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PARTIAL GABA AGONIST ACTIVITY OF SR 95531 ON THE BINDING OF [^{35}S]TBPS, [^{3}H]DMCM AND [^{3}H]LORMETAZEPAM TO RAT BRAIN MEMBRANES

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Abstract—A recently developed series of pyridazinyl–GABA derivatives has been classified as GABA antagonists in electrophysiological, behavioural and biochemical experiments. These substances seemed superior to the classical GABA antagonist bicuculline because of their water-solubility, high potency and apparent selectivity for $GABA_A$ receptors. In the present study the most potent representative of this class, SR 95531 almost completely reversed the stimulatory or inhibitory effect of GABA on [^{3}H]lormetazepam and [^{35}S]TBPS binding, respectively. To a lesser extent, it antagonized the inhibition of [^{3}H]DMCM binding by GABA. However, the interaction of SR 95531 with the GABA receptor seems to be of a complex nature since the compound enhanced the binding of [^{3}H]lormetazepam by 28% at 37° in the presence of 200 mM Cl^-. Bicuculline inhibited [^{3}H]lormetazepam binding under these conditions, presumably by antagonizing the effect of residual endogenous GABA. Similar to GABA and THIP, SR 95531 potently inhibited the binding of [^{3}H]DMCM and [^{35}S]TBPS, suggesting SR 95531 to be a partial agonist at the $GABA_A$ receptor.

γ-Aminobutyric acid (GABA) is considered to be the major inhibitory transmitter in the central nervous system. The investigation of the GABA receptor was greatly facilitated by the availability of the competitive $GABA_A$ antagonist bicuculline, though the usefulness of this compound is limited by its low potency and water solubility [1] and its interaction with the glycine receptor [2]. Other GABA antagonists, like pitrazepin [3] or RU 5135 [4] exhibit greater potencies, but are non-selective as well.

A recently developed series of pyridazinyl–GABA derivatives has been shown to displace [^{3}H]GABA from its recognition site and one representative of this class, SR 95531 exhibits a 200-fold higher affinity than bicuculline [5].

Since SR 95531 is water-soluble, stable [6], of great potency in *in vitro* binding studies and apparently selective for $GABA_A$ receptors it may be a more suitable tool for the investigation of the GABA receptor than the classical antagonist bicuculline.

Findings from electrophysiological and behavioural studies identify SR 95531 to be a GABA antagonist, but the high potency of this compound is observable only by *in vitro* binding experiments. In electrophysiological studies SR 95531 is similar or only two to three-fold more potent than bicuculline [7–9]. Moreover, SR 95531 is even less potent in inducing convulsions in mice than bicuculline [5].

The behavioural data may be explained by assuming that SR 95531 does not penetrate the blood brain barrier easily, but this assumption cannot account for the differing potencies in biochemical and electrophysiological experiments.

The susceptibility of benzodiazepine receptor ligand binding to the modulatory effect of GABAergic compounds can be used to evaluate *in vitro* the agonist or antagonist nature of a substance acting at the GABA recognition site. Thus, GABA agonists like GABA itself or muscimol enhance the binding of radiolabeled benzodiazepine agonists [10] and inhibit the binding of the benzodiazepine receptor inverse agonist DMCM [11] to the GABA–benzodiazepine receptor/chloride channel complex. However, the stimulatory effect of a series of identified GABA agonists like 4,5,6,7-tetrahydroisoxazolo(4,5-c)-pyridin-3-ol (THIP) or piperidin-4-sulfonic acid (PSA) cannot be demonstrated easily; at 0° and in the absence of Cl^- ions these substances fail to enhance the binding of [^{3}H]diazepam but, instead, antagonize the stimulatory effect of GABA or muscimol [12]. Under more physiological assay conditions, i.e. at 37° in the presence of Cl^-, THIP and PSA, like GABA, potently enhanced the binding of [^{3}H]flunitrazepam [13]. In the present study, we investigated the effect of SR 95531 on the binding of some modulators of the GABA–benzodiazepine receptor/chloride channel complex utilizing a dialysed rat brain membrane preparation in the presence and absence of GABA.

MATERIALS AND METHODS

Drugs. [^{35}S]TBPS (tert-butylbicyclophosphorothionate; sp. act. 4.1 TBq/mmol) was purchased from New England Nuclear (Boston, MA). [^{3}H]DMCM (6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylic acid methyl ester; sp. act. 2.7 TBq/mmol) and [^{3}H]lormetazepam (sp. act. 2.6 TBq/mmol) were from Schering (Berlin, F.R.G.). GABA was from Serva (Heidelberg, F.R.G.); SR 95531 from Research Biochemicals Inc. (Natick, MA); picrotoxin from Schuchardt (Munich, F.R.G.) and clonazepam from Hoffmann-LaRoche (Basel).

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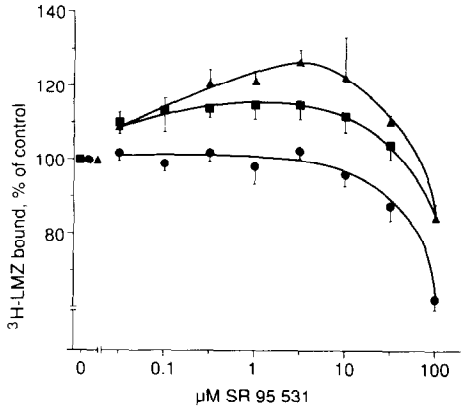

Fig. 1. Temperature-dependent effect of SR 95531 on specific [^{3}H]lormetazepam binding. The values are the means ± SE of four experiments in triplicate, expressed as a percentage of basal binding at the corresponding temperature: (●) 0°; (■) 23°; (▲) 37°.

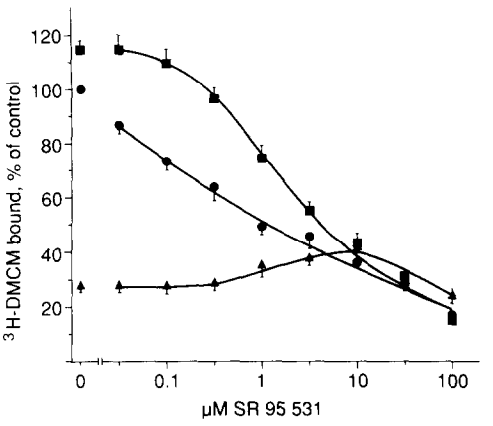

Fig. 2. The effect of SR 95531 on specific [^{3}H]DMCM binding in the absence (●) and presence of 50 μM bicuculline (■) or 10 μM GABA (▲) at 23°. The points are expressed as a percentage of the value without added drug; shown are the means ± SE of four experiments performed in triplicate.

Clonazepam was dissolved in DMSO to a concentration of 1 mM; all other substances were dissolved in buffer.

Tissue preparation. Male Wistar rats (*ca.* 140 g body weight) were decapitated and their forebrains dissected on ice. Individual brains were homogenized in 10 volumes of buffer A (50 mM Tris–citrate, pH 7.1) with an Ultra-Turrax, centrifuged at 30,000 *g* for 15 min, resuspended in buffer A and stored frozen overnight at −20°. The following day, the membranes were thawed, washed twice and resuspended in buffer A containing 1 mM EDTA. After 4 hr of dialysis against five successive portions of 20 volumes of 5 mM Tris–citrate, pH 7.1, the membranes were washed twice by centrifugation, resuspended in buffer and stored frozen at −80° for up to two weeks. On the day of the experiment, the membranes were pelleted and resuspended in 10 volumes of buffer A containing 200 mM NaCl.

Binding experiments. The binding of [^{35}S]TBPS was assayed at a final concentration of 0.5 nM in an incubation volume of 1 ml with a membrane suspension at a dilution of 10 mg tissue/ml and the appropriate dilution of test drugs. The incubation was performed for 120 min at room temperature. [^{3}H]Lormetazepam, 0.65 nM and [^{3}H]DMCM, 0.45 nM were incubated in a final volume of 0.5 and 1 ml, respectively, with membrane suspension derived from 2 mg tissue/tube and the drugs to be tested for 30 min at room temperature, unless otherwise indicated. All binding experiments were performed in triplicate in buffer A with 200 mM NaCl added. Membrane-bound radioligands were separated from free tracer by rapid filtration through Whatman GF/B glass fibre filters with a Brandel manifold, followed by three 3 ml washes of ice-cold buffer. The filters were transferred to minivials and 4 ml of scintillation fluid (Pico-fluor, Packard) was added. After 1 hr of vigorous shaking the radioactivity retained on the filters was measured by liquid scintillation spectrometry. Non-specific binding was defined as the binding in the presence of 50 μM picrotoxin for [^{35}S]TBPS and 1 μM clonazepam for [^{3}H]lormetazepam and [^{3}H]DMCM binding.

Statistics. Comparisons of the effects of SR 95531 (or GABA) with controls were made using a 2-way ANOVA, the two factors being subject and drug concentration. Effects of individual concentrations were tested post hoc using multiple *t*-tests with Bonferroni corrections. A probability level of 5% was taken as being significant.

RESULTS

The binding of the benzodiazepine agonist [^{3}H]lormetazepam was stimulated two-fold in the presence of 10 μM GABA. SR 95531 reversed the increase with an EC_{50} of 0.3 μM. This effect of SR 95531 is in agreement with published data [5]. SR 95531 alone had only a weak effect on the binding of [^{3}H]lormetazepam, but the enhancement of 16% was statistically reliable (see Fig. 1; 23°). At concentrations above 30 μM, SR 95531 inhibited the binding of [^{3}H]lormetazepam, abolishing completely the effect of 10 μM GABA.

The influence of SR 95531 on specific [^{3}H]DMCM binding was more pronounced and significant at all concentrations tested. At 10 μM, the amount of specifically bound [^{3}H]DMCM was reduced to 38% of the control, corresponding to the value to which the inhibitory effect of GABA could be reversed by SR 95531 (Fig. 2). The binding of [^{3}H]DMCM was inhibited by 10 μM GABA to 27% of the control and SR 95531 antagonized this effect only partially to 42% of the control value, but the reversal of the GABA effect was significant in the range of 1 to 10 μM SR 95531. At 100 μM, SR 95531 further inhibited the binding of [^{3}H]DMCM to 18% of the control in the presence, as well as in the absence, of GABA.

To clarify the nature of this inhibitory, apparently GABA-mimetic effect of SR 95531, the effect of the pure GABA antagonist bicuculline on GABA-inhibited [^{3}H]DMCM binding was investigated.

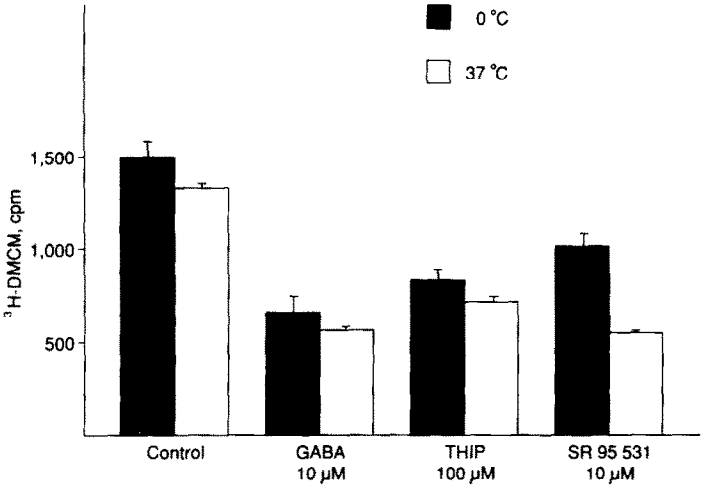

Fig. 3. Inhibition of specific [^{3}H]DMCM binding by GABA receptor ligands at 0 and 37°. Shown are the means ± SE of three experiments performed in triplicate.

Bicuculline alone exerted no inhibition at concentrations from 1.5 to 200 μM, but at 25 μM completely reversed the inhibition of [^{3}H]DMCM binding induced by 10 μM GABA (data not shown). The inhibitory effect of SR 95531 on [^{3}H]DMCM binding was not eliminated in the presence of 50 μM bicuculline, but the concentration–effect curve was shifted to the right (Fig. 2). At 37° SR 95531 was distinctly more effective in inhibiting [^{3}H]DMCM binding than at 0°, whereas THIP and GABA showed no such temperature dependency (Fig. 3). The binding of [^{3}H]lormetazepam is enhanced by the GABA agonist THIP to 120 and 186% of control level at 0 and 37°, respectively. SR 95531 also stimulated the binding of [^{3}H]lormetazepam at 37°, but this effect was not detectable at 0° (Fig. 1). The stimulatory effect of SR 95531 was significant at concentrations in the range of 0.03 to 30 μM at 23° and 37°. In contrast to SR 95531, 150 μM bicuculline inhibited the binding of [^{3}H]lormetazepam in the same membrane preparation to 80% of control at both 0 and 37°.

The binding of [^{35}S]TBPS was reduced to 19% of the control by 10 μM GABA and this effect was reversed only incompletely by SR 95531 up to 100 μM. In the absence of added GABA, SR 95531 inhibited the binding of [^{35}S]TBPS to 51% of control at 3 μM and higher concentrations (Fig. 4A). The inhibition exerted by SR 95531 was significant at each concentration tested. GABA at 1 μM enhanced the binding of [^{35}S]TBPS by 9% only. SR 95531, 3 μM, decreased [^{35}S]TBPS binding to 60% of the control. This inhibition could not be antagonized completely by GABA up to 30 μM (Fig. 4B).

DISCUSSION

The results of the present study show that SR 95531 antagonizes the effect of GABA on [^{3}H]benzodiazepine and [^{35}S]TBPS binding, thus confirming the results obtained by other laboratories [5, 14]. Additionally, we demonstrate that SR 95531 is less effective in reversing the GABA induced inhibition of the benzodiazepine receptor inverse agonist [^{3}H]DMCM binding. Instead, SR 95531 inhibits the binding of [^{3}H]DMCM when tested in the absence of exogenously added GABA.

The high efficacy of SR 95531 in inhibiting [^{3}H]DMCM binding as compared to the effect on [^{3}H]lormetazepam binding may suggest a competitive interaction with the DMCM binding site in addition to a weak partial GABA agonist activity. However, since it is commonly accepted that the benzodiazepine receptor agonists, antagonists and inverse agonists share at least in part a common binding site, the displacing activity of SR 95531 should extend to the benzodiazepine receptor antagonist [^{3}H]Rol5-1788, which is insensitive to GABAergic modulation. Up to 30 μM, we have detected no effect on the binding of this ligand by SR 95531 (unpublished results).

In contrast, SR 95531 stimulated the binding of [^{3}H]lormetazepam. Though this stimulatory effect on benzodiazepine agonist binding is weak and detectable only at 23 and 37° in the presence of Cl^- this effect is opposite to that of the GABA antagonist bicuculline. The repeatedly frozen/thawed, well washed and dialysed membrane preparation we used in our study obviously contained enough residual endogenous GABA to stimulate the binding of [^{3}H]lormetazepam; we interpret the slight inhibitory effect of bicuculline on [^{3}H]lormetazepam binding to be due to an antagonism of this residual GABA. Since SR 95531 is more potent than bicuculline as a displacer of GABA from its binding site [5] it should inhibit rather than enhance [^{3}H]lormetazepam binding, if it were devoid of intrinsic agonist GABAergic activity. The conditions under which SR 95531 enhances [^{3}H]lormetazepam binding are also those required to demonstrate a stimulatory effect of the partial GABA agonist THIP on benzodiazepine agonist binding [13]. In the presence of Cl^- ions, the efficacy of SR 95531 and THIP in enhancing the binding of [^{3}H]lormetazepam is increased by ele-

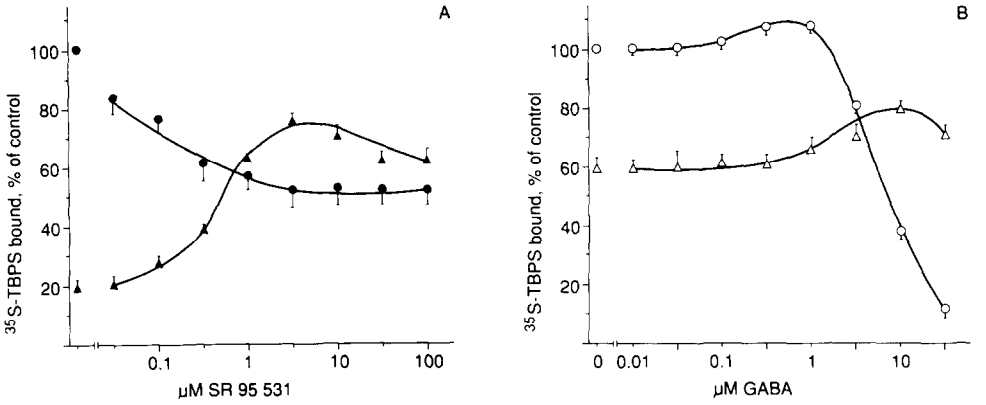

Fig. 4. Dose-dependent modulation of [^{35}S]TBPS equilibrium binding by GABA and SR 95531 at 23°. (A) The effect of SR 95531 on [^{35}S]TBPS binding in the absence (●) and presence (▲) of 10 μM GABA. (B) The effect of GABA on [^{35}S]TBPS binding in the absence (○) and presence (△) of 3 μM SR 95531. The data are expressed as a percentage of the value without added drugs; shown are the means ± SE of four experiments performed in triplicate.

vating the temperature, and the effect of SR 95531 is not detectable in the absence of Cl^- ions (unpublished observation).

Under the conditions employed, the binding of [^{3}H]DMCM seems to be a more sensitive probe for GABA partial agonist activity since the maximal effect of THIP could be detected already at 0°, at which temperature THIP only slightly affects the binding of [^{3}H]lormetazepam. Similarly, SR 95531 inhibited [^{3}H]DMCM binding moderately at 0°, but this effect could be strengthened by elevating the temperature to 37°, thus indicating weaker intrinsic activity compared to THIP.

This effect of SR 95531 is not restricted to benzodiazepine receptor ligands since it also inhibits the binding of [^{35}S]TBPS, a ligand which presumably binds at or near the chloride channel associated with the $GABA_A$ receptor in a GABA sensitive manner [15].

It has been demonstrated that low concentrations of the GABA agonist muscimol stimulate the binding of [^{35}S]TBPS under non-equilibrium conditions [16], and the inhibitory effect of SR 95531 could therefore be due to antagonism of a low level of residual endogenous GABA. In our experiments performed under equilibrium conditions, low concentrations of added GABA only slightly enhanced [^{35}S]TBPS binding, but increasing the concentration of GABA did not reverse the inhibitory effect of 30 μM SR 95531, suggesting that SR 95531 effects the binding of [^{35}S]TBPS independent from GABA antagonism. In contrast to SR 95531, the pure GABA antagonist bicuculline does not inhibit [^{35}S]TBPS binding [15].

Taken together, the results of our study show that in *in vitro* binding assay systems, the proposed GABA antagonist SR 95531 reveals properties consistent with weak partial GABA agonist activity, though this needs to be confirmed in functional tests. The concentrations at which these effects occurred correspond to the EC_{50}s in electrophysiological studies [7] and to the lower affinity site in [^{3}H]SR 95531 binding [17].

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