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Interactions Between Insulin and the Cyclic AMP System of Cloudman S91 Mouse Melanoma Cells

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Insulin inhibits the proliferation of wild-type Cloudman S91 mouse melanoma cells. The effects, which are mediated through specific, high-affinity receptors for insulin, appear to involve interactions with the cAMP system. Our evidence is as follows: 1) Cloudman cells have a cAMP requirement for proliferation and pigmentation. Exposure of cells to insulin results in a lowering of intracellular cAMP levels and inhibition of both cell division and pigment formation. 2) The effects of insulin are reversed by agents which raise cAMP levels, or by the cAMP analogue dibutyryl cAMP. 3) A mutant cell line with a temperature-dependent requirement for cAMP is most sensitive to the growth inhibitory effects of insulin when its requirements for cAMP are maximal. 4) Mutants selected only for alterations in their response to insulin frequently have concomitant alterations in their cAMP systems. 5) The melanotropin-responsive adenylate cyclase system is stimulated following prolonged exposure of cells in culture to insulin. Although we do not know the mechanism(s) for the interactions between the insulin and the cAMP system, our initial findings suggest that protein phosphorylation/dephosphorylation reactions are involved.

Key words: melanoma, Cloudman S91 in culture, cell proliferation, cyclic AMP, genetic complementation, protein phosphorylation, MSH, melanotropin, insulin

Cloudman S91 mouse melanoma cells exhibit multiple responses to alterations in intracellular levels of cAMP. Wild-type cells exposed to melanotropin (MSH) show 5- to 50-fold increases in cAMP 10 min after the hormone is added to the culture medium. A few hours later, tyrosinase is activated, the cells become dendritic, and eventually the rate of division slows or ceases [1]. The effects of MSH on inhibition of growth and increased pigmentation are enhanced by cyclic nucleotide phosphodiesterase inhibitors such as methylisobutylxanthine (MIX), and are mimicked by high levels of dibutyryl cAMP (Bt_2cAMP, 1 mM), or by agents which raise intracellular levels of cAMP such as cholora toxin or prostaglandin E_1 [2]. On the other hand, low levels of Bt_2cAMP (0.01 mM) stimulate growth, and through analyses of mutant cell lines it has been demonstrated that Cloudman cells have a cAMP requirement for

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growth [3,4]. Apparently the cells require an optimum level of cAMP for progression through the cycle—if the intracellular levels of cAMP are above or below this optimum, the cell cycle is lengthened. Our evidence suggests that cAMP is necessary for the activation of a cAMP-dependent protein kinase, indicating that protein phosphorylation/dephosphorylation reactions are involved in the regulation of the cell cycle [3,4].

Insulin has been reported to affect the cAMP system in a number of cell types [5–25]; that is, insulin has been reported to lower cAMP levels, suppress hormone-mediated rises in cAMP, and affect enzyme systems involved with cAMP metabolism and action. Because of the sensitivity of the Cloudman cells to changes in cAMP levels, we were interested in testing the effects of insulin on their proliferation. We reasoned that if insulin should lower cyclic cAMP levels in Cloudman cells, it might inhibit their growth. We found that insulin did inhibit the growth of Cloudman cells and that the effects of insulin were mediated through specific high-affinity receptors for insulin [26]. The inhibition of growth by insulin appeared to result from a lowering of cAMP levels within the cells [4]. In this paper we present additional evidence concerning interactions between insulin and the cAMP system. Using several different approaches we have concluded that insulin, by interacting with the cAMP system, produces multiple effects on cAMP metabolism.

MATERIALS AND METHODS

Cell Culture Conditions

Cloudman S91 mouse melanoma cells were cultured in monolayer in Ham's F10 medium supplemented with horse serum as previously described [27].

Cell Lines

Four Cloudman S91 melanoma cell lines were used in these studies: wild-type (PS1-wt [28], cAMP-dependent (cA^{dep} [2–4], insulin-dependent ("46" [26,31], and insulin-resistant (rev-1 [26,31]). Pertinent characteristics of these cell lines are described.

Determination of Rates of Proliferation

To determine rates of proliferation under various culture conditions, cells (2×10^4) were inoculated into 25-cm^2 Corning tissue culture flasks in 4 ml medium, and medium was changed three times per week. Triplicate cultures were harvested and counted in a Coulter Counter every 2 or 3 days over a 2-week period. Variation within triplicate samples was $\pm$ 15%. The generation time was determined from the rate of division in log phase from the following equation:

$$\text{Generation time} = \frac{0.69\ (h)}{\ln\ (C_2/C_1)}$$

where h = the time in hours between the initial cell number (C_1) and the final cell number (C_2) over the linear portion of a growth curve.

Melanin Content

Melanin content was estimated by visual observations of equal numbers of cells pelleted by centrifugation.

Measurement of Intracellular Levels of cAMP

To measure the effects of various culture conditions on the intracellular levels of cAMP, cells (10^6) were inoculated into 100-mm Falcon tissue culture Petri dishes in unsupplemented culture medium (10 ml). The next day prewarmed, fresh medium supplemented with hormones was added as noted in the figure legends. After various time periods, medium was removed by suction, the cells were rinsed twice with ice-cold NaCl (0.9%) and ice-cold trichloroacetic acid (TCA, 4 ml, 5% vol/vol) containing 500 cpm/ml ^{3}H-cAMP was added to monitor recovery of cAMP. The time from removal of culture medium to addition of TCA was about 10 sec. TCA was extracted with ether, and cAMP was measured by the method of Brown et al [29]. In some experiments, extracts containing cAMP were passed through Dowex-1-formate columns before being assayed, but the results were the same whether or not this extra purification step was included.

Adenylate Cyclase Activity

Cells were collected from flasks with Joklik's modified minimal essential medium containing ethylenediaminetetracetic acid (1 mM). Cells were then pelleted by centrifugation (300g for 10 min), suspended in 0.9% saline, and again pelleted by centrifugation. Cell pellets were suspended in distilled water at a density of 1.0–1.5 $\times$ 10^7 cells/ml on ice and sonicated 3 $\times$ 10 sec at a setting of 3 with an Ultrasonic Sonifier Cell Disrupter, model W185D. The resulting cell homogenates were kept on ice and used immediately after sonication in the cyclase assay. The adenylate cyclase assay used was that of Kreiner et al [30] with some modifications. The assay was run in 40 mM Tris buffer, pH 7.4, with 0.1 mM methylisobutylxanthine (MIX), 2 mM ATP, and ATP-regenerating system of 38 mM creatine phosphate and 80 μg/ml creatine phosphokinase, 4 mM $mgCl_2$, and hormone additions in a final volume of 0.5 ml. The reaction was performed in a 30°C shaking water bath and was begun by the addition of cell lysate. The reaction was stopped by placing tubes in a boiling water bath for 2 min. The samples were then diluted 1:4 with Tris-HCl, pH 7.5, and centrifuged at 16,000g for 30 min. Supernatants were decanted into fresh tubes and aliquots were then assayed for cAMP by the method of Brown et al [29].

RESULTS

Effects of Insulin and cAMP on Proliferation of Wild-type Cells

Proliferation of wild-type Cloudman melanoma cells slows or ceases when insulin is added to the culture medium. The effects of the hormone are observed with concentrations as low as 10^{-10} M [26]. This inhibition of proliferation by insulin was abolished when agents known to increase cAMP levels (MSH or MIX), or the cAMP analogue Bt_2cAMP were also added to the culture medium (Table I). Even if cells had been quiescent for several days following exposure to insulin, they resumed proliferation at the same rate as control cultures when fed medium containing no added insulin, or medium containing insulin plus MSH (data not shown). It should be noted in Table I that MSH or Bt_2cAMP (10^{-4} M), when added to the culture medium in the absence of insulin, caused a reduction in the rate of proliferation. These results suggest that insulin modulates the cAMP system of Cloudman cells. Additional evidence for modulation was obtained from measurements of intracellular levels of cAMP following exposure to insulin, MSH, or a combination of the two hormones.

Table I. Effects of Insulin, Isobutylmethylxanthine, Dibutyryl Cyclic AMP, and MSH on the Generation Time of Cloudman S91 Melanoma Cells

Additions to culture medium	Generation time (hr)
None	39
Insulin only	96
MIX only	40
MIX plus insulin	39
Bt_2cAMP only	51
Bt_2cAMP plus insulin	38
MSH only	63
MSH plus insulin	42

Wild-type cells (2×10^4) were inoculated in 25-cm^2 Corning tissue culture flasks described in Methods. Twenty-four hours later, the culture medium was changed and insulin (10^{-9} M), MIX 10^{-5} M), Bt_2cAMP (10^{-4} M), and MSH (2×10^{-7} M) were supplemented to the medium in combinations listed. Cultures were collected in triplicate every 2 or 3 days over a 12-day period and counted in a Coulter Counter. The generation time was determined as described in Methods. These experiments were repeated three times with similar results each time.

Table II. Effects of Insulin and MSH on Intracellular Cyclic AMP Levels

Additions to culture medium	Cyclic AMP, pmol/10^6 cells
None	3.2
Insulin	2.1
MSH	19.6
Insulin plus MSH	12.3

Cells (10^6) were inoculated as described in Methods. The medium was supplemented with either no additions, insulin (10^{-9} M), MSH (2×10^{-7} M), or insulin plus MSH. Each point represents the average of assays from five different cultures, and the variation within each point was less than ± 15%. The experiments were repeated four times with similar results each time.

Insulin lowered basal levels of cAMP and suppressed the MSH-mediated rise in cAMP within the cells (Table II).

Effects of Insulin on Melanin Content

Exposure of pigmented cells to insulin resulted in a decrease in the basal content of melanin and a partial suppression of the MSH-mediated stimulation of melanogenesis. We concluded this from visual observations of melanin in cells pelleted by centrifugation. It is likely that these effects were due to lowered levels of cAMP since it is well documented that melanogenesis is stimulated through increases in intracellular cAMP content [1].

Effects of Insulin and MSH on cA^{dep} Cells

cA^{dep} cells have a requirement for elevated levels of cAMP in order to proliferate [2–4]. This requirement is absolute at 33°C, but only partial at 37°C (Fig. 1). We tested the effects of insulin on proliferation of these cells at the two temperatures (Table III). At 37°C, the cA^{dep} cells were similar to the wild-type cells shown in Table I; that is, they were inhibited by insulin, but this inhibition was overcome when both MSH and insulin were present in the culture medium. At 33°C, however, the cells were strongly inhibited by insulin, even in the presence of MSH; ie, the cells were most sensitive to insulin under conditions where their requirement for elevated levels of cAMP was maximal.

Effects of MSH and Insulin on an Insulin-Dependent Cell Line

When mutant cells were selected for an altered response to either MSH or insulin, they frequently also had an altered response to the other hormone, even though no selective pressure was applied for the latter trait. This phenomenon occurred in about 20% of the mutants. Since the frequency of appearance of a mutant for any given trait is about 1×10^{-6}, it is highly unlikely that the appearance of the

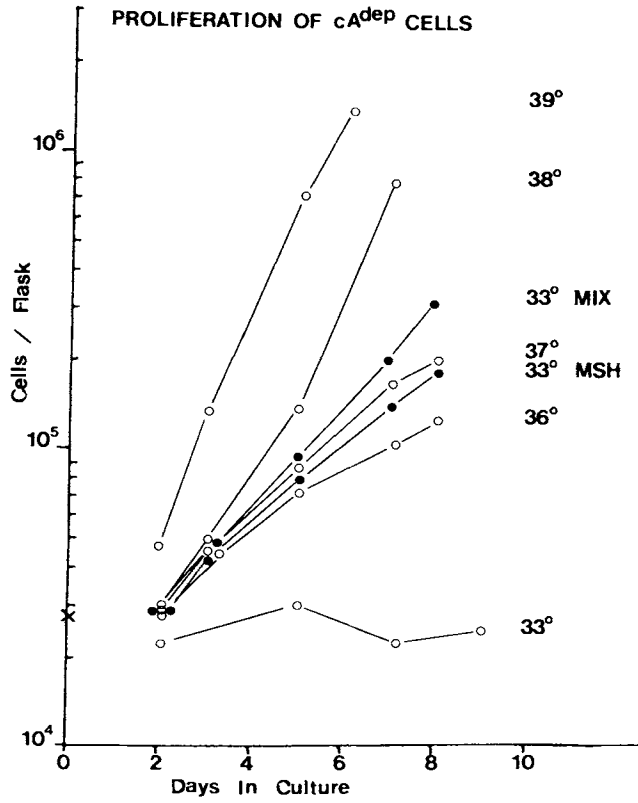

Fig. 1. Proliferation of cA^{dep} cells at various temperatures. MSH (2×10^{-7} M) and methylisobutylxanthine (MIX, 10^{-4} M) were supplemented to the culture medium where noted.

Table III. Effects of Insulin and MSH on Growth of cA^{dep} Cells

Additions to culture medium	Generation time (hr)	
	33°C	37°C
None	no growth	60
MSH (2×10^{-7} M)	72	50
Insulin (6×10^{-8} M)	lysis	137
MSH plus insulin	222	53

Generation times were calculated as described in Methods.

second, unselected trait was a random event. The insulin-dependent mutant line denoted "46" is an example: We isolated a number of variant cell lines by virtue of their ability to proliferate in the presence of insulin [26]. One of these lines, variant 46, not only was resistant to the inhibitory effects of insulin, but actually displayed a requirement for insulin in order to proliferate [26,31]. We found that cell line 46 appeared to have an altered cAMP "system" even though no selective pressure had been applied for this trait. Our observations were as follows: 1) Cells of line 46 did not produce pigment, whether or not MSH was added to the culture medium; 2) proliferation of line 46 was not affected by MSH; 3) intracellular levels of cAMP in line 46 were unaffected by MSH (data not shown).

Effects of MSH and Insulin on Adenylate Cyclase Activity

The above results indicated that the actions of insulin on Cloudman cells are mediated, at least in part, through a suppression of the cAMP-generating system. We therefore examined the effects of exposure of cells to insulin on adenylate cyclase activity. Cells were cultured in the presence or absence of insulin for various time periods. They were then harvested from the culture flasks, and lysed by sonication. The lysates were then assayed for adenylate cyclase activity in the presence or absence of MSH. The results were in paradox to those described above, in that exposure of cells to insulin caused a marked stimulation of MSH-responsive adenylate cyclase activity (Fig. 2). In this experiment, the insulin-dependent line 46 was exposed to insulin (6×10^{-8} M) for 2 days. MSH-responsive adenylate cyclase activity was stimulated maximally by 20 hr. The effects of insulin were seen at concentrations as low as 6×10^{-11} M, and the half-maximal effect was in the nM range (Fig. 3). Exposure of cells to insulin also enhanced adenylate cyclase responsiveness to sodium fluoride and prostaglandin E_1 [32]. Similar results were obtained with wild-type cells and an insulin-resistant variant [32].

DISCUSSION

From a variety of observations we have concluded that insulin interacts with the cAMP system in Cloudman melanoma cells. 1) Cloudman melanoma cells have a cAMP requirement for growth and pigmentation. Insulin lowers cAMP levels and inhibits both growth and pigment formation. 2) The effects of insulin are reversed by agents which raise cAMP levels, or by the cAMP analogue Bt_2cAMP. 3) A temperature-sensitive mutant, cA^{dep}, is most sensitive to the growth-inhibitory effects of

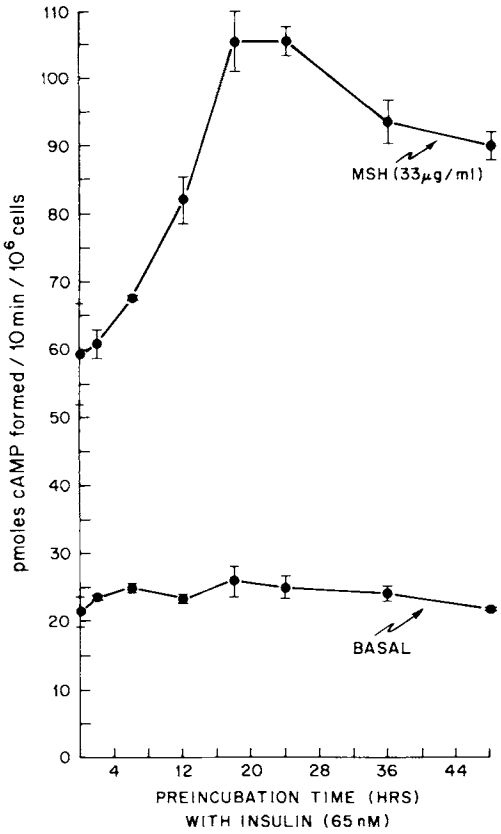

Fig. 2. Time course of the effects of insulin on MSH-responsive adenylate cyclase activity. Cells were exposed to insulin ($6.5\ 10^{-8}$ M) in culture for the time periods indicated. Results shown here are from insulin-dependent cell line 46, which shows no response to MSH in culture. Adenylate cyclase activity was measured in broken cell preparations as described in Methods. Points represent mean ± range of triplicate determinations. Similar results were obtained with wild-type line 1A, and with an insulin-resistant mutant line rev-1.

insulin at the temperature where its requirements for cAMP are maximal. 4) Mutants isolated for alterations in their response to insulin frequently also have altered cAMP systems, even though no selective pressure is applied for these traits. 5) Paradoxically, the MSH-responsive adenylate cyclase system is stimulated when cells are exposed to insulin in culture.

Our evidence suggests that insulin affects multiple aspects of the cAMP system (lowering of intracellular cAMP levels, stimulation of hormone-sensitive adenylate cyclase); however, we know little about the mechanisms by which these interactions occur. There have been several reports of interactions between insulin and the cAMP system in other cells and tissues. It has been known for many years that insulin can cause a decrease in basal cAMP levels and suppress hormone-mediated rises in cAMP. Exposure to insulin has resulted in stimulation of low K_m cyclic nucleotide phosphodiesterase activity [22,23], inhibition of cAMP-dependent protein kinase

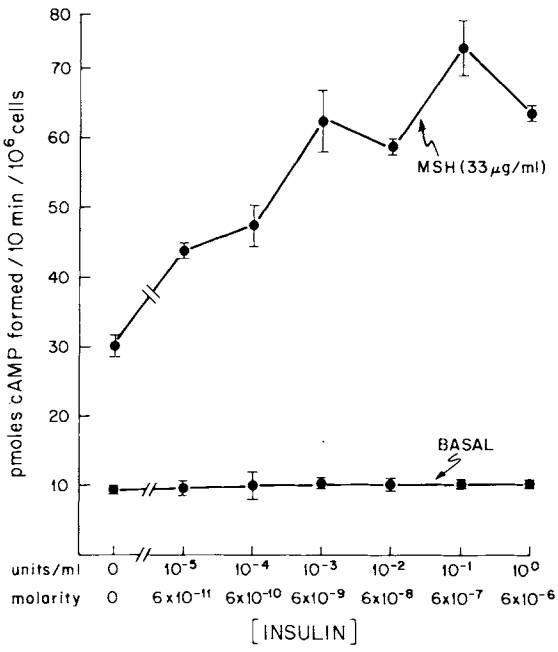

Fig. 3. Effects of exposure of cells to various concentrations of insulin on MSH-responsive adenylate cyclase activity. Cells were preincubated with insulin for 20 hr. Adenylate cyclase activity was measured as described in Methods. Results are from insulin-dependent line 46, which shows no response to MSH in culture. Each point represents the mean and range of triplicate determinations.

activity [20,21], and inhibition of adenylate cyclase activity [10,15,25]. It has also been reported that exposure of human fibroblasts to insulin results in a stimulation of prostaglandin E_1-responsive adenylate cyclase activity [33]. Our findings presented in Figures 2 and 3 may be related to this latter observation.

Many studies suggest that the effects of insulin are mediated through changes in protein phosphorylation/dephosphorylation reactions [eg, 34–43]. In this regard, we have shown through analyses of variant, wild-type, and hybrid cell lines that at least some of the effects of insulin and MSH are mediated through protein phosphorylation/dephorphorylation reactions [44,45]. These observations, in combination with the evidence presented here, suggest there may key regulatory proteins whose state of phosphorylation is determined by interactions between insulin and the cAMP system. Studies are currently underway in hopes of identifying such proteins.

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