Biochem. J. (1974) **143**, 469–471
Printed in Great Britain

Nuclear Protein Kinase Activity in Glucagon-Stimulated Perfused Rat Livers

By WARREN K. PALMER, MONIQUE CASTAGNA and DONAL A. WALSH
Department of Biological Chemistry, School of Medicine, University of California, Davis, Calif. 95616, U.S.A.

(*Received* 8 *July* 1974)

Nuclei isolated from glucagon-stimulated perfused rat livers contained 2–3 times as much protein kinase activity as did nuclei from control animals. In the presence of either the heat-stable inhibitor or the protein kinase regulatory subunit the elevated cyclic AMP-independent enzyme activity from stimulated nuclei was inhibited to an activity equivalent to that found in controls.

It has been shown that cyclic AMP is formed as a result of contact of a variety of hormones with their specific target cells. Many hormonal functions, such as the glucagon stimulation of hepatic glycogenolysis, gluconeogenesis and lipolysis can be mimicked by replacing the hormone with cyclic AMP. The discovery of a cyclic AMP-dependent protein kinase in rabbit skeletal muscle (Walsh *et al.*, 1968) identified an additional step in the pathway linking hormone release with hormone action. Brostrom *et al.* (1970) first postulated a mechanism for the relationship of cyclic AMP to protein kinase as follows. The holoenzyme, R·C, has no known catalytic activity when cyclic AMP is absent. In the presence of the cyclic nucleotide the enzyme dissociates into two distinct subunits. The regulatory subunit, R, binds to cyclic AMP, whereas catalytic subunit (C) is enzymically active: R·C+cyclic AMP$\rightleftharpoons$ R·cyclic AMP+C. Cyclic AMP-dependent protein kinase is widely distributed in mammalian tissue, a fact which led Greengard & Kuo (1970) to propose that protein kinase activity might mediate all cyclic AMP effects in mammalian tissue.

In some cases the response to hormones involves the synthesis of new proteins, a process which can be blocked by inhibitors of RNA synthesis and can be mimicked by exogenous addition of cyclic AMP analogues. These results suggest that cyclic AMP affects nuclear-mediated events. One nuclear alteration that occurs in response to the increase of cyclic AMP in hepatic tissue is the phosphorylation of specific nucleohistone residues (Langan, 1969).

Previous studies (Chen & Walsh, 1971) have shown that over 90% of the cyclic AMP-stimulated protein kinase activity of liver is present in the cytoplasm, and it has proved difficult to exclude the possibility that the small amount of cyclic AMP-dependent protein kinase present in nuclear fractions is not due to cytoplasmic contamination. In contrast, the nucleus contains a cyclic AMP-independent protein kinase that is a distinct entity from either cyclic AMP-dependent holoenzyme or the catalytic subunit derived from it (Walsh & Ashby, 1973). The mechanism by which protein phosphorylation and possibly other nuclear events are altered in response to elevated intracellular cyclic AMP concentrations has not yet been elucidated. In this communication we demonstrate an increased protein kinase activity associated with nuclei in perfused rat liver in response to glucagon stimulation.

Methods

Male Sprague–Dawley rats (120–200g) were starved for 24h before surgery. Animals were anaesthetized with sodium pentobarbital (6mg/100g body wt.) and the livers perfused by the technique of Hems *et al.* (1966). After a 30min equilibration perfusion period sodium lactate (10mM final concn.) was added to both the non-stimulated and glucagon-stimulated preparations. In the latter glucagon was added in a final concentration of 1μM simultaneously with the lactate. Blood samples were withdrawn throughout the entire perfusion for subsequent glucose determinations by the Glucostat technique (Worthington Biochemical Corp., Freehold 2, N.J., U.S.A.). Gluconeogenic activity of the livers was used as a criterion for tissue viability as well as an indicator for a positive glucagon effect. All data utilized were from livers deemed viable by the above criteria. All glucagon-treated livers used showed a positive gluconeogenic stimulation by the hormone of greater than 40% increase in glucose production. At 15min after lactate addition the livers were excised, rinsed in cold 0.9% NaCl and weighed. All subsequent steps were performed at 0–4°C. Nuclei were isolated by a modification of the procedure of Chauveau *et al.* (1956). Tissue was minced and homogenized in a Dounce homogenizer in a solution of cold 2.2M-sucrose–6mM-EGTA [ethanedioxybis(ethylamine)-tetra-acetic acid]–0.1mM-$MgCl_2$ (1g of tissue/10ml of buffer). Homogenates were filtered through a

double layer of cheesecloth and were then centrifuged at 85000*g* for 60min. The nuclear pellet from 5g wet wt. of liver was resuspended in 25ml of buffer containing 100mM-Mes [2-(*N*-morpholino)ethanesulphonic acid], 15mM-magnesium acetate and 1mM-EGTA, pH6.9 (assay buffer), and centrifuged at 20000*g* for 30min. The nuclear fraction was suspended in the assay buffer in a volume of 1ml/g wet wt. of liver by homogenization with a Dounce homogenizer. The cytoplasmic fraction was obtained from the infranatant solution of the initial centrifugation as indicated by Chauveau *et al.* (1956). Protein kinase activity was measured in the cytoplasmic and nuclear fractions by the assay method of Reimann *et al.* (1971) with the following modifications. The assay mixture contained 2μmol of Mes buffer (pH6.9), 0.3μmol of magnesium acetate, 0.02μmol of EGTA, 100μg of histone F2b, 10nmol of [γ-^{32}P]ATP (0.45mCi/mmol) and, when included, 20nmol of cyclic AMP in a final volume of 60μl. The reaction was initiated by the addition of 20μl of tissue extract appropriately diluted so that the reaction kinetics were linear with time and enzyme dilution. The reaction mixture was incubated at 30°C for 10min. The reaction was terminated by spotting a 50μl sample of the incubation on filter-paper squares and the squares were submerged in 10% (w/v) trichloroacetic acid. Papers were washed to remove non-protein-bound label, dried, and counted for radioactivity by appropriate scintillation-counting techniques (Reimann *et al.*, 1971). Corrections were made for phosphorylation of endogenous extract protein.

Lactate dehydrogenase was determined by the method of Kornberg (1955). Protein determinations were by the technique of Lowry *et al.* (1951) with bovine serum albumin as a standard. Purified rabbit skeletal-muscle regulatory subunit of protein kinase was graciously provided by Dr. E. G. Krebs, Dr. J. Beavo and Dr. P. Bechtel. The inhibitor protein was prepared as described previously (Walsh *et al.*, 1971).

From initial reaction rates total protein kinase specific activity ('+cyclic AMP' activity) and cyclic AMP-independent protein kinase specific activity ('–cyclic AMP' activity) measured in cytoplasm and nuclei were statistically compared in glucagon-treated and control livers by Student's *t* test (Steel & Torrie, 1960). Difference between means was considered significant at the 0.05 probability level.

Results

Hepatic nuclear and cytoplasmic protein kinase activity, determined with histone F2b as substrate, in control and glucagon-stimulated liver is given in Table 1. Statistical evaluation of the data confirms that the total protein kinase activity of the cytoplasm is decreased after glucagon administration ($P \leqslant 0.05$). Under the experimental conditions used the cytoplasmic cyclic AMP-independent activity was unaltered by glucagon stimulation, presumably reflecting destruction of endogenous cyclic AMP during subcellular fractionation. Both total nuclear protein kinase activity and nuclear cyclic AMP-independent activity are approximately twofold greater after glucagon stimulation ($P \leqslant 0.001$). To determine whether the increase in nuclear-associated kinase activity could be attributed to differential amounts of cytoplasmic contamination, lactate dehydrogenase activity in the homogenate, cytoplasm and nuclear

Table 1. *Protein kinase activity of nuclear and cytoplasmic fractions from glucagon-stimulated perfused rat livers*

Livers from rats starved for 24h were perfused by the technique of Hems *et al.* (1966). Then 15min after the addition either of 1μM-glucagon plus 10mM-lactate or of lactate alone the livers were removed and nuclear and cytoplasmic fractions prepared as described by Chauveau *et al.* (1956). The protein kinase activity of these fractions was assayed with histone F2b as substrate. Protein kinase activities are expressed as means ±S.E.M. of six experiments. Lactate dehydrogenase activities are the mean of triplicate determinations from two livers.

	Protein kinase activity				Lactate dehydrogenase activity (units/liver)
	(pmol of P_i incorporated/min per mg of protein)		(nmol of P_i incorporated/min per liver)		
	+Cyclic AMP	–Cyclic AMP	+Cyclic AMP	–Cyclic AMP	
Cytoplasmic fraction					
Control	219.6±18.8	52.3±9.7	270.1±36.7	72.5±10.5	559
Glucagon-stimulated	165.4±13.7*	56.3±6.0	211.0±19.3*	72.7±3.2	502
Nuclear fraction					
Control	95.7±9.9	45.9±6.5	6.20±0.64	3.08±0.18	9.2
Glucagon-stimulated	202.0±18.3†	117.5±11.6†	18.35±4.14†	7.54±0.42†	9.5

* Significantly less than control cytoplasm (+cyclic AMP), $P \leqslant 0.05$.
† Significantly greater than control nuclei (±cyclic AMP), $P \leqslant 0.001$.

Table 2. *Cyclic AMP-independent protein kinase activity measured in nuclear extracts of glucagon-stimulated livers in the presence of regulatory subunit and heat-stable protein inhibitor*

The protein kinase activity of nuclei prepared from either control perfused livers or livers stimulated with 1 μM-glucagon was assayed by the standard procedure but in the absence of cyclic AMP. Where added the reaction mixtures contained either 0.15 μg of regulatory subunit or 710 units of inhibitor protein (Ashby & Walsh, 1972). The values expressed are the means of triplicate determinations for two experiments.

		Cyclic AMP-independent protein kinase activity (pmol of P_i/min per mg of protein)		
		No addition	+Regulatory subunit	+Inhibitor
Expt. 1	Glucagon-stimulated	121	45	38
	Control	31	31	32
Expt. 2	Glucagon-stimulated	93	37	32
	Control	39	40	38

fractions was determined. The results showed that under the isolation conditions used less than 2.0% of the total lactate dehydrogenase activity is associated with the nuclear fraction and that this extent of activity is the same in control and glucagon-treated livers.

We have previously shown (Walsh & Ashby, 1973) that one of the principle protein kinases of rat liver nuclei, whose activity is expressed in the absence of cyclic AMP, is not free catalytic subunit derived from the cyclic AMP-dependent holoenzyme. This activity is distinguished on the basis of a lack of interaction with either free regulatory subunit of cyclic AMP-dependent protein kinase or with a heat-stable inhibitor protein of the cyclic AMP-dependent enzyme(s). This nuclear enzyme exhibits more activity with casein and phosvitin than with histone F2b and is often referred to as 'chromatin kinase'. In crude nuclear extracts from control animals the cyclic AMP-independent protein kinase activity is not inhibited by either free regulatory subunit or inhibitor protein (Table 2). Hence this activity is primarily of the 'chromatin (casein) kinase' type. In nuclei prepared from glucagon-stimulated livers the cyclic AMP-independent activity is sensitive to inhibition by either free regulatory subunit or the inhibitor protein and can be titrated by either to the activity observed in control animals (Table 2). Thus whereas in control animals the nuclei contain no detectable free catalytic subunit, the total increment in cyclic AMP-independent activity after glucagon stimulation is accounted for as free catalytic subunit.

The data presented provide a possible explanation for the method by which cyclic AMP modulates nuclear activity. Langan (1970) has characterized in detail the site of phosphorylation that occurs in histone F1 in response to glucagon. It appears to be catalysed by a cyclic AMP-dependent enzyme (or the catalytic subunit derived therefrom). The data in the present communication suggest that this nuclear activity is derived from cytoplasmic protein kinase that has been translocated to the nucleus either as holoenzyme or, after dissociation promoted by the increased amounts of cytoplasmic cyclic AMP, as the free catalytic subunit.

This work was supported by National Institutes of Health grants HL 14780 and AM 13613. D. A. W. is an Established Investigator of the American Heart Association.

Ashby, C. D. & Walsh, D. A. (1972) *J. Biol. Chem.* **247**, 6637–6644
Brostrom, M. A., Reimann, E. M., Walsh, D. A. & Krebs, E. G. (1970) *Advan. Enzyme Regul.* **8**, 191–203
Chauveau, J., Moulé, Y. & Rouiller, C. (1956) *Exp. Cell Res.* **11**, 317–321
Chen, L.-J. & Walsh, D. A. (1971) *Biochemistry* **10**, 3614–3621
Greengard, P. & Kuo, J. F. (1970) *Advan. Biochem. Psychopharmacol.* **3**, 287–306
Hems, R., Ross, B. D., Berry, M. N. & Krebs, H. A. (1966) *Biochem. J.* **101**, 284–292
Kornberg, A. (1955) *Methods Enzymol.* **1**, 441–443
Langan, T. A. (1969) *J. Biol. Chem.* **244**, 5763–5765
Langan, T. A. (1970) *Advan. Biochem. Psychopharmacol.* **3**, 307–323
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
Reimann, E. M., Walsh, D. A. & Krebs, E. G. (1971) *J. Biol. Chem.* **246**, 1986–1995
Steel, R. G. D. & Torrie, J. H. (1960) *Principles and Procedures of Statistics*, pp. 57–75, McGraw-Hill Book Co., New York
Walsh, D. A. & Ashby, C. D. (1973) *Recent Progr. Horm. Res.* **29**, 329–353
Walsh, D. A., Perkins, J. P. & Krebs, E. G. (1968) *J. Biol. Chem.* **243**, 3763–3765
Walsh, D. A., Ashby, C. D., Gonzalez, C., Calkins, D., Fischer, E. H. & Krebs, E. G. (1971) *J. Biol. Chem.* **246**, 1977–1985