Biochemical Pharmacology, Vol. 25, pp. 1793–1797. Pergamon Press, 1976. Printed in Great Britain.

THE EFFECT OF ALKYLATING AGENTS ON ADENOSINE 3′,5′-MONOPHOSPHATE METABOLISM IN WALKER CARCINOMA

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(*Received* 18 *October* 1975; *accepted* 10 *December* 1975)

Abstract—The effect of some alkylating agents on the activity of the enzymes adenylate cyclase and cyclic 3′,5′-nucleotide phosphodiesterase has been studied using Walker carcinoma cells in tissue culture. The monofunctional agent 5-aziridinyl-2,4-dinitrobenzamide (CB 1954), which has previously been shown to elevate the level of adenosine 3′,5′-monophosphate (cyclic AMP) in sensitive Walker cells, has been shown to have no effect on the activity of adenylate cyclase either in the presence or absence of the protecting agent 4-amino-2-phenylimidazole-5-carboxamide (2-phenyl-AIC). Chlorambucil (*p*-*N*,*N*(di-2-chloroethylamino)phenylbutyric acid) (5 μg/ml) while having no effect on either the basal or fluoride-stimulated adenylate cyclase activity caused an inhibition of the high affinity form of the cyclic AMP phosphodiesterase which reached a maximum after 1 hr. This was accompanied by an increase in the intracellular level of cAMP which was proportional to the dose of chlorambucil up to a maximal 2-fold increase at 6.4 μg/ml, a dose which caused complete inhibition of cell growth. Further increases in the concentration of chlorambucil up to 100 μg/ml caused no further increase in cAMP level. Merophan (DL-*o*-*N*,*N*(di-2-chloroethylamino)phenylalanine) (0.5 μg/ml) similarly caused an inhibition of the low K_m form of the phosphodiesterase, but the rate of inhibition was slower than that observed with chlorambucil. The molecular forms of the cAMP phosphodiesterase in Walker cells sensitive or resistant to chlorambucil have been resolved using Sepharose 6B gel chromatography. The resistant lines displayed a reduction in the specific activity of the high affinity form of the enzyme which was accompanied by a shift to lower molecular weight forms. This could explain the lack of effect of chlorambucil on cAMP levels in Walker cells with acquired resistance to this agent.

The anti-tumour alkylating agents have been shown previously to cause an elevation of the intracellular level of adensosine 3′,5′-monophosphate (cAMP) in Walker carcinoma cells sensitive to the cytotoxic effect of these agents [1, 2]. This increase in cAMP level only occurs with bifunctional agents such as chlorambucil and not with the corresponding monofunctional compounds, such as the *N*-ethyl analogue of chlorambucil [1]. The change in the level of cAMP produced by chlorambucil precedes the inhibition of thymidine incorporation into DNA [2].

The increase in the level of cAMP appears to be due to an inhibition of a form of the cAMP phosphodiesterase (3′,5′ cAMP 5′-nucleotidohydrolase, EC 3.1.4.17) with a low K_m value, since only this form of the enzyme is affected and only in cells sensitive to the cytotoxic effects of the alkylating agents [1]. Resistance of Walker cells to a bifunctional alkylating agent is accompanied by changes in the phosphodiesterase, manifested by a reduction in the contribution of the low K_m form of the enzyme to the total activity, by a shift in pH optima, and by different inhibition constants for the competitive inhibitior theophyline [4]. Resistance to the monofunctional aziridine CB 1954 is accompanied by a 70 per cent reduction in the cAMP-binding protein [5]. This could explain the degree of cross resistance observed between this drug and the bifunctional agents.

The intracellular level of cAMP is determined by its rate of synthesis by adenylate cyclase (ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1), its rate of breakdown by cAMP phosphodiesterase and loss by leakage to extracellular fluid. The present study examines in more detail the effect of alkylating agents on adenylate cyclase and cAMP phosphodiesterase. The activities of the multiple forms of cAMP phosphodiesterase have been resolved using Sepharose 6B gel chromatography. In addition, cAMP levels have been measured in sensitive Walker cells and in those resistant to both CB 1954 and chlorambucil after treatment with a range of concentrations of chlorambucil.

MATERIALS AND METHODS

[8-^{3}H]Cyclic AMP (Sp. act. 27.5 Ci/m-mole) was purchased from the Radiochemical Centre, Amersham. Unlabelled cAMP and Sepharose 6B were obtained from Sigma Chemical Co. London. Scintillation fluid NE233 was purchased from Nuclear Enterprises Ltd., Edinburgh and PCS solubilizer from Hopkin and Williams, Romford. 4-Amino-2-phenylimidazole-5-carboxamide (2-phenyl-AIC) was the gift of Dr. J. Heyes of Beecham Research Laboratories, Brockham Park, Surrey. All the alkylating agents used were synthesized at the Chester Beatty Research Institute.

Cell culture. Cell lines 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. A line of Walker carcinoma cells sensitive to alkylating agents (WS), was established *in vitro* as described previously [6]. Two series of cell lines resistant to different concentrations of either chlorambucil (W_{CHL}) or CB1954 (W_R) were developed from the sensitive line. WS cells were suspended at a concentration of 10^5/ml in fresh culture medium and either chlorambucil or CB1954 was added in dimethyl sulphoxide to give final concentrations of 0.1 and 0.001 μg/ml, respectively. These concentrations were approximately twice the ID_{50} concentrations for WS (see below). The treated cells were incubated until a dense suspension was obtained, which was then divided into two portions and diluted with medium to give 10^5 cells/ml. One portion was then treated again with an amount of drug equal to the first dose, while the other received twice this dose. This process was repeated so that some of the cells received regularly increasing doses of drug while others were diverted at each level and subsequently received the same drug dose every 10 days. Three chlorambucil resistant lines and four CB1954 resistant lines were used in this study.

Drug treatment. Cells were taken from rapidly growing cultures and resuspended in fresh medium at 5×10^5 cells/ml. Drug solutions were made up in dimethyl sulphoxide at one hundred times the required concentration and 1 ml of drug solution was added to 100 ml of cell suspension which was then incubated at 37°. The sensitivity of the various cell lines to drug treatment was estimated as previously described [5, 6].

Adenylate cyclase assay. Cells were sedimented by low speed centrifugation and washed with 0.9% NaCl. The cell pellets were suspended in 50 mM Tris–HCl, pH 7.4, containing 25 mM KCl and 5 mM $MgCl_2$ and then disrupted by freezing in an acetone–cardice bath, followed by thawing and homogenization with a Teflon–glass homogenizer. This procedure is necessary because of the difficulty of rupturing Walker cells by conventional methods. The homogenate was centrifuged at 600 *g* for 10 min at 4° and the precipitate was finally resuspended in the Tris–HCl buffer to give a crude membrane suspension with a protein concentration of about 1 mg/ml.

The standard adenylate cyclase assay was similar to that reported by Albano *et al.* [7]. The reaction was initiated at 35° by adding 100 μl of the membrane suspension to 300 μl of 50 mM Tris–HCl, pH 7.4, so as to give final concentrations of 2 mM ATP, 3 mM $MgCl_2$, 10 mM NaCl, 10 mM KCl, 6 mM theophylline, 400 μg phosphocreatine kinase and 20 mM phosphocreatine. Reaction mixtures were incubated for various periods up to 15 min and the incubation was terminated by placing the tubes in boiling water bath for 3 min, followed by cooling and centrifugation at 1250 *g* for 10 min. The supernatant fluid was diluted either 1:1 or 1:3 with 50 mM Tris–HCl, pH 7.5, containing 4 mM EDTA, and the content of cAMP was determined using a kit purchased from the Radiochemical Centre, Amersham. Radioactivity was determined in PCS solubilizer using a Tracer Lab liquid scintillation spectrometer. Protein was determined by the method of Lowry *et al.* [8] using bovine serum albumin as a standard.

Cyclic AMP assay. The determination of cAMP has been described previously [1]. After drug treatment and low speed centrifugation the medium was removed by aspiration and the cell pellet was immediately treated with 1 ml of ice-cold 5% (w/v) trichloroacetic acid. The ether extracted, lyophilized supernatant was purified on a column of Dowex 50W-X8 (200–400 mesh) in the H^+ form, and the amount of cAMP in the eluate was determined using the Radiochemical Centre assay kit. A standard curve was obtained for each group of determinations.

Assay of adenosine 3′,5′-monophosphate phosphodiesterase. The assay of this enzyme has been described previously [3]. At various times after drug treatment cells were removed, centrifuged at 300 g for 3 min, washed with 0.9% NaCl and recentrifuged. The cell pellet was sonicated in 100 mM Tris–HCl, pH 8.1, containing 10 mM $MgSO_4$, with a 20-Kc MSE sonic oscillator. The phosphodiesterase activity of the total sonicated suspension was determined at 37° using [8-^{3}H]cAMP and the 5′ AMP formed was separated from cAMP on a silicic acid thin-layer chromatography (t.l.c.) plate in a solvent system consisting of propan-2-ol, ethyl acetate, 13 M ammonia (59:25:16, by vol). The region of the plate corresponding to 5′ AMP was removed by scraping and the radioactivity was determined in scintillation fluid NE 233. In order to obtain linear reaction kinetics the incubation time was controlled to give less than 10 per cent hydrolysis of the substrate.

The enzyme activity of column eluates was determined using the two-step assay of Thompson and Appleman [9].

Chromatography. Gel filtration was carried out on a Sepharose 6B column with dimensions of 35 × 1.5 cm. The flow rate for the best separation was about 20 ml/hr. Fraction volumes were 1 ml. The buffer conditions were 50 mM Tris–HCl, pH 7.5, containing 10 mM $MgCl_2$ and 10 mM 2-mercaptoethanol. Total sonicated cell suspensions (5×10^7 cells) were applied in buffer (1 ml). Enzyme activity was determined at 5 μM cAMP. Column calibrations were run under the same conditions using myoglobin, chymotrypsinogen, ovalbumin, bovine albumin, human γ-globulin and apoferritin. The column void volume was determined using Dextran 2000.

RESULTS

Drug sensitivity of cell lines. For chlorambucil and CB 1954 the ID_{50} values of the cell lines used in this work are listed in Table 1. WS is extremely sensitive to CB 1954 and less so to chlorambucil. There is cross

Table 1. Sensitivity of Walker cell lines to chlorambucil and CB 1954

Cell line	ID_{50}(μg/ml) Chlorambucil	CB 1954
WS	0.045	0.0005
W_{CHL1}	1.9	0.002
W_{CHL2}	4.6	0.005
W_{CHL3}	9.0	0.008
W_{R1}	0.3	0.016
W_{R2}	1.5	0.03
W_{R3}	1.4	0.35
W_{R4}	1.6	1.9

Table 2. Effect of alkylating agents on the activity of adenylate cyclase from sensitive Walker cells

Treatment	Cyclase activity (pmole/min/mg protein) −F	+F	Stimulation
None	3.37 ± 0.05	4.54 ± 0.07	1.35
5 μg/ml chlorambucil*	3.60 ± 0.06	4.40 ± 0.06	1.23
1 μg/ml CB 1954*	3.54 ± 0.05	4.40 ± 0.06	1.25
1 μg/ml CB 1954 + 100 μg/ml 2-phenyl-AIC*	3.00 ± 0.05	3.10 ± 0.05	1.00
None†	3.36 ± 0.06	4.40 ± 0.06	1.31
5 μg/ml chlorambucil†	3.27 ± 0.05	5.10 ± 0.17	1.56
1 μg/ml CB 1954†	3.20 ± 0.06	4.00 ± 0.08	1.25
100 μg/ml 2-phenyl-AIC†	3.27 ± 0.06	4.60 ± 0.10	1.4
1 μg/ml CB 1954 + 100 μg/ml 2-phenyl-AIC†	4.13 ± 0.06	4.10 ± 0.10	1.0

* Crude membrane preparations were treated with the drug in dimethyl sulphoxide (final concentration 1%) and the activity of the cyclase was determined as described in Methods.
† Cells were treated with drug for 2 hr prior to assay.

resistance between the chlorambucil resistant series W_{CHL1} to 3 and CB 1954 and between the CB 1954 resistant series W_{R1} to W_{R4} and chlorambucil. In both cases the degree of cross-resistance (10-fold and 30-fold, respectively) is similar for all cell lines. This is similar to the results obtained by Goldenberg [10] with L5178Y lympoblasts resistant to nitrogen mustard in which the degree of cross resistance was similar (2 to 3-fold) for a number of alkylating agents.

Effect on adenylate cyclase. The effect of treatment either of crude membrane preparations or of cells in culture with chlorambucil or CB 1954 for 2 hr on the activity of both the basal and fluoride stimulated adenylate cyclase activity is shown in Table 2. Although the activity of adenylate cyclase is known to be increased by fluoride ion [11] the degree of stimulation of Walker cell cyclase is small compared with that observed with some normal cell lines [12, 13], but is of the same order as that observed with a murine mammary carcinoma [14]. The results in Table 1 show that neither chlorambucil nor CB 1954 has any significant effect on the activity of either the basal or fluoride stimulated cyclase. 2-Phenyl-AIC has been shown to protect Walker cells against the toxic effects of CB 1954 [15]. There is no effect on cyclase activity in the presence of 2-phenyl-AIC alone, although there is some stimulation of basal activity produced by the combination of CB 1954 and 2-phenyl-AIC.

Effect on phosphodiesterase. The cAMP phosphodiesterase from Walker carcinoma resembles that from other tissues in showing multiple forms with differing affinities for the substrate [16]. Kinetic analysis suggests the presence of two enzyme activities; a low affinity form (K_m 82.5 μM) and a high affinity form (K_m 1.1 μM) [3], although the results can also be interpreted as showing a negatively cooperative enzyme system [17]. Since the intracellular level of cAMP in Walker carcinoma is about 2 μM probably only the high affinity form of the phosphodiesterase is important in regulating cAMP levels under physiological conditions. The activity of this form of the enzyme at various times after treatment with chlorambucil and merophan is shown in Table 3. Enzyme activity has been measured at a substrate concentration of 5 μM. At this concentration the contribution of the activity of the high K_m form of the phosphodiesterase towards the total activity is 16 per cent and a correction for this contribution has been made to the results in Table 3. The inhibition of this form of the enzyme by chlorambucil is rapid, reaching maximal inhibition after 1 hr. This corresponds to the time when the peak

Table 3. Activity of cyclic AMP phosphodiesterase after treatment with chlorambucil (5 μg/ml) or merophan (0.5 μg/ml).

Time (hr)	Sp. act. (nmole/min/mg protein) after treatment with Chlorambucil	% inhibition	Merophan	% inhibition
0	0.214 ± 0.007	—	0.214 ± 0.007	—
0.5	0.204 ± 0.010	5	0.209 ± 0.010	2
1.0	0.085 ± 0.007	60	0.119 ± 0.007	44
2.0	0.096 ± 0.015	55	0.117 ± 0.010	45
3.0	0.160 ± 0.011	25	0.051 ± 0.009	76
5.0	0.139 ± 0.009	35	—	—
7.0	0.102 ± 0.012	52	0.055 ± 0.0010	74

Walker cells were incubated with the indicated concentrations of drug. At the time periods stated in the table, 10^6 cells were removed, centrifuged, washed with 0.9% NaCl, recentrifuged and suspended in 1 ml of the phosphodiesterase assay buffer. After sonication the total cell supernatant was assayed for phosphodiesterase activity at a substrate concentration of 5 μM. Assays were performed in duplicate, and linearity was established by allowing it to proceed up to 15 min.

Table 4. Effect of chlorambucil on the intracellular level of cyclic AMP in Walker cells sensitive (WS) or resistant to chlorambucil (W_{CHL}) or CB 1954 (W_R) 2 hr after treatment

Cell line	Concentration of chlorambucil (μg/ml) 0	0.3	1.6	6.4	25	100
	Cyclic AMP (pmole/mg protein)					
WS	43 ± 2.5	48 ± 2.0	63 ± 2.5	89 ± 5.0	87 ± 6.0	75 ± 5.0
W_{CHL1}	40 ± 2.5	40 ± 2.0	25 ± 5.5	25 ± 5.5	29 ± 6.0	36 ± 5.5
W_{CHL2}	37 ± 2.0	32 ± 5.0	39 ± 5.0	39 ± 2.5	32 ± 5.0	48 ± 3.5
W_{CHL3}	34 ± 5.0	34 ± 5.0	29 ± 4.5	21 ± 4.5	33 ± 5.0	33 ± 4.5
W_{R1}	43 ± 1.8	—	42 ± 5.0	38 ± 4.0	49 ± 5.0	45 ± 4.5
W_{R2}	42 ± 3.0	—	53 ± 1.5	58 ± 6.0	60 ± 4.5	65 ± 5.0
W_{R3}	43 ± 0.5	—	42 ± 5.0	58 ± 5.0	56 ± 4.5	65 ± 5.0
W_{R4}	51 ± 2.1	—	54 ± 5.0	69 ± 4.0	60 ± 5.0	64 ± 4.5

level of cAMP is found in Walker cells [2]. Thereafter the percentage inhibition of the phosphodiesterase decreases with time. This may reflect new synthesis of the enzyme which is observed in response to elevation of cAMP levels [18, 19]. In contrast the inhibition of the enzyme by merophan is much slower, with maximum inhibition being observed between 3 and 7 hr. Merophan, at a concentration of 0.5 μg/ml, has been shown to cause a 118 per cent increase in the level of cAMP in Walker cells 8 hr after treatment [2].

Effect on cyclic AMP levels. The effect on the intracellular level of cAMP in Walker cells sensitive (WS) or resistant to chlorambucil (W_{CHL1}–W_{CHL3}) or CB 1954 (W_{R1}–W_{R4}) 2 hr after treatment with a range of doses of chlorambucil is shown in Table 4. The basal level of cAMP in all cell lines does not vary appreciably. For WS there is a dose dependent increase in cAMP levels, which reaches a maximal 2-fold increase at 6.4 μg/ml of chlorambucil which corresponds to 100 per cent inhibition of cell growth. Thereafter the level of cAMP remains constant irrespective of the dose of chlorambucil employed. For the chlorambucil resistant cell lines there is no increase in cAMP level at any concentration of chlorambucil and for some of these cell lines there is a decrease in the cAMP content after treatment with chlorambucil. For Walker cells which are resistant to CB 1954, chlorambucil does cause an increase in cAMP at the higher dose levels. Although these cell lines show some resistance to chlorambucil, they are more sensitive towards the drug than the W_{CHL} lines (Table 1). The increase in cAMP in the W_R lines produced by chlorambucil is not, however, as great as for the WS line.

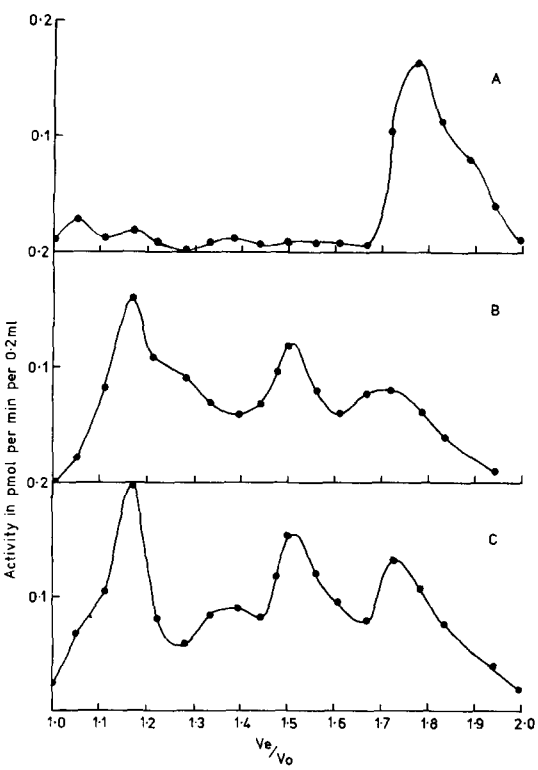

Fig. 1. Sepharose 6B gel filtration of resistant W_{CHL2} (A), W_{CHL1} (B) and sensitive (C) Walker carcinoma.

Sepharose 6B gel filtration of phosphodiesterase. The cAMP phosphodiesterase from total cell sonicated fractions of WS, W_{CHL1} and W_{CHL2} when measured at low substrate concentration gives the activity profiles shown in Fig. 1. Three distinct peaks of phosphodiesterase activity can be detected in WS and W_{CHL1} having apparent mol. wt of approximately 450,000, 350,000 and 225,000. The specific activity of the enzyme in these three cell lines, measured at 5 μM cAMP, decreases in the order WS (0.26 nmole/min/mg protein), W_{CHL1} (0.22 nmole/min/mg protein), W_{CHL2} (0.14 nmole/min/mg protein). This reduction in total enzyme activity is reflected in a reduction in activity of all three peaks of the phosphodiesterase in W_{CHL1}. In W_{CHL2}, however, there is a shift of activity from high mol. wt forms of the enzyme, so that all the activity now resides in the fraction having an apparent mol. wt of 225,000. A similar activity shift to low mol. wt forms of the enzyme has also been found in Walker tumour resistant to melphalan [4].

DISCUSSION

The bifunctional alkylating agent chlorambucil has been shown to exhibit a degree of selectivity in its effect on enzymes which control the intracellular level of cAMP. Thus, while it shows no effect on the activity of adenylate cyclase there is a progressive inhibition of the cAMP phosphodiesterase with time which reaches a maximum within 1 hr. Changes in the activity of the phosphodiesterase in Walker cells resistant to chlorambucil are also consistent with inhibition of this enzyme being of importance in growth inhibition by this agent. Resistance to chlorambucil in

Walker cells is accompanied by a lowering of the activity of the low K_m form of the phosphodiesterase without any concomitant effect on the high K_m form, and in one cell line (W_{CHL2}) this is reflected in an activity shift from high mol. wt to low mol. wt forms of the enzyme. Pichard and Kaplan have shown that an equilibrium exists between three interconvertible forms of cAMP phosphodiesterase in human platelets and that dibutyryl cAMP shifts the equilibrium to the low mol. wt form [20]. A similar lowering of enzyme activity is also found in houseflies resistant to organophosphorus insecticides, susceptible flies having two to three times the enzyme activity of resistant flies [21].

A change of the phsophodiesterase in resistant cells, such that inhibition by bifunctional agents is prevented, is consistent with the lack of effect of chlorambucil on cAMP levels in these cell lines. There is no lowering of the specific activity of the phosphodiesterase in Walker cells resistant to CB 1954 and chlorambucil has been shown to cause an elevation of cAMP levels in these cell lines.

The monofunctional agent CB 1954 has been shown to increase cAMP levels in Walker cells without any concomitant effect on the activity of the phosphodiesterase [1]. The present work indicates that this agent also has no direct effect on the activity of adenylate cyclase either in the presence or absence of the protecting agent 2-phenyl-AIC. The possibility therefore remains that CB 1954 may control cAMP levels through an effect on the rate of efflux of the cyclic nucleotide from the cell. Excretion of this nucleotide probably occurs by an energy-dependent mechanism where the direct source of energy may be ATP [22] and CB 1954 may have an effect on this process via an effect on ATP levels [23]. That cAMP is important in the mechanism of action of CB 1954 is suggested by the 70 per cent loss of binding protein found in Walker cells with acquired resistance to this agent [5]. These cells also a degree of cross resistance to growth inhibition by cAMP.

Experiments are now in progress to determine if the elevation of cAMP produced by the alkylating agents is sufficiently large to cause an activation of the cAMP-dependent protein kinase and also to determine the physiological importance of this alteration in cyclic nucleotide level in the mechanism of action of alkylating agents.

Acknowledgements—The authors wish to thank Professor L. Young for his interest. M. J. Tisdale wishes to acknowledge the receipt of a research grant from the Cancer Research Campaign.

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