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Interaction of Benzodiazepine Receptor Agonists and Inverse Agonists With the GABA Benzodiazepine Receptor Complex

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KAROBATH, M. AND P. SUPAVILAI. *Interaction of benzodiazepine receptor agonists and inverse agonists with the GABA benzodiazepine receptor complex.* PHARMACOL BIOCHEM BEHAV **23**(4) 671–674, 1985.—The effects of the benzodiazepine receptor agonists, antagonists and inverse agonists on the *in vitro* binding of several ligands which label different recognition sites of the GABA benzodiazepine receptor complex are summarized. Also, results with a novel biochemical *in vitro* functional model of the GABA benzodiazepine receptor complex are presented. They are compatible with the concept that drugs which act on benzodiazepine receptors can lead to a bidirectional modulation of the gain of GABAergic neurotransmission.

GABA benzodiazepine receptor complex Benzodiazepine receptor agonists
Benzodiazepine receptor antagonists Inverse benzodiazepine receptor agonists Binding studies
Functional model

SOON after the discovery of brain specific benzodiazepine receptors (BR), it became apparent that these receptors are constituents of certain types of GABA-A receptors, which have been termed the GABA benzodiazepine receptor complex (GBRC). As a consequence of the availability of binding assays for the investigation of the affinity of drugs for the BR, several additional chemical classes of drugs have been discovered which interact with benzodiazepine receptors, but have a pharmacological profile which differs from that of the benzodiazepines [1, 2, 5, 11, 12]. Based on their pharmacological properties, these drugs have been subdivided into three different classes of drugs. One class consists of benzodiazepines, but also of other drugs chemically unrelated to benzodiazepines like zopiclone, which have the well described pharmacological actions of benzodiazepines and drugs with this pharmacological profile have been termed benzodiazepine receptor agonists. Other drugs which exert their pharmacological effects as a consequence of their interaction with the BR, induce the opposite actions when compared to benzodiazepines. These drugs can facilitate or induce seizures, and they can provoke anxiety in animals and man. They have been termed inverse BR agonists [6]. In addition, a third class of BR ligands has been proposed, the BR antagonists [11]. These drugs have few intrinsic pharmacological effects of their own in normal animals, but they are capable of antagonizing the effects of BR agonists, as well as those of inverse BR agonists.

Benzodiazepine binding sites are constituents of a GBRC, and benzodiazepines have been shown to increase GABAergic neurotransmission [7,10]. The discovery of inverse BR agonists indicates that the alterations of GABAergic neurotransmission by BR ligands can be biphasic and result in an allosteric up- or down-regulation of the gain of GABA receptors. The investigation of these novel drugs acting on BR has also provided evidence that there appears to be a continuum ranging from drugs with full agonistic efficacy to partial agonists, antagonists, partial inverse agonists to full inverse agonists [6]. This has raised the possibility that partial BR agonists may provide a new opportunity for drug development, since they could retain the desired therapeutic effects like the anxiolytic or antiepileptic actions, but at the same time they could lack some of the undesired side effects of benzodiazepines such as muscle relaxation, ataxia, the potentiation of CNS depressants or sedation.

If different ligands acting on BR can allosterically up- or down-regulate the gain of the GABAergic system, then these bidirectional effects should also be detectable *in vitro* in binding studies or in a functional biochemical *in vitro* model of the GBRC. In the following these results are briefly summarized.

BINDING STUDIES

In order to explain the various effects seen in binding studies *in vitro* a simplified schematic drawing of the GBRC is given (Fig. 1). Ligands exist which permit to directly investigate the binding to the various recognition sites of the GBRC (Fig. 1). The results of these binding studies indicate that changes in the conformation of the GBRC induced by the various classes of drugs which interact with this receptor can be detected by the investigation of the allosteric perturbation of the other ligand recognition sites of the GBRC. For example, the addition of unlabeled GABA will lead to a competitive inhibition of ^{3}H-muscimol binding, as well as to allosteric alterations of the binding of BR ligands and of the binding of ^{35}S-TBPS.

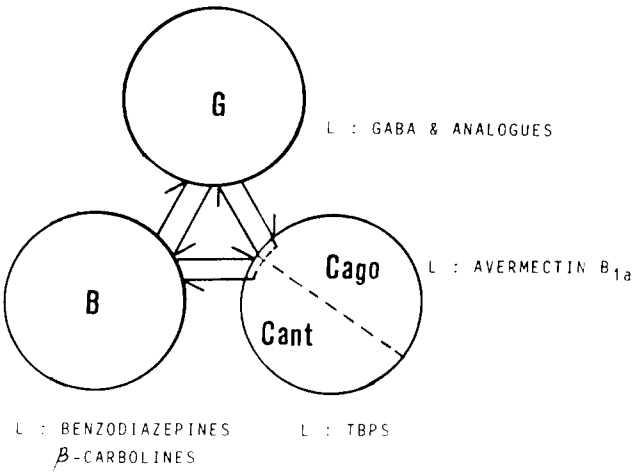

FIG. 1. Simplified schematic drawing of a GABA benzodiazepine receptor complex. G: GABA recognition site; B: Benzodiazepine receptor; Cago: CNS-depressants binding site (the "barbiturate site"); Cant: CNS-convulsants binding site (the "picrotoxinin site"); L: Suitable ligand for the respective binding site. There is increasing evidence that the Cago site and the Cant site are different, but closely associated sites ([9,20], and C. Braestrup, personal communication).

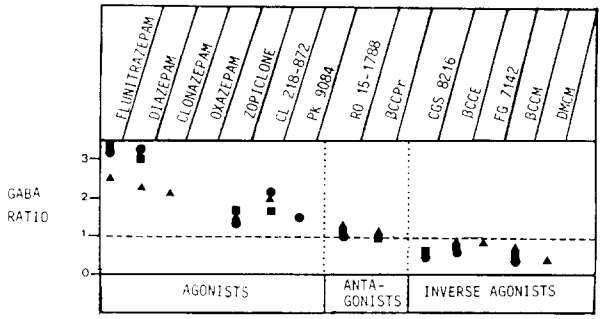

FIG. 2. Ratio of the affinity of BR ligands for the BR in the absence of GABA over that in the presence of GABA or muscimol (IC_{50} [(without GABA)/(with GABA or muscimol)]). ▲Results from Braestrup *et al.* [5], using membranes from rat cerebellum and ^{3}H-diazepam as ligand. ■Results from Mohler and Richards [16], using membranes from rat cerebral cortex and ^{3}H-Ro 15-1788 as ligand. ●Results from Borea *et al.* [4], using membranes from the cerebral cortex of 5 days old rats and ^{3}H-Ro 15-1788 as ligand.

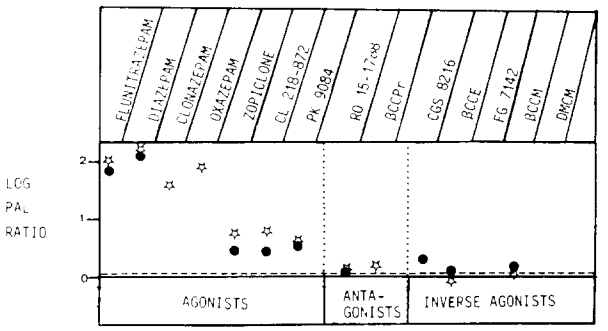

FIG. 3. Log of the ratio of the affinity of BR ligands for the BR in membranes which have been photoaffinity labelled with flunitrazepam over that observed in control membranes. Log PAL ratio is the log (IC_{50} [(in photolabelled membranes)/(not photolabelled membranes)]). ☆Results with membranes from rat cerebellum [13], using ^{3}H-βCCM as ligand; ●Results with membranes from the cerebral cortex of 5-days-old rats using ^{3}H-Ro 15-1788 as ligand [4].

GABA and Barbiturate Effects

GABA, or other GABA-A receptor agonists like muscimol, enhance the affinity of BR agonists for BR, do not markedly alter that of antagonists and decrease that of inverse BR agonists [5,16] (Fig. 2). Certain CNS depressants, like pentobarbital or etazolate, modify the affinity of ligands for BR in a similar mode as GABA does [4]. The actions of the CNS depressants and GABA are additive. This modulation is fairly consistent with the pharmacological profile of the investigated drugs when the binding experiments are performed at more physiological temperatures (~23 to 37°C) and not at 0°C. It has already previously been observed that more physiological temperatures are required for several allosteric interactions to occur within the GBRC [19].

Phtoaffinity Labelling of BR

There is some evidence that a GBRC contains four BR binding sites [15]. When flunitrazepam is irreversibly coupled to one of these four BR binding sites by photoaffinity labelling, the remaining three BR binding sites undergo conformational changes which result in a decreased affinity for BR agonists, but not for BR antagonists or inverse BR agonists [13]. The loss of affinity for BR agonistic benzodiazepines is more pronounced than that of zopiclone, a BR agonist with a different chemical structure. The affinity of BR antagonists and of inverse BR agonists is not markedly altered by photoaffinity labelling (Fig. 3). It has already been pointed out in the initial report that this method permits a distinction of BR agonists from BR antagonists, but it does not allow a distinction between the BR antagonist Ro 15-1788 and the (partial) inverse BR agonists βCCE or βCCM [13]. There have been attempts to explain the differences of the photoaffinity labelling induced affinity changes alternatively by assuming that the binding of different chemical classes of ligands may occur to different domains of the BR [14].

GABA Receptor Binding

Diazepam has been reported to increase ^{3}H-GABA binding to crude synaptosomal membranes [8] and these observations have recently been extended [17]. Using ^{3}H-muscimol as ligand in binding studies performed at 23°C we also have been able to observe small increases of ^{3}H-muscimol binding by several BR agonists ([3], P. Supavilai and M. Karobath, unpublished results). More potent allosteric effects on ^{3}H-muscimol binding by various ligands for the BR can be seen when these investigations are performed in the presence of a CNS depressant like pentobarbital or etazolate (Fig. 4). In these investigations it has been found that BR agonists increase the stimulation of ^{3}H-muscimol binding induced by etazolate, and that inverse BR agonists attenuate the effects of etazolate on ^{3}H-muscimol binding (Fig. 4). The BR antagonist Ro 15-1788 does not markedly alter the actions of etazolate on ^{3}H-muscimol binding but dose dependently antagonizes the effects of a BR agonist and of an inverse BR agonist [3].

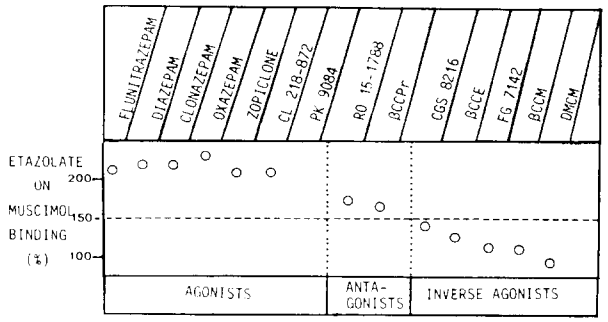

FIG. 4. Perturbation of elazolate enhancement of ^{3}H-muscimol binding by BR ligands. ^{3}H-muscimol binding to membranes from rat cerebral cortex was investigated at 23°C using a centrifugation separation method [3]. ^{3}H-muscimol binding was stimulated by 3 μM etazolate and its perturbation by BR ligands was investigated. Data are expressed as % enhancement of ^{3}H-muscimol binding by etazolate in the absence of other drugs. Three μM etazolate alone stimulates binding by 150% (see dashed line). Ligands for the BR were added in concentrations which gave ≥80% BR occupancy in the assay conditions.

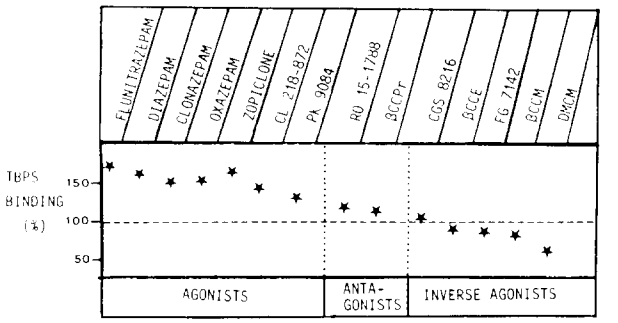

FIG. 5. Perturbation of ^{35}S-TBPS binding by ligands for the BR. Binding experiments were performed with membranes from rat cerebral cortex at 23°C in the presence of 200 mM NaCl [20]. Data are expressed as % binding in the absence of drugs (dashed line). BR ligands were added in concentrations which gave ≥80% BR occupancy in the assay conditions.

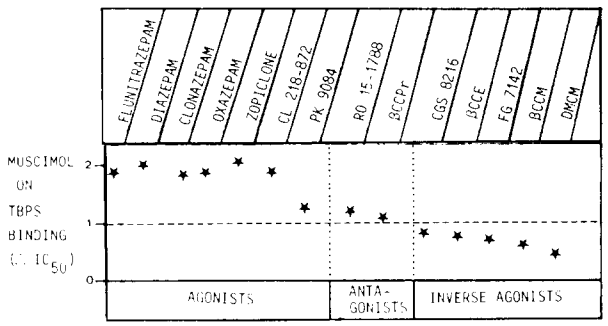

FIG. 6. Perturbation of muscimol inhibition of ^{35}S-TBPS binding to membranes from rat cerebral cortex by ligands for the BR. Displayed is the IC_{50} of muscimol [(without BR ligand)/(with BR ligand)]. BR ligands were added in concentrations which gave ≥80% BR occupancy in the assay conditions.

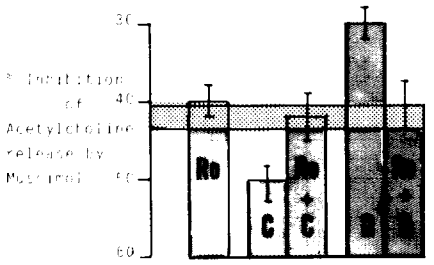

FIG. 8. Antagonism by Ro 15-1788 of the effects of clonazepam or DMCM on muscimol inhibition of electrically induced acetylcholine release from rat striatum slices. The inhibition of electrically induced acetylcholine release by 2 μM muscimol was 42%. This is indicated by the dotted horizontal bar. Shown is the potentiation of the action of muscimol by clonazepam and the attenuation of the action of muscimol by DMCM. Also shown is the antagonism of the modulatory action of these two drugs by Ro 15-1788, which alone is ineffective. The results are expressed as % inhibition of electrically induced acetylcholine release. The results are mean values ±S.E.M.; n=4–10. Ro: 0.5 μM Ro 15-1788 added; C: 0.25 μM clonazepam added; Ro+C: 0.5 μM Ro 15-1788 and 0.25 μM clonazepam added; D: 0.5 μM DMCM added; Ro+D: 0.5 μM Ro 15-1788 and 0.5 μM DMCM added.

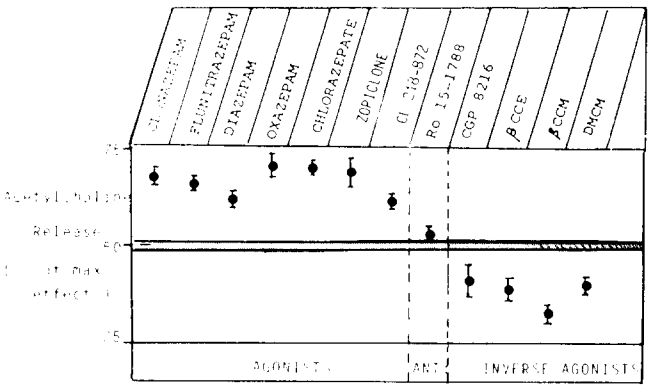

FIG. 7. Potentiation by BR agonists and attenuation by inverse BR agonists of the effect of muscimol on electrically induced acetylcholine release from rat striatum slices. The BR ligands were added in concentrations which give ≥80% BR occupancy in the assay conditions. In the absence of muscimol these drugs did not alter electrically induced acetylcholine release [21]. The release of ^{3}H-acetylcholine derived from prelabelling with ^{3}H-choline was investigated and analyzed as described [21]. Muscimol at 2 μM gave 49% of the maximal inhibitory effect which is a 75% inhibition of electrically induced acetylcholine release. The results are mean values ±S.E.M.; n≥4.

Chloride Channel Antagonist Binding With ^{35}S-TBPS as Ligand

^{35}S-TBPS is a ligand introduced by Squires *et al.* [18], which labels an antagonist binding site of the chloride channel of the GBRC [20]. BR agonists increase, and inverse BR agonists decrease ^{35}S-TBPS binding (Fig. 5). The BR antagonist Ro 15-1788 which does not alter ^{35}S-TBPS binding by itself, however, blocks the allosteric perturbations of a BR agonist and of an inverse BR agonist [20].

Similar results were also obtained when the inhibitory effects of muscimol on ^{35}S-TBPS binding by BR ligands have been investigated [20] (Fig. 6).

Taken together, these results from binding studies *in vitro*, with the possible exception of the photoaffinity labelling studies, support the initial concept formulated by Braestrup [6] that ligands for benzodiazepine receptors can induce

bidirectional allosteric effects on the GBRC. We therefore were interested to investigate these drugs in a functional biochemical *in vitro* model of this receptor.

UP- AND DOWN-REGULATION OF THE GAIN OF A GBRC IN A FUNCTIONAL BIOCHEMICAL IN VITRO MODEL

We have recently demonstrated that the electrically induced release of acetylcholine from rat striatum slices is modulated by a GABA-A receptor which has the characteristics of a GBRC. Thus muscimol or GABA, but not baclofen can inhibit electrically induced acetylcholine release from these slices and this action is sensitive to inhibition by bicuculline, picrotoxin and the cage convulsants [21]. In the presence of clonazepam, which has no actions on acetylcholine release, the concentration effect curve of muscimol is shifted to the left. For example, the concentration of muscimol which yields 10% of the maximal response (ED_{10}) is shifted from 0.27 μM in the absence of clonazepam to 0.03 μM in the presence of 0.1 μM clonazepam. On the other hand the partial inverse BR agonist βCCM at 0.025 μM shifts the ED_{10} for muscimol from 0.27 μM to 0.8 μM. βCCM, in the absence of muscimol does not alter electrically evoked acetylcholine release [21].

These results indicate that in this functional model a BR agonist increases the efficacy of muscimol to exert its functional response whereas the inverse BR agonist has the opposite effect. The action of a number of ligands for the BR on the effect of 2 μM muscimol, a concentration which leads to approximately 50% of its maximal effect on electrically induced acetylcholine release is shown in Fig. 7. It can also be seen that Ro 15-1788 which has weak and not significant agonistic actions (Fig. 7), is able to antagonize the modulatory effects of clonazepam and of DMCM on muscimol inhibition of electrically induced acetylcholine release from rat striatum slices (Fig. 8).

The results with this model provide direct support for the concept proposed initially by Haefely *et al.* [10] and Costa *et al.* [7] that benzodiazepines, in pharmacologically relevant concentration, have no intrinsic pharmacological effects but enhance GABAergic neurotransmission. Further, the observation that BR agonists enhance, and inverse BR agonists attenuate the effectiveness of muscimol at the GBRC whilst the BR antagonist Ro 15-1788 is almost ineffective but blocks the effect of BR agonists and inverse BR agonists, agrees with the concept that the interaction of ligands with the BR can lead to bidirectional functional effects on GABAergic neurotransmission.

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