Adenylate cyclase from synchronized neuroblastoma cells: Responsiveness to prostaglandin E₁, adenosine, and dopamine during the cell cycle

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ABSTRACT Neuroblastoma cells were synchronized by a combined isoleucine plus glutamine starvation. Adenylate cyclase activity [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] was measured under basal conditions and in the presence of dopamine, adenosine, and prostaglandin (PG) E₁. A clear dissociation occurred between the respective evolution patterns of basal and agonist-stimulated adenylate cyclase activities. The magnitudes of the enzyme response to PGE₁, adenosine, and dopamine also exhibited different evolution patterns during the cell cycle. Evolution of adenylate cyclase responsiveness to PGE₁ during the cell cycle exhibited striking similarities with the intracellular 3':5'-cyclic AMP changes observed elsewhere.

Use of theophylline and fluphenazine as specific inhibitors of adenosine and dopamine, respectively, made it possible to demonstrate that adenosine, dopamine, and PGE₁ stimulated adenylate cyclase through independent receptor sites. Furthermore, whatever the stage of the cell cycle, responses to these three agonists were not additive, indicating that the receptors of adenosine, dopamine, and PGE₁ control the same adenylate cyclase moieties. The data suggest that adenylate cyclase cell content and enzyme responsiveness to specific agonists can be independently controlled.

In all synchronized cell types so far studied the intracellular 3':5'-cyclic AMP concentration varies at different stages of the cell cycle, being minimal at the time of mitosis and almost always reaching a maximal value in the early G₁ phase (1-4). More recently it was shown in fibroblasts that at the beginning of the G₁ phase intracellular cyclic AMP drops abruptly while 3':5'-cyclic GMP rises. It was suggested that the changes of cyclic nucleotides regulate the cell cycle and more generally that cyclic nucleotides are involved in the "pleiotropic" control of cell growth (5, 6). Modulation of intracellular cyclic AMP content might result from a modification in the rate of cyclic AMP synthesis by adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] and/or a modification in the rate of hydrolysis by phosphodiesterases. As far as adenylate cyclase is concerned, it is not clear whether enzyme activity is regulated through a change in cell enzyme content or by regulatory signals from extra- or intracellular origin.

The aim of the present work was to analyze the evolution pattern of adenylate cyclase during the cell cycle in neuroblastoma cells and the evolution patterns of the responses to three different regulatory agents: adenosine, dopamine, and prostaglandin (PG) E₁. The latter seems to be a candidate for intracellular regulation because several cell lines, including neuroblastoma, are able to synthesize prostaglandins (7).

Abbreviation: PG, prostaglandin.
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MATERIAL AND METHODS

Mouse neuroblastoma cells were derived from the cholinergic clone NS20 (8). Current properties of these cells are: total protein content is 0.250 mg/10⁶ cells; specific choline acetyltransferase activity is 60 pmol/mg protein per min. Present karyotype: 80% of the cells have 60 chromosomes and 20% have 120.

Cell Culture. Cells were grown on Falcon petri dishes in Eagle's minimal essential medium containing Earle's salts solution supplemented with 10% fetal calf serum (Gibco), penicillin G at 50 international units/ml and streptomycin sulfate at 5 μg/ml. Cells were incubated at 37° in 90% air/10% CO₂. The doubling time of the cell population was close to 20 hr.

Synchronization Procedure. Cells were collected during the exponential phase of growth, plated (10⁶ cells per dish), and grown in normal medium for 40 hr. Cell growth was arrested by aminoacid starvation according to Tobey's method (9). The starvation medium was Eagle's minimal essential medium with Earle's salts, free from glutamine and isoleucine, and supplemented with 10% of extensively dialyzed fetal calf serum. Growth was reinitiated after 36 or 48 hr starvation by addition of complete medium containing isoleucine and glutamine at twice normal concentrations. Cells starved for 48 hr were used to study the first 12 hr of the cell cycle; those starved for 36 hr, to study the last part. In some experiments cells were resynchronized at the end of the first cycle using the following method: 3 hr before mitosis, 0.2 μM Colcemid was added to each dish and cells were collected, washed three times in growth medium at 4°, and replated at a density of 4 X 10⁶ cells per dish.

Determination of DNA Synthesis. Cells were incubated for 30 min in the presence of [³H]thymidine (0.3 μM, 0.3 μCi/ml). Trichloroacetic-acid-insoluble material was separated by filtration through Millipore filters.

Broken Cell Preparation and Adenylate Cyclase Assay. Adenylate cyclase was assayed using a cell lysate obtained as previously described (10). Briefly, cells were rapidly cooled at 4° and collected by centrifugation. They were washed three times with isotonic NaCl solution, incubated for 15 min at 4° in an hypotonotic solution containing Tris-HCl at pH 8.0, 10 mM/EDTA, 0.1 mM. Adenylate cyclase activity was assayed immediately. Microscopic examination indicated that 90% of the cells were disrupted and 20% of the apparently intact cells were permeable to trypan blue.

Adenylate cyclase was assayed at 37° for 15 min in an incubation medium (final volume 50 μl) containing Tris-HCl at pH 8.0, 25 mM/ATP, 0.1 mM/α-[³²P]ATP, 2–3 μCi/MgCl₂, 0.25 mM/papaverine, 0.1 mM/cyclic GMP, 1 mM/creatine phos-
phate, 20 μM/creatine kinase, 50 μg cyclic [3H]AMP, 2-10⁻³ μCi/μg of protein per assay and to incubation time up to 20 min. Reproducibility of the assay method was checked by measuring the activity of 16 cell lysates run in parallel. Means ± SEM of basal or adenosine-100 μM, and PGE1-1 μM stimulated activities were, respectively: 54.9 ± 2.6, 156.8 ± 97, and 437 ± 32 pmol of cyclic AMP/mg of protein per 15 min.

All determinations ([3H]thymidine incorporation, mitotic index, and adenylate cyclase activity) were performed in duplicate or triplicate.

**Chemicals.** Prostaglandin E1 was kindly supplied by John Pike, Upjohn; Colcemid was purchased from Gibco. [3H]Thymidine (1 Ci/mmole) was purchased from the Commissariat à l’Energie Atomique (CEA, Saclay, France); [α-32P]ATP (15–25 Ci/mmole), and cyclic [3H]AMP (13 Ci/mmole) were obtained from New England Nuclear.

### RESULTS

**Studies with Nonsynchronized Neuroblastoma Cells.** Adenylate cyclase obtained from neuroblastoma cells collected in the exponential growth phase was stimulated by adenosine, dopamine, or prostaglandin E1 (Fig. 1). The dose–response curve for PGE1 did not exhibit simple saturation kinetics. For concentrations ranging from 0.01 to 1 μM the dose–response curve can be described adequately by Michaelis kinetics (Fig. 1, insert). The response obtained in this concentration range probably reflects interaction of PGE1 with a limited number of specific receptor sites (apparent Kₘ of 0.18 μM). Increasing PGE1 concentration above 1 μM led to a further and nonsaturable increase in enzyme activity. This effect, which exhibits very poor specificity towards prostaglandins from the different series, might reflect a nonspecific interaction of these highly hydrophobic molecules with the membrane (10). Response to adenosine was obtained over a 1 μM to 100 μM concentration range. The magnitude of maximal activation is about 60% of that induced by 1 μM PGE1. For adenosine concentration above 50 μM an inhibitory effect was apparent in most experiments. Dopamine induced a 3- to 5-fold increase in adenylate cyclase activity with an apparent Kₘ of 1–2 μM. The response to dopamine was blocked by fluphenazine (10 μM) and phentolamine (10 μM) (Fig. 2), partially inhibited by propranolol, and unaffected by a pure adrenergic β-receptor antagonist (pin- dolol). The specificity of dopamine receptors in neuroblastoma cells is comparable to that of dopamine receptors present in the rat caudate nucleus (12). Fluphenazine did not block the response to either adenosine or PGE1; theophylline, a competitive inhibitor of adenosine in neuroblastoma cells (10), did not inhibit responses to dopamine and PGE1 (Table 1).

**Studies with Synchronized Cells.** The isoleucine-glutamine starvation technique resulted in good synchronization of neuroblastoma cells (Fig. 3). As for other cell lines deprived of isoleucine, starved neuroblastoma cells appeared to be blocked at the G₀ restriction point. The first cell cycle following reinitiation of cell growth lasted 21 hr and the relative durations of G₁, S + G₂, and mitosis were 9, 10, and 2 hr, respectively; the G₂ phase was difficult to detect.

The evolution patterns of the responses to the three agonists were very different. Adenylate cyclase activity in presence of PGE1 (1 μM) exhibited the largest variations. It decreased during the G₁ phase, rapidly increased during the first hours of the S phase, and declined before mitosis (Fig. 4). Specific basal adenylate cyclase activity increased linearly over the duration of the entire cell cycle (Fig. 5). When the response to the three agonists tested was measured by the enzyme activation

![Graph](image-url)

**Fig. 1.** Effect of prostaglandin E₁, dopamine, and adenosine on adenylate cyclase activity of neuroblastoma cells. Cells were collected during an exponential phase of growth. Results are expressed as the ratio of stimulated to basal activities. Responses to PGE₁ (G), adenosine (O), dopamine (●). Inset: Eadie plot for PGE₁-stimulated adenylate cyclase activity; apparent Kₘ for high-affinity sites: 0.18 μM.

<table>
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<tr>
<th>Inhibitor</th>
<th>Basal</th>
<th>Dopa-</th>
<th>Adeno-</th>
<th>PGE₁</th>
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<td>50 μM</td>
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<td>±0.7</td>
<td>±1.3</td>
<td>±2</td>
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The agonist-sensitive adenylate cyclase activity represents the difference between the amounts of cyclic AMP formed in the presence and the absence of the agonist (pmol/mg of protein per 15 min). Values are means ± SEM of four experiments.
Fig. 2. Properties of dopamine-sensitive adenylate cyclase activity. (Left) Dopamine dose–response curve. The rate of cyclic AMP production is plotted as a function of dopamine concentration in incubation medium. (Right) Effect of 10 μM fluphenazine, 10 μM phentolamine, 10 μM propranolol, or 10 μM Pindolol, on basal and dopamine- (50 μM) stimulated activities.

Dopamine/[M]

Adenylate cyclase activity per 15 min

Basal activity

Dopamine, 50 μM

No addition

Fluphenazine, 10 μM

Phentolamine, 10 μM

Propranolol, 10 μM

Pindolol, 10 μM

FIG. 3. Cell cycle characteristics of synchronized neuroblastoma cells. Evolution of mitotic index (●, ○) and of [3H]thymidine incorporation into DNA (▲, △). Solid and open symbols correspond to data determined on cells starved for 48 hr or 36 hr, respectively. Mitotic index was determined by counting 500 nuclei.

Our results show that isoleucine and glutamine starvation of neuroblastoma cells led to rapid arrest at a unique stage of the cell cycle. Reinitiation of growth by complete medium resulted in a good synchronization. Comparison of the first two cell cycles following reinitiation of growth suggested that arrest occurred in early G1 phase. In contrast with results obtained with fibroblasts (13), the second cycle was no shorter than the

DISCUSSION

ratio (stimulated/basal activity) (see Figs. 4 and 5), the response to dopamine remained almost constant during the cell cycle; the adenosine response gradually decreased, while the response to PGE1 showed two peaks, one at the beginning of the cycle and the second during the S phase. The cells remained partially synchronized during the second cycle following reinitiation of growth (Fig. 6). The mitotic index was still 45% at the end of the cycle. During the first part of the second cycle, the evolution pattern of PGE1-stimulated adenylate cyclase activity was qualitatively similar to that observed during the first G1 phase. In parallel, cells were resynchronized at the end of the first cycle by Colcemid treatment (Fig. 6). The durations of the first and second cycle following reinitiation of growth were identical and the characteristic evolution pattern of PGE1-stimulated adenylate cyclase activity during the G1 phase was maintained. Depending on the serum batch used for cell culture before starvation, duration of the cell cycle varied from 19 to 21 hr. In addition, both total protein content and basal adenylate cyclase specific activity (expressed as pmol/15 min per mg of protein) increased during the G1 phase only when the initial protein content was low (0.1 mg/10 cells) (results not shown). Whichever serum batch was used, PGE1-stimulated adenylate cyclase activity followed an evolution pattern similar to that shown in Fig. 4.

Comparison of the dose–response curves for adenylate cyclase activation by PGE1 during the different phases of the cell cycle indicated that only the magnitude of maximal activation was affected; there were not any significant changes in apparent K_m values. Similarly, the apparent K_m values for enzyme activation by dopamine or adenosine were constant during the different phases of the cycle (results not shown).

Whatever the phase of the cell cycle considered (0, 7, and 15 hr after reinitiation of growth), the responses to stimulation by two agonists added together were never additive (Fig. 7). At 1 μM PGE1 (a saturating concentration for the high-affinity sites for PGE1) the responses measured in the absence or presence of maximal amounts of adenosine or dopamine appeared to be similar [except when activity in the presence of adenosine and prostaglandin was tested at 7 hr of the cycle (Fig. 7B)]. Furthermore, the threshold dose for PGE1 was not strikingly increased by the presence of dopamine or adenosine.
first, suggesting that starved neuroblastoma cells were able to escape rapidly from the resting phase G0 when optimal growth conditions were restored. In addition, amino-acid-starved neuroblastoma cells did not acquire the morphological differentiation characteristic of quiescent cells obtained by dibutyryl cyclic AMP, PGE₁, or serum deprivation treatments (14, 15). If one considers the close similarity in the evolution patterns of PGE₁-sensitive adenylate cyclase during the two successive G₁ phases (Figs. 4 and 6), it is tempting to consider that the first cycle following reinitiation of growth is representative of a normal cell cycle. However, depending on the batch of serum used, isoleucine plus glutamine starvation led to arrest of cells in different states (modification in cell size and protein content per cell).

The large variations observed in the PGE₁-sensitive adenylate cyclase activity during the cell cycle can hardly be accounted for by variations in the susceptibility of the cells to the treatment used for cell lysis, because more than 90% of the cells were disrupted by the osmotic shock (see Materials and Methods). Furthermore, the observed dissociation in the evolution patterns of basal, and dopamine-, adenosine-, or PGE₁-sensitive adenylate cyclase activities cannot be explained by a modification in the accessibility of ATP to the catalytic site of the enzyme.

Combined stimulations of adenylate cyclase by saturating amounts of either PGE₁ and adenosine, or PGE₁ and dopamine, never led to additive responses. On the other hand, data of Table 1 demonstrate that the three agonists tested exerted their action through three independent receptor sites. The above observations indicate that PGE₁, adenosine, and dopamine are acting on the same cells and suggest that the specific receptors for the three agonists control the same adenylate cyclase system. Furthermore, the observed differences in the evolution patterns of adenylate cyclase responsiveness to PGE₁, adenosine, and dopamine favor the hypothesis that catalytic activity of adenylate cyclase on the one hand, and its sensitivity to specific

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**FIG. 4.** Evolution of stimulated adenylate cyclase activities during the cell cycle of neuroblastoma cells. Adenylate cyclase activity was measured in presence of PGE₁, 1 μM (■, ○); adenosine, 100 μM (▲, △); or dopamine, 100 μM (●, ○). Data are plotted as a function of time following reinitiation of growth. Solid symbols: data obtained from cells starved for 48 hr; open symbols: data obtained from cells starved for 36 hr. Values are means ± SEM of four independent determinations using four separate petri dishes. The duration of the successive phases of the cell cycle is indicated in the figure. The peak value of the mitotic index was 0.7.

**FIG. 5.** Evolution of basal adenylate cyclase activity during the cell cycle of neuroblastoma cells. Basal activity is plotted as a function of time following reinitiation of growth. Solid symbols: cells starved 48 hr; open symbols: cells starved 36 hr.

**FIG. 6.** Evolution pattern of mitotic index, [³H]thymidine incorporation into DNA, and stimulated adenylate cyclase during the second cell cycle following reinitiation of growth. (Upper) The evolution in resynchronized cells. Solid symbols: behavior of cells resynchronized by Colcemid treatment (see Materials and Methods). Open symbols: control cells going through the same treatment without Colcemid. Data are plotted as a function of time following Colcemid washout (Lower) The evolution pattern during the normal second cell cycle of neuroblastoma cells. Adenylate cyclase activity has been tested in the presence of Tris-HCl, 100 mM. This change accounts for the higher activity measured as compared to that shown in Fig. 1.
agonists on the other, can be regulated independently. Modulation of the sensitivity of adenylate cyclase might reflect either modification in the number (or accessibility) of the specific receptor sites coupled to the enzyme, or modification in the efficiency of receptor-enzyme coupling. The first hypothesis would appear more likely, because, in synchronized melanoma cells, it was clearly demonstrated (16) that the number of binding sites for melanocyte-stimulating hormone was maximal during the G2 phase of the cell cycle. The possibility that the number of specific receptor sites and the catalytic activity of adenylate cyclase might be modulated independently favors the hypothesis that the receptor and the catalytic moiety of the enzyme are born by different molecular entities. Dissociation in the evolution of adenylate cyclase activity and of its responsiveness to a specific agonist was also observed in synchronized, catecholamine-sensitive Chang liver cells (17). In various synchronized cell types, intracellular cyclic AMP level has been demonstrated to oscillate (see for review, ref. 18). As shown by Fig. 5, basal adenylate cyclase activity exhibited continuous variations over the whole duration of the cell cycle. If the activity measured in vitro on acellular preparations is representative of the enzymatic content of the intact cells, it seems unlikely that the large variations in cyclic AMP content that have been described elsewhere can be accounted for by parallel changes in adenylate cyclase content. The observed evolution of the PGE2-sensitive activity of neuroblastoma cells during G2 phase and mitosis appeared to be strikingly similar to that of fibroblast intracellular cyclic AMP content (13). It has been shown that PGE2 is able to induce an arrest of cellular growth and that indomethacin, a potent inhibitor of prostaglandin synthesis, stimulates the proliferation of HeLa cells (19). These data, taken into consideration with the evidence that neuroblastoma cells, glial cells, and fibroblasts have the capability of synthesizing prostaglandins (7), raise the possibility that endogenous prostaglandins might participate in the regulation of cyclic AMP production during the cell cycle.

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