Cell Cycle Changes in the Adenylate Cyclase of C_6 Glioma Cells

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ABSTRACT The adenylate cyclase of C_6 glioma cell cultures was characterized for sensitivity to the β-adrenergic agonist isoproterenol, as well as fluoride, and GTP as a function of the cell cycle. The mitotic phase of the cell cycle was emphasized because both the basal cellular cyclic AMP level and the intact C_6 cell's capacity to accumulate cyclic AMP in response to isoproterenol decreased during mitosis. Basal and stimulated adenylate cyclase activities in mitotic cells were decreased relative to the enzyme activities in the G_1, S, and G_2 phases of the cell cycle. Analysis of the β-adrenergic receptor using the radioligand (−) [^{3}H]dihydroalprenolol showed that neither ligand affinity nor receptor density changed during the cell cycle, indicating that the reduced adenylate cyclase activity of the mitotic C_6 cell was not caused by alterations in this hormone receptor. The reduction in the mitotic cell's basal adenylate cyclase activity was more prominent than the decrease in isoproterenol-, fluoride, or GTP-stimulated activities, suggesting that the effectiveness of these enzyme activators (i.e., the efficiency of the coupling mechanism) was not attenuated during mitosis. These studies indicate that the intrinsic catalytic capacity (not the β-adrenergic receptor or the coupling mechanism) of the C_6 adenylate cyclase complex is reduced during mitosis and contributes to the mitotic cell's inability to accumulate and maintain the cyclic AMP concentration at the interphase level.

Intracellular levels of cyclic AMP decrease as cells enter mitosis and then rapidly increase as cells complete mitosis and enter the G_1 phase of the cell cycle (5, 26, 36, 39, 44, 45). To correlate the observed changes in intracellular cyclic AMP levels with cyclic AMP synthesis and hydrolysis, we examined adenylate cyclase (EC 4.6.1.1.) and phosphodiesterase (EC 3.1.4.17.) enzyme activities in synchronized cells. Sodium fluoride or hormone-stimulated adenylate cyclase activity generally was observed to be lower in enzyme preparations from mitotic cells than in those from cells within other phases (G_1, S, G_2)[1] of the division cycle (14, 26, 28). However, basal adenylate cyclase activity increased linearly as cells progressed from early G_1 to the next mitosis (14, 28). Cyclic AMP phosphodiesterase activity was found to increase during mitosis coincident with a decline in adenylate cyclase activity (26). An efflux of cyclic AMP during mitosis has also been observed (44).

These previous studies did not correlate cell cycle-specific alterations in the adenylate cyclase activity with changes in the intact cell's capacity to accumulate cyclic AMP. In the most complete study reported, both basal and hormone-elevated levels of cellular cyclic AMP were assayed during the cell cycle using synchronized Cloudman melanoma cells (42). These studies demonstrated that an increased density of melanocyte-stimulating hormone (MSH) receptors in G_2 was associated with a G_2 phase-specific sensitivity to MSH (41, 42). However, because adenylate cyclase enzyme activity was not measured, a correlation could not be made between the cellular cyclic AMP level and the enzyme activity. Thus, a thorough examination and comparison of the cell cycle phase-dependent changes in cyclic AMP levels, adenylate cyclase specific activities, and hormone receptor characteristics has yet to be performed on a single experimental system.

[1] *Abbreviations used in this paper:* DMEM, Dulbecco's modified Eagle's medium; FMF, flow microfluorimetry; G_1 phase, the period after the completion of mitosis and before the initiation of DNA synthesis; G_2 phase, the period after the S phase and before mitosis; [^{3}H]DNA, (−)[^{3}H]dihydroalprenolol; M phase, mitosis; MSH, melanocyte-stimulating hormone; PBS-CM, Dulbecco's phosphate-buffered saline supplemented with Ca^{+2} and Mg^{+2}; PBS-CMF, Dulbecco's phosphate-buffered saline without Ca^{+2} or Mg^{+2}; S phase, the period of DNA replication in proliferating cells.

We chose the C_6 glioma cell line for this particular study because it is responsive to physiological concentrations of β-adrenergic agonists such as isoproterenol (13). Membranes prepared from these cells possess both a β-adrenergic-sensitive adenylate cyclase activity (4, 13, 20) and measurable β-adrenergic receptor-binding sites for the radiolabeled antagonist (−) [^{3}H]dihydroalprenolol ([^{3}H]DHA) (20, 21). The C_6 cell line seemed especially suited as a model for assaying these activities during the cell cycle because of its apparent tight "coupling" between occupancy of the β-adrenergic receptor site by an agonist molecule and the subsequent activation of the adenylate cyclase moiety (21). We chose to focus on the interval of the cell cycle immediately before and after mitosis because of the suggested importance of cyclic AMP during mitotic events (5, 39).

In this communication we present evidence that both the basal cyclic AMP levels and the accumulation of cyclic AMP in response to the β-adrenergic agonist isoproterenol are depressed in mitotic C_6 cells relative to cells synthesizing DNA, cells in G_2, or cells in early G_1. Furthermore, both basal and stimulated adenylate cyclase-specific activities, but neither [^{3}H]DHA binding site affinity nor density, decrease coincident with intact cell changes in mitosis. Surprisingly, there was an increase in the mitotic cell's stimulation index, which is calculated by dividing the effector-augmented adenylate cyclase activity by the basal enzyme activity.

MATERIALS AND METHODS

Cell Culture

Cultures of C_6 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine and 10% fetal calf serum (the growth medium) in the absence of antibiotics. The fetal calf serum was incubated before use for 18 h at 37°C to hydrolyze serum cyclic AMP and then heat treated at 56°C for 30 min to inactivate complement and phosphodiesterase activities. Cells were maintained at 37°C in a 10% CO_2-90%, air, humidified atmosphere. Cell cultures were judged free of mycoplasma contamination by both measurement of [^{3}H]uridine/[^{3}H]uracil incorporation (35) and scanning electron microscopy.

Broken Cell Preparations

The growth medium was removed and the culture plates were rinsed twice with 10 ml of ice-cold Dulbecco's phosphate-buffered saline without Ca^{+2} or Mg^{+2} (PBS-CMF) and drained well. Subsequent steps were performed at 4°C. The cells were quickly rinsed with 5 ml of 50 mM Tris-HCl, pH 7.4, at 4°C, and the cells scraped from the plates into a chilled tube in 10 mM Tris-HCl, pH 8.0, at 4°C and allowed to swell for 10 min. The enlarged cells were homogenized in a tight-fitting 10-ml glass-Teflon Potter Elvehjem-type tissue grinder by five slow strokes. After at least 90% of the cells were ruptured, the homogenate was used immediately for the determination of adenylate cyclase activity.

Membranes from C_6 cells to be used for characterizing the β-adrenoreceptor binding sites were prepared by bringing the homogenate to 1 mM Mg^{++}. The intact cells and nuclei then were removed by centrifugation for 5 min at 1,200 rpm in a Sorvall HL-4 rotor (DuPont Instruments-Sorvall, DuPont Co., Newtown, Conn.) (max = 200 *g*). The supernate was then centrifuged in a Sorvall SS 34 rotor (max = 30,000 *g*) for 20 min. The pellet was rinsed with 1 ml of 10 mM Tris-HCl and resuspended in a suitable volume of 10 mM Tris-HCl with two strokes of the homogenizer.

Adenylate Cyclase Assay

The final concentration of the components used in the standard adenylate cyclase reaction mixture were: 80 mM Tris-maleate, pH 8.3, at 30°C, 1 mM ATP, 10 mM $MgCl_2$, 1.0 mM theophylline, and a nucleotide-regenerating system consisting of 40 μg (24 U) of pyruvate kinase, 2.5 mM phospho(enol)pyruvate, and 5 mM KCl, in a final volume of 500 μl. Test agents, when used, were included in this reaction volume. The reaction was initiated by the addition of 50 μl of enzyme preparation to the assay tubes, which had been preequilibrated to 30°C for at least three min in a shaking water bath. After incubation for exactly 10 min, the reaction was terminated in a boiling water bath for 3 min. The tube was then placed in an ice-water bath. Experimental blanks were boiled upon lysate addition without prior incubation to assess the effect of additives on the cyclic AMP-binding assay and to detect any endogenous cyclic AMP. All the boiled samples were centrifuged at 1,000 × *g* for 20 min before aliquots of the supernate were assayed, with or without dilution, for cyclic AMP content.

Under these conditions the basal and stimulated adenylate cyclase activities were linear for protein concentration (30-200 μg) and linear for at least 10 min of incubation at 30°C. Better than 85% of the cyclic AMP generated was preserved under these conditions. The activity of adenylate cyclase is reported as picomoles of cyclic AMP formed per minute per milligram protein determined during a 10-min incubation.

Cyclic AMP Analysis

This was modified from the method of Brown et al. (3). Protein kinase was prepared from bovine adrenal glands and was purified using the procedure described in reference 3. Cyclic AMP values were obtained by the linear regression analysis of standard curves constructed from the log of the bound [^{3}H]cyclic AMP (cpm) as a function of the log of the total picomoles of unlabeled cyclic AMP. The coefficient of variation of this competitive binding assay was ±4%. Incubation of a sample with cyclic nucleotide phosphodiesterase eliminated 90-100% of the competition between an unknown and [^{3}H]cyclic AMP for the binding protein.

Cyclic AMP Content of Intact Cells

Intact cell experiments were conducted in a 37°C warm room. Cells on 35-, 60-, or 100-mm culture plates were kept in a 37°C cell culture incubator until they were used. Culture medium was removed with a vacuum pump-aspirator assembly and the cells were quickly rinsed with two 1.8-ml volumes of Dulbecco's phosphate-buffered saline supplemented with Ca^{+2} and Mg^{+2} (PBS-CM). The basal cyclic AMP level was determined after the addition of 1.0 ml of 5% TCA at 4°C. Alternatively, to examine the effects of β-adrenergic agonists on intracellular cyclic AMP levels, we added PBS-CM with the phosphodiesterase inhibitor Ro 20-1724 to cover the cells (1.8 ml). Within 30 s the incubation with or without β-adrenergic agonist and/or antagonist was begun. After an appropriate incubation time the buffer was removed and 1.0 ml of cold 5% TCA was added to extract the cyclic AMP. The plates were then kept at 4°C for ~30 min. TCA was removed from the cell extract by ether extraction. Cyclic AMP was measured as described above.

(−) [^{3}H]DHA Binding to Membrane Preparations

The assay of the [^{3}H]DHA specific binding sites of C_6 cells utilized a reaction mixture identical to that of the adenylate cyclase assay. The reaction was initiated by the addition of the membrane protein (30–100 μg of protein) to the otherwise complete mixture (in polystyrene tubes), giving a final volume of 100 μl. The binding was allowed to proceed for 13–18 min at 30°C in a shaking water bath. To end the binding reaction, the contents of the tube were diluted with 1 ml of ice-cold 50 mM Tris-HCl, 20 mM $MgCl_2$, pH 8 at 0°C (buffer A) and rapidly poured over a glass fiber GF/c filter held in a Millipore rapid-sampling vacuum manifold (Millipore Corp., Bedford, Mass.). The tube was then washed with another 4 ml of buffer A.

After the filtration of all the incubation tubes, the vacuum was increased to remove trapped water containing [^{3}H]DHA. The filters were then placed in scintillation vials and mixed in toluene-based scintillation cocktail (90% toluene, 10% vol/vol Biosolv BBS-3, and 0.4% vol/vol 2a70). The membrane-bound [^{3}H]DHA was determined in a Beckman scintillation counter (35% efficiency) after the filters had clarified (~2 h later). Under these conditions the steady-state association of 10 nM [^{3}H]DHA was attained within 1 min and remained constant for at least 20 min. Binding was proportional to the membrane concentration, was saturable, and could be reversed by the addition of unlabeled alprenolol or propranolol. Specific binding was defined as the difference in [^{3}H]DHA bound in the presence and absence of 10^{-5}M (−) alprenolol (unlabeled), each performed in triplicate. Nonspecific binding (that [^{3}H]DHA bound in the presence of unlabeled [−] alprenolol) was independent of incubation time and dependent on [^{3}H]DHA concentration. Scatchard analysis (31) of specific binding data was utilized to determine the binding capacity and K_D (dissociation constant) of [^{3}H]DHA of cell lysates.

Cell Synchronization

Flasks of stock cells were trypsinized and diluted in the proper volume of medium such that there were 20-30 × 10^4 cells/cm^2 of surface area and, 24 h later, the first of two thymidine blockades commenced.

Cells were exposed to 0.1 mM thymidine in growth medium for ~17 h, washed free of thymidine, and incubated for ~11 h, incubated in 1 mM thymidine for an additional 13 h, and again rinsed free of thymidine. Such exposure to 1 mM thymidine had no effect on the ability of the C_6 cell population to resume proliferation (detected by the incorporation of [^{3}H]thymidine during a 20-h labeling period) compared to unsynchronized cultures; the labeling indices were 91 and 94%, respectively. After the release from the second thymidine block, cells were allowed to progress into S phase for ~4 h. Sterile colcemid (in growth medium) was added to give a final concentration of 0.05 μg/ml (0.13 μM). The incubation was continued for 6 h, allowing an accumulation of up to 70% of the cells in mitosis (late prophase). The colcemid blockade was ended by rinsing the cells two times with DMEM and adding complete growth medium. 1 h later the medium was replaced with fresh growth medium. Less than 5% of the cells remained in mitosis after 1 h in colcemid-free medium.

The mitotic index (MI; cells in late prophase to early telophase) was determined on ethanol-glacial acetic acid- (7:3) fixed cells by counting 400–600 cells using a phase microscope fitted with an ocular grid. The number of cells per culture plate was assessed by counting the trypsinized cultures either with the flow microfluorimeter or in a hemocytometer. Duplicate plates were assayed. Cultures were labeled with [^{3}H]thymidine, fixed, and processed for autoradiography by standard methods (2). These techniques, along with flow microfluorimetry (FMF), were used during an experiment to analyze the extent and duration of the synchrony and the rate at which the cells progressed through the cell cycle.

Materials

The established glioma cell line C_6 was obtained from the American Type Culture Collection. Fetal calf serum from Flow Laboratories Inc. (Rockville, Md.) and L-glutamine (Grand Island Biological Co. [GIBCO], Grand Island, N. Y.) were used to supplement DMEM from GIBCO. ATP, GTP, and cyclic AMP were purchased from Sigma Chemical Co. (St. Louis, Mo.). [^{3}H]thymidine (30-60 Ci/mmol), [^{3}H]cyclic AMP (40–60 Ci/mmol), [^{3}H]cyclic AMP (40–60 Ci/mmol), and (−) [^{3}H]DHA HCl (40–60 Ci/mmol) were obtained from New England Nuclear (Boston, Mass.). ATP, GTP, cyclic AMP, phospho(enol)pyruvate, bovine 3′,5′-cyclic nucleotide phosphodiesterase, pyruvate kinase, L-isoproterenol HCl, L-epinephrine, and L-norepinephrine HCl were purchased from Sigma Chemical Co. L-propranolol HCl and D-propranolol HCl came from Ayerst Laboratories (New York), and D-isoproterenol-D-bitartrate was purchased from Hassel Products, Inc. (Bellport, N. Y.). Norit SG Extra charcoal was purchased from J. T. Baker Chemical Co. (Phillipsburg, N. J.). Whatman GF/C filters were used. Beckman Instruments, Inc. (Fullerton, Calif.) manufactures BioSolv BBS-3, and 2a70 was purchased from Research Products International Corp. (Elk Grove Village, Ill.). Colcemid was obtained in lyophilized form from GIBCO. Calbiochem-Behring Corp., American Hoechst Corp. (San Diego, Calif.) supplied propidium iodide. 4-(3-butoxy-4-methoxy)-2-imidazolidinone (Ro 20-1724) was the gift of Dr. H. Sheppard, Hoffmann-LaRoche, Inc. (Nutley, N. J.).

RESULTS

Cyclic AMP Levels in Synchronized C_6 Cultures

The basal intracellular level of cyclic AMP was measured during the hours before and after mitosis (Fig. 1). The mean cyclic AMP levels of cultures showed a significant and transient decrease at a point coinciding with the peak mitotic index. As the cell population entered G_1, the amount of intracellular cyclic AMP invariably increased to levels meeting or exceeding those found before mitosis.

Responsiveness of Intact, Synchronized C_6 Cells to β-Adrenergic Stimulation

(−) Isoproterenol (0.1 μM) is capable of rapidly elevating the intracellular level of cyclic AMP in cultured C_6 glioma cells (13). This catecholamine stimulation was more pronounced in the presence of the phosphodiesterase inhibitor Ro-20-1724 (15 μg/ml), and was rapidly inhibited upon addition of the β-adrenergic antagonist (−) propranolol (not shown), consistent with the stimulation of adenylate cyclase activity through the β-adrenoreceptor. The cyclic AMP accumulation in response to 0.1 μM (−) isoproterenol reached a maximum in ~20 min (~5,000 pmol cyclic AMP/mg protein) of which the initial 3–5 min were linear (data not shown). Because it was desirable to

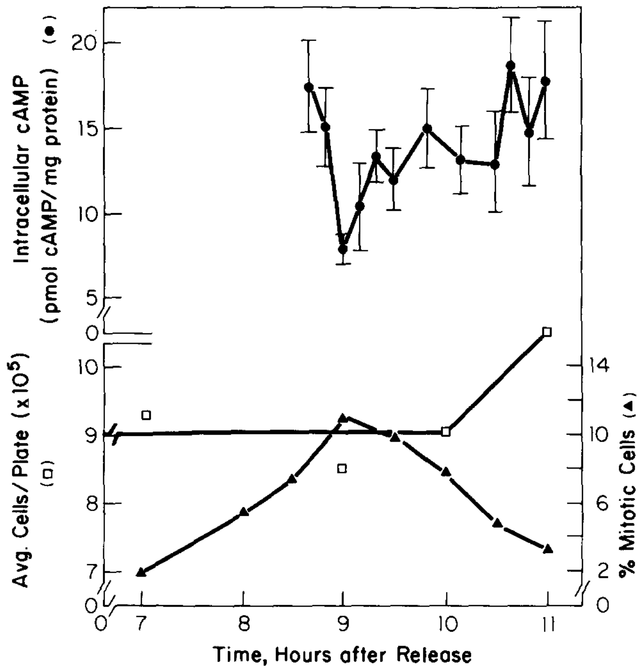

FIGURE 1 Basal cyclic AMP levels during the cell cycle. Basal cyclic AMP was extracted from rinsed cultures with 5% TCA. The mean ± SE of cyclic AMP levels is shown on the graph. Cell counts were by the FMF technique. These results were representative of four different synchrony experiments.

rapidly measure the (−) isoproterenol responsiveness of synchronized C_6 cells before adenylate cyclase desensitization (8, 43) or cyclic AMP efflux (29) occurred, such cells were incubated with hormone for only 2 min before the reaction was terminated with TCA. The measured cyclic AMP was therefore an indication of the initial rate or activity of adenylate cyclase rather than of the total capacity of the C_6 cells to accumulate cyclic AMP. Control studies using multiple cultures confirmed that the experimental variability of the 2-min incubation period was <10%.

The accumulation of cyclic AMP during a 2-min exposure to 0.1 μM (−)isoproterenol was measured in double thymidine synchronized cells that had subsequently been treated with 0.13 μM colcemid during mid-S phase. Experiments performed to examine the effect of colcemid on cellular hormone response showed that a concentration of colcemid (1 μM) eightfold higher than that used for mitotic arrest (0.13 μM) had no significant effect on the isoproterenol responsiveness of exponentially growing C_6 cell cultures. This synchronization technique was employed for the intact cell hormone stimulation studies as well as the subsequent analysis of adenylate cyclase and [^{3}H]DHA-binding activities. The varying responsiveness of the cells to isoproterenol during the cell cycle is shown in Fig. 2, which is a representative experiment using both high and low density cultures. The mean cyclic AMP accumulation in the higher density cultures remained constant during ~5 h before mitosis (regardless of the presence or absence of colcemid) until a significant decrease in sensitivity occurred at 11 h, the time of maximal accumulation of mitotic cells in this experiment. Isoproterenol responsiveness increased after the cells were released from mitotic arrest and had advanced into the G_1 phase of the cell cycle. The low density cultures showed similar changes but exhibited a more dramatic increase as the cells advanced from M to the early G_1 phase.

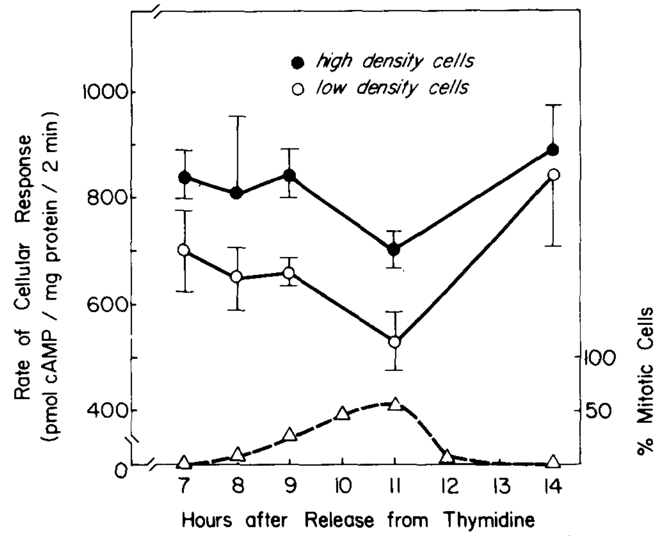

FIGURE 2 Effect of cell cycle traverse on isoproterenol stimulation of intact cells. Cells were synchronized by double-thymidine blockade followed by colcemid addition 5.5 h after removal of the thymidine. The synchronized progression of cells through the cell cycle was assessed by FMF (data not shown) and MI. (maximum = 48% at 11 h). Cyclic AMP levels, after a 2-min stimulation of intact cells by 0.1 μM (−)isoproterenol, were determined on three separate cultures at each time point after ether extraction of TCA as described in Materials and Methods. Colcemid was removed at 11 h and the cultures were incubated with isoproterenol 3 h later at 14 h (early G_1 phase). Cell densities were determined in late S phase: higher density (●) = 3.5×10^6 cells/60-mm plate; low density (○) 1.3×10^6 cells/60-mm plate. Significant differences (Student's *t* test) were observed between isoproterenol responsiveness in mitotic (11 h) cultures and higher density cultures at 9 h ($P < 0.05$) and at 14 h ($P < 0.1$) as well as lower density cultures at 9 h ($P < 0.05$) and at 14 h ($P < 0.05$). The data from FMF analysis and MI determination were used to determine the percentage of cells accumulated in mitosis.

Cell Cycle and Adenylate Cyclase Activity

The adenylate cyclase activity in homogenates of unsynchronized C_6 cells is maximally stimulated by 10 μM GTP (Fig. 3), with half-maximal stimulation of 0.5-1 μM GTP. (−)Isoproterenol, another stimulator of adenylate cyclase activity (Fig. 3), was more effective than GTP alone and showed a biphasic dependence on the GTP concentration. As in intact C_6 cells, isoproterenol stimulation of the adenylate cyclase possessed the characteristics of a β-adrenergic receptor: (−)agonists were more effective cyclase stimulators than (+) agonists; (−)propranolol prevented isoproterenol stimulation at a 100-fold lower concentration than did (+)propranolol; and (−)isoproterenol was an effective agonist at 5- to 10-fold lower concentrations than was (−)epinephrine or (−)norepinephrine. Sodium fluoride, the third activating agent which was used in these cell cycle studies, stimulated the enzyme activity to about the same degree as did GTP (compare Figs. 3 and 4). However, unlike GTP, NaF consistently inhibited the isoproternol stimulation of adenylate cyclase (Fig. 4). These results serve to illustrate the difference in the action and interaction of these agents on the C_6 adenylate cyclase activity.

Adenylate cyclase activity was measured at three different time points within the cell cycle: at approximately t = 6 h, 10 h, and 14 h, corresponding to the late S, M, and early G_1 phase, respectively. Four different plates of C_6 cultures (each yielding separate homogenates) were assayed at each cell cycle time point to measure cyclase activity (Table I). In these experiments, sodium fluoride, GTP, and isoproterenol were capable of stimulating adenylate cyclase to activities 3- to 24-fold above basal enzyme activity. It is evident (Table I) that the stimulated adenylate cyclase enzyme decreased in specific activity as the cells traversed the cell cycle from the latter part of the S phase to mitosis. Moreover, homogenates from G_1 phase cells generally had significantly elevated specific activities relative to the activities observed during mitosis. This is consistent with the observations of reduced levels of basal cyclic AMP and the decreased rate of cyclic AMP synthesis during incubation of mitosis-enriched cells with isoproterenol. Although a combination of GTP and isoproterenol increased the enzyme activity observed in the synchronized cells (not shown), it did not

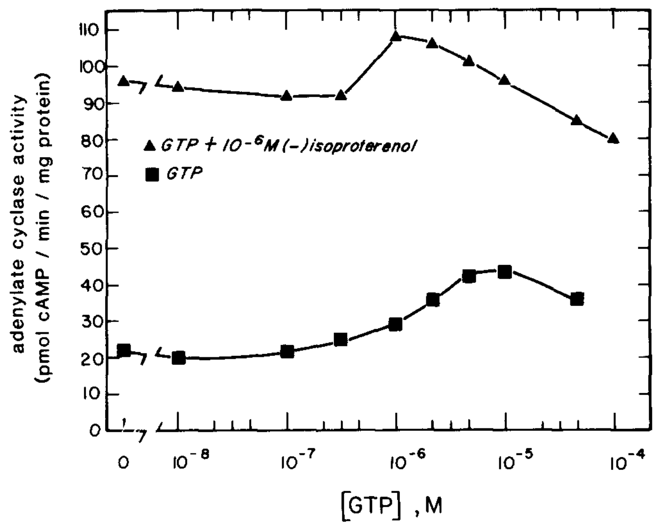

FIGURE 3 Effect of isoproterenol on GTP stimulation of the adenylate cyclase activity of C_6 homogenates. Incubation of homogenates with either no hormone (■) or 1 μM (−)isoproterenol (▲) in the presence of different concentrations of GTP was performed in standard cyclase assay conditions as described in Methods and Materials. Different homogenates were used for each curve; basal activity (pmol cAMP/min · mg protein) was 20 (■) and 22 (▲).

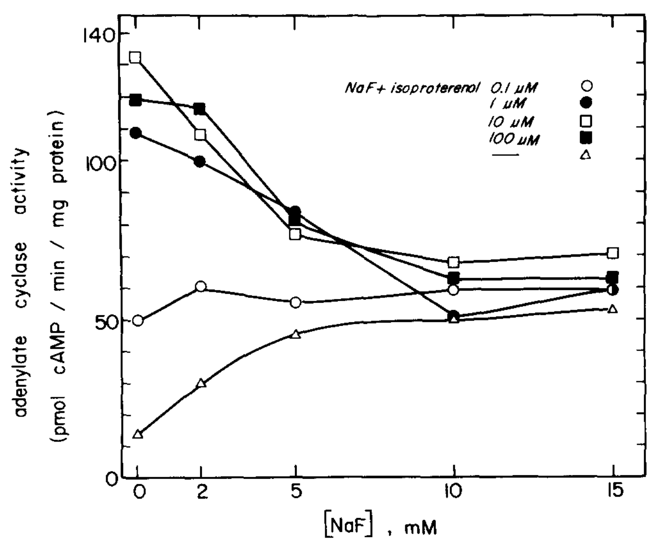

FIGURE 4 Fluoride inhibition of isoproterenol-stimulated adenylate cyclase. Adenylate cyclase activity was determined in the presence of fluoride alone (△), or fluoride plus the following isoproterenol concentrations: 0.1 μM (○), 1 μM (●), 10 μM (□), 100 μM (■). The cyclase assay conditions and detection of cyclic AMP were performed as described in Materials and Methods.

TABLE I

Basal and Stimulated Adenylate Cyclase Activities as a Function of the Cell Cycle

Phase of cell cycle	Adenylate cyclase specific activity			
	Basal	10 μM GTP	10 μM (−)Isoproterenol	10 mM NaF
S	25 ± 1.5 (4)*	82 ± 8.4 (4)*	186 ± 28 (4)*	60 ± 0.8 (4)*
M	3.5 ± 2.2 (4)	35 ± 3.6 (4)	86 ± 11 (3)	16 ± 16 (4)
G_1	11 ± 3.6 (3)‡	61 ± 6 (3)*	98 ± 19 (3)	35 ± 2.3 (3)
US§	14 ± 2 (15)	41 ± 7.0 (12)	116 ± 19 (9)	48 ± 6.1 (34)
		Fold increase over basal (stimulation index)		
S		3.3	7.4	2.4
M		10.0	24.0	4.6
G_1		5.6	8.9	3.2
US		2.9	8.3	3.4

The adenylate cyclase activity in each culture dish was measured in duplicate using standard assay conditions. The activities are arranged within each column as a mean value ± SE for the number of dishes or observations in parentheses. All activities are expressed as pmol cyclic AMP formed/min·mg protein from a 10-min incubation. Within each experiment, adenylate cyclase activities were compared by Student's *t* test between S and M phases or between G_1 and M; asterisks by either S or G_1 activities indicate the statistical significance of the difference in relation to the M phase enzyme activity. These are data from one representative synchrony experiment.

* $P < 0.005$.

‡ $P < 0.01$.

§ Unsynchronized cultures.

reverse the decline in isoproterenol-stimulated specific activity observed as cells progressed from S to M. Therefore, the increased adenylate cyclase activation that can be promoted by GTP and isoproterenol together (Fig. 3) does not reverse the M phase-specific decline in adenylate cyclase activity. However, it was generally observed that M phase homogenates were more responsive to GTP, NaF, and isoproterenol, i.e., there was an increased ratio or index of stimulated adenylate cyclase activity to basal activity in M phase homogenates, relative to such ratios calculated from the S or G_1 phase enzyme activities (see Table I).

Cell Cycle Dependence of [³H]DHA Binding to Membrane Preparations

The β-adrenergic antagonist (−) [^{3}H]DHA was used to characterize the β-adrenergic receptor as a function of the cell cycle. [^{3}H]DHA bound to membrane preparations from unsynchronized C_6 cells in a manner typical of β-adrenergic stimulation: by use of incubation conditions identical to those used for the adenylate cyclase assay to facilitate comparisons, the binding was observed to be rapid and reversible. Competition for the binding sites was stereospecific by both β-agonists and β-antagonists and in the same order of potency as observed for adenylate cyclase activation (see above). Agonist competition was sensitive to GTP as previously reported (19, 20) but not to NaF. The binding was also saturable as examined by measuring the specific binding (see Materials and Methods) of increasing [^{3}H]DHA concentrations to the membrane preparations. The K_D of [^{3}H]DNA binding could then be determined by analyzing these data by the methods of Scatchard (34). Fig. 5 shows the binding isotherm of one such experiment and the linear Scatchard plot generated from these data. The K_D calculated from this experiment was 1.5 nM and the binding capacity of the membranes equalled 0.46 pmol [^{3}H]DHA bound/mg protein. Hill coefficients ranging from 0.9 to 1.2 indicate that C_6 cell membrane β-receptors are neither positively nor negatively cooperative for binding of this tracer ligand.

To determine whether changes in the β-adrenergic receptor might account for the alterations in isoproterenol-responsive adenylate cyclase activity in S vs. M cells, we measured the K_D and binding capacity of the β-adrenergic receptor sites in late S, M, and early G_1 phase cell membranes by saturation binding assays and Scatchard analysis. Table II presents the data from two cell synchrony experiments. There was clearly no change in the receptor K_D for [^{3}H]DHA between synchronized and unsynchronized populations nor within those phases of cell cycle that were examined. There was also little difference in the membrane binding capacity for the radiolabeled antagonist in preparations from thymidine-blocked (G_1/S phase), S phase, or mitotic cells. It is, therefore, doubtful that alterations in the β-receptor sites are the basis for the decreased enzyme activity (or increase in stimulation index) observed between S and M phase cells. The mean binding capacity did appear to increase in early G_1 to a quantity roughly equal to that found in unsynchronized (exponentially growing) cultures and may indicate one mechanism by which the adenylate cyclase activity increases upon progression from the M to G_1 phase. In summary, the only difference in β-receptor sites that was detected

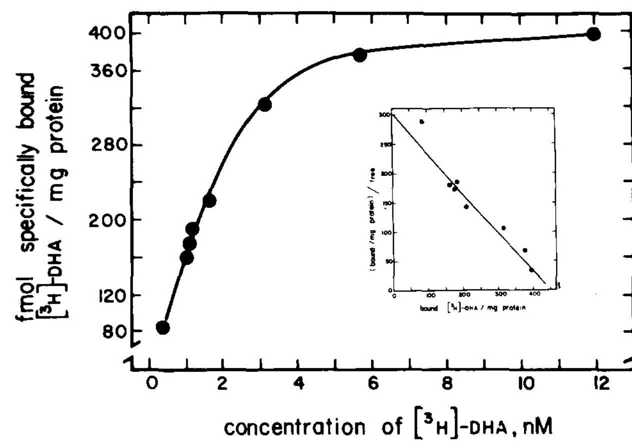

FIGURE 5 Equilibrium saturation binding for [^{3}H]DHA and the Scatchard plot (*inset*) generated from these data. Various concentrations of [^{3}H]DHA were incubated with the C_6 membrane preparation and the binding was terminated as described in Materials and Methods. The K_D and maximal binding capacity evaluated in this experiment are 1.5 nM and 0.46 pmol radioligand bound/mg protein, respectively.

TABLE II

[^{3}H]DHA Binding as a Function of the Cell Cycle

Phase of cell cycle	K_D	Binding capacity
	nM	*pmol specifically bound/mg protein*
G_1/S	1.3	0.23
Mid to late S	1.6 ± 0.3	0.27 ± 0.02
M	1.4 ± 0	0.28 ± 0.06
G_1	1.4	0.36
Unsynchronized*	1.4 ± 0.5	0.38 ± 0.05

The saturation binding of [^{3}H]DHA was determined at equilibrium (18 min at 30°C) as described in Materials and Methods. Each assay was performed on a membrane preparation with six different radioligand concentrations. Both nonspecific and total binding were measured in triplicate. The data are given as the mean ± SD for duplicate observations of the dissociation constant (K_D) and the maximal binding capacity as determined by a Scatchard plot.

* $n = 6$.

in S, M, and G_1 phase cells was a possible increase in the number of specific [^{3}H]DHA-binding sites as cells completed M and entered the G_1 phase.

DISCUSSION

Decreased cyclic AMP levels were found in C_6 cultures enriched for mitotic cells using chemical synchronization methods. The lowest cyclic AMP levels invariably coincided with the peak in MI. At a point after the majority of the cells had completed mitosis, but still early in the G_1 phase, there was a rapid increase in the cellular cyclic AMP content. The present and previous observations (5, 26, 36, 39, 44, 45) that mammalian mitotic cells contain less cyclic AMP per milligram of cell protein than cells in interphase (i.e., the G_1, S, and G_2 phase) indicate the generality of this phenomenon.

We also examined the ability of the β-adrenergic agonist isoproterenol to activate the cyclic AMP synthetic enzyme adenylate cyclase and increase the accumulation of cyclic AMP in whole cells. For these hormone response studies using intact C_6 cells, we chose a 2-min incubation time with isoproterenol because during this short interval the accumulation of cyclic AMP in unsynchronized cultures was linear, reflecting the enzymatic rate of cyclic AMP synthesis. Complications brought on by the secretion of intracellular cyclic AMP in C_6 cells in response to the elevated cyclic AMP levels produced by hormone stimulation could be ignored, as this phenomenon was observed only after longer periods of incubation with hormone (29). Similarly, hormonal desensitization during this period would be negligible (8, 43). The rate of cyclic AMP accumulation in response to isoproterenol stimulation remained relatively constant during the mid to late S phase and early G_2 phase but decreased as cells entered mitosis. After the mitotic phase, as cells entered G_1, the cells exhibited a significantly enhanced responsiveness to isoproterenol. Although hormone responsiveness increases with C_6 cell density,[2] the cell cycle-related changes were density independent. Moreover, in contrast to the previous observation that microtubule-disrupting agents increase the cellular accumulation of cyclic AMP in response to hormones (32), the C_6 cells β-adrenergic hormonal response is not significantly affected by colchicine or colcemid. These data confirm the recent results of Gibbs et al. (12). Thus, the lower response of mitotic C_6 cells to isoproterenol appears to reflect a decreased capacity to synthesize cyclic AMP during mitosis.

Changes in hormonal responsiveness have been previously shown to occur during unusual physiological or pathological conditions. For example, responsiveness to a hormone decreases after extended cellular exposure to that hormone, a phenomenon known as hormonal desensitization (25). Supersensitivity that occurs after an extended period of hormonal deprivation has been associated with an increased density of receptors. Alterations in hormone response that occur upon neoplastic transformation (7, 22, 27, 37) may be related to the receptor changes (38). Other pathological states are known to be caused by changes in hormone receptors and/or hormonal response (9, 11, 15).

We examined the β-adrenergic receptor sites and adenylate cyclase activity in synchronized C_6 cells in an attempt to determine which component(s) of hormone-stimulated adenylate cyclase activity correlates with the decreased cyclic AMP level in the mitotic cell. Such an undertaking, it was hoped, might also indicate the physiologically relevant mechanism(s) by which cells autoregulate their synthesis of cyclic AMP. Direct binding studies using the β-adrenergic radioligand (−)[^{3}H]DHA indicated that no change in β-adrenergic receptor-ligand affinity occurred during the S, M, and early G_1 phases. Receptor density did not differ in S and M phase membranes but increased in early G_1 relative to S and M phase cells, suggesting that β-adrenergic hormone binding sites are maintained at a constant density in the C_6 plasma membrane, just as total cellular protein increases linearly with the cell cycle (31).

The mean specific activity of adenylate cyclase was lower in membrane preparations from mitotic cells than from either S phase or early G_1 phase cells. This was generally true regardless of whether the enzyme activity was assayed in the absence or presence of GTP, fluoride, or isoproterenol (Table I). These changes are consistent with the reduced cyclic AMP levels and the reduced rate of accumulation of cyclic AMP in response to isoproterenol that were observed in whole mitotic C_6 cells. They also confirm earlier reports (14, 26, 28) which indicated that the adenylate cyclase activity of mitotic cells is less than the activity in S or G_1 phase cells, in contrast to one other report (23). The more or less uniform decrease that we observed in the cyclase activity of mitotic cells as well as the subsequent increase in the G_1 enzyme activity are interesting in light of the data (e.g., reference 21, and our Figs. 3 and 4) that each of these adenylate cyclase effector molecules (GTP, NaF, hormone) acts by separate mechanisms to increase the C_6 cell's enzyme activity. Moreover, we found no evidence for an unequal distribution of soluble cytoplasmic substances (e.g., calmodulin) within different phases of the cell cycle (as assayed on sucrose gradient-purified C_6 cell membranes) which could account for the phase-specific changes in cyclase activity.[2]

A most unexpected observation was that, while both the basal and effector-stimulated adenylate cyclase activities decreased during mitosis relative to the S phase and G_1 phase activity, the adenylate cyclase stimulation index (stimulated cyclase activity/basal activity) increased in mitotic cells relative to the S or G_1 phases. Thus, although the basal activity decreased during mitosis, the three enzyme activators (GTP, NaF, and isoproterenol) increased the basal adenylate cyclase activity more efficiently during this phase of the cell cycle.

This paper further substantiates that the plasma membrane of the mitotic cell is functionally altered (1). Structural changes in the membrane have previously been reported in G_2 (41, 42) and mitosis (10, 18, 30). We also observed a quantitative

[2] R. F. Howard and J. R. Sheppard. Unpublished observations.

change, but no qualitative changes, in the membrane-associated β-adrenergic receptor as cells completed mitosis and entered the G_1 phase. This may be partially responsible for the increased hormone responsiveness of the G_1 phase cell. The molecular mechanisms leading to the decreased absolute cyclase activity and the apparent increase in coupling efficiency of the adenylate cyclase in mitotic C_6 cells may involve changes in the G/F (21) or N (6, 17) protein which is required for the stimulation of enzyme activity. This protein and/or the intrinsic catalytic component of the adenylate cyclase membrane complex could be affected by the dynamic physiological changes occurring in the mitotic cell. While the biological importance of this change in membrane function is currently unclear, the decreased basal and stimulated adenylate cyclase specific activities during mitosis could conceivably reflect a relationship between cyclic AMP metabolism and cytoskeletal organization (24, 32, 33, 40).

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