The ability of adenosine to decrease the concentration of fructose 2,6-bisphosphate in isolated hepatocytes

A cyclic AMP-mediated effect

Ramon BARTRONS,* Emile VAN SCHAFTINGEN and Henri-Géry HERS

*Laboratoire de Chimie Physiologique and International Institute of Cellular and Molecular Pathology,
UCL 75.39, avenue Hippocrate 75, B-1200 Brussels, Belgium*

(Received 11 August 1983/Accepted 18 October 1983)

The presence of adenosine (25–250 μM) or of 2-chloroadenosine (2.5–100 μM) in the incubation medium caused a marked decrease in the concentration of fructose 2,6-bisphosphate in isolated hepatocytes. This effect was accompanied by an increase in the concentration of cyclic AMP, an activation of phosphorylase and of fructose 2,6-bisphosphatase, and an inactivation of pyruvate kinase and of 6-phosphofructo-2-kinase. As a rule, the changes in the fructose 2,6-bisphosphate-modifying system were slower but more persistent than those in the activities of phosphorylase and pyruvate kinase. The effect of the nucleoside to decrease the concentration of fructose 2,6-bisphosphate was not affected by an inhibitor of adenosine transport and could not be obtained in a liver high-speed supernatant. These data indicate that the effect of adenosine to decrease the concentration of fructose 2,6-bisphosphate is mediated by the stimulation of adenylate cyclase, secondary to the binding of adenosine to membranous receptors. Like glucagon, 2-chloroadenosine stimulated gluconeogenesis in isolated hepatocytes, whereas adenosine had an opposite effect.

Fru-2,6- P_2 is a metabolic signal formed in liver cells in the presence of glucose and destroyed in the presence of glucagon. The glucagon effect has been explained by the ability of cyclic AMP-dependent protein kinase to phosphorylate PFK 2, the enzyme that forms Fru-2,6- P_2 from Fru-6- P and ATP, as well as FBPase 2, the enzyme that converts Fru-2,6- P_2 back into Fru-6- P and P_i . PFK 2 is inactivated by phosphorylation, whereas FBPase 2 is activated (reviewed by Hers & Van Schaftingen, 1982).

In the course of a systematic investigation of the effect of various metabolites to affect the concentration of Fru-2,6- P_2 in isolated hepatocytes, we have observed that adenosine caused a decrease in this concentration. This effect and the mechanism by which it operates are the subject of this paper.

Abbreviations used: Fru-6- P , fructose 6-phosphate; Fru-1,6- P_2 , fructose 1,6-bisphosphate; Fru-2,6- P_2 , fructose 2,6-bisphosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; PFK 2, 6-phosphofructo-2-kinase (EC 2.7.1.-); FBPase 2, fructose 2,6-bisphosphatase (EC 3.1.3.-).

* Present address: Department of Biochemistry, Faculty of Medicine, University of Barcelona, Casanova 143, Barcelona 36, Spain.

Materials and methods

Chemicals and enzymes

Adenosine (9- β -D-ribofuranosyladenine), 2-chloroadenosine (6-amino-2-chloropurine riboside) and N^6 -(L-2-phenylisopropyl)adenosine were from Sigma Chemical Co. 5-Iodotubercidin {4-amino-5-iodo-7-(β -D-ribofuranosyl)pyrrolo[2,3- d]-pyrimidine} was provided by L. B. Townsend (University of Michigan College of Pharmacy, Ann Arbor, MI, U.S.A.). 2'-Deoxycoformycin {R-3-(2-deoxy- β -D-erythropentofuranosyl)-3,6,7,8-tetrahydro[4,5- d][1,3]diazapin-8-ol} was from Parke-Davis (Detroit, MI, U.S.A.). R-51469 was a gift of Dr. H. Van Belle (Janssen Pharmaceuticals Research Laboratories, B-2340 Beerse, Belgium). Glucagon was from Novo Industri (Copenhagen, Denmark). Cyclic AMP [^{125}I]RIA kit was from New England Nuclear (Boston, MA, U.S.A.). Enzymes and other biochemical reagents were obtained either from Boehringer G.m.b.H. (Mannheim, Germany) or from Sigma Chemical Co. (St Louis, MO, U.S.A.). All chemicals were of analytical grade. Fru-2,6- P_2 (Van Schaftingen & Hers, 1981) and [^{32}P]Fru-2,6- P_2 (Van Schaftingen *et*

al., 1982a) were prepared as described in the references cited.

Preparation and incubation of isolated hepatocytes

Hepatocytes from male Wistar rats fed *ad libitum* were isolated by the procedure described by Hue *et al.* (1978) and incubated at 37°C in the presence of 10 mM-glucose and O₂/CO₂ (19:1) gas phase. In the experiment described in Table 1, 24 h-starved rats were used and glucose was omitted. Adenosine or analogues were added after 30 min of incubation. For the measurement of enzymes, cyclic AMP and Fru-2,6-P₂, samples (0.1 ml) were withdrawn at the appropriate times and immediately frozen in tubes kept in a cooling mixture (solid CO₂ in acetone). These samples were stored at -20°C for no more than 2 days until further processing. Data shown are representative of at least two similar experiments which gave consistently the same results.

Preparation of a liver supernatant

The liver of a fed rat was homogenized in a Potter-Elvehjem tube in the cold with 2 vol. of ice-cold 100 mM-KCl/2 mM-MgCl₂/50 mM-Hepes, pH 7.1. The homogenate was centrifuged for 30 min at 100 000g, and the resulting supernatant was used.

Measurements of enzymes and metabolites

The active form of phosphorylase was assayed as described by Hue *et al.* (1975) in extracts prepared by thawing the cellular suspension in 3 vol. of a solution containing 0.1 M-NaF, 0.5% glycogen, 10 mM-EDTA and 10 mM-glycylglycine and adjusted to pH 7.4. The procedure used for the assay of pyruvate kinase was described by Feliu *et al.* (1977), except that the homogenization buffer was supplemented with 5 mM-P_i. The pyruvate kinase activity ratio ($v_{0.15}/V$) is the ratio of the rates measured at 0.15 mM- and 5 mM-phosphoenolpyruvate respectively. The active forms of PFK 2 and FBPase 2 were measured as described by Bartrons *et al.* (1983). Fru-2,6-P₂ was extracted and measured as described by Van Schaftingen *et al.* (1982b). Cyclic AMP was determined by the method of Steiner *et al.* (1972) with the cyclic AMP [¹²⁵I]RIA kit, without acetylation. Glucose was measured enzymically in neutralized HClO₄ extracts (Huggett & Nixon, 1957).

Results

Effects of adenosine and chloroadenosine on the concentration of Fru-2,6-P₂ in isolated hepatocytes

The addition of adenosine, at concentrations between 10 and 250 μM, to a suspension of isolated hepatocytes caused a time- and dose-dependent de-

crease in the concentration of Fru-2,6-P₂ in the cells (Fig. 1a). At 10 μM, the effect of adenosine was barely detectable and very transient, but the effect of higher doses was sustained for at least 12 min. The relative lack of sensitivity of the adenosine effect could be due to rapid metabolism of the nucleoside by the liver cells. Adenosine is indeed rapidly utilized by adenosine kinase ($K_m = 0.4 \mu M$, according to Miller *et al.*, 1979) and adenosine deaminase ($K_m = 46 \mu M$, according to Arch & Newsholme, 1978a), and it has been reported that, at an initial concentration of 500 μM, the removal of adenosine proceeds linearly at a rate of 0.3 μmol/min per g of cells and is completed in 25 min under conditions similar to those described in Fig. 1 (Lund *et al.*, 1975; Bontemps *et al.*, 1983). We therefore decided to investigate the effect of 2-chloroadenosine, a presumably non-metabolizable derivative of adenosine (Clarke *et al.*, 1952). Fig. 1(b) shows that the effect of 2-chloroadenosine was similar to that of adenosine, with the main difference that it was obtained at 5–10 times lower concentrations.

Role of adenylate cyclase and of membrane receptors in the nucleoside effect

Because of the known effect of glucagon to cause the disappearance of Fru-2,6-P₂, a likely explanation for the adenosine effect was an activation of adenylate cyclase, which had actually been demonstrated by Londos *et al.* (1980) with a liver plasma-membrane preparation. We show in Fig. 2 that 100 μM-adenosine or -2-chloroadenosine caused, similarly to glucagon, the formation of cyclic AMP, the inactivation of pyruvate kinase and of PFK 2, the activation of phosphorylase and of FBPase 2 and the disappearance of Fru-2,6-P₂. As a rule, the effects of chloroadenosine were more persistent than those of adenosine, but more transient than those of a saturating concentration of glucagon. Accordingly, the increase in cyclic AMP concentrations observed at 2 min was much greater with small doses of chloroadenosine than of adenosine (Fig. 3). The effects of another adenine derivative, N⁶-phenylisopropyladenosine, were similar to that of 2-chloroadenosine (results not shown). Furthermore, the addition of AMP (2 mM), which is rapidly converted into adenosine by membrane 5'-nucleotidase (Arch & Newsholme, 1978b), also caused the disappearance of Fru-2,6-P₂ and the inactivation of pyruvate kinase. ATP (2 mM) had an effect similar to that of AMP. By contrast, 2 mM-adenine or -inosine, or 1 mM-guanosine, had no effect on the concentration of Fru-2,6-P₂ in the hepatocytes (results not shown).

The similarity of the effect of glucagon and that of adenosine and its derivatives includes the previously described (Bartrons *et al.*, 1983) slower

Fig. 1. Effect of adenosine (a) and of 2-chloroadenosine (b) on the concentration of Fru-2,6-P₂ in isolated hepatocytes obtained from a fed rat

The effects of 5 μM-adenosine (▼) (a) and of 0.5 μM-2-chloroadenosine (△) (b) were barely perceptible.

modification of the Fru-2,6-P₂-modifying system as compared with the modification of pyruvate kinase and phosphorylase (Fig. 2). Furthermore, there were also great differences in the persistence of the effects. It is indeed remarkable that the activation of phosphorylase and the inactivation of pyruvate kinase by adenosine were no longer apparent after 6–12 min, whereas the inactivation of PFK 2 persisted. This might explain why, with regard to the decrease in Fru-2,6-P₂ concentration, chloroadenosine and even adenosine were nearly as efficient as glucagon (Fig. 2).

Since the effects of adenosine and 2-chloroadenosine were similar to those of glucagon, it was decided to check the potential interrelationship of the two groups of effectors. We show in Fig. 4 that there was no synergism between the effect of the nucleosides and that of the hormone. Indeed, the half-maximal concentration of glucagon required to cause the disappearance of Fru-2,6-P₂ was the same in the presence or in the absence of the nucleosides.

Finally, we have also investigated the effect of R-51469, a compound which is known to inhibit adenosine transport in erythrocytes (Van Belle *et al.*, 1982) at very low concentrations and also in hepatocytes at rather higher concentrations (F. Bontemps, personal communication). We found that, at a concentration of 0.5 mM, which is sufficient to decrease the penetration of adenosine into the cells by 80%, the inhibitor did not decrease the effect of adenosine to cause the disappearance of

Fru-2,6-P₂. This indicates that intracellular transport of adenosine is not required for its metabolic effect, which therefore does not result from a direct interaction of the nucleoside with PFK 2 or FBPAse 2.

Effect on gluconeogenesis

Because glucagon is known to stimulate gluconeogenesis, whereas adenosine has been reported by Lund *et al.* (1975) to inhibit the same metabolic conversion, we decided to investigate the effects of both adenosine and 2-chloroadenosine on the formation of glucose from lactate alone (as in the experiment of Lund *et al.*, 1975) and from a more physiological 10:1 mixture of lactate and pyruvate. We show in Table 1 that the effect of chloroadenosine was to stimulate gluconeogenesis by about 20–25%, whereas adenosine had a large inhibitory effect, identical with that described by Lund *et al.* (1975).

Experiments with cell-free extracts

It has been checked that 100 μM-adenosine, in the presence or absence of 10 μM-deoxycoformycin, which totally inhibits liver adenosine deaminase (Van den Berghe *et al.*, 1980), or of 10 μM-iodotubercidin (an inhibitor of adenosine kinase; Henderson *et al.*, 1972; Bontemps *et al.*, 1983), had no effect on the rate of disappearance of Fru-2,6-P₂ in a liver high-speed supernatant incubated at 30°C in the presence of 50 mM-Hepes, 100 mM-KCl, 2 mM-MgCl₂ and 4 μM-Fru-2,6-P₂ at pH 7.1, with or

Fig. 2. Time course of the effect of 100 μ M-adenosine (■), 100 μ M-2-chloroadenosine (●) or 0.1 μ M-glucagon (Δ) on the conversion of phosphorylase, pyruvate kinase, PFK 2 and FBPase 2 and on the concentrations of cyclic AMP and Fru-2,6- P_2 in isolated hepatocytes obtained from a fed rat
Bars represent S.E.M. for three experiments: ○, control.

Table 1. Effect of adenosine, 2-chloroadenosine and glucagon on rates of gluconeogenesis from lactate in isolated liver cells
Cells were prepared from livers of rats starved for 24h and incubated for 30min at 37°C with or without 10mM-lactate or 10mM-lactate and 1mM-pyruvate. The cell concentration was about 50mg/ml. The values shown are the difference in glucose production observed with and without precursors. They are means $\pm$ S.E.M. for four experiments.

	Lactate		Lactate/pyruvate	
	Glucose formed (μ mol/min per g)	(%)	Glucose formed (μ mol/min per g)	(%)
Control	0.53 $\pm$ 0.05	100	0.50 $\pm$ 0.04	100
Glucagon (0.1 μ M)	0.77 $\pm$ 0.04	145	0.72 $\pm$ 0.05	144
Adenosine (100 μ M)	0.50 $\pm$ 0.04	94	0.56 $\pm$ 0.02	112
(500 μ M)	0.29 $\pm$ 0.06	55	0.26 $\pm$ 0.11	52
2-Chloroadenosine (100 μ M)	0.66 $\pm$ 0.01	125	0.60 $\pm$ 0.06	120
(500 μ M)	0.61 $\pm$ 0.05	115	0.62 $\pm$ 0.05	124

Fig. 3. Dose-response curve of the effect of adenosine and 2-chloroadenosine to increase the concentration of cyclic AMP

Hepatocytes obtained from a fed rat were incubated for 2min in the presence of the nucleosides.

Fig. 4. Dose-response curve of the effect of glucagon to decrease the concentration of Fru-2,6- P_2 in isolated hepatocytes

The experiment was performed in the presence of 10 μM-adenosine (■) or of 5 μM-2-chloroadenosine (●), or in their absence (○). Fru-2,6- P_2 was measured 6 min after the addition of the effectors. Bars indicate the concentration of glucagon causing a half-maximal effect.

Discussion

Our discovery that adenosine causes a decrease in the concentration of Fru-2,6- P_2 in isolated hepatocytes is a chance observation that was not expected from the known effects of adenosine on carbohydrate metabolism in the liver. We wish to discuss both the mechanism of this effect and its potential biological significance.

Mechanism of the adenosine effect

Most of the data presented in this paper allow the conclusion that the effect of adenosine on the concentration of Fru-2,6- P_2 occurs by an activation of adenylate cyclase, itself resulting from the binding of adenosine to receptors as proposed by Londos *et al.* (1980). The participation of adenylate cyclase and cyclic AMP is clearly indicated by the increases in cyclic AMP and by the changes in activity of a series of enzymes which are known to be substrates for cyclic AMP-dependent protein kinase. The similarity between the effect of glucagon and of the nucleosides extends to the slower modification of PFK 2 and FBPase 2 as compared with pyruvate kinase, a difference that has been explained by a slower activity of cyclic AMP-dependent protein kinase on the first substrates (Bartrons *et al.*, 1983). It is also remarkable that, after the brief stimulation caused by 100 μM-adenosine, the effect on the Fru-2,6- P_2 -modifying system persisted longer than the activation of phosphorylase and the inactivation of pyruvate kinase. As previously discussed by Bartrons *et al.* (1983), these differences in the rates of phosphorylation and of dephosphorylation of enzymes are in agreement with the hypothesis that PFK 2 and FBPase 2 are part of a single protein (Van Schaftingen *et al.*, 1982a; El-Maghrabi *et al.*, 1982), and also suggest that the phosphatase that dephosphorylates that protein has a slow action. The participation of extracellular receptors is indicated by the lack of effect of transport inhibitors. This is in agreement with the observation of Londos *et al.* (1980) on the ability of adenosine and some of its derivatives to activate the adenylate cyclase present in a membrane preparation. The fact that, with that preparation, adenosine was slightly more efficient than N^6 -phenylisopropyladenosine is in contradiction with our observation on isolated hepatocytes. It is, however, in agreement with our interpretation that the poor efficiency of low doses of adenosine results from their rapid metabolism by cytosolic enzymes, namely adenosine deaminase and adenosine kinase.

It remains striking, however, that adenosine and 2-chloroadenosine were nearly as efficient as a maximal concentration of glucagon in their ability to decrease Fru-2,6- P_2 concentration in the

without 5 mM-ATPMg. Attempts to demonstrate an inhibition of PFK 2 by adenosine under similar conditions also yielded negative results.

hepatocytes, although not in their ability to affect the other parameters shown in Fig. 2. This indicates that the nucleosides could also affect the activity of PFK 2 or FBPase 2, independently of their phosphorylation state. We have, however, obtained no indication that this was the case.

Metabolic effects

One well-known effect of the addition of adenosine to liver cells is to increase their content of ATP. This is because adenosine is rapidly phosphorylated by adenosine kinase, and this reaction has been demonstrated to be part of a physiological metabolite futile cycle operating between AMP and adenosine (Bontemps *et al.*, 1983). One role of adenosine kinase appears therefore to maintain a very low intracellular concentration of adenosine, accounting for the fact that the formation of adenosine from AMP by 5'-nucleotidase is not part of the physiological mechanism of AMP conversion into uric acid and allantoin. It is therefore probable that the endogenously formed adenosine plays no role in the activation of adenylate cyclase and in the control of the concentration of Fru-2,6- P_2 .

Other effects of adenosine and its derivatives are greatly dependent on the concentration of the nucleosides and the duration of the experiments. Claus *et al.* (1982) have reported the inactivation of pyruvate kinase by $10\mu\text{M}$ - N^6 -phenylisopropyl-adenosine, although not by $10\mu\text{M}$ -adenosine. These data are in agreement with those reported in Fig. 2, since the inactivation of pyruvate kinase by $100\mu\text{M}$ -adenosine was transient and was terminated after 12 min of incubation, which was the duration of the experiment performed by Claus *et al.* (1982). Marchand *et al.* (1979) have incubated hepatocytes in the presence of 1 mM -adenosine and found that after 30 min the concentrations of cyclic AMP and of glycogen were increased, whereas that of Fru-1,6- P_2 and the consumption of glucose were decreased. Except for the glycogen effect, which might be related to the brevity of the phosphorylase activation, these facts are in agreement with our finding. The decrease in glucose consumption is indeed explained by the decrease in Fru-2,6- P_2 concentration. Finally, we were able to confirm the finding by Lund *et al.* (1975) that adenosine inhibits gluconeogenesis from lactate in hepatocytes obtained from starved rats. The mechanism of this effect is badly understood. By contrast, 2-chloroadenosine stimulated gluconeogenesis, similarly to glucagon, in agreement with the known effect of cyclic AMP-dependent protein kinase on several enzymes involved in the regulation of this pathway (reviewed by Hers & Hue, 1983). We have no explanation for the failure of

Fain & Shepherd (1977) to observe an increase in cyclic AMP concentration in hepatocytes incubated with $200\mu\text{M}$ -adenosine.

We also stress the point that AMP and ATP, when present in the incubation medium, are converted into adenosine and therefore cause a stimulation of liver adenylate cyclase. Those nucleotides are present in some culture media and can also originate from broken cells.

We are grateful to Dr. G. Van den Berghe and Dr. F. Bontemps for helpful suggestions. This work was supported by the Belgian Fonds de la Recherche Scientifique Médicale and by the U.S. Public Health Service (grant AM 9235). R. B. is recipient of a long-term European Molecular Biology Organization Fellowship. E. V. S. is Chargé de Recherches du Fonds National de la Recherche Scientifique.

References

- Arch, J. R. S. & Newsholme, E. A. (1978a) *Biochem. J.* **174**, 965–977
- Arch, J. R. S. & Newsholme, E. A. (1978b) *Essays Biochem.* **14**, 82–123
- Bartrons, R., Hue, L., Van Schaftingen, E. & Hers, H. G. (1983) *Biochem. J.* **214**, 829–837
- Bontemps, F., Van den Berghe, G. & Hers, H. G. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 2829–2833
- Clarke, D. A., Davoll, J., Philips, F. S. & Brown, G. B. (1952) *J. Pharmacol. Exp. Ther.* **106**, 291–302
- Claus, T. H., Anand-Srivastava, M. B. & Johnson, R. A. (1982) *Mol. Cell. Endocrinol.* **26**, 269–279
- El-Maghrabi, M. R., Claus, T. H., Pilkis, J., Fox, E. & Pilkis, S. J. (1982) *J. Biol. Chem.* **257**, 7603–7607
- Fain, J. N. & Shepherd, R. E. (1977) *J. Biol. Chem.* **252**, 8066–8070
- Feliu, J. E., Hue, L. & Hers, H. G. (1977) *Eur. J. Biochem.* **81**, 609–617
- Henderson, J. F., Paterson, A. R. P., Caldwell, I. C., Paul, B., Chan, M. C. & Lau, K. F. (1972) *Cancer Chemother. Rep. Part 2* **3**, 71–85
- Hers, H. G. & Hue, L. (1983) *Annu. Rev. Biochem.* **52**, 617–653
- Hers, H. G. & Van Schaftingen, E. (1982) *Biochem. J.* **206**, 1–12
- Hue, L., Bontemps, F. & Hers, H. G. (1975) *Biochem. J.* **152**, 105–114
- Hue, L., Feliu, J. E. & Hers, H. G. (1978) *Biochem. J.* **176**, 791–797
- Huggett, A. St. G. & Nixon, D. A. (1957) *Biochem. J.* **66**, 12P
- Londos, C., Cooper, D. M. F. & Wolff, J. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 2551–2554
- Lund, P., Cornell, N. W. & Krebs, H. A. (1975) *Biochem. J.* **152**, 593–599
- Marchand, J. C., Lavoine, A., Giroz, M. & Matray, F. (1979) *Biochimie* **61**, 1273–1282
- Miller, M. L., Adamczyk, D. L., Miller, W. H., Koszalka, G. W., Rideout, J. L., Beacham, L. M., Chao, E. Y., Haggerty, J. J., Krenitsky, T. A. & Elion, G. B. (1979) *J. Biol. Chem.* **254**, 2346–2352

- Steiner, A. L., Parker, C. W. & Kipnis, D. M. (1972) *J. Biol. Chem.* **247**, 1106–1113
- Van Belle, H., Xhonneux, R., Borgers, M. & Flameng, W. (1982) *Abstr. Int. Symp. Adenosine, Charlottesville*
- Van den Berghe, G., Bontemps, F. & Hers, H. G. (1980) *Biochem. J.* **188**, 913–920
- Van Schaftingen, E. & Hers, H. G. (1981) *Eur. J. Biochem.* **117**, 319–323
- Van Schaftingen, E., Davies, D. R. & Hers, H. G. (1982a) *Eur. J. Biochem.* **124**, 143–149
- Van Schaftingen, E., Lederer, B., Bartrons, R. & Hers, H. G. (1982b) *Eur. J. Biochem.* **129**, 191–195