DETECTION OF ACETYLCHOLINESTERASE ACTIVITY AND GAMMA-AMINOBUTYRIC ACID BINDING SITES IN *DICROCOELIUM DENDRITICUM*

GIMÉNEZ-PARDO C*, ROS-MORENO R.M.*, ARMAS-SERRA C. DE* & RODRIGUEZ-CAABEIRO F.*

Summary :

In the present study we report the presence of acetylcholinesterase activity and gamma-aminobutyric acid binding sites in crude extracts of *Dicrocoelium dendriticum*. This indirectly demonstrates the presence of acetylcholine and GABA. The presence of these neurotransmitters could indicate the existence of two systems implicated in the neurotransmission of the Digenea.

KEY WORDS : *Dicrocoelium dendriticum*, neurotransmitters.

Résumé : DÉTECTION D'UNE ACTIVITÉ ANTICHOLINESTÉRASIQUE ET DE SITES DE FIXATION DE L'ACIDE GAMMA-AMINOBUTYRIQUE CHEZ *DICROCOELIUM DENDRITICUM*

Cette étude rapporte la présence d'une activité anticholinestérasique et de sites de fixation de l'acide gamma-aminobutyrique (GABA) dans des extraits de *Dicrocoelium dendriticum*. Ceci prouve indirectement la présence d'acétylcholine et de GABA, et permet de poser l'hypothèse de l'existence de deux systèmes impliqués dans la neurotransmission des Digènes.

MOTS CLÉS : *Dicrocoelium dendriticum*, neurotransmetteurs.

INTRODUCTION

Acetylcholine is known to influence mobility in platyhelminths and to activate the metabolism in trematodes and cestodes. Conversely, as recent work has shown, GABA exerts an inhibitory effect in platyhelminths. Most of our present knowledge comes from studies on *Schistosoma* spp. and *Fasciola hepatica* (Geary *et al.*, 1992; Erikson & Panula, 1994; Walker Brooks & Holden-Dye, 1996), but little is known about the molecules implicated in neurotransmission in *Dicrocoelium dendriticum*, one of the most common pathogens of domestic animals. Since certain drugs are believed to affect receptors, these molecules might be proposed as attractive targets for chemotherapy in parasitic diseases. The present study has *i)* characterized acetylcholinesterase activity in crude extracts of *Dicrocoelium dendriticum*, indicating indirectly the presence of acetylcholine (Walker & Holden-Dye, 1991) and *ii)* made a preliminary study of GABA receptors in whole extracts from this parasite using binding assays. The object was to collect new data on the molecules implicated in neurotransmission in flatworms and in platyhelminths generally.

* Parasitology Laboratory, Faculty of Pharmacy, University of Alcalá, Crtra. Madrid-Barcelona Km. 33.600, 28871 Alcalá de Henares, (Madrid) Spain.

Correspondence: Consuelo Giménez Pardo.

Tel: +34 1 8854636 – Fax: +34 1 8854663.

E-mail: consuelo.gimenez@uah.es

MATERIALS AND METHODS

COLLECTION AND PREPARATION OF CYTOSOLIC FRACTION

Adult *D. dendriticum* were removed from the gallbladder and bile ducts of sheep at the municipal slaughterhouse in Alcalá de Henares (Spain). The worms were washed several times with 10mM PBS pH 7.2 with added penicillin (1 mg/ml) and streptomycin sulphate (2 mg/ml). Homogenates were prepared using a glass Potter-Elvehjem homogenizer and centrifuged twice at 100.000 xg for 30 min. The final supernatant was considered to be the cytosolic fraction and once the protein concentration had been determined by the method of Lowry *et al.* (1951), it was adjusted to 2 mg/ml. Aliquots were used immediately or frozen and stored at – 80° C.

ENZYMATIC ACTIVITY

Spectrophotometric determinations

Cholinesterase (ChE) activity was determined spectrophotometrically by a modified version of the Ellman procedure (Ellman *et al.*, 1961; Rathaur *et al.*, 1987) with acetylthiocholine iodide (ATCI) as a substrate. Next, 260 µl of 0.1 M PBS buffer (pH 8), 10 µl of 10 mM 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) solution (39.6 mg 5,5'-dithio-bis-(2-nitrobenzoic acid)) (DTNB), 15 mg of sodium bicarbonate and 10 µl of 0.1 M PBS (pH 7.2), 2.5 µl of 75 mM substrate and 25 µl of the sample was added to a microplate and the OD at

405 nm measured every minute. The increases in OD were converted to units per litre (Ellman *et al.*, 1961). One unit was equivalent to a 1 μ mol of substrate hydrolyzed per minute per mg of protein. Inhibition assays were performed in microplates using the same method described above, except that samples (20 μ l of cytosolic fraction plus 5 μ l of each inhibitor) were first incubated at 37°C for 10 min before measuring the increase in OD. The final concentrations and inhibitors used were 400×10^3 nM, 2×10^3 nM, 2×10^2 nM and 4 nM of 1,5-bis(4-allyl(dimethylammoniumphenyl)pentan-3-one dibromide) (BW284C51), and 50 mM, 5 mM, 0.5 mM and 0.05 mM of tetraisopropylpyrophosphoramidate (isoOMPA). One unit was the equivalent of 1 μ mol of substrate hydrolyzed per minute per mg of protein, and the inhibition was calculated as the percentage of ChE activity in the controls.

Electrophoretic assays

Electrophoresis was performed according to the method of Laemli (1970) using a 6 % polyacrylamide gel without SDS (100-200 μ g protein diluted 3:1 in glycerol per lane). The gels were incubated in 20 mg of ATCI dissolved in 26 ml of 0.1 M PBS buffer (pH 6) for 2 h, to which the following were added sequentially: 2 ml of 0.1 M sodium citrate, 4 ml 30 mM $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 4 ml distilled water and 4 ml 5 mM potassium ferricyanide. ChE activity produced brown bands in the gel after 2 h. The bands were then fixed in 5 % acetic acid.

^3H -GABA BINDING ASSAYS

In the ^3H -GABA binding assays performed, 1 ml of 6.29 mg/ml of *D. dendriticum* adult extract was homogenized at 4°C for 10 minutes in two volumes of 20 mM Tris-HCl buffer (pH 7.4) as previously described by Pong & Wang (1980). The homogenate was centrifuged at 1.000 g for five minutes, the pellet (P_1) was resuspended in fresh buffer (Tris-HCl buffer pH 7.4) and the supernatant (S_1) was centrifuged at 60.000 g for 15 minutes. The final pellet (P_2) was resuspended in fresh buffer and was regarded as the membrane fraction. P_2 fraction was incubated with increasing concentrations of ^3H -GABA (specific activity 0.0441 Ci/mmol): 1×10^{-8} M, 5×10^{-8} M and 10×10^{-8} M at 34°C for 30 minutes in the presence (non-specific binding) or absence (total binding) of a 1.000 fold molar excess of unlabeled GABA in Eppendorf tubes. In order to reduce the number of dpm, and thus radioactivity, the ^3H -GABA concentrations were prepared from a mixture of 9 μ l of 27 mM ^3H -GABA, 4.5 μ l of unlabeled GABA and 455 μ l of 20 mM Tris-HCl buffer (pH 7.4). Each tube designated "non-specific binding" contained a final volume of 250 μ l, in which 100 μ l of ^3H -GABA was incubated with 100 μ l of P_2 fraction, 10 μ l of

100 mM amino oxalacetic acid (AOAA) and 40 μ l of unlabeled GABA. Each tube designated "total binding", contained a final volume of 250 μ l comprising 100 μ l of the P_2 fraction, 100 μ l of ^3H -GABA, 10 μ l of 100 mM amino oxalacetic acid (AOAA) and 40 μ l of 20 mM Tris-HCl buffer (pH 7.4). All assays were performed simultaneously with their respective controls. The incubation was terminated by rapid filtration through rinsed three times with 15 ml ice-cold 20 mM Tris-HCl (pH 7.4). After incubation the filters were placed into plastic vials containing 2 ml Aquasol II and the radioactivity was determined by liquid scintillation spectrometry using a Wallac 1410 liquid scintillation counter operating at 42 % efficiency. The specific binding was calculated by subtraction of the non-specific binding from the total binding.

RESULTS AND DISCUSSION

Our results demonstrated the presence of cholinesterases in the cytosolic fraction of *D. dendriticum* adult extract (Fig. 1). Since the gels employed in electrophoresis were not copolymerized with SDS and the sample buffer did not contain the detergent either because SDS inhibits enzymatic activity, it was impossible to determine the molecular weight of the enzyme. However, ChE activity was also quantified by the method of Ellman *et al.* (1961) and the rate was 2.11 U/mg. In inhibition assays, the effect of specific inhibitors (BW284C51 and iso-OMPA) corroborated that to break down ATCI as substrate the parasite uses both acetylcholinesterases and pseudo-cholinesterases, as shown in Tables I and II. Additionally, *D. dendriticum* adults are known to contain embryonated eggs, so the activity detected might be attributable to miracidia. In previous work, cholinesterase activity has also been found present in embryonated eggs (unpublished data). Activity by that

Fig. 1. – Electrophoretic profile of cholinesterase activity in adult crude extract of *Dicrocoelium dendriticum* employing acetylthiocholine iodide (ATCI) as substrate.

BW284C51 (nM)	ChE activity (U/mg) (1)	
	X $\pm$ SD	% inhibition
0	2.11 $\pm$ 0.37	0
400.000	1.71 $\pm$ 0.35	62.96*
2.000	1.28 $\pm$ 0.19	72.22*
200	1.71 $\pm$ 0.40	66.66*
4	0.47 $\pm$ 0.019	90.74*

* $p \leq 0.05$.

Table I. – Cholinesterase activity in crude extract of adult *Dicrocoelium dendriticum* using acetylthiocholine iodide (ATCI) as substrate: inhibition by BW284C51. (1) The data value are the mean of three experiences $\pm$ the standard deviation.

isoOMPA (mM)	ChE activity (U/mg) (1)	
	X $\pm$ SD	% inhibition
0	2.11 $\pm$ 0.37	0
50	0	100*
5	0	100*
0.5	0.85 $\pm$ 0.025	88.57*
0.05	1.6 $\pm$ 0.42	80*

* $p \leq 0.05$.

Table II. – Cholinesterase activity in crude extract of adult *Dicrocoelium dendriticum* on acetylthiocholine iodide (ATCI) as substrate: inhibition by isoOMPA. (1) The data value are the mean of three experiences $\pm$ the standard deviation.

same enzyme was recorded using the technique of Karnovsky *et al.* (1964), though only faint bands that could not be photographed appeared. Quantification according to the method of Ellman *et al.* (1961) revealed that the ChE rate was 0.44 U/mg protein. The GABA-receptor dissociation constant was $K_D = 1.20$ nM and the receptor density $B_{max} = 1.60$ pmol/mg protein for the ^3H -GABA-specific binding site in *D. dendriticum* membranes as shown in Figures 2A,B. Affinity and receptor density were similar to the levels observed in the free-living nematode *Caenorhabditis elegans* ($K_D = 37$ nM; $B_{max} = 2.25$ pmol/mg protein) (Schaeffer & Bergstrom, 1988). However, affinity was lower than that observed in the parasitic nematode *Trichinella spiralis* ($K_D = 1.20$ μ M), though receptor density was very similar (4.78 pmol/mg protein) (Ros-Moreno *et al.*, 1999). In this study we have demonstrated the presence of ChE in the cytosolic fraction in both adults and embryonated eggs of the digenetic parasitic flatworm *D. dendriticum*. The enzymatic activity indirectly demonstrated the presence of acetylcholine. Additionally, this is the first discovery that GABA binding sites have been detected in the membranes of *D. dendriticum* adults. These are new data concerning the physiology of this parasite, whose habitat is the gall-bladder and bile ducts of sheep. As other workers have postulated in other helminths, it may be that GABA is

Fig. 2. – ^3H -GABA specific binding in *Dicrocoelium dendriticum* membranes. A. Incubation of *D. dendriticum* with increasing concentrations of ^3H -GABA (each data point being the mean of three determinations). B. Scatchard plot of the specific binding data shown in A.

a beneficial inhibitory neurotransmitter in the environment low in oxygen and could be the cornerstone of an inhibitory system depressing energy consumption more efficiently at low levels of oxygen (Nilson & Wienberg, 1993; Eriksson *et al.*, 1995). Further research may elucidate the existence of two separate neurotransmission systems depending on oxygen level, and this is our next point of action.

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