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Biochemistry

Regulation of hepatic nuclear guanylate cyclase

(immunofluorescence/cGMP/cAMP/glucagon)

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ABSTRACT **In immunohistochemical studies of rat liver tissue slices and purified nuclei, adenosine 3′:5′-cyclic monophosphate (cAMP) and guanosine 3′:5′-cyclic monophosphate (cGMP) immunofluorescence on the nuclear membrane are sequentially increased after glucagon administration. An explanation for the increased cGMP immunofluorescence was sought in experiments in which guanylate cyclase [GTP pyrophosphate-lyase (cyclizing), EC 4.6.1.2] activity of hepatic subcellular fractions was determined. The results showed that a nuclear guanylate cyclase exists which can be distinguished from the soluble and crude particulate guanylate cyclases. The activity of the nuclear enzyme was increased by 35% in nuclei isolated from rats 30 min after glucagon injection, the time at which maximal nuclear membrane cGMP immunofluorescence is observed. Because glucagon altered both cAMP location and levels prior to the observed changes in nuclear cGMP metabolism, the hypothesis that cAMP acted as the second messenger was tested. *In vitro* incubation of nuclei isolated from control rats with 10^{-5} M cAMP produced a 25% increase in nuclear guanylate cyclase activity. With nuclei isolated from glucagon-treated rats, no significant increase in enzyme activity was observed; this indicates that maximal stimulation of nuclear guanylate cyclase by cAMP occurred at levels that are obtained *in vivo* after glucagon administration. These findings suggest that hepatic nuclear cGMP content may be regulated by a specific organelle guanylate cyclase and that cAMP may be one of the determinants of this enzyme's activity.**

The precise description of the role of adenosine 3′:5′-cyclic monophosphate (cAMP) in modulating procaryote DNA transcription has stimulated interest in the cyclic nucleotides as potential regulators of nuclear processes (1, 2). The relationship between cyclic nucleotides and growth has been an area of particular emphasis but the current data do not support any unified hypothesis of the involvement of cAMP or guanosine 3′:5′-cyclic monophophate (cGMP) in growth regulation. In cultured cell lines, and in lymphocytes transformed by lectins, high levels of cAMP are correlated with cessation (3–5) or absence of DNA synthesis (6, 7). Conditions that stimulate growth induce increased levels of cGMP (8, 9), although this has not been a universal finding (10). In other model systems, measurement of tissue levels of cyclic nucleotides has revealed that growth is correlated with increased cAMP either alone [rat liver regeneration (11, 12)] or in concert with increased cGMP [salivary gland (13), rabbit lens epithelium cells (14)].

These previous studies have relied on the measurement of total cyclic nucleotide content after a defined growth stimulus. However, it is possible that changes in the concentration of cGMP or cAMP in a critical location or organelle might have biologic significance without producing altered tissue levels. This communication describes two approaches to the possibility that the cGMP content of rat liver nuclei might be regulated at the organelle level. Immunofluorescence techniques that specifically localize cGMP have demonstrated that nuclear membrane cGMP immunofluorescence is increased after glucagon injection, a treatment which also increases cAMP immunofluorescence and tissue content. The observed alteration in nuclear membrane cGMP staining led to experiments to determine the activity of guanylate cyclase [GTP pyrophosphate-lyase (cyclizing), EC 4.6.1.2] in rat liver subcellular fractions. These experiments revealed that, in addition to the soluble and crude particulate guanylate cyclases (15, 16), a specific nuclear guanylate cyclase exists. The activity of the nuclear guanylate cyclase is increased after *in vivo* treatment with glucagon and can be stimulated *in vitro* by cAMP. Thus, in rat liver, a potential mechanism exists through which the nuclear content of cGMP may be independently regulated. The data indicate that cAMP may be among the adenine nucleotides and ions whose local concentrations seem to control the activity of specific guanylate cyclases (17).

Abbreviations: cAMP, adenosine 3′:5′-cyclic monophosphate; cGMP, guanosine 3′:5′-cyclic monophosphate; TS, 0.01 M Tris·HCl, pH 8.0/0.25 M sucrose.

MATERIALS AND METHODS

Male Sprague-Dawley rats (150–180 g) received food and water ad lib. Rats were injected with saline (controls) or 0.5 mg of glucagon (Eli Lilly) intraperitoneally and killed by cervical dislocation at the indicated times. Liver samples for measurement of cGMP or cAMP levels were immediately frozen in liquid nitrogen.

Cyclic Nucleotide Immunofluorescence. Hepatic tissue for cGMP and cAMP immunofluorescence determination was frozen in a well containing optimal cutting temperature compound (Miles Laboratory) submerged in acetone and Dry Ice. Tissue, sliced to 4-μm thickness, was processed on slides as previously described (18, 19). Briefly, rabbit antiserum to cGMP or cAMP was applied to the tissue section, and the section was incubated. The slide was washed with phosphate-buffered saline, pH 7.2, and fluorescein-labeled goat antiserum to rabbit immunoglobulin was added; the slide was then incubated again and washed. The specificity of the cyclic nucleotide staining was tested by using normal rabbit serum as the first antibody and by competitively inhibiting the specific immunofluorescence by preincubating the antiserum with 10^{-6} M cGMP or cAMP. Purified nuclei were spread on a glass slide, air-dried overnight, and stained by the procedure noted above. The slides were examined with a Leitz ultraviolet microscope with photographic attachment. All samples (control and experimental) from one experiment were photographed at the same exposure time, to give a relative estimate of immunofluorescence.

Tissue Fractionation. Soluble and crude particulate fractions for guanylate cyclase determinations were prepared by homogenizing 1 g of liver in 20 ml of 0.01 M Tris·HCl, pH 8.0 (4°)/0.25 M sucrose (TS) with a Teflon-glass homogenizer. This and subsequent steps were performed at 4°. Homogenates were strained through gauze and centrifuged in a 50 Ti rotor at 105,000 × *g* for 1 hr. The supernatant was used for soluble enzyme assays. The pellet was washed three times with TS and resuspended in a volume equal to the original volume. Imme-

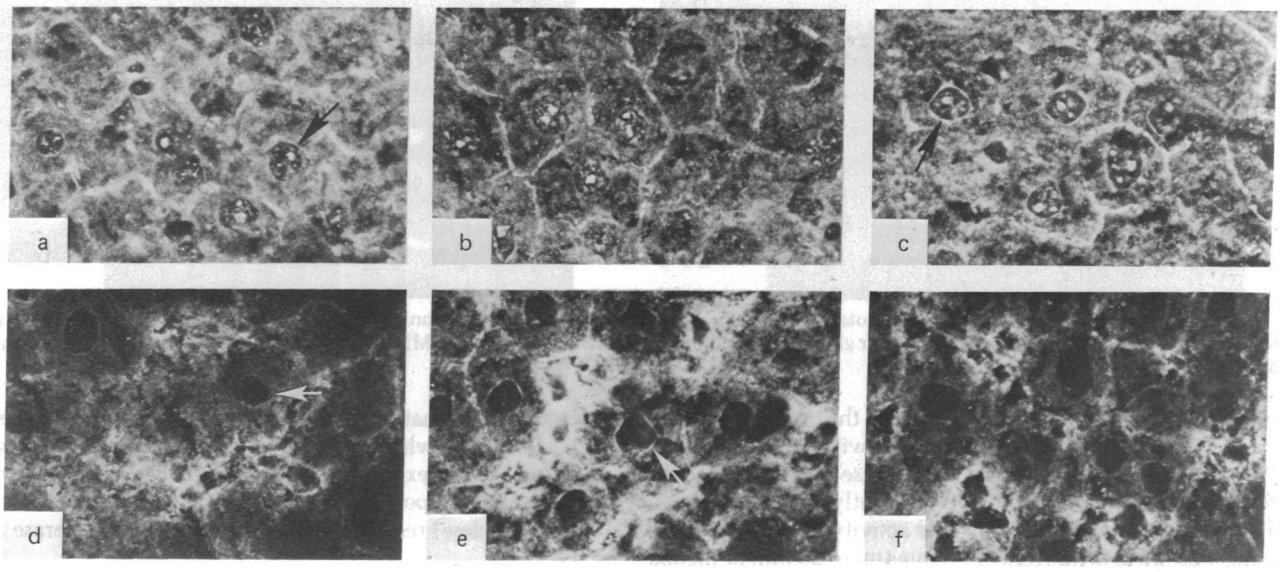

FIG. 1. Pattern of cGMP and cAMP immunofluorescence before and after glucagon administration. All photographs are at the same magnification (×550) and were taken with the same exposure time. In the control rat the nuclear membrane (arrow) exhibited low intensity immunofluorescence for both cGMP (*a*) and cAMP (*d*). Five minutes after glucagon injection, nuclear membrane cGMP immunofluorescence had not increased appreciably (*b*) but cAMP staining (*e*) had increased (arrow). Thirty minutes after glucagon treatment, nuclear membrane cGMP staining (*c*) had increased (arrow) while cAMP immunofluorescence (*f*) had returned to control levels.

diately prior to assay the particulate fraction was finely dispersed with a Brinkman Polytron (ST-10 probe, setting 4, 15 sec). Nuclei were purified by a modification of the method of Blobel and Potter (20). One liver (8–10 g) was homogenized in 35 ml of TS containing 3 mM $MgCl_2$ in a Teflon-glass homogenizer and strained. The preparation was centrifuged at 800 × *g*, resuspended in TS/$MgCl_2$, and respun at 800 × *g*. The pellet was resuspended in 35 ml of 2.2 M sucrose containing 3 mM $MgCl_2$ and 0.01 M Tris and layered over 10 ml of the same solution. The discontinuous gradient was centrifuged at 100,000 × *g* for 1 hr in an SW 27 rotor. The purified nuclear pellet at the bottom of the clear 10-ml layer was separated from the gradient interface with a tube slicer. After aspiration of the 2.2 M sucrose, the pellet was resuspended in 15 ml of TS/$MgCl_2$. The nuclei were spun twice at 800 × *g* and resuspended in TS. This fraction was used for guanylate cyclase, adenylate cyclase, and glucose-6-phosphatase (D-glucose-6-phosphate phosphohydrolase, EC 3.1.3.9) activity assays, as well as for immunofluorescence studies. Electron micrographs of the preparation were prepared by resuspending this fraction in Epon and fixing with glutaraldehyde and osmium tetroxide.

Guanylate Cyclase Assay. Guanylate cyclase activity was assayed by the method of Kimura and Murad (15). The final reaction mixture (volume, 150 μl) contained 50 mM Tris·HCl (pH 7.8 at 37°), 3 mM $MnCl_2$, 0.2 mM methylisobutylxanthine, a GTP-regenerating system (15 mM phosphocreatine, 20 μg of creatine phosphokinase), and 30 μl of tissue sample diluted to the appropriate concentration (30–100 μg of protein). The reaction was started by adding 1 mM GTP. When designated, 1 mM NaN_3, 10^{-6} or 10^{-5} M cAMP, or 10^{-5} M 5′-AMP was added to the reaction mixture and preincubated with the enzyme preparation for 10 min prior to the addition of GTP. The reaction was stopped, either immediately after GTP addition (0 time) or after 10 min of incubation at 37°, by adding 0.9 ml of 0.05 M Na acetate (pH 4.0) and heating (90° for 2 min). The cGMP produced was measured by radioimmunoassay (21). The cGMP product was linear in the immunoassay and completely degraded by phosphodiesterase. Exogenous cGMP added to the guanylate cyclase reaction mixtures and incubated was less than 5% hydrolyzed. The activity of the guanylate cyclases studied was linear with respect to time and protein concentration. Cyclic nucleotide tissue levels were measured in the radioimmunoassay after ether extraction of the supernatant from a 6% trichloroacetic acid tissue homogenization.

Adenylate cyclase activity was assayed by the method of Salomon *et al.* (22). Glucose-6-phosphatase activity was measured by the method of Swanson (23) based on the conversion of glucose-6-phosphate to inorganic phosphate. Protein and DNA concentrations were measured by the methods of Lowry *et al.* (24) and Burton (25), respectively.

RESULTS

Fig. 1 demonstrates the changes in hepatic nuclear membrane cGMP and cAMP immunofluorescence that occur after glucagon injection. Tissue from the control rat exhibited low-intensity nuclear membrane staining for cGMP and cAMP. Five minutes after glucagon injection, cGMP immunofluorescence was only minimally changed but cAMP staining on the nuclear membrane has increased. At 30 min after glucagon injection there was a marked increase in cGMP nuclear membrane immunofluorescence but the cAMP pattern was similar to that of the control. Thus, glucagon produced a sequential increase in cAMP and cGMP nuclear membrane immunofluorescence.

The effect of glucagon on cGMP immunofluorescence was still demonstrable after the isolation of hepatic nuclei (Fig. 2). The immunofluorescence of nuclei isolated from a rat 30 min after glucagon injection was brighter than that of control nuclei isolated simultaneously and photographed with the same exposure time. Four such experiments confirmed this observation.

The mean (±SEM, $n = 5$ rats) control level of cAMP (0.56 ± 0.1 pmol/mg wet weight) was increased by 6-fold at 5 min after glucagon injection (3.40 ± 1.0 pmol/mg wet weight, $n = 5$ rats). cGMP levels were more variable; however, a small but significant increase over the control cGMP level (13 ± 1.1 fmol/mg wet weight, 29 rats) was seen 30 min after glucagon injection (18 ± 1.2 fmol/mg wet weight, 29 rats).

To determine whether or not cGMP is synthesized by hepatic nuclei, guanylate cyclase activity was measured in subcellular fractions. The activities in the soluble fraction (37.6 ± 1.61 pmol

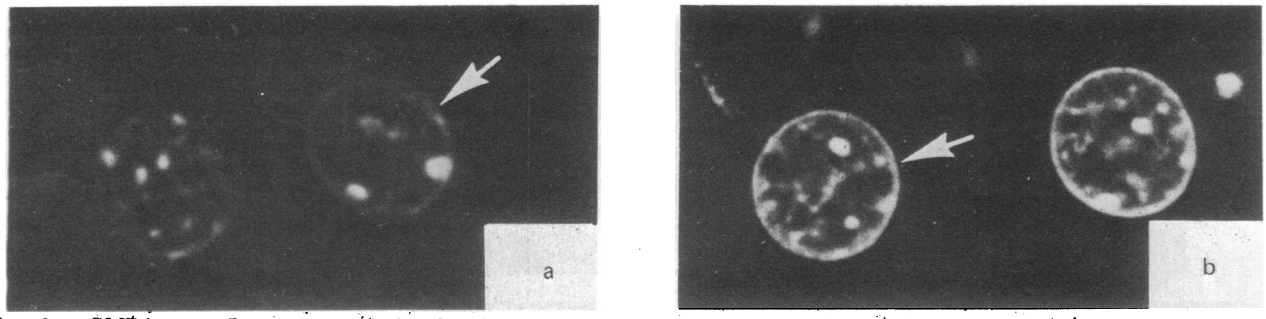

FIG. 2: cGMP immunofluorescence of isolated rat liver nuclei. Nuclei isolated from a control rat (*a*) exhibited a low level of cGMP staining whereas nuclei isolated from a rat 30 min after glucagon injection (*b*) had a higher level of cGMP immunofluorescence, particularly on the nuclear membrane. (×1600.)

of cGMP/mg of protein per min) and in the crude particulate fraction (5.32 ± 0.39) are in agreement with published values (26). The activity of the guanylate cyclase in the purified nuclear preparation (4.10 ± 0.67) was slightly lower than that in the crude particulate fraction. The activity of the nuclear enzyme was linear with respect to time (up to 20 min of incubation) and protein (30–120 μg).

The possibility that the nuclear preparations were contaminated by crude particulate guanylate cyclase was ruled out by the following observations. (*i*) Photomicrographs and electron micrographs showed the population of nuclei to be highly purified. (*ii*) The protein-to-DNA ratio of the preparation was 2.1:1. (*iii*) The basal adenylate cyclase activity of the nuclear preparations was <1 pmol of cAMP/mg per min and the fluoride-stimulated activity was less than 15% of that in the crude particulate fraction (nuclear guanylate cyclase activity is 80% of the crude particulate activity). (*iv*) Glucose-6-phosphatase activity (a microsomal enzyme marker) was undetectable in the nuclear fractions but was 68.2 μg/mg of protein per 10 min in the particulate fraction (this represented 85% recovery of the whole homogenate activity).

The three hepatic guanylate cyclases were tested to determine if their *in vitro* responsiveness to NaN_3 differed. [NaN_3 is one of a class of compounds that are known to stimulate the activity of certain guanylate cyclase preparations (27).] The increase in soluble guanylate cyclase activity was 10- to 20-fold. The particulate fraction was stimulated 180 ± 14% but the nuclear guanylate cyclase was only stimulated by 35 ± 7%, a small but significant effect ($P < 0.01$). The blunted response to NaN_3 seems to distinguish the nuclear and particulate enzymes. However, it is known that the stimulation of soluble guanylate cyclase by NaN_3 depends on a factor that can be dissociated from the core enzyme (27).

The following experiments were performed to determine whether the "NaN_3 factor" is lost during the preparation of nuclei. Crude particulate preparations were dispersed with the Polytron apparatus in 10 ml of 2.2 M sucrose and layered at the bottom of an SW 27 centrifuge tube. A 1.8 M sucrose solution was layered on top and the preparation was centrifuged at 100,000 × *g* for 1 hr. At the end of centrifugation, particulate guanylate cyclase activity was found in the layer floating at the top surface. This activity was fully stimulated by NaN_3 (180%). Therefore, it is reasonable to assume that the decreased response to NaN_3 is a property of the nuclear guanylate cyclase and not a preparative artifact. The total nuclear activity represented 6–8% of the total crude particulate activity.

Experiments were performed to compare the guanylate cyclase activity of nuclei isolated from glucagon-treated and control rats. Fig. 3 demonstrates that glucagon treatment increases the nuclear guanylate cyclase activity by 35% ($P < 0.01$). To test whether this effect was an artifact due to differential contamination with phosphodiesterase, experiments were performed in which the recovery of exogenous cGMP was assessed. These experiments showed that none of the *in vivo* treatments (reported above) or the *in vitro* incubations (presented below) resulted in increased phosphodiesterase activity.

Glucagon increased hepatic cAMP levels as well as nuclear membrane cAMP immunofluorescence (Fig. 1*e*) prior to its effect on nuclear guanylate cyclase. These facts suggested that cAMP might be an intermediate in guanylate cyclase activation. To test this supposition, nuclei isolated from control rats were preincubated with and without 10^{-5} M cAMP prior to the guanylate cyclase assay. Table 1 shows that 10^{-5} M cAMP increased the activity by 25% ($P < 0.01$). Incubation of control nuclei with 10^{-6} M cAMP did not consistently increase guanylate cyclase activity. This effect was specific for cAMP because when 10^{-5} M 5′-AMP was preincubated with control nuclei, the activity did not increase (data not shown). The guanylate cyclase activity of nuclei isolated from glucagon-treated rats was not significantly increased by 10^{-5} M cAMP. This indicates that the maximal stimulatory effect of cAMP is obtained at levels attainable *in vivo*. NaN_3 increased the control guanylate cyclase activity by 35%. The combination of NaN_3 and 10^{-5} M cAMP increased control activity by 70%, an additive effect greater than either alone ($P < 0.01$). In nuclei prepared from glucagon-treated rats, NaN_3 increased the activity by 70% and cAMP did not produce any additive effect. This result again

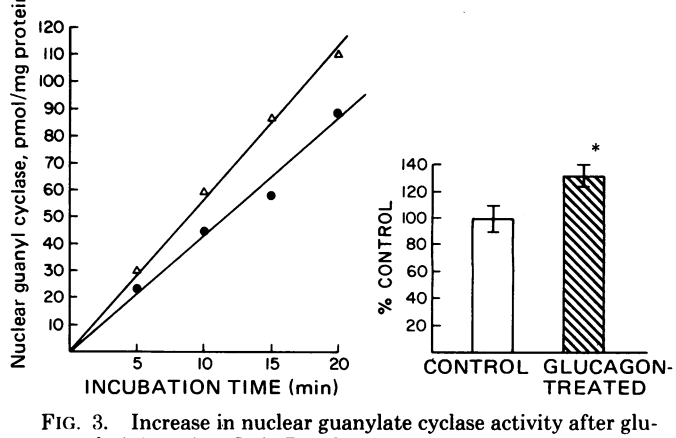

FIG. 3. Increase in nuclear guanylate cyclase activity after glucagon administration. *Left.* Results of one experiment in which the guanylate cyclase activity of nuclei isolated from a rat treated with glucagon (△) and from a control rat given a saline injection (●) 30 min prior to sacrifice are compared at different incubation times. *Right.* Mean (±SEM) cumulative data (10 observations for each bar) expressed as percentage increase in activity of the nuclei isolated from the glucagon-treated rat compared to the matched control (*, $P < 0.01$).

Table 1. Effect of *in vitro* additions on nuclear guanylate cyclase from rats

Condition	% of basal activity					
	Control rats			Glucagon-treated rats		
	n	Mean	SEM	*n*	Mean	SEM
Basal	29	100	15	10	100	14
+ 10^{-3} M NaN_3	29	135*	7	10	178*	23
+ 10^{-3} M NaN_3 & 10^{-5} M cAMP	24	170*	10	9	186*	21
+ 10^{-5} M cAMP	21	125*	7	7	110	11

In each experiment, nuclei were preincubated for 10 min prior to addition of GTP either alone (basal activity) or with NaN_3 or cAMP at the indicated concentrations. Glucagon was injected 30 min before the treated rats were killed. *n* = number of experiments. The combined effect of 10^{-3} M NaN_3 and 10^{-5} M cAMP is additive in the control rats but not in the glucagon-treated rats.
* For difference from basal, $P < 0.01$.

demonstrates that the effect of cAMP is already fully expressed—i.e., further incubation with cAMP *in vitro* does not add to the *in vivo* activation brought about by glucagon injection.

The difference between crude particulate guanylate cyclase and the nuclear guanylate cyclase is also demonstrated by the response to glucagon and cAMP. The injection of glucagon did not alter the activity of the crude particulate guanylate cyclase isolated from rats 30 min after injection. The preincubation of cAMP with particulate guanylate cyclase from control rats did not stimulate enzyme activity.

DISCUSSION

The present experiments document a mechanism by which the nuclear content of cGMP might be regulated locally without major changes in the total tissue content. The alteration in nuclear membrane cGMP immunofluorescence after glucagon injection has been observed in tissue slices and in isolated nuclei. The results could be explained by an increased binding of cGMP at the nuclear membrane or by increased production of the nucleotide at this site. The biochemical approach to this question supports the latter explanation, a nuclear guanylate cyclase, but does not rule out the former. The nuclear guanylate cyclase differs from the other cellular cyclases not only in location but also in its biochemical properties. The nuclear location of the enzyme is attested to by the recovery of adenylate cyclase, glucose-6-phosphatase, and guanylate cyclase activities from the various fractions. The blunted response to *in vitro* stimulation by NaN_3 distinguishes the nuclear and crude particulate enzymes as does the different response of the two enzymes to glucagon and cAMP. The correlation between the increased activity of the enzyme and the immunocytochemical location of cGMP after glucagon treatment suggests localized control of cGMP production. A similar correlation between increased nuclear membrane cGMP immunofluorescence and increased nuclear guanylate cyclase activity has been detected during the course of rat liver regeneration after partial hepatectomy (Y. Koide, H. S. Earp, and A. L. Steiner, unpublished results). The isolation of nuclear guanylate cyclase activity from rat uterus has also recently been reported (28).

The discovery of cGMP (29) has stimulated conjecture concerning coordinated regulation of biochemical processes by the two nucleotides, cAMP and cGMP. Many combinations of an effect of one nucleotide on the synthesis, destruction, or action of the other have been postulated (30, 31). The present studies suggest yet another form of interaction—a positive effect of cAMP on the activity of a specific guanylate cyclase The data include: the sequential increase in nuclear membrane cAMP and cGMP immunofluorescence that follows glucagon administration; an increase in nuclear guanylate cyclase activity after glucagon injection; and an increase in the activity of nuclear guanylate cyclase from control rats when these nuclei are incubated *in vitro* with cAMP. The observed *in vitro* stimulation is small and somewhat variable; however, the correlation with the *in vivo* data and the failure of 5′-AMP to mimic the effect makes it likely that cAMP is one of the determinants of nuclear guanylate cyclase activity. Generalization, to all tissues, of any constant relationship between the two nucleotides has been fraught with risk. However, certain model systems exhibit the following temporal relationships: increases in cAMP level, increases in cGMP level or nuclear cGMP immunofluorescence, and DNA synthesis. During rat liver regeneration, increased cAMP concentrations are detected (11, 12) and are followed by an increase in nuclear cGMP immunofluorescence and DNA synthesis (Y. Koide, H. S. Earp, and A. L. Steiner, unpublished results). Isoproterenol is known to result in serial elevation of cAMP, cGMP, and DNA synthesis in the rat salivary gland (13). A recent report indicates that, in rabbit lens epithelial cells, epidermal growth factor rapidly increases cAMP and cGMP (14); 24 hr later, DNA synthesis occurs. These examples suggest that in certain tissues a positive correlation among cAMP, cGMP, and DNA synthesis may exist.

The stimulation of a nuclear guanylate cyclase by cAMP may be one mechanism by which the two nucleotides interact; however, the functional significance of this interaction on the modification of DNA synthesis is unknown at this time. It is probable that glucagon and cAMP are not the only determinants of rat liver nuclear guanylate cyclase activity. Preliminary experiments have shown that pharmacologic doses of insulin increase nuclear membrane cGMP immunofluorescence. Both glucagon (32) and insulin (32, 33) have been shown to increase cGMP levels in *in vitro* liver preparations. In the present experiments the injection of glucagon would also increase pancreatic insulin secretion and thus some of the effects seen may be a product of glucagon and insulin action. However, the demonstration of the *in vitro* effect of cAMP on nuclear guanylate cyclase suggests a primary role for glucagon.

The two most likely mechanisms by which cAMP might alter the activity of the nuclear guanylate cyclase are directly by interaction with the enzyme or indirectly by activating a cAMP-dependent protein kinase. The direct interaction of adenine nucleotides with guanylate cyclases has been demonstrated (17). ATP can stimulate or inhibit the activity of sea urchin sperm guanylate cyclase, depending on the *in vitro* assay conditions (34). Thus, a direct action of cAMP is possible. However, most if not all actions of cAMP in eucaryotes are mediated by specific protein kinases (35). Therefore, cAMP

might increase nuclear guanylate cyclase activity by promoting phosphorylation of the enzyme.

Although the intranuclear role of cGMP is not presently known, several investigators have reported modification of nuclear proteins (36) and nucleic acid synthesis (30) by cGMP. The nuclear guanylate cyclase might serve to regulate the intranuclear content of cGMP. The intense cGMP immunofluorescence on the nuclear membrane suggests another potential role for cGMP: the regulation of transport across the nuclear membrane. Alterations in the nuclear membrane cGMP content might modulate the influx of ions such as calcium or the outward movement of ribonucleotide-protein complexes.

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