REVISTA ESPAÑOLA DE FISIOLOGIA, 40, 375-380. 1984

The Chloride Channel Opening by GABA as an Energy Dependent Process

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(Received on February 3, 1984)

C. M. SANCHEZ, M. C. TOLEDO and M. P. GONZALEZ. *The Chloride Channel Opening by GABA as an Energy Dependent Process.* Rev. esp. Fisiol., 40, 375-380, 1984.

4-aminobutyric acid (GABA) induced an increase in the chloride ion uptake into synaptosomes from rat brain. The synaptosomal fraction used maintained its integrity and metabolic functionality. The chloride uptake was potentiated by the presence of succinate, reaching an increase of 46.5 % and 76.4 % without or with ADP respectively. These results were parallel to those obtained in the assays of oxygen consumption. These results suggest the energy dependence of the chloride uptake process.

Key words: Glucose, Bilirubin, Glucuronyl transferase.

There is increasing evidence that 4-aminobutyric acid (GABA) is the major neurotransmitter that mediates inhibitory synaptic transmission in the central nervous system of vertebrates and in the peripheral nervous system of invertebrates (5, 13). The GABA exerts its inhibitory action when bound to specific receptors in the postsynaptic membrane. This interaction includes the ability of GABA to enhance the membrane permeability for chloride ion (10, 14). Nevertheless, at the moment, there is not clear evidence for a physical coupling between GABA receptor binding and chloride ion channel opening.

Likewise, it is not known the mechanism by which the ion channel opening occurs, although there is the possibility that it could be mediated by a membrane phosphorilation process (1, 15). In the present work we have studied some aspects of the GABA receptor chloride ion channel interaction using synaptosomes from rat brain as an experimental model.

Materials and Methods

Synaptosomes preparation. Fresh rat brains were homogenized in 10 volumes of 1 mM Tris-HCl buffer pH 7.4 containing 0.32 M sucrose and 1 mM

EDTA (Medium A) in a glass Potter fitted with Teflon pestle. The homogenate was centrifuged for 10 min at 700 × *g*. The pellet was discarded and the supernatant fraction was centrifuged at 11.000 × *g* for 20 min. The pellet was then resuspended in Medium A and layered on top of 4 volumes of Medium A containing 6 % of Ficoll and centrifuged for 30 min at 11.000 × *g*. The supernatant was discarded and the fluffy layer was carefully taken and washed twice with 20 ml of 5 mM of potassium phosphate buffer pH 7.8 containing 0.32 M sorbite and 0.1 mM EDTA (Medium B). Then, it was suspended in assay medium. This fraction was considered as the synaptosomal one and was used in the chloride uptake assays.

Synaptosomal integrity assays. They were followed by measuring the LDH activity in the synaptosomal fraction according to LAI and CLARK (6), in the absence and in the presence of 0.1 % of Triton-X-100. The LDH activity in the presence of Triton-X-100 was considered to be the total activity (100 %) and the one obtained in its absence the activity due to free LDH in the medium. The synaptosomal integrity was expressed as the percentage of LDH activity into synaptosomes. It was calculated by subtracting the activity in the absence of Triton-X-100 from the total activity.

Synaptosomal Functionality assays. They were followed according to LAND *et al.* (7) by measuring the oxygen consumption polarographycally at 25° C in a final volume of 1 ml. The incubation mixture containing 5 mM KCl; 225 mM manitol; 75 mM sucrose; 0.1 mM EDTA; 10 mM Tris-phosphate buffer pH 7.4 and 10 mM Tris-HCl buffer pH 7.4 and synaptosomal aliquots (2.5-3 mg proteins). State III was induced by addition of ADP Calculations were carried out according to CHANCE and WILLIANS (2).

Chloride uptake assays. Aliquots of synaptosomal fraction (about 1 mg proteins) were incubated in triplicate for 4 min at 37° C, without and with 200 μM GABA. The incubation medium contained 0.25 M sucrose, 2 mM 2-mercaptoethanol; 1 mM EDTA, 121 μM $^{36}Cl^-$ (0.41 μCi/μMol) and the buffer indicated in each case. At the end of the incubation, the synaptosomes were rapidly trapped on Whatman GF/C filters. An additional 2 ml of Medium A were added twice to the incubation vials and poured onto the filter. The filters were placed into glass vials, dried and after the addition of 5 ml of toluene containing 0.6 % naftol and 0.5 % PPO they were counted in a Packard Tri-Carb model 2425. Proteins were determined according to LOWRY *et al.* (8).

Results

Table I shows the influence of different media and the effect of GABA on the chloride uptake into synaptosomes. In all media assayed this neurotransmit-

Table I. *Effect of GABA on chloride uptake.* The uptake assays were carried out as described in Materials and Methods, with the synaptosomal fraction suspended in the assay medium indicated in each case. Data are expressed as the mean ± s.d. from four separate experiments, each one performed in triplicate. In all cases the differences, in respect to data in absence of GABA, were statistically significant by the t-Student test.

	Chloride uptake (pmol/mg prot.)		
Assay Medium (mM)	— GABA	+ GABA	% Inc.
Tris-Citrato, 50	850± 91	1426± 70	67.8
Tris-Succinato, 50	992± 60	1345± 4	35.6
Tris-HCl, 1	1378±124	2486±250	80.4

Table II. *Respiratory assays.*
The assays were carried out as described in Materials and Methods with the synaptosomal fraction suspended in Medium A. Both, substrates and ADP (in State 3) were added directly to the reaction mixture in concentrations of 10 mM and 1 mM respectively, in a final volume of 600 μl. Data are expressed as the mean ± s.d. of three separate experiments, each one performed by triplicate.

	Oxygen consumption (nat. O_2/mg prot/min)		
Substrate	State 1	State 2	State 3
Succinate	7.3±0.1	21.3±1.6	27.5±4.0
Glucose	7.3±0.8	7.2±0.9	6.4±1.6
Citrate	7.7±0.5	8.4±0.7	8.9±0.8

Table III. *Effect of storage on the integrity and metabolic functionality of synaptosomes.*
Synaptosomal fractions were stored at 0-5° C suspended in Medium A in a concentration of 4.6 mg prot/ml. Its integrity and functionality were measured daily during a week, as described in Materials and Methods. Succinate was used as energetic substrate. Data are expressed as the mean ± s.d. of three separate experiments each one performed by triplicate.

		Oxygen consumption (nat.O_2/mg prot/min)		
Days	% Integrity	State 1	State 2	State 3
0	75.0±5.8	—	—	—
1	71.5±3.3	1.9±0.11	14.1±0.13	33.8±0.60
2	82.2±0.9	3.5±0.29	23.5±0.14	36.3±1.73
3	78.7±1.6	4.2±0.25	20.7±0.01	32.9±0.01
4	83.3±1.5	5.5±0.01	24.6±0.64	35.3±0.01

ter produced an increase in Cl^- uptake in the presence of GABA. These results are in agreement with those obtained by ionophoretic studies (9, 12). The optimal results were reached with Tris-HCl medium. The results from LDH suggest intact synaptosomes and those from respiratory activity indicated a good functional state. The best respiratory medium was that with succinate as substrate. The presence of ADP produced an increase in the oxygen consumption which did not occur with the other substrates (table II).

Table III shows that both parameters, integrity and metabolic functionality of synaptosomes were maintained for, at least, four days after the synaptosomal obtention when these were stored at 0° C and suspended in Medium A. Therefore, probably the chloride uptake assay could be carried out in this time space. Preliminar results (data not shown) seem to help this hypothesis.

When the chloride uptake assays were carried out in the presence of both, GABA and succinate, the uptake, in respect to the control, was increased by 46.5 %, reaching an increment of 76.4 % when ADP was added. These

Table IV. *Relation between chloride uptake and oxygen consumption.*
Both experiments were performed in parallel, one day after the synaptosomal obtention, in the conditions described in Materials and Methods, in 1 mM Tris-HCl buffer pH 7.4. Each condition corresponds to the previous one with the addition of the compound specified. Data are expressed as the mean ± s.d. of three separate experiments, each one performed by triplicate. Res. Inc. = Respiratory increase when compare to control (Chloride).

	Chloride uptake		Oxygen consumption	
Conditions	pmol/mg prot.	% Inc.	nat.O_2/mg/min	Res.Inc.
Chloride	2500 ± 199	—	2.7 ± 0.36	1.0
+ GABA	2713 ± 399	8.5	—	—
+ Succinate	3662 ± 74*	46.5	14.7 ± 1.08	5.4
+ ADP	4409 ± 391*	76.4	25.6 ± 1.08	9.4

* $P < 0.01$ in respect to data in the absence of GABA (t-Student test).

increases were also observed in the oxygen consumption (table IV). Therefore, it could be inferred that the chloride uptake is proportional to the oxygen consumption.

Discussion

Studies about chloride uptake into the postsynaptic cells, mediated by the GABA action, have been performed only in the peripheral nervous system of invertebrate (4, 11) by using either complete tissue or slices, while studies on characteristics of GABA receptors are done with synaptosomal membrane preparations from rat brain. In the present work, one have studied the GABA action by measuring the chloride uptake into synaptosomes from rat brain with a radiometric procedure.

Results in table I indicate that GABA increases the chloride uptake into synaptosomes. These results are in agreement with those obtained by GHIASUDDIN and MATSUMURA (4) with complete tissue. The fact that the optimal results were obtained with Tris-HCl buffer could indicate a chloride ion dependence of the uptake process; dependence mediated by GABA. This assumption was proved with posteriority (data not shown).

The suitability of synaptosomes as an experimental model for studies on chloride uptake was verified when it was observed that they maintained their integrity and metabolic functionality (table III), keeping in that way the optimal conditions to the assay until, at least two days after the synaptosomal obtention, as indicated above.

The GABA action on Cl^- uptake was potentiated by succinate. This effect was increased by ADP (table IV). The fact that the chloride uptake to be related to the oxygen consumption could indicate a parallel relation between Cl^- uptake and energetic state of synaptosomes. This seems to indicate an intimate relation between the opening of the chloride channel and the ATP synthesis.

These results could serve as support of the hypothesis proposed by some authors which consider that the opening of the chloride channel is mediated by a phosphorilation of membrane proteins. Nevertheless, we cannot eliminate the possibility of the use of ATP as an energy source for a conformational change in the synaptic membrane phospholipid bilayer structure (3). To clarify this point further studies are now being undertaken.

Resumen

El ácido 4-aminobutírico induce un incremento en la entrada del ion cloro a los sinaptosomas procedentes de cerebro de rata, los cuales mantienen su integridad y funcionalidad metabólica. Este efecto se potencia por la presencia de succinato, alcanzando un incremento del 46,5 o del 76,4 % si está presente el ADP. Estos resultados son paralelos a los obtenidos en los ensayos de consumo de oxígeno, lo que sugiere la posible dependencia energética del proceso de entrada del cloro.

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