s) in the supernatant depresses some inhibitory factors of adenyl cyclase. The alternative explanation is that the stimulatory substance(s) directly activates the accumulation of cyclic AMP. Further experiments are necessary to clarify this phenomenon.

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