SERUM MODIFICATION OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE FORMS INDEPENDENT OF PROTEIN SYNTHESIS

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SUMMARY

Addition of 10% fetal calf serum to BHK cells made quiescent by maintenance for 48 hours in sub-optimal serum (0.5%) caused rapid changes in cyclic AMP phosphodiesterase activity (increased maximum velocity and affinity) even in the presence of inhibitors of protein synthesis. Activity changes were associated with an alteration in the number of forms of cyclic AMP phosphodiesterase identified by Agarose gel filtration. Three forms of cyclic nucleotide phosphodiesterase were apparent after serum addition whereas only two forms were resolved in quiescent BHK cells. The initial rapid increase in cyclic AMP phosphodiesterase activity seen when serum was added to quiescent cells was followed temporally by a much slower increase in cyclic AMP phosphodiesterase activity that could be prevented by cycloheximide or actinomycin D.

INTRODUCTION

Analyses of cyclic nucleotide phosphodiesterases (E.C. 3.14.17c) with kinetic and physical techniques have documented the presence of multiple forms of this enzyme in a wide variety of mammalian tissues. The regulation of this enzyme system is complex involving a number of different control mechanisms including hormones (1-6), endogenous protein activators (7-11), certain ions (12,13), genetic controls (14-18), cyclic nucleotides themselves (19-21) and possibly enzyme interconversions (22-24). Tissue culture systems have aided the study of cellular regulation of cyclic nucleotide phosphodiesterase. For example, experiments with cultured astrocytoma, neuroblastoma or fibroblast cells have shown that cyclic AMP phosphodiesterase activity increases following treatment with agents that elevate cyclic nucleotide levels (2, 25-28). In such studies, presumably *de novo* protein synthesis was involved since cycloheximide and actinomycin D prevented the increase in enzymatic activity. Trypsin has been shown by Russell and Pastan (29) to alter the kinetic properties of a particulate cyclic AMP phosphodiesterase form in chick embryo fibroblast cells. In recent reports we suggested that the cyclic nucleotide phosphodiesterase system may control cellular cyclic nucleotide levels by a mechanism affecting phosphodiesterase catalysis through an alteration in the substrate affinity of the enzyme (30). These changes in enzyme activity occurred independent from protein synthesis and were mediated by cell-cell interactions

which altered the growth properties of the cells (31). The studies reported here extend our previous observations on serum induced activity changes by demonstrating concomitant modification in the number of physical forms of cyclic nucleotide phosphodiesterase in the absence of *de novo* protein synthesis.

MATERIALS AND METHODS

BHK 21/C13 cells were obtained from the American Type Culture Collection. The cells, when grown in an atmosphere of air in plastic tissue flasks (75 cm^2) or glass roller bottles (Bellco^R; 1580 cm^2), Eagles minimal essential medium (MEM), 15 mM HEPES (N-2-hydroxyethyl piperazine-N-2-ethane sulfonic acid) buffer, and 10% fetal calf serum (FCS), had a doubling time of 15 hours (31). Cells became "quiescent" after culture for 48 hours in suboptimal 0.5% FCS as described previously (30). The quiescent condition was substantiated by analyses of growth curves and measurements of *in vitro* incorporation of radioactive precursors of macromolecular synthesis under identical culture conditions of serum deprivation. Approximately 5 x 10^5 cells were incubated for 15 min in 60 mm x 15 mm culture plates with 5 μCi of [^{3}H] thymidine, uridine, or valine (Schwarz-Mann) in 5 ml of medium and 0.5% serum. After pulse labeling, the culture medium was decanted and the cells were resuspended in 3.0 ml of 0.4 N perchloric acid and centrifuged at 500 x g. The acid soluble precipate was washed three times with 3 ml of 0.4 N perchloric acid and then hydrolyzed in 0.3 N NaOH. Radioactivity was measured in a 0.5 ml aliquot of the base-treated extract and data calculated as cpm/1 x 10^6 cells/min.

To study phosphodiesterase, cells were removed from the substrata (tissue flasks or roller bottles) with a rubber scraper. The harvested cells were washed in serum-free medium and homogenized (100 x 10^6 cells/ml) in a buffer consisting of 5 mM MES (2-(n-morpholine) ethane sulfonic acid; pH 6.2), 50 mM sodium acetate and 2% ethylene glycol. The homogenate, prepared with 5-10 rotations with a Duall glass homogenizer and Teflon pestle, was then centrifuged at 105,000 x g for 30 minutes. The resulting supernatant (0.3 ml) was assayed directly and the remaining complement of phosphodiesterase activity applied to a previously equilibrated Agarose A 0.5 M column (1.5 x 30 cm; bed volume 72 ml). Enzyme activities were eluted with the homogenizing buffer at 0.25 ml/min in 3.3 min fractions. Individual fractions were assayed for cyclic AMP (0.25 μM) and cyclic GMP (1 μM) hydrolytic activity by a modification (32) of the method of Thompson and Appleman (33). Total cyclic AMP phosphodiesterase activity was measured with 200 μM cyclic AMP using the method of Weiss et al. (34).

RESULTS

Cell numbers and cyclic nucleotide phosphodiesterase activities were compared at 24 hour intervals after harvesting BHK cells from confluent cultures and re-seeding them into culture dishes containing minimal essential medium and optimal (10%) or sub-optimal (0.5% fetal calf serum. Cells plated in the sub-optimal serum concentration maintained their initial cell density for the next 72 hours whereas those cultured in 10% serum commenced growing by 24 hours (Figure 1). The cyclic nucleotide phosphodiesterase activities of the serum-deprived cells remained relatively constant between 24 and 72 hours. In contrast, the cells growing in 10% FCS showed increases in phosphodiesterase activities paralleling growth.

Moreover, when 10% FCS was added to the quiescent BHK cultures, a rapid increase in cyclic AMP phosphodiesterase was detectable within minutes after serum addition (Figure 2); the initial increase in enzyme activity in response to serum was not affected by the presence of cycloheximide in the culture medium, however, the increase in phosphodiesterase activity at

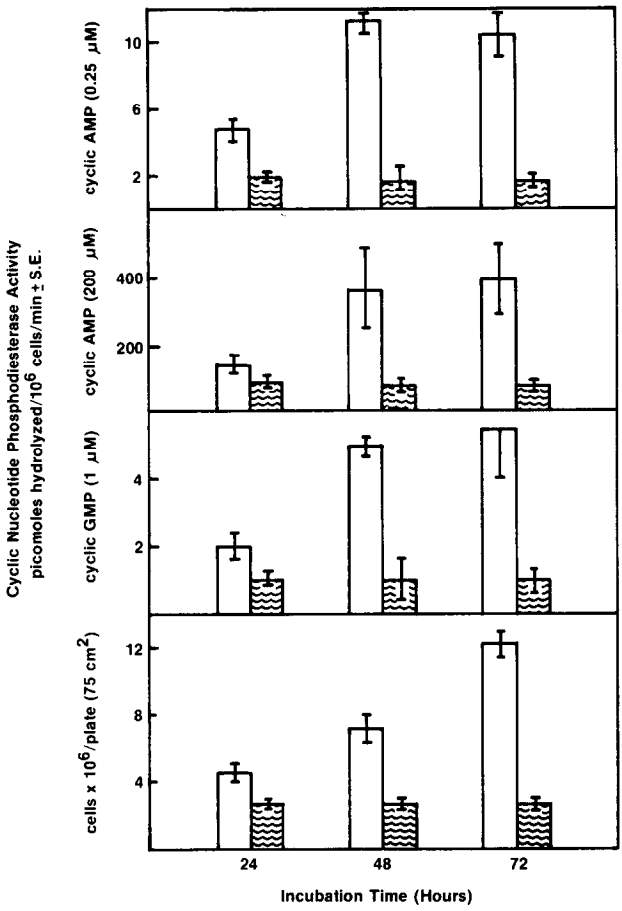

Figure 1: BHK cells were grown as surface cultures to a high cell density (20 x 10^6 cells/75 cm^2) and then removed from the substratum with trypsin treatment (0.2%). The harvested cells were plated at a cell concentration of 2.5 x 10^6 cells/75 cm^2 culture dish in MEM containing either 10% (open bars) or 0.5% (hatched bars) fetal calf serum and were incubated at 37°C for 72 hours with subsequent media changes at 24 hour intervals. At the indicated times, cells were harvested by scraping and cyclic nucleotide phosphodiesterase activities were determined using 0.25 μM or 200 μM cyclic AMP or 1 μM cyclic GMP as substrates. Results are expressed in picomoles of cyclic nucleotide hydrolyzed/10^6 cells/min ± S.E.

later times could be prevented by cycloheximide or Actinomycin D (Figure 2 and reference 30).

Cyclic nucleotide phosphodiesterase forms were analyzed by gel filtration techniques for BHK cells growing in log phase, cells rendered quiescent by incubation for 48 hours in sub-optimal serum, and in serum-deprived cultures replenished with fresh 10% serum. Phosphodiesterase activity from cells in log growth phase showed three peaks of activity (Figure 3). The highest molecular weight fraction (peak L) demonstrated appreciable low Km cyclic AMP phosphodiesterase activity

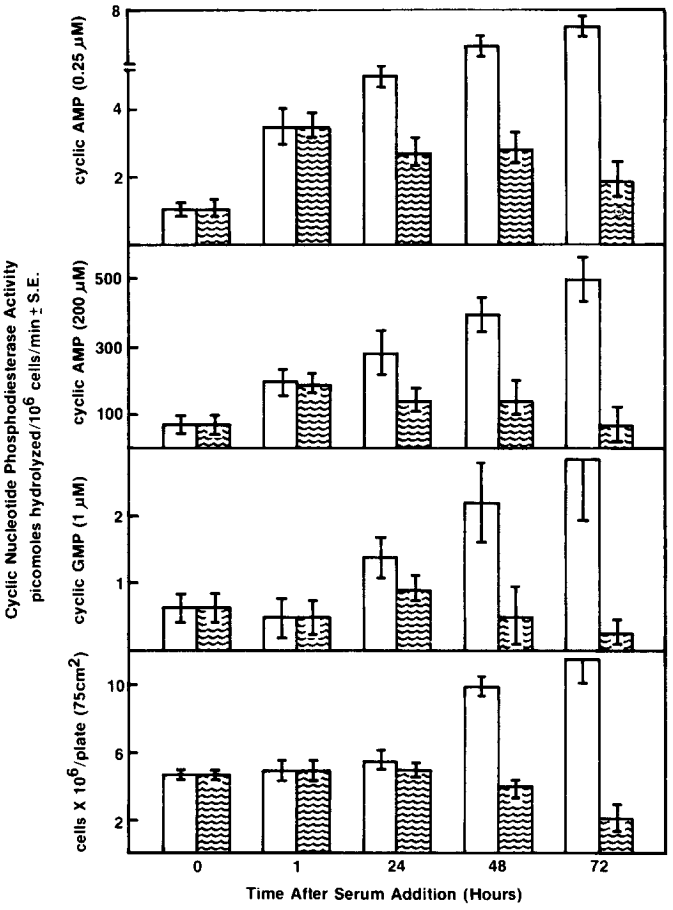

Figure 2: 4 x 10^6 cells were plated in 75 cm^2 plastic culture dishes and were incubated for 48 hours in 0.5% serum at 37°C. 48 hours later, fresh medium containing 10% serum was added to the quiescent cultures. At indicated times cells were harvested by scraping and cyclic nucleotide phosphodiesterase activities determined in the cell homogenate. Incubations in 10% serum were performed in the absence (open bars) or presence (hatched bars) of 0.2 mM cycloheximide with medium changes every 24 hours. Cell counts were monitored and are indicated in the lower panel. Cell viability, measured by trypan blue exclusion, was greater than 90% for each condition. Enzyme activities were determined with 0.25 μM and 200 μM cyclic AMP, and 1 μM cyclic GMP. Results are expressed in picomoles of cyclic nucleotide hydrolyzed/10^6 cells/min ± S.E.

(i.e. activity measured at 0.25 μM cyclic AMP) and negligible cyclic GMP hydrolysis. An intermediate form (peak M) showed significant hydrolysis of cyclic AMP at high substrate concentrations (200 μM) with some cyclic GMP phosphodiesterase activity. The third phosphodiesterase activity fraction (peak S) demonstrated a higher degree of specificity for cyclic GMP as substrate.

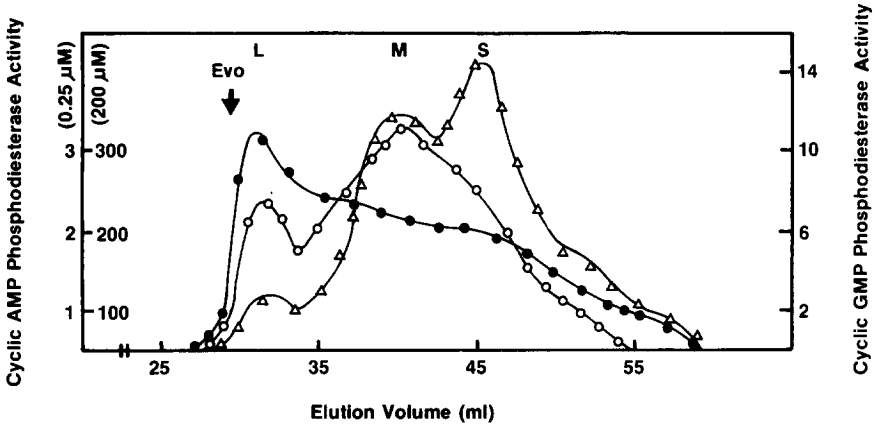

Figure 3: Agarose gel filtration profile of cyclic nucleotide phosphodiesterase activities of a 105,000 x g supernatant fraction obtained from BHK cells growing in MEM with 10% serum. Details of the fractionation procedure are provided in Methods. Activities are expressed as picomoles of cyclic nucleotide hydrolyzed/min/fraction with 0.25 μM cyclic AMP (○), 200 μM cyclic AMP (●), 1.0 μM cyclic GMP (△) as substrates.

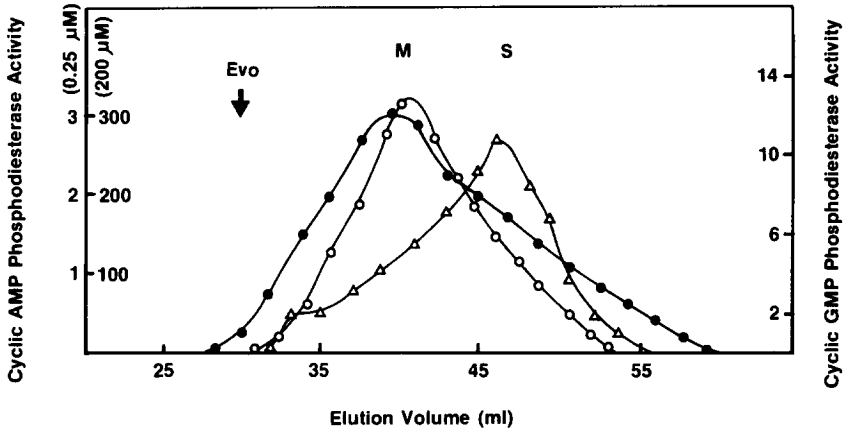

Figure 4: Gel filtration profile of cyclic nucleotide phosphodiesterase activities of a 105,000 x g supernatant fraction prepared from BHK cells made quiescent by maintenance for 48 hours in sub-optimal (0.5%) serum. Activities are expressed in picomoles of cyclic nucleotide hydrolyzed/min/fraction as described in the legend to Figure 2.

When quiescent BHK cells were analyzed, only two fractions of cyclic nucleotide phosphodiesterase activity were resolved by gel filtration. Quiescent cells showed only the intermediate peak M form that had significant cyclic AMP phosphodiesterase activity but negligible cyclic GMP hydrolysis (Figure 4) and the smaller molecular weight form that contained

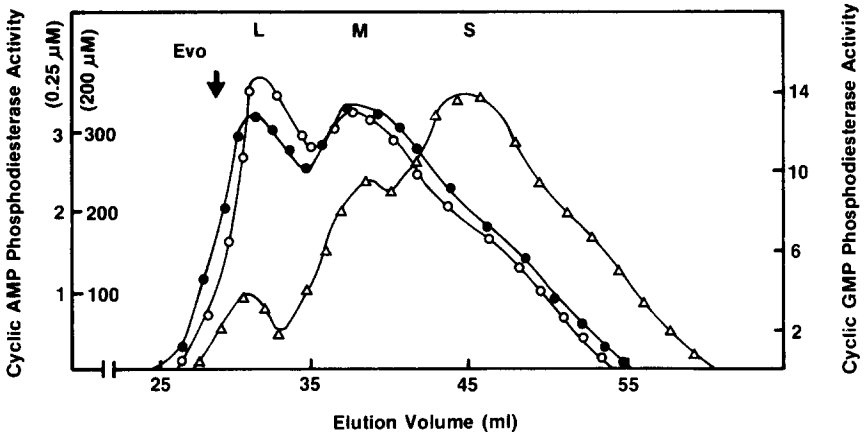

Figure 5: Agarose gel profile of the cyclic nucleotide phosphodiesterase activities of a 105,000 x g supernatant fraction of BHK cells incubated for 1 hr in 10% serum after maintenance for 48 hours in 0.5% serum. Other details are identical to that described in the legends to Figures 3 and 4.

predominately cyclic GMP phosphodiesterase activity; the larger fraction seen in cells from log phase (Figure 3) was not apparent in the quiescent cells (Figure 4).

Agarose gel fractionation of cyclic nucleotide phosphodiesterase of quiescent BHK cells 60 minutes after addition of 10% FCS again showed the presence of the larger molecular weight form (Figure 5). Preincubation of quiescent BHK cells for 3 hours with cycloheximide (0.2 mM) prior to the serum addition failed to modify the appearance of this enzyme form nor did it alter the activity of the other forms.

DISCUSSION

Intracellular concentrations of cyclic nucleotides reflect the physiologic balance between their synthetic and hydrolytic enzymes and mechanisms of extracellular transport. Tissue culture techniques have provided a useful approach to study each process as they are linked to mechanisms of growth and differentiation. The evidence supporting the involvement of cyclic nucleotides in growth and development is largely indirect (17, 35-37). For example, when serum is added to nutritionally deprived quiescent cultures, cellular levels of cyclic AMP decrease prior to the onset of growth induced by serum addition (30, 38-40). A mechanism involving changes in properties of adenylyl cyclase and/or nucleotide phosphodiesterases and/or extracellular transport could mediate the reduction in cyclic nucleotide content. We previously reported that specific activities of cyclic AMP phosphodiesterase of cultured fibroblasts can be markedly and rapidly altered through enzymic mechanisms not dependent on protein synthesis (30,31). Serum addition to

quiescent BHK cells produced a rapid increase in apparent low Km cyclic AMP phosphodiesterase activity (caused by a modification of its kinetic properties) coincident with a decrease in the intracellular cyclic AMP concentration. We have now shown rapid changes in cyclic nucleotide phosphodiesterase forms in these cells. Studies such as these strengthen the importance of cyclic nucleotide phosphodiesterase in the regulation of intracellular cyclic nucleotide levels. Cyclic nucleotide phosphodiesterase activities may be regulated by an external factor(s) present in serum or by internal processes activated by cell-cell interactions. The proposition that both serum and cell-cell contact are important receives support from previous observations showing that the specific activity of BHK cyclic AMP phosphodiesterase was rapidly altered after cell dilution due to a serum dependent change in the apparent Km of the enzyme system. Other studies have indicated that cyclic AMP may regulate its own metabolic rate through mechanisms associated with *de novo* protein synthesis (2, 14, 16, 18); however, as shown here, changes in phosphodiesterase activity clearly can occur in the absence of protein synthesis. The results of this study provide additional evidence that the enzyme system in BHK cells can be regulated through modulation of specific phosphodiesterase enzyme forms independent of protein synthesis within the cell.

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