Notes

Activation of Adenyl Cyclase and Adenosine 3',5'-Monophosphate Phosphodiesterase in Rat Brain Synaptosomes

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When the 105000 g supernatant from rat brain cerebral cortex was fractionated using chromatography on a Sephadex G-25 Superfine, similar low molecular factors activated adenyl cyclase and adenosine 3',5'-monophosphate (cyclic AMP) phosphodiesterase in rat brain synaptosomes. Therefore a comparative study of the effects of these factors with those of sodium fluoride (NaF) or imidazole was carried out. These factors additively enhanced NaF stimulated adenyl cyclase and did activate the particulate (synaptosomal) cyclic AMP phosphodiesterase but not soluble enzyme. These results suggest that these factors act on a distinctly different site from that of NaF and imidazole.

Adenosine 3',5'-monophosphate (cyclic AMP) has been implicated as a second messenger which mediates the cellular effects of a variety of hormone and neurohormonal agents.[2] The enzymes involved in the synthesis and hydrolysis of cyclic AMP occur in the mammalian central nervous system and are present in particulary high concentrations in the synaptosomal fraction of brain homogenates.[3]

We have recently reported that the addition of the 105000 g supernatant from rat brain to adenyl cyclase system of synaptosomes increased the accumulation of cyclic AMP significantly.[4] In the course of the investigation of this stimulatory factor, we found that synaptosomal cyclic AMP phosphodiesterase was also activated by an eluate from Sephadex G-25 Superfine column. Therefore, a study was undertaken to compare the effects of these factors with those of sodium fluoride or imidazole on adenyl cyclase or on cyclic AMP phosphodiesterase in rat brain synaptosomes.

Experimental

Cerebral cortical grey matter was obtained from young rats of an inbred Sprague-Dawley strain (CLEA Japan, Inc.). Homogenates were prepared in nine volumes of ice-cold solution containing 0.32M sucrose and 3 mM $MgSO_4$ and were centrifuged at 1000 g for 20 min to remove nuclei and cell debris. The crude mitochondrial fraction was obtained by centrifuging the 1000 g supernatant fraction at 10000 g for 20 min. This preparation was subfractionated into the fractions which contained synaptosomes, myeline and mitochondria by the method of Whittaker.[5]

Adenyl cyclase activity was determined by the conversion of [^{3}H]ATP to [^{3}H]cyclic AMP based on the method of Krishna, et al.[6] Unless otherwise described, the standard incubation medium (0.6 ml) contained the following components in the final concentrations indicated: 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. Incubation was conducted at 30° in air for 15 min.

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3) a) E.W. Sutherland, T.W. Rall, and T. Menon, J. Biol. Chem., 237, 1220 (1962); b) E. De Robertis, G.R.D. Arnaiz, M. Alberici, R.W. Butcher, and E.W. Sutherland, J. Biol. Chem., 242, 3487 (1967).
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Cyclic AMP phosphodiesterase activity was determined by the method of Pöch[7] with a cyclic AMP concentration of 3.6×10^{-4}M. Enzymatic activity was measured as the rate of hydrolysis of cyclic AMP in a standard reaction medium (0.5 ml) containing [^{14}C]cyclic AMP (0.05 μCi), 3.0 mM Mg-acetate, 2.0 mM 5'-AMP, 100 mM Tris-HCl buffer (pH 7.4) and enzymes. Incubation was run at 30° for 15 min in air.

Results and Discussion

As previously reported,[4] the 105000 *g* supernatant contained the soluble stimulatory factor which increased the accumulation of cyclic AMP in synaptosomes from rat brain cerebral cortex. The results prompted us to determine whether this accumulation of cyclic AMP resulted from an inhibition of cyclic AMP phosphodiesterase in synaptosomes. Therefore, we have investigated the effects of this stimulatory fraction on this enzyme. Contrary to our expectation, synaptosomal cyclic AMP phosphodiesterase was also activated by this fraction, indicating that increased cyclic AMP accumulation with the stimulatory factor might be due to increased cyclic AMP formation by adenyl cyclase rather than decreased rate of degradation by cyclic AMP phosphodiesterase. The following experiments were next carried out. As can be seen in Fig. 1, when an aliquot (100 μl) of the Sephadex G-25 column fraction was examined for the ability to activate the synaptosomal cyclic AMP phosphodiesterase as well as adenyl cyclase, coincident peak of activating factor was found (fraction 23) for the two enzymes. The first peak fraction (fractions 12—14) is likely to be dependent on cyclic AMP phosphodiesterase contained in the 105000 *g* supernatant, because this peak fraction was not observed when the boiled supernatant was fractionated. These stimulations were found
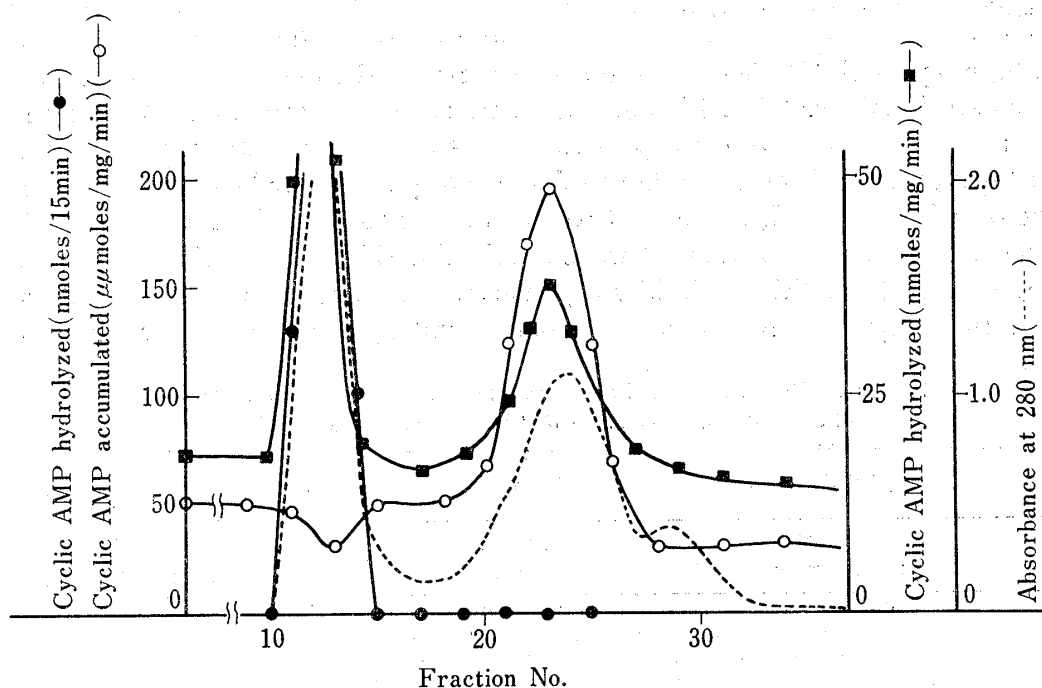

Fig. 1. Chromatography of the 105000 *g* Supernatant from Rat Cerebral Cortex on Sephadex G-25 Superfine

Rat brain cerebral cortex was homogenized in five volumes of ice-cold 10 mM Tris-HCl buffer (pH 7.4). The homogenates was centrifuged; 1000 *g* for 20 min, 10000 *g* for 20 min and 105000 *g* for 60 min. The resulting supernatant was lyophilized and then applied to a column of Sephadex G-25 superfine (1.5 × 43 cm) equilibrated previously in 50 mM Tris-HCl buffer (pH 7.4). The column was eluted with same buffer in 2.5 ml fraction at a flow rate of 10 ml per hour. An aliquot (100 μl) of each fraction was assayed for its cyclic AMP phosphodiesterase activity (—●—) and its ability to activate the synaptosomal adenyl cyclase (—○—) or cyclic AMP phosphodiesterase activity (—■—). Assays of adenyl cyclase and cyclic AMP phosphodiesterase activities are described in the Experimental. The protein content of synaptosomes was 200 μg in each assay. Column buffer alone has no effects on adenyl cyclase and cyclic AMP phosphodiesterase activities.

7) G. Pöch, *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **268**, 299 (1971).

in a fraction of molecular weights of 1000—1300 as previously reported.[4]

In order to examine the relation of the stimulatory effects between adenyl cyclase and cyclic AMP phosphodiesterase, sodium fluoride (NaF), a potent activator of adenyl cyclase in various tissue homogenates,[8] was added to each incubation medium, and then both adenyl cyclase and cyclic AMP phosphodiesterase activities were assayed. As shown in Fig. 2, we observed that the plot of adenyl cyclase activity to NaF concentration in synaptosomes was sigmoidal in nature in accordance with the observations of Birnbaumer, et al.[8a] for rat fat cell ghost and of Perkins, et al.[8b] for rat cerebral cortex homogenates. However, the addition of various concentrations of NaF to cyclic AMP phosphodiesterase system did not affect the synaptosomal enzyme activity at all. Previous studies[4] showing that the stimulatory factor in the 105000 g supernatant enhanced NaF stimulated adenyl cyclase additively suggest that different mechanisms are responsible for the stimulatory effects of these two agents. These results indicate that there is little relationship between the stimulation of adenyl cyclase and that of cyclic AMP phosphodiesterase.

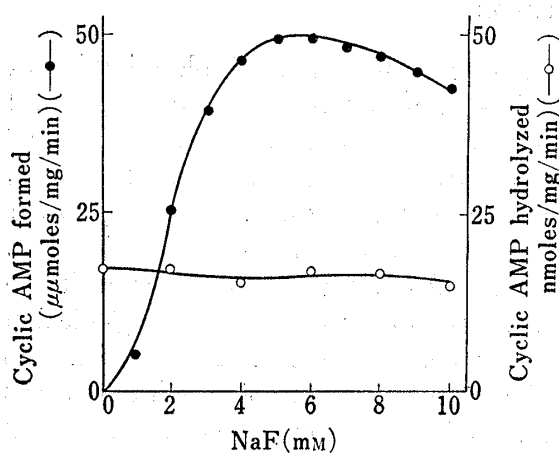

Fig. 2. Effect of Concentration of NaF on Adenyl Cyclase (—●—) and Cyclic AMP Phosphodiesterase Activities(—○—) in Synaptosomes from Rat Cerebral Cortex

Conditions of incubations and assays are described in the Experimental. The protein content of synaptosomes was 200 μg in each assay.

The brain cyclic AMP phosphodiesterase was reported to be distributed in both soluble and particulate fractions.[3b,9] Although the relationship between these two enzymes is not fully understood, a comparative study of the effects of the stimulatory factor and imidazole on cyclic AMP phosphodiesterase in both soluble and synaptosomal enzymes was carried out. As can be seen from Table I, in contrast to membrane-bound cyclic AMP phosphodiesterase, the enzyme in the soluble supernatant was not stimulated by the addition of the stimulatory factor. However, the addition of imidazole, an activator for cyclic AMP phosphodiesterase,[10] stimulated the enzyme activities in both soluble and synaptosomal enzymes (Table II).

TABLE I. Effect of the Stimulatory Factor(s) on Soluble Supernatant and Synaptosomal Cyclic AMP Phosphodiesterase Activities

Enzyme preparations	Control	With the stimulatory factor(s)
Supernatant	42.8±5.1	39.8±4.0
Synaptosomes	46.6±5.1	84.1±7.0[a]

Rat cerebral cortex was homogenized in 50 mM Tris-HCl buffer (pH 7.4). The homogenate was centrifuged at 1000 g for 20 min, 10000 g for 20 min and 105000 g for 60 min. The 105000 g supernatant was then dialyzed extensively against the same buffer. An aliquot of this supernatant fluid used a source of cyclic AMP phosphodiesterase in the supernatant. The synaptosomes were prepared as described in the text. One-tenth ml of the stimulatory fraction (23 fractions) from a Sephadex G-25 superfine in Fig. 1 was added to the enzyme system as the stimulatory factor(s). Protein concentration in μg per 0.5 ml of the reaction mixture: supernatant, 30; synaptosomes, 220. Activity is expressed as mμ moles of cyclic AMP hydrolyzed per 15 min. The values reported are the average results of single experiment carried out in triplicate. The ranges of values are represented by average deviations.
a) The addition of the stimulatory factor(s) significantly ($p < 0.01$) increased cyclic AMP phosphodiesterase activity of the synaptosomes.

8) a) L. Birnbaumer, S.L. Pohl, and M. Rodbell, J. Biol. Chem.. 244, 3468 (1969); b) J.P. Perkins and M.M. Moore, J. Biol. Chem., 246, 62 (1971); c) G.I. Drummond, D.L. Severson, and L. Duncan, J. Biol. Chem., 246, 4166 (1971).
9) W.Y. Cheung and L. Salganicoff, Nature, 214, 90 (1967).
10) R.W. Butcher and E.W. Sutherland, J. Biol. Chem., 237, 1244 (1962).

TABLE II.   Effects of Various Concentrations of Imidazole
on Soluble Supernatant and Synaptosomal Cyclic AMP
Phosphodiesterase Activities

Addition imidazole (mM)	Supernatant	Synaptosomes
0	35.3	54.9
5	40.6	52.8
10	41.5	55.1
20	51.7	77.1
40	54.9	82.4
80	44.6	73.7

The enzyme preparations of the supernatant and the synaptosomes were
prepared as described in Table I and the text, respectively.   Protein concent-
ration in μg per 0.5 ml of the reaction mixture: supernatant, 30; synaptosomes
220.   Activity is expressed as mμ moles of cyclic AMP hydrolyzed per 15 min.
The solution of imidazole was adjusted to pH 7.4 with acetic acid.

These results indicate that the mode of action of this stimulatory factor on the synapto-
somal cyclic AMP phosphodiesterase is different from that of imidazole.   These results also
suggest that this stimulatory factor of cyclic AMP phosphodiesterase would be different from
those obtained by various investigators.[11]

Recent evidence showed that the cyclic AMP system may be associated with the synaptic
transmission physiologically and that cyclic AMP plays a prominent role in sympathetic
nervous function.[12] Although the physiological significance of these stimulatory factors has
not yet been established, our results suggest that these factors may play an important role in
the regulation of cyclic AMP levels in synaptosomes.

11)   S. Kakiuchi and R. Yamazaki, Biochem. Biophys. Res. Commun., 41, 1104 (1970); W.Y. Cheung, Biochem.
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        gard, Science, 171, 1156 (1971).