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γ-Aminobutyric Acid B Receptors Are Negatively Coupled to Adenylate Cyclase in Brain, and in the Cerebellum These Receptors May Be Associated with Granule Cells

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SUMMARY

Baclofen and γ-aminobutyric acid (GABA) are shown to inhibit basal adenylate cyclase activity in brain of rat. The response is mediated through the $GABA_B$ receptor, and the rank order of potency for agonists is (−)-baclofen (EC_{50} = 4 μM) > GABA (EC_{50} = 17 μM) > muscimol > (+)-baclofen. $GABA_A$ agonists are not effective inhibitors of cyclase activity. The response is bicuculline-insensitive, and diazepam does not modify the GABA or (−)-baclofen inhibition of adenylate cyclase. Studies with neurologically mutant mice correlated a loss in $GABA_B$ receptor-mediated inhibition of cyclase with a loss in cerebellar granule cells. Thus, the $GABA_B$ receptor is negatively coupled to adenylate cyclase in various brain areas, and, in the cerebellum, data suggest a granule cell localization of this activity.

INTRODUCTION

Baclofen, β-(4-chlorophenyl)-γ-aminobutyric acid, is an analogue of GABA.[1] In spite of baclofen's close structural similarity to GABA, it does not mimic the actions of GABA or GABA agonists, e.g., muscimol and isoguavacine (1, 2). Baclofen is shown to reduce skeletal muscle tone, most probably by inhibiting spinal mono- and polysynaptic reflex activity (3); thus, it is a useful antispastic agent in man (4). Because these actions of baclofen are not antagonized by bicuculline, a specific GABA antagonist, it was believed that baclofen was not acting through a GABA receptor. Subsequently, baclofen was shown to reduce the evoked release of neurotransmitters from peripheral sympathetic nerve terminals and from brain slices (5, 6). These effects were mimicked by GABA but were not blocked by bicuculline. It appeared that baclofen could be a GABA-mimetic which acts at a site different from the classical GABA receptor. Furthermore, receptor-binding studies, which used radiolabeled GABA and baclofen as ligands at the GABA recognition site(s), also suggested the existence of a novel GABA receptor, now termed $GABA_B$ (7, 8), that is pharmacologically and anatomically distinct from the classical, bicuculline-sensitive GABA ($GABA_A$) site (for reviews see refs. 9 and 10). In contrast to the $GABA_A$ site, the $GABA_B$ site has a low affinity for GABA; baclofen is a potent agonist, and bicuculline is not a $GABA_B$ antagonist. We show that the $GABA_B$ receptors in brain are coupled to adenylate cyclase in an inhibitory manner, and that there is a nonhomogeneous distribution of baclofen-sensitive adenylate cyclase activity in the central nervous system.

METHODS

Preparation of tissue. Adult male Sprague-Dawley rats (Zivic Miller; Allison Park, Pa.) or neurological mutant mice (Jackson Laboratories, Bar Harbor, Me.) were killed by decapitation and the brain regions were rapidly removed. Crude synaptic membranes were immediately prepared by homogenization in a glass/Teflon grinder, 9–10 strokes, with 90 volumes of 10 mM imidazole buffer (pH 7.4) containing 1 mM EDTA and 310 mM sucrose. The homogenate was centrifuged at 1,000 × *g* for 10 min and the resulting supernatant was centrifuged at 12,000 × *g* for 20 min. The pellet (P2) was washed by resuspending in 10 mM imidazole buffer (pH 7.4) containing 1 mM EDTA and then centrifuged at 30,000 × *g* for 20 min. The final pellet was resuspended in 10 mM imidazole buffer (pH 7.4) to a protein concentration between 0.2 and 0.4 mg/ml.

Adenylate cyclase assay. Adenylate cyclase was determined by the enzymatic conversion of [α-^{32}P]ATP to cyclic [^{32}P]AMP. The incubation medium contained 50 mM Tris-HCl (pH 7.4), 2 mM $MgCl_2$, 50 μM ATP, an ATP-regenerating system consisting of 6.3 mM phosphocreatine and creatine phosphokinase (60 μg/sample) (C3755, Sigma), approximately 0.5 μCi of [α-^{32}P]ATP, 1 mM cyclic AMP, 10 μM GTP, 0.2 mM papaverine, and 0.5 mM 3-isobutyl-1-methylxanthine in a final volume of 150 μl. Experiments represented in Fig. 2 used forskolin (344270, Calbiochem) at concentrations from 10 nM to 100 μM. The reaction was initiated by adding 50 μl of tissue and continued for 6 min at 37°. Reactions were terminated by adding 200 μl of a solution containing 2% (w/v) sodium dodecyl sulfate, 45 mM ATP, and 1.3 mM cyclic AMP. Labeled cyclic AMP was isolated according to the method of Salomon *et al.* (11). Proteins were determined by the method of Lowry *et al.* (12).

Cerebellar lesions. Two strains of neurologically mutant mice and their heterozygous, wild-type littermates (control) were obtained from Jackson Laboratories. The nervous mice (C3HeB/FeJ background)

[1] The abbreviation used is: GABA, γ-aminobutyric acid.

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were similar to their littermates at birth; however, during maturation, the cerebellar Purkinje cells begin to degenerate. Two months after birth, only 10–20% of the Purkinje cells remain (13). The weaver mice (B6CBA-A background) differ from the nervous mice in the type of cerebellar cells lost. For the weaver mutation, the cerebellar granule cells degenerate (14). Both mutants are distinguishable from their littermates behaviorally by their instability of posture and gait, and anatomically by their small cerebella with fewer folia. All experiments were performed with cerebella from nervous and weaver mice 2 months old or older.

The cerebellar climbing fibers, one of two types of afferents, were also chemically lesioned with 3-acetylpyridine injection to rats. The procedure was described by Llinas *et al.* (15) and consists of an injection of 3-acetylpyridine (75 mg/kg, i.p.) followed by harmaline (15 mg/kg, i.p. 3 hr later) and a final injection of niacinamide (300 mg/kg, i.p.) 4.5 hr after the 3-acetylpyridine injection. It was necessary to feed these animals by intragastric injection for the next 3 days. The cerebella from these treated rats were studied 14 days later.

RESULTS

(−)-Baclofen inhibited adenylate cyclase activity, to varying degrees, in crude synaptosomal membrane preparations from every rat brain region studied (Table 1). The greatest responses were seen in striatum and cerebellum. The greater sensitivity to baclofen in these areas might result from a higher density of receptors through which the cyclase activity is mediated or a more effective coupling of receptor occupancy to inhibition of catalytic activity in these areas.

Because the percentage inhibition of basal adenylate cyclase activity was the greatest in cerebellum, further characterization of this inhibitory receptor was performed in this brain region. In crude synaptosomal membranes, the rank order of potency for the GABA agonists was (−)-baclofen (EC_{50} = 4 μM), GABA (EC_{50} = 17 μM), muscimol (EC_{50} > 1 mM), and (+)-baclofen (EC_{50} > 3 mM) (Fig. 1). The GABA$_A$ agonists isoguvacine, 3-aminopropanesulphonic acid, nipecotic acid, and 4,5,6,7-tetrahydroisoxazole[5,4-*c*]pyridin-3-ol, tested at 0.1 and 1 mM, did not significantly inhibit basal adenylate cyclase activity. This rank order of agonist potencies is the reverse order observed at the GABA$_A$ site (17, 18), but is characteristic of the GABA$_B$ site (7, 8, 18, 19). Furthermore, the amino acids L-glutamate and L-glycine did not mimic the response on cyclase by GABA or (−)-baclofen. This inhibitory activity on adenylate cyclase by (−)-baclofen was enhanced in the presence of forskolin, an activator of cyclase activity (20); however, the percentage inhibition of basal activity remained approximately 30% (Fig. 2 and *inset*). Other differences between the GABA$_A$ and GABA$_B$ receptors were also observed. The inhibitory response to adenylate cyclase by GABA and (−)-baclofen could not be blocked by the GABA$_A$ antagonist bicuculline methiodide (Table 2A), nor was there any facilitation of this inhibitory response with diazepam (Table 2B).

Since a GTP requirement is a common property shared by receptors which inhibit adenylate cyclase (e.g., the *alpha*$_2$-adrenergic, dopamine D-2, adenosine A-1, and muscarinic cholinergic receptors; see reviews in refs. 21 and 22), we also tested whether GABA or (−)-baclofen inhibited adenylate cyclase in a GTP-dependent manner. Table 3 shows that, without the addition of GTP, neither compound could decrease basal cyclase activity; however, in the presence of 10 μM GTP, both drugs were able to decrease adenylate cyclase activity.

Cerebella that were deficient in Purkinje cells, granule cells, or climbing fibers were tested for their responsiveness to (−)-baclofen. If the GABA$_B$ receptor is mainly associated with one of these three cell types, then the loss of that cell group would result in an attenuation of

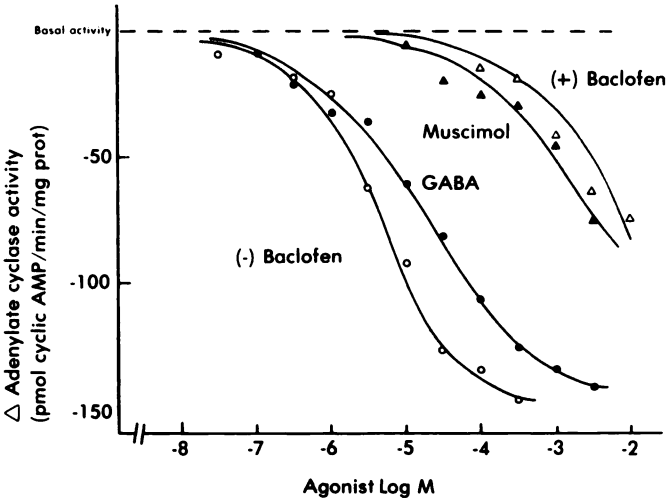

FIG. 1. *Log concentration-response curves for various GABA agonists in crude synaptosomal membrane preparations from rat cerebellum*

Since the extent of inhibitory activity from basal cyclase was consistent, the results are presented as the amount of cyclase activity inhibited from basal activity by the agonists and are the averages of at least four separate experiments performed in triplicate. The half-maximal concentrations (EC_{50}) were 4 ± 0.4 μM (−)-baclofen, 17 ± 1 μM GABA, 1 mM muscimol, and 3 mM (+)-baclofen (mean ± standard error). The EC_{50} values were determined by log-probit plots of each experiment. The averaged basal activity was 486 ± 17 pmoles of cyclic AMP per minute per milligram of protein (all following enzyme activities are represented as means ± standard error and have the same units).

TABLE 1

Distribution of baclofen-sensitive adenylate cyclase

The ability of (−)-baclofen to inhibit adenylate cyclase was measured in crude synaptosomal membranes prepared from various regions of rat central nervous system. Approximately four different regions were studied simultaneously, and the analyses of the brain regions were staggered over many days so that four or five separate results were accumulated for each region. Statistical evaluations compared the individual basal activities from each experiment with the activities obtained with (−)-baclofen. The test was the paired *t*-test (16). The results shown are the averages (± standard error) of all experimental values, three or four experiments performed in triplicate, on each brain region.

Structure in rat brain	Adenylate cyclase activity		
	Basal	(−)-Baclofen, 100 μM	Difference
	pmoles cyclic AMP/min/mg protein		
Striatum	1761 ± 58	1464 ± 33[a]	−297
Cerebellum	546 ± 22	392 ± 20[a]	−154
Frontal cortex	378 ± 8	298 ± 6[a]	−80
Thalamus	341 ± 30	268 ± 25[a]	−73
Hippocampus	343 ± 11	284 ± 7[a]	−59
Hypothalamus	797 ± 29	756 ± 29[a]	−41
Spinal cord, cervical	193 ± 20	175 ± 22[a]	−18

[a] $p < 0.05$.

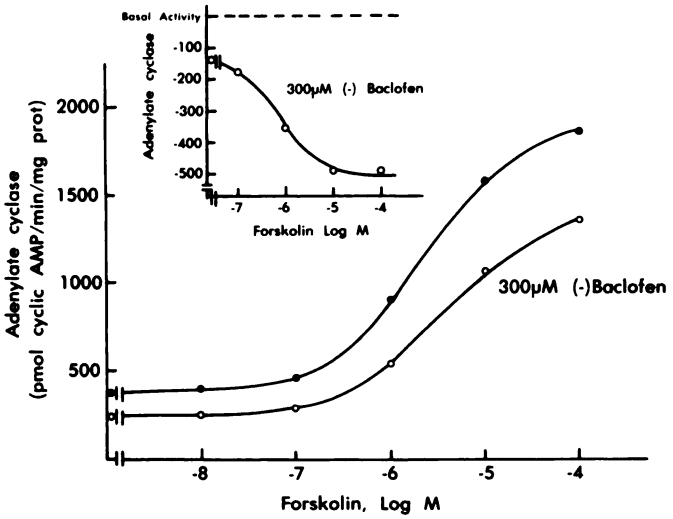

FIG. 2. *Log concentration-response curves to forskolin alone (●) and to forskolin and 300 μM (−)-baclofen (○) in a membrane preparation from rat cerebellum*

The values represent the averages of two experiments performed in triplicate in which the standard deviations for the points were less than 3% of the average activity. The differences in activities at each concentration of forskolin are plotted in the *inset, showing baclofen's inhibitory activity increases in a dose-response relationship to increasing concentrations of forskolin.*

baclofen's inhibition of adenylate cyclase. Figure 3 depicts baclofen concentration-response curves for each of the three lesions and for their respective controls. Neither the nervous mutants (Purkinje cell deficient) nor the 3-acetylpyridine-treated rats (loss of climbing fibers) showed any difference from the control group (Fig. 3A and B). However, the Weaver mutant (granule cell-deficient) showed a significant loss of responsiveness to (−)-baclofen (Fig. 3C). Because the reduced responsiveness to baclofen could result either from a loss of granule cells or from a genetic defect of the $GABA_B$ receptor or some component of the adenylate cyclase system, we determined whether the response to baclofen was similar

TABLE 2

Effect of bicuculline and diazepam on $GABA_B$ receptors

The (−)-baclofen- and GABA-mediated inhibition of adenylate cyclase in a crude membrane preparation from cerebellum was tested in the presence of bicuculline methiodide (A) and diazepam (B). Conditions for the cyclase assay are as reported under Methods. The results represent means ± standard error for four separate experiments performed in triplicate.

	Adenylate cyclase activity	
A.	Control	+ Bicuculline, 100 μM
	pmoles cyclic AMP/min/mg protein	
Basal activity	280 ± 13	260 ± 12
(−)-Baclofen, 100 μM	208 ± 12	200 ± 11
GABA, 300 μM	209 ± 11	203 ± 11
B.	Control	Diazepam, + 10 μM
	pmoles cyclic AMP/min/mg protein	
Basal activity	396 ± 6	394 ± 6
(−)-Baclofen, 100 μM	286 ± 7	279 ± 7
GABA, 300 μM	288 ± 7	285 ± 6

TABLE 3

GTP dependency for $GABA_B$ response

GTP requirement for $GABA_B$ inhibition of adenylate cyclase in a crude membrane preparation from cerebellum of rat. Results are means ± standard error of experiments performed in triplicate. Statistical analysis was performed on the group without and with GTP by the one-way analysis of variance with comparisons made by the Dunnett test.

	Adenylate cyclase activity	
	Control	+ GTP, 10 μM
	pmoles cyclic AMP/min/mg protein	
Basal activity	138 ± 3	303 ± 13
(−)-Baclofen, 100 μM	136 ± 2	228 ± 12[a]
GABA, 300 μM	136 ± 2	234 ± 11[a]

[a] $p < 0.01$ compared with basal activity + 10 μM GTP.

in the striatum from Weaver and control mice. In both control and Weaver mice, the striatal responses to baclofen were similar (inhibition of striatal cyclase by 300 μM (−)-baclofen in control animals was −272 ± 11 pmoles of cyclic AMP per minute per milligram of protein and for the Weaver mice, −302 ± 9 pmoles of cyclic AMP per minute per milligram of protein; mean ± standard error for $N = 5$; average basal activities were 1.7 ± 0.1 nmoles of cyclic AMP per minute per milligram of protein for both groups). Thus the $GABA_B$ receptors may be associated with the cerebellar granule cells.

DISCUSSION

It is evident from our results in either the presence or absence of forskolin that the $GABA_B$ receptor is negatively coupled to adenylate cyclase. Several properties distinguish $GABA_A$ and $GABA_B$ receptors. The $GABA_A$ receptor has a higher affinity for GABA than does the $GABA_B$ receptor. The order of potencies for various GABA mimetics are almost the reverse order for the two receptors. For example, at the $GABA_A$ site, muscimol is more potent than GABA, which is far more potent than baclofen (17, 18), whereas at the $GABA_B$ site, (−)-baclofen is the most potent, followed closely by GABA and then muscimol, a poor agonist (Fig. 1) (1, 7, 8, 18). Furthermore, baclofen shows stereoselectivity at the $GABA_B$ site, where the (−)-isomer is approximately 1000 times more potent than the (+)-isomer (Fig. 1) (18, 19, 22, 23). Stereoselectivity for baclofen at the $GABA_A$ site is not observed (17). Differences also exist with receptor antagonists. Bicuculline is an effective $GABA_A$ receptor antagonist (24); however, it cannot block the $GABA_B$ receptor (Table 2A) (5–7, 24). A specific $GABA_B$ receptor antagonist is still not known. Recently, a study on the GABA- or (−)-baclofen-mediated inhibition of a smooth muscle twitch proposed 5-amino-valeric acid as a $GABA_B$ antagonist (25). We tested 5-amino-valeric acid at 300 μM and 1 mM and found that neither concentration prevented the inhibition of adenylate cyclase by various concentrations of baclofen. Another distinction between $GABA_A$ and $GABA_B$ receptors is that an interaction exists between GABA and diazepam at the $GABA_A$ site (9), whereas diazepam has no effect at the $GABA_B$ receptor (Table 2). Diazepam reportedly increases the number of $GABA_A$ sites without changing the $GABA_A$ affinity

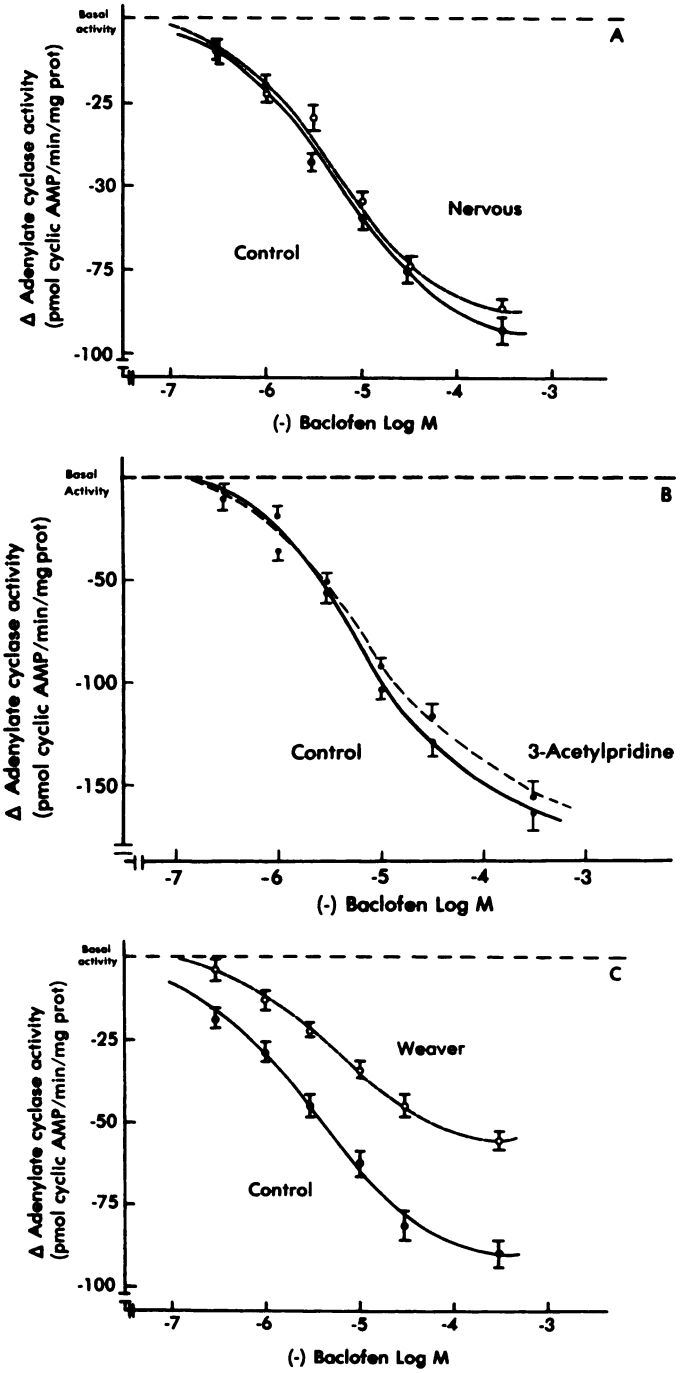

FIG. 3. *(−)-Baclofen concentration-response curves in cerebella deficient in specific cell types*

All curves represent the difference in cyclase activity from basal (no baclofen) activity.

A. Cerebella from nervous mutant mice (loss in Purkinje cells) were compared with cerebella from littermate control mice (N = 4 experiments in triplicate). Basal activity for the nervous mice was 297 ± 12; for the control, 281 ± 8.

B. Baclofen's responsiveness in cerebella from 3-acetylpyridine-treated rats (lesion of climbing fibers) was compared with the response from control cerebella (N = 8 experiments). Basal activity for the lesioned cerebella was 480 ± 12; for the control cerebella, 509 ± 21.

C. Cerebella from Weaver mutant mice (deficient in granule cells) were compared with cerebella from littermate control mice (N = 5 experiments). Statistical comparisons by the three-way analysis of variance showed the Weaver group to differ from the control group (p < 0.005. Basal activity for the Weaver group was 252 ± 6; for the control group, 278 ± 2.

for labeled GABA. The final difference between the two receptor types is that the GABA$_B$ receptor is coupled to adenylate cyclase in an inhibitory manner. A common property of most receptors coupled to adenylate cyclase is the requirement of GTP to elicit the receptor-mediated response on cyclase (21). Table 3 shows that the inhibition of basal cyclase activity by GABA or (−)-baclofen is GTP-dependent. We could not detect any stimulation of adenylate cyclase with GABA, although one report demonstrated that GABA increases cyclic AMP content in ovarian cells and that this response is mediated through the GABA$_A$ receptor (26).

In this study, the regional distribution in rat brain shows the highest inhibitory activity with (−)-baclofen to be in the striatum and cerebellum. These results suggest that the striatum and cerebellum have the highest densities of GABA$_B$ receptors and/or that receptor-cyclase coupling is of higher efficiency in these regions. The possibility that the distribution of baclofen-sensitive adenylate cyclase activity is an indirect determinant of baclofen binding sites is supported by the observation that the cerebellum has the highest binding for [^{3}H] baclofen when compared with the cerebral cortex, brain stem, and mid- plus forebrain regions (8). [^{3}H]Baclofen binding was not examined in striatum.

We wished to determine the cellular location of the GABA$_B$ receptor in the central nervous system. Even though the striatum was the area most responsive to baclofen, the high basal activity made the striatum difficult to study. Thus, the cerebellum was chosen for those studies aimed at identifying with which cell type the GABA$_B$ receptors were associated. Our approach was to use animals with lesions of various cerebellar cell types and to correlate any possible loss in responsiveness to baclofen with the lesioned cerebellar cell. We were able to examine cerebella from rats and mice (both species exhibited GABA$_B$ receptor-mediated inhibition of adenylate cyclase) in which the climbing fibers, Purkinje cells, or granule cells were missing. These results indicated that the GABA$_B$ receptors may be associated with cerebellar granule cells (Fig. 3). Since autoradiographic studies of the cerebellar GABA$_B$ sites have shown binding sites in the molecular layer and not the granular layer (8), we believe that the GABA$_B$ sites could be located on the granule cell nerve terminals (parallel fibers) present in the molecular layer.

The physiological and biochemical relevance for hormone inhibition of adenylate cyclase is still unknown. However, there are interesting similarities between the responses reported with baclofen and other transmitters which are inhibitory to cyclase. One similarity is that baclofen, acetylcholine, and adenosine can reduce excitatory postsynaptic potentials (27–31). Another similarity is that many inhibitory transmitters to cyclase will reduce the stimulated release of excitatory transmitters (6, 22, 23, 32), which may explain the reduced excitatory postsynaptic potentials observed.

Clinically, baclofen is effective in the treatment of spasticity seen in patients with multiple sclerosis or with spinal cord injury (4, 33). Oral administration of baclofen lessens the exaggerated tonic stretch reflex and relieves the pain accompanying muscle spasms. Baclofen is proposed to inhibit the release of excitatory transmitter

from the primary afferent fibers of the stretch reflex loop in the dorsal horn of the spinal cord. Even though our results show a low response to baclofen in the spinal cord, the $GABA_B$ receptors may be highly concentrated in the dorsal gray of the cord. Since it appears that baclofen's antispastic action can be mediated through the $GABA_B$ receptor, we tested whether the other antispastic drugs (33), e.g., methocarbamol, mephenesin, chlorphenesin, and carisoprodol, could also elicit their therapeutic effects by stimulating the $GABA_B$ receptor. None of these drugs was able to mimic baclofen's inhibition of adenylate cyclase activity when compared with baclofen on cerebellar crude synaptosomal membranes at concentrations from 300 nM–3 mM.

In summary, baclofen stimulates $GABA_B$ receptors and inhibits adenylate cyclase activity in the brain. Baclofen elicits the greatest inhibitory activity on basal adenylate cyclase in striatum and cerebellum. Studies on animals with cerebellar lesions suggest that the $GABA_B$ receptors may be associated with the granule cells.

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