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INTERACTION OF CYCLOPHOSPHAMIDE AND ITS METABOLITES WITH ADENOSINE 3′,5′-MONOPHOSPHATE BINDING PROTEINS

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Abstract—The sensitivity for recognition of cyclophosphamide and its metabolites by adenosine 3′,5′-monophosphate (cAMP) specific proteins has been investigated. A 4-hydroxyl substituent in the 1,3,2-oxazaphosphorine ring is required for inhibition of cAMP binding to both cAMP phosphodiesterase and the regulatory subunit of the cAMP protein kinase holoenzyme. Binding to the latter causes an activation of the kinase and results in a dissociation into regulatory and catalytic subunits. The inhibitor constant K_i for the inhibition of cAMP binding (0.19 mM) correlates well with that for inhibition of the low K_m form of the phosphodiesterase (0.19 mM). In both cases inhibition is of the competitive type. Although 4-ketocyclophosphamide resembles 4-hydroxycyclophosphamide in electron donating properties it is inactive with respect to binding to cAMP specific sites. This probably results from the difference in conformation of the rings of these two compounds.

Cyclophosphamide [2-bis(2-chloroethyl)aminotetrahydro-2H-1,3,2-oxazaphosphorine-2-oxide] (I) is an antitumour agent used clinically in the treatment of various types of human neoplasms [1]. Although inactive as such it is known to be activated by enzymatic oxidation by liver microsomes [2]. The initial step appears to involve hydroxylation at the C-4 position of the 1,3,2-oxazaphosphorine ring to give 4-hydroxycyclophosphamide (II), which is in equilibrium with the ring opened aldehyde (III) [3–5]. Further oxidation of (II) to 4-ketocyclophosphamide (IV) and the ring-opened carboxylic acid (V) probably represents a deactivation pathway, since these forms are cytostatically less active than cyclophosphamide *in vivo* [6]. *N,N*-Bis (2-chloroethyl)phosphorodiamidic acid (VI) probably represents the final active metabolite of cyclophosphamide [7].

There is a similarity between the biochemical effects produced by cyclophosphamide and those produced by the intracellular mediator, adenosine 3′,5′-monophosphate (cAMP). Cyclophosphamide injected into rats bearing Jensen sarcomas produces a hyperglycemia which lasts up to 72 hr [8] and reduces the total amounts of both lipid and phospholipid [9]. This is equivalent to the well known glycogenolytic and lipolytic effects of cAMP [10]. Cyclophosphamide lowers plasma and erythrocyte cholesterol in rats [11]. This effect could also be mediated via cAMP since the latter has been shown to inhibit the conversion of acetate to cholesterol in broken cell preparations from liver [12]. Cyclophosphamide causes a rise in tyrosine transamminase in the livers of rats [13], in ornithine carboxylase in plasmacytomas of hamsters [14] and in alkaline phosphatase activity [15]. These enzymes are thought to be induced by cAMP [16–18]. The incorporation of precursors into DNA is inhibited by both cyclophosphamide and cAMP treatment [19–20]. Interferon production in response to Sendai virus in mice is inhibited during the early stages of the disease with cyclophosphamide [21]. Production of interferon is also prevented by exposure of activated lymphocytes to substances which elevate cAMP [22]. All agents that inhibit histamine release also suppress T cell-mediated cytolysis and cause in a parallel manner increases in the cAMP content of the lymphocyte effector cell population [23]. In this context the formation of histamine decreases up to 50 per cent following administration of cyclophosphamide to rats [24] as does also the graft-versus host reaction [25]. Cyclophosphamide has been shown to be highly effective in reducing the immune response to sheep erythrocytes [26]. This inhibition of humoral immunity is thought to be mediated by cAMP [27].

In view of this similarity of biochemical effects the interaction between cyclophosphamide and its metabolites and cyclic AMP binding proteins has been determined.

MATERIALS AND METHODS

[8-^{3}H]Cyclic AMP, ammonium salt (sp. act. 27 Ci/m-mole) and [γ-^{32}P]ATP (sp. act. 1.78 Ci/m-mole) were purchased from the Radiochemical Centre, Amersham. Histone (type II-A) and unlabelled cAMP were obtained from Sigma Chemical Co., London. Scintillation fluid NE233 was purchased from Nuclear Enterprises Ltd., Edinburgh. Cellulose ester filters were from Millipore Corp., London. Cyclophosphamide and its metabolites were kindly supplied by the Chester Beatty Research Institute.

Cell culture. Walker cells were maintained in static suspension culture in Dulbecco's modified Eagle's medium, supplemented with 10% foetal calf serum, under an atmosphere of 10% CO_2 in air.

Competition binding with [^{3}H]*cAMP.* Cells were removed from tissue culture medium by centrifugation and washed with 0.9% NaCl. The cell pellet was sonicated in 10 mM Tris–HCl, pH 7.6, containing 250 mM sucrose and 1 mM $MgCl_2$ and particulate

materials were removed by centrifugation at 100,000 *g* for 1 hr. The protein in the supernatant fraction, which was used in the binding experiments was determined according to the method of Lowry *et al.* [28] using bovine serum albumin as standard.

Competition binding with [^{3}H]cAMP (75,000 counts/min per assay) was performed in a final assay vol. of 0.3 ml. The inhibitor (in dimethyl sulphoxide so that the final concentration in the assay was 1%) in 100 μl of 50 mM potassium phosphate buffer, pH 6.5, was added to 100 μl of the soluble protein, at a concentration of 150–250 μg per assay, and allowed to equilibrate for 15 min at 4° before addition of [^{3}H]cAMP. After incubation for at least 2 hr at 4°, the reaction mixture was diluted with 1 ml of cold 20 mM potassium phosphate buffer, pH 6.0, filtered through a Millipore filter (0.45 μm), presoaked with potassium phosphate buffer and washed twice with a total vol. of 15 ml of the same buffer. The filters were placed in scintillation vials, dissolved in 1 ml of 2-(methoxy)-ethanol and the radioactivity was determined in 10 ml of scintillation fluid (2-(methoxy)-ethanol, toluene, PPO).

Corrections for non-specific binding by the filter were made as described previously [29].

Assay of phosphodiesterase activity. The determination of phosphodiesterase activity has been previously described [30]. Incubations were carried out at 35° for a time interval which gave less than 10% hydrolysis of the substrate. [8-^{3}H]Cyclic AMP and [8-^{3}H]5′-AMP were separated by a thin-layer chromatographic procedure and radioactivity was determined in NE 233 scintillation fluid. Compounds to be tested as inhibitors of phosphodiesterase activity were dissolved in dimethyl sulphoxide such that the final concentration in the enzyme assay was 1% or less. Control experiments showed that this had no effect on enzyme activity.

Protein kinase activity. This was measured as described previously [31]. Washed cells were suspended in 10 mM potassium phosphate buffer, pH 6.5, containing 10 mM EDTA, 0.5 mM 1-methyl-3-isobutylxanthine and 50 mM NaCl and disrupted by freezing, thawing and homogenization. The homogenate was centrifuged at 12,000 *g* for 20 min at 4° and the protein kinase activity of the supernatant was assayed in the presence of various concentrations of the compounds under test. The reaction mixture (80 μl final vol.) contained final concentrations of 0.21 mM [γ-^{32}P]ATP (sp act. 100 counts/min per pmole), 25 mM NaF, 0.5 mg histone, 0.125 mM 1-methyl-3-isobutylxanthine and 3.75 mM magnesium acetate in 50 mM potassium phosphate buffer, pH 6.8. The mixture was incubated at 30° and the reaction was initiated by the addition of 20 μl of the supernatant fluid. After 5 min incubation the reaction was terminated by pipetting 50 μl of the mixture onto Whatman 3 MM filter paper discs. The discs were washed in trichloroacetic acid, ethanol and ether and the radioactivity was determined in NE233 scintillation fluid. Assays were performed in triplicate.

Sephadex chromatography. The supernatant fraction derived from a tumour cell homogenate was incubated at 30° for 5 min in either the presence or absence of 2 mM 4-hydroperoxycyclophosphamide and was then chromatographed on a Sephadex G-100 column using 10 mM potassium phosphate buffer, pH 6.5 containing 10 mM EDTA and 500 mM NaCl. Fractions (0.5 ml) were collected and the enzyme activity (+ or − cAMP) was determined in 20 μl of each fraction.

RESULTS

Binding by protein kinases. The affinity of cyclophosphamide and its metabolites towards the regulatory subunit of cytosolic protein kinase from Walker cells was determined by investigating the competitive inhibition of binding of [8-^{3}H]cAMP at a range of concentrations of both inhibitor and cAMP. The results shown in Table 1 refer to the percentage inhibition of specific binding of the control assay found at each inhibitor concentration. 4-Hydroperoxycyclophosphamide (VII) has been employed as a suitable congener of 4-hydroxycyclophosphamide (II). Both compounds exhibit a similar high cytostatic activity in both *in vitro* and *in vivo* experiments [4]. Of the metabolites of cyclophosphamide only VII produced a substantial inhibition of cAMP binding. A similar effect was seen with the 4-hydroxy derivative of

Table 1. Effect of cyclophosphamide and its metabolites on the binding of cAMP to specific receptor proteins*

Compound	Inhibitor concentration (mM) 5	2.5	1.0	0.5	0.1	0.05	cAMP (nM)
Cyclophosphamide	3.0	0	—	0	—	—	133
	6.5	4.7	—	5.9	—	—	33.25
4-Ketocyclophosphamide	1.0	0	—	0	—	—	133
	—	6.6	1.0	1.0	—	—	33.25
4-Hydroperoxycyclophosphamide	—	—	35.0	25.0	13.0	8.0	266
	—	—	25.0	17.0	8.0	4.5	133
	—	—	39.0	26.0	11.5	8.0	66.5
	—	—	18.0	11.0	6.4	0	33.25
Cytoxyl alcohol	0	0	0	0	0	0	266
	0	0	0	0	0	0	33.25
4-Hydroxy-4-methyl-cyclophosphamide	—	—	33.0	30.0	28.0	0	266
	—	—	27.0	19.0	14.0	0	133
	—	—	3.0	2.0	0	0	66.5
	—	—	2.0	1.0	0	0	33.25

* Figures refer to per cent inhibition of binding of [^{3}H]cAMP.

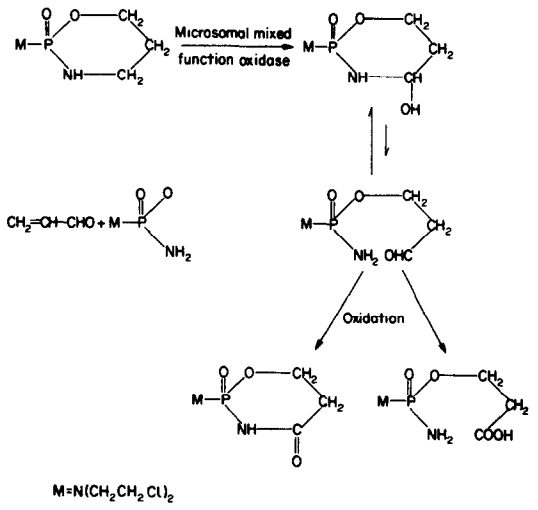

Fig. 1. Scheme for the metabolic activation of cyclophosphamide (I).

4-methylcyclophosphamide which is considered to be the activated form of this compound [32]. An oxygenated substituent at position 4 alone is not sufficient for interaction with the cAMP binding protein since 4-ketocyclophosphamide (IV) is a much less potent inhibitor than VII. The data from the binding with VII at cAMP concentrations of 33.25, 133 and 266 nM is expressed as a Dixon plot [33] in Fig. 2. Inhibition is seen to be of the competitive type with a K_i value of 0.19 mM. The binding protein from Walker cells has been shown to possess two sites with differing affinities for the substrate [29]. Since inhibition by VII is greatest at a high substrate concentration this indicates that competition with cAMP is largely at the low affinity site.

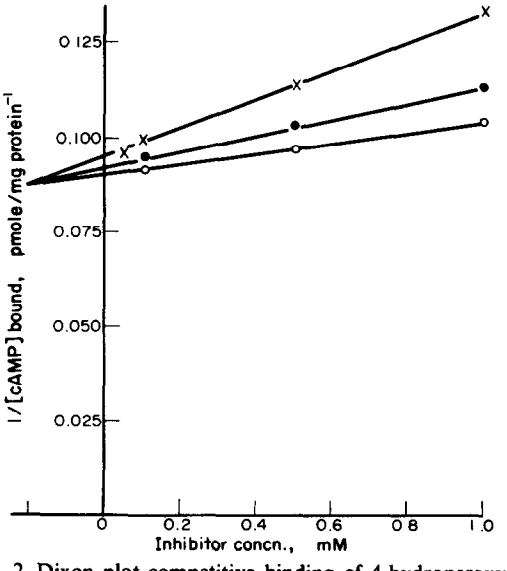

Fig. 2. Dixon plot competitive binding of 4-hydroperoxycyclophosphamide (VII) with [^{3}H]cAMP. The reciprocal amount of bound [^{3}H]cAMP is plotted against the concentration of VII at three different constant concentrations of [^{3}H]cAMP. Total [^{3}H]cAMP concentration was ×——× 266 nM; ●——● 133 nM; ○——○ 33.25 nM.

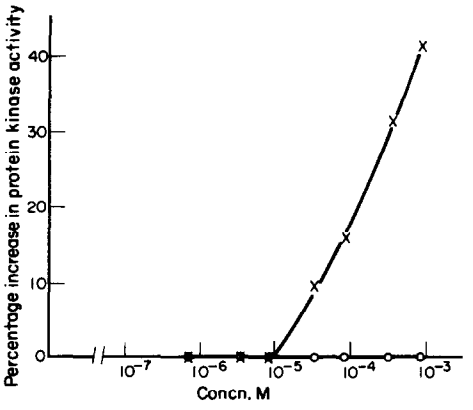

Fig. 3. Effect of cyclophosphamide and its metabolites on cytosolic protein kinase. ○——○ cyclophosphamide; ×——× 4-hydroperoxycyclophosphamide. For the sake of clarity the results with 4-ketocyclophosphamide are not shown.

Stimulation of protein kinase. The affinity towards protein kinase can also be demonstrated by determining the ability of the compounds under test to stimulate the phosphotransferase activity of these enzymes. The results in Fig. 3 show the percentage increase in protein kinase activity with varying concentrations of cyclophosphamide and 4-hydroperoxycyclophosphamide. Of the metabolites investigated only VII causes an activation of protein kinase and there is a linear relationship between the inhibition of binding of cAMP and the activation of protein kinase with a slope of 0.75 (Fig. 4). This suggests that this metabolite binds to the regulatory subunit of the protein kinase causing dissociation of the latter into an active catalytic subunit in a similar manner to that caused by cAMP. This was further confirmed by the experiment in Fig. 5. This shows the elution profile of cAMP-dependent protein kinase from Walker cell cytosol on a Sephadex G-100 column either with or without treatment with 4-hydroperoxycyclophosphamide. In Fig. 5A about 50 per cent of the cAMP-dependent protein kinase is in the R–C form (fractions 47–55) and 50 per cent in the C form (fractions 57–64). This compares well with the measured −cAMP/+cAMP ratio of 0.46. As shown in Fig. 5B

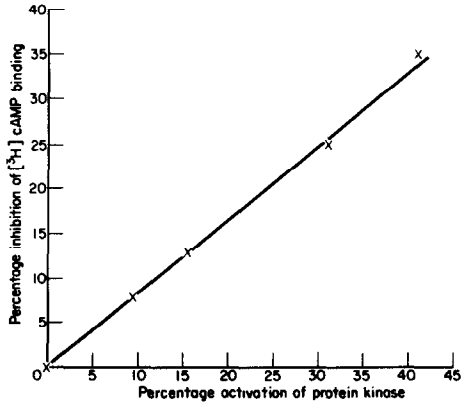

Fig. 4. Relationship between inhibition of [^{3}H]cAMP binding at 266 nM cAMP and activation of cytosolic protein kinase by VII.

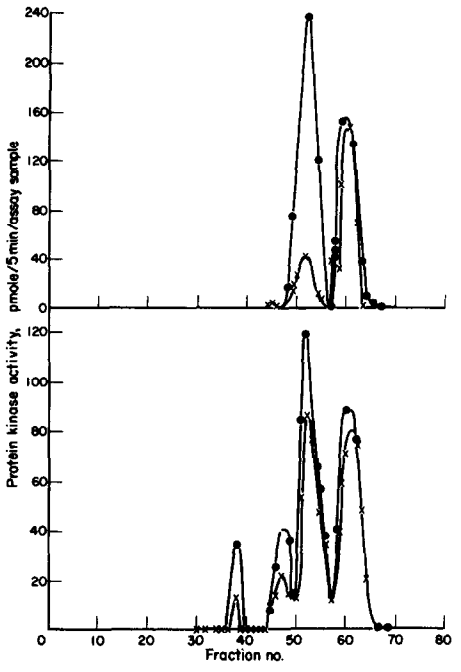

Fig. 5. Sephadex G-100 chromatography of Walker cell protein kinase 5 min after incubation at 30° alone (A) or with 2 mM VII (B). ×——× −cAMP; ●——● +2 μM cAMP.

there is less cAMP-dependent protein kinase in the R–C form and a corresponding increase in the C form after treatment with VII. The ratio of the peak areas of 0.74 compares favourably with the −cAMP/+cAMP ratio of 0.61. Since this experiment was performed with a broken cell preparation the dissociation of the protein kinase holoenzyme could not have been due to an elevation of endogenous cAMP and must be due to direct interaction between VII and the protein kinase R subunit. In contrast neither 4-ketocyclophosphamide or cyclophosphamide itself have any effect on either protein kinase activation or dissociation even at concentrations up to 1.5 mM. That the activation of protein kinase is not due to an alkylation mechanism is shown by the fact that

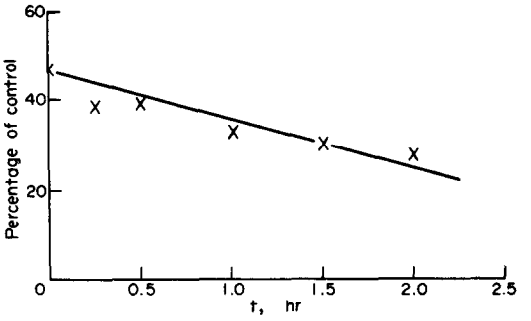

Fig. 7. Effect of contact time of VII with phosphodiesterase on enzyme inhibition.

neither VI or chlorambucil have any effect on the protein kinase activity of broken cell supernatant fractions at concentrations up to 1 mM (results not shown).

Inhibition of phosphodiesterase. The active site of cAMP-specific phosphodiesterase provides a further recognition site for cAMP to compare cyclophosphamide and its metabolites. Walker cell supernatant was used as a source of the enzyme since the kinetics have been reported in detail [30]. The results presented in Fig. 6 show that both cyclophosphamide and 4-hydroperoxycyclophosphamide act as competitive inhibitors of the low K_m form of this enzyme though the K_i value for inhibition by the former (2.5 mM) is much higher than for the latter agent (0.19 mM). 4-Ketocyclophosphamide showed no inhibition of the enzyme even at concentrations as high as 2 mM. The inhibition data may be interpreted as reflecting the affinities of the drugs towards the active site of phosphodiesterase.

The results presented in Fig. 7 show that 4-hydroperoxycyclophosphamide causes a progressive inhibition of enzyme activity as the contact time increases. This inhibition follows pseudo-first order kinetics. There was no inactivation of the enzyme control over the time period of the experiment. These results suggest that the interaction between VII and the enzyme consists of a rapid reversible equilibration followed by a slower irreversible inactivation.

$$E + I \underset{k-1}{\overset{k+1}{\rightleftharpoons}} (EI)rev \xrightarrow{k_2} (EI)irev$$

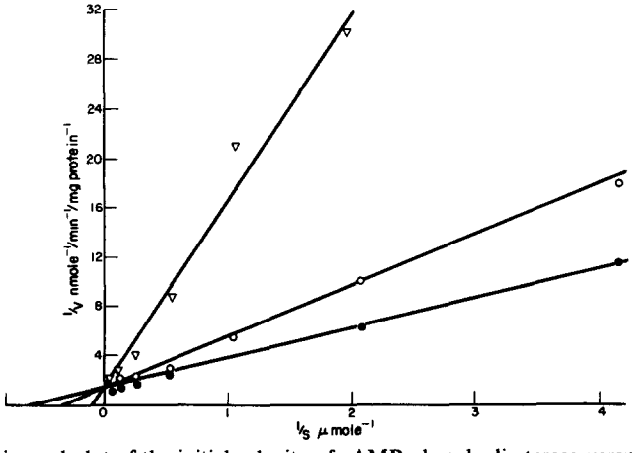

Fig. 6. Double reciprocal plot of the initial velocity of cAMP phosphodiesterase versus cAMP concentration with no inhibitor present (●——●); 2.04 mM I (○——○); or 1.06 mM VII (△——△) at low concentration of cAMP.

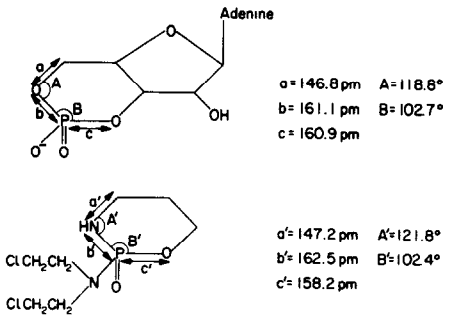

Fig. 8. Bond distances and angles of the cyclic phosphate moiety of cAMP and cyclophosphamide.

The latter reaction probably results from the β-elimination of the reactive VI from VII which alkylates a nucleophilic site on the enzyme.

DISCUSSION

The data in Fig. 8 shows the bond distances and angles of the cyclic phosphate moiety of cAMP and cyclophosphamide [34]. This data illustrates the strong similarity in these parameters for these two compounds. Insertion of a hydroxyl group at position 4 of cyclophosphamide would be expected to produce a hydrogen bonding site equivalent to the ribose ring oxygen, but to have little effect on bond distances and angles. It therefore seemed a possibility that 4-hydroxycyclophosphamide could substitute for cAMP as regards recognition by its coordinate proteins and this has been vindicated by the results presented in this paper. Although 4-ketocyclophosphamide also had a potential electron-donor oxygen atom at C-4 it is unable to compete with cAMP for specific protein binding. Although the bond lengths and angles in 4-ketocyclophosphamide are all very similar to the corresponding ones in cyclophosphamide the P–N ring bond is significantly longer in the keto derivative (1.668 vs 1.625 Å) [35] owing to withdrawal of electrons from this bond by the neighbouring carbonyl group. This also reflects a significant difference between 4-ketocyclophosphamide and cAMP in which the corresponding bond is 1.611 Å. However, the difference in the conformation of the rings is probably the major factor in the inactivity of the keto compound. 4-Ketocyclophosphamide is planar [35] whereas in hydroxycyclophosphamide C-4 would have a tetrahedral configuration and the six-membered ring would be expected to exist in the chair conformation. The cyclic phosphate ring of cAMP is known to be locked in the chair conformation [36] and this is probably essential for binding to its coordinate proteins.

Thus of the metabolites of cyclophosphamide only the 4-hydroxylated derivative (VII), which is thought to be the active cytotoxic form *in vivo* competes with cAMP for its specific receptor sites. The similarity in the mode of action of cAMP and VII is illustrated with regard to the protein kinase when binding of VII to the regulatory subunit of the holoenzyme causes a dissociation into catalytic and regulatory subunits. It is unlikely that 4-hydroxycyclophosphamide would produce any *in vivo* effect as a direct result of competition with cAMP specific proteins at physiological concentrations since the K_i value for inhibition of both cAMP phosphodiesterase and cAMP binding at 0.19 mM is fifty-five times as high as the ID_{50} value towards HeLa cells *in vitro* [4]. However, binding of VII to phosphodiesterase causes a time dependent inactivation of the enzyme probably due to the release of VI which is a highly reactive alkylating agent, and in time would cause a complete irreversible inactivation of the enzyme. Thus the low affinity reversible binding of VII to phosphodiesterase could act as highly efficient mechanism for enzyme inhibition. Another alkylating agent, chlorambucil, has also been shown to cause an inactivation of phosphodiesterase by direct reaction, and also to cause an increase in the intracellular level of cAMP in Walker cells [37]. This increase in cAMP level may potentiate the cytotoxic effect of the alkylating agents.

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