Vol. 96, No. 3, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 16, 1980 Pages 1312-1316

HISTOCHEMICAL LOCALIZATION OF ORNITHINE DECARBOXYLASE WITH A LABELLED SUICIDAL ENZYME INHIBITOR

Gad M. Gilad and Varda H. Gilad

Department of Isotope Research, The Weizmann Institute of Science,

Rehovot, Israel.

Received July 23,1980

SUMMARY: We describe a new technique for cytochemical localization of ornithine decarboxylase by the use of a synthesized conjugate of rhodamine bound to α-difluoromethylornithine a suicidal inhibitor of the enzyme. The labelled inhibitor retained its specificity and irreversibility towards ornithine decarboxylase inhibition. Using this technique we have localized the enzyme in specific regions of the developing rat cerebellum. This novel technique may be generally applicable to other enzymes.

INTRODUCTION

Ornithine decarboxylase (EC 4.1.1.17, ODC) is the enzyme catalyzing the first step in the synthesis of polyamines which appear to be intricately involved in growth processes (1). The activity of ODC has been shown to increase rapidly in tissues prior to onset of rapid growth or regeneration (2,3) as well as in several tissues after hormonal treatment (4,5). In spite of the general interest in this enzyme it has been never localized histochemically so far. The present study therefore describes the use of a newly developed technique for the histochemical localization of ODC. In this technique we have conjugated the fluorescent molecule rhodamine with α-dFMO, a newly synthesized enzyme activated irreversible inhibitor (i.e. suicidal inhibitor) of ODC (6). The synthesized conjugate has retained its inhibitory activity and been used successfully for the histological localization of ODC in the developing

Abbreviations used are: ODC, ornithine decarboxylase; α-dFMO, α-difluoromethylornithine; SAM-DC, S-adenosylmethionine decarboxylase; GAD, glutamic acid decarboxylase; PLP, pyridoxal-5'-phosphate; DTT, dithiothreitol.

0006-291X/80/191312-05$01.00/0

rat cerebellum, where the developmental changes in ODC activity are well characterized (7).

MATERIALS AND METHODS

Preparation of labelled inhibitor: The inhibitor α-dFMO (a generous gift from the Centre de Recherche Merrel International, Strasbourg, France) was dissolved in H_2O and reacted with excess cupric carbonate. The inhibitor- copper chelate was mixed with rhodamine-B-isothiocyanate (1:1 molar ratio) in a 0.4M sodium carbonate-bicarbonate buffer, pH 8.4, overnight at 4°C. The mixture was then brought to pH 3 1N HCl and the precipitate formed washed and redissolved in 0.1M Tris-HCl buffer pH 7.2. The preparation was passed through a column of sepharose-coupled lysine (a gift from Dr. M. Wilchek, Department of Biophysics, Weizmann Institute of Science, Israel) and the eluate, cleared from excess free rhodamine, used for biochemical and histochemical studies.

Enzyme assays: Enzyme activities were assayed in homogenates of cerebellum from 7d old sprague-Dawley rats by established methods: ODC according to Oka and Perry (11); SAM-DC (EC 4.1.1.50) as described by us before (7), and GAD (EC 4.1.1.15) by the method of Roberts and Simonsen (12).

Cytochemistry: Rats were perfused transcardially with phosphate buffered saline (ph 7.4) containing 0.32 sucrose (PBS), followed by a fixative solution of PBS containing 1.25% glutaraldehyde (vol./vol.) and 1% formaldehyde (wt./vol.). Following excision the brains were immersed in PBS until sunk and then cut frozen. This treatment resulted in a 30% reduction of *in vitro* assayable enzyme activity. Sections, affixed to gelatin (0.5% wt./vol. in H_2O) coated glass slides were briefly washed in phosphate buffered saline, then incubated in a humid chamber for 1-2h at 37°C with 2-50μM rhodamine-labelled inhibitor preparation containing 60μM PLP, washed with phosphate buffered saline and mounted in glycerol: PBS, pH 7.2 (4:1 vol./vol.). Control sections were pre-incubated with unlabelled (free) inhibitor.

RESULTS

Table 1 demonstrates the retaining of both specificity (towards ODC) and irreversible inhibitory activity of the newly synthesized rhodamine-labelled α-dFMO. This preparation has been used for histochemical staining of the developing rat cerebellum (Fig. 1). Intensive fluorescence staining appeared in the external granule layer of the developing cerebellar cortex and just beneath the Purkinaje cell layer. No staining appeared in control sections pre-incubated with unlabelled inhibitor (Fig. 1). Adult cerebellum was devoid of staining.

TABLE 1. Specific inhibition of ODC activity by labelled inhibitor

Reagent	Enzyme activity (% of control)		
	ODC	SAM-DC	GAD
Before Dialysis			
α-dFMO	5.0	98.0	103.5
Rhodamine labelled α-dFMO	8.5	98.5	106.0
After Dialysis			
α-dFMO	7.5	107.5	91.5
Rhodamine labelled -dFMO	9.5	97.0	95.5

The homogenates were pre-incubated for 0.5h at 37 C with 20 μM of the corresponding inhibitor compound and then dialyzed overnight against 0.1M Tris-HCl buffer pH 7.4 containing 60 μM PLP and 5 mM DTT. Controls, incubated in the presence of 20μM of rhodamine, were completely devoided of innibitory activity. Control values (n=6) were: for ODC, 14.1±1.0 nmole $^{14}CO_2$/g wet weight/h; for SAM-DC, 35.0± 4.1 nmole $^{14}CO_2$/g wet weight/h; and for GAD, 11.1± 0.5 μmole $^{14}CO_2$/g wet weight/h. Results are the mean values from two separate determinations done in duplicates.

DISCUSSION

The cytochemical localization of ODC using this novel technique appears to be highly specific, and indicates the cellular elements which are the source for the high ODC activity detected in the developing cerebellum in previous studies (7). Furthermore, this technique could have several general important applications: Firts, several other α-fluoromethyl amino acids inhibitors of amino acid decarboxylases are now available (9) and this technique should be readily applicable for these enzymes; second, by using enzyme activated irreversible inhibitors the localization should be highly specific (8) and can be used in conjunction with immunocytochemical localization when antibodies against the corresponding enzymes are available; third, the involvement of polyamines in tissue growth and reaction to various hormonal stimuli (1,5) makes the cytochemical localization of ODC more generally important, and fourth, the involvement of ODC in neuronal growth and regeneration

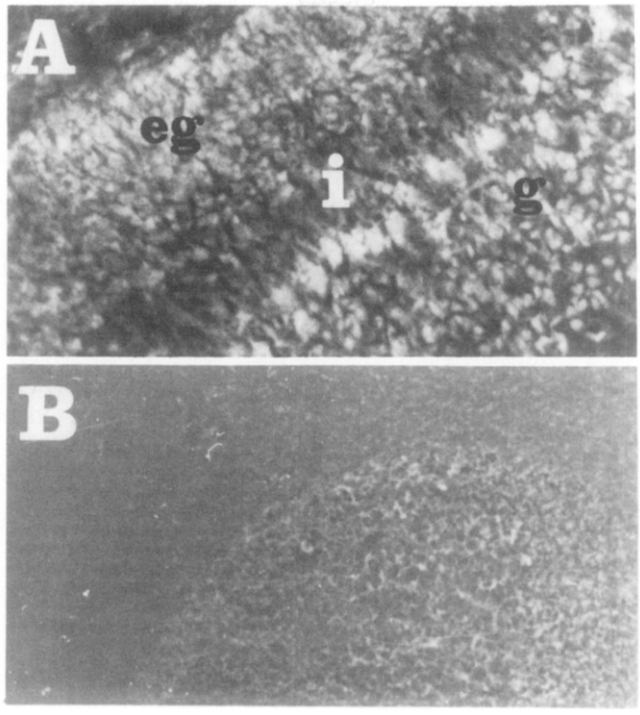

Figure 1. Photomicrographs of ornithine decarboxylase localization in frozen cross sections (16 m thick,) of 7d old rat cerebellar cortex. A. A section stained with the labelled inhibitor. Histofluorescence is apparent in cells of the external granule layer (eg) and in the developing granular layer (g). The intermediate zone (i) and deep areas of the cortex are devoided of staining. The picture was taken with epi-illumination in a Zeiss microscope equipped with rhodamine optics. B. Control section pre-incubated with unlabelled inhibitor before staining with rhodamine-labelled inhibitor X 160.

(7,10) makes this technique of immediate application in studies of the latter.

A detailed description of this newly developed methodology, studies of other tissues and different inhibitor-conjugates which will enable to visualize the reaction in the electron microscope, is in preparation.

ACKNOWLEDGEMENT

We thank Prof. Meir Wilchek for his help and valuable suggestions.

REFERENCES

1. Russell, D.H. (ed.) (1973), Polyamines in Normal and Neoplastic Growth, Raven Press, New York.
2. Russell, D.H. and Snyder, S.H. (1968), Proc. Natl. Acad. Sci. USA, 60, 1420-1427.

3. Anderson, T.R. and Schanberg, S.M. (1972), J. Neurochem. 19, 1471-1481.
4. Russell, D.H. and Taylor, R.L. (1971), Endocrinology 88, 1397-1407.
5. Oka, T. and Perry, J.W. (1974), Nature 250, 660-661.
6. Metcalf, B.W., Bey, P., Danzin, C., Jung, M.J., Casara, P. and Vevert, J.P. (1976), J. Am. Chem. Soc. 100, 2551-2553.
7. Gilad, G.M. and Kopin, I.J. (1979), J. Neurochem. 33, 1195-1204.
8. Rando, R.R. (1973), Science 185, 320-324.
9. Kollonitsch, J., Patchett, A.A., Marburg, S., Maycock, A.L., Perkins, L.M., Doldouras, G.A., Duggan, D.F. and Aster, S.D. (1978), Nature 274, 906-908.
10. Gilad, G.M. and Kopin, I.J. (1979), Int. Soc. Neurochem., Abstr. 7th Meeting, 353.
11. Oka, T. and Perry, J.W. (1976), J. Biol. Chem. 251, 1738-1744.
12. Roberts, E. and Simonsen, D.J. (1963), Biochem. Pharmacol. 12, 113-134.