Guanylate Cyclase Activity and Cyclic Nucleotide Concentrations during Liver Regeneration after Experimental Injury

By CHRISTO GORIDIS,*§ JEAN ZWILLER† and WERNER REUTTER‡

*C.N.R.S.–CBM4, 13274 Marseille Cedex 2, France, †Centre de Neurochimie, 67085 Strasbourg Cedex, France, and ‡Biochemisches Institut der Universität Freiburg, 78 Freiburg, Germany

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Cyclic nucleotide concentrations and guanylate cyclase activity were measured in regenerating rat liver. Previous work has shown that in livers of partially hepatectomized rats the activity of a membrane-bound guanylate cyclase increases considerably during the early replicative phase [Kimura & Murad (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 1965–1969; Goridis & Reutter (1975) *Nature (London)* **257**, 698–700]. Over the same time period after partial hepatectomy, increased tissue concentrations of cyclic GMP were found when the rats were killed under pentobarbital anaesthesia, but not when anaesthesia was omitted. The results obtained on hepatectomized livers were compared with the changes in guanylate cyclase activity and cyclic nucleotide concentrations during the response to galactosamine treatment. Here, a peak of guanylate cyclase activity and of cyclic GMP concentrations occurred at 8 h, that is before the beginning of the proliferative response. Both parameters were normal at the time of increased DNA synthesis. There does not, therefore, seem to be a consistent correlation between changes in guanylate cyclase activity or concentrations of cyclic GMP and an increase in liver DNA synthesis. A modest rise in cyclic AMP concentrations was found, however, in livers of galactosamine-treated rats, which was coincident with the time of DNA synthesis.

Considerable attention has been focused recently on the role that cyclic nucleotides may play in regulating cell proliferation. It has been postulated that cyclic GMP may function as a positive intracellular signal in the regulation of growth of a variety of fibroblasts and peripheral lymphocytes, and that cyclic AMP acts as a negative intracellular signal in the growth process of these cells (for references, see Goldberg *et al.*, 1974; Sheppard & Bannai, 1974; Pastan *et al.*, 1975).

One of the models for studying cell growth *in vivo* is the compensatory hyperplasia of the liver, which occurs after partial hepatectomy (Bucher, 1963). From studies on the effects of exogenous cyclic nucleotides on hepatic DNA synthesis, Short *et al.* (1975) concluded that cyclic AMP, but not cyclic GMP, plays an important role in the regulation of liver cell proliferation after partial hepatectomy. We have reported an increase in liver guanylate cyclase [GTP pyrophosphate-lyase (cyclizing) EC 4.6.1.2] activity which accompanied the beginning of the replicative phase after partial hepatectomy

(Goridis & Reutter, 1975). This increase in guanylate cyclase activity concerned only the membrane-bound enzyme, the soluble activity being unchanged.

Before one can seriously consider cyclic GMP as a regulator of DNA synthesis in regenerating liver, it is, however, necessary to show that changes in the tissue concentrations of this cyclic nucleotide occur after partial hepatectomy. Whitfield *et al.* (1976) and Fausto & Butcher (1976) have measured cyclic GMP concentrations in regenerating liver, but only during the early phases after partial hepatectomy before the increase in guanylate cyclase activity reaches its maximum. Over the time course that they studied, cyclic GMP concentrations were stable and were not different from the controls. In the present study, we show that elevated cyclic GMP concentrations only coincide with the increased guanylate cyclase activity after partial hepatectomy if the rats are killed under anaesthesia.

Apart from partial hepatectomy, liver damage caused by chemical agents can also induce compensatory hyperplasia. This therefore gives us the opportunity to test whether an increased guanylate cyclase activity is an obligatory part of the proliferative response or is specific for the condition after partial hepatectomy. After injection of galactosamine,

§ To whom requests for reprints should be addressed.
Present address: Centre d'Immunologie de Marseille, Luminy, 13288 Marseille Cedex 2, France.

which is a treatment known to induce DNA synthesis and mitosis in adult liver (Reutter *et al.*, 1968; Lesch *et al.*, 1970), changes in total and particulate guanylate cyclase activities occurred, as shown in the present report. But after injection of galactosamine the phase of parenchymal proliferation was accompanied neither by increased activity of membrane-bound guanylate cyclase nor by elevated concentrations of cyclic GMP.

Three peaks of cyclic AMP concentrations occur during the pre-replicative and early-replicative phase of liver hyperplasia after partial hepatectomy (Thrower & Ord, 1974; Whitfield *et al.*, 1976). In agreement with this observation, we found moderate increases in cyclic AMP concentrations in regenerating liver which occurred during the time of increased DNA synthesis after injection of galactosamine.

Materials and Methods

Animals

Female Wistar rats (Ivanovas, Kissleg, Germany) weighing 150–200 g were fed on Altromin R diet (Altromin G.m.b.H., Laage, Germany) containing 19% (w/w) protein and water *ad libitum*. For partial hepatectomy, two-thirds of the liver was removed under diethyl ether anaesthesia by the procedure of Higgins & Anderson (1931). Sham-operated rats were used as controls. When the response to galactosamine injury was to be studied, a single injection of D-galactosamine hydrochloride (375 mg/kg) in 0.9% NaCl was given intraperitoneally. Control animals received NaCl only. Animals were hepatectomized and galactosamine was administered between 08:00 and 09:00 h.

Guanylate cyclase

To determine guanylate cyclase activity, livers were removed under diethyl ether anaesthesia after perfusion with ice-cold 0.9% NaCl containing 0.5 mM-CaCl₂. Plasma membranes were prepared by a modification of the method of Neville (1960) as described previously (Emmelot *et al.*, 1964). The purity of each fraction was ascertained by measuring the following marker enzymes: 5'-nucleotidase (EC 3.1.3.5), succinate dehydrogenase [succinate-2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl-tetrazolium oxidoreductase (EC 1.3.99.1)] and glucose 6-phosphatase (EC 3.1.3.9) (Emmelot *et al.*, 1964). Crude soluble and particulate fractions were obtained from 25% (w/v) homogenates prepared in 10 mM-Tris/HCl buffer (pH 7.5) by centrifugation at 100 000 *g*_{av} for 60 min. The pellet was taken up in the same buffer and recentrifuged at 100 000 *g*_{av} for 60 min. Guanylate cyclase activity was determined as reported previously (Helwig *et al.*, 1975; Goridis &

Reutter, 1975). Reaction mixtures (130 μ l) contained: 15 mM-phosphocreatine, 75 μ g of creatine kinase, cyclic [³H]GMP (5 mM, 5.7×10^4 d.p.m.), 8 mM-MnCl₂, 0.1 mM-papaverine, [α -³²P]GTP (0.185 mM, 1.45×10^6 – 2.9×10^6 d.p.m.), 60 mM-Tris/HCl (pH 7.5) and enzyme (40–80, 75–150 and 5–10 μ g of protein for total homogenate, particulate and plasma-membrane fractions respectively) and were incubated at 37°C for 10 min. The reaction was stopped by adding unlabelled GTP (500 nmol) in 4 mM-EDTA and immersion into a boiling-water bath for 3 min. No measurable hydrolysis of added cyclic GMP occurred in any fraction. Product formation was linear with time for up to 20 min. Two to three dilutions were tested to assure that the reaction velocity was proportional to enzyme concentration. Cyclic GMP was separated from other labelled compounds by successive chromatography on alumina and Dowex 1 (X2) columns. After the boiling step, 0.1 ml of ice-cold 0.06 M-Tris/HCl buffer (pH 7.5) was added and the denatured protein was removed by centrifugation (20 min at 2000 *g*). Portions (200 μ l) of the supernatants were transferred to columns (1.5 g of dry alumina, column diameter 0.5 cm), the columns were washed with 2 ml of 0.06 M-Tris/HCl and the cyclic GMP was eluted with 2 ml of 0.6 M-Tris/HCl (pH 7.5). The eluate from the alumina columns was transferred to columns (0.5 cm $\times$ 3.5 cm) of Dowex 1 (X2; formate form). The columns were washed with 2 ml of water, followed by 8 ml of 0.5 M-formic acid. Then cyclic GMP was eluted with 3 ml of 8 M-formic acid directly into counting vials containing 13 ml of Monophase 40 and the radioactivity determined in an Intertechnique SL 4000 liquid-scintillation counting system with the pre-set ³H/³²P double-radioisotope counting window. All results were corrected for recovery of cyclic [³H]GMP (60–70%). Plasma membranes had to be assayed freshly; all other fractions could be stored at –40°C for up to 10 days without detectable loss of activity. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Cyclic nucleotides

For determination of cyclic nucleotide concentrations, livers were removed by a freeze-stop technique (Wollenberger *et al.*, 1960) under pentobarbital anaesthesia (45 mg/kg). In some cases, no pentobarbital was given and the rats were stunned by a blow to the head. Freeze-clamped tissue (1 g wet wt.) was homogenized in 4 ml of 0.9 M-HClO₄. After centrifugation at 1000 *g* for 10 min, the supernatants were neutralized with solid KHCO₃, recentrifuged under identical conditions and the supernatants stored at –40°C until determination of their cyclic nucleotide content. Cyclic GMP and cyclic

AMP were measured by radioimmunoassay after succinylation (3-carboxypropionylation) as described by Cailla *et al.* (1973, 1976). Cyclic AMP could be assayed directly after succinylation of the neutralized tissue extracts as judged from the following criteria. (1) The immunoinhibitory cyclic AMP-like activity was always proportional to the amount of tissue extract over a 4-fold range of dilutions; (2) virtually identical values were obtained before and after purification of the cyclic AMP by successive chromatography on alumina and Dowex 1 (X2) columns. Some samples, however, contained unknown material, which interfered with the cyclic GMP assay. Therefore for the determination of cyclic GMP, all samples were passed through alumina and Dowex 1 (X2) columns. The method described above for the guanylate cyclase assay was followed, with some modifications. The alumina columns were loaded with 1 ml of neutralized extract and the washing step was omitted. The eluate from the Dowex 1 (X2) column was freeze-dried, the residue was taken up in 0.5 ml of water and the cyclic GMP determined after succinylation. Trace amounts of cyclic [^3H]GMP (2×10^4 d.p.m.) were added to the extracts to measure recovery. This purification scheme was effective in eliminating the interfering material, as shown by the following criteria. The cyclic GMP-like activity was proportional to the amount of tissue extract. Phosphodiesterase digestion decreased the immunoinhibitory activity to less than 10% of the original value. Succinylation of the tissue extract resulted in a 50-fold increase in the sensitivity of the method towards native cyclic GMP (Cailla *et al.*, 1976). Since only cyclic nucleotides are efficiently succinylated under our conditions, the presence of immunoreactive material other than cyclic nucleotides should be indicated by measurable cyclic GMP-like activity without succinylation. However, omission of the succinylation step completely abolished any immunoreactivity of the tissue samples. For cyclic AMP and cyclic GMP determinations at least three dilutions of each sample were tested and the mean values were recorded.

Incorporation of [^3H]thymidine into DNA

Rats were given an intraperitoneal injection of 2 mCi of [^3H]thymidine/kg. The livers were removed 1 h after injection and were homogenized in ice-cold 0.1 M-Tris/HCl buffer (pH 7.5). DNA was extracted and its concentration was measured as described by Ceriotti (1952). A portion of the extract was used for determination of the radioactivity.

Chemicals

Cyclic [$8\text{-}^3\text{H}$]GMP (20 Ci/mmol), cyclic [$8\text{-}^3\text{H}$]AMP (30 Ci/mmol) and [$6\text{-}^3\text{H}$]thymidine (40 Ci/

mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. [$\alpha\text{-}^{32}\text{P}$]GTP (2–8 Ci/mmol) was from NEN, Dreieichenhain, Germany. Monophase 40 was from Packard International (Zürich, Switzerland), alumina (W 200-neutral) from ICN, Eschwege, Germany, and D-galactosamine from C. Roth, Karlsruhe, Germany. Phosphocreatine, creatine kinase (25 units/mg) and unlabelled nucleotides were purchased from Boehringer, Mannheim, Germany. Bovine serum albumin was obtained from Sigma, St. Louis, MO, U.S.A., and papaverine from E. Merck, Darmstadt, Germany. The 2'-O-succinyl cyclic nucleotide, tyrosine methyl esters and the anti-(succinyl cyclic nucleotide) antibodies used for the cyclic nucleotide radioimmunoassay had been prepared in the laboratory of M. A. Delaage (CNRS-CBM4, Marseille, France). All other reagents were commercial preparations of the highest purity available.

Results

Changes in cyclic GMP concentrations after partial hepatectomy

Our earlier experiments had shown that guanylate cyclase activity in crude liver homogenates started to increase 16 h after partial hepatectomy. It reached peak activities at 24 h and then declined slowly over the next 72 h (Goridis & Reutter, 1975). We measured the endogenous cyclic GMP content over the same time period after partial hepatectomy. Cyclic GMP concentrations were increased at around 13 h after partial hepatectomy (Fig. 1). There was a second elevation of the cyclic GMP concentration at 24 h and 36 h. The peak of cyclic GMP content coincided with the peak of guanylate cyclase activity in purified plasma-membrane fractions, although the enzyme activity declined more slowly. By contrast, cyclic GMP concentrations fell below control values at 60 h.

The periods of elevated tissue concentrations were associated with a wide spread of values, especially at 24 h. One group of four rats, killed 24 h after hepatectomy, showed especially high concentrations of cyclic GMP of around 350 pmol/g of liver. Although these values represented authentic cyclic GMP, as judged from the criteria given in the Materials and Methods section, we did not include these results in the value computed in Fig. 1. The rest of the values were obtained from ten rats, which were processed in two groups. Cyclic GMP concentrations ranged from 12.7 to 60.2 pmol/g wet wt., with five values higher than 15.3 (i.e. the control value + 2 s.d.). This variability could be caused by rapid oscillations during this time after hepatectomy. In view of the incomplete synchronization of the events in regenerating liver (Bucher, 1963), we regard

Fig. 1. Cyclic GMP content of rat liver (a) and cyclic GMP formation by liver plasma membranes (b) at various times after partial hepatectomy

(a) For determination of endogenous cyclic GMP concentration, livers were freeze-clamped under pentobarbital anaesthesia and cyclic GMP concentrations measured as described in the Materials and Methods section. Groups of four to ten rats were used for each time point. The results were expressed as pmol of cyclic GMP/g wet wt. of liver; the mean $\pm$ S.D. is given. Student's *t* test was used for statistical analysis; * indicates values significantly different from controls ($P < 0.05$). ▲, Partially hepatectomized rats; △, sham-operated rats. (b) Plasma membranes were prepared and guanylate cyclase activity was measured as described in the Materials and Methods section. Results shown are means $\pm$ S.D. for four rats, except where only two experiments had been performed. ●, Partially hepatectomized rats; ○, sham-operated rats.

this possibility as highly unlikely. The variability could also be the result of an amplified response to exogenous stimuli which were not controlled precisely. Since influences of anaesthesia on the liver content of cyclic nucleotides have been reported (Kimura *et al.*, 1974), those stimuli could be provided by the pentobarbital anaesthesia. Therefore we omitted the pentobarbital anaesthesia usually applied before freeze-clamping the liver and instead stunned the animals by a blow to the head. Then cyclic GMP concentrations at 24 h after partial hepatectomy were not significantly different ($P = 0.2$) from the control values and varied only slightly from one animal to another (results not shown).

After partial hepatectomy, the protein content of the liver declines by about 20% during the first 24 h and increases slowly over the next days (Bauer *et al.*, 1976). When the cyclic GMP concentrations are expressed per mg of protein, similar results are obtained at all times except for the 60 h value which is not significantly different from control values.

Changes in guanylate cyclase activity and cyclic nucleotide concentrations after galactosamine injection

We extended our studies to include the response of rat liver guanylate cyclase activity and cyclic nucleotide concentrations to chemical injury as caused by a single injection of galactosamine. Where-

Fig. 2. Incorporation of [³H]thymidine into the acid-insoluble DNA fraction at different times after galactosamine injection

[³H]Thymidine was given 1 h before killing the rats. Five animals were used for each time point. The mean $\pm$ S.E.M. is given. For details see the Materials and Methods section.

as in partially hepatectomized livers, the DNA synthesis reaches peak values around 24 h after surgery (Bucher, 1963; Lesch *et al.*, 1973), restitution of the damaged liver tissue follows a slower time course after galactosamine injection (Lesch *et al.*, 1970). The [³H]thymidine incorporation into DNA was not yet significantly elevated at 24 h and maximum values were attained only 48 h after the administration of galactosamine (Fig. 2). The guanylate

Fig. 3. Rat liver guanylate cyclase activity at various times after galactosamine injection

Guanylate cyclase activity in total homogenates (▲) and crude particulate fractions (●) was determined as described in the Materials and Methods section. Results shown are expressed as pmol of cyclic GMP formed/min per mg of protein; the mean ± s.d. for three to six rats is given. The control activities are given by the horizontal bars; they did not change over the time indicated. * indicates values that differ significantly from the control value ($P < 0.01$).

Table 1. Guanylate cyclase activity in liver plasma membranes from galactosamine-treated rats

Guanylate cyclase activity is expressed as pmol of cyclic GMP formed/min per mg of protein. Either the mean ± s.d. for three rats is given or the individual values, where only two rats had been used.

Time after galactosamine injection (h)	Activity
Control	21.6 ± 1.2
5	32.3, 25.3
8	45.8, 41.6
24	23.4 ± 3.8

cyclase activity changed rapidly during the early phase after galactosamine administration, which corresponds to the development of liver-cell necrosis and inflammatory infiltration (Reutter *et al.*, 1968; Keppler *et al.*, 1968). The total and particulate activity started to increase at 5h, and reached a peak at 8h, followed by a rapid decline (Fig. 3). Whereas the absolute changes were roughly the same in total homogenates and in crude particulate fractions, the percentage increase in the membrane-bound activity was much greater. During the later stages, that is during restitution of the liver damage, the particulate activity was still slightly elevated at 23h, but not at 48 and 72h. Total activity was sig-

Fig. 4. Cyclic AMP (●) and cyclic GMP (▲) content of rat liver after galactosamine injection

Livers were freeze-clamped under pentobarbital anaesthesia and cyclic nucleotide concentrations measured as described in the Materials and Methods section. Groups of four to eight rats were used for each time point. The results are given as pmol of cyclic nucleotide/g wet wt. of liver (mean ± s.d.); * indicates values significantly different from the controls ($P < 0.05$). The control values are plotted at zero time; cyclic nucleotide concentrations in livers of rats killed at 0 and 8h were not significantly different.

nificantly elevated only at 72h (Fig. 3). Control values did not change over a 12h period. From some livers, plasma membranes were prepared and guanylate cyclase was assayed. Increased activity was found at 8h but not at 24h after galactosamine injection (Table 1). Tissue concentrations of cyclic GMP were elevated at around 8h after galactosamine administration. During the later stages, the concentrations did not differ from control values (Fig. 4). Cyclic AMP concentrations were significantly elevated over control values only at 48 and 72h, when the proliferative activity reaches its maximum. This increase may correspond to the elevated values seen during the early-replicative phase after partial hepatectomy (Thrower & Ord, 1974). A significantly ($P < 0.01$) decreased value was found at 3h after galactosamine injection.

Comparable results are obtained when the cyclic nucleotide concentrations are expressed per mg of protein, since the protein content of the liver decreases by only 20% after galactosamine injection (W. Reutter, unpublished work).

Discussion

In several systems, cyclic GMP has been evoked as an intracellular signal stimulating cell proliferation (Goldberg *et al.*, 1974; Rudland *et al.*, 1974a), and it has been suggested that stimulation of a plasma-membrane-bound guanylate cyclase might be involved in the stimulation of cultured fibroblasts by a growth

factor (Rudland *et al.*, 1974b). The increase in particulate guanylate cyclase activity that we and others (Kimura & Murad, 1975; Goridis & Reutter, 1975) have observed during compensatory hyperplasia of partially hepatectomized livers comes too late to be implicated in the primary events after hepatectomy which trigger the proliferation of the liver remnant. The rise in plasma-membrane-bound guanylate cyclase activity could still be an essential component of the proliferative response, although no evidence has been obtained that particulate guanylate cyclase or cyclic GMP are directly involved in the alteration leading to DNA synthesis and mitosis.

If elevated activities of membrane-bound guanylate cyclase were an obligatory part of liver cell growth, similar changes should occur during liver-cell proliferation induced by different means. After galactosamine administration, however, no increase in guanylate cyclase activity was seen during the time of increased DNA synthesis. Apparently, the increased particulate guanylate cyclase activity found after partial hepatectomy and in foetal liver (Kimura & Murad, 1975) is not a general phenomenon which accompanies all modes of liver-cell proliferation. In agreement with this conclusion, we found not an increase but a great decrease in guanylate cyclase activities in several hepatoma lines (Goridis & Reutter, 1975), which are another system of rapidly growing liver tissue.

A further result which emerges from the present study is that a rise in guanylate cyclase activity does not always lead to a measurable increase in the intracellular concentrations of cyclic GMP. At 24h after partial hepatectomy, when guanylate cyclase activities were at their maximum, elevated cyclic GMP concentrations were found if the rats had been killed under pentobarbital anaesthesia, but not if anaesthesia was omitted. These results suggest that the increased guanylate cyclase activity in broken-cell preparations leads to elevated intracellular concentrations only under certain conditions. Clearly, the way of killing the animal has profound influences on the endogenous cyclic GMP concentrations.

After galactosamine injection, the elevated guanylate cyclase activities at around 8h correspond to a peak of the cyclic GMP content of the liver. These changes, however, precede the beginning of the proliferative response. It remains to be established whether these early changes in guanylate cyclase activities and cyclic GMP concentrations can be linked to the hepatitis-like response (Reutter *et al.*, 1968; Lesch *et al.*, 1970) to galactosamine administration.

Increased cyclic AMP concentrations have been shown to occur in partially hepatectomized livers before and coincident with the phase of DNA replication (MacManus *et al.*, 1972; Thrower & Ord, 1974; Whitfield *et al.*, 1976; Fausto & Butcher,

1976). We observed an increase in the hepatic cyclic AMP content of galactosamine-treated animals only at the time when DNA was actively synthesized. Apparently, elevated tissue concentrations of cyclic AMP during the replicative phase are more consistently associated with induced liver-cell growth than are changes in cyclic GMP concentrations or elevated cyclic AMP concentrations before the onset of DNA synthesis.

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