Norbornan, a New Irreversible Ligand of the $GABA_A$-Receptor Chloride Channels

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In vitro experiments demonstrate the high affinity of the toxic convulsant norbornan [2,2-di-(trifluoromethyl)-3,3-dicyano-5,6-dichloronorbornane] for the $GABA_A$-receptor chloride channels of membranes from rat brain. Repeated washing and centrifugation do not restore the ionophore density on norbornan-treated membranes, suggesting that norbornan may be counted among the agents acylating the chloride channels.

Key Words: *norbornan; $GABA_A$ receptors; chloride ionophore; GABAlytics; acylation*

GABA-lytics represent a large group of compounds inhibiting neurotransmission in the GABAergic synapses, among which are bicuculline, benzodiazepine receptor antagonists, and blockers of the chloride channels of the $GABA_A$ receptor [4,6]. The last group is the most abundant and includes picrotoxin, 2,6,7-trioxabicyclo[2,2,2]-octanes, tetramethylenedisulfotetramine (TETS), norbornan (NB, Middleton ether), penicillin, furacillin, etc. [1,3,4,6]. GABAlytics are indispensable "pharmacological probes" for investigation of the structure and function of the GABA/benzodiazepine/ionophore complex [10]. Among them tertbutylbicycloorthobenzoate (TBOB) analogs, irreversible ligands of the chloride channels, are considered to be the most promising agents. The interaction between NB and the ionophore protein has not yet been studied and therefore this was the subject of the present study.

MATERIALS AND METHODS

The experiments were carried out on male albino rats weighing 180-200 g. Preparation of the synaptic membranes from the brain cortex and the procedure of radioligand assay with ^{3}H-TBOB (29.5 Ci/mM, Amersham) were described previously [2].

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The possibility of irreversible inhibition of the chloride channels with GABAlytics was studied *in vitro*. To this end the washed synaptic membranes were incubated (30 min, 0°C) in the presence of NB, TETS, and picrotoxin (Serva) and then 5 times washed and centrifuged at 20,000 g for 15 min. The chloride ionophore density was assessed with ^{3}H-TBOB (5 nM). An equivalent volume of solvent (dimethyl sulfoxide) was added to the control samples. Radioactivity was measured on a Rack-beta 1217-802 counter.

RESULTS

The ability of NB, TETS, and picrotoxin to expel ^{3}H-TBOB from its binding sites on synaptic membranes is shown in Fig. 1. All convulsants effectively blocked the binding of radiolabeled ligand (IC_{50}=46 and 700 nM for NB and TETS, respectively), while the classic chloride channel blocker picrotoxin was less effective (IC_{50}=5000 nM).

Bearing in mind that ^{3}H-TBOB binds specifically to the $GABA_A$-receptor ionophore protein, we can assume that NB is a high-affinity ligand of the chloride ionophore.

In a special experimental series the specific binding of ^{3}H-TBOB was studied after GABAlytic pretreatment of the synaptic membranes followed by a 5-fold washing and centrifugation procedure (Table 1).

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Preincubation with NB considerably reduced the number of chloride channels (^{3}H-TBOB binding sites) on the membranes. We were unable to restore the binding of ^{3}H-TBOB with the membranes after a 5-fold washing procedure even at inhibitor concentrations of 4, 8, and 16 nM (21, 22, and 29% inhibition, respectively). The specific binding of the radioligand constituted 22 and 18% of the control at NB concentrations of 100 and 250 nM and was practically abolished after pretreatment with 500 and 1000 nM NB (93 and 97% inhibition, respectively). Under these conditions neither picrotoxin nor TETS at a concentration of 100-1000 nM affected the binding of ^{3}H-TBOB.

The apparent NB concentration inducing irreversible inhibition of 50% of the $GABA_A$-receptor chloride channels was calculated by regression analysis using the method of least squares; it was 39 nM and is seen to be close to IC_{50}. It should, however, be noted that IC_{50} was measured in the presence of both the inhibitor and the radioligand in the incubation medium, whereas the NB concentration inducing irreversible inhibition of 50% of the chloride ionophores was evaluated after the toxin had been removed by repeated washing.

The experiments demonstrated a high affinity of NB to the $GABA_A$-receptor chloride ionophore protein. As is evidenced from the experiments with toxin pretreatment followed by repeated washout, NB, unlike TETS and picrotoxin, irreversibly inhibited these channels. This phenomenon is termed alkylation or acylation. The terms are not identical, but both point to the formation of a covalent bond between the GABAlytic molecule and some site of the chloride ionophore.

The time characteristics of acylation of the chloride channel protein were also studied. To this end the membranes were incubated for 5, 10, 30, and 60 min in the presence of NB (100 nM). The samples were 5 times washed and centrifuged and the preserved ^{3}H-TBOB binding sites were determined. The apparent time of half-acylation was calculated by regression analysis using the method of least squares and constituted 28±6 min.

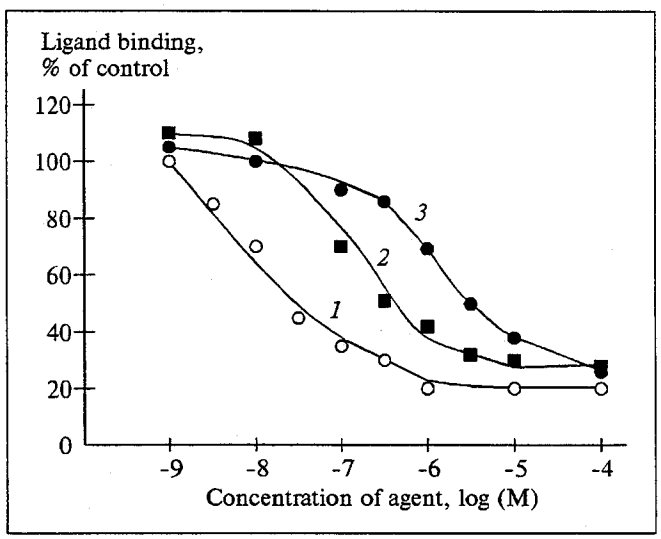

Fig. 1. Effect of norbornan (*1*), TETS (*2*), and picrotoxin (*3*) on binding of ^{3}H-TBOB (5 nM) with synaptic membranes from rat cortex. Each value was obtained in 3 independent experiments performed in triplicate; ^{3}H-TBOB binding in the control was 187±8 fmol/mg protein.

There is evidence that the ability of acylating ligands to compete for specific binding sites is more pronounced at low concentrations of membrane protein. And, conversely, a high concentration of membrane protein in the incubation mixture has been found to lower the affinity of the toxin for the target receptor [8]. This phenomenon is thought to result from conformational changes in the receptor complex upon binding the acylating agent.

To detect this phenomenon we measured IC_{50} for NB and TETS in terms of the specific binding of ^{3}H-TBOB with membranes from the whole brain except for the cerebellum at various protein concentrations (Table 2).

An elevation of the protein content in the incubation medium is seen to lower the affinity of NB for chloride ionophore, which manifested itself in higher IC_{50} values, IC_{50} for TETS being unchanged under these conditions. This is indicative of irreversible inhibition of the $GABA_A$-receptor chloride channel by NB.

TABLE 1. Effect of GABAlytic Pretreatment of Synaptic Membranes from the Cerebral Cortex of Intact Rats Followed by 5-Fold Washout on the Binding of ^{3}H-TBOB (5 nM), % of Control

Ligand	Concentration, nM							
	4	8	16	64	100	250	500	1000
Norbornan	79*	78*	71*	56*	22**	18**	7**	3**
TETS	-	-	-	-	102	101	96	102
Picrotoxin	-	-	-	-	103	104	98	101

Note. *$p<0.01$, **$p<0.001$ in comparison with the control (to the control samples an equivalent amount of dimethyl sulfoxide was added and then they were treated in parallel with the experimental samples); binding in the control was 163±7 fmol/mg protein.

TABLE 2. IC_{50} for Ligands of the $GABA_A$ Receptors as a Function of the Content of Membrane Protein of Rat Brain in the Incubation Medium

Compound	IC_{50}, nM, at different concentrations of membrane protein, mg				
	0.8	1.5	2.0	2.5	3.0
Norbornan	43±8	45±4	68±10	87±12*	110±14**
TETS	652±94	680±52	647±91	683±100	691±73

Note. *$p<0.01$, **$p<0.001$ in comparison with IC_{50} obtained in the presence of 1.5 mg membrane protein. Each value is representative of two independent experiments carried out in triplicate.

There are now other irreversible ionophore inhibitors, 2,6,7-trioxabicyclo[2,2,2]octane derivatives [5,7-9]. In particular, preincubation of membranes from rat cortex with para-isothiocyanato-t-butylbicycloorthobenzoate (250 nM) followed by 5-fold washout reduced the number of ionophores by 60-70% [8], while NB in the same concentration acylated practically all ion channels. The acylation ability of NB evidently surpasses that of TBOB analogs.

Some authorities believe that the use of acylating ligands of chloride channels in neurochemical studies will help in understanding the structure and function of the GABA-receptor complex and, in particular, its ionophore [5,7-9].

The exact binding sites for these agents (inside or outside the channel) remain unclear, as well as which subunit(s) of the receptor interacts with the agent. It is assumed that the agent covalently binds to an amino group of an amino acid (or acids) of the protein [9]. These same considerations may also apply to NB.

Thus, *in vitro* experiments showed that the convulsant NB possesses a high affinity for the $GABA_A$-receptor chloride channel protein, surpassing that of TETS and picrotoxin. In synaptic membranes from the cortex of intact rats the density of chloride channels (^{3}H-TBOB binding sites) cannot be restored by washout. This allows this GABAlytic to be counted among the irreversible ionophore inhibitors.

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