STUDIES OF THE MECHANISM OF ACTION OF 3', 5'-CYCLIC NUCLEOTIDES ON HEPATIC GLUCOSE PRODUCTION*

H. O. Conn*, I. S. Karl, A. Steiner, and D. M. Kipnis

Metabolism Division, Department of Medicine
Washington University School of Medicine,
St. Louis, Missouri and
Yale University School of Medicine
New Haven Connecticut

Received August 12, 1971

SUMMARY: In order to elucidate the mechanism of action of 3'5' cyclic nucleotide-induced hepatic glucose production, isolated rat livers were perfused with a variety of cyclic nucleotides. Hepatic levels of cyclic AMP, which were increased 2 to 3 fold by glucagon perfusion, did not exceed control levels (1.12±20 picomoles/g) after perfusion with cyclic GMP or cyclic IMP, which were equipotent to cyclic AMP in stimulating glucose production, or with pyrimidine 3'5' cyclic nucleotides (cyclic UMP, cyclic CMP, cyclic TMP), which were less potent. Measurement of the glycolytic intermediates demonstrated that cyclic GMP and cyclic IMP, like glucagon and cyclic AMP, induced a characteristic gluconeogenic stimulation of the pyruvate to phosphoenolpyruvate sequence. Pyrimidine cyclic nucleotides induced non-specific patterns of glycolytic intermediary metabolism.

The actions of many hormones, including glucagon stimulation of hepatic glucose production, appear to be mediated by adenosine 3'5' monophosphate (cyclic AMP), a so-called secondary messenger (1-4). Recent studies in this laboratory have shown that a number of purine 3'5' cyclic nucleotides (cyclic GMP, cyclic IMP) and pyrimidine 3'5' cyclic nucleotides (cyclic UMP, cyclic CMP), in addition to cyclic AMP, stimulate glucose release in the isolated perfused rat liver (5). Although the purine cyclic nucleotides were more potent than the pyrimidine nucleotides, the relative glucogenic activities of the purine and pyrimidine nucleotides were similar whether based on their ability to accelerate glycogenolysis or stimulate gluconeogenesis. It was not determined, however, whether these activities represented direct effects of the cyclic nucleotides or were mediated by changes in hepatic levels of cyclic AMP. In the present paper, data are presented which demonstrate that the various purine and pyrimidine cyclic nucleotides exhibiting gluconeogenic activity do so without causing significant changes in cyclic AMP. In addition, the steady-state levels of the various glycolytic intermediates have been measured in order to identify the site(s) in the gluconeogenic sequence affected by these nucleotides.

*Supported in part by USPHS Grant A-1921, USPHS Special Fellowship (Dr. Conn) IF3AM-35-658-01, The Irwin Strasburger Memorial Medical Foundation and The Stratfield Fund.

Materials and Methods

Livers were obtained 20 minutes after induction of amobarbital anesthesia in 24 hour fasted male Sprague-Dawley rats weighing 250-300 g. The liver perfusion system used was a modification of the recirculation technique of Williamson et al (6) as previously described (5). After two consecutive 45 minute perfusion periods, the first without substrate and the second with 20 mM lactate, either glucagon (3.3×10^{-8}M) or one of the cyclic nucleotides (1×10^{-3}M) or N^6, 2'-O-dibuteryl cyclic AMP (1×10^{-6}M) was added to the perfusate and the perfusion continued for an additional 45 minutes. Tissue samples were obtained at the end of the experimental period but before stopping the perfusion. All tissue specimens were frozen immediately in liquid nitrogen and stored at -80° C until analyzed.

Cyclic AMP was determined by the radioimmunoassay procedure of Steiner et al (7) and the various glycolytic and tricarboxylic acid cycle intermediates by modifications of the microenzymatic fluorimetric methods of Lowry et al (8-10).

Results

Cyclic AMP levels in perfused rat liver. The cyclic AMP concentration in control liver perfusions (i.e. 20 mM lactate) was 1.12 ± 0.18 picomoles/g wet wt. (Table I). Similar levels were observed before perfusion (0.96 picomoles/g) and slightly lower levels after perfusion for 135 minutes without added lactate (0.82 ± 0.15 picomoles/g wet wt.), but these differences were not statistically significant ($p > 0.1$). Glucagon produced a two and one half fold increase in liver cyclic AMP content (2.64 ± 0.54 picomoles/g wet wt.; $p < 0.01$). The cyclic AMP concentration of livers perfused with cyclic AMP or dibuteryl cyclic AMP, both of which stimulated glucose production, were enormously elevated due to the presence of high cyclic AMP levels in the perfusate. None of the cyclic purine or pyrimidine nucleotides caused a significant change in hepatic cyclic AMP (Table I).

Effect of glucagon and cyclic nucleotides on glycolytic intermediates. The levels of glycolytic intermediates in livers perfused with lactate were similar to those reported by Exton and Park (11). The major difference was the higher tissue lactate level and, as a result, the higher lactate:pyruvate ratio, both of which were consequences of the high concentration of lactate in the perfusate.

The addition of glucagon resulted in the characteristic gluconeogenic pattern of the glycolytic intermediates characterized by elevations of phosphoenolpyruvate (PEP), 2-phosphoglycerate (2-PG) and 3-phosphoglycerate (3-PG) above control levels (Table II)(2,11). This is demonstrated graphically by the cross-over plot which indicates stimulation of the conversion of pyruvate to PEP (Fig. 1). None of the subsequent intermediates of the glycolytic pathway were significantly different from control levels.

The patterns observed with each of the three purine cyclic nucleotides (cyclic AMP, cyclic GMP, cyclic IMP) were qualitatively and quantitatively similar to

TABLE I

EFFECT OF CYCLIC 3',5' NUCLEOTIDES ON CONCENTRATION OF CYCLIC AMP IN PERFUSED RAT LIVER

Experimental Condition	cyclic AMP Concentration picomoles/g Mean ± SEM
No addition (9)*	1.12 ± 0.20
Glucagon (10)+	2.64 ± 0.54
cGMP (10)+	0.97 ± 0.21
cIMP (5)	0.74 ± 0.13
cUMP (6)	0.63 ± 0.08
cCMP (7)	0.87 ± 0.16
cTMP (6)	0.95 ± 0.17

*Number of studies given in parentheses. All perfusions contained 20 mM lactate in the 2nd and 3rd perfusion periods.

+Glucagon perfusate concentration 3.3×10^{-8}M; all nucleotide perfusate concentrations 1×10^{-3}M.

that observed with glucagon (Fig. 1). A similar pattern was also observed with dibuteryl cyclic AMP. In each instance the elevations above control levels were statistically significant for PEP ($p < 0.01$) and 3-PG ($p < 0.05$).

Different patterns were observed, however, with the three pyrimidine cyclic nucleotides (Table II). Cyclic UMP and cyclic CMP were associated with identical patterns in which none of the metabolites differed significantly from control levels (Fig. 2). With both cyclic UMP and CMP the glucose-6-phosphate levels were slightly but insignificantly elevated. Cyclic IMP was associated with non-specific elevations of most of the intermediary metabolites.

Effect of glucagon and cyclic nucleotides on citric cycle intermediates. The pattern of livers perfused with glucagon differed from lactate-perfused controls by small insignificant decreases of all of the citric acid cycle intermediates, especially of isocitrate and acetyl CoA (Table III). The patterns induced by the cyclic purine nucleotides were similar to those of glucagon. The cyclic pyrimidine nucleotide-induced patterns differed from those seen with glucagon, but they varied non-specifically with the individual nucleotides.

TABLE II

EFFECT OF VARIOUS 3',5' CYCLIC NUCLEOTIDES ON GLYCOLYTIC INTERMEDIATES

Experimental Condition	G6P	FDP	DHAP	xGP	3-PG	2-PG	PEP	PYR	LAC	$\frac{xGP}{DHAP}$	$\frac{LAC}{PYR}$
None (7)	53.4 ±8.1	28.8 ±3.0	40.1 ±9.3	193 ±15	237 ±25	46.9 ±9.1	132 ±18	357 ±29	7083 ±1294	5.3 ±0.6	19.9 ±3.2
Glucagon (7)	45.7 ±8.1	26.6 ±2.6	33.2 ±2.0	154 ±42	406 ±81	63.6 ±8.2	231 ±30	278 ±66	5750 ±1250	3.1 ±0.7	21.2 ±2.8
cAMP (9)	43.1 ±4.8	29.6 ±3.7	32.0 ±3.5	258 ±57	325 ±22	54.4 ±9.5	230 ±13	255 ±63	5968 ±1105	5.3 ±0.7	28.7 ±3.3
cGMP (7)	44.7 ±9.7	23.6 ±2.0	25.1 ±3.2	155 ±23	332 ±34	49.8 ±9.6	236 ±19	246 ±39	4990 ±420	5.0 ±0.9	22.7 ±3.3
cIMP (5)	81.1 ±18.5	18.8 ±2.3	26.5 ±2.2	192 ±53	444 ±41	65.3 ±7.0	329 ±32	273 ±40	4153 ±349	7.2 ±4.5	13.7 ±1.7
cUMP (5)	66.3 ±13.3	23.3 ±4.0	34.7 ±6.4	168 ±30	252 ±38	42.4 ±5.3	167 ±16	301 ±13	5108 ±1565	5.1 ±0.7	16.6 ±4.6
cCMP (5)	73.5 ±18.0	21.2 ±1.5	27.0 ±1.6	157 ±16	262 ±46	41.9 ±8.2	173 ±32	341 ±66	5230 ±869	5.9 ±0.6	15.9 ±1.4
cTMP (4)	54.5 ±1.0	41.4 ±6.8	43.4 ±2.0	212 ±11	368 ±51	63.2 ±7.1	143 ±24	364 ±29	4349 ±1193	5.0 ±0.2	11.5 ±2.3
5' AMP (5)	89.4 ±20.8	63.9 ±2.4	36.0 ±4.5	228 ±9	284 ±50	47.4 ±4.9	178 ±21	376 ±54	6854 ±783	6.7 ±0.7	20.4 ±4.2

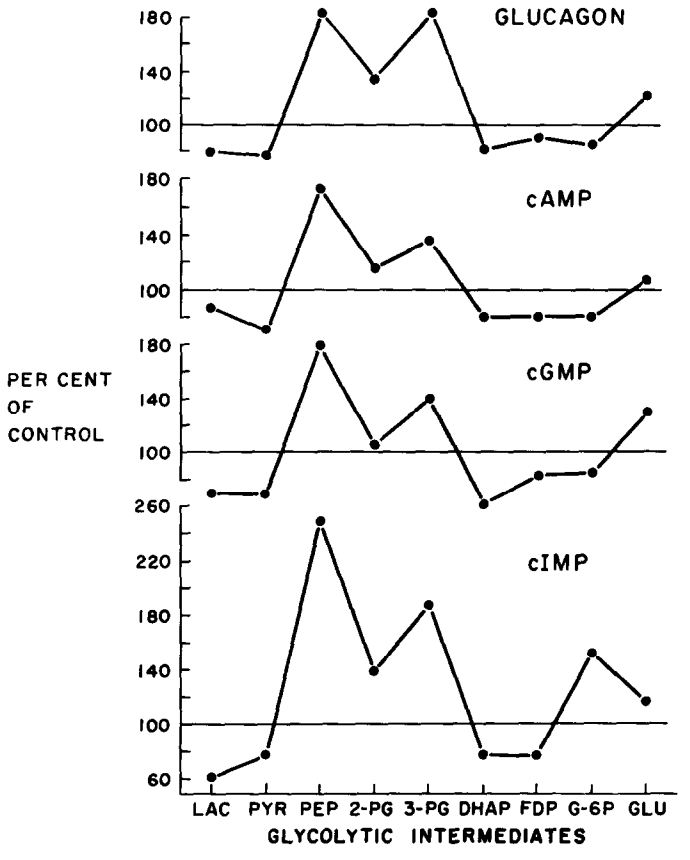

Fig. 1. Effects of 3',5' Cyclic Purine Nucleotides on Concentration of Metabolic Intermediates of the Glycolytic Cycle in Perfused Liver Tissues. In each of the crossover plots the horizontal line represents control levels (100%) of intermediates (abbreviated along the abscissa) observed in livers perfused with 20 mM Na lactate. The dotted curves represent the concentration of each of the intermediates after perfusion with the test substance identified at the right expressed as percent of lactate-perfused control levels. A typical gluconeogenic pattern after glucagon is shown in the top curve. Almost identical patterns were observed with cAMP, cGMP and cIMP. Abbreviations: LAC, lactate; PYR, Pryuvate; PEP, phosphoenolpyruvate; 2-PG, 2-phosphoglycerate; 3-PG, 3-phosphoglycerate; DHAP, dihydroxyacetone phosphate; FDP, fructose diphosphate; G-6P, glucose 6-phosphate; and GLU, glucose.

Discussion

The demonstration of glucagon-induced elevations of cyclic AMP levels in liver tissue confirms that cyclic AMP mediates the glycoregulatory action of this hormone and illustrates the capability of the radioimmunoassay to detect physiologically-stimulated tissue levels of cyclic AMP. The failure to demonstrate an increase in hepatic cyclic AMP levels after perfusion with non-adenine cyclic nucleotides despite potent stimulation of glucose production confirms the observa-

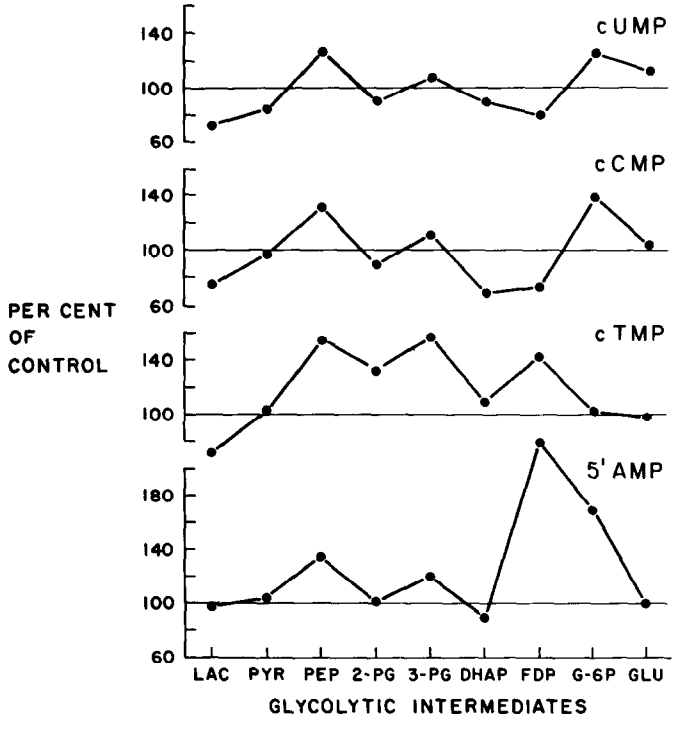

Fig. 2. Effects of 3',5' Cyclic Pyrimidine Nucleotides on Concentration of Metabolic Intermediates of the Glycolytic Cycle in Perfused Rat Livers. The method of plotting is the same as in Figure 1. The patterns of cUMP and cCMP are similar. The non-specific pattern after cTMP shows increased concentration of practically all intermediates in the pathway.

tions of Glinsmann and Exton and their associates (12,13) that perfusion with cyclic GMP does not increase hepatic levels of cyclic AMP. It is conceivable that cyclic GMP-induced stimulation of hydrolysis of cyclic AMP (14) resulted in decreased cyclic AMP levels. The similarity of hepatic cyclic AMP levels after perfusion with a variety of cyclic nucleotides, some of which do not stimulate phosphodiesterase activity, argues against this possibility. These observations suggest that 3',5' cyclic nucleotides other than cyclic AMP directly stimulate hepatic gluconeogenesis.

Furthermore, similarity of the patterns of glycolytic intermediary metabolites induced by each of the three cyclic purine nucleotides suggests that cyclic AMP, GMP and cyclic IMP stimulate gluconeogenesis at the same metabolic control site, presumably at the pyruvate-phosphoenol pyruvate-carboxykinase sequence, the first major control site in the gluconeogenic pathway from lactate. The qualitative and quantitative similarity of the tissue intermediary metabolite pattern following administration of the purine cyclic nucleotides parallels the equipotent stimulation of hepatic glucose production induced by these substances (5).

TABLE III

EFFECT OF VARIOUS 3',5' CYCLIC NUCLEOTIDES ON TRICARBOXYLIC ACID CYCLE INTERMEDIATES

Experimental Condition	OA	CIT	ISO	MAL	FUM	AcCoA
			μ moles/kg wet weight			
None (7)	3.89 ±0.38	885 ±70	36.0 ±6.4	265 ±28	39.1 ±3.7	54.4 ±11.9
Glucagon (7)	3.56 ±1.09	721 ±58	22.5 ±4.6	236 ±39	35.3 ±5.1	33.7 ±10.6
cAMP (9)	1.87 ±0.45	685 ±50	19.4 ±3.8	310 ±35	47.6 ±8.2	32.5 ±4.9
cGMP (7)	3.20 ±0.25	942 ±38	40.2 ±3.9	302 ±29	33.7 ±3.7	39.6 ±5.9
cIMP (5)	3.99 ±0.35	719 ±67	37.8 ±1.6	275 ±47	36.2 ±6.6	30.1 ±1.0
cUMP (5)	5.23 ±0.92	855 ±93	32.9 ±5.0	245 ±44	28.2 ±8.7	38.4 ±4.2
cCMP (5)	4.44 ±0.54	961 ±153	37.2 ±6.4	258 ±21	41.0 ±5.2	28.9 ±419
cTMP (4)	6.8 ±1.9	829 ±57	47.1 ±1.4	184 ±21	24.2 ±3.1	33.8 ±4.9

The infusion of pyrimidine cyclic nucleotides induced variable and non-specific patterns in which the site of stimulation of gluconeogenesis was not evident. Clearly the control site was not the pyruvate-PEP sequence and was, presumably, distal to triosephosphate. The patterns observed with cyclic UMP and cyclic CMP were similar. It is consistent that these two cyclic nucleotides, which stimulate hepatic glucose production equally, have such similar patterns of tissue metabolites. The pattern of metabolic intermediates following perfusion with cyclic TMP, which stimulated neither gluconeogenesis or glycogenolysis (5), showed small elevations of all of the glycolytic cycle intermediates, a non-specific pattern suggestive of decreased gluconeogenesis.

As would be expected, there were no specific patterns noted in the citric cycle intermediates or in the levels of the adenine nucleotides after perfusion with various 3'5'-cyclic nucleotides.

References

1. Sutherland, E.W., G.A. Robinson: Pharm.Rev. 18:145, 1966.
2. Exton, J.H., L.S. Jefferson, R.W. Burcher, C.R. Park: Am.J.Med. 40:709, 1966.
3. Menehan, L.A., O.Wieland: Biochem.Biophys.Res.Comm. 29:880, 1967.
4. Levine, R.A., S.E. Lewis: Biochem.Pharm. 18:15, 1969.
5. Conn, H.O., D.M. Kipnis: Biochem.Biophys.Res.Comm. 37:319, 1969.
6. Williamson, J.R., A.E. Garcia, A.E. Renold, G.F. Cahill: Diabetes 15:183, 1966.
7. Steiner, A.L., D.M. Kipnis, R. Utiger, C. Parker: Proc.Nat.Acad. Sci. 64: 367, 1969.
8. Lowry, O.H., J.V. Passonneau: J.Biol.Chem. 239:31, 1964.
9. Goldberg, N.D., J.V. Passonneau, O.H. Lowry: J.Biol.Chem. 241:3997, 1966.
10. Karl, I.E., N. Voyles, L. Recant: Diabetes 17:374, 1968.
11. Exton, J.H., C.R. Park: J.BiolChem. 244:1424, 1969.
12. Glinsmann, W.H., E.P. Hern, L.G. Linarelli, R.V. Farese: Endocrinology 85:74, 1969.
13. Exton, J.H., J.G. Hardman, T.F. Williams, E.W. Sutherland, C.R. Park: J.Biol.Chem. 246:2658, 1971.
14. Beavo, J.A., J.G. Hardman, E.W. Sutherland: J.Biol.Chem. 246:3841, 1971.