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Presence of Glutamate Decarboxylase in Bovine Adrenal Medullary Cells

J. M. Fernández-Ramil, J. Sánchez-Prieto and M. P. González

Departamento de Bioquímica
(Centro Coordinado del C.S.I.C.)
Facultad de Farmacia
Madrid-3 (Spain)

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J. M. FERNANDEZ-RAMIL, J. SANCHEZ-PRIETO and M. P. GONZALEZ. *Presence of Glutamate Decarboxylase in Bovine Adrenal Medullary Cells.* Rev. esp. Fisiol., 38, 91-96. 1982.

Data confirming the presence of GAD in bovine adrenal medullary cells are reported. The subcellular distribution of this enzyme seems to be cytosolic and mitochondrial. The possibility of two GAD, GAD I and GAD II, is discussed. The mitochondrial enzymatic activity has been checked by both the ^{14}C-CO_2 liberated and the presence of ^{14}C-GABA formed.

Two different glutamate decarboxylase (GAD) enzymes have been identified in nervous tissues of animals, both are designed in the literature as GAD I and GAD II. GAD I is found primarily and perhaps exclusively, in the nervous system and is associated with the synaptosomal fraction of nervous tissues (3, 13, 14). It requires pyridoxal phosphate (PLP) as a cofactor (12). GAD II in nervous tissues appears to be localized in the mitochondrial fraction (5) and in glial cells (6). It is also found in nonneural tissues such as kidney (7, 9) and does not appear to require PLP as cofactor (6). At the present paper we report data confirming the presence and localization of GAD in bovine adrenal medulla, tissue of ectodermic origin.

Materials and Methods

Bovine adrenal glands were supplied by the local slaughter house, immediately placed in cold 0.32 M sucrose and dissected in the hour following the death of animals.

Subcellular fraction. The method for subcellular fractionation was based on that of AUNIS *et al.* (1). The adrenal were defatted and, after fine dissection, the medulas were suspended in 10 volumes of 0.32 M sucrose containing 0.1 mM PLP, 5 mM EDTA-Na and 0.01 M reduced glutathione. The mixture was homogenized in a mechanical Potter-Elvehjem with teflon pestle and then centrifuged for 10 minutes at 800 $\times$ *g*. The supernatant

was passed through cheese-cloth in order to remove floating fat, this fraction was considered as homogenate. The homogenate was centrifuged at 11,000 × *g* for 20 minutes. The supernatant was considered as soluble fraction, and the pellet as crude mitochondrial fraction. This pellet was suspended in the homogenization medium (1 ml/2 g of starting material). The suspension was layered on 1.6 M sucrose (5 ml suspension on 10 ml 1.6 M sucrose), in Swing-out tubes and centrifuged for 90 minutes at 100,000 × *g*. Two bands were obtained: one, on the top with 1.6 M sucrose, referred as purified mitochondria and another, at the botton, referred as chromaffin granules.

Enzyme extraction. Purified mitochondria and chromaffin granules were separated, resuspended in the homogenate medium and centrifuged for 20 minutes at 15,000 × *g*. Each pellet was homogenized in a Potter-Elvehjem, with emery glass postle, in the presence of 0.12 M potassium phosphate buffer, pH 6.5, containing 0.1 mM PLP; 0.01 M reduced glutathione; 5 mM EDTA-Na^+ and 0.25 % Triton X-100. These homogenates called «pure mitochondria» (PM) and «Chromaffin granules» (CG) were used as enzymatic preparations.

GAD activity. The incubation mixture contained 6 μmoles U-^{14}C-glutamate (1,360,000 c.p.m.); 120 μmoles potassium phosphate buffer, pH 6.5, 0.1 μmol PLP; 10 μmoles reduced glutathione; 5 μmoles EDTA-Na^+ and 5 mg protein preparation, in a total volumen of 1.2 ml. Incubations were carried out in Warburg vessels, with stirring at 37° C and under anaerobic conditions (under N_2) and during 1 hour. The ^{14}C-CO_2 released was trapped on 0.2 ml hyamine hydroxide in methanol. The reaction was stopped by addition of 0.2 ml of 4 M $HClO_2$ and then 0.2 ml of 35 mM $KHCO_3$ was injected in order that the CO_2 released from $KHCO_3$ helps to expel the small amounts of ^{14}C-CO_2 produced from GAD reaction mixture. After stopping the reaction, it was kept 1 hour with shaking before measuring the radioactivity from hyamine hydroxide.

Evaluation of labeled GABA. The GAD incubation medium, after reaction was neutralized with OHK and centrifuged; then it was passed through a Lewatit-S-100 column, chloride form (25 × 1 cm). Aminoacids were retained by the column and eluated from it with 1 M of ammonium; fractions of 2 ml were collected. Samples of 10 μl, from each fraction, were taken to measure the radioactivity. The remain from the ammonium fractions, which presented radioactivity, was mixtured and evaporated by vacuum until dryness. The dry extract was redissolved in 0.5 ml of distilled water and ^{14}C-GABA identified by both, paper electrophoresis and chromatography on column.

Paper electrophoresis was carried out at pH 10 in 4 ml borate buffer. The paper strip (2.4 × 40 cm) was cut out in 0.5 ml pieces and placed in a counting vial which contained 2 ml of scintillation counting liquid.

The chromatography was performed on Dowex-50-X-1 (100-200 mesh) in the chloride form. This Dowes-50-X-1 chloride form was converted to the acetate form by washing it with 2 M sodium acetate and then placed in a small column (0.5 × 3 cm). The dry extract previously described was applied on this column and the labeled GABA obtained from the column by elution with 10 ml of water. The labeled glutamate was then eluated from the column by washing it with 2 M CHl. In both cases, fractions of 100 μl were collected on counting vials and radioactivity checked as before.

Proteins were assayed by Biuret method (4).

Results

The bovine adrenal medulle GAD is preferently localized in soluble fractions

Table I. *Subcellular distribution of glutamate decarboxylase activity in adrenal medullary cells.*

Fraction	Total activity (nmol/minute)	Specific activity (nmol/m/mg prot.)
Homogenate	99±8	0.04±0.003
Soluble (S)	54	0.04
Purified mitochondria (PM)	5±0.05	0.15±0.014
Cromaffin granules (CG)	2±0.05	0.05±0.002
ΣS + PM + CG	61	
% Recovery	62	

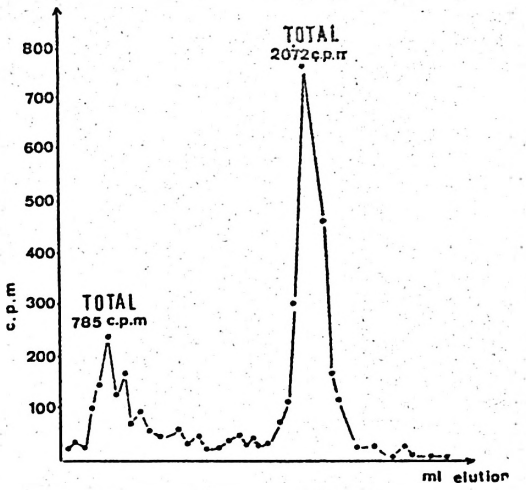

Fig. 1. *Chromatography on Lewatit S-100 of incubation medium after enzymatic GAD reaction.*

(87 %) although a small amount of GAD is also present in mitochondria (8 %). The mitochondrial activity cannot be attributed to cytosol contamination since the lactate dehydrogenase (LDH) found in this fraction was 0.33 % respect to soluble fraction (unpublished results). The 4 % of activity found in chromaffin granules may be due to mitochondrial and/or soluble fraction according to the results for fumarase and LDH (unpublished results). The highest enzymatic activity (specific activity) was found in the mitochondrial fraction. The enzyme recovery was low, about 62 %, and this may be due to inactivation of enzyme because of high instability (table I).

As the enzyme was assayed by measuring or ^{14}C-CO_2 released during reaction and as ^{14}C-U-glutamate was used, we checked the presence of ^{14}C-GABA in the mitochondria incubation in order to eliminate the presence of ^{14}C-CO_2 was due to glutamate metabolism via Krebs cycle. On figure 1 one can observe the presence of two peaks after passing the incubation medium through a Lewatit S-100 column. The first peak, probably, corresponds to labelled ketoacids formed during reaction, and the second one to the aminoacids glutamate and GABA. On figure 2 and 3 it is clearly possible to identify the presence of GABA in the incubation mitochondria medium. The first peak corresponds to GABA and the second one to glutamate (fig. 2), the profile of electrophoregrame where two peaks are differentiated presenting the radioactivity due to glutamate and GABA respectively (figure 3).

Table II shows data on effect of aminooxiacetic acid (AOAA) on GAD from soluble and mitochondrial fractions. The behaviour of soluble and mitochondrial

Table II. *Effect of aminooxiacetic acid on glutamate decarboxilase activity from different subcellular location of adrenal medulla.* AOAA was assayed at concentration of 1 mM in the incubation medium.

Fraction	% Activity
Soluble fraction (S)	100
S + AOAA	78
Purified mitochondria (PM)	100
PM + AOAA	225

GAD is opposed, that is, GAD from soluble fraction is inhibited by AOAA while the enzyme from mitochondria is activated.

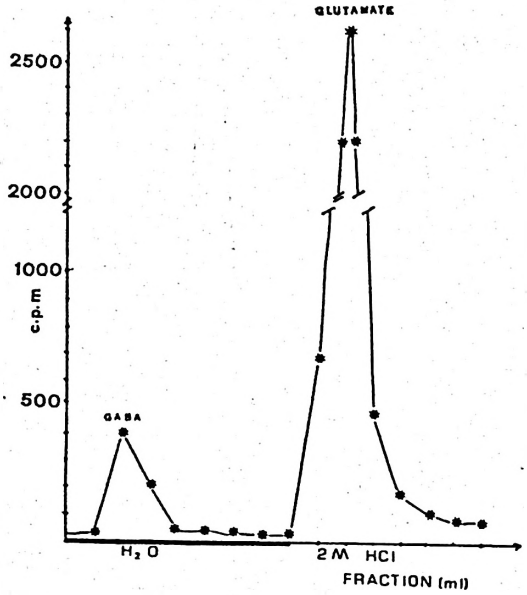

Fig. 2. *Chromatography on Dowex X-1 column of aminoacids from GAD reaction.*

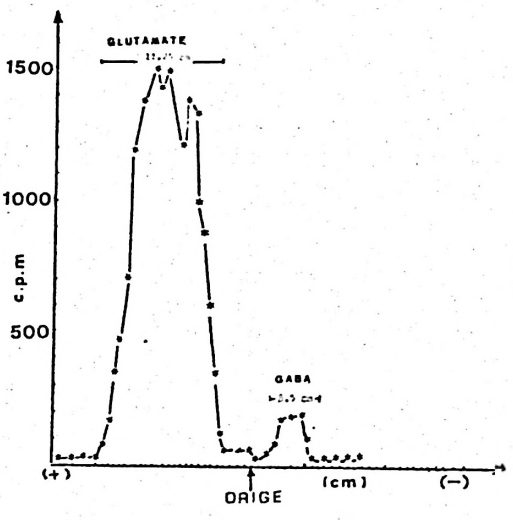

Fig. 3. *Distribution of radioactivity in aminoacids from GAD reaction along paper electrophoresis.*

Discussion

The present results seem to indicate that in medulla cells, as in brain, there are two GAD, one in soluble fraction, GAD type I, inhibited by AOAA and another in mitochondria, GAD II, activated by AOAA (9). What it is not possible to infer whether GAD I from madulla is similar to that from nervous tissue because further studies are necessary to confirm that. However, the enzymes localization and their behaviour against AOAA may indicate that, probably, both enzymes are present in this tissue. This could be possible because medullary cells are of neural origin, ectodermic origin, and these cells may be considered as catecholaminergic neurons because they are able to release catecholamines following appropriated stimuli. Besides that, the chromaffin granules, show several characteristics similar to those of cholinergic vesicles (10, 11). Although in brain GAD I seems to be localized in GABA-ergic neurons there is no evidence to confirm the absence of this enzyme in other types of neurons. Actually, several controversies exist on this aspect since there is evidence for neurons containing more than one transmitter (14). So, recently, HATANAKA *et al.* (8) have demonstrated the presence of GAD in PC 12 cell line which are known to be able to synthesized both neurotransmitters, acetylcholine and catecholamines. On the other hand, the amount of GAD specific activity in this tissue is about 1/3 of that found in brain by BLINDERMAN *et al.* (2) which may suggest some importance of this bypass in adrenal medulla. Nevertheless, the role of this bypass in these cells is of interest since its study could serve as a model in the knowledge of the role of GABA synthesis in neurons no GABA-ergic and the possible relationship between GABA-ergic and catecholaminergic system.

Resumen

Se confirma la presencia de glutamato descarboxilasa (GAD) en células de médula adrenal bovina. La localización subcelular de este enzima parece ser citosólica y mitocondrial. Se discute la posibilidad de la presencia de dos GAD, la GAD I y la GAD II. La actividad de la GAD mitocondrial se demuestra no sólo por el desprendimiento de ^{14}C-CO_2, sino también por la presencia de ^{14}C-GABA formado por la descarboxilación del glutamato-U^{14}C.

References

1. AUNIS, D., MIRAS-PORTUGAL, M. T. and MANDELL, P.: *Biochem. Biophys. Acta*, **327**, 313-327, 1973.
2. BLINDERMAN, J. M., MAITRE, M., OSSOLA, L. and MANDELL, P.: *Eur. J. Biochem.*, **86**, 143-152, 1978.
3. FONNUM, F.: *Biochem. J.*, **106**, 401-417, 1968.
4. GONNAL, A. G., BARDAWILL, C. S. and DAVIZ, N. N.: *J. Biol. Chem.*, **177**, 751-755, 1949.
5. HABER, B., KURIYAMA, K. and ROBERTS, E.: *Brain Res.*, 22, 105-112, 1970.
6. HABER, B., KURIYAMA, K. and ROBERTS, E.: *Science*, **168**, 598-599, 1970.
7. HABER, B., KURIYAMA, K. and ROBERTS, E.: *Biochem. Pharmacol.*, **19**, 1119-1136, 1970.
8. HATANAKA, H., TANAKA, M. and AMANO, T.: *Brain Res.*, **183**, 490-493, 1980.
9. LANCASTER, G., MOHYUDDIN, F., SCRIVER, CH. R. and WHELAN, D. T.: *Biochem. Biophys. Acta*, **297**, 229-240.
10. LEGERCRANTZ, H.: *Neuroscience*, **1**, 81-92, 1976.
11. PALFREYMAN, M. G., HUOT, S., LIPPERT, S. and SCHECHTER, P. J.: *Eur. J. Pharmacol.*, **50**, 325-336, 1978.
12. ROBERTS, E. and SIMONSEN, D. G.: *Biochem. Pharmacol.*, **12**, 113-134, 1964.
13. SALGANICOFF, L. and ROBERTS, E.: *J. Neurochem.*, **12**, 287-309, 1965.
14. VAN KEMPER, G. M. J., VAN DEN BERG, C. J., VAN DER HELM, H. J. and VELDSTRA, H.: *J. Neurochem.*, **12**, 581-583, 1965.