Cyclic Adenosine 3′:5′-Monophosphate and the Induction of Deoxyribonucleic Acid Synthesis in Liver*

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SUMMARY

A mixture containing glucagon and thyroid hormone was previously devised that enhances markedly nuclear DNA replication and mitosis in the parenchymal liver cells of the unoperated rat. It is now shown that the glucagon of the stimulatory solution can be completely replaced by a mixture of a butyryl derivative of cyclic adenosine 3′:5′-monophosphate and theophylline. Cyclic guanosine 3′:5′-monophosphate and its butyryl derivatives and insulin and high levels of glucose are inactive. The inactivity of N2-monobutyryl cyclic guanosine 3′:5′-monophosphate cannot be ascribed to rapid breakdown in the animal or to the impenetrability of the liver cell since the compound elevates the rate of hepatic amino acid transport and the activity of ornithine decarboxylase. The observation of others (MacManus, J. P., Franks, D. J., Youdale, T. & Braceland, B. M. (1972) Biochem. Biophys. Res. Commun. 49, 1201-1207) that the level of cyclic adenosine 3′:5′-monophosphate is raised during most of the prereplicative period after 70% hepatectomy is confirmed. The evidence supports a positive role for adenosine 3′:5′-monophosphate in regulating DNA synthesis in the liver.

The stimuli that lead to the multiplication of parenchymal liver cells in vivo are unknown. As one approach to this problem, efforts were made to induce hepatic DNA synthesis with bio-chemicals (1). An infusate was devised that contains triiodothyronine,1 amino acids, glucagon, and heparin (to be referred to as the TAGH solution) and that markedly increases nuclear DNA formation in the hepatocytes of the unoperated rat. Mitosis ensues and liver growth can be measured after three treatments with the stimulatory solution. Each component of the TAGH solution contributes to the enhancement of DNA synthesis but the two hormones are the most important constituents.

We have now enquired whether the glucagon of the stimulatory solution can be replaced by a cyclic nucleotide. A mixture of a butyryl derivative of CAMP and theophylline is as effective as glucagon in helping to induce nuclear DNA replication in the liver of the intact rat. On the other hand, cGMP and its derivatives and insulin and glucose are without activity. These results, taken together with the observation of McManus et al. (2) that the concentration of CAMP is elevated in the liver during most of the prereplicative period after 70% hepatectomy and after infusion of intact animals with the TAGH solution, are consistent with a role for the cyclic adenosine nucleotide in stimulating DNA synthesis in liver.

EXPERIMENTAL PROCEDURE

Labeled compounds were from New England Nuclear, an 8.5% solution of 15 amino acids (Freamine) was from McGGaw, glucagon was from Lilly, insulin was from Squibb, heparin (Liquasem) was a product of Organon, and all other compounds were from Sigma.

Female albino rats, Fischer 344, were from Charles River and were kept in a room at 23 ± 0.05°C. They were freely given food and water at all times and, unless otherwise indicated, were used when they weighed 140 to 150 g. All injections were in the tail vein. Partial hepatectomy was the removal of 70% of the liver. Sham hepatectomized animals were treated in the same way but no liver was excised.

Solutions for infusion (10 ml) were freshly prepared and were given in the tail vein from 20-ml hypodermic syringes fixed in a horizontal position in stainless steel plates with a moving carriage assembly acting on the plungers to deliver a constant flow of 3.3 ml per hour. After infusion, the animals were returned to their cages. The TAGH solution (pH 7.2), in 0.15 M NaCl, contained 100 µg of triiodothyronine, 200 mg of amino acids (Freamine), 1 mg of glucagon, and 100 U.S.P. units of heparin. The infusate without glucagon will be referred to as the TAH solution.

DNA synthesis was measured by the incorporation of [3H]-thymidine. Nuclei were isolated by repeated homogenization of liver in 0.1 M citric acid and sedimentation in a sucrose (0.3 M) CaCl2 (0.2 mM) solution. DNA, purified from the nuclei essentially as described by Zamenhof (3), was estimated with diphenylamine (4) and counted in a hyamine phosphor solution.

To measure the rate of amino acid uptake by liver cells, each rat was given 0.3 µCi of α-[14C]aminobutyrate. At the end of the labeling period, blood flow to a portion of the liver (about 0.9 g of...
the left lateral lobe) was stopped with a hemostat and blood was taken from the hepatic portal vein. The liver sample was then cut off, blotted to remove excess blood, and homogenized in 7% sulfosalicylic acid. The supernatant liver fractions and the portal sera were counted in Instage (Packard). The size of the hepatic extraacellular space was measured by the distribution of [carboxyl-14C]insulin between liver and portal serum (taken to be identical with the extracellular fluid). A value of 18% was found with rats infused (10 ml in 3 hours) with 0.15 M NaCl, theophylline (10 mg), or a mixture of theophylline and BtscAMP (5 μmol). The α-[14C]aminoisobutyrate inside the liver cells was calculated from the radioactivity of the liver supernatant fraction by subtracting 18% of the counts of the corresponding sample of portal serum.

Ornithine decarboxylase was estimated with [1-14C]ornithine by the release of 14CO2 exactly as described by Jänne and Williams-Ashman (6). For the assay of cAMP, liver samples were taken with liquid nitrogen-cooled tongs and the frozen liver was lyophilised to dryness at −50°. Estimation of the cyclic nucleotide was exactly as described by Kuo and Green gard (6). To maintain low blank values, it was found to be essential to repurify the [32P]ATP before each assay. Purification was on a column (2 x 0.5 cm) of QAE-Sephadex A-25, formate form. After adsorption of the ATP, the column was washed with 10 ml of 0.2 M ammonium formate and [32P]ATP was eluted with 4 ml of 0.4 M ammonium formate. The ATP was used without further treatment.

RESULTS

Replacement of Glucagon with Mixture of BtscAMP and Theophylline—Tested in combination with the TAH solution, a mixture of BtscAMP and theophylline was as effective as glucagon in stimulating nuclear DNA synthesis in the liver of the unoperated rat (Table I). 5’-AMP was unable to substitute for the cyclic nucleotide.

Requirement for Triiodothyronine—The mixture of amino acids, glucagon, and heparin, without thyroid hormone, is only about one-tenth as effective in stimulating DNA replication in the intact liver as the TAH solution (1). Table II shows that triiodothyronine was also essential for the full activity of the infusate in which glucagon was replaced with BtscAMP and theophylline.

Comparison of Abilities of BtscAMP, BtscAMP, and cAMP to Induce DNA Synthesis—Supplementation of the TAH-theophylline infusate with 1 or 2 μmol of BtscAMP or BtscAMP caused a large additional increase in DNA synthesis in the intact liver (Table III). The nonesterified cyclic nucleotide (10 μmol) was inactive.

Inability of cGMP and Derivatives, Insulin, or Glucose to Substitute for BtscAMP—Neither cGMP, its butyryl derivatives, insulin, nor glucose was able to replace BtscAMP for the stimulation of DNA formation in the intact liver (Table IV). As the table shows, none of the compounds blocked the action of BtscAMP.

Effects of cAMP, cGMP, and Derivatives on Rate of Transport of α-[14C]Aminoisobutyrate into Liver Cells—The inability of cAMP, cGMP, and the butyryl derivatives of the cyclic guanine nucleotide to stimulate liver DNA synthesis could have stemmed from their rapid destruction in the animal or from their exclusion from the liver cell. To look at these possibilities, the effects of the cyclic nucleotides on hepatic processes other than DNA replication were examined.

Glucagon elevates the rate of transport of the nonmetabolizable amino acid, α-aminoisobutyrate, by liver cells in vivo (7, 8) and in the perfused organ (9). With liver slices, BtscAMP is as active as the pancreatic hormone (10).

The rate of uptake of α-aminoisobutyrate by liver cells in the rat was stimulated by BtscAMP, BtscAMP, and consistently, albeit less well, by BtscGMP (Table V). cAMP, cGMP, and BtscGMP were inactive.

Table I

| Infusate                  | DNA synthesis |
|---------------------------|--------------|
| None                      | 120 (45–100) |
| NaCl                      | 150 (95–240) |
| BtscAMP                   | 340 (240–450) |
| Theophylline              | 150 (120–310) |
| BtscAMP + theophylline    | 615 (340–1000) |
| Glucagon                  | 570 (290–660) |
| TAH solution              | 550 (340–1050) |
| + BtscAMP                 | 1800 (990–2100) |
| + Theophylline            | 1200 (860–1500) |
| + BtscAMP + theophylline  | 4500 (2600–6600) |
| + 5’-AMP + theophylline   | 1400 (1100–1600) |
| + Glucagon                | 4300 (2600–6100) |

Table II

| Triiodothyronine | Amino acids and heparin | Additions DNA synthesis |
|-----------------|-------------------------|------------------------|
|                 |                         | +                      |
|                 |                         | + BtscAMP + theophylline |
|                 |                         | + BtscAMP + theophylline |
|                 |                         | + BtscAMP + theophylline |
|                 |                         | + Glucagon |

Stimulation of Ornithine Decarboxylase Activity in Liver by BtscGMP—The activity of hepatic ornithine decarboxylase can be elevated by treating intact rats with BtscAMP (11), L-histidine (12), or growth hormone (12). The amino acid is without effect in hypophysectomized rats (12) suggesting that it acts via the pituitary hormone.

Infusion of intact animals with a mixture of theophylline and BtscGMP raised the activity of liver ornithine decarboxylase at least as well as the mixture with BtscAMP (Table VI). The table shows that the cyclic guanine nucleotide, unlike L-histidine, had almost the same effect in hypophysectomized as in intact rats. The activity of BtscGMP was not dependent upon the adrenal glands.
Levels of AMP in Liver after Partial Hepatectomy—In confirmation of the results of McManus et al. (2), 70% hepatectomy (70-g rats) caused a biphasic increase in the level of AMP in the liver that extended over the entire prereplicative period (about 12 hours). Thus, the levels of the cyclic nucleotide at 0, 4, 7, 10, and 12 hours after the operation were 1.8, 4.3, 2.7, 4.3, and 2.8 pmol per mg of dry liver, respectively.

**TABLE III** Titration of BtgAMP and effects of BtgAMP and cAMP on DNA synthesis in intact liver

Unoperated rats were infused with TAH solution containing 10 mg of theophylline and supplemented with cAMP or a butyrylated derivative, as shown. Nuclear DNA synthesis was measured by the incorporation of \(^{14}C\)thymidine as for Table I. The values shown are the averages of the results obtained, and the ranges of the individual determinations and the numbers of rats tested are given in the parentheses.

| BtgAMP | BtgAMP | cAMP | DNA synthesis |
|--------|--------|------|---------------|
| pmol  | pmol  | cpm/mg DNA |
|       |       |           |
| 0.1   | 1200 (510-1750) (4) |
| 1     | 2900 (1200-4200) (6) |
| 2     | 3200 (2800-3800) (3) |
| 5     | 6000 (1800-15,000) (13) |
| 10    | 7600 (4000-10,000) (3) |
| 2     | 2500 (1800-3500) (3) |
| 5     | 4600 (3350-5800) (3) |
| 10    | 1400 (1100-1900) (4) |
| 5     | 5300 (2800-5900) (4) |

**TABLE IV** Inability of cGMP and butyryl derivatives, insulin, or glucose to substitute for BtgAMP in stimulation of DNA synthesis

Unoperated rats were infused with TAH solution supplemented, as shown, with theophylline (10 mg), cGMP or a derivative (10 pmol), insulin (5 units), glucose (2.3 mmol), and BtgAMP (5 pmol). Nuclear DNA synthesis was measured as for Table I. The values shown are the averages of the results obtained, and the ranges of the individual determinations and the numbers of rats tested are given in the parentheses.

| Additions to TAH solution | DNA synthesis |
|-------------------------|---------------|
|                         | cpm/mg DNA |
| None                    | 570 (380-720) (8) |
| Theophylline            | 910 (545-1600) (11) |
| cGMP + theophylline     | 1020 (650-1600) (3) |
| cGMP + BtgAMP + theophylline | 3900 (2400-5400) (3) |
| BtgAMP                  | 550 (320-750) (6) |
| BtgAMP + theophylline   | 1000 (600-1300) (4) |
| BtgAMP + BtgAMP + theophylline | 4400 (2100-7100) (5) |
| BtgAMP + theophylline   | 830 (410-970) (5) |
| BtgAMP + BtgAMP + theophylline | 4100 (2900-5200) (5) |
| Insulin                 | 520 (240-670) (4) |
| Insulin + theophylline  | 920 (500-1200) (4) |
| Insulin + BtgAMP + theophylline | 4200 (2100-6100) (4) |
| Glucose                 | 580 (300-770) (4) |
| Glucose + theophylline  | 1000 (720-1500) (4) |
| Glucose + BtgAMP + theophylline | 4900 (2100-6600) (4) |
| BtgAMP + theophylline   | 5100 (1900-8250) (12) |

**TABLE V** Effects of cAMP, cGMP, and butyrylated derivatives on rate of transport of \(\alpha\)-[\(^{14}C\)]aminoisobutyrate into liver cells

Unoperated rats were infused (10 ml in 3 hours) with a 0.15 M NaCl solution that contained theophylline (10 mg) and cyclic nucleotides, as shown. At 4 hours from the start of the infusion, each rat was given 0.3 \(\mu\)Ci of \(\alpha\)-[\(^{14}C\)]aminoisobutyrate and portal blood and a sample of liver were taken 2.5 or 5 min later. The radioactivities of the portal serum and the total liver homogenate and the calculation of the counts in the liver cell were as described under "Experimental Procedure." The values shown are the averages of the results obtained, and the ranges of the individual determinations and the numbers of rats tested are given in the parentheses.

| Theophylline | Cyclic nucleotide | Labeling period | Portal serum | Liver cell |
|-------------|------------------|-----------------|--------------|------------|
|             | \(\mu\)mol | \(\mu\)mol | cpm/\(\mu\)g wet liver | cpm/\(\mu\)g wet liver |
| +           | 2.5     | 7.5 (6.8-7.9) | 2.6 (2.3-2.7) (3)<sup>a</sup> |
| +           | 5       | 5.8 (3.6-6.1) | 4.9 (3.6-6.4) (7)<sup>a</sup> |
| +           | 10      | 6.1 (5.7-6.9) | 6.0 (4.9-6.7) (4) |
| +           | 5       | 4.8 (4.0-5.4) | 10 (5.6-14) (11) |
| +           | 0.1 BtgAMP | 5 | 5.6 (5.3-5.9) | 13 (11-18) (4) |
| +           | 1 BtgAMP  | 2.5 | 4.5 (4.3-4.9) | 18 (12-23) (3) |
| +           | 1 BtgAMP  | 5 | 3.1 (2.7-3.5) | 31 (10-40) (5) |
| +           | 5 BtgAMP  | 2.8 (2.5-3.6) | 40 (27-54) (9) |
| +           | 5 BtgAMP  | 2.7 (2.5-3.6) | 41 (30-49) (3) |
| +           | 10 cAMP   | 5 | 4.6 (4.8-6.2) | 8.1 (5.9-12) (3) |
| +           | 10 BtgGMP | 5 | 4.0 (4.7-5.2) | 8.7 (4.6-12) (4) |
| +           | 10 BtgGMP | 5 | 4.1 (3.8-4.7) | 19 (15-25) (6) |
| +           | 10 cGMP   | 5 | 4.9 (3.9-5.7) | 11 (10-14) (4) |
| +           | 10 BtgAMP + 5 | 3.3 (2.4-3.6) | 34 (22-41) (4) |

<sup>a</sup> Values are in thousands.
<sup>b</sup> The rats were infused with 0.15 M NaCl.

**TABLE VI** Stimulation of ornithine decarboxylase activity in liver by BtgGMP

Unoperated, hypophysectomized (2 days after the operation), and adrenalectomized (5 days after the operation) rats were infused (10 ml in 3 hours) with 0.15 M NaCl containing theophylline (10 mg), BtgAMP (5 pmol), BtgGMP (10 pmol), and L-histidine (1.4 mmol), as shown. At 4 hours from the start of infusion, liver samples were removed and ornithine decarboxylase was estimated as described under 'Experimental Procedure.' The values shown are the averages of the results obtained, and the ranges of the individual determinations and the numbers of rats tested are given in the parentheses.

| Operation | Theophylline | Additions | Ornithine decarboxylase |
|-----------|--------------|-----------|-------------------------|
|           | \(\mu\)mol | \(\mu\)mol | \(\mu\)COCO<sub>4</sub>/mg protein/hr |
| None      | 0.01 (0.01-0.01) (4) | 0.01 (0.01-0.01-0.1) (8) |
| +         | BtgAMP       | 0.69 (0.41-0.93) (6) |
| +         | BtgGMP       | 0.90 (0.17-1.7) (11) |
| +         | L-Histidine  | 0.52 (0.33-0.76) (4) |
| +         | 0.01 (0.02-0.02) (3) |
| Hypophysectomy |   | + BtgAMP | 0.08 (0.02-0.12) (3) |
|     | + BtgGMP   | 0.60 (0.29-0.92) (4) |
|     | + L-Histidine | 0.10 (0.02-0.46) (5) |
|     | + BtgAMP  | 0.34 (0.19-0.51) (3) |
| Adrenalectomy | + BtgAMP | 1.3 (1.1-1.7) (4) |
nuclear DNA and cell division. With cultured cells, it is usually assumed that all alterations that follow the application of a stimulus for multiplication are directly involved in release from the resting state. In the case of systems in vivo, for example, the regenerating liver after partial hepatectomy, this is clearly an unsafe assumption. With such a system, it is necessary to distinguish between events that are requisite for the subsequent synthesis of DNA from those that may be necessary for the survival of the animal.

The mixture containing triiodothyronine and glucagon (1) that stimulates DNA formation in the hepatocytes of the unoperated rat provides some means for assessing the relationship of a pre-replicative change to DNA synthesis. The occurrence of the same change after partial hepatectomy and in the stimulated, intact liver would suggest a role for the alteration in leading to DNA replication.

McManus et al. (2) described a biphasic increase in liver cAMP during the prereducptive period after 70% hepatectomy of the rat and almost identical changes in unoperated animals that had been infused with the TAGH mixture. The present finding that the glucagon of the mixture can be replaced completely by a butyrated cAMP and theophylline provides additional evidence that the cyclic nucleotide plays a critical role in the induction of hepatic nuclear DNA synthesis.

With the whole animals, it is not possible to know whether the effects of administered glucagon or a butyrated cAMP are primary results or are secondary responses, for example, to the action of the insulin that is released (13, 14). Insulin and proteins with insulin-like activity have been shown to stimulate multiplication of various cultured cells (15). Arguing against a secondary stimulus, however, is the inability of insulin and glucose to substitute for the glucagon of the TAGH solution. It may be noted here that the insulin-like activity and the cell division-promoting activity of serum for a cultured fibroblast have now been completely dissociated (16).

The question also arises whether the stimulation of hepatic DNA formation by glucagon or a cAMP derivative is by their direct action on the liver cell. That a direct effect is likely to be involved is suggested by the elevated level of cAMP in liver after partial hepatectomy and treatment of unoperated rats with the TAGH mixture (2) and by the fact that at least one of the liver changes that is seen after the operation (17) and in intact animals given glucagon (7, 8) or Bt2cAMP (7), an increase in the rate of amino acid transport, can also be produced by the cyclic nucleotide in vitro (10).

From observations with some lines of cultured cells, cAMP has been assigned a role of limiting cell multiplication. Thus, growth of cells to high density is associated with an elevation in adenylate cyclase activity (18, 19) whereas transformation with virus causes a reduction in the activity (20); an inverse correlation is found between growth rate and cellular levels of cAMP (21–24); and Bt2cAMP inhibits the growth of transformed fibroblasts (20, 25) and of stimulated, untransformed cells (24). The added cyclic nucleotide does not act, however, by blocking DNA replication (26). Instead, multiplication seems to be lowered by inhibition of a process involved in cell division, and some cell death occurs.

It is clear that there is no universal relationship between cAMP and the control of cell multiplication. The level of the cyclic nucleotide becomes elevated, not reduced, in normal liver that has been stimulated to make DNA and divide (2) and it is higher than normal in all the hepatomas that have been examined (27); added cAMP has no effect on the rate of multiplication of cultured monkey kidney cells (28) and Bt2cAMP enhances the growth of cultured chick fibroblasts (29) and of rat lens cells (30); density-restriction of the multiplication of 3T3 mouse fibroblasts is not accompanied by an increase in the level of cellular cAMP (31); and finally, there is no difference in the level of the cyclic nucleotide in stationary and exponentially multiplying cultures of murine lymphoblasts (32).

More recently, a primary role has been proposed for cGMP in the stimulation of DNA replication and cell division (33–35). It has been suggested (36) that cGMP and cAMP represent opposing cellular forces and that a high level of the one is accompanied by a low level of the other. This is not the case, however, with lung slices in which 3- to 4-fold elevations in both cyclic nucleotides simultaneously follow treatment with bradykinin (37).

cGMP is able to mimic many of the effects produced by cAMP in the perfused liver (38–40). It is not surprising, therefore, that in the animal, Bt2GMP, just as the butyrated derivatives of cAMP, causes increases in the rate of amino acid transport and in the activity of ornithine decarboxylase in liver.

Whether or not a change in the level of cGMP is related to the regulation of DNA formation in hepatocytes is not yet known. The inability of Bt2GMP to substitute for glucagon or butyryl cAMP for the induction of DNA replication would suggest that the cyclic guanine nucleotide plays no role in regulating hepatic DNA synthesis but the possibility cannot be excluded that elevations in the levels of both cyclic nucleotides are essential.

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