MODIFICATIONS OF ADENYLATE AND GUANYLATE CYCLASE ACTIVITIES DURING MULTIPLICATION OF KB CELLS

Annie GUIRAUD-SIMPLOT
Louis COLOBERT

Laboratoire de Chimie Biologique et Médicale
Université Claude-Bernard, 8 avenue Rockefeller
69373 Lyon Cedex 2

Received February 15,1977

SUMMARY

Adenylate and guanylate cyclase activities do not vary in concert during the multiplication of KB cells. Adenylate cyclase activity is low and slightly increases at cell confluency, guanylate cyclase activity, great in sparce cells, decreases during cell multiplication period. These variations are not caused by a modification of catalytic sites because the apparent Km for ATP or GTP is not changed, but by a modification of the dependance on Mg^{++} or Mn^{++} ions. Fresh serum increases guanylate cyclase activity but does not affect adenylate cyclase.

INTRODUCTION

The relationship between changes in intracellular levels of cyclic AMP and cyclic GMP to cell proliferation is not clear. It has been proposed that the action of cyclic GMP is in opposition to that of cyclic AMP. This hypothesis is based on the following observations : cyclic GMP levels increase when cells growth is stimulated whereas cyclic AMP levels decrease (1, 2) ; fibroblast growth factor which promotes the growth of cultured cells, can activate guanylate cyclase and increase cyclic GMP levels (3), cyclic GMP treatment of BALB 3T3 cells blocks the effects of prostaglandin E1 on amino-acid transport (4), this effect is thought to be mediated by cyclic AMP, and thirdly SV40 transformed 3T3 cells have higher cyclic GMP levels than the untransformed parent cells (5). Opposingly, it was been recently demonstrated that guanylate cyclase changes in a similar manner to that of adenylate cyclase and the intracellular concentration of both cyclic nucleotides tends to vary in concert (6, 7). To better understand the relationship of the metabolism of these two cyclic nucleotides in transformed cells, a study was initially performed on adenylate

ISSN 0006-291X

and guanylate cyclase activities as well as their enzymatic characteristics in KB cells over a period of culture.

MATERIALS AND METHODS

Cell Culture. KB cells were grown in 75 cm culture surface Falcon flasks containing 20 ml of Eagle's minimal essential medium supplemented with 10 % calf serum, 0,25 % sodium bicarbonate and 0,005 % aureomycin pH 7.1. Approximately 8 x 10^6 cells were seeded from confluent cells dispersed by scraping with glass taws. Change of culture media were made 42 hours later. In these conditions, confluency was obtained 48 hours later.

Adenylate and guanylate cyclase assays. The flasks were emptied and cells were harvested by scraping in 25 mM Tris-HCl, pH 7.6 supplemented with 1 mM $MgCl_2$ and 250 mM saccharose for adenylate cyclase assay, and in 50 mM Tris HCl, pH 7.6 supplemented with 1 mM $MgCl_2$, 200 mM KCl' and 250 mM saccharose for guanylate cyclase assay. Cell viability was tested by eosine dye exclusion. Cells were broken in a Sorval RF-1-Ribi Refrigerated Cell Fractionnator (temperature +4°C, pressure 16000 psi). Breakage was controled by phase contrast microscopy. Adenylate cyclase assay reaction constituents (8) included 2 mM (α ^{32}P) ATP 1 µCurie, 1 mg/ml creatine phosphokinase, 20 mM creatine phosphate, 1 mg/ml bovine serum albumin, 10 mM $MgCl_2$, 10 mM theophylline, 25 mM Tris-HCl pH 7.6 and approximately 400 µg protein in a final volume of 100 µl. Guanylate cyclase assay reaction constituents included 2 mM ($\alpha^{32}P$) GTP (1.2 µCurie), 6 mM $MnCl_2$, 1 mg/ml bovine serum albumin, 2,5 mM cyclic GMP, 20 mM caffeine, 50 mM Tris-HCl pH 7.6 and approximately 200 µg protein in a final volume of 100 µl. The reactions were initiated by addition of enzyme at 4°C, were incubated in a shaking water bath at 37°C 10 minutes for adenylate cyclase assay, and 5 minutes for guanylate cyclase assay. The reaction was stopped by immersion in boiling water for 3 minutes and addition of stop solution containing 20 mM ATP and 6 mM (8^3H) cyclic AMP (5000 cpm) as chromatographic tracer for adenylate cyclase assay and 100 mM sodium pyrophosphate and (8^3H) cyclic GMP for guanylate cyclase assay. Cyclic nucleotides were isolated and measured as described by White et Zenzer (9). Blanks consisted of immersing the samples and adding the stop solution prior to the addition of enzyme. Blank values which were at most 20 % of the lowest experimental value were substracted from each experimental value. The recovery of tritiated carriers varied from 50 to 60 %. Each enzyme assay was performed in duplicate.

In absence of ATP or GTP regenerating system in the assay medium, only 50 % to 62 % of the added triphosphate nucleotide remains intact after 10 minutes of incubation. The rest is broken down to diphosphate nucleotide by enzymatic systems like ATPase or GTPase. The presence of a regenerating system to reconstitute triphosphate nucleotide from diphosphate nucleotide so that the rate of substrate does not risk to be limiting, seems to be usefull. In the adenylate cyclase assay, such a system gave a large stimulation of activity (40 %). In the opposite, the presence of a regenerating system of GTP slightly decreased guanylate cyclase activity (20 %). ATP added to the guanylate cyclase assay medium partly protected GTP from break-downing but we have observed that 0,75 mM ATP inhibits guanylate cyclase by decreasing its Vm and by increasing the apparent Km for GTP from 0,5 mM up to 1,4 mM. Routinely an ATP regenerating system was used for adenylate cyclase assay, but neither ATP nor a regenerating system was used for guanylate cyclase assay ; for this reason, incubation was carried out for only 5 minutes to limit GTP breakdown. The reactions were linear with respect to protein concentrations up to 1 mg protein and to incubation time up to 20 minutes for adenylate cyclase and 8 minutes for guanylate cyclase, with all the samples of cells over a period of culture of 72 hours.

TABLE I. Subcellular distribution of adenylate and guanylate cyclase in sparce and confluent KB cells.

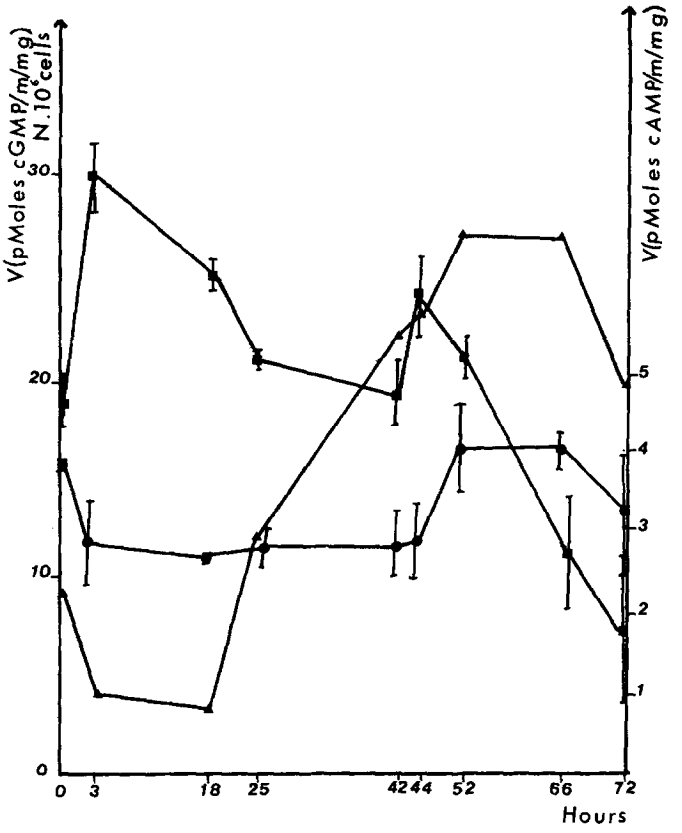
	Sparce cells (3 hours after seeding)						Confluent cells (52 hours after seeding)					
	Adenylate cyclase			Guanylate cyclase			Adenylate cyclase			Guanylate cyclase		
	T.Act. (pM/m)	%	Spec.Act. (pM/m/mg)	T.Act. (pM/m)	%	Spec.Act. (pM/m/mg)	T.Act. (pM/m)	%	Spec.Act. (pM/m/mg)	T.Act. (pM/m)	%	Spec.Act. (pM/m/mg)
Homogenat	94±4	100	1,4	403±12	100	6,7	143±7	100	2,2	290±9	100	4,8
1,000 g pellet (10 minutes)	84±4	89	2,6	60±5	15	3,1	125±9	87,5	3,9	38±6	13	1,4
7,000 g pellet (10 minutes)	2±3	2	-	8±3	2	0,8	8±1	5,5	0,8	13±1	4,5	1,1
105,000 g pellet (100 minutes)				-	-					3±1	1	-
105,000 g supernatent				301±7	73	29,5				206±5	71	20,5
Recovery %		91			90			93			89,5	

KB cells were grown, harwested and broken as described. Breakage was controled by phase contrast microscopy. At each step of centrifugation, the pellet was resuspended in the breakage medium and resedimented as before, the two supernatents were combined for the following step. Each assay was made in duplicate in the standard conditions and results were the mean values of 4 experiments. Total activity (T. Act.) was expressed as picomoles of cyclic nucleotides formed/minute/120 x 10^6 cells, Specific Activity (Spec. Act.) as picomoles of cyclic nucleotides formed/minute/mg protein.

RESULTS

Subcellular distribution of adenylate and guanylate cyclase. Table I shows that adenylate cyclase activity is located in the 1.000 g pellet which collects whole membrane enveloppes, large membrane fragments and broken cells. Guanylate cyclase seems to be soluble or solubilized by the breakage method, because the activity is mainly located in the 105,000 x g supernatent, but 13 % of the activity is located in the particulate fraction which contains adenylate cyclase activity. KCl present in the breakage medium prevents soluble enzymes from adhering to particles, we notice that, in absence of KCl in the breakage medium, guanylate cyclase activity increases up to 18 % in the 1,000 x g pellet. However, if the KCl concentration was increased up to obtain enzyme denaturation, or if 1 % triton is used for detergent treatment, the activity does not decrease down to 13 %, therefore, it can be assumed that there is probably a real particulate guanylate cyclase fraction and it does not just adhere to membrane fragments.

Adenylate cyclase activity is greater in confluent cells than in sparce cells (but 10 mM NaF added to the assay medium increases slightly stronger the



. *Fig.1.* Adenylate and guanylate cyclase activity as a function of growth in KB cells. Three flasks were pooled for each assay which is carried out in duplicate in the standard conditions. Results are mean ± SD of three experiments. Adenylate cyclase activity —●—, guanylate cyclase activity —■— Number of living cells (10^6) —▲—

activity in sparce cells (46 ± 4 pM/m/mg) than in confluent cells (32 ± 3pM/m/mg) ; opposingly, guanylate cyclase activity is higher in sparce cells than in confluent cells, however for each enzyme the subcellar distribution is the same in sparce and confluent cells. For further experiments, assays were carried out in the fractions where the specific activities were the greatest : 1,000 x g pellet for adenylate cyclase, 105,000 x g supernatent for guanylate cyclase. Activities were linear with respect to protein concentrations and incubation times as in crude homogenate.

Dependance on Mg^{++} or Mn^{++} ions. Mn^{++} ions are absolutely required for guanylate cyclase activity, but Mg^{++} ions may be replaced by Mn^{++} ions for adenylate cyclase activity which is slightly decreased (20 %). In the latter case, kine-

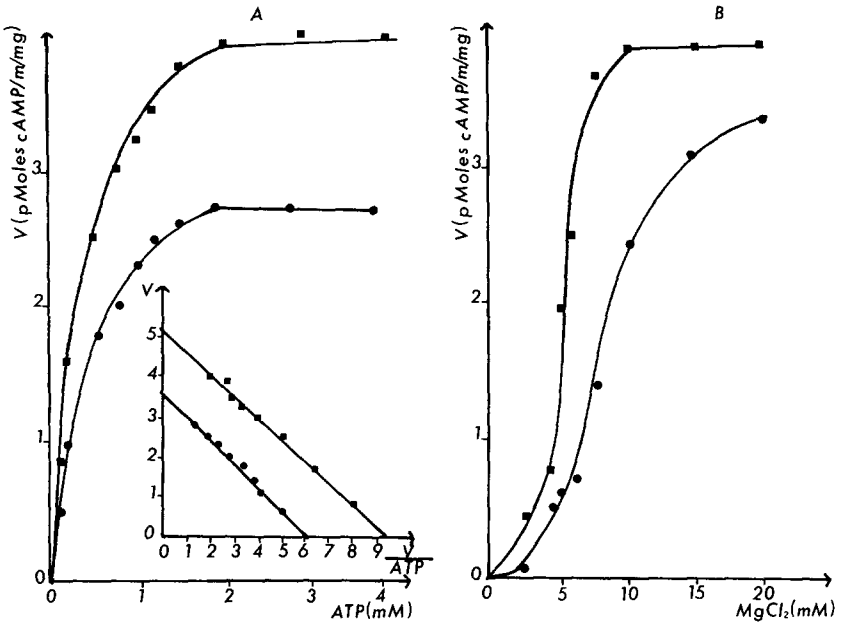


Fig. 2. Kinetic properties of adenylate cyclase in sparce cells (3 hours after seeding) —●— and in confluent cells (52 hours after seeding) —■— Ten flasks were pooled for each assay which is carried out in duplicate. A. Influence of ATP concentration on activity when Mg^{++} concentration is established at 10 mM. B. Influence of Mg^{++} concentration on activity when ATP concentration is established at 2 mM.

tics were the same with Mg^{++} ions. At set ATP or GTP concentrations, Mg^{++} or Mn^{+} ions effect activities by increasing Vm without changing the apparent Km for ATP or GTP (unpublished figure). In the standard medium triphosphate nucleotide and ion concentrations corresponded to our highest activities found in sparce cells as well as in confluent cells.

Adenylate and guanylate cyclase activities at growth stages of KB cells. Figure I shows that these activities do not vary in the same manner. Adenylate cyclase activity slightly decreases after seeding, remains almost constant during cell multiplication and slightly increases at confluency. Guanylate cyclase activity is stimulated just after seeding then decreases during cell multiplication. Addition of fresh serum in the medium, which is changed 42 hours after seeding, increases guanylate cyclase activity, but does not influence adenylate cyclase. It might be supposed that in absence of a regenerating system of GTP in the assay medium, a phosphatase activity varying over the period of culture, could modify the GTP degradation, the assay conditions and so the assaied guanylate cyclase

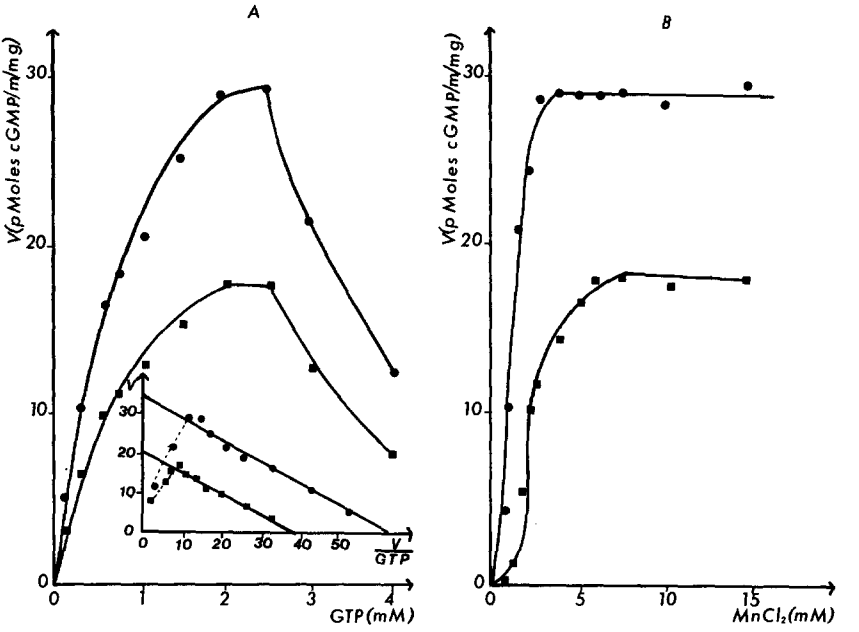


Fig.3. Kinetic properties of guanylate cyclase in sparce cells (3 hours after seeding) and in confluent cells (52 hours after seeding) Ten flasks were pooled for each assay which is carried out in duplicate. A. Influence of GTP concentration on activity when Mn^{++} concentration is established at 6 mM. B. Influence of Mn^{++} concentration on activity when GTP concentration is established at 2 mM.

activity. To eliminate this hypothesis, we have tested that the activity is linear with respect to time up to 8 minutes in sparce as in confluent cells and in each sample over the period of culture.

Effects of cell density on the kinetic properties of adenylate and guanylate cyclases are shown in fig. 2 and 3. The apparent km for ATP is not changed (fig. 2 A slope of Eadie plot) and although the GTP degradation can decrease the precision of the guanylate cyclase assay in presence of low GTP concentration, the apparent km for GTP does not seem to be changed by cell contact (fig. 3 A slope of Eadie plot); reversly the dependance on Mg^{++} or Mn^{++} ions is modified (fig. 2 B and 3 B). In presence of standard Mn^{++} concentrations, guanylate cyclase activity is decreased by elevated GTP concentrations (fig. 3A). It is possible that free GTP, non complexed with Mn^{++}, is an inhibitor, but an increase of $MnCl_2$ concentrations up to 35 mM did not alter this inhibition. This effect is not observed with elevated ATP concentrations

in adenylate cyclase assay although it was been shown in other type of cells (10). This inhibition might be caused by GDP resulting from breakdown of GTP at elevated concentration in assay medium, effectively GDP concentrations greater than 1 mM decreased very strongly guanylate cyclase activity.

DISCUSSION

In KB, the characteristics of adenylate cyclase correspond to that is usually described concerning transformed cells : activity is low and it varies little with cell density (11,12). Opposingly, guanylate cyclase which is known to vary in concert with adenylate cyclase in normal and transformed fibroblasts (6,7), changes in an opposite manner in KB cells. Adenylate cyclase activity is slightly increased at cell confluency but guanylate cyclase, high in sparce cells, decreases during the cell multiplication period. It should be noted that the elevated guanylate cyclase activity in sparce cells may be related to fresh serum of seeding medium, effectively, the change of medium made 42 hours later, for feeding of cultures, causes a similar but slighter activation.It was shown that serum added to fibroblast cultures causes a rapid increase in cyclic GMP levels by decreasing the total cyclic GMP phosphodiesterase activity, and that it causes decrease in the intracellular concentration of cyclic AMP by increasing the total cyclic AMP phosphodiesterase activity (13). Serum is also known to decrease adenylate cyclase activity (14). In KB, cells, we observe that serum increases guanylate cyclase activity, but has no influence on adenylase cyclase activity.

Between sparce cells and confluent cells, the differences in cyclase activities are not produced by a change in apparent affinity of the enzymes for ATP ou GTP but by a modification of the dependance on Mg^{++} or Mn^{++} ions. It can be assumed that cell contact may induce modifications of membrane receptors of adenylate cyclase, but it is more difficult to explain the influence of cell contact for guanylate cyclase which is located in the 105,000 x g supernatent, therefore additional studies will be needed to elucidate relationship between guanylate cyclase and cellular membrane.

To determine and compare enzyme activities, at different growth stages, optimal assay conditions were used to measure maximal activity, even though they may negate other regulatory properties of the enzyme. Changes in cyclic nucleotide metabolism occuring in cell cycle (15) are concealed by the use of asynchronous cell population. Cell disruption may also alter enzyme activity as well as regulatory parameters. The close interelationship between adenylate and guanylate cyclase and their enzymatic parameters seems strictly characterize conditions of culture, agents of cell transformation and appa-

rently types of cells. It has been suggested (16) that a viral gene is closely related to the specific changes in the kinetic parameters of adenylate cyclase and that the mechanisms of reduction in enzyme activity during transformation vary with the agents of transformation (11, 16). Yet, in spite of difficulties relative to the numerous variables effecting the experimental conditions, the data presented in this paper support more the hypothesis for an opposite synthesis of the two cyclic nucleotides than a production in concert during the growth of KB cultures.

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