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THE INTERACTION OF 4-ALKYL DERIVATIVES OF 2,6,7-TRIOXA-1-PHOSPHABICYCLO [2,2,2] OCTANES WITH CYCLIC ADENOSINE 3′,5′-MONOPHOSPHATE PHOSPHODIESTERASE, AND WITH CYCLIC ADENOSINE 3′,5′-MONOPHOSPHATE BINDING PROTEINS

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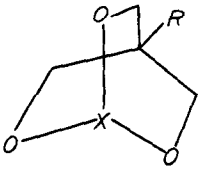
Abstract—4-Alkyl-2,6,7-trioxa-1-phosphabicyclo [2,2,2] octanes are weak competitive inhibitors of the high-K_m form of cyclic adenosine 3′,5′-monophosphate phosphodiesterase (PDE) and non-competitive inhibitors of the low-K_m form enzyme. The possibility that inhibition of the low-K_m form enzyme by the bicyclic organo-phosphates may contribute to their toxic action is discussed. The compounds do not affect the binding of adenosine 3′,5′-monophosphate (cyclic AMP) with specific binding proteins, and are unlikely to have any effect on cyclic AMP receptors.

The 4-alkyl-2,6,7-trioxa-1-phosphabicyclo [2,2,2] octanes (I) with X as P, $P(O)$ or $P(S)$ are a class of highly toxic organophosphorus esters[1] whose toxicity is not mediated through cholinesterase inhibition [2]. Animals poisoned by these compounds die with convulsive seizures indicative of action on the CNS [3], but do not show the characteristic symptoms of the excessive parasympathetic stimulation typical of poisoning with anticholinesterases. The similarities between the symptoms of poisoning by the inhibitors of cyclic adenosine 3′,5′-monophosphate diesterase (E.C. 3.1.4.17) (PDE), caffeine and theophylline [4], together with the structural resemblance of the bicyclic phosphates and adenosine 3′,5′-monophosphate (cyclic AMP) suggested that interference with the control of the physiological levels of cyclic AMP might account for the toxicity of the organophosphates.



(I)

Thus, the interaction of some bicyclic compounds (I with $X = P(O)$, R = methyl (Ia), ethyl (Ib) and isopropyl (Ic) and I with $X = P$, R = isopropyl (Id)) with PDE whose activity will influence the levels of cyclic AMP, was investigated. The effects of the compounds on the binding of cyclic AMP to proteins with a specific binding capacity for this nucleotide [5, 6] were also investigated to determine whether the bicyclic compounds might act like cyclic AMP at a cyclic AMP receptor site which would be an alternative basis for the mechanism of action.

MATERIALS AND METHODS

Adenosine 3′,5′-monophosphoric acid was supplied by Sigma Chemical Co., and [8-^{3}H] adenosine 3′,5′-monophosphate (ammonium salt, 27 Ci/m-mole) was supplied by the Radiochemical Centre, Amersham. Cyclic AMP binding protein from adrenal cortex and cyclic AMP binding protein from bovine skeletal muscle were supplied by British Drug Houses and Calbiochem, respectively. The bicyclic organophosphates were synthesised at the Chemical Defence Establishment. All other chemicals, which were either AR or scintillation grade, were obtained from British Drug Houses.

Enzyme preparation

A preparation of PDE from sheep cerebral cortex was used. The brains were removed as soon as possible after death, chilled and cleaned of extraneous blood vessels and meninges. The cortical tissue was homogenised in distilled water (1 part of tissue to 3 of water). The homogenate was filtered through cheese cloth and, after adjustment of its pH to 5.9 with 6 M acetic acid, the filtrate was centrifuged at 13 000 *g* for 30 min. The resulting supernatant was filtered through glass wool to remove lipid material and the pH of this filtrate was adjusted to 7.0 with 1 M ammonium hydroxide. Stepwise ammonium sulphate fractionation of the filtrate with solid ammonium sulphate gave a protein precipitate which was rich in PDE at 40–50% saturation with ammonium sulphate. This precipitate was dissolved in a minimum vol. of Tris-HCl buffer (20 mM) and the solution dialysed against the same buffer. The dialysate was centrifuged at 18 000 *g* for 30 min and the supernatant freeze dried. All the above operations were carried out at 4°. The purification is a modified version of that described by Cheung [7].

Enzyme assays

(i) *Tritrimetric method for PDE activity.* This method is based on the continuous titration of the acid produced during the hydrolysis of cyclic AMP to adenosine 5′-monophosphate (AMP) by the enzyme PDE [8], and is applicable for the high-K_m form of the enzyme. The freeze dried enzyme preparation was dissolved in phosphate buffer (5 mM; pH 7.4) containing sodium chloride (100 mM) and magnesium chloride (1 mM). Enzyme solution (1 ml ≡ 0.5 unit or 1 mg of protein) (where 1 unit of PDE will convert 1 μmole of cyclic AMP to 5 AMP per min at pH 7.5 at 30°) was mixed with sodium chloride (9 ml; 100 mM) containing magnesium chloride (1 mM) in the titration vessel of a Radiometer automatic titrator, and the mixture was maintained at 37° under an atmosphere of nitrogen. The pH of the mixture was adjusted to 7.4 with sodium hydroxide solution (10 mM) and cyclic AMP added. The acid produced was titrated with 10 mM sodium hydroxide using a twin syringe technique which allowed not only the pH to be maintained constant, but also the substrate concentration by the addition of 10 mM substrate with the second syringe.

Assays with a fixed substrate concentration 0.5 mM showed a linear relationship between the rate of acid production and the concentration of PDE. Substrate concentrations between 10^{-5} and 10^{-3} M were used for the determination of K_m values, which were calculated graphically using the Hofstee plot (values obtained were reproducible to ±10 per cent).

The inhibition of the enzyme was examined by monitoring the rate of acid production before and after the addition of a solution of the compound being tested to the assay system. This was done under two regimes: one in which the substrate concentration was constant and the concentration of the compound being tested was varied, and the other in which the concentration of cyclic AMP was varied and the concentration of the compound was constant.

(ii) *Radiometric method for PDE activity.* This method is based on the measurement of the conversion of radio-labelled cyclic AMP to radio-labelled AMP [9], and allows the enzyme activity to be assayed at low substrate concentrations viz. 2.5×10^{-6} M to 10^{-7} M.

Tris buffer (0.9 ml; 50 mM; pH 7.4) containing magnesium chloride (1 mM) and cyclic AMP (10^{-3} M to 10^{-7} M) were mixed with a solution of radio-labelled cyclic AMP (50 μl; 0.25 μCi) in small test tubes (10 mm × 75 mm). A solution of enzyme (100 μl; 0.1 mg protein in 1 ml 50 mM Tris buffer; pH 7.4) was added to start the reaction. The tubes were incubated at 37° for 10 min, then placed in a boiling water bath for 3 min to stop the reaction. After cooling, each reaction mixture was thoroughly mixed with 50 μl of a solution containing cyclic AMP (100 mM) and AMP (100 mM). A sample (50 μl) from each tube was streaked onto a glass plate (10 cm × 20 cm) coated with silica gel GF_{254} (0.5 mm thick) for thin layer chromatography. The chromatograms were developed in a solvent system of ethylacetate:ethyl alcohol: pH 7.4 Tris buffer (50:35:15) for 2.5 h, dried and examined under UV light. The non-fluorescing areas of silica gel containing AMP ($R_f = 0$) and cyclic AMP ($R_f = 0.3$) were located and transferred to glass vials. Dioxane-based scintillation mixture (15 ml) was added to each vial and the radioactivity was determined in a Packard Tris Carb Scintillation Counter. The number of moles of cyclic AMP hydrolysed was calculated from the fraction of radioactivity present as tritiated AMP and the concentration of cyclic AMP utilised. Propan-2-ol solutions of the compounds tested as inhibitors and aqueous solutions of caffeine and theophylline were added to the reaction mixture before the addition of substrate.

(iii) *Assay of other enzyme activities.* The activities of other enzymes present in the PDE preparation were assayed using a titrimetric method similar to that described in (i).

Acetyl β-methyl choline was used as substrate for acetylcholinesterase, benzoyl choline for cholinesterase, 5′ AMP was used for 5′ nucleotidase and bisnitrophenyl phosphoric acid for non-specific phosphodiesterases.

Binding studies

Binding studies using cyclic AMP and the bicyclo organophosphate (Ic) to displace cyclic [8-^{3}H] AMP were carried out with the binding protein from skeletal muscle according to the method of Gilman [5]. Studies using the preparation from adrenal cortex were according to the method of Brown [6], except that the mixtures were filtered through GSWP Millipore filters, not adsorbed on charcoal.

Protein assay

Protein was assayed spectrophotometrically using bovine serum albumin as standard [10].

Protein activator

The protein activator was obtained from sheep brain using the method described by Cheung [9], but was not purified beyond the ammonium sulphate fractionation stage.

RESULTS

The supernatant from the homogenate before purification contained the following enzyme activities per 10 mg protein: PDE 1.2 units (using cyclic AMP as substrate), acetylcholinesterase 0.6 units (using acetyl choline as substrate). Non-specific phosphodiesterase and 5′ nucleotidase were not detected. After partial purification the only enzyme activity found was for cyclic AMP.

The PDE activity of the supernatant used for the purification of the enzyme was 1100 units and ammonium sulphate fractionation of the supernatant gave 0–30%, 30–40%, 40–50%, 50–60% ammonium sulphate stauration fractions with 100, 160, 420 and 120 units of PDE, respectively. The 40–50% saturation fraction gave 850 mg of protein with a sp. act. of 0.46 units/mg after freeze drying. The activities of the various protein fractions of the purification procedure were not enhanced by the addition of the protein activator. A typical Hofstee plot for the activity of the sheep brain preparation with cyclic AMP as substrate is shown in Fig. 1. The titrimetric method gave $K_m = 1.7 \times 10^{-4}$ M, $V_{max} = 0.46$ μmoles/mg protein/min whilst the values from the radiometric method

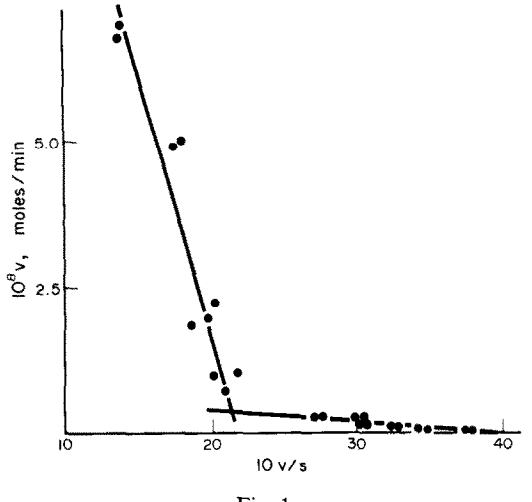


Fig. 1.

were K_m 1.0×10^{-4} M, V_{max} 0.22 μmoles/mg protein/min and K_m 2.2×10^{-6} M, V_{max} 0.008 μmoles/mg protein/min, indicating the presence of two forms of the enzyme. The proportion of the low-K_m form enzyme was only 4 per cent in freshly prepared solutions of the enzyme, but this proportion was found to be as high as 15 per cent after the solution had been stored at −20 to −25°.

The results of the inhibition studies wtih caffeine, theophylline and the bicyclic organophosphates are summarised in Table 1. The inhibition for the high-K_m form was competitive; however it was not possible to determine K_i values for the bicyclic organophosphates because of their low solubility in water. The inhibition was non-competitive for the low-K_m form enzyme as indicated by a plot of $\log_{10} V/V_m - V$ against $\log_{10}$ [cyclic AMP].

The binding studies showed that the bicyclic organophosphates bound at least 10^6 times less strongly than cyclic AMP to both preparations.

DISCUSSION

The bicyclic organophosphates (I) are weak competitive inhibitors of the high-K_m form of PDE, and fifty-fold less effective than theophylline. The toxicity data [1, 3, 4] indicate that bicyclic organophosphate (Ic) is several hundred times more toxic than theophylline or caffeine. It is therefore unlikely that the toxicity of the bicyclic organophosphates can be explained on the basis of their inhibition of the high-K_m form of PDE.

Table 1. Inhibition of PDE by caffeine, theophylline and bicyclic organophosphates

Compound	$10^4 \cdot K_i$ (M) High-K_m PDE	Low-K_m PDE
Caffeine	30	
Theophylline	4.8	3.3
Ia	< 10	
Ib	< 10	
Ic	< 10	1.5
Id*	< 10	

* This compound is hydrolysed rapidly and undergoes ring opening in aqueous solution at 37°, pH 7.4. K_i is that of a product which has not been definitely isolated.

The bicyclic organophosphate (Ic) inhibits the PDE with a high affinity for cyclic AMP (low-K_m form) non-competitively, with a K_i value similar to that for theophylline. Since theophylline and the organophosphate have similar signs of poisoning [3, 4] the possibility still exists that inhibition of the low-K_m form of PDE could account for the toxicity of the organophosphates. Supporting evidence comes from recent observations that rat adrenal PDE is inhibited by this compound at a concentration of 10^{-6} to 10^{-8} M, and that the brain levels of cyclic AMP of rats convulsing after the administration of the compound showed significant changes [11]. The low-K_m form PDE is probably important for the control of the physiological levels of cyclic AMP because, not only are the normal tissue levels of cyclic AMP low (ca. 10^{-7} M) [12], but it is also the form of the enzyme which increases in activity in animals treated with β-adrenergic agonist, PDE inhibitors or mixtures of these [13].

The higher toxicity of Ic compared to theophylline may be explained in terms of comparative transport, membrane penetration and metabolism. Recent work has shown that the bicyclic organophosphates are not readily metabolised, and that the hydrophobic character of the 4-alkyl group is important regarding their toxicity [14]. The bicyclic organophosphates are more hydrophobic than theophylline, and thus may be able to penetrate more easily to its probable site of action in the CNS.

The lack of binding of the bicyclic organophosphates to the cyclic AMP binding proteins suggests that the toxic action is unlikely to be mediated through interaction with cyclic AMP receptors, which would be expected to show a similar specificity to the cyclic AMP binding proteins.

The data does indicate a possible mechanism of toxic action for the bicyclic organophosphates, but further work is necessary to establish that the basis of their toxic action is inhibition of the low-K_m PDE.

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