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CYCLIC NUCLEOTIDE METABOLISM IN WALKER CARCINOMA CELLS RESISTANT TO ALKYLATING AGENTS

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Abstract—Walker carcinoma cells resistant to growth inhibition by 5-aziridinyl-2,4-dinitrobenzamide (CB 1954) also show some degree of cross-resistance to the cytotoxic effect of N^6,$O^{2'}$-dibutyryl adenosine 3',5'-monophosphate (dbcAMP). Using DEAE-cellulose chromatography and a linear salt gradient the cAMP-dependent protein kinase, binding protein and phosphodiesterase from sensitive and resistant cells has been resolved into multiple forms. A type 1 enzyme which elutes at 0.03 M KCl is present only in sensitive cells and those with a low resistance to CB 1954. A type 2 enzyme which elutes between 0.15 and 0.28 M KCl is present in all cell lines. There is a decrease in specific activity of the cAMP-dependent protein kinase, binding protein and phosphodiesterase with increasing resistance to CB 1954. The binding proteins from resistant cells were more sensitive to temperature than those from sensitive cells, suggesting a difference in conformation of the receptor. Both 2-mercaptoethanol and 5,5'-dithiobis(2-nitrobenzoic acid) increase the temperature sensitivity of the proteins with 2-mercaptoethanol producing a greater effect on the proteins from the resistant lines. The cAMP-dependent protein kinase of resistant Walker cells exhibits an apparent K_a for activation by cAMP 2.5-fold greater than that of sensitive cells. Heterologous reconstituted enzymes using separated subunits from sensitive and resistant cells show defects in both R and C subunits in resistant cells.

Although the alkylating agents are presumed to exert their cytotoxic effect as a result of their inhibition of DNA synthesis, the exact mechanism by which this is achieved has not been fully elucidated. Treatment of sensitive Walker carcinoma cells with physiological doses of bifunctional alkylating agents results in an elevation of the intracellular level of adenosine 3',5'-monophosphate (cyclic AMP) which reaches a maximum within 1 hr of treatment and thereafter decreases [1, 2]. This is accompanied by a stimulation of a cyclic AMP-dependent protein kinase (ATP: protein phosphotransferase; EC 2.7.1.37), which presumably mediates the cyclic AMP directive [3]. This increase in cyclic AMP level is specific for the alkylating agents and is not a general feature of cell death [1]. Also a monofunctional analogue of an active bifunctional agent fails to produce such a response [1].

When Walker cells become resistant to growth inhibition by the bifunctional alkylating agents no increase in cyclic AMP is observed except at very high dose levels [4]. Such resistance is accompanied by a reduction in the specific activity of the high affinity form of the cyclic AMP phosphodiesterase (3,5 cyclic AMP 5'-nucleotidohydrolase, EC 3.1.4.17) [5]. Inhibition of this enzyme most probably accounts for the cyclic AMP increase produced by the bifunctional alkylating agents such as chlorambucil, since only this form of the enzyme is inhibited and only in cells sensitive to the alkylating agents [1, 4]. This is achieved without a concomitant inhibition of adenylate cyclase (ATP pyrophosphate-lyase (cyclizing, EC 4.6.1.1) [4]. Of the monofunctional agents investigated only 5-aziridinyl-2,4-dinitrobenzamide (CB 1954) produces a rise in the level of cyclic AMP. This is not, however, accomplished by inhibition of the phosphodiesterase [1].

In eukaryotes the major, if not exclusive, mode of implementation of cyclic AMP directives involves protein phosphorylation [6, 7]. Cyclic AMP binds to the regulatory subunit (R) of the inactive holoenzyme(RC) causing dissociation of the latter into active catalytic subunits (C). Mouse lymphoma cells resistant to the cytotoxic effect of cyclic AMP have been shown either to contain less of the R subunit or to display a mutation of the structural gene coding for the regulatory subunit, such that a higher concentration of cyclic AMP is required for dissociation [8, 9].

The present experiments were undertaken to study the changes in both the cyclic AMP binding proteins and protein kinases in Walker cells with induced resistance to alkylating agents with the aim of elucidating the possible role of cyclic AMP in alkylating agent induced cell kill.

MATERIALS AND METHODS

[γ-^{32}P] ATP (sp.act. 1.78 Ci/m-mole) and [8-^{3}H] cyclic AMP (sp.act. 27.5 Ci/m-mole) were purchased from the Radiochemical Centre, Amersham. Histone (type 2A), unlabelled cyclic AMP and dibutyryl cyclic AMP were obtained from Sigma Chemical Co., London. Scintillation fluid NE 233 was purchased from Nuclear Enterprises Ltd., Edinburgh. Cellulose ester filters were from Millipore

Corp., London and ATP and N^6 monobutyryl cyclic AMP from Boehringer Corporation, London. 1-Methyl-3-isobutylxanthine was purchased from Aldrich Chemical Co., London. 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 Eagles medium, supplemented with 10% foetal calf serum, under an atmosphere of 10% CO_2 in air. The conditions for the establishment and maintenance of Walker cells resistant to alkylating agents has previously been described [10].

[^{3}H]*cAMP binding assay.* This was carried out as described previously [10]. Assays were performed in duplicate in a 200 μl final volume containing 10 μmoles potassium phosphate, pH 6.5, 0.6 μmole theophylline, 40 pmole [^{3}H]cAMP and 200 μg protein (determined by the method of Lowry *et al.* [11]) which was incubated for at least 2 hr at 4°. The [^{3}H] cAMP–protein complex was collected on a Millipore filter (0.45 μm pore size), dissolved in 2-(methoxy)ethanol and counted in 10 ml of a scintillation mixture containing 2-(methoxy)ethanol, toluene and PPO. Corrections for non-specific binding were made as described [10].

Assay of temperature sensitivity of cAMP binding proteins. Cells were sonicated in 100 mM Tris–HCl, pH 8.1 and centrifuged at 100,000 *g* for 1 hr at 4°. The supernatant fluid was incubated at 2–45° for 15 min, cooled at 2° for 10 min and then incubated overnight at 4° with 100 nM [^{3}H] cAMP. In some

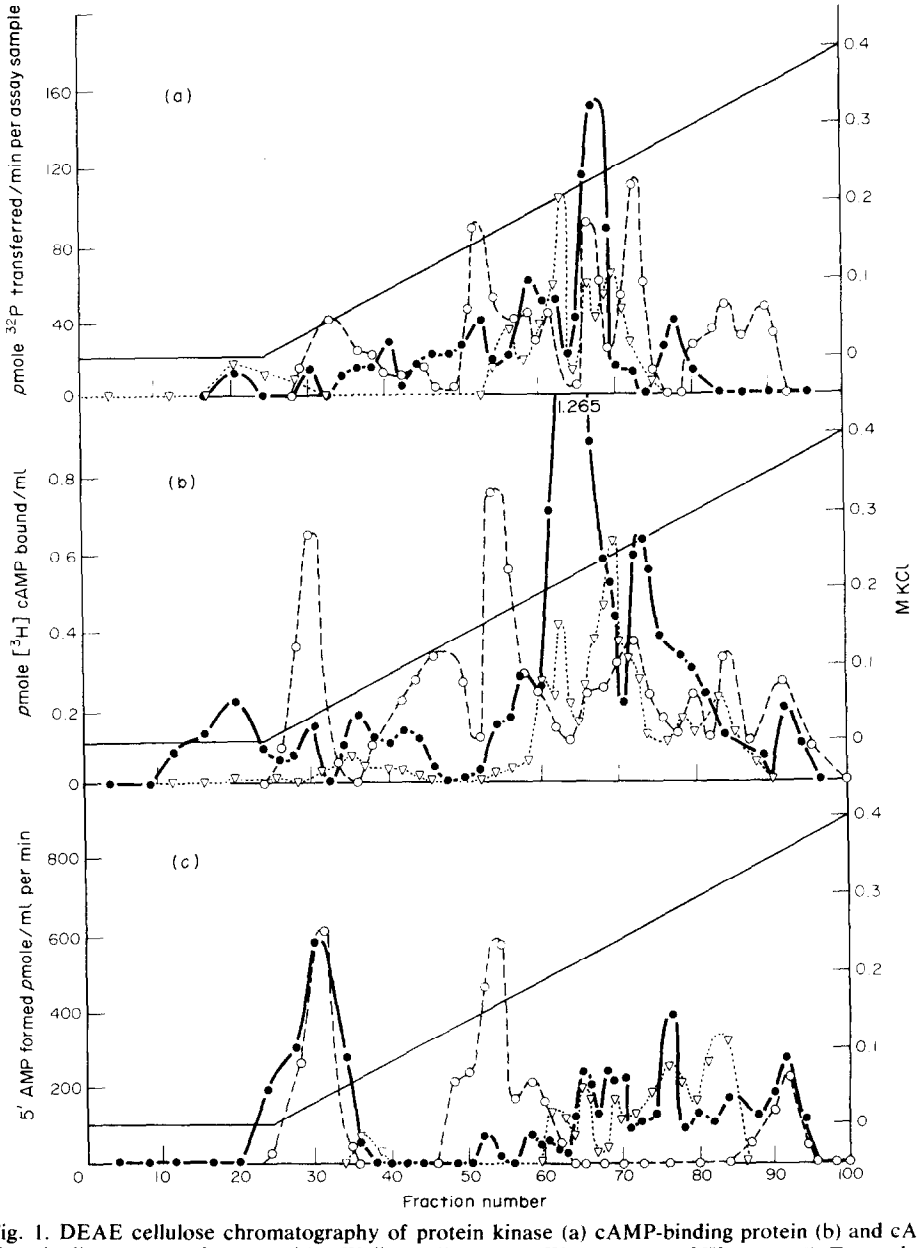

Fig. 1. DEAE cellulose chromatography of protein kinase (a) cAMP-binding protein (b) and cAMP phosphodiesterase (c) from sensitive Walker cells (●—●), W_{R1} (○—○) and W_{R5} (▽—▽). For each cell line 4.3 mg of total cell protein in 1 ml of the homogenization buffer was applied to the column and fractions of 1 ml were collected. Enzyme activity was determined as in Methods.

experiments 8 mM 2-mercaptoethanol or 3 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were also added before incubation at different temperatures. The amount of [^{3}H]cAMP bound was determined as above.

Protein kinase assay. This was determined as described previously [3]. Assays were performed in triplicate in a final reaction volume of 80 μl containing 16.8 nmoles [γ^{32}P] ATP (sp.act. 100 cpm/pmole), 2 μmole NaF, 0.5 mg histone (type 2A), 10 nmoles 1-methyl-3-isobutylxanthine, 300 nmoles magnesium acetate, 4 μmoles potassium phosphate, pH 6.8, an aliquot (20 μl) of protein kinase and 160 pmoles cAMP. After incubation at 30° for 5 min the reaction was terminated by pipetting 50 μl of the reaction mixture onto Whatman 3 MM discs which were washed in ice-cold 10% trichloroacetic acid, ethanol and ether and the radioactivity was determined in NE 233 scintillation fluid.

Assay of phosphodiesterase activity. This was determined by the method of Thompson and Appleman [12]. An aliquot of the enzyme solution (200 μl) was mixed with the substrate (100 μl) containing [^{3}H] cAMP (final concentration 5 μM). After incubation at 35° for 10 min, the reaction was terminated by boiling for 1 min. Snake venom (0.1 mg) was added and the incubation continued for a further 10 min at 35°. The [^{3}H] adenosine remaining in solution was determined after removal of [^{3}H] cAMP with Amberlite CG 400 type 1 ion exchange resin.

Preparation of cAMP-binding and catalytic units from the cAMP-dependent protein kinase. Cells were removed from culture medium by centrifugation at 300 *g* for 3 min, followed by washing in 0.9% NaCl and recentrifugation. All subsequent operations were carried out at 0–4°. The cell pellets were sonicated with a 20 KHz MSE sonic oscillator in 10 mM Tris–HCl, pH 7.6, containing 250 mM sucrose, 1 mM $MgCl_2$ and 0.5 mM dithiothreitol. The supernatant fraction obtained after centrifugation at 100,000 *g* for 1 hr was applied to a column (17 × 1 cm) of DEAE cellulose in the above buffer without sucrose and fractions of 1 ml were collected. Flow rate 40 ml/hr. After 24 ml had been eluted from the column a linear salt gradient from 0–0.4 M KCl was applied such that the total volume eluted from the column was 100 ml. Fractions 63–68 from the elution profile of WS shown in Fig. 1a and fractions 52–58 from W_{R1} were dialysed against 20 mM potassium phosphate buffer, pH 6.7, containing 5 mM 2-mercaptoethanol and 100 mM KCl, and further chromatographed on a Sephadex G-150 column (1.5 × 90 cm) equilibrated with the dialysis buffer. Fractions of 1 ml were collected and assayed for protein kinase and binding activity. Those fractions containing both cAMP-binding and kinase activity were pooled and concentrated by vacuum dialysis, dissociated by incubation with 10 μM cAMP for 15 min at 4° and re-chromatographed on the same Sephadex G-150 column. Fractions (1 ml) were collected and assayed for both cAMP-binding and kinase activity. Recombination experiments were performed on the isolated catalytic and regulatory subunits after dialysis for 10 hr against 20 mM Tris–HCl, pH 7.4, containing 100 mM KCl and 5 mM 2-mercaptoethanol.

Table 1. Effect of 2-mercaptoethanol and DTNB on temperature stability of cAMP-binding proteins

Cell line	Condition of treatment: Temperature °C	Condition of treatment: Compound added	Percentage of [^{3}H] cAMP bound*
WS	4	None	100 ± 2.1
	27	None	168 ± 2.6
	37	None	136 ± 1.0
	37	2-mercaptoethanol	121 ± 1.5
	37	DTNB	32 ± 2.2
	37	DTNB†	73 ± 1.0
	45	None	122 ± 2.8
	45	2-mercaptoethanol	122 ± 0.1
W_{R1}	4	None	100 ± 1.0
	27	None	100 ± 1.0
	37	None	100 ± 8.6
	37	2-mercaptoethanol	79 ± 1
	37	DTNB	32 ± 1.1
	37	DTNB†	70 ± 0.1
	45	None	75 ± 1.6
	45	2-mercaptoethanol	57 ± 9.0
W_{R5}	4	None	100 ± 2.5
	27	None	95 ± 6.7
	37	None	94 ± 1.4
	37	2-mercaptoethanol	71 ± 6.3
	37	DTNB	25 ± 1.3
	37	DTNB†	33 ± 1.0
	45	None	51 ± 4.7
	45	2-mercaptoethanol	50 ± 4.0

* Percentage of control at 4° ± S.E. of the mean.
† Measured at 0.56 nM cAMP.

RESULTS

Temperature sensitivity of binding proteins. The 100,000 *g* supernatant fraction of Walker carcinoma contains components which bind cAMP. The specific activity of these cAMP-binding proteins has been shown to be reduced in parallel with increasing resistance to alkylating agents [10]. This may also be accompanied by differences in receptor structure. This has been investigated by determining the temperature sensitivity of the cAMP-binding proteins from sensitive and resistant (W_{R1} and W_{R5}) Walker 100,000 *g* supernatant fraction. The binding activity measured at 100 nM [^{3}H] cAMP is shown in Table 1. The binding proteins from the CB 1954 resistant lines are more temperature sensitive than those from sensitive cells. After 15 min incubation at the indicated temperatures the activity of the binding protein in sensitive cells increases over that observed at 4°. There is, however, a peak in activity at 27° and further increase in temperature causes a decrease in activity. This suggests that in sensitive Walker cells there may be an inhibitor of cAMP binding which is more temperature sensitive than the binding protein itself. For the two resistant lines there is no appreciable loss of binding activity at temperatures below 37°. Above this temperature there is a loss of binding activity, which is more pronounced in the more resistant line (W_{R5}). Both DTNB and 2-mercaptoethanol increase the temperature sensitivity of the binding proteins, the former agent producing the most pronounced increase (Table 1). There is a greater effect of 2-mercaptoethanol on the

proteins from the resistant lines, than from the sensitive, while DTNB produces a similar effect with all three cell lines. When assayed at 0.56 nM cAMP 2-mercaptoethanol has no effect on the temperature sensitivity of cAMP-binding (results not shown), while DTNB has a more pronounced effect on W_{R5} at 37° than the other cell lines. The binding protein from WS and W_{R1} appears to be much less sensitive to DTNB at this cAMP concentration. These results suggest that the molecular structure of the protein kinase regulatory subunits are different in sensitive and resistant cells.

DEAE cellulose chromatography of cAMP dependent protein kinase, binding protein and phosphodiesterase in sensitive and resistant Walker cells. In order to investigate possible differences in molecular structure of cAMP dependent binding proteins in sensitive and resistant cells supernatant fractions from these cell lines were subjected to DEAE cellulose chromatography with a linear salt gradient from 0–0.4 M KCl. The elution profiles of cAMP dependent protein kinase, cAMP binding protein and cAMP phosphodiesterase from sensitive Walker cells (WS), those with a 32-fold resistance to CB 1954 (W_{R1}) and those with a 16,000-fold resistance to CB 1954 are shown in Fig. 1. Since both sensitive and resistant cells contain the same amount of protein (ca 250 $\mu g/10^6$ cells) the specific activities can be compared directly. For all three cell lines the main peaks of cAMP-binding protein are coincident with the peaks of protein kinase and phosphodiesterase activity. However, the peaks of activity for the individual cell lines differ in the salt concentration at which they elute suggesting that they differ in ionic charge. Increase in resistance of the cell lines is accompanied by a decrease in the activity of cAMP-binding protein, protein kinase and phosphodiesterase and in W_{R1} this is accompanied by a shift in the main peaks of activity, so that elution occurs at a lower salt concentration than that for sensitive cells. For both WS and WR_5 elution of the main peaks of protein kinase activity occurs between 0.20 and 0.28 M KCl (type 2 protein kinase), whereas in W_{R1} elution occurs between 0.15 and 0.26 M KCl. A peak of activity eluting at low salt concentration (0.03 M KCl; type 1 protein kinase) is present only in WS and W_{R1}.

Protein kinase. It has previously been shown that the degree of stimulation of cytosolic protein kinase decreased and the apparent dissociation constant

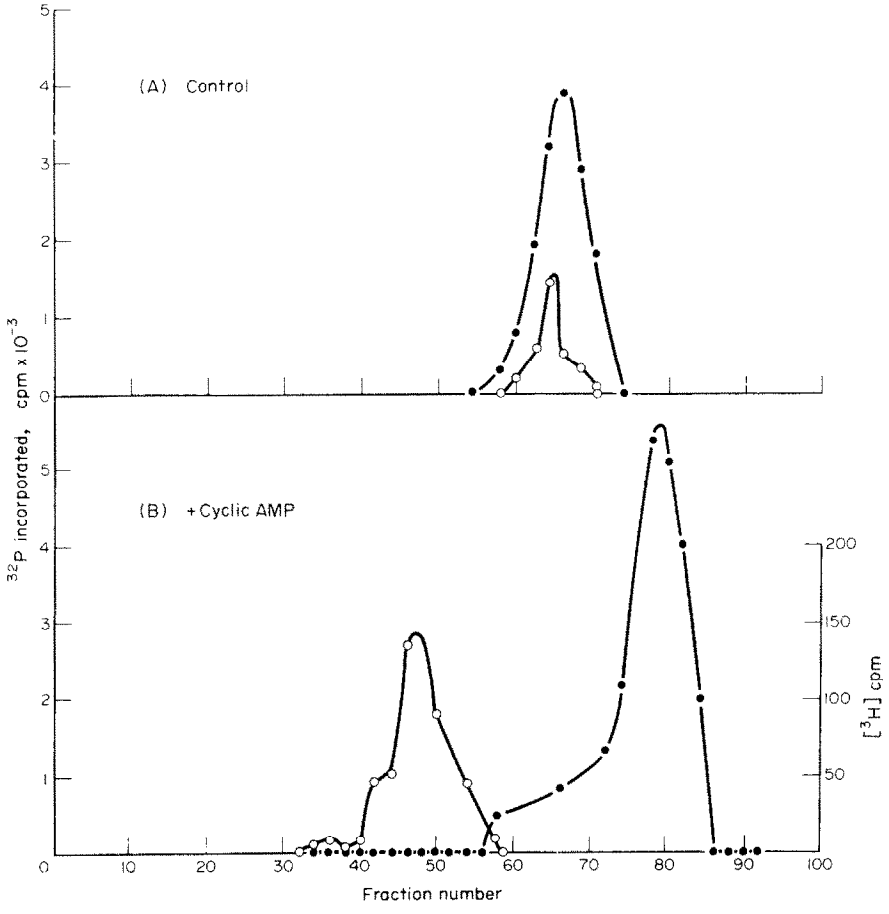

Fig. 2. Gel filtration of cAMP-dependent protein kinase from WS on Sephadex G-150. (a) Undissociated protein kinase (fractions 63–68 from Fig. 1a) activity measured in the absence (○—○) or presence (●—●) of cAMP. (b) Rechromatography of activity peak from (a) after dissociation with 10 μM cAMP. Protein kinase activity (●—●) and [^{3}H] cAMP bound (○—○) were determined as in Methods.

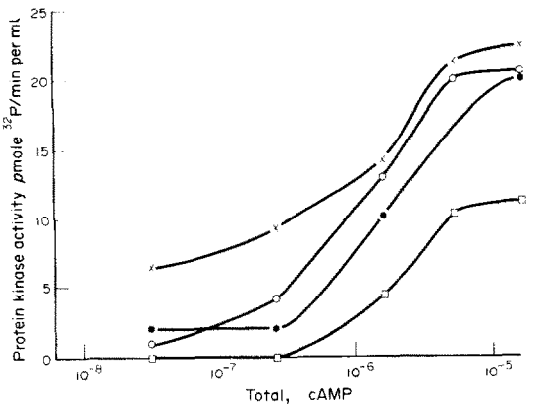

Fig. 3. Stimulation by various cAMP concentrations of homologous and heterologous reconstituted kinase holoenzymes. (×—×) WS–C + WS–R; (○—○) WS– C + W_{R1}–R; (●—●) W_{R1}–C + W_{R1}–R; (□—□) W_{R1}–C + WS–R. The concentration of R:C subunits used were in the ratio 4:1. This was the minimum ratio of combination at which activation by cAMP occurred. There was no increase in the kinase activity of C subunits alone in the presence of cAMP.

for cAMP bound to protein kinase increased with increasing resistance of Walker cells to alkylating agents [3]. Either an altered regulatory or an altered catalytic subunit could be responsible for this difference. This question was resolved by determining the K_a for cAMP of heterologously reconstituted holoenzymes composed of R and C subunits derived either from WS or W_{R1} and comparing this with homologously reconstituted enzymes with both subunits derived from the same cell type. The elution profile in Fig. 2a shows the main peak of protein kinase (fractions 63–68 from WS) from the DEAE cellulose column shown in Fig 1 after rechromatography on a Sephadex G-150 column. A single peak of activity is obtained which contains both [^{3}H] cAMP-binding and cAMP-dependent protein kinase activity. After treatment with 10 μM cAMP and rechromatography on Sephadex G-150 the [^{3}H] cAMP-binding and kinase ativities were completely separated as shown in Fig. 2b. Similar results were obtained with W_{R1}. These peaks of activity after dialysis and concentration were used as a source of subunits for the recombination experiments. The R and C fractions were mixed and incubated overnight at 4°. The R:C ratio was arbitrarily chosen in order to suppress catalytic activity completely (see legend to Fig. 3). The results in Fig. 3 show the kinase activity of the recombined holoenzymes exposed to various concentrations of cAMP. The apparent K_a values for activation of the kinase by cAMP are shown in Table 2. These results indicate a deficiency in both the catalytic and regulatory subunits of the protein kinase in the resistant cells. Thus a combination of the WS–C subunit with the W_{R1}–R subunit caused a decrease in the affinity of the heterologous holoenzyme towards cAMP, which was similar to that observed with the homologous W_{R1} holoenzyme. Also the WS–R subunit failed to increase the affinity of the WR–C subunit towards cAMP and this preparation also had a much lower maximal stimulation of kinase activity at saturating concentrations of cAMP.

Table 2. Apparent K_a' values for activation of protein kinase by cAMP using homologous and heterologous reconstituted kinase holoenzymes

Source of C subunit	Source of R subunit	$K_a' \times 10^{-6}$ M
WS	WS	0.8 ± 0.2
WS	WR_1	1.1 ± 0.15
WR_1	WR_1	2.0 ± 0.2
WR_1	WS	3.5 ± 0.3

K_a' refers to the concentration of cAMP required to cause an increase of protein kinase activity to one-half of its maximum value.

DISCUSSION

Two cytosolic protein isoenzymes, called types 1 and 2, which differ in their charge and dissociability by salt, have been described in mammalian tissues [13]. Tissues may contain either predominantly a type 1 or type 2 protein kinase of a mixture of both. The activities of these kinase isoenzymes vary during the cell cycle [14]. In the present experiments elution profiles of the cAMP-binding, protein kinase and phosphodiesterase activities after DEAE cellulose chromatography demonstrate differences between cytosols from Walker cells sensitive and resistant to CB 1954. For all parameters investigated there is a decrease in specific activity with increasing resistance to CB 1954. Sensitive Walker cells possess both a type 1 and type 2 protein kinase, while those with a high resistance to CB 1954 (W_{R5}) possess only a type 2 kinase. The type 1 enzyme is generally the more cAMP-dependent of the two, which may explain the decreased stimulation of protein kinase by cAMP in this cell line. For each of the cell lines the individual peaks of activity elute at different salt concentrations suggesting that they differ in net charge.

Two classes of binding site for cAMP have previously been demonstrated in Walker carcinoma cells, one of low affinity but high capacity and one of high affinity, but low capacity [10]. The data can, however, be equally interpreted as the existence of one site which exhibits negative cooperativity. Binding at 100 nM cAMP is representative primarily of low affinity sites. Gharrett *et al.* [15] observed that the cAMP-binding activity from SV 3T3 cells was substantially more heat resistant than the binding activity in 3T3 cytosol and suggested that thermolability measurements may be a more sensitive probe of differences between species of cAMP-dependent protein kinases than either specific activity or K_d measurements. The observations presented in this report that the temperature-sensitive proteins were more temperature resistant in the presence of 2-mercaptoethanol and DTNB at 0.56 nM cAMP than at 100 nM cAMP suggests a difference in the conformation of the two binding sites. Simantor and Sachs [16] have reported 2-mercaptoethanol to decrease the temperature sensitivity of the binding proteins from neuroblastoma cells. For

both sensitive and resistant Walker cells, however, the opposite situation is observed. Simantor and Sachs also reported that the binding proteins from neuroblastoma cells resistant to the cytotoxic effect of dibutyryl cAMP were more sensitive to temperature than the binding proteins from non-resistant cells [16]. The experiments described in the present report show that the binding proteins from Walker cells with induced resistance to CB 1954 are also more sensitive to temperature than the binding proteins from sensitive cells, which suggests a difference in conformation of the receptor proteins from these cell lines in addition to the reported decrease in binding activity with increasing resistance [10]. However, it is also possible that the binding subunit from W_R contains an aberrant modulator substance which is distinct from R itself. Some endogenous inhibition of cAMP binding in WS is suggested by the increased binding activity with increasing temperature.

The results from the combination experiments suggest that Walker cells with induced resistance to CB 1954 have both a defective catalytic and regulatory subunit of protein kinase compared with the sensitive cells. Hochman *et al.* [9] have shown that mutant S.49 mouse lymphoma cells which exhibit a 10-fold resistance to the biological effects of cAMP show an altered R component of the protein kinase holoenzyme, which leads to an apparent K_a' for activation by cAMP, which is 10-fold greater than for the wild type. The results presented here indicate that resistance of Walker cells to the cytotoxic effect of CB 1954 results in an increased Ka for activation which is 2.5-fold greater than that for sensitive cells.

The reduction of binding protein per cell as well as alterations in the molecular structure of the regulatory and catalytic components of the protein kinase holoenzyme explain the cross-resistance of these cell lines to dibutyryl cAMP and suggest a fundamental role for cAMP in the mechanism of action of the alkylating agents.

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