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Possible Involvement of Low and High Affinity GABA Receptors in the Chloride Influx into Synaptosomes

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Experiments carried out in the absence or presence of GABA using a synaptosomal fraction from which endogenous GABA was as far as possibly eliminated, seem to indicate that both GABA receptors are involved in the chloride channel opening. This hypothesis is supported by results obtained in the presence of GABA agonist (muscimol) or drugs which are related to the complex GABA receptor-ionophore (diazepan and phenobarbital).

Key words: GABA binding; Cl^- uptake, Diazepan, Muscimol, Phenobarbital.

The major inhibitory neurotransmitter in mammalian brain γ-aminobutyric acid (GABA), exerts its effect by increasing postsynaptic membrane permeability to chloride ion (6, 21, 23). The GABA receptor and associated chloride ion channel appear to be part of a protein complex containing receptor sites for GABA, benzodiazepines and picrotoxin/barbiturates, as well as the chloride ionophore (24). The GABA receptor site, according to GUIDOTTI *et al.* (11, 13) seems to involve two specific GABA receptors with different physiological, anatomical and biochemical characteristics which TOFFANO *et al.* (30) called receptor $GABA_1$ or low affinity (K=128 nM and Bmax=3.7 pmol/mg protein) and receptor $GABA_2$ or high affinity receptor (Kd=21 nM and Bmax=0.96 pmol/mg protein). The binding of GABA to the latter appears to be blocked by the existence of an endogenous inhibitor which could be eliminated by treatment with detergents (7, 9) or by intensive washes (10). The identity of this endogenous inhibitor has been argued, and some authors think that it could be GABA.

After the characterization of two different specific GABA receptors the question arises if both of them are involved in the neurotransmitter action of GABA. Based on this information the present study has, therefore, been directed to the examination of whether both GABA receptors are involved in the opening of the chloride channel. The

GABA implication in Cl^- permeability (2, 3, 19) has been demonstrated by using electrophysiologic techniques (2, 3) but due to methodological limitations, biochemical studies of chloride transport in brain have generally been unsuccessful. Recently, the GABA mediated transport of ^{36}Cl has been reported in rat hippocampal slices (31), in intact cultured embryo chick cerebral neurons (12, 25, 28, 29), in synaptoneurosome (26) and in membrane vesicles (14). The synaptosomal fraction has been used because several studies on GABA receptors which employ synaptosomal mitochondrial membrane fractions (13, 24, 29) exist and because GALLO *et al.* (8) reported that the presynaptic effect mediated by GABA might well be chloride dependent.

Material and Methods

Synaptosomal preparation. It was prepared by a modification of the LAI and CLARK methods (8). Fresh Wistard albino rat brains were homogenized with 10 volumes (w/v) of 1 mM Tris-HCl buffer pH 7.4 containing 0.32 M sucrose and 1 mM EDTA-K (Medium A) in a homogenized glass with Teflon pestle. The homogenate was centrifuged for 10 min at 700×*g* and the supernatant centrifuged for 20 min at 11.000×*g*. The pellet was resuspended in medium A and placed in 4 volumes of 6 % Ficoll in medium A (w/v) and centrifuged for 20 min at 11.000×*g*. The supernatant was discarded and the pellet was washed twice with 20 ml of 5 mM potassium phosphate buffer pH 7.8 containing 0.32 M sorbitol and 0.1 mM EDTA-K (Medium B) and then suspended in assay medium. This suspension was denominated «synaptosomal fraction» and it was used in the uptake assays.

Endogenous GABA elimination. This was performed by incubation of the synaptosomal fraction with GABASE. The synaptosomal fraction (15 ml) was incubated at 37 °C with 1 IU of GABASE in a medium containing 0.1 M sodium pirophosphate pH 8.6, 0.32 M sucrose, 10 mM EDTA-K, NADPH and 2-oxoglutarate (100 mM of both). After the incubation time the mixture was centrifuged for 20 min at 11.000×*g* and the pellet was washed twice with medium B and finally, the pellet was resuspended in the assay medium.

Chloride uptake assays. The synaptosomal fraction (0.8-1.2 mg protein) was incubated in 50 mM Tris-succinate pH 7.4 containing 0.25 M sucrose, 2 mM 2-mercaptoethanol, 1 mM EDTA and 242 μM of ^{36}Cl (specific activity 0.1 μC/μmol) in the absence and presence of GABA or other drugs at the concentration indicated in each case. The assays were terminated by filtration under vacuum, through Whatman GF/C filters. The washed filters were dried and counted using liquid scintillation counter. The counting efficiency was about 90 %. The uptake was determined by measuring the amount of radioactivity retained by the filters. When the assays were carried out in the presence of GABA agonist or other drugs, the synaptosomal fraction was preincubated for 12 min with the drug, in both, the absence and presence of 300 nM of GABA in the medium. Blanks were defined by experiments carried out in the presence of Triton X-100 in the medium (1 %).

Determination of synaptosomal integrity and functionality. It was determined by measuring the LDH activity in the absence and presence of Triton X-100 (0.1 %) according to the procedure of LAI and CLARK (16). The metabolic functionality was carried out by measuring the O_2 consumption in the absence and presence of 10 mM succinate and 1 mM ADP according to LAND *et al* (17). Proteins were measured by the LOWRY *et al.* method (18) with bovine serum albumin as standard.

Results

The incubation with GABASE did not eliminate the total endogenous GABA but decreased it from 20 μM to 3.5 μM (table I). This reduction of endogenous GABA increased the effect of exogenous GABA on chloride uptake.

The effect on chloride uptake of external GABA or other drugs, with action on GABA receptors, were checked. Exogenous GABA, at a concentration of 300 nM, increased the chloride uptake by 28 %. Muscimol, a GABA agonist, enhanced the chloride uptake by 46 % and 48 % in the absence and the presence of GABA respectively (table II). Diazepan, a drug, which according to several authors enhances the GABA binding to its high affinity receptor (31), at a concentration of 300 nM increased the chloride uptake by 53 % in the absence of exogenous GABA and by 52 % in its presence. Phenobarbital, a drug involved in the GABA-receptor-ionofore complex (24) produced an enhancement of chloride uptake of 116 % in the absence of GABA and of 166 % in its presence, this effect being dose dependent (table II). The synaptosomal integrity was about 85 % and the O_2 consumption was about 21.3 $\pm$ 1.6 *nat* oxygen/mg protein using succinate as substrate.

Discussion

The similarity of the results obtained in the absence and presence of GABA may be due to an interference of endogenous GABA in the synaptosomal preparation, as other authors proposed (10, 20, 22). The chloride uptake in the absence of exogenous GABA could be due: 1) to an unspecific chloride uptake or 2) to an uptake mediated by endogenous GABA.

To find out whether both GABA receptors or only one of them are involved in the opening of the chloride channel, the experiments were carried out with synaptosomal fractions from which endogenous GABA was nearly eliminated and in the presence of high concentrations of chloride ions in the medium. When endogenous GABA was partially, eliminated by GABASE, the Cl^- uptake, in the absence of exogenous GABA, decreased in respect to that obtained without incubation with GABASE. On the other hand, the % of chloride uptake mediated by exogenous GABA was higher in preparations treated with GABASE. These results could suggest that both GABA receptors (high and low affinity receptors) are involved in the opening of the channel. In order to support this hypothesis experiments in the presence of diazepan were performed.

Diazepan does not compete with GABA for a common binding site, so that its effect will be probably alosteric by binding to its specific receptor (5, 11, 27) either by eliminating the endogenous inhibitor from the GABA high affinity receptor (11) or by increasing the affinity of the low affinity GABA recep-

Table I. *Effect of incubation with GABASE on endogenous GABA elimination.* Experiments were performed in triplicate. Values between parenthesis refer to % with respect to the values in absence of GABA.

Conditions	GABA μM	Specific chloride (nm Cl^-/mg prot.) — GABA	+ GABA
Synaptosomal fraction	20.0	2403 $\pm$ 363 (100)	3833 $\pm$ 148 (160)
+ Incubation with GABASE	3.5	2059 $\pm$ 1285 (100)	4461 $\pm$ 556 (216)

Table II. *Effect of diazepan, muscimol and phenobarbital on chloride uptake.* Values between parenthesis refer to % with respect to the values in the presence of GABA.

Conditions	Specific Cl^- uptake (nmol Cl^-/mg prot.)		% Increment	
	— GABA	+ GABA (300 nM)	— GABA	+ GABA (300 nM)
No drugs	3587 ± 257	4586 ± 344*	100	128 (100)
+ Diazepan, 300 nM	5497 ± 902*	6950 ± 451*	153	194 (152)
+ Muscimol, 40 nM	5254 ± 599*	6780 ± 236*	146	189 (148)
+ Phenobarbital	—	—	—	—
100 μM	—	9278 ± 431*	—	259 (202)
400 μM	7754 ± 633*	12216 ± 828*	216	341 (266)

* $p < 0.05$.

tor (27). According to the present results diazepan, per se, is observed to produce an increment of 53 % in chloride uptake in the absence of exogenous GABA. As diazepan is not a GABA agonist, its effect on chloride uptake could be explained in terms of its action on the high affinity GABA receptor. In the presence of exogenous GABA, the diazepan effect was 52 %, similar to the one in its absence, which supports the hypothesis that diazepan acts via the high affinity receptor, which in such a case, will be involved in the opening of the chloride channel.

The action of muscimol on chloride uptake was similar to that of diazepan, although muscimol is a GABA agonist bound to a specific GABA receptor (4). Thus the effect of muscimol cannot be explained in terms of an effect on GABA binding. It should be noted that the enhancement of muscimol in the opening of the chloride channel was of 46 % in the absence of GABA and 48 % in its presence. Thus a possible explanation could be that muscimol acts by mimicking the GABA effect on Cl^- uptake. These results are in agreement with MATHERS *et al.* (19) who say that muscimol is about twice as effective as GABA in increasing membrane conductance to chloride ions.

In respect to phenobarbital, a drug that according to several authors (1, 4, 5) stimulates both GABA and benzodiazepine binding, an increment in chloride uptake of 116 % in the absence of GABA and of 166 % in its presence was found. This could indicate: 1) that phenobarbital increases the response on both GABA receptors or 2) that phenobarbital, per se, acts directly on the opening chloride channel. The effects of barbital found in the present study are in accordance with HUANG *et al.* (15) who consider that the barbiturate effect on GABA receptors may be relevant in the prolongation of the chloride channel opened by GABA. Therefore, according to all these results, the existence of a receptor - ionophore complex, characterized by others (1, 23) appears evident in the synaptosomal fraction used in the present study and the authors think that the method used in this study on the chloride uptake could provide great help in the study of the characteristics of the GABA receptor-ionophore complex.

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Resumen

De los resultados obtenidos sobre la incorporación de Cl^{36} en ausencia y presencia de GABA parece deducirse que tanto el receptor de alta afinidad para el GABA como el de baja afinidad están implicados en la apertura del canal del cloro. Esta hipótesis se apoya también en los efectos encontrados sobre dicho transporte al ensayar drogas agonistas (muscimol) o drogas relacionadas con el complejo receptor del GABA ionóforo (diazepan y fenobarbital).

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