Biochemical Pharmacology, Vol. 26, pp. 2163–2167. Pergamon Press, 1977. Printed in Great Britain.

THE INHIBITION OF BRAIN ARYL ACYLAMIDASE BY 5-HYDROXYTRYPTAMINE AND ACETYLCHOLINE

ANNA OOMMEN and A. S. BALASUBRAMANIAN

Neurochemistry Laboratory, Department of Neurological Sciences, Christian Medical College Hospital, Vellore-632004, India

(*Received* 13 *December* 1976; *accepted* 20 *April* 1977)

Abstract—Sheep brain aryl acylamidase (aryl-acylamide amidohydrolase, EC 3.5.1.13) was partially purified. Of a number of amines tested at 1 mM the enzyme was maximally inhibited by 5-hydroxytryptamine (5-HT) and to a lesser extent by tryptamine and practically unaffected by tyramine, histamine, noradrenaline, dopamine and benzylamine and a number of amino acids including tryptophan. Choline derivatives at 1 mM were inhibitory to the enzyme in the order of butyrylcholine > succinylcholine > benzoylcholine > choline > acetylcholine > acetylthiocholine > acetyl-β-methylcholine > propionylcholine. The inhibition by 5-HT and acetylcholine was further studied. Both inhibit the enzyme in a noncompetitive manner. The inhibition could be reversed by removal of the inhibitors from the enzyme by gel filteration. A number of metal ions, EDTA, high concentrations of sodium chloride, the thiol reagents *p*-chloromercuribenzoate, *N*-ethyl-maleimide and iodoacetamide were found to have no effect on the inhibition. Dithiothereitol as well as neuraminidase treatment did not alter the extent of inhibition of the enzyme by 5-HT or acetylcholine. Nitration of the enzyme with tetranitromethane led to approximately a 50 per cent drop in enzyme activity as well as a significant decrease in the extent of inhibition by 5-HT and acetylcholine. This suggested the possibility of the involvement of tyrosine residues both at the catalytic site as well as at the site(s) of inhibition by 5-HT and acetylcholine. Mixed inhibitor studies favoured a common inhibition site for both 5-HT and acetylcholine on the brain enzyme. The sheep liver enzyme was not inhibited by either 5-HT or acetylcholine.

The enzyme aryl acylamidase (aryl-acylamide amidohydrolase, EC 3.5.1.13) is known to be present in plant [1], bacterial [2, 3] and mammalian [4–6] systems. The role of this enzyme in plants in the detoxification of herbicides [1] is well documented. In the mammalian species Fujimoto [6] has suggested that the enzyme may serve to hydrolyze some anti-pyretic and analgesic drugs which are aryl acylamides. Recently Fujimoto [6] demonstrated the 5-hydroxytryptamine (5-HT) sensitivity of this enzyme from rat brain but not from rat liver and suggested that the brain aryl acylamidase could serve as a model for the study of the mechanism of action of 5-HT in the mammalian central nervous system. In the present work it is demonstrated that not only 5-HT but also acetylcholine and several of its analogues inhibit the partially purified aryl acylamidase from sheep brain. The insensitivity of the sheep liver acylamidase to these inhibitors is also noted. Factors which influence the inhibition and attempts at modifications of the brain enzyme to locate the 5-HT and acetylcholine sensitive sites are also presented.

MATERIALS AND METHODS

5-Hydroxytryptamine creatinine sulphate, tryptamine hydrochloride, histamine dihydrochloride, tyramine hydrochloride, dopamine hydrochloride, benzylamine, benzoylcholine chloride, succinylcholine chloride, butyrylcholine chloride, acetylthiocholine iodide, propionylcholine chloride, *p*-chloromercuribenzoate (pCMB), tetranitromethane, Tris (hydroxymethyl) aminomethane, glycine, tryptophan and neuraminidase (Cl. perfringens) and bovine serum albumin were from Sigma Chemical Co. Acetyl-β-methylcholine bromide and acetylcholine iodide were from Cal Biochem.; choline chloride from E. Merck Ag. Darmstadt; L-glutamine, L-histidine, iodoacetamide and gamma amino butyric acid from Nutritional Biochemicals Corporation; L-tyrosine from BDH, England; *N*-ethylmaleimide from Schwarz Bioresearch Inc.; dithiothreitol from Seikagaku Fine Biochemicals; Sephadex from Pharmacia Fine Chemicals and Triton X-100 from Rohm and Hass. All other chemicals used were of the highest purity available.

Sheep brain and liver were obtained fresh immediately after killing the animal from the slaughter house and stored frozen at −18° until required.

Preparation of o-*nitroacetanilide.* *o*-Nitroaniline, 1 gm, was refluxed with 5.0 ml of acetic acid and 5.0 ml of acetic anhydride for 30 min. The resulting solution was then poured while hot and while stirring into approximately 25 ml of ice-cold water. After leaving aside for 1 hr, the product was filtered under suction, washed with a minimum amount of water and recrystallized thrice from hot water. The *o*-nitroacetanilide so prepared had a m.p. of 93–94°.

Partial purification of aryl acylamidase from sheep brain. All operations were performed at 0–4° unless otherwise mentioned. Frozen sheep brain, 40 g, was homogenized in a Potter–Elvehjem homogenizer with 80 ml of 0.05 M potassium phosphate buffer pH 7.0 containing 0.5% (v/v) Triton X-100. The homogenate was kept at 0° for 60 min and then centrifuged at 12,000 *g* for 30 min. The supernatant was brought to 30% ammonium sulphate saturation by the addition of 12 g of ammonium sulphate and centrifuged for 30 min at 12,000 *g*. The precipitate was discarded and the supernatant was raised to 65% ammonium sulphate saturation by the addition of 12.4 g of

ammonium sulphate, centrifuged for 30 min at 12,000 *g* and the precipitate was dissolved in 5.0 ml of 0.05 M potassium phosphate buffer pH 7.0. Aliquots of 2.0 ml were applied to a Sephadex G-200 column (39 × 2.3 cm) equilibrated with 0.05 M potassium phosphate buffer pH 7.0 containing 0.1 M NaCl and eluted with the same buffer. Fractions of 5.0 ml were collected. The active fractions appearing in the void volume were pooled and centrifuged at 12,000 *g* for 30 min. The resultant supernatant was dialyzed against 10 vol. of 0.05 M potassium phosphate buffer pH 7.0, for 14 hr with 2 changes of buffer. The enzyme was concentrated about 5.5-fold by lyophilization followed by dialysis against 50 vol. of 0.05 M potassium phosphate buffer pH 7.0 for 14 hr with 2 changes of buffer. The final dialyzed fraction was centrifuged at 12,000 *g* for 30 min and the supernatant was used as the enzyme source from sheep brain. The enzyme thus prepared could release 0.34 μmoles of *o*-nitroaniline/mg protein/hr under the standard assay conditions. Sheep liver was subjected to similar treatment when used as the source of enzyme. The liver enzyme liberated 0.25 μmoles of *o*-nitroaniline/mg protein/hr under the standard assay conditions.

Neuraminidase treatment. Sheep brain enzyme 0.43 ml (2.9 mg protein) was incubated with 0.2 ml (0.28 units) of Cl. perfringens neuraminidase and 70 μmoles of sodium acetate buffer pH 5.0 in a total vol. of 0.7 ml at 37° for 60 min and chilled in ice for 5 min before use. Controls without neuraminidase were incubated under the same conditions.

Tetranitromethane treatment. Sheep brain enzyme, 0.45 ml (3 mg protein) was mixed with 0.05 ml 0.084 M tetranitromethane (a solution in 95% ethanol). The mixture was shaken for 60 min at room temperature and chilled in ice for 5 min. To separate the excess tetranitromethane from the enzyme, 0.2 ml of the mixture was applied to a Sephadex G-50 (20 × 1 cm) column equilibrated with 0.05 M Tris–HCl buffer pH 8.0 containing 0.1 M NaCl and 1.0 ml fractions were collected. The active fractions were pooled and used as the source of tetranitromethane treated enzyme [7].

Assay of aryl acylamidase. The assay mixture consisted of 50 μmoles potassium phosphate buffer pH 7.0, 4.5 μmoles *o*-nitroacetanilide and 0.05 to 0.1 ml enzyme in a total vol. of 0.5 ml. After incubation for 2 hr at 37° the reaction was stopped by the addition of 2.5 ml of 0.19 N perchloric acid and the liberated *o*-nitroaniline was measured at 430 nm within 10 min of addition of perchloric acid.

The reaction rate was linear with respect to time and enzyme concentrations in all the assays. The velocity was linear for a minimum of 2.5 hr up to a maximum protein concentration of 0.45 mg (0.15 ml enzyme).

Protein estimation. Protein was estimated according to the method of Lowry *et al.* [8] using crystalline bovine serum albumin as standard.

RESULTS

Triton X-100 was essential for maximal extraction of the enzyme which suggested that the aryl acylamidase of the brain was a membrane-bound enzyme. Solubilization with Triton X-100 followed by ammonium sulphate fractionation and gel filtration gave an enzyme from sheep brain that was 6-fold purified with a recovery of 23 per cent.

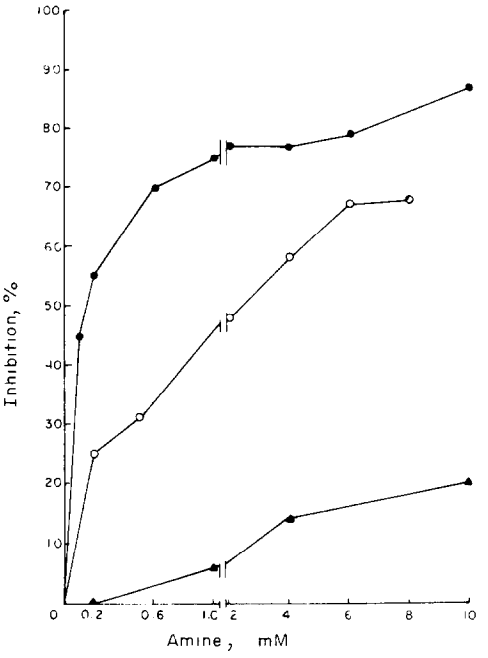

Fig. 1. The inhibition of brain aryl acylamidase by various amines. (●) 5-HT (○) Tryptamine (▲) Dopamine. Tyramine, histamine and benzylamine (not shown in the figure) were non-inhibitory up to concentrations of 10 mM. Results are the average of values from duplicate experiments which did not differ from each other by more than 5 per cent.

Inhibition by amines. The enzyme was inhibited to about 75 per cent by 5-HT and to a lesser degree (45 per cent inhibition) by tryptamine at 1 mM concentrations, but practically unaffected by tyramine, histamine, noradrenaline, dopamine and benzylamine at the same concentrations (Fig. 1). Dopamine at concentrations above 1 mM was slightly inhibitory but none of the other amines was inhibitory up to 10 mM concentrations. The enzyme was also unaffected by a number of amino acids such as tryptophan, histidine, glycine, glutamine and gamma aminobutyric acid at concentrations up to 10 mM.

Inhibition by acetylcholine and other choline derivatives. The brain enzyme was also inhibited by acetylcholine and a number of its analogues and homologues (Fig. 2). Butyrylcholine was the most potent inhibitor while succinylcholine, benzoylcholine and choline, acetylcholine, acetylthiocholine, acetyl-β-methylcholine and propionylcholine inhibited to a lesser extent.

Nature of the inhibition. Figures 3 and 4 indicate the non-competitive nature of the inhibition by 5-HT and acetylcholine respectively as determined by the Lineweaver–Burke plots. K_i values for 5-HT and acetylcholine were 0.55 mM and 2.5 mM respectively as determined by the method of Dixon [9]. 5-HT and acetylcholine inhibition was also found to be reversible because the enzyme preincubated with either of these inhibitors upon gel filtration on Sephadex G-50

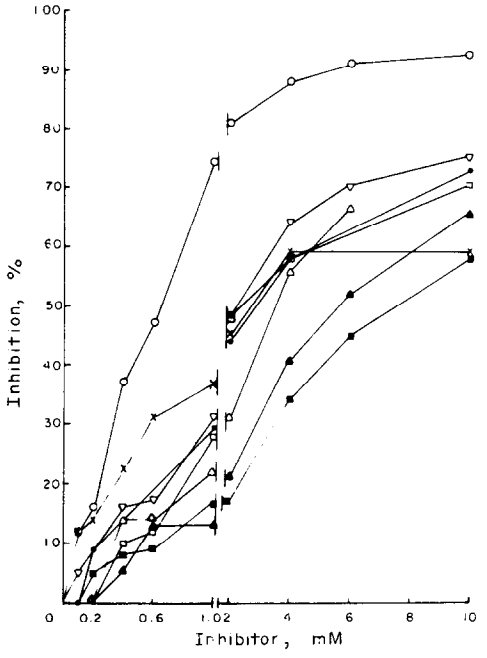

Fig. 2. The effect of various cholines on brain aryl acylamidase. (○) butyrylcholine (▽) benzoylcholine (△) acetylthiocholine (●) choline (□) acetylcholine (▲) propionylcholine (×) succinylcholine, (■) acetyl-β-methylcholine. Results are the average of values from duplicate experiments which did not differ from each other by more than 5 per cent.

to remove the small molecular weight inhibitors regained almost its full original activity.

Other studies on the inhibition of the sheep brain enzyme. Except Hg^{2+} which almost completely inhibited the enzyme, the metal ions Cu^{2+}, Zn^{2+}, Ca^{2+}, Mg^{2+}, Mn^{2+} and Fe^{2+} and EDTA at 2 mM had no significant effect on the enzyme. The presence of EDTA, Ca^{2+} and Fe^{2+} did not influence the inhibition of the enzyme by either 5-HT or acetylcholine. The sheep brain aryl acylamidase exhibited a broad pH optimum ranging from pH 6.5 to pH 8.0. A study of 5-HT and acetylcholine inhibition at various pH indicated that these substances were inhibitory at all the pH tested and that the extent of inhibition increased from the acid to the alkaline range (Fig. 5).

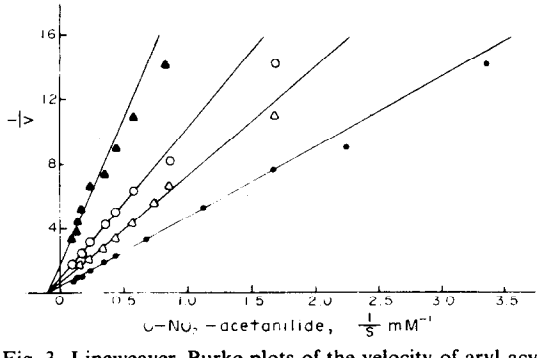

Fig. 3. Lineweaver–Burke plots of the velocity of aryl acylamidase as a function of the concentration of *o*-nitroacetanilide measured in the absence of 5-HT (●), in the presence of 5×10^{-5} M 5-HT (△), 1×10^{-4} M 5-HT (○) and 4×10^{-4} M 5-HT (▲).

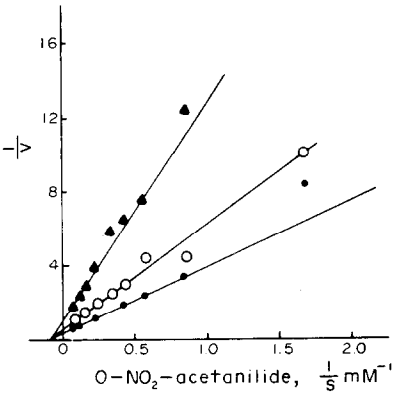

Fig. 4. Lineweaver–Burke plots of the velocity of aryl acylamidase as a function of the concentration of *o*-nitroacetanilide measured in the absence of acetylcholine (●) in the presence of 2×10^{-3} M acetylcholine (○) and 6×10^{-3} M acetylcholine (▲).

NaCl up to concentrations of 0.3 M did not affect aryl acylamidase activity significantly nor its inhibition by either 5-HT or acetylcholine. Inhibition of the enzyme by both 5-HT and acetylcholine was found to decrease by 20 and 30 per cent respectively when the temperature of incubation was increased from 4° to 38°. Preincubation of the enzyme with the thiol reagents pCMB, *N*-ethyl-maleimide and iodoacetamide at 10 mM concentrations for 10 min. at 37° resulted in neither loss of activity nor alteration in the extent of inhibition by 5-HT or acetylcholine. Treatment with 10 mM dithiothreitol for 20 min at 37° although reduced enzyme activity by about 20 per cent was not found to affect either 5-HT or acetylcholine inhibition.

Neuraminidase treatment affected neither enzyme activity nor the inhibition by either 5-HT or acetylcholine. Tetranitromethane treatment of the enzyme resulted in about 50 per cent decrease in the enzyme activity. The extent of inhibition of this nitrated enzyme was tested in the presence of 5-HT at concentrations ranging from 0.2 mM to 0.4 mM and by acetylcholine at 2 mM to 10 mM. The inhibitions were

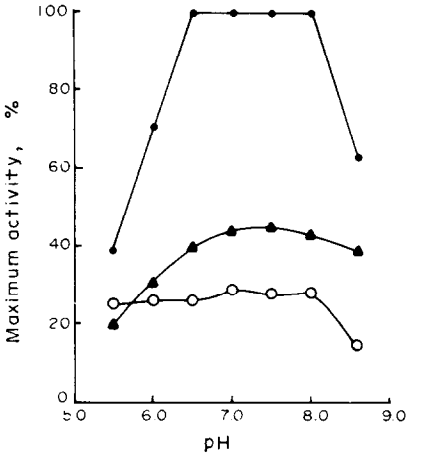

Fig. 5. pH optima profiles of brain aryl acylamidase in the absence of inhibitor (●), in the presence of 0.2 mM 5-HT (○) and in the presence of 4 mM acetylcholine (▲).

Table 1. Mixed inhibitor studies

5-HT mM	Acetylcholine mM	Butyrylcholine mM	% Inhibition of aryl acylamidase Observed	Expected*
0.2	—	—	56	—
—	1.0	—	20	—
0.2	1.0	—	59	76
—	—	0.4	35	—
0.2	—	0.4	63	91
0.125	—	—	43	—
—	7.5	—	69	—
0.125	7.5	—	72	100
—	—	1.0	53	—
0.125	—	1.0	64	96
0.3	—	—	64	—
—	4.0	—	41	—
0.3	4.0	—	72	100

Results are the average of values from duplicate experiments which did not differ from each other by more than 5 per cent.

* Sum of the percentage inhibition caused by each of the inhibitors at the concentrations used. It is the inhibition that would be expected if the inhibitors were to act at independent sites on the enzyme without mutual competition.

found to be reduced by about 50 and 20 per cent respectively by 5-HT and acetylcholine as compared to the untreated enzyme.

Mixed inhibitor studies. Inhibition studies were carried out in the presence of both 5-HT and acetylcholine or 5-HT and butyrylcholine at different concentrations. The observed inhibitions were less than the sum of the individual inhibitions (Table 1).

Sheep liver aryl acylamidase. Studies on the aryl acylamidase prepared from sheep liver showed that the enzyme was not affected by any of the amines or acetylcholine analogues and homologues which were inhibitory to the brain enzyme and which are described under Figs 1 and 2.

DISCUSSION

The observation that aryl acylamidase from sheep brain similar to the enzyme from rat brain [6] was strongly inhibited by 5-HT prompted us to study the effect of other neurotransmitters on the enzyme. Amongst the amines tested only 5-HT and tryptamine showed significant inhibition at low concentrations. A number of other amines and amino acids did not inhibit the enzyme while acetylcholine, butyrylcholine and other choline derivatives were found to be potent inhibitors of this enzyme. The observation that 5-HT and tryptamine are the only two potent inhibitors of the enzyme among the amines tested suggested that the basic 2-(3-indolyl) ethylamine structure was essential for inhibition. Substitution in the side chain of the above structure with a carboxyl group as in tryptophan appeared to make the compound non-inhibitory. The inhibition by acetylcholine, its various analogues, homologues and choline chloride indicated that the choline moiety was required for the observed inhibition. Further studies were then concentrated on the inhibition of the brain enzyme by both 5-HT and acetylcholine. Lineweaver–Burke plots indicated the non-competitive nature of these inhibitions and gel filtration experiments showed that the inhibition was reversible. The increase in inhibition found with a pH change from pH 5.5 to pH 8.0 may be suggestive of basic groups involved at the site of inhibition. The lack of significant alterations in the level of inhibition in the presence of NaCl up to concentrations of 0.3 M rules out the possibility of an ionic interaction between either 5-HT or acetylcholine and the enzyme. Again the lack of any effect on either enzymatic activity or on the inhibition by a number of metal ions apart from Hg^{2+} as well as EDTA seems to suggest that these metal ions are not involved in either the enzyme activity or the inhibition. The strong inhibition of enzymatic activity by Hg^{2+} could be suggestive of -SH group(s) at the active site, though the total lack of any effect of the three thiol reagents, pCMB, *N*-ethyl-maleimide and iodoacetamide on enzyme activity rules out this possibility. In this aspect, the sheep brain enzyme differs from the enzyme from bacterial [10] and plant [11] sources, both of which are reported to be sensitive to thiol reagents. The absence of active -SH groups at the site of inhibition by either 5-HT or acetylcholine is indicated by the insensitivity of these inhibitions to the above mentioned thiol reagents. Although treatment with dithiothereitol slightly reduced the enzyme activity it did not affect either 5-HT or acetylcholine inhibition of the enzyme, presumably because no disulfide bonds are operative at the inhibition site(s). The failure of neuraminidase to alter either enzyme activity or the extent of inhibition by either 5-HT or acetylcholine points to the non-involvement of sialic acid residues in either process. Inhibition of enzymatic activity by tetranitromethane could suggest involvement of tyrosine residues for maximal activity. The definite drop in inhibition by both 5-HT and acetylcholine of the tetranitromethane treated sheep brain enzyme could also be attributed to a requirement of tyrosine residues at the inhibition site(s).

The numerous similarities in the inhibition of the aryl acylamidase from sheep brain by 5-HT and acetylcholine suggest that both these inhibitors act on either the same or similar sites on the enzyme. The probability that both inhibitors act at the same site

was strengthened after mixed inhibitor studies. The observed inhibition in the presence of both 5-HT and acetylcholine should be the sum of the individual inhibitions if independent inhibition sites are envisaged, while if only one inhibition site is operative the observed inhibition will be less than the sum of the individual inhibitions due to competition between the inhibitors. The data in Table 1 support the latter possibility.

In accordance with the observation of Fujimoto [6] that the rat liver enzyme unlike the rat brain enzyme was totally unaffected by 5-HT or tryptamine, the present studies on the sheep liver enzyme also indicated that it was unaffected by not only 5-HT and tryptamine but also by acetylcholine and its analogues and homologues.

The marked inhibition of aryl acylamidase by 5-HT and acetylcholine may prove useful in determining this enzyme activity as an indirect index of 5-HT and acetylcholine concentrations in the brain tissue.

Acknowledgement—We thank Prof. James Verghese for the generous supply of *o*-nitroaniline and Prof. A. N. Radhakrishnan for dopamine hydrochloride. The work was supported by a grant from the Council of Scientific and Industrial Research, India. A. O. is a junior research fellow of the Council of Scientific and Industrial Research.

REFERENCES

1. C. C. Still and O. Kuzirian, *Nature, Lond.* **216,** 799 (1967).
2. G. Engelhardt, P. R. Wallnoefer and R. Plapp, *Appl. Microbiol.* **22,** 284 (1971).
3. K. P. Hsiung, S. S. Kuan and G. G. Guilbault, *Biochem. biophys. Res. Commun.* **66,** 1225 (1975).
4. H. A. Krebs, W. O. Sykes and W. C. Bartley, *Biochem. J.* **41,** 622 (1947).
5. C. H. Williams and K. H. Jacobson, *Toxic. appl. Pharmac.* **9,** 495 (1966).
6. D. Fujimoto, *Biochem. biophys. Res. Commun.* **61,** 72 (1974).
7. F. Riordan and B. L. Vallee in *Methods in Enzymology* (Eds C. H. W. Hirs and S. N. Timasheff) p. 515. Academic Press, New York (1972).
8. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193,** 265 (1951).
9. M. Dixon and E. C. Webb in *Enzymes* (Eds M. Dixon and E. C. Webb) 2nd ed. p. 328. Longman Green, London (1964).
10. G. Engelhardt, P. R. Wallnoefer and R. Plapp, *Appl. Microbiol.* **26,** 709 (1973).
11. D. S. Frear and G. G. Still, *Phytochemistry* **7,** 913 (1968).