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RELATIONSHIP BETWEEN THE INHIBITION OF ADENYLATE CYCLASE BY PENTOBARBITAL AND THE FUNCTIONAL COUPLING OF N_s AND THE CATALYTIC UNIT

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SUMMARY: The effect of barbiturate on adenylate cyclase system was examined in rat brain. Pentobarbital inhibited the enzyme activities in both synaptic membrane and solubilized catalytic unit of the system in dose and time-dependent manners. The inhibitory effect of pentobarbital was more potent on the activation of the system by NaF-$AlCl_3$ than on the basal activity. The inhibitory effect, however, was less in the synaptic membrane in which the catalytic unit was prestimulated through coupling with N_s by the treatment with NaF-$AlCl_3$. Similar results were obtained with the solubilized preparation which was pretreated with guanylyl-5'-imidodiphosphate before solubilization. On the other hand, the effect of pentobarbital was not modified by the treatment of the synaptic membrane with pertussis toxin. These findings indicate that barbiturates suppress primarily the activation of the catalytic unit through the coupling with guanine nucleotide-binding stimulatory protein (N_s) without affecting the inhibitory protein (N_i).

It has been suggested that the change in the cyclic AMP level in the central nervous system is related to the epileptic symptoms. The nucleotide level in the cerebrospinal fluid of epileptic patients is generally higher than that in normal subjects (1). Intraventricular injection of dibutyryl cyclic AMP is epileptogenic in experimental animals (2). High level of cyclic AMP is observed in the brain after the administration of convulsants or electroshock, and the increase is suppressed by anticonvulsants (3-8). Furthermore, clonidine, an α_2-adrenergic agonist, and propranolol, a β-adrenergic antagonist, show the anticonvulsant effect (9,10). These findings suggest that brain adenylate cyclase system plays some role in the regulation of epilepsy and is affected by anticonvulsant drugs, though no evidence is available at present for the site of the drug action on the enzyme.

Abbreviations: N_s (N_i), guanine nucleotide-binding stimulatory (inhibitory) regulatory protein in adenylate cyclase system; GppNHp, guanylyl-5'-imidodiphosphate; GABA, γ-aminobutyric acid.

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In this study, we attempted to examine the effect of barbiturates, especially of pentobarbital, on adenylate cyclase system in rat brain using the synaptic membrane and solubilized catalytic unit of the system. We report here that barbiturates inhibit the activity of the catalytic unit by affecting the functional coupling of N_s and the catalytic unit.

MATERIALS AND METHODS

[^{3}H]ATP was purchased from Amersham International Ltd. All other drugs and chemicals are of reagent grade from standard commercial sources.

Synaptic membrane was prepared from brains of male Wistar rats (150-200 g). The brains were homogenized in 10 volumes of 320 mM sucrose solution containing 5 mM Tris-HCl and 1 mM EDTA (pH 7.4) with a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 800 x g for 10 min to remove cell nuclei and debris. The post-nuclear supernatant was centrifuged at 10,000 x g for 30 min. The pellet was resuspended in 20 volumes of 5 mM Tris-HCl buffer (pH 7.4). After standing for 30 min, the suspension was centrifuged at 20,000 x g for 30 min. The resulting pellet was suspended in 250 mM sucrose-50 mM Tris-HCl (pH 7.4).

The catalytic unit of adenylate cyclase system was prepared from the above-mentioned synaptic membrane according to the method of Ross (11). In brief, adenylate cyclase was solubilized with 250 mM sucrose solution containing 50 mM Tris-HCl, 15 mM $MgCl_2$, 1 mM dithiothreitol, 0.7 % sodium cholate and 0.6 M ammonium sulfate (pH 8.0). After standing for 30 min, the mixture was centrifuged at 100,000 x g for 30 min. To the resulting supernatant, ammonium sulfate powder was added to a concentration of 35 % saturation. After standing for 30 min, the mixture was centrifuged at 20,000 x g for 30 min. The pellet was suspended in 50 mM Tris-HCl containing 8 mg/ml lecithin (pH 8.0) to stabilize the catalytic unit.

Pertussis toxin treated synaptic membrane was prepared according to the method of Kurose and Ui (12), by treating the synaptic membrane with 20 μg/ml activated pertussis toxin which had been incubated with 25 mM dithiothreitol in the presence of 1 mM NAD and 1 mM ATP at 30°C for 30 min.

Adenylate cyclase activity was measured according to the method of Salomon et al. (13) with some modifications (14). The standard reaction mixture consisted of 62.5 mM Tris-HCl, 10 mM $MgCl_2$, 0.2 mM cyclic AMP, 62.5 μM [^{3}H]ATP (5×10^5 cpm), 15 mM phosphocreatine, 50 unit/ml creatine phosphokinase and 5 mM theophylline (pH 7.4) in a final volume of 200 μl. In cases indicated, NaF, $AlCl_3$, GppNHp, and/or drugs were added to the reaction mixture. After incubation at 30°C for 20 min, the reaction was stopped by adding 200 μl of 10 % SDS solution containing 10 mM EDTA. Produced [^{3}H]cyclic AMP was separated by Dowex 50W x 4 and neutral alumina chromatographies, and measured for the radioactivity with a liquid scintillation spectrometer. Protein was determined by the method of Lowry et al. (15) using bovine serum albumin as the standard.

RESULTS AND DISCUSSION

Synaptic membrane of rat brain was pretreated with pentobarbital and then adenylate cyclase activity was measured in the presence or absence of NaF-$AlCl_3$. Fig.1 shows that pentobarbital inhibits both basal and NaF-$AlCl_3$-stimulated adenylate cyclase activities in dose- and time-dependent manners.

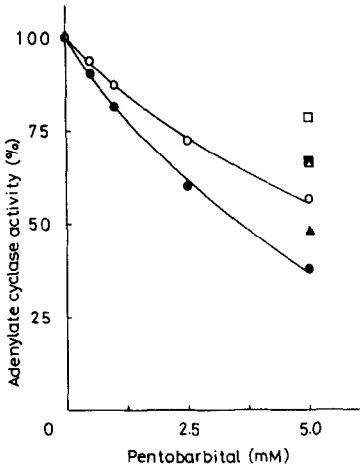


Fig. 1. Inhibition of adenylate cyclase activity in synaptic membrane by pentobarbital. The synaptic membrane was preincubated with indicated concentrations of pentobarbital in 125 mM Tris-HCl buffer (pH 7.4) for 15 min (■, □), 1 hr (▲,△), and 2 hr (●,○), and then measured for adenylate cyclase activity in the presence (■,▲,●) or absence (□,△,○) of 5 mM NaF and 200 μM $AlCl_3$. Without pentobarbital, adenylate cyclase activities were 37.2 and 118.2 pmol/min/mg protein in the absence and presence of NaF-$AlCl_3$, respectively.

The inhibition of the basal and NaF-$AlCl_3$-activated activities by 5 mM pentobarbital are approximately 40 and 60 %, respectively, indicating that the latter activity is more sensitive to pentobarbital. Although millimolar concentrations of pentobarbital are required to inhibit the enzyme activities, the concentrations are coincident with those reported by Lohse, et al. (16). They observed that only higher concentrations of pentobarbital inhibited both the basal and 5'-N-ethylcarboxamidoadenosine-stimulated adenylate cyclase activities of NIE 115 neuroblastoma cells. The results suggest a possibility that pentobarbital inhibits not only the activity of the catalytic unit itself but also the functional coupling between N_s and the catalytic unit in brain adenylate cyclase system.

Therefore, we attempted to examine if the interaction between N_s and the catalytic unit was actually affected by pentobarbital. The synaptic membrane was first incubated with various concentrations of NaF in the presence of 200 μM $AlCl_3$ for 15 min to stimulate the catalytic unit through the association with α-subunit of N_s, and then treated with 5 mM pentobarbital. The higher was the concentration of NaF for the prestimulation of the catalytic unit, the

Table 1. Relationship between stimulation of adenylate cyclase activity by pretreatment with NaF-$AlCl_3$ and inhibition of the activity by pentobarbital

Pretreatment NaF (mM)	Adenylate cyclase activity (pmol/min/mg protein)		Inhibition (%)
	control	pentobarbital	
0	29.7	17.6	40.7
0.1	28.6	16.9	40.9
0.2	40.5	24.2	40.2
0.4	63.0	38.2	39.3
0.8	104.8	68.2	34.9
1.25	142.3	104.2	26.8
2.0	167.7	132.0	21.3
5.0	175.8	148.7	15.4

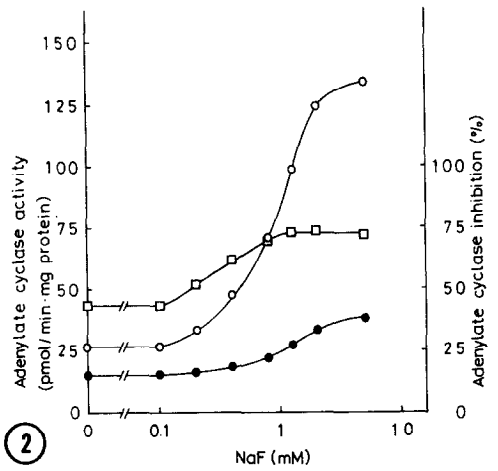
The synaptic membranes were preincubated with indicated concentrations of NaF in the presence of 200 μM $AlCl_3$ for 15 min at 30°C, then treated with or without 5 mM pentobarbital for 2 hr, and measured for adenylate cyclase activity. Inhibition percent of the activity was calculated as

$$\frac{[\text{activity without pentobarbital}] - [\text{activity with pentobarbital}]}{[\text{activity without pentobarbital}]} \times 100$$

Values shown are the average of duplicate determinations.

lower was the inhibitory effect of pentobarbital (Table 1). On the other hand, pretreatment of the membrane with 5 mM pentobarbital reduced the stimulation of adenylate cyclase system by NaF-$AlCl_3$ (Fig. 2). These findings strongly suggest that the site of action of pentobarbital is the N_s-coupling domain on the catalytic unit. To ascertain the possibility, we prepared the catalytic unit according to the method of Ross (11) from the synaptic membrane which had been treated with or without 100 μM GppNHp in the presence of 20 mM $MgCl_2$. As shown in Fig. 3, pentobarbital inhibited the enzyme activity of the catalytic unit isolated from the control membrane more markedly than that of the unit isolated from the GppNHp-pretreated membrane in which the catalytic unit and N_s had been coupled by the pretreatment. The result indicates that the enzyme activity of the catalytic unit already associated with N_s is insensitive to pentobarbital inhibition, supporting the above-mentioned possibility. The figure also indicates that the effect of pentobarbital is more significant in the isolated catalytic unit than in the synaptic membrane.

Since there was another possibility that pentobarbital stimulated the function of N_i, we examined the effect of pertussis toxin on the pentobarbital action. In the membrane pretreated with pertussis toxin, adenylate cyclase



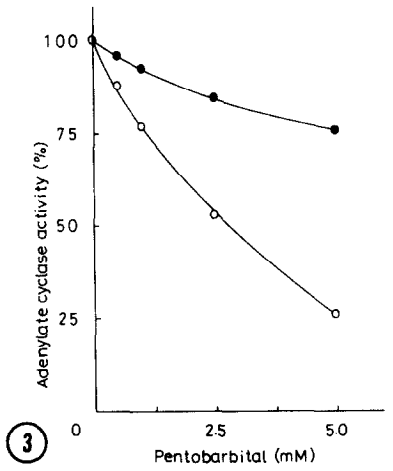


Fig. 2. Effect of NaF-$AlCl_3$ on adenylate cyclase activity in the synaptic membrane pretreated with pentobarbital. The synaptic membrane was pretreated with (●) or without (○) 5 mM pentobarbital for 2 hr at 30°C, then incubated with indicated concentrations of NaF in the presence of 200 μM $AlCl_3$ for 15 min, and measured for adenylate cyclase activity. Expression of the inhibition percent of the activity (□) is the same as in Table 1.

Fig. 3. Effect of pentobarbital on adenylate cyclase activity of the isolated catalytic unit. The catalytic unit solubilized from GppNHp-pretreated (●) or control (○) synaptic membranes was incubated with indicated concentrations of pentobarbital for 2 hr at 30°C, and then measured for adenylate cyclase activity. Without pentobarbital, the enzyme activities were 113.3 and 29.6 pmol/min/mg protein for GppNHp-pretreated and control preparation, respectively.

activity was increased approximately 1.4 times as the results of the inhibition of the N_i function. But, no difference was observed in the effect of pentobarbital between the pertussis toxin-treated and control membranes (Table 2).

Table 2. Effect of pentobarbital on adenylate cyclase activity in the pertussis toxin-treated synaptic membrane

Treatment 1st incubation pertussis toxin	2nd incubation pentobarbital	Adenylate cyclase activity (pmol/min/mg protein) basal	NaF	GppNHp
-	-	35.5 ± 2.2	135.6 ± 5.5	98.8 ± 7.9
-	+	18.8 ± 0.6	48.0 ± 2.5	29.2 ± 0.6
		(0.53)	(0.35)	(0.30)
+	-	48.0 ± 0.5	185.6 ± 7.6	133.8 ± 4.6
+	+	26.6 ± 1.1	67.0 ± 3.4	43.8 ± 1.0
		(0.55)	(0.36)	(0.33)

Synaptic membranes which had been first treated with or without pertussis toxin were subsequently incubated with or without 5 mM pentobarbital for 2 hr at 30°C, and then measured for adenylate cyclase activity in the basal state as well as in the presence of 5 mM NaF-200 μM $AlCl_3$ or 100 μM GppNHp. Values are the mean ± S.D. (n=4). Values in parentheses are the ratio of the activity with pentobarbital treatment to that without the treatment.

Throughout these experiments, similar results were obtained with phenobarbital and pentobarbital, though the former was less effective than the latter (data not shown). These results indicate that barbiturates modify the functional coupling between N_s and the catalytic unit of rat brain adenylate cyclase system without affecting N_i function, resulting suppression of the enzyme activity. It is not clear, however, if the present findings for the action mechanism of barbiturates are actually concerned with the anticonvulsant effect of barbiturates through Cl^- channel-coupled GABA receptors (17). But, it can be concluded at least that barbiturates may be useful for the study on the functional interactions between components of rat brain adenylate cyclase system.

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