β-Adrenergic agents increase the phosphorylation of phosphofructokinase in isolated rat epididymal white adipose tissue

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Pieces of rat epididymal adipose tissue were incubated in medium containing [^{32}P]phosphate for 2 h to achieve steady-state labelling of intracellular phosphoproteins and then with or without hormones for a further 15 min. Phosphofructokinase was rapidly isolated from the tissue by use of either Blue Dextran–Sepharose chromatography or immunoprecipitation with antisera raised against phosphofructokinase purified from rat interscapular brown adipose tissue. Similar extents of incorporation of ^{32}P into phosphofructokinase were measured by both techniques. Exposure of the tissue to adrenaline or the β-agonist isoprenaline increased phosphorylation by about 5-fold (to about 1.4 mol of phosphate/mol of enzyme tetramer). No change in phosphorylation was detected with the α-agonist phenylephrine, but exposure to insulin resulted in an approx. 2-fold increase. The increased phosphorylation observed with isoprenaline was found to be associated with a decrease in the apparent K_a for fructose 2,6-bisphosphate similar to that observed on phosphorylation of phosphofructokinase purified from rat epididymal white adipose tissue with the catalytic subunit of cyclic AMP-dependent protein kinase. These results support the view [Sale & Denton (1985) Biochem. J. **232**, 897–904] that an increase in cyclic AMP in adipose tissue may result in an increase in glycolysis through the phosphorylation of phosphofructokinase by cyclic AMP-dependent protein kinase.

INTRODUCTION

Evidence has been presented in the preceding paper (Sale & Denton, 1985) that purified phosphofructokinase from white and brown adipose tissue can be phosphorylated by the catalytic subunit of cyclic AMP-dependent protein kinase and that the phosphorylation may be associated with an increase in enzyme activity. The increase in activity could be at least a partial explanation of the increase in glycolysis observed in rat epididymal white adipose tissue exposed to adrenaline (Katz *et al.*, 1966; Halperin & Denton, 1969; Saggerson & Greenbaum, 1970).

In the present paper we demonstrate that phosphofructokinase is phosphorylated in rat epididymal adipose tissue and that the β-agonist isoprenaline caused a marked increase in the extent of phosphorylation. The low concentration of phosphofructokinase in rat epididymal white adipose tissue makes this technically difficult. It can be calculated from the activity in extracts (equivalent to about 0.3 unit/g wet wt. of tissue), assuming a specific activity of 80 units/mg of protein for purified phosphofructokinase (Sale & Denton, 1985), that the concentration of phosphofructokinase in the tissue is about 3 μg/g wet wt. of tissue. This is much less than the concentration of the major phosphoproteins in the tissue, such as acetyl-CoA carboxylase, ATP citrate lyase and pyruvate dehydrogenase, which are present in the range 50–100 μg/g wet wt. of tissue. In the present study rat epididymal-adipose-tissue pieces have been incubated in medium containing [^{32}P]phosphate and then phosphofructokinase was rapidly isolated from tissue extracts by two different techniques. One technique was based on the Blue Dextran–Sepharose chromatography as described in the preceding paper (Sale & Denton, 1985); the other employed immunoprecipitation with antisera raised against phosphofructokinase purified from brown adipose tissue (Sale & Denton, 1985). We also demonstrate that exposure of rat epididymal adipose tissue to the β-agonist isoprenaline leads not only to an increase in phosphorylation but also to activation of the enzyme in a manner similar to that found on phosphorylation of the purified rat epididymal-adipose-tissue enzyme by the catalytic subunit of cyclic AMP-dependent protein kinase.

EXPERIMENTAL

Animals and materials

These were as given by Sale & Denton (1985), with the following additions. [^{32}P]Phosphate was from Amersham International, Amersham, Bucks, U.K. Insulin, adrenaline and phenylephrine were from Sigma (London) Chemical Co., Poole, Dorset, U.K., and isoprenaline was from Kodak, Kirkby, Liverpool, U.K.; the proteinase inhibitors pepstatin, antipain and leupeptin were obtained from Cambridge Research Biochemicals, Harston, Cambridgeshire, U.K. Protein phosphatase 1A purified from rabbit skeletal muscle by the method of Hemmings *et al.* (1983) was kindly given by Professor P. Cohen, Department of Biochemistry, University of Dundee. Antiserum raised against brown-adipose-tissue phosphofructokinase was prepared as described by Sale & Denton (1985).

Tissue incubations

Rat epididymal fat-pads (about 0.3 g) were each cut into three or four pieces and preincubated for 30 min at

37 °C in bicarbonate-buffered medium (Krebs & Henseleit, 1932) containing 1.25 mM-$CaCl_2$, 5 mM-glucose and 1 mM-potassium phosphate and gassed with O_2/CO_2 (19:1). For the study of the phosphorylation of phosphofructokinase and ATP citrate lyase, the pieces were transferred to fresh medium (about 5 ml/g wet wt.) of the same composition but containing [^{32}P]phosphate (initially 0.2 mM and about 300 d.p.m./pmol). The pieces were then incubated for 2 h before addition of hormones as indicated, and incubations were continued for a further 15 min. For the study of the effects of isoprenaline on the kinetic properties of phosphofructokinase, the pieces were transferred directly to fresh medium of the original composition, but also containing 2.7 μg of isoprenaline/ml, and incubations continued for 10 min.

Purification of phosphofructokinase and ATP citrate lyase from epididymal fat-pad pieces previously incubated in medium containing [^{32}P]phosphate, by using Blue Dextran–Sepharose

After incubation as above, fat-pad pieces (4–6 g wet wt.) were quickly blotted and extracted in 6–10 ml of phosphate-buffered medium (5 mM-potassium phosphate/5 mM-$MgSO_4$/1 mM-EDTA/0.5 mM-dithiothreitol, pH 7.1, containing pepstatin, leupeptin and antipain each at 1 μg/ml) by using a Polytron P10 homogenizer. Supernatant fractions (30000 *g* for 45 min) were prepared and subjected to chromatography on 2 ml columns of Blue Dextran–Sepharose as described by Sale & Denton (1985). Fractions containing phosphofructokinase activity were pooled, concentrated, assayed for phosphofructokinase activity and treated with 10% (v/v) trichloroacetic acid. Precipitated proteins were then separated by SDS/polyacrylamide-gel electrophoresis. Overall recovery of phosphofructokinase was approx. 30%.

Fractions enriched with ATP citrate lyase were also recovered from the Blue Dextran–Sepharose columns immediately after elution of phosphofructokinase, by addition of ATP (10 mM) plus citrate (50 mM) to the eluting buffer. The enzyme was eluted in about 10 ml under these conditions, together with some acetyl-CoA carboxylase. The fractions were concentrated and separated by SDS/polyacrylamide-gel electrophoresis as described for the fractions containing phosphofructokinase. Yield of ATP citrate lyase was about 40%. The enzyme represented about 25% of the protein in these final preparations, as judged by both Coomassie Blue staining of the proteins separated by SDS/polyacrylamide-gel electrophoresis and its specific activity, which was about 2.5 units/mg of protein. Pure ATP citrate lyase has specific activity of 5–10 units/mg of protein (Redshaw & Loten, 1981; Brownsey *et al.*, 1984).

Use of immunoprecipitation to separate phosphofructokinase from epididymal fat-pad pieces previously incubated in medium containing [^{32}P]phosphate

After incubation (as above), fat-pad pieces (about 0.7 g) were quickly blotted and extracted in about 1 ml of sucrose-based extraction medium (0.25 M-sucrose/20 mM-Tris/HCl/20 mM-KF/7.5 mM-GSH/5 mM-EDTA/5 mM-EGTA, pH 7.4, containing pepstatin, leupeptin and antipain each at 1 μg/ml). A high-speed-supernatant fraction was prepared by centrifugation at about 100000 *g* for 10 min in a Beckman Airfuge. Phosphofructokinase was immunoprecipitated from a 200 μl sample of this supernatant by incubation with 9 μl of antiserum raised against brown-adipose-tissue phosphofructokinase for 90 min at 0 °C, followed by centrifugation at about 100000 *g* for 10 min. The immunoprecipitate was washed by suspension in 200 μl of the sucrose-based extraction medium, and the centrifugation was repeated. Pellets were finally resuspended in 20 μl of the same medium, the activity of phosphofructokinase was determined and the remainder analysed by SDS/polyacrylamide-gel electrophoresis.

Assay of phosphofructokinase and ATP citrate lyase activities

Phosphofructokinase was assayed at 30 °C as given in Sale & Denton (1985). Unless otherwise stated, assays were carried out under conditions of maximal activity (i.e. in the presence of 5 mM-potassium phosphate, 5 mM-fructose 6-phosphate, 0.5 mM-ATP and 2 mM-AMP).

ATP citrate lyase was assayed at 30 °C as described by Martin & Denton (1970).

Other methods

Assays of protein, SDS/polyacrylamide-gel electrophoresis and radioautography were as given by Sale & Denton (1985). Incorporation of ^{32}P into phosphoproteins was determined by densitometric scanning of the radioautographs with a Joyce–Loebl Chromoscan 3 instrument. Traces were produced and analysed for calculation of peak heights and peak areas with a Hewlett–Packard 98455 computer, for which programs were written by Dr. Andrew Halestrap and Dr. Paul England of this laboratory.

RESULTS

Effects of hormones on the phosphorylation of phosphofructokinase in rat epididymal adipose tissue

Tissue pieces were incubated in medium containing 0.2 mM-[^{32}P]phosphate for 2 h to achieve steady-state labelling of intracellular phosphoproteins, and then hormones were added as appropriate and incubations continued for a further 15 min (Belsham *et al.*, 1982). Under these conditions, it is well established that exposure to either adrenaline or insulin results in marked changes in the phosphorylation of a number of proteins. In particular, both hormones cause a 2–4-fold increase in the phosphorylation of ATP citrate lyase (subunit M_r 130000), which is easily demonstrated as an increase in ^{32}P associated with a protein band of M_r 130000 separated from other ^{32}P-labelled proteins from whole-cell extracts by SDS/polyacrylamide-gel electrophoresis (Ramakrishna & Benjamin, 1979; Avruch *et al.*, 1982). No changes in ^{32}P associated with a protein band of M_r 78000, and thus possibly corresponding to phosphofructokinase, are evident in such studies. This is not surprising, because of the low concentration of the enzyme in white adipose tissue and the presence of other labelled phosphoproteins of similar subunit size, including triacylglycerol lipase (subunit M_r 84000; Nilsson *et al.*, 1980). Phosphofructokinase was therefore separated from tissue extracts either by chromatography on Blue Dextran–Sepharose (Fig. 1) or by immunoprecipitation

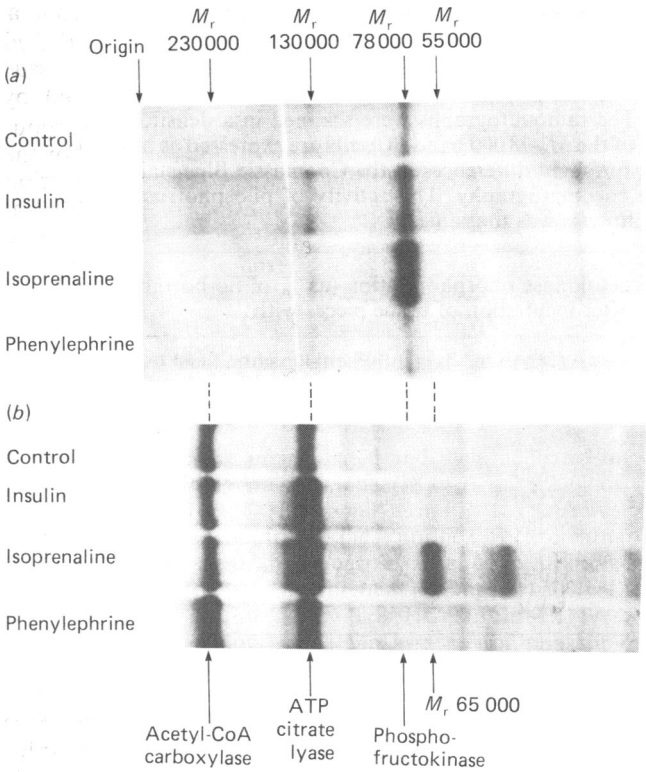

Fig. 1. Effects of insulin, isoprenaline and noradrenaline on ^{32}P incorporated into phosphofructokinase and ATP citrate lyase purified from rat epididymal adipose tissue by chromatography on Blue Dextran–Sepharose and SDS/polyacrylamide-gel electrophoresis

Fat-pad pieces (about 4 g in total) were preincubated at 37 °C for 30 min in bicarbonate-buffered medium and then incubated for 2 h in medium containing [^{32}P]phosphate (initially 0.2 mM and about 300 d.p.m./pmol). Additions were made as follows: no further additions (control), insulin (0.5 μg/ml), isoprenaline (2.7 μg/ml) and noradrenaline (1 μg/ml), and incubations continued for 15 min. Fractions enriched in (*a*) phosphofructokinase and (*b*) ATP citrate lyase were prepared by chromatography on Blue Dextran–Sepharose (see the Experimental section). Samples corresponding to about 250 munits of phosphofructokinase and 50 munits of ATP citrate lyase were treated with 10% trichloroacetic acid, and the precipitated protein was separated by SDS/polyacrylamide-gel electrophoresis on parallel tracks of approx. 8% polyacrylamide gels. The gels were stained with Coomassie Blue, dried and radioautographed for (*a*) 7 days or (*b*) 2 days.

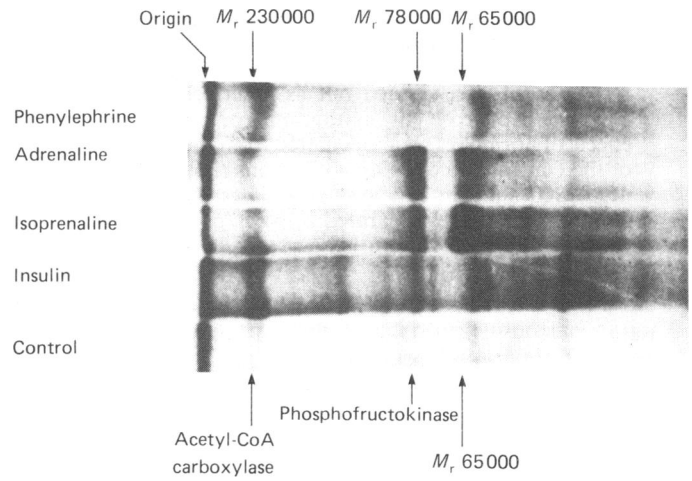

Fig. 2. Effects of hormones on ^{32}P incorporation into phosphofructokinase isolated from rat epididymal adipose tissue by immunoprecipitation and SDS/polyacrylamide-gel electrophoresis

Fat-pad pieces (total weight about 0.7 g) were preincubated and incubated as in Fig. 1. The concentration of adrenaline was 2.2 μg/ml. Immunoprecipitation was carried out as described in the Experimental section, and antibody pellets were analysed in parallel tracks of 8% -polyacrylamide gels. The gels were then stained with Coomassie Blue, dried and radioautographed.

(Fig. 2), followed in each case by separation by SDS/polyacrylamide-gel electrophoresis.

The fraction containing phosphofructokinase eluted from Blue Dextran–Sepharose was found to contain one major labelled protein which co-migrated with phosphofructokinase (subunit M_r 78000) on SDS/polyacrylamide-gel electrophoresis (Fig. 1*a*). There was also evidence of a second, minor, labelled protein with a subunit M_r of 130000, which probably represented a small amount of ATP citrate lyase in the fraction. Most of the ATP citrate lyase was eluted subsequently with buffer containing ATP plus citrate. This fraction when analysed by SDS/polyacrylamide-gel electrophoresis was found to contain a number of labelled phosphoproteins in addition to ATP citrate lyase (subunit M_r 130000) (Fig. 1*b*). These included acetyl-CoA carboxylase (M_r 230000), since this phosphoprotein was precipitated by antisera raised against the purified enzyme (Brownsey *et al.*, 1977) and an unidentified protein of M_r 65000. Recovery of ATP citrate lyase activity in this fraction was close to 40% and was apparently unaffected by the previous treatments with hormones. In contrast, the recovery of acetyl-CoA carboxylase was less than 25% and variable. Incorporation of ^{32}P into ATP citrate lyase was increased about 4-fold after exposure of the tissue to either insulin or isoprenaline and about 2-fold after exposure to phenylephrine. There were no appreciable differences in the Coomassie Blue staining of the protein bands corresponding to ATP citrate lyase (Fig. 1*b*). Additionally, the phosphoprotein of M_r 65000 exhibited very large increases in phosphorylation after exposure of the tissue to isoprenaline, but not to insulin, and probably corresponds to a protein of M_r 65000 which has been found by many laboratories to exhibit increased phosphorylation after exposure of fat-cells to adrenaline and other β-agonists (Avruch *et al.*, 1982; Brownsey *et al.*, 1979; Benjamin & Singer, 1975). The elution of this protein by buffer containing ATP suggests that it might also be a kinase. These above changes in phosphorylation served as an excellent check that the tissue responded to the added hormones as expected and that a phosphoprotein can be purified by this chromatographic procedure without major changes in its phosphorylation.

Hormone treatment had no apparent effect on the maximum activity of phosphofructokinase. Overall recovery of phosphofructokinase activity through the Blue Dextran–Sepharose purification procedure was determined in each experiment and was close to 30% in each case. Purification was in excess of 1000-fold. The

Table 1. Summary of the effect of hormones on the steady-state incorporation of ^{32}P from phosphate in the medium into phosphofructokinase of rat epididymal fat-pad pieces

Experiments were carried out as described in Fig. 1 or Fig. 2 in which phosphofructokinase was isolated either by Blue Dextran–Sepharose chromatography or by immunoprecipitation. The radioautographs were scanned in a densitometer, and effects of hormones were calculated from the changes in peak height of the M_r-78000 band. Results are expressed as a percentage of the control value. Small corrections (less than 15%) were made for slight differences in the Coomassie Blue staining of the M_r-78000 band in fractions purified by Blue Dextran–Sepharose chromatography. The activity of phosphofructokinase in immunoprecipitates separated by SDS/polyacrylamide-gel electrophoresis was the same.

Expt. no.	Procedure	Change in phosphofructokinase phosphorylation (as % of no-hormone control) after incubation of tissue pieces with: Isoprenaline	Adrenaline	Phenylephrine	Insulin
1	Blue Dextran	330	–	110	150
2	Blue Dextran	690	–	–	120
3	Immunoprecipitation	680	620	140	400
4	Immunoprecipitation	510	520	100	190
5	Immunoprecipitation	370	380	160	190
	Overall mean (±S.E.M.) for no. of observations given in parentheses:	520±75 (5)	510±70 (3)	130±15 (4)	210±50 (5)

purified phosphofructokinase was separated by SDS/polyacrylamide-gel electrophoresis and the Coomassie Blue stain associated with the M_r-78000 protein was measured by densitometric scanning. Differences in loading were less than 15%. As shown by the radioautograph depicted in Fig. 1(*a*), exposure of the tissue to isoprenaline markedly increased the phosphorylation of phosphofructokinase. Insulin treatment also resulted in an increase in phosphorylation, but to a lesser extent, whereas phenylephrine had no appreciable effect. Similar results with isoprenaline and insulin were observed in a separate experiment carried out in the same way. Values for the increased ^{32}P associated with phosphofructokinase in response to hormones are summarized in Table 1.

Separation of phosphofructokinase by immunoprecipitation was found to have a number of advantages over the use of chromatography. It was simpler, quicker, required less tissue, and the overall recovery of phosphofructokinase in the immunoprecipitates was greater than 70%. There was also less possibility of dephosphorylation, because EDTA and NaF could be present throughout the procedure. This approach gave very similar results to those obtained with the separation of phosphofructokinase by chromatography (Fig. 2 and Table 1).

Combining the results of the two approaches, the mean increase in phosphorylation of phosphofructokinase, expressed as a percentage of control values, after exposure to isoprenaline or adrenaline was about 400%, to insulin about 100% and to phenylephrine less than 40% (Table 1).

Estimates of the extent of phosphorylation of phosphofructokinase in fat-cells as mol of ^{32}P incorporated/mol of enzyme tetramer were also made. Incorporation of ^{32}P into phosphofructokinase was determined by cutting out the appropriate piece of gel and determining the radioactivity by scintillation counting after digestion of the gel with H_2O_2 (see the Experimental section). The amount of phosphofructokinase was calculated from the activity of phosphofructokinase in the immunoprecipitates by assuming a specific activity of 90 units/mg of protein (Sale & Denton, 1985), or alternatively from the Coomassie Blue staining of the protein band when the enzyme was purified by Blue Dextran–Sepharose chromatography. The initial specific radioactivity of phosphate in the medium was also measured. Separate studies in this laboratory (T. J. Hopkirk & R. M. Denton, unpublished work) have shown that under the conditions of the present study the specific radioactivity of the γ-phosphate of ATP reaches a steady-state value of 30% of that of phosphate in the initial medium. This value is not altered by more than 3% by the presence of insulin, adrenaline, isoprenaline or phenylephrine. A similar value has been found in isolated cells by Lawrence & James (1984). On this basis, the calculated extent of phosphorylation of phosphofructokinase in tissue incubated in the absence of hormones is about 0.2 mol of phosphate/mol of enzyme tetramer, rising on exposure to adrenaline or isoprenaline to about 1.4 mol of phosphate/mol of enzyme tetramer. Insulin treatment of fat-pads resulted in an increase in the phosphorylation of phosphofructokinase, to about 0.4 mol of phosphate/mol of enzyme tetramer. It should be noted that these values are estimates involving a number of assumptions, in particular with regard to the amount of phosphofructokinase protein. However, the extent of phosphorylation of phosphofructokinase in fat-pad pieces exposed to adrenaline or isoprenaline appear to be broadly similar to that achieved on phosphorylation of the purified white-adipose-tissue enzyme by cyclic AMP-dependent protein kinase, as calculated by using a similar estimate of enzyme protein (Sale & Denton, 1985).

Effects of exposure of epididymal fat-pad pieces to isoprenaline on the activity of phosphofructokinase

As mentioned above, no appreciable changes were found in maximum activity of phosphofructokinase after exposure of tissue to hormones when activity of the enzyme was assayed either in crude tissue extracts or after purification on Blue Dextran–Sepharose. This is in contrast with the small decreases in maximum activity

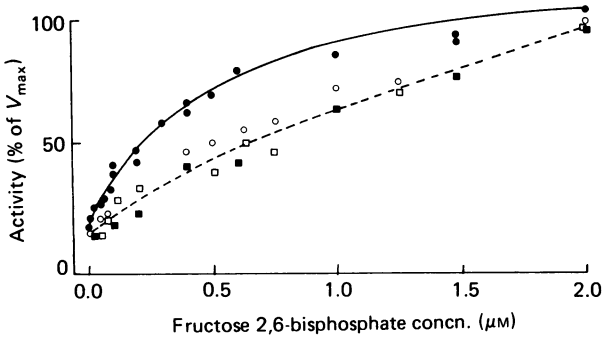

Fig. 3. Effect of incubation of rat epididymal-fat-pad pieces with isoprenaline on the sensitivity of phosphofructokinase to activation by fructose 2,6-bisphosphate

Fat-pad pieces were preincubated and then incubated in medium containing no hormone (■, □) or 2.7 μg of isoprenaline/ml (●, ○) for 15 min as in Fig. 1. Tissue was extracted and phosphofructokinase purified by chromatography on Blue Dextran–Sepharose. After concentration, the enzyme was dialysed overnight at 4 °C against 500 vol. of Tris buffer, pH 7.0 (50 mM-Tris/HCl/0.1 mM-EDTA/2 mM-mercaptoethanol) and then assayed for phosphofructokinase activity before (■, ●) and after incubation with protein phosphatase 1A (30 munits/ml) for 10 min at 3 °C (□, ○). The concentration of phosphofructokinase during this treatment was 150 munits/ml. Phosphofructokinase assays were carried out at pH 7.1 with 0.2 mM-fructose 6-phosphate, 1 mM-potassium phosphate, 1 mM-ATP and various concentrations of fructose 2,6-bisphosphate.

found in extracts of fat-cells previously exposed to adrenaline (Sooranna & Saggerson, 1982; Lederer & Hers, 1984). We can offer no entirely satisfactory explanation for this difference. However, the assay of phosphofructokinase in whole-cell extracts has a number of problems which make it very difficult to carry out precise kinetic studies in crude extracts of adipose tissue. We therefore investigated the effects of isoprenaline treatment on the kinetic properties of the enzyme purified by chromatography on Blue Dextran–Sepharose.

It was found that the enzyme from tissue exposed to the hormone exhibited increased sensitivity to activation by fructose 2,6-bisphosphate. In the experiment depicted in Fig. 3, the calculated K_a was decreased by 70%, from 0.81 ± 0.21 μM to 0.25 ± 0.02 μM. Similar results were obtained in two other separate experiments in which decreases of 69 and 43% in the apparent K_a values were obtained. These changes in kinetic properties are in line with those found after phosphorylation by the catalytic subunit of cyclic AMP-dependent protein kinase of phosphofructokinase purified from epididymal white adipose tissue incubated in the absence of hormones (Sale & Denton, 1985). Furthermore, when the purified preparations of phosphofructokinase from control and isoprenaline-treated tissue were incubated with protein phosphatase 1A, the difference in apparent K_a for fructose 2,6-bisphosphate was no longer apparent (Fig. 3). Both enzymes gave K_a values similar to that observed with the untreated enzyme from control tissue. Phosphofructokinase from control and isoprenaline-treated tissue exhibited little difference in their sensitivity to inhibition by ATP (results not shown).

DISCUSSION

Previous studies with purified fat-tissue phosphofructokinase indicated that the enzyme was phosphorylated by the catalytic subunit of cyclic AMP-dependent protein kinase, and that phosphorylation was associated with an increase in enzyme activity, in particular with a decrease in the apparent K_m for fructose 2,6-bisphosphate (Sale & Denton, 1985). The present studies show that this can occur within intact tissues. Not only has it been demonstrated that both adrenaline and isoprenaline (apparently acting through β-receptors) increase phosphorylation of phosphofructokinase, but also it has been found that this increase in phosphorylation in tissue exposed to isoprenaline is associated with a decrease in the apparent K_a for fructose 2,6-bisphosphate. This would appear to offer at least a partial explanation for the increase in glycolysis observed in fat tissue incubated with adrenaline (Katz *et al.*, 1966; Halperin & Denton, 1969; Saggerson & Greenbaum, 1970). It may be of more general significance, since, in most tissues other than the liver, increases in cyclic AMP are associated with increases in glycolysis. However, it should be emphasized that this change in apparent sensitivity may not be the most important effect of phosphorylation. It is quite possible that sensitivity to other effectors or combination of effectors may be altered, including sensitivity to inhibition by fatty acids (Lederer & Hers, 1984) or the recently described protein regulator (Kellett & Robertson, 1984). It should be noted that the kinetic properties of phosphofructokinase may be critically dependent on the concentration of the enzyme (Bosca *et al.*, 1985).

These studies have also shown for the first time that insulin can result in an increase in phosphorylation of the enzyme. The increase was smaller than that observed with the β-adrenergic agonist, but may nevertheless be of interest. It has subsequently been found that phosphofructokinase from skeletal muscle is phosphorylated by purified insulin receptor; the rate of phosphorylation is stimulated by insulin and occurs almost exclusively on tyrosine residues (E. M. Sale, M. White & C. R. Kahn, unpublished work).

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