Involvement of the Inhibitory GTP-Binding Regulatory Protein and a Low-Affinity Benzodiazepine Receptor in the Inhibitory Effect of Diazepam on Rat Brain Adenylate Cyclase System

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Abstract—The effect of diazepam on the adenylate cyclase system was studied in rat synaptosomal membranes. Micromolar concentrations of diazepam inhibited the cyclase activities in the presence or absence of guanylyl-5'-imidodiphosphate (GppNHp). The inhibitory effect of diazepam was greater on the cyclase activity in the presence of GppNHp than on that in the basal state. This effect of diazepam was not antagonized by Ro15-1788, an antagonist of a high affinity benzodiazepine receptor in the central nervous system. Furthermore, micromolar concentrations of Ro15-1788 had no inhibitory effect on cyclase activities in the presence or absence of GppNHp. In addition, the bromide ion enhanced the inhibition by diazepam of the cyclase activity in the presence of GppNHp, but not the basal activity, although the bromide ion had no effect on both activities in the absence of diazepam. On the other hand, the pretreatment of synaptosomal membranes with GppNHp increased the K_D value for [3 H]diazepam binding from 98 μ M to 198 μ M. These data led us to conclude that diazepam inhibits rat brain adenylate cyclase through the effects on both a low affinity benzodiazepine receptor coupled with the inhibitory GTP-binding regulatory protein (G_i) and the catalytic protein.

It is well-known that seizures lead to the increase of cyclic AMP level in the central nervous system (1). In addition, propranolol, a β -adrenergic antagonist, has an anticonvulsant effect (2), and dibutyl cyclic AMP produces seizures in animals when injected intraventricularly (3). These previous studies indicate that brain adenylate cyclase system is involved in the regulation of epilepsy.

On the other hand, the binding affinity of benzodiazepine agonists to their receptors is modulated by guanine nucleotide (4, 5) as generally seen in a hormone sensitive adenylate cyclase system (6, 7). In addition, the presence of at least three types of benzodiazepine receptors, a high affinity type with K_D of 3.6 nM for diazepam (8), a low affinity type with K_D of 45 μ M for diazepam (9) and a peripheral type with K_D 1.1 nM for Ro5-4864, an apparently specific peripheral

benzodiazepine binding site ligand (10), was observed in rat brain. At present, however, there is no evidence that benzodiazepine receptors could be coupled with the adenylate cyclase system.

In this study, we examined the effect of diazepam on the adenylate cyclase system in rat synaptosomal membrane. We report here that a low affinity benzodiazepine receptor is coupled with the inhibitory GTP-binding protein (G_i), which in turn inhibits adenylate cyclase activity and that this effect of diazepam is enhanced by bromide ions. Furthermore, we report that diazepam also has a direct inhibitory effect on the basal activity of this enzyme system.

Materials and Methods

Materials: Ro15-1788 was kindly donated by Nippon Roche K.K. [3 H]ATP was pur-

chased from Amersham International, Ltd., and [methyl- ^3H]diazepam was from New England Nuclear. All other drugs and chemicals were of reagent grade from standard commercial sources.

Preparation and solubilization of synaptosomal membranes: Synaptosomal membranes were prepared from brains of male Wistar rats (150–200 g). The brains were homogenized in 10 vol. of 0.32 M sucrose solution containing 5 mM Tris-HCl and 1 mM EDTA (pH 7.4) with a Potter-Elvehjem homogenizer. The homogenate was centrifuged at $800\times g$ for 10 min, and then the post-nuclear supernatant was centrifuged at $10,000\times g$ for 30 min. The pellet was resuspended in 20 vol. of 5 mM Tris-HCl buffer (pH 7.4). After standing for 30 min, the suspension was centrifuged at $20,000\times g$ for 30 min. The resultant pellet was washed twice, and the final pellet was suspended in 0.25 M sucrose–50 mM Tris-HCl buffer (pH 7.4). The membranes were solubilized with 50 mM Tris-HCl buffer (pH 8.0) containing 0.25 M sucrose, 1 mM DTT, 0.7% sodium cholate, 0.6 M ammonium sulfate and 15 mM MgCl_2 . Then, the suspension was centrifuged at $100,000\times g$ for 30 min.

Measurement of [^3H]diazepam binding to solubilized synaptosomal membranes: [^3H]diazepam binding was measured by the method of Bowling and DeLorenzo (9) using sodium cholate-solubilized synaptosomal membranes. The reaction mixtures containing sodium cholate-solubilized membranes and [^3H]diazepam (final concentrations of 50–500 μM) were incubated at 4°C for 2 hr. The samples were filtered through cellulose nitrate filters (0.45 μm pore size) and immediately washed twice with ice-cold 50 mM Tris-HCl buffer (pH 7.4). Nonspecific binding was determined in the presence of 2.5 mM unlabeled diazepam and subtracted from total binding to give specific binding.

Measurements of adenylate cyclase activity and protein: Adenylate cyclase activity was measured according to the method of Salomon et al. (11) with some modifications (12). The assay system consisted of 50 mM Tris-HCl (pH 8.0), 10 mM MgCl_2 , 8 mM theophylline, 15 mM phosphocreatine, 50 units/ml creatine phosphokinase and 0.1 mM [^3H]ATP (5×10^5 cpm) as a substrate in a

final volume of 200 μl . After incubating at 30°C for 20 min, the reaction was terminated by the addition of 200 μl of 10% sodium dodecyl sulfate/10 mM EDTA solution. In the cases indicated, either 100 μM GppNHP or 5 mM NaF/200 μM AlCl_3 , which promoted the effect of NaF (13), was added to the reaction mixture. Protein was determined by the method of Lowry et al. (14) using bovine serum albumin as the standard.

Results

Effects of diazepam on adenylate cyclase activity: Synaptosomal membranes from rat brain were pretreated with 400 μM diazepam for 2 hr at 30°C , and then adenylate cyclase activity was measured in the presence or absence of GppNHP (Fig. 1). It should be noted that the degree of the inhibition by diazepam of the cyclase activity in the presence of GppNHP (36%) was greater than that of the basal activity (24%). The inhibition by diazepam of the cyclase activity was dependent on its concentration (data not

Fig. 1. Inhibitory effect of diazepam on adenylate cyclase activity in rat synaptosomal membranes. The synaptosomal membranes were pretreated with or without 400 μM diazepam for 2 hr at 30°C , and then measured for adenylate cyclase activity in the presence or absence of 100 μM GppNHP. Each column represents the mean $\pm$ S.E. of 6 determinations obtained in two separate experiments. *: significant difference from the activity without GppNHP in the absence of diazepam at $P < 0.01$. **: significant difference from the activity with GppNHP in the absence of diazepam at $P < 0.001$.

Table 1. Effect of Ro15-1788 on the inhibition by diazepam of synaptosomal adenylate cyclase activity

Treatment	Adenylate cyclase activity (pmol/min/mg protein)	
	basal	NaF-AlCl ₃
Control	35.5±0.4 (1.00)	144.1±3.1 (1.00)
Diazepam	26.1±0.7 (0.74)*	86.0±1.8 (0.60)**
Ro15-1788	36.5±0.6 (1.03)	141.2±3.7 (0.98)
Diazepam+Ro15-1788	26.6±0.6 (0.75)*	87.3±1.3 (0.61)**

Synaptosomal membranes were treated with or without 400 μ M diazepam and/or Ro15-1788 for 2 hr at 30°C, and then measured for adenylate cyclase activity in the presence or absence of 5 mM NaF/200 μ M AlCl₃. Values are the mean±S.E. of 6 determinations obtained in two separate experiments. Values in parentheses are the ratio of the activity treated with diazepam and/or Ro15-1788 to the control activity. * and **: significant difference from the control in the absence or presence of NaF/AlCl₃ at $P < 0.001$, respectively.

shown. (Moreover, the cyclase activity in the absence of diazepam was almost unchanged during the incubation up to 3 hr (data not shown). In order to further eliminate the nonspecific effect of diazepam because the concentrations of diazepam required to inhibit the cyclase activity were relatively high, attempts were made to examine the effect of Ro15-1788, an antagonist of a high affinity receptor in the central nervous system, at the same concentrations as that of diazepam. The data shown in Table 1 indicated that 400 μ M Ro15-1788 had no effect on adenylate cyclase activities in the presence or absence of NaF-AlCl₃. Furthermore, the effect of diazepam was not antagonized by Ro15-1788. We also observed that diazepam had little inhibitory effect on adenylate cyclase activity in peripheral tissues (data not shown).

Effect of GppNHp on specific [³H]-diazepam binding: Specific [³H]diazepam binding was examined using solubilized synaptosomal membranes, which had been treated with or without 100 μ M GppNHp for 30 min at 30°C. Scatchard analysis of [³H]-diazepam binding showed K_D values of 195 μ M and 98 μ M in solubilized synaptosomal membranes pretreated with or without GppNHp, respectively, although the B_{max} did not differ between the control and GppNHp treatment (Fig. 2).

Enhancing effect of bromide ions on the inhibition by diazepam of adenylate cyclase activity in the presence of GppNHp: Synaptosomal membranes were pretreated in the presence or absence of 250 μ M diazepam for

Fig. 2. Scatchard analysis of [³H]diazepam binding to sodium cholate-solubilized synaptosomal membranes. Synaptosomal membranes preincubated with (●) or without (○) at 100 μ M GppNHp in the presence of 20 mM MgCl₂ were solubilized as described in Materials and Methods. Solubilized membranes (0.7 mg/assay) were incubated with increasing concentrations of [³H]diazepam.

2 hr at 30°C with or without the halide salts of sodium, and then adenylate cyclase activity was measured in the presence or absence of 100 μ M GppNHp. In the control membranes, chloride and bromide salts of sodium had no effect on the enzyme activities in either the presence or absence of GppNHp. In diazepam treated membranes, 50 mM NaBr caused a significant enhancement of the inhibition by diazepam of the cyclase activity in the presence of GppNHp but not the basal

Table 2. Effect of bromide ion on the inhibition by diazepam of synaptosomal adenylate cyclase activity

Treatment	Adenylate cyclase activity (pmol/min/mg protein)	
	basal	GppNHp
Control	29.7±1.4 (1.00)	92.4±1.5 (1.00)
+NaCl	28.0±0.5 (0.92)	87.9±0.5 (0.95)
+NaBr	28.0±0.4 (0.92)	87.3±1.3 (0.94)
Diazepam	22.7±1.3 (0.76)*	59.1±2.0 (0.64)**
+NaCl	21.3±1.8 (0.72)*	58.2±2.0 (0.63)**
+NaBr	22.3±0.7 (0.75)*	42.7±0.8 (0.46)*****

Synaptosomal membranes were treated with or without 250 μ M diazepam in the presence or absence of 50 mM NaCl and NaBr for 2 hr at 30°C, and then measured for adenylate cyclase activity in the presence or absence of 100 μ M GppNHp. Values are the mean±S.E. of 6 determinations obtained in two separate experiments. Values in parentheses are the ratio of the activity treated with diazepam and/or various ions to the control activity. *: significant difference from control in the absence of GppNHp at $P<0.01$. **: significant difference from control in the presence of GppNHp at $P<0.001$. ***: significant difference from GppNHp-stimulated enzyme activity with diazepam in the absence of a halide salt at $P<0.001$.

activity (Table 2). In contrast, 50 mM NaCl had no effect on the inhibition by diazepam of both enzyme activities.

Discussion

The results presented here show that micromolar concentrations of diazepam inhibited adenylate cyclase activity in the presence or absence of GppNHp in rat synaptosomal membranes. Indeed, the degree of the inhibition by diazepam of the cyclase activity in the presence of GppNHp (36%) was greater than that of the basal activity (24%) (Fig. 1). Furthermore, the K_D value for [3 H]-diazepam was significantly increased 2-fold by the treatment with GppNHp without a change of B_{max} (Fig. 2). These data, together with our recent studies (15) showing that the preincubation of the membranes with pertussis toxin suppressed the effects of micromolar concentrations of diazepam on the inhibition of the cyclase activity only in the presence of GppNHp, suggest that micromolar concentrations of diazepam act on at least two sites: one is a low affinity benzodiazepine receptor, linked to the inhibitory GTP-binding regulatory protein (G_i), and the other is the catalytic protein of the adenylate cyclase system in rat synaptosomal membranes. Although the action of some neurotransmitters has been known to be mediated by G_i , which in turn causes the inhibition of adenylate cyclase activity (16,

17), the mode of the action of benzodiazepine is unclear on adenylate cyclase system.

Recently, Fung and Fillenz (5) reported that GTP reduced the binding affinity and the capacity of [3 H]flunitrazepam to rat hippocampal synaptic membranes. In addition, they proposed the model that benzodiazepine receptors seemed to be coupled to G_i , although the inhibition of adenylate cyclase activity by benzodiazepine had not been demonstrated. Furthermore, our present results obtained using sodium cholate-solubilized synaptosomal membranes from whole rat brain are consistent with data reported by Fong et al. (4) who showed that GTP decreased the binding affinity of [3 H]-flunitrazepam to bovine cerebral cortical membranes without a change in B_{max} . On the other hand, plasma concentrations of diazepam to produce the pharmacological effects in men and rats are 0.1–50 μ M (18), and the concentrations of benzodiazepines such as lorazepam in rat brain are significantly higher than those in the corresponding blood (19). In addition, since the same concentration of Ro15-1788, an antagonist of a high affinity benzodiazepine receptor in the central nervous system, as that of diazepam had no effect on adenylate cyclase activity and failed to antagonize the effect of diazepam (Table 1), it seemed to be more likely that the inhibitory effect of diazepam was mediated by a low affinity benzodiazepine receptor, but not by

a high affinity receptor. Bowling and DeLorenzo (9) had reported a low affinity benzodiazepine receptor with a K_D of 45 μ M for diazepam. The affinity (K_D of approximately 100–200 μ M) shown in the present study was lower than that previously reported. As one of the pharmacological characteristics of a low affinity benzodiazepine receptor, although this type of receptor seemed to play an inhibitory role on depolarization dependent Ca^{2+} uptake in rat brain synaptosomal membranes (20), the concentrations of diazepam required for the half-maximal inhibition of depolarization dependent Ca^{2+} uptake was approximately 200 μ M. This K_i value is consistent with that in our present study. On the other hand, File et al. (21) failed to observe this type of receptor in rat synaptosomal membranes. Thus, although the existence of this receptor remains controversial, our present studies are in line with the model proposed by Fung and Fillenz (5).

The present study further indicated that bromide ions enhance the effect of diazepam on the inhibition of adenylate cyclase activity in the presence of GppNHp, although bromide ions had no effect on the cyclase activity in the absence of diazepam irrespective of the presence of GppNHp (Table 2). These data are consistent with those of Martin and Candy (22) who reported that [3H]diazepam binding was markedly increased by the bromide ion in rat brain membranes, indicating that the effect of diazepam on the inhibition of adenylate cyclase activity seems to be mediated through its receptor. In contrast, chloride ion had no enhancing effect on the inhibition by diazepam of cyclase activities in the presence or absence of GppNHp. These findings, together with the previous study that chloride ion facilitated [3H]diazepam binding to a high affinity receptor (23), indicate that a low affinity benzodiazepine receptor is likely to be involved in the inhibition of adenylate cyclase activity. Although there is still a possibility that diazepam nonspecifically inhibits brain adenylate cyclase activity, other anticonvulsants such as barbiturates and phenytoin at micromolar concentrations have no inhibitory effect on this cyclase activity (data not shown). In addition, it remains to be

elucidated whether the inhibition by diazepam of adenylate cyclase activity in the presence of GppNHp in synaptosomal membranes is actually related to the anticonvulsant effect of diazepam in the central nervous system. However, our present findings can be interesting information about the effect of benzodiazepine on the adenylate cyclase system in the central nervous system. Furthermore, since diazepam inhibited the basal cyclase activity, we have an interest in the direct effect of diazepam on the catalytic protein of adenylate cyclase system in rat brain. In the future, further clarification is required as to the functional interaction between a low affinity benzodiazepine receptor and G_i .

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