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Molecular Mechanisms Regulating the Interactions Between the Benzodiazepines and GABA Receptors in the Central Nervous System

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GUIDOTTI, A., M. BARALDI, J. P. SCHWARTZ AND E. COSTA. *Molecular mechanisms regulating the interactions between the 1,4-benzodiazepines and GABA receptors in the central nervous system.* PHARMAC. BIOCHEM. BEHAV. **10**(5) 803–807, 1979.— Using radioreceptor assay techniques to measure the kinetics of GABA and diazepam receptors, a relationship between GABA and benzodiazepine receptors has been firmly established in membranes of brain and neuroblastoma NB_{2a} clonal cell lines. Occupancy of benzodiazepine receptors uncovers a new population of GABA receptors ($GABA_2$ receptors) endowed with high affinity for GABA. Moreover, stimulation of GABA receptors increases the affinity of 1,4-benzodiazepine receptors for 1,4-benzodiazepines. This reciprocal interaction appears to be mediated by an endogenous regulatory protein (for details on this protein see [14 and 29]) which allosterically regulates $GABA_2$ receptors while it competitively interacts with benzodiazepines for their specific binding sites. The rank order of potency of the various 1,4-benzodiazepines to block the action of this protein inhibitor on GABA receptors is related to their capacity to displace ^{3}H-diazepam binding. These data suggest that the interaction between the 1,4-benzodiazepine receptors and the endogenous protein modulator of $GABA_2$ receptors might play a role in the pharmacological action of the 1,4-benzodiazepines.

Molecular mechanisms GABA receptors 1,4-benzodiazepines

NEUROPHARMACOLOGICAL, behavioral and neurochemical evidence support the notion that 1,4-benzodiazepines facilitate the action of GABA at the synaptic level [1, 4, 6, 8–11, 18, 23, 26, 30]. While GABAergic transmission is considered to be operative in the muscle relaxant, anticonvulsant and ataxic actions of benzodiazepines, the participation of GABA in their anxiolytic action is still controversial [23, 26, 27, 30]. The action of 1,4-benzodiazepines on behavioral depression elicited by conditioned fear is inhibited by GABA receptor antagonists [23, 26, 30], while inhibitors of GABA degradation act similarly to the benzodiazepines [23,30]. However, some perplexity was generated by the finding that muscimol, a GABA receptor agonist, possesses benzodiazepine-like anticonflict activity which was not dose-dependent [27]. It is probable that the sedative effect [27] or other peculiarities of the profile of this GABA receptor agonist may, in due time, explain the apparent discrepancies between the anticonflict effect of 1,4-benzodiazepines and the atypical action of muscimol, a prototype of the GABA receptor agonists. The facilitation of GABA transmission by 1,4-benzodiazepines requires an optimal storage of GABA and the presence of nerve impulses. Based on these observations, it has been postulated that the benzodiazepines act by releasing GABA from presynaptic storage sites [15]. However, a lack of increase of GABA turnover rate following 1,4-benzodiazepines tends to exclude this possibility [19]. Moreover, a series of recent studies supports the view that the action of 1,4-benzodiazepines is postsynaptic and occurs at the level of the GABA receptor [2, 5, 6, 10, 14, 18, 26]. The discovery that 1,4-benzodiazepines bind with high affinity to a specific receptor site in brain [21,25] has stimulated a great interest in looking for an endogenous agonist of this receptor and in studying at the membrane level, the molecular mechanisms of the interactions between 1,4-benzodiazepines and GABA at the postsynaptic GABA receptors. While numerous attempts to isolate the postulated endogenous neurotransmitter agonist of the receptor for 1,4-benzodiazepines have been unconvincing so far [2, 5, 14, 16, 20], a relationship between GABA and the benzodiazepine receptors has been firmly established [5, 14, 28]. In vitro occupancy of the benzodiazepine receptors located in brain membranes uncovers a new population of GABA receptors endowed with high affinity for GABA [5,14]. Moreover, stimulation of GABA receptors increases the affinity of the benzodiazepine receptors for 1,4-benzodiazepines [28]. This reciprocal interaction appears to be

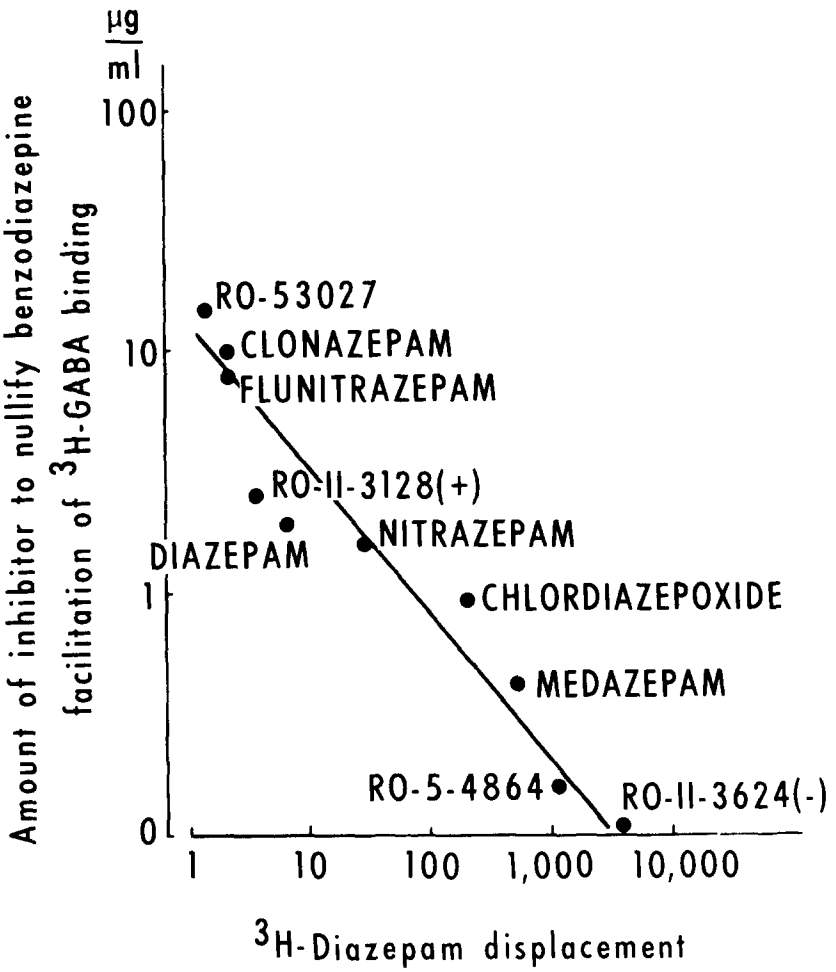

FIG. 1. Inverse correlation between the amount of inhibitor required to nullify benzodiazepine facilitation of ^{3}H-GABA binding and the potency of the benzodiazepines in displacing ^{3}H-diazepam binding. The μg/ml of endogenous protein modulator required to produce 50% inhibition of ^{3}H-GABA binding was determined by measuring the binding of 20 pmoles of ^{3}H-GABA to 200 μg of frozen + Triton X-100 treated rat brain membranes in the presence of different amounts of 500-fold purified endogenous protein modulator (for details see [11]). Each benzodiazepine (10^{-6}M) was preincubated with membranes for 15 minutes before adding the endogenous protein inhibitor. The K_i (mM) values for the displacement of ^{3}H-diazepam by different benzodiazepines are from Mohler and Okada [21]

mediated by an endogenous regulatory protein which allosterically regulates the number of GABA and benzodiazepine receptors and competitively interacts with 1,4-benzodiazepines [5,14]. This protein was isolated and purified and *in vitro* recombination experiments have shown that this protein can competitively obliterate the high affinity GABA receptors that were expressed in the presence of the benzodiazepines or in membranes deprived of this inhibitor protein by washing, freezing and treatment with Triton X-100 [14,29]. The rank order of potency of the various 1,4-benzodiazepines to block the action of the protein inhibitor on GABA receptors is related to their capacity to displace ^{3}H-diazepam binding (see Fig. 1).

Evidence now available strongly indicates that in brain there are two populations of GABA receptors [5, 7, 14, 22, 29] and that only one class of these receptors is the site of action for the 1,4-benzodiazepines. The evidence also indicates that the ratio of the two receptor populations changes in various brain areas [13]. Therefore, one can provisionally suggest that in brain there are $GABA_1$ low affinity and $GABA_2$ high affinity receptors. Only $GABA_2$ receptors are regulated by the inhibitor protein and by the 1,4-benzodiazepines. Based on these results, it can be proposed that 1,4-benzodiazepines, by binding to specific receptors allosterically increase the affinity of $GABA_2$ receptors for GABA, and thereby displace the endogenous protein modulator which forms a part of the supramolecular complex constituting the $GABA_2$ receptors including, at least, the recognition sites, the ionophores and the allosteric regulator protein.

TABLE 1

^{3}H-GABA BINDING AND THE ENDOGENOUS PROTEIN MODULATOR IN A NEUROBLASTOMA CLONAL CELL LINE AND RAT BRAIN

Membranes	S.B. ^{3}H-GABA Fresh		Frozen & Triton Low affinity		Frozen & Triton High affinity		Endogenous Protein Modulator
	K_D	B_{max}	K_D	B_{max}	K_D	B_{max}	Unit/mg membrane proteins
NB_{2a}-Neuroblastoma	200	0.39	300	0.9	19	0.22	25
Brain cortex	220	2.5	130	4.0	15	0.75	10

Mouse NB_{2a} neuroblastoma cells were grown to confluency in Dulbecco's modified Eagle medium containing 10% fetal calf serum [24]. ^{3}H-GABA binding was determined in fresh or frozen+Triton X-100 treated membranes prepared as previously described [29]. The endogenous protein modulator released from the membranes after extraction with H_2O and 50 mM Tris citrate was processed as described by Toffano *et al.* [29] and then assayed with 20 nM ^{3}H-GABA and 200μg of brain synaptic or neuroblastoma NB_{2a} membranes devoid of endogenous modulator.

1 unit=20% inhibition of ^{3}H-GABA binding; K_D=nM; Bmax=pmol/mg protein; SB=specifically bound ligand.

Each value is the mean of three separate experiments. Variation from the mean was never greater than 15%.

EVIDENCE FOR THE EXISTENCE OF THE 1,4-BENZODIAZEPINE RECEPTOR AND THE ENDOGENOUS PROTEIN MODULATOR AS PART OF THE GABA IONOPHORE SUPRAMOLECULAR COMPLEX

The location of the benzodiazepine receptors in brain has been studied using neuroanatomical or histochemical approaches. Although it is clear from these studies that the benzodiazepine receptors may be located on both neurons and glial cells, a location in specific brain cells could not be documented (for a review see [17]). A certain similarity in the brain distribution of GABA and benzodiazepine receptors [7, 21, 25], some analogies in the pharmacological profile of GABA mimetics and the benzodiazepines [1, 4, 8, 10, 14, 30] and some ontogenetic considerations (1,4-benzodiazepines and GABA receptors and the endogenous protein modulator are low at birth but they rise in a parallel fashion to reach adult levels 3 to 4 weeks after birth, Toffano, personal communication) provide indirect support for a possible location of these three molecules on the same cell membrane. However, one still wonders whether the neurons containing the benzodiazepine receptors also contain the endogenous protein modulator and the $GABA_2$ receptors, or whether the interaction shown in vitro in brain synaptic membranes is merely the result of an homogenization artifact. Moreover, it was important to establish whether these three entities were a part of a supramolecular organization constituting the GABA receptor ionophore complex. To answer these questions, we directed our attention to the mouse neuroblastoma NB_{2a} cells because the use of clonal cell lines had provided a suitable model to study the supramolecular organization of catecholamine and opiate receptors. These studies have indicated that NB_{2a} cell lines might represent an adequate substrate to study the supramolecular regulation of the GABA receptor ionophore complex. As shown in Table 1, NB_{2a} cells have a measurable amount of GABA recognition sites. The Scatchard analysis of the saturation curve for GABA binding obtained from freshly prepared membranes reveals only one receptor component. However, as was shown for brain membranes, the Scatchard analysis reveals a high affinity $GABA_2$ and a low affinity $GABA_1$ binding site following freezing, thawing, washing with buffer and treatment with Triton X-100 [29] of NB_{2a} membranes.

Though the density of binding sites is lower than that measured in membranes prepared from brain homogenates the K_D values are similar. The binding of GABA is saturable, it is displaced by muscimol ($IC_{50}=5\times10^{-4}$M) and bicuculline ($IC_{50}=10^{-5}$M) and its pH optimum is between 7 and 8. The membranes of NB_{2a} cells also possess high affinity, saturable benzodiazepine receptor sites (see Table 2). The K_D values

TABLE 2

^{3}H-BENZODIAZEPINE BINDING TO NEUROBLASTOMA CLONAL CELL LINE AND RAT BRAIN

Membranes	S.B. ^{3}H-Benzodiazepines Diazepam		Clonazepam		Flunitrazepam	
	K_D	B_{max}	K_D	B_{max}	K_D	B_{max}
NB_{2a}-Neuroblastoma	9.2	0.24	3.7	0.23	3.4	0.23
Brain cortex	6.9	0.84	2.2	1.2	3.6	0.98

Binding was determined as previously described [14]. Mean of 3–5 separate experiments.

K_D=nM; B_{max}=pmol/mg protein.

TABLE 3

INTERACTION OF DIAZEPAM WITH DIFFERENT AMOUNTS OF ENDOGENOUS PROTEIN MODULATOR

Endogenous Protein Modulator μg	% Inhibition of ^{3}H-GABA Binding Solvent	Diazepam (10^{-6}M)
None	—	—
0.1	9.5	1.0
0.25	30.0	1.5
0.50	55.0	—
0.75	72.0	17
1.00	90.0	50
2.50	100.0	—

Frozen-triton X-100 treated crude synaptic membranes (200μg protein) from the neuroblastoma cell line were used. The endogenous protein modulator was obtained from neuroblastoma cells and purified approximately 250-fold before use [29]. Specifically bound ^{3}H-GABA using 20 nM ^{3}H-GABA was 42 ± 3.8 fmol/mg protein.

TABLE 4

EFFECT OF GABA ON ^{3}H-DIAZEPAM BINDING

Membranes	GABA 10^{-4}M	S.B. ^{3}H Diazepam (fmol/mg protein)	% Increase
NB_{2a}-Neuroblastoma	−	135	
	+	170*	30
Rat Brain cortex	−	465	
	+	600*	29

^{3}H-Diazepam binding was performed using 6 nM ligand. Each point is the mean of 3 separate experiments. *$p<0.05$.

for ^{3}H-diazepam, ^{3}H-clonazepam and ^{3}H-flunitrazepam are similar to those measured in brain. The only difference so far determined between the membranes prepared from NB_{2a} and brain homogenates is that in NB_{2a} membranes, ^{3}H-diazepam is displaced by small concentrations of the benzodiazepine derivative, RO-4864 which displaces ^{3}H-diazepam from brain membranes only in high concentrations. In addition, an unusually high amount of the endogenous protein modulator (Table 1) can be extracted from the membranes of NB_{2a} cells.

As in brain, the NB_{2a} cell membranes contain the endogenous modulator protein that allosterically inhibits the Na^+ independent high affinity binding of GABA (Table 3); diazepam competitively antagonizes the action of the modulator protein in blocking high affinity binding of GABA (Table 3): the stimulation of GABA receptors increases the binding of ^{3}H-benzodiazepines (Table 4). Thus, these data taken together with preliminary evidence indicating that in intact cells, GABA or muscimol can increase the Cl^- conductance, provide direct evidence that the benzodiazepine receptors and the modulator protein are a part of the supramolecular complex constituting the GABA receptor ionophore unit. In addition, the data reported in this study indicate for the first time that the same membranes contain two types of binding sites for GABA: $GABA_1$ (low affinity) and $GABA_2$ (high affinity receptors). Whether these two sites represent transitional states of the same receptors or two separate entities remains to be elucidated. Indirectly, one could infer that there are probably two receptors because the total number of receptors for GABA increases whenever $GABA_2$ receptors are expressed. Another question that remains unanswered is that of the molecular nature

of the allosteric mechanism whereby the protein modulator inhibits the affinity of $GABA_2$ receptors for GABA. Finally, the reason that occupation of GABA receptors modifies 1,4-benzodiazepine binding and vice versa must be determined. To initiate these studies, more precise information on the nature of GABA and 1,4-benzodiazepine receptors is needed.

It is well established at this time that transitional changes in membrane fluidity and intramembrane rearrangement of integral and peripheral membrane proteins can be triggered by receptor occupancy and by concurrent changes in lipid metabolism or protein phosphorylation. In this regard, it is important to state that the endogenous protein modulator of GABA and the benzodiazepine receptors also possesses the ability to block protein phosphorylation in cell membranes [12]. How the observed inhibition of membrane protein phosphorylation by the endogenous protein modulator relates to the interaction between the benzodiazepines and GABA receptors and whether the proposed interaction between the benzodiazepine receptors and the endogenous protein modulator plays a role in determining the level of anxiety remains to be elucidated.

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