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^{3}H-tert-BUTYLBICYCLOORTHOBENZOATE – A NEW LIGAND FOR THE GABA$_A$-RECEPTOR GATED ION CHANNEL

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The specific ligand ^{35}S-*tert*-butylbicyclophosphorothionate (^{35}S-TBPS) has been used in recent years to study the chloride ion channel of the GABA$_A$-receptor. For this same purpose, ^{3}H-*tert*-butylbicycloorthobenzoate (^{3}H-TBOB) has been used. Agonists of GABA$_A$-receptors have an allosteric action on specific binding of ^{35}S-TBPS with mammalian brain membranes. Antagonists abolish these effects. As regards ^{3}H-TBOB, no such information is available. The present investigation was undertaken to remedy this omission.

EXPERIMENTAL METHOD

Noninbred male albino rats weighing 180-220 g were used. The synaptosomal-mitochondrial P_2 fraction, isolated from the brain (excluding the brain stem and cerebellum) was homogenized in 50 volumes of 10 mM Tris-HCl, pH 7.4, containing 1 mM EDTA. The suspension was incubated for 10 min at 0°C and centrifuged for 20 min at 20,000g. This procedure was repeated 3 times. The final residue was frozen for 18 h at −18°C. After thawing the membranes were washed twice in 50 volumes of 10 mM Tris-HCl, pH 7.4, followed by centrifugation for 20 min at 20,000*g*. The residue was suspended in 50 mM Tris-HCl, pH 7.4, containing 500 mM NaCl, it being assumed that 1 ml contained 1.8-1.9 mg protein.

Radioligand Analysis. The incubation medium included the membrane preparation (900-950 μg protein), 50 mM Tris-HCl buffer, pH 7.4, containing 500 mM NaCl, 5 nM ^{3}H-TBOB (Amersham International, England, 30.6 Ci/mmole), as well as different concentrations of GABA (Reanal, Hungary) and bicuculline (Sigma, USA). The bicuculline was added to 10 μl of DMSO. The total volume of the incubation medium was 2 ml. Samples were incubated for 30 min with continuous shaking at a temperature of +25°C. The contents of the tubes were transferred to GF/B and GF/C filters (Whatman, England) and washed twice with 5 ml of ice-cold buffer. Binding of ^{35}S-TBPS (2 nM, from NEN, Germany, 87.1 Ci/mmole) was estimated by the method described previously. Some samples were washed on filters without preliminary incubation, so that nonspecific binding of the radioactive label by the material of the filters could be determined. The filters were placed in scintillation flasks containing ZhS-8 scintillation fluid, radioactivity was counted on a 1214 RackBeta II counter. Each value was determined from the results of three separate experiments, conducted in three parallel series. The protein concentration in the samples was determined by Lowry's method. The numerical results were subjected to statistical analysis by Student's t test.

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TABLE 1. Binding of Radioactive Preparations by Filters

Filters	^{3}H-TBOB (5 nM)		^{35}S-TBPS (2 nM)	
	total binding, fmoles/mg protein X ± m	binding by filters, % of total, X ± m	total binding, fmoles/mg protein, X ± m	binding by filters, % of total, X ± m
GF/B	248.1±7.0	32.6±2.2	70.1±5.8	8.3±3.2
GF/C	217.5±2.0	29.5±2.5	61.4±9.2	9.3±2.1

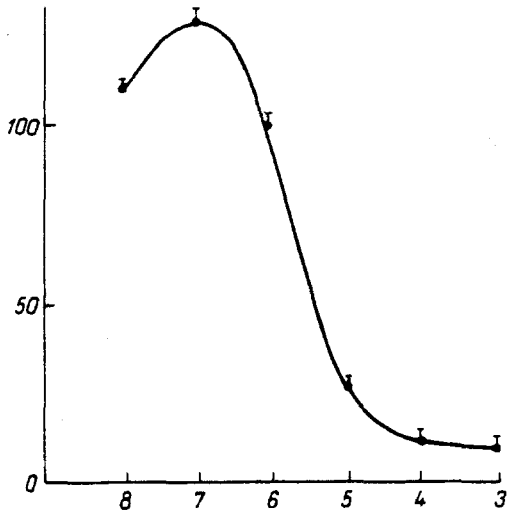

Fig. 1. Effect of GABA on binding of ^{3}H-TBOB (5 nM) with rat brain synaptic membranes. Abscissa, GABA concentration, −log (M); ordinate, binding of ^{3}H-TBOB (% of control). Binding of ^{3}H-TBOB assessed allowing for adsorption of ligand by material of filters.

EXPERIMENTAL RESULTS

Table 1 gives the results of a study of the ability of glass fiber GF/B and GF/C filters to bind radioactive ligands of the chloride ion channel. The filters actively bound ^{3}H-*tert*-butylbicycloorthobenzoate, whereas sorption of ^{35}S-TBPS was significantly less. In experiments to determine specific binding of ^{3}H-TBOB with synaptic membranes, this fact must evidently be taken into account.

The study of binding of the radioactive ligands with the material of the filters has been the subject of several investigations. It has been shown, for instance, that GF/B filters actively adsorbed the ^{3}H-imipramine marker of serotonin receptors. This nonspecific binding could be reduced after preliminary treatment of the filters with polyethylenimine, but the use of this compound is undesirable because of its toxicity. It has been suggested that the glass-fiber filters be treated with polycations: protamine, methylated BSA, etc. This procedure greatly improved the quality of radioligand analysis of receptors of vasoactive intestinal peptide.

Data obtained by the study of the effect of GABA on binding of ^{3}H-TBOB with rat brain synaptic membranes and the ability of bicuculline to prevent the effects of GABA are given in Figs 1 and 2. Within the nanomolar range of concentrations GABA caused increased binding of the ligand of the chloride ion channel. For instance, a significant increase in binding was observed at a concentration of the amino acid of 100 nM (by 29%). A further increase in the GABA concentration in the incubation mixture led to the opposite effect – reduced binding of the ligand. EC_{50}, determined from the results of three separate experiments, was 6.7 ± 0.3 μM. Consequently, the GABA recognition site in the $GABA_A$-receptor, benzodiazipine receptor, ionophore complex, modulated binding of the chloride ion channel marker ^{3}H-TBOB. Convincing

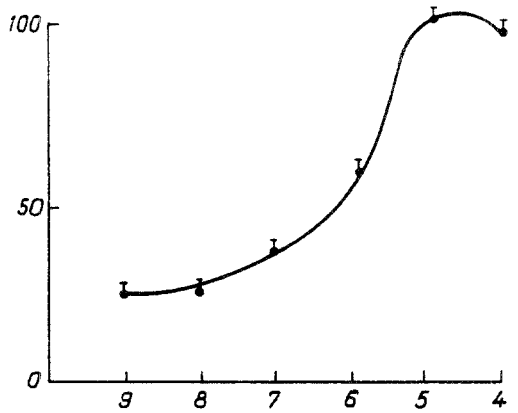

Fig. 2. Inhibition by bicuculline of changes in ^{3}H-TBOB (5 mM) binding induced by GABA (10 μM). Abscissa, concentration of bicuculline, −log (M). Ordinate, binding of ^{3}H-TBOB (% of control). Binding of ^{3}H-TBOB was estimated allowing for adsorption of the ligand by material of the filters. Bicuculline in concentrations of 10^{-9}-10^{-4} M had a weak effect on ^{3}H-TBOB binding (varying from 94 to 101%).

proof was obtained of a similar effect of GABA-agonists on binding of ^{35}S-TBPS. The effects of GABA on specific binding of ^{3}H-TBOB have received less study. It has been shown, for example, that the amino acid inhibits binding of the ligand with brain membranes of chick embryos and chickens. Our data show that GABA has a similar effect also in the mammalian brain.

Specificity of the modulating effect of GABA on binding of the ligand of the chloride ion channel is indicated by the results of experiments showing the ability of bicuculline, a $GABA_A$-receptor antagonist, to prevent the effects of the amino acid (Fig. 2). Bicuculline completely abolished inhibition of ^{3}H-TBOB binding induced by 10 μM GABA. EC_{50} was 1.08 ± 0.18 μM. There is no information in the literature on antagonism of bicuculline and GABA in relation to binding of ^{3}H-TBOB with brain membranes. Our findings bridge this gap. Meanwhile, investigations have shown that bicuculline can prevent effects of GABA on binding of ^{35}S-TBPS. The convulsant steroid R-5135 and patrazepine, which is a mono-N-arylpiperazine, have been found to be more active than bicuculline in this respect.

The results of this investigation demonstrate that ^{3}H-TBOB, a new ligand of the chloride ion channel of the $GABA_A$-receptor is adsorbed to a greater degree by glassfiber GF/B and GF/C filters than the traditional marker ^{35}S-*tert*-butylbicyclophosphorothionate. This phenomenon must be taken into account when the binding characteristics of this new ligand are determined.

The GABA recognition site of the receptor—ionophore complex influences binding of ^{3}H-TBOB allosterically. This is proved by the fact that GABA had a biphasic action on binding of the ligand with synaptic membranes of the rat brain, but bicuculline completely abolished the effects of the amino acid. It must be postulated that reduction of the modulating effect of endogenous GABA can be achieved by thorough washing or by dialysis of membrane preparations.