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ASSAY OF GLYCOGEN PHOSPHORYLASE IN ISOLATED FAT CELLS OF THE RAT*

D. O. ALLEN and J. ASHMORE

Department of Pharmacology, Indiana University School of Medicine, Indianapolis, Ind. 46202, U.S.A.

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Abstract—A sensitive fluorometric assay for glycogen phosphorylase (alpha-1,4-glucon: orthophosphate glucosyl transferase; E.C. 2.4.1.1) was applied to the rat isolated fat cells. Phosphorylase activity was proportional to time and the amount of tissue. Maximum activity was observed at pH values of 6·5 and 7·0. The assay had an absolute requirement for glycogen and inorganic phosphate, thus appearing to be specific for glycogen phosphorylase. Activation of phosphorylase by catecholamines, adrenocorticotropic hormone (ACTH) and the dibutyryl derivative of cyclic 3′,5′-adenosine monophosphate was observed when these agents were incubated with intact fat cells. Serotonin failed to activate the enzyme. When epinephrine was incubated with broken cells, no activation of phosphorylase occurred.

IN THE past few years many reports have been published dealing with metabolic regulation in adipose tissue. Glucose utilization,[1–3] lipolysis[4,5] and lipogenesis[6–8] have received a great deal of attention, and hormonal control of these processes has been extensively investigated. One hormonally controlled process which has not been adequately investigated in adipose tissue is glycogenolysis. Although intensively studied in such tissues as skeletal muscle,[9] very few reports exist dealing with this process in fat and fewer with its hormonal regulation in this tissue.[10,11]

The rate of glycogenolysis is under the control of the enzyme glycogen phosphorylase (alpha-1,4-glucon: orthophosphate glucosyl transferase; EC 2.4.1.1), the activity of which can be altered by a variety of hormones.[12] Previous reports dealing with assay of this enzyme in adipose tissue have used a method which measures the inorganic phosphate produced by the reversal of the reaction from glucose 1-phosphate to glycogen.[10,11] Hardman *et al.*[13] have described a fluorometric assay for cardiac glycogen phosphorylase which measures the reaction in the forward direction. In this assay the product of the phosphorylase reaction, glucose 1-phosphate, was enzymatically converted to glucose 6-phosphate and then to 6-phosphoglyconolactone with the resulting reduction of NADP. The appearance of this reduced pyridine nucleotide was followed fluorometrically. The present report deals with the application of the procedure of Hardman *et al.*[13] to the assay of glycogen phosphorylase in isolated fat cells. Phosphorylase activity was found to be linear with time and amount of tissue, pH dependent, and sensitive to the presence of a variety of hormones

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MATERIALS AND METHODS

Fed, male, Cox–Holtzman rats weighing 150–200 g were stunned by a blow to the head and killed by exsanguination. The epididymal fat pads were removed and isolated fat cells prepared by the method of Lech and Calvert.[14] Aliquots of the fat cells were incubated for various periods of time in Krebs–Ringer bicarbonate buffer (pH 7·4) with and without appropriate drugs. Incubations were carried out at 37° with gentle shaking in an atmosphere of 95% O_2–5% CO_2 in a final volume of 2·0 ml.

The cells were broken by homogenization with a Polytron homogenizer (type Pt-10). The resulting homogenates were transferred to glass centrifuge tubes and spun in a clinical centrifuge for 1 min. The fat cake which accumulated at the top was removed by aspiration, and the infranatant solution was used for the assay of glycogen phosphorylase activity. Tissue was maintained at 0° during the process, and the time from beginning of the homogenization to the start of the assay never exceeded 2 min. Protein content of this material was determined by the method of Lowry *et al.*[15]

Aliquots (usually 200 μl) of the infranatant solution were transferred to glass fluorometer tubes containing glycogen (100 μg), phosphoglucomutase (6 Units), glucose 6-phosphate dehydrogenase (1·25 Units), magnesium chloride (9 μmoles), NADP (250 nmoles), dibasic sodium phosphate (50 μmoles) and triethanolamine buffer (0·25 M; pH 6·5). The final volume was 1·32 ml. The solution was mixed, and the rate of formation of reduced NADP was followed at 27° using a Farrand filter fluorometer. In control experiments the production of reduced NADP over a 30-min period was proportional to the amount of added glucose 1-phosphate up to at least 100 nmoles. Phosphorylase activity is expressed as millimicromoles of glucose 1-phosphate formed per milligram of protein per 30 min.

All values for P were calculated by using the Student's *t*-test for paired comparisons.

Glucose 6-phosphate dehydrogenase, phosphoglucomutase, NADP, triethanolamine, dibutyryl cyclic AMP,* serotonin (creatine sulfate complex) and glucose 1-phosphate were purchased from Sigma Chemical Company, St. Louis, Mo. Glycogen was from General Biochemicals, Chagrin Falls, Ohio. The adrenocorticotropic hormone (ACTH) was purchased from Parke, Davis & Company, Detroit, Mich. *l*-Epinephrine, *l*-norepinephrine, and *dl*-isoproterenol HCl were the kind gifts of Sterling Winthrop Research Institute, Rensselaer, N.Y.

RESULTS

Isolated fat cells were prepared and incubated in the presence or absence of epinephrine (10^{-4} M) for 15 min. The incubation mixtures were handled as described in Methods, and phosphorylase activity was assayed on various sized aliquots of the infranatant solutions. Both basal and epinephrine-stimulated enzymatic activities were proportional to the amount of added adipose tissue protein. In other experiments it was demonstrated that, using this assay system, enzymatic activity was proportional to the time of incubation up to 30 min. This proportionality existed with both basal and epinephrine-stimulated tissue. The pH optimum was between 6·5 and 7·0. As would be expected in the absence of either adipose tissue extract or NADP, no fluorescence developed. The omission of either glycogen or inorganic phosphate

* Abbreviations used: dibutyryl cyclic AMP, N^6,O^2-dibutyryl adenosine 3′,5′-cyclic monophosphate; cyclic AMP, adenosine 3′,5′-cyclic monophosphate.

from the assay system resulted in a development of fluorescence which was 4 per cent of the control.

In six experiments, the activation of adipose tissue phosphorylase by epinephrine occurred very rapidly (Fig. 1). Intact fat cells were incubated in the presence of epinephrine (10^{-4} M) for varying lengths of time, after which the cells were homogenized and phosphorylase activity was assayed. Significant activation of the enzyme occurred within 30 sec after the addition of epinephrine, with maximum activation

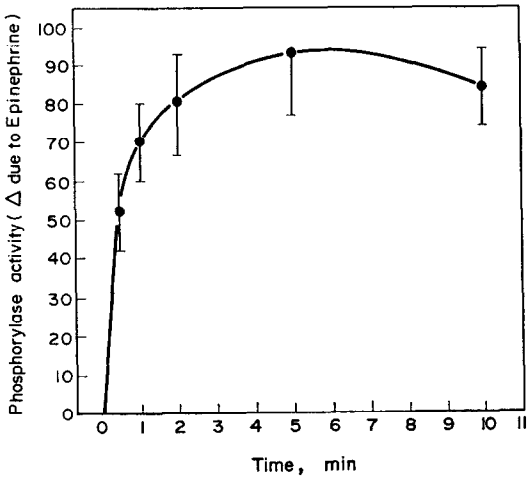

FIG. 1. Time course of activation of phosphorylase by epinephrine in intact fat cells. Intact fat cells were incubated in the presence of epinephrine (10^{-4} M) for varying periods of time after which phosphorylase was assayed. Results are expressed as the increase produced by epinephrine above control levels. Phosphorylase activity at zero time was 54 ± 12 nmoles glucose 1-phosphate/mg protein/30 min. If no epinephrine was added to the cells, the activity did not change significantly ($P > 0{\cdot}1$) for the duration of the experiment. Results are the means ± S.E.M. for five experiments.

having been obtained by 5 min. After 10 min of incubation in the presence of epinephrine, the degree of activation of phosphorylase was not significantly different from that at 5 min ($P > 0{\cdot}05$). In control samples saline was added to the intact fat cells, and the incubation continued for an identical time to those samples to which epinephrine had been added. The phosphorylase activity assayed at these various time periods did not differ significantly from that at zero time ($P > 0{\cdot}05$).

That an intact fat cell is necessary for epinephrine activation of phosphorylase was determined in a series of eight experiments. Intact fat cells were incubated in the presence and absence of 10^{-4} M epinephrine. Another aliquot of the same cell suspension was homogenized and then incubated with epinephrine (10^{-4} M). After a 5-min incubation period, all samples were homogenized and phosphorylase activity was assayed. The addition of epinephrine to intact cells resulted in a doubling of the phosphorylase activity, while the addition of epinephrine to the broken cell preparation had no significant ($P > 0{\cdot}05$) effect on the activity of this enzyme (Table 1). Addition of 5′-AMP (2×10^{-3} M) to the assay mixture greatly increased the activity of both the basal and epinephrine-stimulated phosphorylase (Table 2). The presence of 5′-AMP did not alter the degree of activation produced by epinephrine.

TABLE 1. EFFECT OF EPINEPHRINE ON PHOSPHORYLASE ACTIVITY IN INTACT AND BROKEN FAT CELLS*

Assay conditions	Phosphorylase activity†
Control (intact cells)	52 ± 15
Epinephrine (added to intact cells)	101 ± 15
Epinephrine (added to homogenized cells)	44 ± 8

* Intact fat cells were incubated in the presence and absence of epinephrine (10^{-4} M) for 5 min. Another aliquot of the same cell suspension was homogenized and then incubated with epinephrine (10^{-4} M) for 5 min. All samples were treated as described in Methods, and phosphorylase activity was assayed. Results are the means ± S.E.M. of eight experiments.

† Millimicromoles of glucose 1-phosphate formed per milligram of protein per 30 min.

TABLE 2. EFFECT OF 5′-AMP ON PHOSPHORYLASE ACTIVITY*

Assay conditions	Phosphorylase activity†		
	Basal	Plus epineprine	Δ
Without 5′-AMP	94 ± 14	228 ± 28	134 ± 16
With 5′-AMP	168 ± 18	322 ± 35	154 ± 22
P	< 0·001	< 0·001	> 0·10

* Isolated fat cells were incubated in the presence and absence of epinephrine (10^{-5} M) for 5 min, then treated as described in Methods. Phosphorylase was assayed in the presence and absence of 5′-AMP (2×10^{-3} M). Results are expressed as means ± S.E.M. of eight experiments.

† Millimicromoles of glucose 1-phosphate formed per milligram of protein per 30 min.

In a series of ten experiments, cells were incubated with or without epinephrine (10^{-5} M), after which they were homogenized in the presence of 0·1 M sodium fluoride and phosphorylase activity was assayed. The control activity (114 ± 12·9 nmoles/mg protein/30 min; mean ± S.E.M.) was somewhat higher than values obtained in the absence of sodium fluoride. The activity from the epinephrine-treated cells (217 ± 6·2 nmoles/mg protein per 30 min; mean ± S.E.M.) was significantly ($P < 0·001$) greater than control. The degree of activation was similar to that seen in cells homogenized in the absence of sodium fluoride.

In eight experiments, epinephrine increased adipose tissue phosphorylase activity in a concentration-dependent manner. Aliquots of fat cells were incubated in the presence of epinephrine at concentrations of 10^{-7} to 10^{-4} M. The results of these experiments are plotted in Fig. 2. A sigmoidal-shaped concentration–response curve resulted with the maximum response occurring at 10^{-5} M epinephrine.

Other agents also stimulated phosphorylase (Table 3). l-Norepinephrine, *dl*-isoproterenol and ACTH all produced significant increases in activity. The dibutyryl analogue of cyclic AMP also stimulated the enzyme. Serotonin failed to significantly increase the activity in nine experiments ($P > 0·1$).

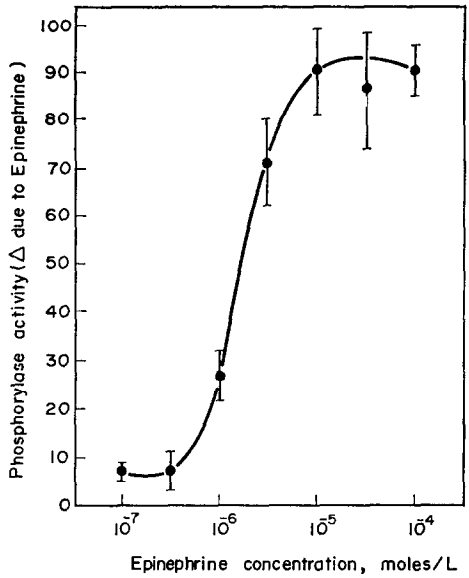

FIG. 2. Relationship between phosphorylase activity and concentration of epinephrine. Isolated fat cells were incubated for 5 min in the presence or absence of various concentrations of epinephrine, after which phosphorylase was assayed. Results are expressed as the increase produced by epinephrine above control levels. Phosphorylase activity in the absence of epinephrine was 94 ± 11 nmoles glucose 1-phosphate/mg protein/30 min. Results are the means ± S.E.M. of eight experiments.

TABLE 3. EFFECT OF VARIOUS AGENTS ON PHOSPHORYLASE ACTIVITY*

	Additions	Phosphorylase† activity	P
I	Control	74 ± 4·5	
	Epinephrine (10^{-4} M)	133 ± 8·6	< 0·001
	ACTH (0·1 U/ml)	126 ± 9·8	< 0·001
	DbcAMP (10^{-4} M)‡	132 ± 8·0	< 0·001
II	Control	80 ± 6·4	
	Norepinephrine (10^{-4} M)	145 ± 12·8	< 0·001
	Isoproterenol (10^{-4} M)	124 ± 10·9	< 0·005
III	Control	77 ± 6·8	
	Epinephrine (10^{-4} M)	135 ± 18·5	< 0·02
	Serotonin (10^{-4} M)	86 ± 6·6	> 0·10

* Isolated fat cells were incubated in the presence and absence of the various agents for 5 min, then treated as described in Methods, and assayed for glycogen phosphorylase activity. Results are expressed as means ± S.E.M. of 27 experiments (I), 12 experiments (II), or 9 experiments (III).

† Millimicromoles of glucose 1-phosphate formed per milligram of protein per 30 min.

‡ Dibutyryl cyclic AMP.

DISCUSSION

In previous reports the assay of adipose tissue glycogen phosphorylase was accomplished by measuring inorganic phosphate or glycogen produced by reversal of the phosphorylase reaction.[10,11] The present report describes the use of the sensitive fluorometric method of Hardman *et al.*[13] for assaying glycogen phosphorylase in the forward direction of the reaction. The reaction was found to be linear with time and amount of tissue and pH dependent. Standard assay conditions were chosen that provided for the assay of glycogen phosphorylase in aliquots of tissue from isolated fat cells.

That the assay was specific for glycogen phosphorylase was established by the omission of single components from the assay mixture and the subsequent measurement of fluorescence development. As would be expected, the assay had an absolute requirement for the presence of tissue and NADP. The omission of either glycogen or inorganic phosphate from the assay mixture reduced the activity by better than 95 per cent. Based on the fact that both glycogen and inorganic phosphate are necessary for the enzymatic formation of glucose 1-phosphate, the product of the phosphorylase reaction, these results indicate that the glucose 1-phosphate was being formed by glycogen phosphorylase. These pieces of information taken collectively strongly support the contention that the assay as designed specifically measured the activity of glycogen phosphorylase. The fact that both basal and hormone-stimulated phosphorylase activity were linear with time indicates that the activity of the enzyme is constant at least for the duration of the assay.

Using standard conditions based on the results reported here, non-stimulated phosphorylase activity varied between 52 $\pm$ 15 and 94 $\pm$ 14 nmoles glucose 1-phosphate/mg protein/30 min. These values are similar to, but somewhat higher than the values reported for rat adipose tissue by Villar-Palasi and Larner,[16] who also assayed phosphorylase activity in the forward direction.

Similar to results reported for liver, skeletal muscle and cardiac muscle,[12,17] epinephrine increases the activity of glycogen phosphorylase in isolated fat cells. This activation occurs very rapidly, being significant within 30 sec and maximal within 5 min of the addition of the hormone. Although previous reports have demonstrated that single doses of epinephrine stimulate phosphorylase in fat pads, the present report represents the first demonstration of a dose–response relationship between epinephrine concentration and phosphorylase activity.

Based on the work of Sutherland *et al.*,[12,17] it is generally assumed that the hormonal activation of phosphorylase in a variety of tissues is secondary to the activation of adenylate cyclase and the subsequent increase in intracellular levels of cyclic AMP. It must be tentatively assumed that the same process is operating in adipose tissue. Consistent with this assumption is the finding that the enzyme was activated by epinephrine, norepinephrine, isoproterenol, and ACTH, all four of which are known to increase intracellular levels of cyclic AMP in isolated fat cells.[18] In addition, the enzyme was markedly activated by the dibutyryl analogue of cyclic AMP.

In contrast, glycogen phosphorylase was not activated by serotonin. These results are contrary to the findings reported by Vaughan[10] and Frerichs and Ball.[19] Conceivably this discrepancy can be explained on the basis that the present work was done with isolated fat cells, while that of the other workers was conducted with intact epididymal fat pads. It is known that qualitative differences exist between responses

in isolated fat cells and in fat pads. For example, Butcher and Baird[20] have demonstrated that prostaglandin E_1 was able to elevate levels of cyclic AMP in the intact fat pad but produced no change in the levels of the cyclic nucleotide in the isolated fat cell.

Although Vaughan[10] and later Frerichs and Ball[19] were able to demonstrate that serotonin activated glycogen phosphorylase in adipose tissue, these authors and others[21,22] were unable to demonstrate an increase in lipolytic activity with this agent. These two pieces of information appear to be inconsistent with the premise that both phosphorylase activation and stimulation of lipolysis are mediated by an increase in cyclic AMP levels. As reported here with the isolated fat cell, serotonin does not activate glycogen phosporylase. The inconsistency in responses to serotonin would thus appear to be resolved, in that with the use of the isolated fat cell preparation serotonin not only lacks lipolytic activity but also fails to activate glycogen phosphorylase.

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