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A FLOW MICROCALORIMETRIC STUDY OF THE INHIBITION OF ACETYLCHOLINESTERASE BY CATECHOLAMINE DERIVATIVES

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ABSTRACT

A flow microcalorimetric method has been applied to the determination of acetylcholinesterase activities in crude tissue homogenates. This simple and sensitive method, which measures the heat generated by a reaction, is also suitable for the study of the inhibition of the enzyme by specific compounds. In the present work this method was used to determine the effect of catecholamines and their derivatives on the acetylcholinesterase activity. The results indicate that epinephrine, norepinephrine and dopamine, in concentrations up to 2.5 mM, do not inhibit acetylcholinesterase, whereas metanephrine (0.8 mM), normetanephrine (0.8 mM), 3-methoxydopamine (0.6 mM) and L-DOPA (0.7 mM) decrease the enzyme activity by 37, 75, 50 and 20%, respectively. The apparent inhibition rate constants determined for these compounds are 0.09, 0.34, 0.18 and 0.19 mM^{-1} min^{-1}. A comparison of the chemical structure and inhibitory potency of the catecholamine derivatives suggests that a 3-methoxy group is responsible for the inhibition of the enzyme and that a α-carboxyl group also reduces the acetylcholinesterase activity. The comparative assays for the determination of acetylcholinesterase activities, using a flow microcalorimeter and a pH-meter, indicate that flow microcalorimetry is a useful method for enzyme kinetic studies because it has a high sensitivity and can be applied whenever heat exchange is involved in an enzymatic reaction.

INTRODUCTION

Flow microcalorimetry is becoming an important analytical technique in the field of biochemistry, allowing the study of many biological systems with different levels of complexity. This simple and sensitive method, which relies only on the heat generated by a reaction, provides both thermodynamic and kinetic information for a wide variety of systems [1–4]. The calorimetric technique does not require time-consuming separation procedures which are often needed for current analytical methods in biochemistry; it is not

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restricted to the use of chromophoric or isotopically labelled substrates and it is independent of the optical properties and the buffer effect of the system under study [1].

An area of special interest for the application of flow microcalorimetry is the study of enzyme-catalysed reactions. Apart from its precision and sensitivity, flow microcalorimetry is also a non-destructive technique, thus allowing complete recovery of the enzymes after the reactions. The fact that flow microcalorimetry can be directly applied to tissue homogenates and that it can be adapted for automatic data acquisition are also favourable characteristics of this method.

With flow microcalorimetry a quantitative assay for an enzymatic activity can be obtained by measuring the heat effect generated by the substrate-saturated enzymatic system [5,6]. The inhibition of enzymes by specific compounds can also be investigated as a decrease in the rate of heat output upon addition of an inhibitor will be directly proportional to the amount of inhibition occurring [7].

In the present study, using acetylcholinesterase (AchE) as a model enzyme, we report the application of flow microcalorimetry to the determination of AchE activities in sheep brain tissue homogenates and to the study of the influence of several catecholamine derivatives on enzyme activity. These compounds were chosen because they may have a modulatory effect upon the enzymes associated with cholinergic activities [8–11]. Moreover, it is known that O-methylation is an important process for the biological inactivation of catecholamine neurotransmitters [12]. Therefore, the influence of some catecholamines and O-methyl derivatives on AchE activities may be of interest in the regulation of neuronal function.

The calorimetric results obtained for the AchE activity were compared with the values of the enzyme activity determined by the more conventional potentiometric-pH method.

It is the purpose of this report to demonstrate that flow microcalorimetry is a general analytical procedure because it can be applied directly to crude tissue homogenates to estimate the kinetic parameters of all enzymatic reactions whose enthalpy variations are sufficiently high.

EXPERIMENTAL

Materials and equipment

Acetylcholine chloride, dopamine, L-epinephrine, L-norepinephrine, 3-methoxydopamine, 3,4-dihydroxyphenylalanine (L-DOPA), DL-metanephrine and DL-normetanephrine were purchased from Sigma Chemical Co. (St. Louis) and were used without further purification. The AchE used for calibration (type IX, from bovine erythrocytes) and the AchE-specific in-

hibitor BW284C51 (1,5-bis(4-allyldimethylammoniumphenyl) pentan-3-one dibromide) were also Sigma products.

Stock solutions of AchE (30 units/ml) and of aqueous substrate (acetylcholine chloride 0.1 M) were stored at 4° C and used within two days after preparation. Catecholamine stock solutions (10 mM or 100 mM) were prepared immediately before use in a 5 mM Tris–HCl buffer and 0.1% ascorbic acid, if necessary.

Acetylcholinesterase activities were measured in the microsomal fraction of bovine caudate nucleus membranes which was isolated in 15 mM Tris–HCl buffer (pH 7.4), following the procedure of Hajós [13]. Protein was determined by the Biuret method using bovine serum albumin as a standard [14].

Enzymatic assays were performed at 37° C in 5 mM Tris–HCl buffer (pH 7.4) containing 100 mM NaCl and 10 mM $MgCl_2$. The total volume in the reaction cell was 10 ml in the calorimetric method and 2.5 ml in the potentiometric–pH method. The final concentration of acetylcholine (Ach) in the medium was 8 mM in both procedures.

The calorimetric experiments were performed in an LKB 2277 model microcalorimeter ("Bioactivity Monitor") operating in the flow-through mode. The reaction mixtures were pumped into the measuring cell with an LKB 2232 microperspex-S peristaltic pump at a flow rate of 20 ml h^{-1}.

The potentiometric–pH measurements were made with a Crison-Digilab 517 apparatus equipped with an Ingold 9811 glass electrode.

Experimental procedure

Microcalorimetric method

Each series of experiments was started by pumping buffer and substrate through the calorimeter in order to establish the instrument baseline.

For enzymatic activity measurements, an aliquot of the enzyme solution or suspension was mixed with buffer and excess substrate, and the mixture was immediately pumped through the calorimeter until a steady state heat effect signal was reached. At this stage the experimental voltage curve was parallel to the instrument baseline. The baseline displacement, which is proportional to the heat effect, constitutes a direct measure of the enzymatic activity under the conditions of the experiment. One unit of AchE activity (u) is defined as the amount of enzyme that hydrolysis 1 μmole of Ach per minute at 37° C and pH 7.4.

To study the effect of catecholamine derivatives upon AchE activity, an aliquot of a catecholamine stock solution was added to the reaction mixture containing buffer, substrate and enzyme, after a steady state had been reached. Upon addition of an enzyme inhibitor the heat effect was reduced and another steady state level was obtained. The procedure was repeated with different aliquots of the catecholamine stock solution. The final con-

centrations of these compounds in the reaction vessel varied between 0.15 mM and 2.5 mM.

Potentiometric–pH method

For AchE activity measurements, buffer and substrate were placed in a thermostatted vessel and the reaction was initiated by the addition of enzyme. The decrease in pH due to the hydrolysis of Ach was then monitored. Experiments without enzyme were also made to eliminate the effect of non-enzymatic hydrolysis of Ach. During the enzymatic reaction the pH of the medium was always within the pH range where the enzyme is known to be at its maximal activity [15].

In order to determine the amount of H^+ produced per minute (which is equal to the amount of Ach hydrolysed per minute), calibration experiments were made by adding aliquots of a standard HCl solution (1 M) to the reaction medium. With this method, AchE activities were expressed as the number of μmoles of substrate hydrolysed per minute at 37 °C and pH 7.4.

Determination of inhibition rate constants

Based on the calorimetric experiments, and assuming a pseudo-first-order kinetics for the inhibition reaction, the inhibition rate constants were calculated as follows. For each concentration of inhibitor tested, plots of $\ln(P_t - P_\infty)$ vs. time were made, where P_t is the value of the heat effect signal at time t after addition of the inhibitor and P_∞ is the value of the steady state heat-effect signal when complete inhibition was reached. These plots were linear and their slopes represent values of pseudo-first-order rate constants (k_i'). For each compound, values of k_i' were plotted against concentration of inhibitor. The slopes of the lines obtained gave the values of the second-order inhibition constants for the reactions (k_i).

RESULTS AND DISCUSSION

In order to measure acetylcholinesterase activities in the tissue homogenates with the flow microcalorimetric technique, calibration experiments were first performed with a stock solution of a solubilised enzyme from bovine erythrocytes with a known activity. The results show a linear relationship between the heat effect signal and the total enzymatic activity in the medium (Fig. 1).

For the assay of AchE in the tissue extracts, calorimetric curves of the type shown in Fig. 2 were obtained. In order to test whether the calorimetric signal was attributable only to the AchE activity, a specific inhibitor of the enzyme (BW284C51) at a concentration of 10 μM was added to the reaction medium. This compound reduced the heat signal to the baseline level,

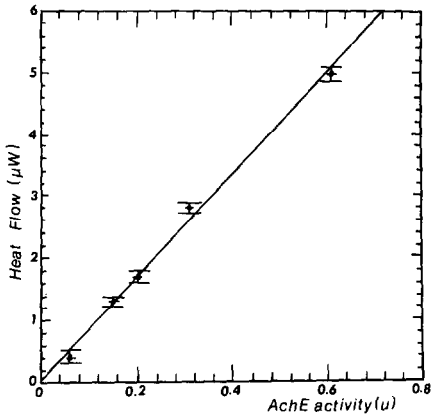


Fig. 1. Calibration curve for the determination of AchE activities in tissue homogenates. Experimental medium was 50 mM Tris–HCl buffer (pH 7.4) with 100 mM NaCl, 10 mM $MgCl_2$ and 8 mM Ach. Activity of the stock solution of bovine erythrocytes AchE was 30 u/ml. The results are means of triplicates ± SD ($r = 0.997$).

providing evidence that, under the experimental conditions used, the AchE-catalysed reaction is the only heat source contributing significantly to the heat measured. The results depicted in Fig. 3 show that there is a rectilinear relationship between heat flow and protein added. The values plotted represent the mean of three different measurements of the same enzyme sample.

Because substrate inhibition is a well known characteristic of AchE [16], the effect of the concentration of acetylcholine on the enzyme activity was analysed. As shown in Fig. 4, in the range of concentrations investigated, the AchE activity was greater for concentrations of Ach between 2 and 8 mM, and was significantly reduced for higher concentrations of substrate. Based on these results and on the report that the optimum substrate concentrations for the enzyme are about 4–7 mM [16], all subsequent experiments were performed using an Ach concentration of 8 mM in order to establish a

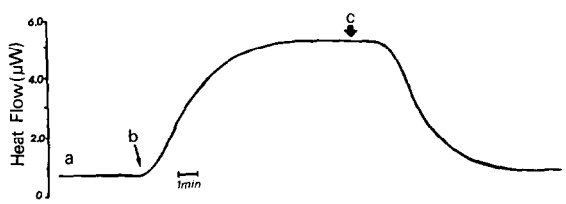


Fig. 2. Calorimetric curve of the inhibition of AchE activity by the compound BW284C51: sensitivity, 10 μW full scale; flow rate, 20 ml h^{-1}. The baseline (a) was established by pumping the experimental medium (5 mM Tris–HCl buffer, pH 7.4, with 100 mM NaCl and 10 mM $MgCl_2$ + 8 mM Ach) into the calorimeter. Caudate nucleus microsomal fraction (20 μg/ml of protein) was added at the time indicated by the arrow at (b). Inhibitor (10 μM) was added at the time indicated by the arrow at (c).

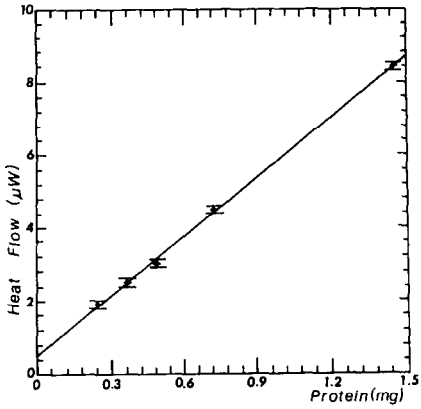


Fig. 3. Linearity of the microcalorimetric method for the assay of AchE activities. Experimental conditions are the same as in Fig. 2. The values shown are the means ± SD of three independent determinations ($r = 0.996$).

compromise between substrate saturation of the enzyme and non-inhibition of AchE by excess Ach.

The microcalorimetric results of AchE activity were compared with those obtained with a potentiometric–pH method. Under similar experimental conditions, the values of enzymatic activity determined by microcalorimetry were identical to those obtained with the pH measurement technique (Fig. 4).

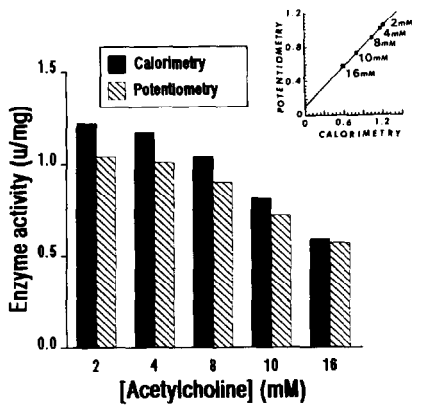


Fig. 4. Comparison of the AchE activities obtained with the microcalorimetric and potentiometric–pH methods. The incubation medium for enzyme activity measurements is the same as that reported in Fig. 2. Enzyme activity, in the presence of increasing concentrations of Ach, was measured by a calorimetric method and by a potentiometric–pH method as described in the text. The insert shows the correlation between the microcalorimetric and potentiometric–pH values of AchE activity (u/mg) obtained at various concentrations of Ach ($r = 0.999$).

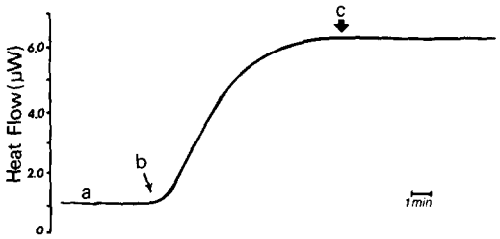


Fig. 5. Calorimetric curve of the effect of epinephrine on AchE activity: sensitivity, 10 μW full scale; flow rate, 20 ml h^{-1}. The baseline (a) was established by pumping the experimental medium (5 mM Tris–HCl buffer, pH 7.4, with 100 mM NaCl and 10 mM $MgCl_2$ + 8 mM Ach) into the calorimeter. Caudate nucleus microsomal fraction (20 μg/ml of protein) was added at the time indicated by the arrow at (b). Epinephrine (2.5 mM) was added at the time indicated by the arrow at (c).

Flow microcalorimetry was also used to study the influence of some catecholamines and related compounds on the AchE activity. From the calorimetric curve represented in Fig. 5 one can see that epinephrine (2.5 mM) has no effect on the AchE activity of the microsomal fraction of bovine caudate nucleus membranes. Similar results were obtained with norepinephrine and dopamine (2.5 mM).

In contrast, the O-methyl catecholamines (metanephrine, normetanephrine and 3-methoxydopamine) inhibit AchE (Fig. 6). In the presence of 1.5 mM metanephrine, the heat effect signal is reduced by 2.2 μW, corresponding to a decrease of 0.26 u in the enzyme activity. When 0.15 mM normetanephrine is added to the reaction medium, the heat-output rate is lowered by 2.0 μW, which is equivalent to an inhibition of 0.24 u in the AchE activity. Similarly, with 0.32 mM 3-methoxydopamine a 1.7 μW decrease in the heat effect signal is observed, which corresponds to an AchE inhibition of 0.2 u. L-DOPA (0.46 mM) also decreases the heat effect from 5.0 to 4.4 μW, which is equivalent to a reduction of 0.07 u in the enzyme activity. The experimental curves illustrated in Fig. 6 show the effect of these compounds on the AchE activity.

Increasing the concentration of these inhibitors resulted in a progressive increase in the inhibition of AchE. The experimental results which are depicted in Fig. 7 indicate that normetanephrine has the greatest inhibitory potency ($IC_{50} = 0.3$ mM), followed by 3-methoxydopamine ($IC_{50} = 0.58$ mM) and metanephrine ($IC_{50} = 1.5$ mM). L-DOPA is the least potent of the inhibitors tested, and AchE inhibition by this compound does not reach 50% in the range of concentrations analysed. The IC_{50} values reported here are apparent values because they depend on the concentration of substrate. These values were calculated for an acetylcholine concentration of 8 mM.

The effect of pre-incubation of the enzyme for one hour with each of the catecholamines was also analysed. It was found that this procedure did not enhance the inhibition of the compounds studied (data not shown).

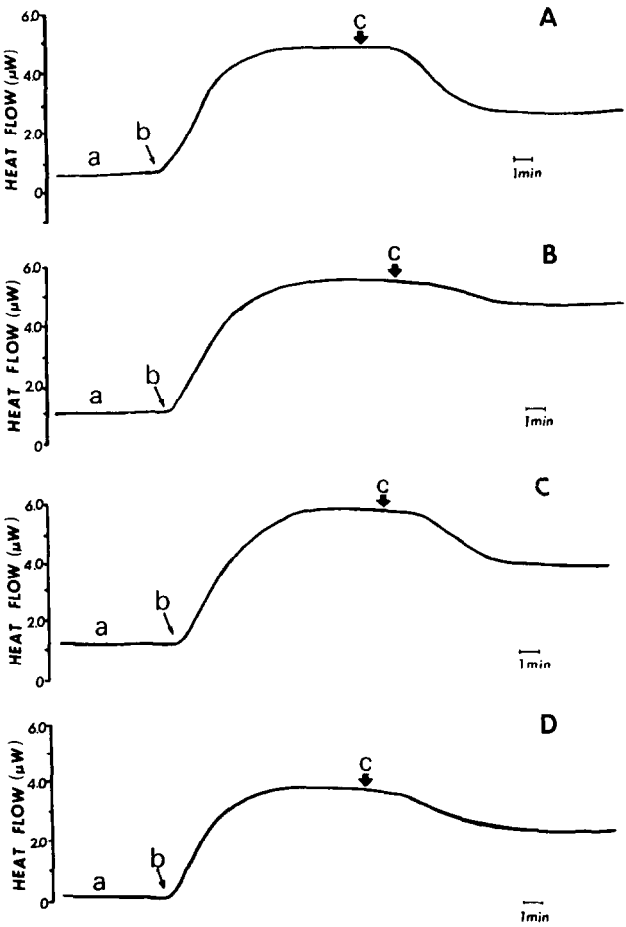


Fig. 6. Thermograms of AchE inhibition by catecholamine derivatives: sensitivity, 10 μW full scale; flow rate, 20 ml h^{-1}. The baseline (a) was established by pumping the experimental medium (5 mM Tris–HCl buffer, pH 7.4, with 100 mM NaCl and 10 mM $MgCl_2$ + 8 mM Ach) into the calorimeter. Caudate nucleus microsomal fraction (20 μg/ml of protein or 16 μg/ml of protein, in the case of 3-methoxydopamine) was added at the time indicated by the arrow at (b). Inhibitor was added at the time indicated by the arrow at (c): (A), metanephrine (1.5 mM); (B), L-DOPA (0.46 mM); (C), normetanephrine (0.15 mM); and (D), 3-methoxydopamine (0.32 mM).

From the calorimetric data, apparent inhibition rate constants for the catecholamine derivatives were evaluated. Our results indicate that normetanephrine reduces the AchE activity more rapidly ($k_i = 0.34$ mM^{-1} min^{-1}) than 3-methoxydopamine ($k_i = 0.18$ mM^{-1} min^{-1}) or L-DOPA ($k_i = 0.19$ mM^{-1} min^{-1}), these two compounds having similar rates of inhibition of the enzyme. On the other hand, the decrease in AchE activity induced by metanephrine occurs at a much slower rate ($k_i = 0.09$ mM^{-1} min^{-1}).

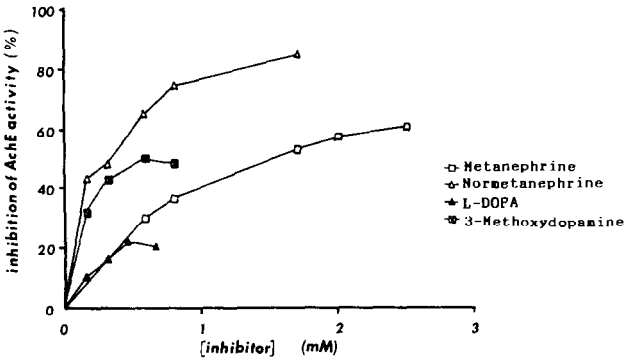


Fig. 7. Effect of inhibitor concentration on AchE activity. Experimental conditions: 5 mM Tris–HCl buffer (pH 7.4) with 100 mM NaCl and 10 mM $MgCl_2$; [Ach] = 8 mM; [inhibitor stock solution] = 100 mM.

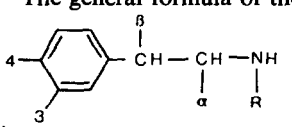
A summary of the experimental data obtained with the catecholamines and related compounds and their effect on the AchE activity is given in Table 1. We conclude from these results that the catecholamine derivatives with a 3-methoxy group inhibit the AchE from the microsomal fraction of bovine caudate nucleus membranes and that an α-carboxyl group also reduces the enzyme activity to some extent.

TABLE 1

Inhibitory potency (IC_{50}) and kinetic data (k_i) of catecholamines and related compounds [a] on the AchE activity

Compound	4	3	β	α	R	IC_{50} (mM)	k_i (mM^{-1} min^{-1})
Epinephrine	OH	OH	OH	H	CH_3	– [b]	– [b]
Norepinephrine	OH	OH	OH	H	H	– [b]	– [b]
Dopamine	OH	OH	H	H	H	– [b]	– [b]
Metanephrine	OH	OCH_3	OH	H	CH_3	1.5	0.09
Normetanephrine	OH	OCH_3	OH	H	H	0.3	0.34
3-Methoxydopamine	OH	OCH_3	H	H	H	0.58	0.18
L-DOPA	OH	OH	H	COOH	H	– [c]	0.19

[a] The general formula of the compounds studied is



[b] Not determined because no inhibition has been observed.

[c] Not determined because 50% inhibition was not reached.

CONCLUSIONS

Flow microcalorimetry constitutes a very simple and sensitive method for the direct assay of acetylcholinesterase activity in tissue homogenates, and one can easily follow the inhibitory action of 3-methoxy catecholamines on the enzyme activity with this technique.

The inhibitory effect of the catecholamine derivatives is of interest because O-methylcatecholamines are the main products of catecholamine metabolism and, thus, these compounds may regulate the catecholaminergic/cholinergic balance and, in this way, influence neuronal function.

The high sensitivity and the simplicity of this analytical procedure make possible the widespread application of flow microcalorimetry in the pharmacological area.

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