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Cyclic nucleotide metabolism in HeLa and Chinese hamster cells after treatment with chlorambucil

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Bifunctional alkylating agents have been shown to cause an increase in the basal level of adenosine 3',5'-monophosphate (cAMP) in Walker carcinoma cells in tissue culture [1, 2]. This is accompanied by a reduction in the amount of cAMP binding protein measured with saturating concentrations of [^{3}H]cAMP and an activation of a cAMP-dependent protein kinase [3]. Walker cells resistant to the cytotoxic effect of such agents show no increase in cAMP levels after treatment [1]. Such cells have reduced levels of cAMP phosphodiesterase [1], cAMP binding protein [4] and protein kinase [3], a reduction in the degree of stimulation of cytosolic protein kinase by saturating concentrations of cAMP, an increased dissociation constant for cAMP bound to protein kinase [3] and are cross-resistant to N^6,$O^{2'}$-dibutyryl cAMP (dbcAMP) [4]. These results suggest that cyclic nucleotides participate in the toxic action of these agents in Walker cells. Caffeine has been found to potentiate the lethal action of mitomycine C [5] and alkylating agents [6] towards mouse L-cells, and of alkylating agents towards Chinese hamster V79 cells [7]. Caffeine was shown to be effective in rendering cytoxan resistant plasmacytomas sensitive to the action of cyclophosphamide and X-rays [8]. In the presence of caffeine Chinese hamster cells treated with *cis* platinum (II) diamminedichloride (*cis* Pt II) synthesized low molecular weight DNA [9]. Furthermore, the rate of DNA synthesis, measured by the incorporation of [^{3}H]thymidine into acid precipitable material, rapidly returned to control levels in the presence of caffeine. Inhibition of so called post replication repair of alkylated DNA is thought to explain the potentiating effect of caffeine on the toxic and mutagenic effect of many agents. Since low doses of caffeine do not inhibit excision repair in mammalian cells [7] this has been attributed to a postreplication repair system [9]. Caffeine is a more potent inhibitor of guanosine 3',5'-monophosphate (cGMP) hydrolysis from pig coronary arteries than of cAMP hydrolysis [10]. This suggests that the actions of caffeine could be mediated by cGMP. The tumour promoters 12-O-tetradecanoylphorbol-13-acetate and phorbol-12,13-dibenzoate have been shown to cause a rapid increase in cGMP levels in human amnion cells [11], while having no effect on cAMP levels.

In the present experiments the effect of chlorambucil on the cAMP level and the cAMP-dependent protein kinase activity of two other cell lines HeLa and Chinese hamster V79-379A has been studied in order to investigate the generality of this phenomenon previously observed with Walker cells. Since caffeine potentiates the effect of alkylating agents the effect of combinations or caffeine and chlorambucil on cyclic nucleotide levels has also been determined.

MATERIAL AND METHODS

[8-^{3}H]Cyclic AMP (sp. act. 27.5 Ci/m-mole), and [γ-^{32}P]ATP (sp. act. 2.25 Ci/m-mole) were purchased from the Radiochemical Centre, Amersham. Chlorambucil was synthesized at the Chester Beatty Research Institute, London.

Cell culture and treatment conditions. Chinese hamster lung V79-379A and HeLa cells were maintained in static suspension culture as previously described [12]. Walker cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum under an atmosphere of 10% CO_2 in air. For measurement of cyclic nucleotide levels exponentially growing cultures were treated with 15 μM chlorambucil in ether with or without 750 μM caffeine. At various times after treatment the cells were sedimented by centrifugation, the culture medium carefully removed and 1 ml of cold 5% (w/v) trichloroacetic acid was added within 30 sec. The centrifuge tubes were rinsed with a further 0.5 ml of trichloroacetic acid and the two extracts were pooled.

Cyclic nucleotide assay. The trichloroacetic acid extracts of the cell pellets was purified as previously described [1]. The concentration of cAMP was determined by means of an assay kit (Radiochemical Centre, Amersham). A standard curve was performed for each determination. Radioactivity was measured in PCS solubilizer. For the determination of cGMP the precipitated protein was removed

Table 1. Effect of chlorambucil (15 μM), caffeine (750 μM) and chlorambucil (15 μM) plus caffeine (750 μM) on the cAMP level of Chinese hamster V79-379A and HeLa cells

Cell line	HeLa			V79–379A		
Treatment	cAMP(pmoles/mg protein)			cAMP(pmoles/mg protein)		
Time hr	Chlorambucil	Caffeine	Combination	Chlorambucil	Caffeine	Combination
0	7.0 ± 0.7*	7.0 ± 0.98	7.4 ± 0.8	8.1 ± 1.5	6.9 ± 0.95	7.8 ± 0.8
1	7.75 ± 0.8	7.35 ± 1.34	—	6.7 ± 2.4	6.7 ± 0.7	5.0 ± 1.4
2	7.5 ± 0.5	5.9 ± 1.34	6.0 ± 0.95	12.4 ± 0.8	6.9 ± 0.7	—
3	10.6 ± 0.8	5.3 ± 0.85	11.1 ± 1.6	13.9 ± 1.2	9.9 ± 1.5	18.4 ± 2
4	13.3 ± 1.2	6.2 ± 1.06	13.55 ± 1.2			
5	14.4 ± 1.2	6.25 ± 1.8	11.1 ± 2			

* Mean ± S.D. of mean.

by centrifugation at 10,000 *g* for 10 min and the supernatant fluid after acidification with 1 N NCl was extracted six times with water saturated ether and then lyophilized. The residue was dissolved in 50 mM Tris–HCl, pH 7.5, containing 4 mM EDTA. After addition of NaOH to bring the pH to 7.5 the cGMP content was determined with a radioimmunoassay kit purchased from the Radiochemical Centre. Approximately 10^5 times as much cAMP as cGMP is needed to give an equivalent reduction in the binding of [^{3}H]cGMP using this system. Protein was determined by the method of Lowry *et al.* [13] using bovine serum albumin as a standard.

Protein kinase activity ratio. The method of Corbin and Reimann [14] was used to determine the protein kinase activity ratio. Assays were performed in triplicate in a reaction volume of 80 μl containing 16.8 nmoles (γ-^{32}P) ATP (sp. act. 100 c.p.m./pmole), 2 μmol NaF, 0.5 mg histone (type IIA), 10 nmole 1-methyl-3-isobutylxanthine, 300 nmoles magnesium acetate, 4 μmoles potassium phosphate, pH 6.8 and an aliquot (20 μl) of protein kinase in the presence or absence of 160 pmoles cAMP. After incubation at 30° for 5 min the reaction was terminated by pipetting 50 μl of the mixture onto a Whatman 3 MM filter disc which was washed in ice-cold 10% trichloroacetic acid, ethanol and ether and the radioactivity was determined in scintillation fluid NE 233.

RESULTS

Effect on cyclic nucleotide levels. The effect of chlorambucil (15 μM) on the intracellular level of cAMP of Chinese hamster V79-379A and HeLa cells is shown in Table 1. Continuous treatment with this dose of chlorambucil would be expected to reduce survival to not less than 10 per cent control [16]. The basal level of cAMP in these two cell lines is similar and is much lower than that previously found with Walker cells [1]. Treatment with chlorambucil causes approximately a doubling of cAMP levels in both cell lines, though the time scale of this effect is slightly different. Thus the maximal elevation is reached much sooner in V79 than in HeLa cells. Similar conclusions are reached if the results are expressed in terms of cell number. Caffeine alone at a concentration of 750 μM is non-toxic to Chinese hamster cells [15], but potentiates the toxicity caused by chlorambucil. Since caffeine has been shown to be an inhibitor of both cAMP and cGMP phosphodiesterase [10] its effect on cyclic nucleotide levels, both alone and in combination with chlorambucil was investigated. The results in Table 1 show that caffeine has no effect on the cAMP level in HeLa cells, either alone or in combination with chlorambucil. In V79 cells caffeine produces a small rise in the basal level of cAMP 3 hr after treatment and also enhances the elevation of basal levels caused by chlorambucil. Its effect on cGMP levels in this cell line is, however, much more pronounced than its effect on cAMP levels, causing a doubling 3 hr after treatment (Table 2). Chlorambucil alone has no effect on the cGMP level of this cell line.

Effect on protein kinase activity. The increase in the intracellular level of cAMP corresponds well with the increase in cAMP-dependent protein kinase activity for HeLa (Fig. 1) and Chinese hamster V79-379A (Fig. 1) cell lines. The protein kinase activity ratio (−cAMP/+cAMP) is the ratio of the protein kinase activity of the supernatant fraction assayed in the absence of added cAMP to that measured in the presence of saturating concentrations of cAMP, using total histone as substrate. Under the conditions of the assay the incorporation of [^{32}P]phosphate from [γ-^{32}P]ATP into histone increases linearly with reaction time 0–15 min. For HeLa cells cAMP-dependent protein kinase activity reaches a maximum 174 per cent of control between 4–5 hr after administration of chlorambucil. Continuous treatment of HeLa cells with chlorambucil has a maximum effect on cell survival after approximately 4 hr [16]. For Chinese hamster cells cAMP–dependent protein kinase activity reaches a maximum 195 per cent of control 3 hr after treatment. Control cultures showed no increase in the protein kinase activity ratio during the time course of the experiments.

DISCUSSION

The increase in the intracellular level of cAMP in Chinese hamster lung V79-379A and HeLa cells in response to chlorambucil is similar to that previously observed with Walker carcinoma [1] and corresponds approximately to a doubling of cAMP levels. However, the time to produce the maximum effect on cAMP levels differs for each cell line being much longer for Chinese hamster and HeLa than for Walker cells. The time for the maximum increase of cAMP levels produced by chlorambucil in HeLa cells corresponds with the time that this agent has its maximum effect on cell survival [16]. This suggests that cAMP may be involved in its cytotoxic effect. A linear relationship between the reciprocals of the intracellular level of cAMP produced by a given dose of chlorambucil and the percentage inhibition of cell growth has previously been observed with Walker cells [1].

The major if not exclusive, mode of action for cAMP-mediated directives appears to result via protein phosphorylation. Thus cAMP binds to the regulatory subunit (*R*) of the inactive protein kinase holoenzyme (*RC*) causing a dissociation into active catalytic (*C*) subunits, which then phosphorylate some cellular macromolecule. Using whole histone as substrate the increase in cAMP level in Chinese hamster and HeLa cells in response to chlorambucil causes an increase in the protein kinase activity ratio (−cAMP/+cAMP) the time course of which is similar to the time course for change in basal levels of cAMP. Measurement of cAMP-dependent protein kinase is more reproducible than cAMP determinations as the standard error for the latter is much greater than for protein kinase. This suggests that a protein kinase is activated *in vivo* after treatment with chlorambucil.

Although caffeine has been shown to be an inhibitor of both cAMP and cGMP hydrolysis in a number of cellular preparations, the present results demonstrate that it has little effect on the basal level of cAMP or the stimulation of the basal level produced by chlorambucil in either Chinese hamster V79 or HeLa cells. This suggests that caffeine does not enhance the cytotoxicity of the alkylating agents by an alteration of endogenous cAMP. In contrast

Table 2. Effect of caffeine (750 μM) and chlorambucil (15 μM) on the cGMP level of Chinese hamster V79–379A cells.

Treatment	Caffeine		Chlorambucil	
Time hr	pmoles/mg protein	pmole/10^6 cells	pmole/mg protein	pmoles/10^6 cells
0	0.36 ± 0.01*	0.11 ± 0.01	0.34 ± 0.01	0.1 ± 0.01
1	0.34 ± 0.02	0.14 ± 0.03	0.42 ± 0.04	0.1 ± 0.009
2	0.36 ± 0.01	0.14 ± 0.03	0.36 ± 0.01	0.1 ± 0.015
3	0.64 ± 0.05	0.25 ± 0.03	0.35 ± 0.02	0.1 ± 0.008

* Mean ± S.D. of mean.

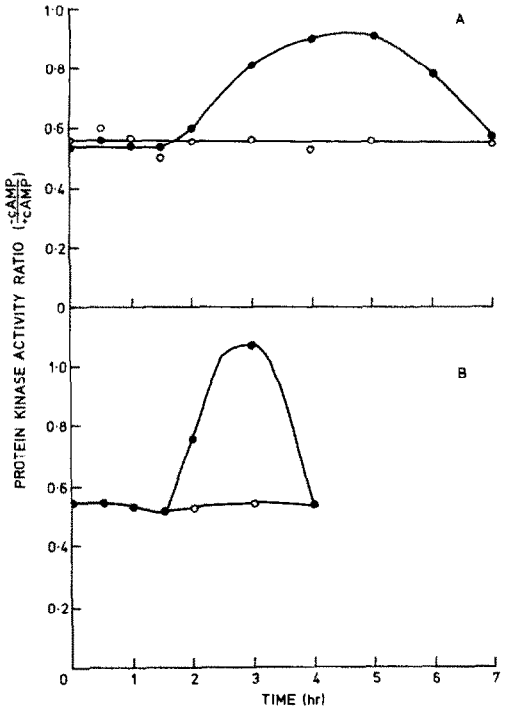

Fig. 1(A). Changes in the protein kinase activity ratio in HeLa cells after treatment with 15 μM chlorambucil (●——●). Control cultures (○——○) received solvent alone. (B) Changes in the protein kinase activity ratio in Chinese hamster V79-379A cells after treatment with 15 μM chlorambucil.

caffeine produces a 75 per cent increase in cGMP levels in Chinese hamster cells 3 hr after treatment with a concentration of 750 μM, which is the concentration employed for inhibition of a postreplication repair mechanism in this cell line [9]. At this concentration it also produces a 2-fold increase in the cGMP level of Walker cells 3 hr after treatment [17], cGMP resembles caffeine in reversing to control levels the inhibition of [^{3}H]thymidine into acid precipitable material after chlorambucil administration [17]. Both caffeine and cGMP have also been shown to cause an induction of TMP synthetase activity in *Tetrahymena pyriformis*. Since caffeine has been shown to inhibit the ligation of newly synthesized DNA on a *cis* Pt (II) [9] or a 7 DMBA [19] damaged template it suggests that cyclic nucleotides may also be involved in this process.

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