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FACTORS AFFECTING SOLUBILIZED DOPAMINE-SENSITIVE ADENYLATE CYCLASE

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Abstract—Bovine striatal adenylate cyclase was solubilized with sodium cholate and assayed for its responsiveness to a variety of agents. Magnesium ions (0–20 mM), guanylyl-imidodiphosphate (10–50 μM), and striatal lipids were effective in increasing enzyme activity. The adenylate cyclase could be stimulated by dopamine, and neuroleptic drugs inhibited the effect of dopamine (50 μM) with potencies that paralleled their clinical potencies. The IC_{50} values for spiroperidol, haloperidol and chlorpromazine were 0.2, 3 and 500 nM respectively.

In our recent reports we described the solubilization of dopamine-sensitive adenylate cyclase and [^{3}H]spiroperidol binding sites from bovine striata [1–3]. In the present paper we have examined in detail the factors involved in maintaining the enzyme activity following solubilization. The various factors known to affect adenylate cyclase activity in particular or membrane enzymes in general were investigated.

We examined the effects of Mg^{2+}, guanylyl-imidodiphosphate (GPP(NH)P), which is a nonhydrolyzable analogue of GTP, and striatal tissue lipids on solubilized adenylate cyclase, with emphasis on the maintenance of its sensitivity to dopamine. As it has been observed in several laboratories that neuroleptic drugs inhibit dopamine-sensitive adenylate cyclase in homogenized preparations [4–6], we studied the response of the solubilized form of the enzyme to the drugs and determined IC_{50} values for them.

MATERIALS AND METHODS

Whole beef brains were obtained at a local slaughterhouse. Upon arrival at the laboratory, the striata were removed immediately and stored individually at −70°.

The ^{3}H-cyclic AMP used in the cyclic AMP/kinase binding assay was obtained from the New England Nuclear Corp., Boston, MA. Spiroperidol was supplied by Janssen Pharmaceutica, Beerse, Belgium; haloperidol by McNeil, Toronto, Ontario; and fluphenazine and chlorpromazine by Poulenc, Montreal, Quebec. The tetralithium salt form of GPP(NH)P was purchased from Boehringer Mannheim, Montreal, Quebec. All of the biochemicals (e.g. dopamine hydrochloride, sodium cholate, maleic acid and crude soybean phospholipid) were obtained from the Sigma Chemical Co., St. Louis, MO. The soybean phospholipids, consisting mainly of phosphatidylcholine, were acetone-washed and dried under nitrogen before use. Inorganic salts were reagent grade.

Homogenization and solubilization

The homogenization and solubilization steps were modified from those of Hoffmann [7]. During the 16–20 hr prior to homogenization, the striata that were to be used were stored at −20°. After being weighed, the striata were cut up and added to 3 vol. of homogenizing buffer (75 mM Tris maleate and 20 mM EGTA‡, pH 7.4) and then were homogenized by taking 20 strokes with a manual Teflon homogenizer. To every 100 ml of homogenate the following chilled ingredients were added: 3.0 ml of 2.0 M Tris maleate, pH 7.5; 3.8 ml of 0.5 M sodium cholate; 5.0 ml of 0.3 M $MgSO_4 \cdot 7H_2O$; 50 ml of saturated ammonium sulfate; and 38 mg of dopamine hydrochloride. GPP(NH)P was added to a final concentration of 1 μM. The mixture was stirred for 20 min on ice and then centrifuged at 200,000 *g* for 20 min. The supernatant fraction was brought to 39% of saturation with cold saturated ammonium sulfate, and GPP(NH)P was added again (1 μM). (The floating lipid layer was stored on ice temporarily and sonicated and used as described later.) The solution was stirred for 20 min on ice and then centrifuged as indicated previously. The supernatant fraction was increased to 49% of saturation, and again GPP(NH)P was added (1 μM). After stirring and centrifuging as before, the 39–49% fraction (pellet) was resuspended in 100 mM Tris maleate and 2 mM EGTA, pH 7.4, and 1 μM GPP(NH)P by grinding gently with a rounded smooth-glass rod. Some of the experiments included addition of dopamine and lipids after the grinding, and they will be described later.

Dialysis

To remove residual cholate and ammonium sulfate the resuspension was dialyzed immediately at 4° in 100 vol. of 10 mM Tris maleate and 0.2 mM EGTA,

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‡ EGTA = ethyleneglycolbis(amino-ethylether)tetra-acetate.

pH 7.2, 15 mM $MgSO_4$ and 1 μM GPP(NH)P, while being stirred vigorously. After 1 hr the solution was replaced, and again after another hour. During dialysis the preparation changed from a translucent yellow solution to opaque light yellow-brown particulate matter. In agreement with the findings of Hoffmann [7], we observed that particulate formation was necessary in order to obtain enzyme activity.

Adenylate cyclase assay

After being dialyzed for at least 2.5 hr, the preparation was assayed using a modification of the method that we have used always in our homogenate experiments [8]. Unless indicated otherwise, the following assay conditions were employed. The total assay volume of 100 μl contained, in final concentrations, the following reagents: 80 mM Tris maleate buffer, pH 7.4; 10 mM theophylline; 10 mM $MgSO_4$; 10 μM GPP(NH)P; 10 μl of dialyzed enzyme preparation; 5 μl of dopamine solution or any other drug tested; and lipid (described later). The tubes were incubated at 30° for 10 min. The ATP (0.5 mM) was added, and the tubes were incubated at 30° for another 10 min. The reactions were stopped by placing the tubes in a boiling-water bath for 2.5 min. Low speed centrifugation for 10 min removed particulate matter. From the clear colourless supernatant fractions 10 μl aliquots were removed and frozen for cyclic AMP assay by the method of Brown and Makman [9], which employs the protein kinase binding method devised by Gilman [10]. Protein concentrations were determined by the method of Lowry *et al.* [11].

Triplicate tubes were incubated for every condition tested, and at least three preparations were made for every condition tested. The results of the *t*-tests and analyses of variance performed on the data will be described in Results.

Variations

Lipids added to assays. Hoffmann [7] indicated that it was necessary to add sonicated phospholipids (16 mM) to the assays in order to demonstrate adenylate cyclase activity. We used that concentration in the tests reported in our previous papers [2, 3], and here compare the effects of those soybean phospholipids with striatal lipids.

The floating lipid layer obtained from the first 200,000 *g* centrifugation was added to the Tris maleate incubation buffer, pH 7.4, in a concentration of 40 mg/ml. The mixture was sonicated on ice for 30 min. The same steps were carried out for the crude soybean phospholipids. The final concentration of either lipid solution in the assay tubes was 10 mg/ml.

Magnesium ions in the assays. In experiments with homogenate [8] the final Mg^{2+} concentration was always 2 mM. Hoffmann [7] used 10 mM in his solubilization experiments. Therefore, we decided to compare a range of Mg^{2+} concentrations (0–20 mM) in our assays.

Dopamine and lipids added prior to dialysis. In these experiments 2 ml of the final resuspended pellet was added to a tube containing 1 mg of dopamine hydrochloride and then vortexed. Then 1 ml of the contents of the tube was removed to a second tube that contained 0.5 ml of sonicated floating lipid layer and was vortexed. Also, 1 ml of the final resuspended pellet was added to 0.5 ml of sonicated floating lipid layer. Samples from the three different tubes were used in a comparison study.

Neuroleptic drugs in the assays. Once dopamine stimulation (50 μM) of adenylate cyclase has been observed repeatedly, some neuroleptic drugs were tested for their ability to inhibit the stimulation. Spiroperidol was dissolved in warm 0.01 N acetic acid. Haloperidol was dissolved in warmed 0.1% tartaric acid. Other drugs were dissolved readily in water.

RESULTS

Lipids added to assays

A comparison of the effectiveness of soybean phospholipids and striatal lipids in increasing enzyme activity is shown in Table 1. In all conditions significant stimulation by dopamine (DA) occurred. The highest activity (3.13 nmoles cyclic AMP per mg protein per 10 min) was observed when the assay tubes, containing sonicated striatal lipids (10 mg/ml)

Table 1. Effects of lipids on adenylate cyclase*

Incubation lipids (10 mg/ml)	cAMP [nmoles·(mg protein)$^{-1}$·(10 min)$^{-1}$] GPP(NH)P (10 μM)	GPP(NH)P + DA (10 μM) (50 μM)
No lipids	0.17 ± 0.005	0.23 ± 0.026
Crude soybean phospholipids	0.20 ± 0.024	0.64 ± 0.203
Floating lipid layer	1.02 ± 0.100	1.32 ± 0.058
Floating lipid layer; 10-min preincubation	1.81 ± 0.162	3.13 ± 0.343

* All comparisons of values, horizontally or vertically, are significant at $P < 0.01$ or better. The value shown for crude soybean phospholipids includes the results of assays with or without a 10-min preincubation. Each value is the average of four separate experiments ± S.E.M.

Table 2. Effect of Mg^{2+} and GPP(NH)P on adenylate cyclase*

Mg^{2+} (mM)	cAMP [nmoles·(mg protein)$^{-1}$·(10 min)$^{-1}$] Basal	GPP(NH)P (10 μM)
0	0.13 ± 0.012	0.84 ± 0.260
2	0.18 ± 0.005	1.13 ± 0.235
4	0.28 ± 0.010	2.18 ± 0.030
10	0.66 ± 0.107	7.04 ± 0.033
20	0.61 ± 0.035	16.40 ± 0.059

* The basal value obtained with 10 mM Mg^{2+} is not significantly different from that obtained with 20 mM Mg^{2+}. All other comparisons are significant, at $P < 0.01$ or better. Each value is the average of five separate experiments ± S.E.M.

and all other reagents, were incubated for 10 min before the substrate, ATP, was added. Note that both GPP(NH)P- and dopamine-stimulated enzyme activities were increased significantly ($P < 0.001$) in the presence of the striatal lipids, which had been obtained from the first ultrahigh centrifugation.

Some tests were conducted in which two concentrations of striatal lipids (10 and 20 mg/ml) were compared. As the higher concentration more consistently maintained dopamine sensitivity of the enzyme, the following experiments were conducted with striatal lipids (20 mg/ml).

Magnesium ions in the assays

Tests were conducted in which the Mg^{2+} concentration was varied (Table 2). The results indicate that Mg^{2+} (10 mM) produced the maximal basal activity. The optimum concentration of GPP(NH)P was found to be 10 μM.

Dopamine and lipids added prior to dialysis

Table 3 shows that including 0.5 mg dopamine hydrochloride for each ml of preparation to be dialyzed (2.64 mM dopamine) increased significantly GPP(NH)P- and dopamine-sensitive adenylate cyclase. Addition of sonicated striatal lipids (20 mg/ml) decreased the sensitivity of adenylate cyclase to dopamine and GPP(NH)P. However, the dopamine/lipid combination produced a greater increase in dopamine-sensitive adenylate cyclase (1.89 vs 0.93 nmoles cyclic AMP/per mg protein per 10 min; $P < 0.001$) than did dopamine alone (2.31 vs 1.44 nmoles cyclic AMP per mg protein per 10 min; ($P < 0.05$). Therefore, in subsequent experiments both dopamine and sonicated striatal lipids, in the concentrations indicated here, were added to the solubilized preparations immediately prior to dialysis.

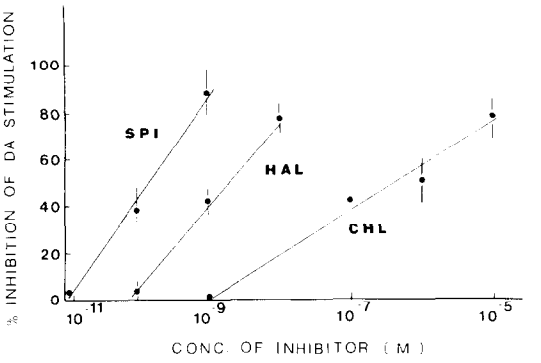

Fig. 1. Percentage inhibition of dopamine (50 μM) stimulated adenylate cyclase by neuroleptic agents. The data points represent the means ± S.E.M. of three separate experiments. Abbreviations: SPI, spiroperidol; HAL, haloperidol; and CHL, chlorpromazine. The estimated IC_{50} values are SPI 0.2 nM; HAL, 3 nM; and CHL, 500 nM. The basal and dopamine-stimulated adenylate cyclase activities were 6.42 ± 0.850 and 11.84 ± 1.024 nmoles cyclic AMP per mg protein per 10 min.

Neuroleptic drugs in the assays

Based upon the results of the preceding experiments, tests were set up to examine solubilized dopamine-sensitive adenylate cyclase activity and its inhibition by neuroleptic drugs.

Initially, five different solubilized preparations were tested on different days to generate enzyme activity data using the improved, closer to optimal, conditions. GPP(NH)P-sensitive activity was 6.86 nmoles cyclic AMP per mg protein per 10 min, and the addition of dopamine (50 μM) increased the value to 9.01 nmoles cyclic AMP per mg protein per 10 min; these results were significantly different by analysis of variance ($P < 0.05$). Basal activity was always less than one-tenth of the GPP(NH)P activity.

With dopamine (50 μM) in the assay, the neuroleptic drugs spiroperidol, haloperidol, and chlorpromazine were tested in the assays to determine their potencies in inhibiting the enzyme. Figure 1 illustrates that spiroperidol most strongly inhibited

Table 3. Effect of dopamine and striatal lipids added prior to dialysis*

Assay additions	cAMP [nmoles·(mg protein)$^{-1}$·(10 min)$^{-1}$] DA (a)	Lipid (b)	DA + lipid (c)
(1) Basal	0.17 ± 0.01	0.09 ± 0.003	0.09 ± 0.01
(2) GPP(NH)P	1.44 ± 0.16	1.03 ± 0.07	0.93 ± 0.10
(3) GPP(NH)P + DA	2.31 ± 0.63	1.01 ± 0.07	1.89 ± 0.16

* Assay conditions: GPP(NH)P, 10 μM; DA, 50 μM. The statistical comparisons are as follows: 1a vs 2a $P < 0.001$; 2a vs 3a $P < 0.05$; 1b vs 2b $P < 0.001$; 1c vs 2c $P < 0.001$; 2c vs 3c $P < 0.001$; 1a vs 1b $P < 0.005$; 1b vs 1c NS; 2a vs 2b $P < 0.05$; 2b vs 2c NS; 3a vs 3b $P < 0.001$; and 3b vs 3c $P < 0.002$. NS = not significant. Each value is the average of five separate experiments ± S.E.M.

dopamine-sensitive activity (IC_{50} = 0.2 nM), and chlorpromazine was the weakest inhibitor (IC_{50} = 500 nM). Haloperidol (IC_{50} = 3 nM) was closer to spiroperidol in effectiveness. Although some of the values indicate fairly wide standard errors of the mean, none of the values overlaps any other value.

The effects of various neuroleptic drugs were also compared in solubilized and homogenized adenylate cyclase assays in parallel experiments. The results are shown in Table 4. The dopamine-stimulated adenylate cyclase acitivity was inhibited by all neuroleptic drugs tested, and propranolol, a β-receptor blocking agent, did not have a significant effect in either the homogenized or the solubilized system.

DISCUSSION

In this paper we have examined some of the factors involved in the regulation of adenylate cyclase, and its sensitivity to stimulation by dopamine, once the enzyme has been solubilized from striatal membranes. The refinements made in these experiments produced an approximately 10-fold increase in enzyme activity as compared with the results in our previous paper [2].

Earlier reports have described the dependence of adenylate cyclase in striatal homogenate, on divalent cations, particularly Mg^{2+} [12, 13]. Recently, detailed kinetics of its involvement in dopamine and GPP(NH)P stimulation of the enzyme have been presented [14]. Our results demonstrate now the dependence of adenylate cyclase on Mg^{2+} when the enzyme is in a solubilized form. However, further tests will be necessary to disclose the molecular characteristics of the interaction. Multiple sites may be involved. The adenylate cyclase activity in the presence of Mg^{2+} in the solubilization experiments probably resulted at least in part from Mg^{2+} interaction with lipid/protein associations essential in the reconstitution of membrane enzyme activity [15].

Several reviews have discussed the role of guanine nucleotides in hormonal activation of adenylate cyclase [12, 16, 17]. Their interactions with several of the enzyme stimulants have been examined more closely, including NaF [18], catecholamines [19, 20] and combination treatments [21]. Furthermore, a guanine nucleotide regulatory component that serves to reconstitute hormonally stimulated adenylate cyclase has been purified recently [22]. We have found it necessary to include GPP(NH)P at every solubilization step in order to obtain dopamine stimulation of the enzyme, perhaps because the nucleotide does function as part of the receptor/enzyme coupling mechanism, in addition to having a stimulation site on the enzyme. Table 2 shows that GPP(NH)P stimulation of adenylate cyclase correlates well with the Mg^{2+} concentration in the assay.

Table 4. Effect of neuroleptic drugs on adenylate cyclase in homogenate and after solubilization*

Drug	% Inhibition	
	Homogenate	Solubilized
Spiroperidol (1 μM)	46 ± 8	97 ± 2
Haloperidol (1 μM)	33 ± 4	87 ± 1.6
Fluphenazine (1 μM)	87 ± 6	72 ± 2.6
Chlorpromazine (1 μM)	74 ± 8	57 ± 3
Propanolol (1 μM)	10 ± 2	5 ± 0.8

* Each value is the average of four separate experiments ± S.E.M. The basal and dopamine (50 μM) stimulated adenylate cyclase values were 0.367 and 0.580 nmoles cyclic AMP per mg protein per 10 min in homogenate and 6.970 and 11.103 in solubilized system. The S.E.M. were less than 10%. All drugs caused significant ($P < 0.005$) inhibition except propanolol.

It has been observed that membrane phospholipids affect the activity of hormonally activated adenylate cyclase [12, 16]. Reports have shown that the kinds of phospholipids that are effective depend upon the tissue source of the enzyme [23–26]. Phospholipids determine the extent of fluidity of membranes, which in turn affects the positioning and alignment of membrane proteins. It is likely that, as fluidity changes, the coupling of membrane receptors to membrane enzymes (e.g. adenylate cyclase) changes. Such an occurrence was observed recently for β-catecholamine stimulation of adenylate cyclase [27]. In addition, drug-induced changes in fluidity have been reported to alter significantly the ability of guanine nucleotides to stimulate adenylate cyclase [28, 29].

Our results have shown that striatal lipids are more effective than crude soybean phospholipid in increasing the dopamine-sensitive adenylate cyclase. This preference of floating striatal lipids could be due to the fact that this floating lipid layer is actually a mixture of lipids and hydrophobic protein [30]. We also have found it to contain 3.5% protein of the floating mass of lipids. The importance of this lipid–protein complex in the [^{3}H]spiroperidol binding for the dopamine receptor has been reported by Boyan-Salyers and Clement-Cormier [30].

In the second half of our experiments, we examined the effect of antipsychotic drugs on solubilized dopamine-sensitive adenylate cyclase. These results are presented in Fig. 1 and Table 4. Data from many laboratories have indicated a close correlation between clinical potencies of neuroleptic agents and their abilities to inhibit [^{3}H]haloperidol binding to receptors in striatal homogenate preparations [31]. There has also been a correlation between clinical potencies and the abilities of phenothiazine neuroleptics to inhibit striatal homogenate dopamine-sensitive adenylate cyclase; no such correlation has been apparent for butyrophenones, which inhibited the enzyme in high concentrations only. Our initial studies [2, 3] and, more importantly, our present study show that, when a solubilized form of the enzyme is tested, the inhibitory potencies of at least four neuroleptics (two butyrophenones and two phenothiazines) match their inhibitory potencies in [^{3}H]haloperidol binding and, therefore, their clinical potencies as well. The IC_{50} values fall in the same range for either test. The β-receptor blocking agent, propranolol, was without effect either in solubilized or homogenate adenylate cyclase. The reversal of potency of neuroleptic drugs in the solubilized system was not due to the experimental conditions since parallel experiments carried out in homogenate and solubilized preparation showed the same pattern of inhibition. These results are in excellent agreement with Joo and Wollemann [32]. They have also observed haloperidol to be the strongest inhibitor

in the nanomolar range in solubilized bovine retinal adenylate cyclase. Apparently, solubilization removes the disparate membrane interactions displayed by the neuroleptics in homogenate tests. Alternatively, there may be a positive coupling of dopamine D-2 receptor during solubilization and reconstitution. D-2 receptors have been shown to be negatively coupled to adenylate cyclase in anterior pituitary gland [33].

One final consideration is that in the homogenate experiments phenothiazines are more effective inhibitors of dopamine-sensitive adenylate cyclase than are butyrophenones, and in solubilization tests the potencies are reversed, as mentioned previously. Henn and Henn [34] have indicated that, like in our solubilization tests, haloperidol is a more potent inhibitor than chlorpromazine in striatal astroglial homogenate preparations of dopamine-sensitive adenylate cyclase, but in micromolar rather than nanomolar concentrations. Therefore, the relative contributions of solubilized neuronal adenylate cyclase and solubilized astroglial adenylate cyclase to our results should be examined.

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