Enhancement of the Anti-Anabolic Response to Adenosine 3':5'-Cyclic Monophosphate during Development

THE INHIBITION OF HEPATIC PROTEIN SYNTHESIS

By ARUNEE KLAIPONGPAN, DAVID P. BLOXHAM and MUHAMMAD AKHTAR
Department of Physiology and Biochemistry, University of Southampton, Southampton SO9 3TU, U.K.

(Received 29 March 1977)

Cyclic AMP causes an age-dependent inhibition of protein synthesis in rat liver. The onset of inhibition is about 10–12 days after birth. The developmental response to cyclic AMP is associated with a change in the microsomal component of the protein-synthesizing system.

Cyclic AMP, and hormones that increase its intracellular concentration, has been shown to stimulate gluconeogenesis (Exton & Park, 1968; Exton *et al.*, 1970) and glycogenolysis (de Werve *et al.*, 1977) and to inhibit the biosynthesis of cholesterol, fatty acid and protein in rat liver (Exton *et al.*, 1972; Bloxham & Akhtar, 1972; Bricker & Levey, 1972a; Klein & Weiser, 1973; Capuzzi *et al.*, 1974; Sellers *et al.*, 1974; Harris, 1975; Klaipongpan *et al.*, 1975; Hoshino *et al.*, 1977). We have been especially interested in the latter aspect of cyclic AMP action and have suggested that the anti-anabolic role of cyclic AMP could contribute towards enhanced gluconeogenesis by virtue of channeling gluconeogenic precursors away from biosynthetic routes towards gluconeogenesis (Bloxham & Akhtar, 1972; Sellers *et al.*, 1974). In the present paper we investigated whether the simultaneously high rates of gluconeogenesis (Ballard, 1971; Clark *et al.*, 1974) and protein synthesis in developing animals would alter the response of liver protein synthesis to externally added cyclic AMP. The result shows that cyclic AMP-dependent inhibition of hepatic protein synthesis is virtually absent from newborn animals and that the onset of inhibition occurs about day 8–12 after birth. A preliminary account of this work has been presented (Klaipongpan *et al.*, 1976).

Materials and Methods

All labelled compounds were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Wistar albino rats were used throughout this work and laboratory chow was fed *ad libitum*.

Hepatocytes

Hepatocytes from mature animals were prepared by using the recirculating collagenase-digestion procedure described by Zahltén & Stratman (1974). For hepatocytes from 4-day-old animals this pro-

cedure was unsuitable, and a simple non-recirculating system was used. Animals were anaesthetized with Nembutal and a loose ligature was tied around the inferior vena cava just above the right renal vein. The thorax was now exposed and a cannula was inserted into the inferior vena cava via the right atrium and held in place with a single ligature. The outflow from the inferior vena cava was now stopped by closing the ligature above the renal vein, and the hepatic portal vein and hepatic artery were cut. The liver was left *in situ* and perfusion was commenced with Ca²⁺-free Krebs bicarbonate Ringer (as below minus Ca²⁺) (37°C) at 10 ml/min for 5 min. After this 50 ml of 0.15% (w/v) collagenase in Ca²⁺-free Krebs bicarbonate Ringer was perfused. By the end of this stage the liver was close to disintegration and liver cells were then isolated as described by Zahltén & Stratman (1974) by using volumes scaled down appropriately for the amount of tissue. Isolated hepatocytes from mature and 4-day-old rats were suspended in Krebs bicarbonate Ringer containing Ca²⁺ [0.9% (w/v) NaCl; 1.15% (w/v) KCl; 0.11 M-CaCl₂; 3.82% (w/v) MgCl₂; 2.11% (w/v) KH₂PO₄ and 1.3% (w/v) NaHCO₃, in the proportions 100:4:1.5:1:1:20, by vol.] and glucose (0.1%, w/v).

Protein synthesis in hepatocytes

Hepatocyte suspensions (2 ml; approx. 20 mg of protein) in Krebs bicarbonate Ringer were incubated with 2 μ Ci of L-[4,5-³H]leucine (46 Ci/mmol) under O₂/CO₂ (19:1) in a stoppered 25 ml flask with constant shaking at 37°C for 30 min. The reaction was terminated by the addition of 3 ml of cold 10% (w/v) trichloroacetic acid. The suspension was left at 4°C for 1 h, then the protein was washed four times with cold 5% (w/v) trichloroacetic acid solution containing 0.1 M-leucine, once with hot 5% (w/v) trichloroacetic acid (90°C for 30 min) and the lipids were extracted twice with acetone at room temperature. The protein pellet was dissolved in 0.9 ml of

Protosolve and 0.3ml samples were counted in 15ml of Triton X-100-based scintillation fluid [30% (v/v) Triton X-100 in toluene containing 0.3% 2,5-di-phenyloxazole and 0.035% 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene] acidified with 0.25ml of acetic acid.

Protein synthesis in liver slices

Weighed liver slices (approx. 100–300mg wet wt.) were incubated in 2ml of phosphate/saline buffer, pH 7.4 [0.09% (w/v) NaCl; 1.15% (w/v) KCl; 0.11M-CaCl₂; 3.82% (w/v) MgCl₂; 0.1M-sodium phosphate buffer, pH 7.4; 100:4:3:1:20, by vol.], with 2μCi of L-[4,5-³H]leucine (46Ci/mmol) at 37°C for 30min in either the presence or the absence of cyclic AMP. The reaction was terminated by placing the flasks on ice. The liver slices were washed in cold water, blotted on damp tissue paper and homogenized in 4ml of cold 10% (w/v) trichloroacetic acid solution. The suspension was left at 4°C for 1h, then the protein pellet was collected by centrifugation (1000g, for 5min) and the radioactive incorporation was analysed by the procedure described for the hepatocyte system.

Protein synthesis in cell-free system

The preparation of the post-mitochondrial supernatant, washed microsomal fraction and the post-microsomal supernatant was carried out as described by Sellers *et al.* (1974) in 25mM-potassium phosphate, pH 7.0, containing 25mM-KCl and 10mM-MgCl₂. The cell-free incubation mixture contained 5mM-phosphoenolpyruvate, 3mM-ATP, 2μCi of L-[4,5-³H]leucine (46Ci/mmol) and the subcellular fraction(s), with or without cyclic AMP, in a total volume of 1ml. Incubations were for 15min at 37°C. The reaction was terminated by cooling in ice, followed by the addition of 5ml of 12% (w/v) cold trichloroacetic acid solution containing 0.15M-leucine. The incorporation of the radioactive precursor into protein was measured as described above.

Determination of protein

The concentration of delipidated protein samples was determined by the biuret method, with bovine serum albumin (1–10mg/ml) as a standard (Cleland & Slater, 1953).

Presentation of results

All the experiments reported in this work are the minimum of four experimental observations. The standard error of the radioisotopic incorporation was never greater than $\pm 5\%$. To compare differences the unpaired *t* test was used and significance was at least $P < 0.01$ compared with the control. Where the result were not significantly different they are marked N.S.

Results

Inhibition of protein synthesis in isolated hepatocytes from young and mature rats

Fig. 1 shows the effect of increasing concentrations of either cyclic AMP or cyclic GMP on protein synthesis from L-[4,5-³H]leucine in hepatocytes from mature rats. Whereas cyclic AMP caused a pronounced inhibition of protein synthesis, cyclic GMP was virtually without effect, showing that there is a high degree of specificity for the cyclic adenosine nucleotide. The maximum inhibition of protein synthesis was about 60%, which corresponds to the magnitude of inhibition observed with liver-slice incubations (Bloxham & Akhtar, 1972). This occurred despite the fact that the incorporation was much higher in the hepatocyte preparation (i.e. 30000 d.p.m./30min per mg of protein) compared with the incorporation in liver slices (i.e. 850 d.p.m./30min per mg of protein).

To study the effect of cyclic AMP on protein synthesis in young animals, hepatocytes were prepared from livers of 4-day-old rats by using the non-circulating perfusion method. When hepatocytes from either mature or 4-day-old animals were incubated with different concentrations of cyclic AMP, then only protein synthesis in the hepatocytes

Fig. 1. Dose-response curve for the inhibition of protein synthesis by cyclic AMP in hepatocytes from young and mature rats

Protein synthesis in hepatocytes from 4-day-old (□) and mature (Δ) rats was measured as described in the Materials and Methods section in the presence of different concentrations of cyclic AMP. The result also shows the effect of cyclic GMP on protein synthesis in mature hepatocytes (○). The control rates of synthesis in the absence of added cyclic nucleotide were 30000 and 90000 d.p.m./30min per mg of protein in the mature and young animals respectively.

from the mature rats was inhibited, whereas protein synthesis in hepatocytes from young animals was unaffected (Fig. 1). The control rate of incorporation in hepatocytes from young animals was 3-fold higher. Clearly this experiment introduces the concept of a differential response of hepatic protein synthesis to cyclic AMP depending on the state of development of the animal.

Development of the cyclic AMP-dependent inhibition of hepatic protein synthesis

To assess the onset of inhibition of protein synthesis by cyclic AMP during development, liver-slice preparations and cell-free protein-synthesizing systems were used. In agreement with the results observed in hepatocytes, cyclic AMP inhibited protein synthesis in liver slices and also in cell-free systems from mature animals, whereas those from the 4-day-old were unaffected (Fig. 2). The maximum inhibitory response developed with increasing age of the rats. In liver-slice preparations there was no statistically significant inhibition of synthesis before 12 days after birth, and the onset of the inhibitory response was at about day 14. The response then increased progressively and had attained its maximum extent by 24 days after birth. In the cell-free system the inhibition was apparent about days 8–10, and the response was fully established by 16 days after birth.

Fig. 2. *Development of the cyclic AMP-dependent inhibition of hepatic protein synthesis*

The inhibition of protein synthesis by cyclic AMP was estimated by using either liver-slice preparations (Δ) or post-mitochondrial supernatant cell-free protein-synthesizing systems (○) derived from animals of different ages. For each system a complete dose-response curve was performed; however, the result only shows the maximum inhibition with either 1 mM-cyclic AMP (Δ) or 100 μM-cyclic AMP (○). The control rates of hepatic protein synthesis were 1850 and 1300 d.p.m./15 min per mg of protein for the cell-free system from 4-day-old and mature rats and 2180 and 850 d.p.m./30 min per mg of protein for liver-slice incubations from 4-day-old and mature animals.

It appears that the cell-free system responds to cyclic AMP approx. 4 days earlier than the liver-slice system.

In addition, adrenaline (30 μM) also produced an inhibition of protein synthesis in liver slices from mature but not from young animals. The lack of inhibitory response to cyclic AMP in young animals was not dependent on the amino acid precursor, since similar developmental patterns of inhibition were obtained with different radioactive amino acid precursors, including glycine, serine, isoleucine, alanine, glutamic acid and methionine. The observation that there was a similar developmental pattern of response in both liver-slice and cell-free preparations also eliminates the permeability barrier to cyclic AMP as the cause for the age-dependent differences in the response to cyclic AMP.

Finally, the inhibition of synthesis in adult liver was not accompanied by a detectable change in the rate of transport of L-[4,5-³H]leucine into the water-soluble fraction of the liver cell. For the experimental conditions used in Fig. 2 the uptake in mature liver slices corresponded to 6500 d.p.m./30 min per mg wet wt. of liver in the presence or absence of cyclic AMP. This eliminates a cyclic AMP-dependent action on amino acid transport as being responsible for the inhibition of protein synthesis.

Changes in the microsomal component of the protein-synthetic system during development

The results so far indicate that there must be some change in one or more components of the system through which cyclic AMP inhibits protein synthesis. If the inhibition occurs through a phosphorylation mechanism (Monier *et al.*, 1972; Sellers *et al.*, 1974), then the change in response during development could be accounted for either by alteration of hepatic protein kinase or by alteration of the protein-synthetic system.

To investigate whether the changes take place in the protein-synthetic system or in the soluble component, the microsomal fractions prepared from both the young and the mature animals were reconstituted with either the homologous soluble fraction or the soluble fraction from animals of different age groups. The washed microsomal fraction from the 4-day-old rat reconstituted with the soluble component from either the young (4-day) or mature animals did not respond to cyclic AMP (Table 1). In contrast, the washed microsomal fraction prepared from mature animals was inhibited by cyclic AMP when supplemented with the soluble fraction from either 4-day-old or mature rats. The result indicates that the microsomal protein-synthetic system in the mature animal is different from that of the young with regard to the ability to respond to cyclic AMP.

Table 1. *Inhibition of cell-free protein synthesis by cyclic AMP: cross-matching experiment*

A portion (0.5 ml) of the washed microsomal fraction (M_2) from mature (5 mg of protein/ml) or 4-day-old rats (1 mg of protein/ml) supplemented with 0.2 ml of the cytosolic fraction (S_{100}) from either the mature (26 mg of protein/ml) or 4-day-old animal (9 mg of protein/ml) was incubated under the conditions described by Klaipongpan *et al.* (1975) in either the presence or the absence of 0.5 mM-cyclic AMP. The incorporation of radioactive precursor in each system in the absence of energy-regenerating system [720 and 2500 d.p.m./15 min per mg of microsomal protein for M_2 (mature) and M_2 (4-day-old) supplemented with the S_{100} fraction respectively] was subtracted from each experimental values and thus the results shown represent the net energy-dependent protein-synthetic rates. The numbers in parentheses show the *P* values in the unpaired *t* test; NS, not significant.

Protein-synthetic system	Protein synthesis (d.p.m./15 min per mg of microsomal protein)		Inhibition by cyclic AMP (%)
	–Cyclic AMP	+Cyclic AMP	
M_2 (mature) + S_{100} (mature)	5550 ± 150	2200 ± 80	60 (0.01)
M_2 (mature) + S_{100} (4-day-old)	7000 ± 200	4000 ± 150	43 (0.001)
M_2 (4-day-old) + S_{100} (4-day-old)	4700 ± 200	4350 ± 175	8 (NS)
M_2 (4-day-old) + S_{100} (mature)	1950 ± 50	1750 ± 50	10 (NS)

Discussion

The hormone-stimulated increase in hepatic cyclic AMP in mature animals may be regarded as a generally anti-anabolic signal which specifically channels liver metabolism towards glucose output either by enhanced gluconeogenesis or by increased glycolysis (Exton & Park, 1968, 1969; Exton *et al.*, 1970; de Werve *et al.*, 1977). Glucagon, cyclic AMP or dibutyl cyclic AMP inhibit a number of biosynthetic pathways in the liver, including fatty acid synthesis (Exton *et al.*, 1972; Bricker & Levey, 1972a; Bloxham & Akhtar, 1972; Harris, 1975), protein synthesis (Bloxham & Akhtar, 1972; Monier *et al.*, 1972; Sellers *et al.*, 1974) and cholesterol synthesis (Bricker & Levey, 1972a; Capuzzi *et al.*, 1974). The current work shows that protein synthesis in hepatocytes is also inhibited by cyclic AMP. Since the gluconeogenic capacity of the isolated hepatocytes more closely resembles that of the parenchymal cell *in vivo* than does that of the liver slice, this is evidence that cyclic AMP-dependent inhibition of protein synthesis might be a physiologically important contribution to enhanced gluconeogenesis. Certainly the inhibition of net protein synthesis is understandable in terms of the diversion of substrates towards gluconeogenesis. The current observation that the magnitude of the cyclic AMP inhibition depends on the age of the rats adds an interesting dimension to this problem. Another difference in the anti-anabolic response to cyclic AMP that may be related to cellular development has been reported by Bricker & Levey (1972b), since they showed that in certain rapidly growing hepatomas the inhibition of fatty acid synthesis and cholesterol synthesis by cyclic AMP was lost.

During development, the inhibition by cyclic AMP is first detected between 8 and 12 days of age and is maximally established by 16–24 days after birth. Our result from the cross-matching experi-

ment indicates that this development with increasing age of the inhibitory response to cyclic AMP is associated with a change in the microsomal component of the protein-synthetic system.

In our laboratory, rats are weaned towards laboratory chow at 18 days of age. Therefore the onset of the cyclic AMP response begins before weaning; however, the response is maximum at weaning. It is well known that weaning coincides with a profound alteration in hepatic metabolism, which involves an increase in fatty acid synthesis and a decrease in gluconeogenesis (Ballard & Hanson, 1967; Taylor *et al.*, 1967; Ballard, 1971; Clark *et al.*, 1974; Foster & Bailey, 1976). Since lactate is a major precursor for both of these pathways (Exton *et al.*, 1970; Salmon *et al.*, 1974) it is clear that weaning results in a switching of metabolic roles for lactate. If we suggest that the inhibition of protein synthesis by cyclic AMP is involved in diverting amino acids away from protein synthesis and towards gluconeogenesis, then the observed developmental pattern would be most consistent with a minor role of amino acids in gluconeogenesis before 10 days of age and an increasing role for amino acids thereafter. Ferre *et al.* (1977), using an inhibitor of gluconeogenesis, have come to the similar conclusion that amino acids are poor precursors for gluconeogenesis before weaning. The gluconeogenic role of amino acids is well established in the livers of mature animals (Mallette *et al.*, 1969; Exton *et al.*, 1970). How much an inhibition of protein synthesis by cyclic AMP could contribute to enhanced gluconeogenesis must be speculative; however, Woodside (1976) has shown that, in perfused livers, if protein synthesis was, inhibited by 93% by 18 μ M-cycloheximide then gluconeogenesis from endogenous sources was enhanced 3-fold.

In conclusion, the present work demonstrates that the ability of cyclic AMP to inhibit hepatic

protein synthesis develops progressively between 10 and 20 days after birth. It is proposed that this might be related to a changing role for amino acid utilization for gluconeogenesis during development.

We thank the British Council for a Scholarship to A. K., who was granted study leave by the Thailand Government.

References

- Ballard, F. J. (1971) *Biochem. J.* **121**, 169–178
- Ballard, F. J. & Hanson, R. W. (1967) *Biochem. J.* **104**, 866–871
- Bloxham, D. P. & Akhtar, M. (1972) *Int. J. Biochem.* **3**, 294–308
- Bricker, L. A. & Levey, G. S. (1972a) *J. Biol. Chem.* **247**, 4914–4915
- Bricker, L. A. & Levey, G. S. (1972b) *Biochem. Biophys. Res. Commun.* **48**, 362–365
- Capuzzi, D. M., Rothman, V. & Margolis, S. (1974) *J. Biol. Chem.* **249**, 1286–1294
- Clark, M. G., Bloxham, D. P., Holland, P. C. & Lardy, H. A. (1974) *J. Biol. Chem.* **249**, 279–290
- Cleland, K. N. & Slater, E. C. (1953) *Biochem. J.* **53**, 547–556
- de Werve, G. V., Hue, L. & Hers, H. G. (1977) *Biochem. J.* **162**, 135–142
- Exton, J. H. & Park, C. R. (1968) *J. Biol. Chem.* **243**, 4189–4196
- Exton, J. H. & Park, C. R. (1969) *J. Biol. Chem.* **244**, 1424–1433
- Exton, J. H., Mallette, L. E., Jefferson, L. S., Wong, E. H. A., Friedmann, N., Miller, T. B. & Park, C. R. (1970) *Rec. Progr. Horm. Res.* **26**, 411–459
- Exton, J. H., Corbin, J. G. & Harper, S. C. (1972) *J. Biol. Chem.* **247**, 4996–5003
- Ferre, P., Pegorier, J. P. & Girard, J. (1977) *Biochem. J.* **162**, 209–212
- Foster, P. C. & Bailey, E. (1976) *Biochem. J.* **154**, 49–56
- Harris, R. A. (1975) *Arch. Biochem. Biophys.* **169**, 168–180
- Hoshino, J., Kuhne, U., Studinger, G. & Kroger, H. (1977) *Biochem. Biophys. Res. Commun.* **74**, 663–669
- Klein, G. J. & Weiser, P. C. (1973) *Biochem. Biophys. Res. Commun.* **55**, 76–83
- Klaipongpan, A., Bloxham, D. P. & Akhtar, M. (1975) *FEBS Lett.* **58**, 81–84
- Klaipongpan, A., Bloxham, D. P. & Akhtar, M. (1976) *Biochem. Soc. Trans.* **4**, 1068–1069
- Mallette, L. E., Exton, J. H. & Park, C. R. (1969) *J. Biol. Chem.* **244**, 5713–5723
- Monier, D., Santhanam, K. & Wagle, S. R. (1972) *Biochem. Biophys. Res. Commun.* **46**, 1881–1886
- Salmon, D. M. W., Bowen, N. L. & Hems, D. A. (1974) *Biochem. J.* **142**, 611–618
- Sellers, A., Bloxham, D. P., Munday, K. A. & Akhtar, M. (1974) *Biochem. J.* **138**, 335–340
- Taylor, C. B., Bailey, E. & Bartley, W. (1967) *Biochem. J.* **105**, 717–722
- Woodside, K. H. (1976) *Biochim. Biophys. Acta* **421**, 70–79
- Zahlten, R. N. & Stratman, F. W. (1974) *Arch. Biochem. Biophys.* **163**, 600–608