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Expression of β-Adrenergic Receptors in Synchronous and Asynchronous S49 Lymphoma Cells. II. Relationship between Receptor Number and Response

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SUMMARY

We have used two experimental approaches—receptor inactivation with an irreversible antagonist and changes in receptor expression during passage of cells through the cell cycle—to explore the relationship between β-adrenergic receptor number and response in intact S49 lymphoma cells. β-Receptors in asynchronous cultures of S49 cells were blocked to varying degrees with the irreversible antagonist bromoacetylalprenololmenthane (BAAM). Blockade by BAAM was noncompetitive and did not alter the affinity of receptors for the agonist isoproterenol. Intracellular accumulation of cAMP in response to 1 μM isoproterenol was proportional to receptor number both at times of initial and maximal accumulation. In contrast, when intracellular accumulation of cAMP in response to isoproterenol was measured in synchronized cultures of S49 cells (obtained by centrifugal elutriation), a notably different relationship was observed. Cells were least responsive, that is, receptors appeared "uncoupled," during S phase of the cell cycle. This attenuation of response was not due to alterations of receptor number, receptor affinity for agonist, or expression of the catalytic unit of adenylate cyclase. Use of the antibiotic mycophenolic acid, a selective inhibitor of the synthesis of GTP, elicited response patterns in asynchronous cells similar to those seen in synchronized cells. These results confirm that wild-type S49 cells do not possess spare receptors. In addition to the importance of total receptor number in determining maximal response to isoproterenol, receptors may show differential efficacy in promoting cAMP accumulation as cells traverse the cell cycle. Changes in cellular levels or utilization of GTP during the cell cycle may serve to regulate the coupling of receptors to the stimulation of adenylate cyclase.

Many studies of β-adrenergic receptors linked to the activation of adenylate cyclase have focused on understanding the underlying molecular interactions between receptor, G_s, and adenylate cyclase during activation by agonists (1–3). In addition, much recent attention has been directed toward the cellular regulation of these receptors, as well as aspects and consequences of hormone stimulation in intact cells. Clonal cell lines, which have provided useful model systems for such studies, have allowed experiments to be conducted with cells of uniform genetic background in well controlled environmental milieus. These cell lines fall broadly into two classes: 1) continuously growing cells in suspension culture, and 2) cells that exhibit confluence, or contact inhibition, of growth. Experiments with contact-inhibited cultures are often performed at confluence, to minimize the number of actively dividing cells, and thereby gain an additional degree of experimental homogeneity. In experiments performed with cells that grow continuously, cells are asynchronous with respect to the different phases of their cell cycle (4). Thus, an accurate interpretation of experimental results obtained from asynchronous cultures of continuously growing cells depends upon a detailed knowledge of the characteristics of cells within each phase of the cell cycle.

Most previous studies concerned with the cell cycle-dependent changes of adenylate cyclase activity have focused on the role of cyclic nucleotides in the control of cell growth (5–8) or on stimulation of adenylate cyclase by agonists such as epinephrine, prostaglandin E_1, adenosine, and dopamine (9–11). Because many of these studies were performed on membrane preparations or on "broken" cells, the relevance of observed alterations in stimulation of adenylate cyclase to regulatory

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ABBREVIATIONS: R, receptor; G_s, guanine nucleotide-binding stimulatory protein; C, adenylate cyclase; DME/HS, Dulbecco's modified Eagle's medium containing 10% inactivated horse serum; DME/H/BSA, Dulbecco's modified Eagle's medium containing 20 mM 4-(2-hyrdoxyethyl)-1-piperazineethanesulfonic acid in lieu of $NaHCO_3$ and 1 mg/ml bovine serum albumin; BAAM, bromoacetylalprenololmenthane; ICYP, iodocyanopindolol; HPLC, high pressure liquid chromatography; MA, mycophenolic acid.

mechanisms in the intact cell were unclear. In the absence of a knowledge regarding changes in expression and properties of receptors during the cell cycle, an additional degree of uncertainty was added. By contrast, other studies have investigated the cell cycle-dependent binding to receptors for insulin (12), epidermal growth factor (13), melanocyte-stimulating hormone (14), and radiolabeled β-adrenergic antagonists (15), but in these studies the relationship between receptor expression and hormonal responsiveness could only be surmised.

In order to overcome the limitations of interpretation in earlier studies of cell cycle-specific expression of receptors, we have conducted experiments on the function of β-adrenergic receptors linked to adenylate cylase in both asynchronous and synchronous cultures of S49 lymphoma cells (16). The present studies were undertaken in asynchronous cells, using receptor inactivation methodology, and in cell-enriched populations of wild-type S49 cells to define more clearly the relationship between β-receptor expression and response to agonists.

Materials and Methods

Cell lines and cell culture. Wild-type (24.3.2) S49 lymphoma cells were grown at 37° in suspension culture in Dulbecco's Modified Eagle's medium containing 10% horse serum (DME/HS) in a 10% CO_2:90% air environment, as described (16, 17).

Cellular cAMP accumulation. Cells in DME/HS were centrifuged at 300 × *g* for 5 min at room temperature and resuspended in DME/H/BSA at 37° to a final concentration of 1–2 × 10^6 cells/ml. Superoxide dismutase and catalase (10 μg/ml each) were included to prevent catecholamine oxidation (18). Cells were incubated for 10 min with 100 μM Ro 20-1724, a potent phosphodiesterase inhibitor, followed by the addition of either (−)-isoproterenol or the diterpene forskolin at the indicated concentrations for an additional 15 min. In some experiments (initial accumulation), incubation with (−)-isoproterenol was carried out for 15 sec. Cell suspensions were then rapidly pelleted (20 sec) by microcentrifugation, the medium was removed by aspiration, and cell pellets were resuspended in 50 mM sodium acetate buffer, pH 4.0, containing 0.2 mM isobutylmethylxanthine followed by boiling for 5 min. Intracellular cAMP was measured by a competitive protein binding assay (17).

Receptor inactivation protocols. Bromoacetylalprenololmenthane (BAAM) was used to alkylate receptors exactly as described in the preceding article (16). Ethanol, the vehicle used to dissolve BAAM, was added to control cell cultures. Cells were then washed by repeated centrifugation and used immediately as described.

β-Adrenergic receptor binding assay. β-Adrenergic receptor number on intact S49 cells was determined using the radiolabeled β-adrenergic antagonist [^{125}I]ICYP, as previously described (16, 17).

Centrifugal elutriation of S49 cells and determination of cell cycle phase distribution. Centrifugal elutriation was used to isolate cell cycle-specific fractions of S49 cells (16, 19). The protocol used was that described in the preceding article (16). Total cellular DNA content (analyzed by flow cytofluorometry, Refs 16, 20, and 21) was used to determine cell cycle phase position in elutriated fractions.

Analysis of nucleotide triphosphates in S49 cells. Replicates (two) of S49 cells (~2.0 × 10^7) were pelleted by centrifugation (300 × *g*, 5 min) and resuspended with 500 μl of 0.4 N perchloric acid (4°). After vigorous vortexing, the acid-precipitate was separated by centrifugation (1000 × *g*, 5 min, 4°) and the supernatant was transferred to 12 × 75 mm glass tubes. Soluble perchloric acid was removed from the sample by 2:1 extraction with alamine:freon (5.6 g/30 ml) and subsequent centrifugation (1000 × *g*, 5 min) to clarify the aqueous (nucleotide-containing) and alamine-freon-perchloric salt phases. These samples (upper phase) were carefully collected and stored at −70° until HPLC analysis.

Samples were separated by HPLC on a Partisil PXS 10/25 SAX column (Whatman). Typically, 100-μl samples were analyzed. A single step buffer jump was used to separate mono- and diphosphates (10 mM KH_2PO_4, pH 3.46) from resolved triphosphates (250 mM KH_2PO_4, 0.5 M KCl, pH 3.77). Nucleotide levels were assessed by comparison to standards (ATP, GTP, CTP, and UTP) separated at the beginning and end of each day's run.

Materials. BAAM was the generous gift of Dr. Joseph Pitha, Gerontology Research Center, National Institute of Aging, the Francis Scott Key Medical Center (Baltimore, MD). (±)-Cyanopindolol (Dr. G. Engel, Sandoz), (−)-isoproterenol (Sterling-Winthrop), (−)-propranolol (Ayerst), and mycophenolic acid (Dr. J. Seegmiller, University of California, San Diego) were also gifts. All other reagents were of highest quality and were obtained from standard sources.

Results

Properties of cAMP accumulation in wild-type S49 cells after β-receptor Inactivation by BAAM. To define the relationship between β-receptor number and receptor-mediated cAMP generation in intact asynchronous cultures of S49 cells, we conducted experiments in which the amount of intracellular cAMP accumulation was measured after blockade of receptors by BAAM. Isoproterenol-stimulated cAMP accumulation in S49 cells, performed in the presence of a cAMP phosphodiesterase inhibitor, attains a maximum intracellular level of cAMP by 15 min (17). In order to determine whether the inactivation of β-receptors had an effect on this peak response, S49 cells were treated with various concentrations of BAAM for 30 min at 37°, washed, and then assayed for cAMP accumulation by increasing concentrations of (−)-isoproterenol (Fig. 1). Reduction of (−)-isoproterenol-stimulated cAMP accumulation by BAAM was noncompetitive, i.e., only maximal cAMP accumulation was affected, not potency of the agonist. Similar results were obtained when initial (<1 min) response to isoproterenol was measured. The reduction in cAMP re-

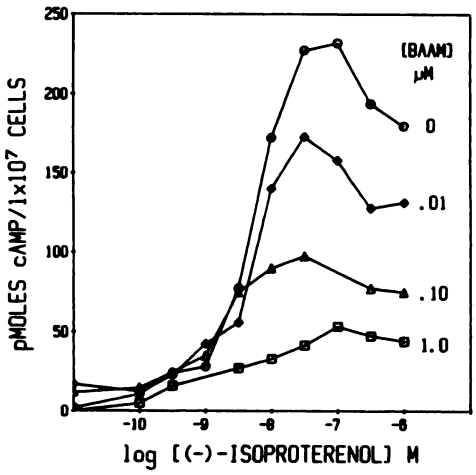

Fig. 1. Dose response of cellular cAMP accumulation to (−)-isoproterenol in BAAM-treated S49 cells. Cells (0.5–1.0 × 10^6) were incubated in the absence (○——○) or presence of either 0.01 μM (◇——◇), 0.1 μM (△——△), or 1.0 μM (□——□) BAAM for 1 hr. Cells were washed and stimulated with the indicated concentrations of (−)-isoproterenol for 15 min and then terminated, and cAMP was measured as described in Materials and Methods. Data shown are the mean of dupliate determinations. K_{act} values for isoproterenol in control and BAAM-treated cells were 5.0 nM (0.01 μM BAAM), 1.8 nM (0.1 μM BAAM), and 3.2 nM (1.0 μM BAAM), respectively. K_{act} values were determined graphically from the 50% value of the mean of the two highest cAMP values for each curve.

sponse closely parallels the degree of inactivation by BAAM of receptor binding sites for the β-adrenergic antagonist [^{125}I] ICYP (Ref. 16 and Fig. 2). The data shown in Fig. 2 are the mean values from three to four experiments and are expressed as the percentage of cAMP accumulation and receptor number in untreated control cells. Thus, in S49 cells, maximal cAMP generation at times of either initial or peak accumulation seems to be linearly dependent upon the number of receptors detectable with [^{125}I]ICYP.

Reduction of cAMP response following treatment of intact cells with BAAM could have resulted from a direct, dose-dependent effect on adenylate cyclase which has a known sensitivity to reagents that act on free sulfhydryls (22). To rule out this possibility, cells incubated with increasing concentrations of BAAM for 30 min at 37° were washed and assayed for cAMP accumulation to (−)-isoproterenol or the diterpene forskolin, a hormone-independent activator of adenylate cyclase activity in these cells (23). Inactivation of β-adrenergic receptors with BAAM had no effect on the ability of 50 μM forskolin to stimulate cAMP accumulation (Fig. 3). This result confirms that the reduction of cAMP response following receptor blockade was attributable to irreversible receptor occupancy by BAAM rather than to the inactivation of adenylate cyclase.

Characteristics of isoproterenol-stimulated cAMP accumulation in synchronized S49 cells. In order to determine whether the proportional relationship observed for receptor number and cAMP response in asynchronous cultures of S49 cells reflected a similar relationship for cells in different phases of the cell cycle, synchronized populations of S49 cells were obtained by centrifugal elutriation (16, 19, 24). As shown in the preceding article (16), receptor number increases in proportion to cell size and transition through the cell cycle from G_1 to mitosis.

In contrast to the expression of β-adrenergic receptors on synchronized S49 cells during their progression through the cell cycle, isoproterenol-stimulated cAMP accumulation showed a strikingly different pattern (Fig. 4). When assayed under conditions of maximal cAMP accumulation (1 μM (−)-isoproterenol for 15 min in the presence of a phosphodiesterase inhibitor), there was approximately equal response in G_1 and G_2/M cells. A progressive decline in response (to 35% of G_1 cells) was observed as G_1 cells entered and progressed through S phase. The data shown are the average of three experiments. Although the absolute picomoles of cAMP/10^7 cells varied between experiments (e.g., 200–700 pmol of cAMP for G_1 cells),

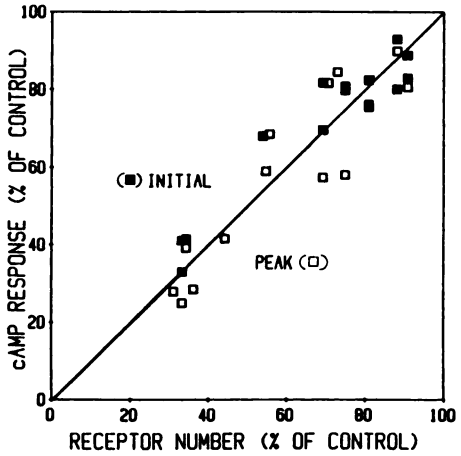

Fig. 2. Relationship of cAMP response and receptor number in S49 cells after BAAM treatment. For initial rate of accumulation, cells were stimulated with 1.0 μM (−)-isoproterenol for 15 sec followed by rapid (20 sec) micorcentrifugation. Supernatants were aspirated (10 sec) and cell pellets were terminated as described in Materials and Methods. Peak accumulation was measured at 15 min. Ro 20-1724 (100 μM) was included in all samples. Data shown are the pooled mean values of three replicates from four experiments for both initial and peak accumulation and are expressed as percentage of untreated control cells. Receptor number was determined by specific [^{125}I]ICYP binding to cells and is expressed as a percentage of control cells as described (17). The *line* shown represents a theoretical case of absolute proportionality between receptor number and response.

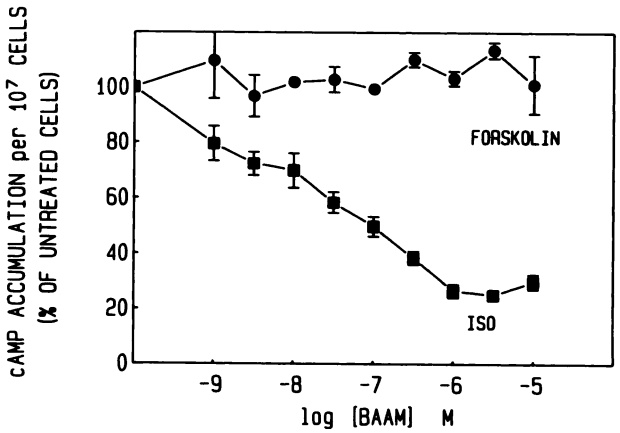

Fig. 3. Intracellular cAMP accumulation in response to 1.0 μM (−)-isoproterenol or 50 μM forskolin in S49 cells after treatment with BAAM. Cells (0.5–1.0 × 10^6) were treated with the indicated concentrations of BAAM for 30 min in DME/HS at 37°. After four washes, cells were resuspended in DME/H/BSA containing 10 μg/ml each of superoxide dismutase and catalase. Intracellular cAMP was assayed after stimulation of 1.5 × 10^6 cells in triplicate for 15 min (in the presence of 100 μM Ro 20-1724) with either 1.0 μM (−)-isoproterenol or 50 μM forskolin. At 1.0 μM, (−)-isoproterenol stimulation ranged from 330–650 pmol of cAMP/1 × 10^7 untreated cells, and 50 μM forskolin stimulation varied from 1300–2000 pmol of cAMP/1 × 10^7 cells between experiments. Data shown are expressed as the percentage of the maximum cAMP accumulation (n = 4, mean ± SE) compared to untreated control.

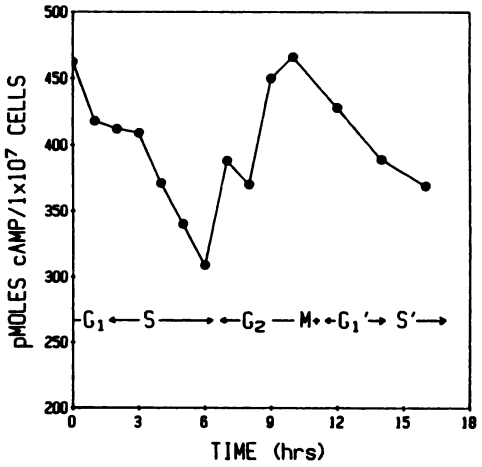

Fig. 4. Isoproterenol-stimulated cAMP accumulation in synchronized S49 cells during the cell cycle. S49 cells synchronized in G_1 were prepared by centrifugal elutriation as described in Materials and Methods and returned to culture in DME/HS. At the times indicated, aliquots of cells were removed and assayed for intracellular cAMP accumulation in response to 1 μM (−)-isoproterenol for 15 min; 100 μM Ro 20-1724 was included to inhibit phosphodiesterase activity. Typically, cells were assayed with three to six replicates. Data shown are the mean values from three experiments. Cell cycle position at the time of the isoproterenol stimulations is indicated at the *bottom*.

this same pattern was observed in each experiment. Therefore, as G_1 cells traverse through S phase, a period of continued increase in receptor expression, cAMP accumulation declined. In addition, cells in G_2/M which express almost twice the number of receptors as G_1 cells showed approximately equal response to G_1 cells. This relationship, implying a dissociation between receptor number and response in the synchronized cells, is in contrast to what we observed in experiments conducted in asynchronous S49 cells treated with BAAM (Fig. 2).

Three possibilities that might account for this heterogeneity in cAMP response during the cell cycle were pursued in experiments performed on cells enriched in G_1, S, and G_2/M phases of the cell cycle: 1) differences might exist in the affinity (K_{act}) of isoproterenol for the stimulation of cAMP accumulation, 2) accumulation of cAMP at 15 min might not represent the peak time of response for each phase of the cell cycle, and 3) despite the presence of phosphodiesterase inhibitor, residual cAMP degradation or extrusion (25) from cells might be responsible for these results. The data for experiments testing these possibilities are summarized in Table 1. Neither the affinity of isoproterenol to stimulate cAMP, the time selected for the measurement of cAMP accumulation, nor differences in the cellular degradation and/or extrusion of cAMP could account for the heterogeneity observed in cells within various compartments of the cell cycle. This suggested that alterations at the level of adenylate cyclase must be responsible for the changes in β-adrenergic stimulation of cAMP accumulation during the cell cycle.

Use of forskolin to assess interactions between β-adrenergic receptors, guanine nucleotide regulatory protein, and adenylate cyclase during the cell cycle of S49 cells. Differences in the properties of the interaction of agonist with receptors on G_1, S, and G_2/M fractions of cells thus appeared not to explain the dissimilarity between receptor expression and response during the cell cycle. Experiments were therefore designed using the diterpene forskolin to explore the patterns of hormone-independent and forskolin/isoproterenol-synergized stimulation of adenylate cyclase in intact S49 cells as they progressed through the cell cycle. Forskolin is able to directly stimulate the catalytic unit of adenylate cyclase (C) in intact S49 cells, although we (23) and others (26, 27) have shown that maximum stimulation appears to require the nucleotide regulatory protein, G_s, and functional G_s-C coupling. In addition, forskolin can act to potentiate the response to maximally effective concentrations of isoproterenol, and this potentiation requires interaction between receptor and G_s. Thus, forskolin could be used to explore both agonist-independent and agonist-dependent G_s-C interaction during the cell cycle in intact S49 cells.

A homogenous population of S49 cells in G_1 phase was returned to the incubator for a longitudinal study of the intracellular accumulation of cAMP in response to stimulation by 50 μM forskolin alone (Fig. 5, *top panel*). Under these conditions of a relatively high concentration of forskolin [EC_{50} = 5–10 μM (Ref. 23)], differences were observed from the pattern of cAMP accumulation in response to isoproterenol: 50 μM forskolin-stimulated cAMP accumulation increased as cells moved through S phase, while parallel stimulation of cells with isoproterenol in this experiment was reduced through this period (not shown; for reference, see Fig. 5, *bottom panel:* ISO-alone curve). In addition, a drop in cAMP accumulation occurred during mitosis. Thus, a lack of functional activity of C is not responsible for the lowering of response to isoproterenol during S phase.

Moreover, when a maximum concentration of (−)-isoproterenol (1 μM) and a potentiating concentration of forskolin (1 μM) were incubated together, the decrease in cAMP accumulation observed for isoproterenol during transition through S phase was also not observed (Fig. 5, *middle panel*). Thus, it would appear that when forskolin was used to potentiate the capacity for hormone-stimulated cAMP accumulation, the attenuation in cAMP accumulation during S phase was diminished.

The intracellular cAMP accumulation achieved with high concentrations (50–100 μM) of forskolin is in excess (3–10-fold) of that achieved by stimulation with isoproterenol. Therefore, a low concentration of forskolin (1 μM) was used to stimulate cAMP accumulation, because at this concentration cAMP accumulation was comparable to that observed in response to 1 μM isoproterenol (Fig. 5, *bottom panel*). In contrast to the pattern observed with a higher concentration of forskolin, cAMP accumulation by 1 μM forskolin was similar to that observed for isoproterenol, including the prominent attenuation in response as cells progressed through S phase. Forskolin-stimulated cAMP accumulation declined during mitosis, however, in a manner similar to that observed at the higher (50 μM) concentration of forskolin. Taken together, these data suggest that the expression of C activity in two phases of the cell cycle, S phase and mitosis, are regulated in different ways. In certain instances (increasing forskolin concentration and potentiating agonist-mediated stimulation), forskolin appears able to overcome the regulation in S phase.

Intracellular GTP as a potential regulator of agonist-mediated accumulaton of cAMP during S phase. GTP is well known as an obligatory regulator in the β-adrenergic-mediated activation of adenylate cyclase in membrane preparations of S49 cells (28). In the data obtained from the use of forskolin, the variation in agonist-stimulated cAMP accumulation during S phase seemed unlikely to be the consequence of either an uncoupling of receptor from G_s (based on the experiment test forskolin plus isoproterenol) or the lack of C activity and G_s-C interaction (50 μM forskolin experiment). We reasoned that lowered availability of GTP, which would be involved in the activation of C during S phase might account for the pattern of agonist-stimulated cAMP accumulation. In intact cell studies with two different cultured cell lines, a selective decrease in GTP levels had been shown cause attenuation of β-adrenergic receptor-mediated cAMP generation (29, 30). GTP levels in S49 cells can be selectively decreased without

TABLE 1
Cellular cAMP accumulation

	G_1	S	G_2/M
ISO K_{act} (nM)[a]	5.9 ± 0.5	6.8 ± 1.8	5.7 ± 0.7
Time to peak response (min)[b]	15	15	15
Intracellular cAMP decay[c] (min^{-1})	−0.067	−0.069	−0.064

[a] Isoproterenol (ISO) values were obtained at 15 min in the presence of 100 μM Ro 20-1724 and estimated graphically (n = 3).
[b] N = 2; data obtained as in Ref. 17.
[c] Rate constant of a single exponential decay was obtained from data expressed as percentage of remaining pmol of cAMP/10^7 cells after block of 1 μM (−)-isoproterenol-stimulated cAMP accumulation at 15 min by the addition of 1 μM (−)-propranolol.

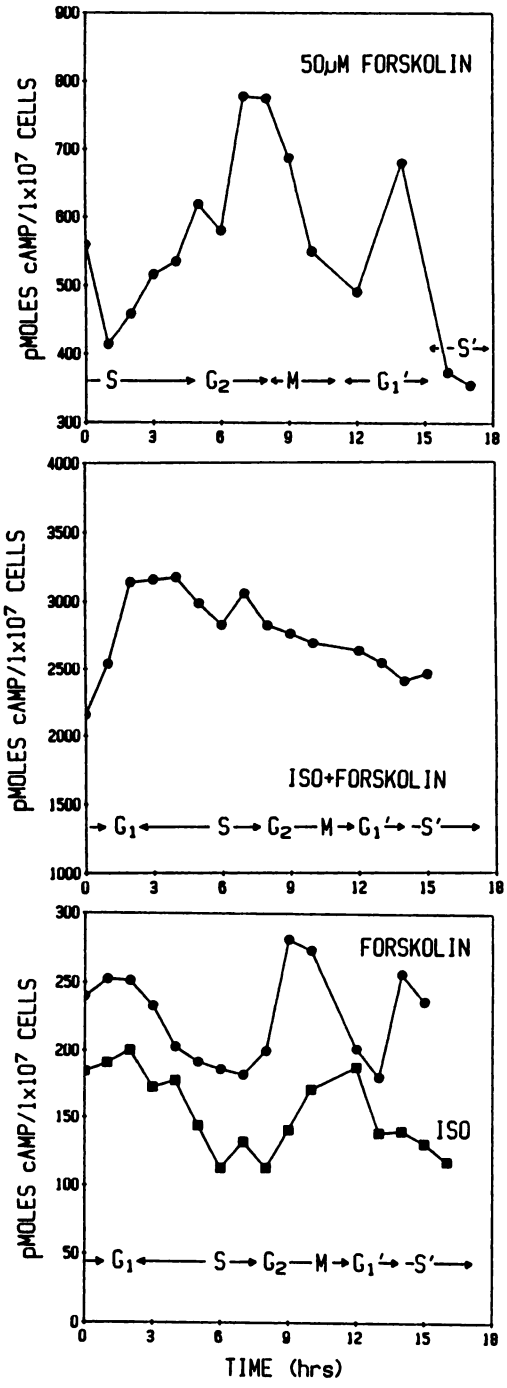

Fig. 5. Isoproterenol, forskolin, and forskolin-potentiated cAMP accumulation in synchronized S49 cells during the cell cycle. G_1 cells were prepared by centrifugal elutriation and returned to growth conditions as in Fig. 4. At the times indicated, aliquots were taken for cAMP accumulation assays (15 min in the presence of a phosphodiesterase inhibitor) in response to the following agents. *Top panel*, cAMP accumulation in response to stimulation with 50 μM forskolin. *Middle panel*, cAMP accumulation in response to stimulation with 1 μM (−)-isoproterenol and 1 μM forskolin (potentiated stimulation). *Bottom panel*, cAMP accumulation in response to stimulation by either 1 μM (−)-isoproterenol or 1 μM forskolin. Cell cycle positions are indicated at the *bottom* of each *panel*. Data points shown are the mean of triplicate stimulations performed at each time point. Data in the *top panel* are from a separate experiment in which isoproterenol stimulation was performed in parallel as a control (not shown). Levels of basal (unstimulated) cAMP accumulation did not contribute to these patterns, although low basal activity during S phase and mitosis was observed (data not shown).

lowering other nucleotides by incubation of cells with the antibiotic, MA (31, 32). This decrease in cellular GTP is attributable to the arrest by MA of *de novo* GMP biosynthesis through the inhibition of IMP dehydrogenase. Thus, experiments were performed to alter intracellular GTP in asynchronous S49 cells and to measure the effects on agonist-dependent and agonist-independent stimulation of intracellular cAMP accumulation.

Treatment of S49 cells with 1.2 μM MA has been reported to be optimal for the reduction of GTP (31). To confirm these findings, S49 cells were incubated for periods of up to 3 hr at this concentration and no effect on cell viability, as assessed by trypan blue exclusion, was observed (control viability: 95%, treated: 92%; $n = 2$). When intracellular levels of nucleotide triphosphates were analyzed by HPLC, a selective decrease in GTP levels was seen (>80% by 2 hr) compared to control untreated cells (Fig. 6, *top*). Cells treated with 1.2 μM MA for 3 hr showed no alteration in their ability to bind [^{125}I]ICYP (control cells: $K_D = 11$ pM, $B_{max} = 1377$ sites/cell; treated: $K_D = 14$ pM, $B_{max} = 1422$ sites/cell).

Control and MA-treated (1.2 μM, 3 hr) S49 cells were assayed for their ability to accumulate cAMP in response to either 1 μM (−)-isoproterenol, 1 μM forskolin, 1 μM (−)-isoproterenol plus 1 μM forskolin (synergy) or 100 μM forskolin (Fig. 6, *bottom*) under assay conditions identical to those used for synchronized cells. At all times of MA treatment, response to isoproterenol alone or 1 μM forskolin was affected more by the drop in GTP levels than were the responses to potentiated stimulated or stimulation with 100 μM forskolin. In another series of experiments, cells were treated with 1.2 μM MA for 1 hr, and cAMP accumulation in response to 1 μM isoproterenol, 1 μM forskolin, 1 μM isoproterenol plus 1 μM forskolin, and 100 μM forskolin was measured. MA-treated cells had a 58% and 48% decrease in cAMP accumulation in response to stimulation with 1 μM isoproterenol or 1 μM forskolin, respectively. Potentiated response in the presence of 1 μM isoproterenol and 1 μM forskolin was unaffected by MA, and cAMP accumulation in response to 50 μM forskolin was decreased only 15%. Thus, alteration in available GTP in asychronous S49 cells shows differential effects on the type of stimulation of cAMP accumulation in a manner very similar to that of S phase cells, thus supporting the hypothesis that cellular GTP level may act as a modulator of cAMP response during the S phase of the cell cycle. Further evidence supporting this idea is provided by independent analyses, obtained by another laboratory,[1] of GTP levels in G_1, G_1/S, and late S/G_2 S49 cells (by "cross-sectional" fractions obtained by elutriation). GTP levels were found to drop significantly from 300 nmol/10^9 cells in G_1 cells to 232 nmol/10^9 cells in a 50/50 population of late G_1/early S cells. GTP levels had returned to 262 nmol/10^9 cells in the late S/G_2 fraction. This value for the G_2/S fraction may represent an underestimate of how low GTP levels may drop in S cells due to contamination with G_1 cells.

Discussion

In the preceding article (16), we reported that the number of β-adrenergic receptors in S49 lymphoma cells increased in proportion to cell size throughout the cell cycle and that apparent affinity for the agonist isoproterenol and for [^{125}I]ICYP

[1] V. Groppi, B. Ullman, and S. Erickson, personal communication.

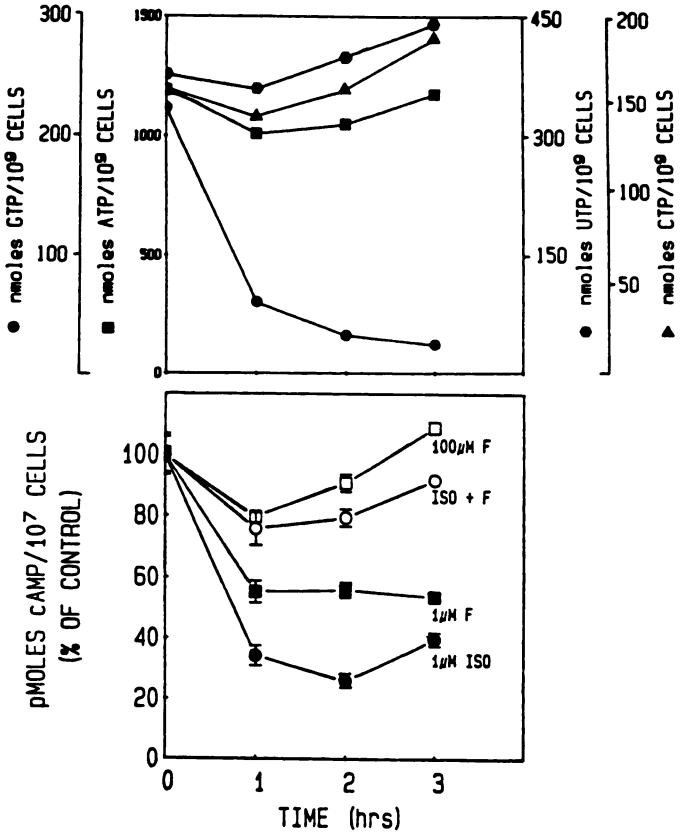

Fig. 6. Nucleotide levels and cAMP accumulation in asynchronous S49 cells after treatement with AM. Asynchronous S49 cells were treated with 1.2 μM MA in DME/HS for the times indicated. Cells were then prepared for the analysis of nucleotide levels (*top*) or for analysis of cAMP accumulation (*bottom*). Nucleotide levels were quantitated by comparison to standards for ATP, GTP, CTP, and UTP run on HPLC and normalized according to cell number as described in Materials and Methods. In addition, cells (~1 × 10^6) were assayed for cAMP accumulation in response to 1 μM (−)-isoproterenol (*1 μM ISO*), 1 μM forskolin (*1 μM F*), 1 μM (−)-isoproterenol plus 1 μM forskolin (*ISO + F*), and 100 μM forskolin alone (*100 μM F*). Data shown are the means (±SE) of triplicate stimulations. No loss in cell viability was observed at any time point. The "zero time" values for cAMP accumulation were (in pmol/10^7 cells): 1 μM isoproterenol, 655 ± 38; 1 μM forskolin, 1452 ± 35; 1 μM isoproterenol + 1 μM forskolin, 6449 ± 432; and 100 μM forskolin, 6775 ± 620.

was unaltered during tranverse of the cell cycle. We have extended these findings in this report to address the relationship of receptor number to agonist-stimulated increase in intracellular cAMP. With the use of the irreversible β-adrenergic antagonist BAAM on asynchronous cultures of wild-type S49 cells, we have found a proportional relationship between receptor number and cAMP accumulation. These results with BAAM support previous data indicating that the full complement of β-adrenergic receptors is required to generate maximal β-adrenergic-stimulation cAMP accumulation in S49 cells (33, 34).

A different conclusion, however, was obtained with respect to the relationship between receptor number during the S49 cell cycle and the accumulation of cAMP in response to stimulation by isoproterenol. S49 cells in different phases of the cell cycle did not respond equally to isoproterenol, nor could the magnitude of this response be explained by changes in the number of cellular receptors. This type of heterogeneity of cAMP response would not be detectable in studies with asynchronous S49 cells, as in the series of experiments using irreversible blockade of receptors with BAAM. In particular, two observations stand out. 1) As cells entered and progressed through S phase, the ability to accumulate cAMP in response to isoproterenol decreased. This was apparently not due to the lack of functional C activity because a high concentration of forskolin revealed an increase in activity through this period. 2) During mitosis, the capacity to respond to agonist remained blunted with respect to the number of receptors present. The activity of C, measured at both high and low concentrations of forskolin, was likewise diminished. Thus, two separate mechanisms of attenuation of responsiveness of adenylate cylase may occur in different periods of the cell cycle of S49 cells.

Low expression of the activity of C during mitosis has been reported in a number of cells. Basal activity is lowest during mitosis (5, 6, 8, 35), both in studies with intact cells and in membrane preparations. Sodium fluoride- and hormone-stimulated adenylate cyclase activity in membranes prepared from synchronized cells has also generally been reported to be lowest in mitotic cells (6, 10, 35). Thus, distinct differences may exist in either the quantity or the regulation of the catalytic unit in M phase cells compared to other phases of the cell cycle.

The observation that β-adrenergic stimulation of adenylate cyclase activity was low during S phase has been reported in unwashed Chang liver cell lysates (9). By contrast, in homogenates of C6 cells (35), the specific activity of adenylate cyclase (stimulated by GTP, NaF, or isoproterenol) was highest in an S-enriched fraction of cells; however, the authors reported that the fold-increase in stimulation was lowest in S phase cells.

Synchronized populations of S49 cells appear "uncoupled" during periods within the cell cycle. Is this uncoupling a consequence of a defective interaction between receptors and G_s? The data obtained using forskolin both in synchronized S49 cells and in asychronous cells treated with MA suggest that this uncoupling may occur between G_s and C, perhaps as a consequence of altered levels of GTP. Data obtained with S49 cells that support the hypothesis that altered response during S phase may be due to impaired G_s-C interaction from lowered GTP levels include the following.

1) In studies with S49 variants having lesions in R-G_s-C interactions, forskolin was able to restore response to agonist in variants having lesions between G_s and C but not to those with lesions between R and G_s (23).

2) In intact S49 cells, maximum stimulation of C by forskolin requires intact G_s-C interaction (23, 36). Since high concentrations of forskolin "override" the attenuation through S phase, a lack of G_s cannot explain this attenuation.

3) Activation of C by forskolin is hormone independent, yet forskolin (at 1 μM) exhibited a pattern similar to that of hormone-stimulated activity during S phase. The differential response to low forskolin concentration and to forskolin-potentiated hormone stimulation during S phase is qualitatively similar to responses in an S49 variant, H21a, that has impaired G_s-C interaction (23).

4) GTP binding and hydrolysis regulate the β-agonist-stimulated activation of C in membranes. Selective alterations in the GTP levels in intact cell studies (29, 30) and shown here for S49 cells markedly effect β-agonist stimulated activity of C as evidenced by the decrease in cAMP accumulation. Thus, it would appear that GTP availability in intact cells is critical for a maximal activation of C by β-agonists such as isoproterenol.

5) When S49 cells are selectively depleted of GTP, nucleic acid synthesis, particularly DNA, is substantially reduced (31). This action of MA is restricted to S phase cells. A role of GTP

in the synthesis of guanosine-containing RNA primers has been proposed. Thus, it may be reasonable that GTP levels available to adenylate cylase or total cellular GTP fall during S phase as a consequence of either a redistribution of metabolic pools of GTP or from an increased demand for guanosine, respectively.

How forskolin acts at high concentrations alone or at low concentrations in potentiated response with hormones to overcome GTP depletion is unclear. Perhaps forskolin increases the affinity of G_s for binding of GTP. This may represent an additional site of interaction on G_s or a consequence of binding to the catalytic unit in the intact cell. Recent evidence demonstrating sites of high ($<\mu M$) and low ($>\mu M$) affinity for forskolin representing G_s-C interaction and C alone respectively, support this hypothesis (37, 38).

In summary, studies on the response of β-adrenergic receptors to catecholamines in synchronized populations of S49 lymphoma cells have provided insight into potential regulatory mechanisms of cAMP generation in the intact cell. This regulation was masked in studies on asynchronous cell cultures, where direct proportionality between receptor number and response was observed. The results suggest a novel means—changes in availability of GTP—as a determinant of responsiveness of cells to β-agonists. Moreover, the findings emphasize that observations on the regulation of the hormonal or drug responsiveness of continuously growing cells may fail to detect regulatory events that affect cellular responsiveness during various phases of the cell cycle.

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