Comparison of the effects of insulin and adrenergic agonists on the phosphorylation of an acid-soluble 22 kDa protein in rat epididymal fat-pads and isolated fat-cells

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1. Earlier studies have shown that exposure of fat-cells to insulin results in the rapid increased phosphorylation of an acid-soluble 22 kDa protein and that increases in phosphorylation were also evident in cells exposed to adrenaline [Belsham & Denton (1980) *Biochem. Soc. Trans.* **8**, 382–383; Belsham, Brownsey, Hughes & Denton (1980) *Diabetologia* **18**, 307–312]. 2. The effects of adrenaline are shown to be brought about through β -adrenergic receptors and to be mimicked by other agents which increase cell cyclic AMP concentrations. The maximum extent of phosphorylation is about 60 % of that observed with insulin. Increased phosphorylation is also observed in fat-cells exposed to vasopressin, oxytocin and phorbol esters, but not to α -adrenergic agonists. 3. No changes in the phosphorylation of the protein are evident in epididymal fat-pads from fat-fed, starved or starved/refed animals, despite the large changes in protein composition of fat-cells which accompany these nutritional alterations. This suggests that the protein is not closely involved in lipogenesis or associated metabolic pathways, but rather that it may play a more general regulatory role. 4. The 22 kDa protein migrates as a doublet on SDS/PAGE even after purification to apparent homogeneity by sequential use of Mono Q chromatography, SDS/PAGE and h.p.l.c. The amino acid compositions of the two components are very similar and share features in common with a number of proteins, including inhibitor-1, inhibitor-2, dopamine- and cyclic-AMP-regulated phosphoprotein (DARPP-32), and G-substrate, which may be involved in the regulation of protein phosphatase activity. 5. Phosphopeptide mapping and phosphoamino acid analysis reveals that insulin increases the phosphorylation of two distinct peptides within the protein (in one peptide insulin increases the amount of phosphothreonine, whereas in the other the hormone increases the amounts of phosphothreonine and phosphoserine). Both components of the doublet exhibit similar changes in phosphorylation, and hence the differences in migration are not the result of differences in phosphorylation, as suggested previously [Blackshear, Nemenoff & Avruch (1983) *Biochem. J.* **214**, 11–19]. The pattern of phosphorylation observed with the β -adrenergic agonist isoprenaline was similar to that observed with insulin. 6. The possible role and regulation of the 22 kDa protein are discussed.

INTRODUCTION

The phosphorylation of a number of fat-cell proteins on serine/threonine residues is increased within a few minutes of exposure of the cells to insulin (for review, see Denton, 1986). Amongst these proteins is a 22 kDa protein which migrates as a doublet on SDS/PAGE (Belsham & Denton, 1980; Belsham *et al.*, 1980, 1982; Blackshear *et al.*, 1982). The role of this protein has not been established, but it has a number of properties which suggests that it may be important in cell regulation. The protein is heat- and acid-soluble and appears to be poorly stained by Coomassie Blue. Together with its apparent molecular mass on SDS/PAGE, these are properties shared in varying degrees by a number of important regulatory proteins in cells, including calmodulin, myosin light chains, G-substrate, phosphoprotein phosphatase inhibitors 1 and 2, DARPP-32 and members of low-molecular-mass guanine nucleotide-binding proteins (Cheung, 1970; Huang & Glinemann, 1976; Nimmo & Cohen, 1978; Schlichter *et al.*, 1980; Aswad & Greengard, 1981a; Blackshear *et al.*, 1982; Hemmings *et al.*, 1984; Kamata *et al.*, 1987). Further studies have indicated that the 22 kDa protein may be present in muscle, liver and various cultured cell lines (Vargas *et al.*, 1982; Blackshear *et al.*, 1983, 1984; Blackshear, 1986; Witters *et al.*, 1988) and that its phosphorylation may also be increased by exposure of appropriate cells to epidermal growth factor, platelet-derived growth factor and phorbol esters (Blackshear *et al.*, 1983, 1985). Most recently we have shown not only that the 22 kDa protein from fat-cells may be a substrate for casein

kinase 2, but also that insulin increases the activity of this protein kinase in fat-cells (Diggle *et al.*, 1991). The observed increased phosphorylation of the 22 kDa protein in fat-cells exposed to insulin may therefore be brought about, at least in part, through the activation of casein kinase 2 (Diggle *et al.*, 1991).

A number of related issues concerning the 22 kDa protein are tackled in the present paper. These are: (i) the extent to which adrenergic agents and other hormones can influence the level of phosphorylation of the protein in fat-cells [this is a matter of controversy, since Belsham & Denton (1980) and Belsham *et al.* (1980, 1982) reported that adrenaline can elicit the increased phosphorylation of the 22 kDa protein, but Blackshear *et al.* (1982, 1983) failed to observe this effect]; (ii) the extent to which the phosphorylation of the protein is altered by prior alterations in the nutritional status of the animal that cause substantial changes in the protein composition of fat-cells, especially of enzymes and other proteins closely involved in the main metabolic pathways; and (iii) a detailed study of the relationship of the components of the doublet separated by SDS/PAGE by amino acid analysis of the purified protein, two-dimensional electrophoresis, phosphopeptide mapping and phosphoamino acid composition. Since it is clearly shown in the present paper that β -adrenergic agonists [and other hormones which increase cell cyclic AMP (cAMP) concentrations] increase the phosphorylation of the 22 kDa protein, but to a lesser extent than that observed with insulin, the patterns of phosphorylation of the protein obtained in fat-pads exposed to insulin and the β -adrenergic agonist isoprenaline are compared.

Abbreviations used: TCA, trichloroacetic acid; cAMP, cyclic AMP.

Fig. 1. Effects of insulin, adrenergic agonists and antagonists, and okadaic acid on the phosphorylation of the 22 kDa protein bands in rat epididymal fat-cells

Fat-cells were incubated in medium containing [32 P]P_i, followed by treatment with hormone, antagonists and okadaic acid, preparation of 1.5–15% TCA fraction, SDS/PAGE and radioautography as described in the Experimental section. (a) Effects of 10 min incubation with insulin (83 nM), isoprenaline (9 μ M) or adrenaline (5 μ M), with or without 10 min preincubation with propranolol (2 μ M) or prazosin (2 μ M). (b) Effects of 10 min incubation with insulin or isoprenaline after 15 min preincubation with or without okadaic acid (1 μ M).

EXPERIMENTAL

Materials

Usually, male Wistar rats (160–200 g) were fed *ad libitum* up to the time of killing on a stock laboratory diet (CRM; Bioshore, Lavender Mill, Manea, Cambs., U.K.). In some experiments, animals were fed on a high-fat diet consisting of 28.2% (w/w) casein and 71.8% (w/w) fat, in the form of peanut oil and lard at a ratio of 0.43:1. The amount of carbohydrate present was less than 0.1% (w/w). Kodak X-Omat S film for radioautography was obtained from Eastman Kodak (Kirby, Liverpool, U.K.). All chemicals and biochemicals were from Sigma Chemical Co. or BDH (both of Poole, Dorset, U.K.), except for the proteinase inhibitors pepstatin A, antipain and leupeptin (Cambridge Research Biochemicals, Harston, Cambridge, U.K.), okadaic acid (Fujisawa Chemical Co., Tokyo, Japan), collagenase (Worthington Diagnostic Systems, Freehold, NJ, U.S.A.) and [γ - 32 P]ATP which was from Amersham International (Amersham, Bucks., U.K.).

General techniques

SDS/PAGE was performed by the method of Laemmli (1970) adapted to a Bio-Rad Protean II mini-gel system using 15% (w/v) polyacrylamide gels. Radioautography was performed at

–80 °C using pre-flashed film in cassettes with intensifying screens (Hughes *et al.*, 1980). Densitometry was performed with a Joyce-Loebl Chromoscan 3 linked to a Hewlett-Packard 9000/300 computer (Brownsey *et al.*, 1979).

Incubation of rat epididymal fat-pads and cells

Fat-cells were prepared from epididymal fat-pads and then preincubated in a bicarbonate-buffered medium, pH 7.4 (Krebs & Henseleit, 1932) containing BSA (10 mg/ml), glucose (11 mM), Hepes (10 mM) and [32 P]P_i (initially 0.4 mM and 1000 c.p.m./pmol), gassed with CO₂/O₂ (1:19) as described by Diggle *et al.* (1991). Cells were incubated in the same medium with further additions as indicated, at approx. 15–20% (vol. of packed cells/vol. of buffer). After incubation, cells were quickly washed in 2 ml of the same buffer minus BSA and further additions, and extracted by vortex-mixing in a glass tube (Martin & Denton, 1970) in 1 ml of ice-cold 10 mM-Tris, pH 7.4, containing 20 mM-Mops, 250 mM-sucrose, 2 mM-EGTA, 1 mM-GSH, the proteinase inhibitors pepstatin, antipain and leupeptin (1 μ g/ml) and benzamidine (2 mM), and the phosphatase inhibitors NaF (10 mM) and Na₂P₂O₇ (20 mM). Before use, this extract was centrifuged initially at 3000 *g* for 90 s and then twice at 10000 *g* for 5 min.

When used intact, epididymal fat-pads were incubated as described for the isolated cells, except that albumin was omitted and the specific radioactivity of [32 P]P_i was 500 c.p.m./pmol. Pads were extracted by homogenizing two pads (Polytron PT10) for 10 s in 2 ml of ice-cold extraction buffer (Borthwick *et al.*, 1990).

All incubations were at 37 °C, and centrifugations were carried out at 4 °C.

Preparation of a 1.5–15% (w/v)-TCA fraction containing the 22 kDa protein

Acid-soluble fraction containing the 22 kDa protein was prepared from the fat-free supernatant fractions of extract prepared from epididymal fat-pads. TCA was added to a final concentration of 1.5% (w/v) and the samples were incubated overnight at 4 °C, followed by centrifugation at 10000 *g* for 5 min to remove precipitated proteins. Further TCA was added to a concentration of 15%, and the samples were stored on ice for 1–2 h, followed by centrifugation (10000 *g* for 5 min). The pellet containing the 22 kDa protein was washed in 400 μ l of diethyl ether to remove residual triacylglycerol and phospholipids and then solubilized in 62.5 mM-Tris buffer, pH 6.8, containing 20% (w/v) sucrose, 10% (w/v) SDS and 5% (v/v) β -mercaptoethanol before boiling and separation by SDS/PAGE, unless otherwise indicated.

Large-scale preparation of 22 kDa protein for amino acid analysis

Epididymal fat-pads (from 120 rats) were incubated in the presence of insulin but no added [32 P]P_i and extracted as described above. The 22 kDa protein in the extracts was partially purified by Mono Q chromatography performed as described by Borthwick *et al.* (1990). In this system the 22 kDa phosphoprotein is eluted at approx. 80 mM-NaCl. Extract prepared from 20 fat-pads incubated under the same conditions except for the addition of [32 P]P_i (0.4 mM and 500 c.p.m./pmol) was added to these fractions, and a 1.5–15% TCA fraction was prepared and separated on a series of SDS/polyacrylamide gels. The 22 kDa protein was revealed by radioautography and eluted from appropriate slices of gel by incubation in h.p.l.c.-grade water containing 1 mM-DTT. The eluted sample was concentrated to 0.5 ml by centrifugation using a Centriprep-10 and further decreased to about 20 μ l by using a Savant Speedivac before

Table 1. Phosphorylation of a 22 kDa protein in rat epididymal fat-cells

Conditions are given in the legend to Fig. 1. Cells were incubated with all hormones and phorbol 12-myristate 13-acetate (PMA) for 10 min. Where appropriate, adrenergic antagonists were introduced 10 min before addition of adrenergic agonists. Incorporation of ^{32}P into the 22 kDa protein doublet was determined by scanning densitometry (625 nm) of radioautographs. Results are given as either means $\pm$ S.E.M. for the numbers of observations on separate preparations of cells given in parentheses, or as individual observations where less than three have been made. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ versus no-treatment control.

Treatment	^{32}P incorporation (% of no-treatment control)
Insulin (83 nM)	464 $\pm$ 61 (16)***
Isoprenaline (9 μM)	269 $\pm$ 31 (13)***
Isoprenaline (9 μM) + propranolol (2 μM)	121 $\pm$ 13 (4)
Adrenaline (5 μM)	258 $\pm$ 32 (9)**
Adrenaline (5 μM) + propranolol (2 μM)	90 $\pm$ 10 (3)
Adrenaline (5 μM) + prazosin (2 μM)	164, 212
Noradrenaline (5 μM)	187, 217
Noradrenaline (5 μM) + propranolol (2 μM)	81, 123
Propranolol (2 μM)	90 $\pm$ 10 (6)
Prazosin (2 μM)	98 $\pm$ 3 (4)
Glucagon (1 nM)	284 $\pm$ 48 (4)*
8-(4-Chlorophenylthio)cAMP (1 μM)	178, 188
Phenylephrine (20 μM)	95, 105
PMA (0.1 μM)	375, 453
PMA (20 nM)	159, 205
Vasopressin (200 nM)	233 $\pm$ 24 (3)*
Oxytocin (200 nM)	150, 162
Oxytocin (1 μM)	409

separation by h.p.l.c. (Zorbax Protein Plus; 4.6 mm $\times$ 25 cm). Recovery of radiolabelled 22 kDa protein from the gel pieces was in excess of 90 %.

Tryptic digestion and two-dimensional separation of tryptic peptides

Samples for tryptic digestion were incubated in 100 mM-*N*-ethylmorpholine, pH 8.2, containing 10 μg of trypsin (treated with tosylphenylalanylchloromethane) for 16 h at 30 °C, after which time another 10 μg of trypsin was added for a further 4 h at 30 °C. The reaction was terminated and the sample separated in two dimensions as described by Tavaré & Denton (1988). Dinitrophenyl-lysine (1 μg) was included as an internal standard.

Phosphoamino acid determinations

Samples were hydrolysed with 6 M-HCl, separated in one dimension by electrophoresis at pH 1.9 (formic acid/acetic acid/water, 25:78:897, by vol.) for 1.5 h at 400 V and revealed by radioautography. Non-radioactive phosphoserine, phosphothreonine and phosphotyrosine (1 μg of each) were included with each sample as carrier and standards.

Amino acid analysis

Samples for amino acid analysis were hydrolysed in 300 μl of 6 M-HCl (constant boiling) containing phenol under argon for 1.5 h at 150 °C. The sample was then freeze-dried and washed and re-freeze-dried with 20 μl of methanol/triethanolamine/water (2:2:1, by vol.) before derivative formation for 20 min at room temperature with 20 μl of methanol/triethanolamine/phenyl isothiocyanate/water (39:5:1:5, by vol.). Tubes were evaporated to dryness before resuspension in 5 mM-phosphate buffer, pH 7.4, containing 5 % (v/v) acetonitrile. Amino acid

phenylthiohydantoins were separated by reversed-phase h.p.l.c. by a modification of the method of Bidlingmeyer *et al.* (1984) on a Waters system with two model 510 pumps.

RESULTS

Effects of insulin, adrenergic agonists and other agents on the phosphorylation of the 22 kDa protein in fat-cells from normal fed rats

In agreement with earlier studies, insulin was found to increase markedly the phosphorylation of an acid-soluble protein of about 22 kDa in both fat-cells and intact fat-pads (Belsham & Denton, 1980; Belsham *et al.*, 1980; Blackshear *et al.*, 1982) (Fig. 1, Table 1). Under the conditions used in the present studies, it was the main radiolabelled phosphoprotein in the 1.5–15 %-TCA fraction and usually ran on SDS/PAGE as a doublet. Adrenaline also increased the phosphorylation of the protein, but the maximum extent of the effect of this hormone was about 60 % of that of insulin (Fig. 1a, Table 1); this is in close agreement with Belsham *et al.* (1980), but is in direct contrast with Blackshear *et al.* (1982).

The effect of adrenaline on fat-cells appeared to be exerted solely through β -receptors, as the β -agonist isoprenaline had a similar effect, whereas the α_1 -agonist phenylephrine had none, and the effects of adrenaline and isoprenaline were blocked by propranolol (β -antagonist), but only marginally inhibited by prazosin (α_1 -antagonist) (Fig. 1a, Table 1). In support of this view, 8-(4-chlorophenylthio)cAMP and glucagon also caused similar increases in the phosphorylation of the protein (Table 1). The half-maximal effect with isoprenaline was observed at about 10 nM (results not shown). Isoprenaline was also found to increase the phosphorylation of the protein within intact fat-pads. For example, exposure of pads to 9 μM -isoprenaline for 10 min resulted in an increase to 159 $\pm$ 14 % (7 observations); again this was less than that observed with insulin in parallel studies (248 $\pm$ 22 %; 13 observations).

As is evident in Fig. 1(a), the 22 kDa phosphoprotein usually runs on SDS/PAGE as a poorly resolved doublet. The predominant effect of insulin was to increase the phosphorylation of the upper component, whereas the β -adrenergic agonists appeared to increase the phosphorylation of both components to a more similar extent. In about half the experiments (e.g. Fig. 1b) the doublet was not resolved.

The phosphoprotein phosphatase inhibitor okadaic acid was found also to cause a marked increase in phosphorylation of the 22 kDa protein (Fig. 1b). At a concentration that results in maximal effects on many fat-cell processes (Haystead *et al.*, 1989; Rutter *et al.*, 1991), the increase in phosphorylation approached twice that observed with insulin and obliterated the effects of both insulin and isoprenaline. These observations suggest that the 22 kDa protein may be a substrate for phosphoprotein phosphatases 1 and/or 2A.

Blackshear *et al.* (1983, 1985) have reported increases in the phosphorylation of the 22 kDa protein in 3T3-L1 adipocytes with a number of growth factors (epidermal growth factor, fibroblast growth factor, platelet-derived growth factor) and phorbol esters. In agreement with these observations, we have found that phorbol 12-myristate 13-acetate caused a similar increase in the phosphorylation of the 22 kDa protein in fat-cells to that observed with insulin (Table 1). Moreover, the effects of phorbol 12-myristate 13-acetate could be mimicked by vasopressin and oxytocin. Both these hormones appear to act in fat-cells via receptors linked to phospholipase C, and hence are likely to lead to activation of protein kinase C (Augert & Exton, 1988).

Fig. 2. Effects of feeding a high-fat diet on the phosphorylation of the 22 kDa protein in rat epididymal fat-pads

Rat epididymal fat-pads were incubated in medium containing [32 P] P_i , then incubated with or without insulin (83 nM), followed by extraction, preparation of 1.5–15% TCA fraction, SDS/PAGE, radioautography and densitometric scanning as described in the Experimental section. (a, b) Densitometric scans of radioautographs: (a) normal diet; (b) high-fat diet; pads incubated in the absence (—) or presence (·····) of insulin. (c, d) Densitometric scans of Coomassie-Blue-stained gels: (c) pads incubated in the absence of hormone and (d) pads incubated in the presence of hormone from animals fed on a normal (—) or a high-fat diet (·····). Abbreviations: FAS, fatty acid synthase; ACL, ATP citrate-lyase.

Effects of dietary manipulation on the phosphorylation of the 22 kDa protein

Many enzymes and other proteins in the metabolism of fat-cells exhibit substantial changes in concentration with changing nutritional status. For example, many enzymes involved in fatty acid synthesis are largely repressed in the fat-cells of fasting animals or of animals fed on a high fat-diet (Saggerson & Greenbaum, 1970; Stansbie *et al.*, 1976). However, we have not detected any changes in the extent of phosphorylation of the 22 kDa protein under such conditions. For example, feeding rats on a high-fat diet for 18 days had no discernible effect on the incorporation of [32 P] P_i into the protein when intact epididymal fat-pads were incubated in either the absence or the presence of a saturating concentration of insulin (Figs. 2a and 2b). In contrast, the amounts of fatty acid synthase (250 kDa) and ATP citrate-lyase (130 kDa) were greatly diminished (Figs 2c and 2d).

Similar results were obtained when the phosphorylation of the 22 kDa protein was compared in the pads of animals fasted for 2 days with that in the pads of animals fasted for 2 days and then fed on the stock laboratory diet for 2 days. The effect of insulin on the phosphorylation of the 22 kDa protein was 3.03-, 3.1- and 2.6-fold from fed, fasted and fasted-refed animals respectively. On the other hand, fasting resulted in the expected decreases in the amounts of fatty acid synthase and ATP citrate-lyase and refeeding to a reversal of these changes (results not shown).

It is possible that changes in the total amount of the 22 kDa protein may occur with these dietary changes. The protein appears to be very poorly immunogenic, and we have not been able to raise any polyclonal or monoclonal antisera to allow direct quantification. However, if major changes in amounts of

the 22 kDa protein occurred, it would be expected that corresponding changes in phosphorylation would be observed.

Purification and amino acid composition of the 22 kDa protein from epididymal fat-pads

The 22 kDa phosphoprotein was purified to apparent homogeneity by the sequential use of Mono Q chromatography, SDS/PAGE and h.p.l.c. chromatography. Fig. 3 shows the single peak of 32 P-labelled protein corresponding to the 22 kDa protein eluted at 40–45% (v/v) acetonitrile. When this peak was re-run on SDS/PAGE, essentially all the protein migrated as a radiolabelled doublet of molecular mass close to 22 kDa (see Fig. 3 insert).

Table 2 gives the amino acid composition of the protein eluted from h.p.l.c., and also of the upper and lower bands after further separation on SDS/PAGE. The compositions are very similar. The main features were the high contents of proline, glycine and acidic residues, and low amounts of tyrosine, methionine and phenylalanine and complete absence of cysteine.

An estimate can be made of the stoichiometry of phosphorylation in the purified 22 kDa protein from fat-pads incubated in the presence of insulin, from the molar amounts of protein calculated from the amino acid analysis, assuming a molecular mass of 22 kDa, and the 32 P incorporation assuming that the specific radioactivity of cell ATP at the end of the incubation was 50% of that of the initial medium P_i (Hopkirk & Denton, 1986). The mean value ($\pm$ S.E.M.) obtained in 11 separate experiments was 1.0 ± 0.2 mol/mol. The amount of 22 kDa protein purified from each rat epididymal fat-pad was $0.18 \pm$

Fig. 3. Purification of the 22 kDa protein bands by reverse-phase h.p.l.c.

The 22 kDa phosphoprotein was prepared from the fat-pads of 130 rats incubated in the presence of insulin by a combination of Mono Q chromatography and SDS/PAGE (see the Experimental section), subjected to chromatography on a reverse-phase h.p.l.c. column (Zorbax Protein Plus) equilibrated in 0.1 % (v/v) trifluoroacetic acid and developed with a gradient of 0–60 % (v/v) acetonitrile from 5 to 45 min, A_{212} (·····); — ^{32}P). Total radioactivity loaded was 99 700 c.p.m., with a recovery of over 50 % in the peak eluted at 40–45 % acetonitrile. The insert shows the Coomassie-Blue-stained 22 kDa protein purified by h.p.l.c. and re-separated by SDS/PAGE. Results are typical of at least three separate experiments.

0.03 μg (mean $\pm$ S.E.M. for 11 observations), which corresponds to a minimum concentration of about 2 μM in the cytoplasm of fat-cells, assuming that the volume of cytoplasm is about 20 $\mu\text{l/g}$ wet wt. of tissue (Denton *et al.*, 1966).

Characterization of the sites phosphorylated within the 22 kDa protein by tryptic-peptide mapping and phosphoamino acid analysis

Fig. 4 (upper panels) shows typical phosphopeptide maps derived from the 22 kDa protein doublet separated by one-dimensional SDS/PAGE from fat-cells incubated in the absence of hormones and after incubation in the presence of insulin or isoprenaline. Two major [^{32}P]phosphopeptides were denoted peptide (a) and peptide (b). In four separate experiments performed as in Fig. 4, both insulin and isoprenaline increased the incorporation of ^{32}P into both peptides. Phosphoamino acid analysis was carried out on the separated peptides (a) and (b) (Fig. 4, lower panels). This indicated that peptide (a) was phosphorylated on both threonine and serine residues, whereas peptide (b) was phosphorylated only on threonine residues. The pattern of phosphorylation appeared to be the same in samples from fat-cells exposed to either insulin or isoprenaline. Very similar results to those shown in Fig. 4 were obtained with intact epididymal fat-pads (results not shown).

Previous studies by Blackshear *et al.* (1982, 1983) on the 22 kDa protein from insulin-treated rat fat-cells and 3T3-L1 adipocytes have indicated that three major species of the protein can be separated by two-dimensional electrophoresis; the pattern shown in Fig. 5 (left-hand centre panel) for insulin-treated intact epididymal fat-pads is in broad agreement with this, and we have denoted the three species (i)–(iii) with increasing apparent molecular mass and decreasing pI (range 5.5–5.1). Blackshear *et al.* (1982, 1983) reported that insulin decreased the phosphorylation of a species corresponding to species (i) while causing a corresponding increase in the phosphorylation of the higher-molecular-mass species (ii) and (iii). However, we find that, although insulin may cause a greater increase in the phosphorylation of species (ii) and (iii), which correspond to the upper band of the poorly resolved doublet in one-dimensional SDS/PAGE, there was still an increase in the phosphorylation of

Table 2. Comparison of amino acid analyses of h.p.l.c.-purified 22 kDa protein and after further separation by SDS/PAGE

Amino acid analysis of the 22 kDa protein bands was performed as described in the Experimental section. Sample (a) was prepared by h.p.l.c. as in Fig. 3. Samples (b) and (c) were prepared from the sample shown in Fig. 3; each band was sliced from the gel, electroeluted in 20 mM-Tris buffer, pH 8.5, containing 1 mM-EDTA and 1 mM-dithiothreitol, and dialysed extensively against h.p.l.c.-grade water before amino acid analysis. Values were calculated by direct comparison with their respective standard amino acid quantities.

Amino acid	Composition of purified 22 kDa protein (mol/100 mol)		
	(a) H.p.l.c.- purified	(b) SDS/PAGE upper band	(c) SDS/PAGE lower band
Aspartate/asparagine	10.6	10.6	10.6
Glutamate/glutamine	18.2	23.7	19.3
Serine	7.5	8.3	7.7
Glycine	14.9	11.3	10.8
Histidine	1.3	1.4	2.6
Arginine	4.2	4.4	4.8
Threonine	3.9	5.4	5.4
Alanine	12.0	7.7	7.1
Proline	10.1	8.2	7.1
Tyrosine	1.0	1.2	1.6
Valine	4.4	5.0	6.2
Methionine	1.1	0.8	1.1
Cysteine	0	0	0
Isoleucine	1.3	2.0	3.3
Leucine	3.8	4.3	5.8
Phenylalanine	1.4	1.4	2.2
Lysine	4.4	4.4	4.4

Fig. 4. Tryptic-peptide mapping of the 22 kDa protein bands separated in one dimension (SDS/PAGE)

Fat-cells were incubated, extracted, and samples (equivalent to 1.5 ml of packed cells) of 1.5–15% TCA fractions were separated by SDS/PAGE as in Fig. 1. The radiolabelled 22 kDa phosphoprotein doublet was revealed by radioautography (of the unstained gel), eluted from appropriate gel slices with water and dried in a Savant Speedivac. The samples were then digested with trypsin and the phosphopeptides separated by two-dimensional analysis as given in the Experimental section. Upper panels are radioautographs (48 h) of samples from cells incubated in the absence of hormones (control), or in the presence of 83 nM-insulin or of 9 μ M-isoprenaline together with a key showing the major peptides, (a) and (b). These peptides were recovered and phosphoamino acid analysis was performed as described in the Experimental section (lower panels). Abbreviation: DNP-, dinitrophenyl-.

species (i). Isoprenaline was also found to result in an increase in the phosphorylation of all species, but the extent of increase in the phosphorylation of species (ii) and (iii) tended to be less than that observed with insulin. Essentially identical results were obtained in studies using isolated fat-cells (results not shown).

In an attempt to shed some light on the relationship between the three major species of 22 kDa protein, tryptic-peptide mapping was performed on the species obtained from tissue incubated without hormones, with insulin and with isoproterenol (Fig. 5, right panels). In every case, two phosphopeptides corresponding to those denoted peptides (a) and (b) in Fig. 4 were obtained. Phosphoamino acid analysis of the peptides was subsequently performed, and this revealed that the effect of both insulin and isoprenaline on all three species was to increase the amount of radiolabelled phosphoserine and phosphothreonine in peptide (a) and the amount of radiolabelled phosphothreonine in peptide (b).

DISCUSSION

Blackshear *et al.* (1983) have concluded that the multiple

forms of the 22 kDa protein from 3T3-L1 adipocytes separated by electrophoresis are due to changes in the degree of phosphorylation. Our present studies involving protein purification, amino acid analysis, phosphopeptide mapping and phosphoamino acid measurements certainly support the view that all the components represent closely related proteins, but, as the phosphopeptide maps of the various components are indistinguishable, our results are incompatible with this conclusion. At present, we cannot offer any firm conclusions as to the reasons for the multiple forms separable by electrophoresis. The species may represent differences in post-translational modifications other than phosphorylation or closely related isoforms of the protein. It is of note that inhibitor-1 and G-substrate, which are also heat- and acid-stable proteins, exhibit multiple phosphorylated isoforms which may not necessarily be explained by differences in the extent of phosphorylation (Aswad & Greengard, 1981a; Nimmo & Cohen, 1978).

The amino acid composition of the adipose tissue 22 kDa protein shows a number of interesting features, including high amounts of proline, glycine and acidic residues and low amounts of tyrosine, phenylalanine, methionine and cysteine. This is

Fig. 5. Comparison of tryptic-phosphopeptide maps obtained from the three major species of the 22 kDa protein separated by two-dimensional gel electrophoresis

Fat-pads were incubated, extracted, and 1.5–15% TCA fractions were prepared as in Fig. 2. Samples (equivalent to 20 pads) were separated by two-dimensional gel electrophoresis (see Diggle *et al.*, 1991). Left-hand column radioautographs (4 h) showing the major radiolabelled species (i–iii) from extracts of pads incubated in the absence of hormones (control), or with insulin (83 nM) or isoprenaline (9 μ M) respectively. Each of these species was recovered by water elution (see Fig. 4), digested with trypsin, and the peptides were separated in two dimensions (see Fig. 4). Radioautographs (48 h) are shown. Abbreviation: IEF, isoelectric focusing.

similar to phosphoprotein phosphatase inhibitors 1 and 2 from rabbit skeletal muscle (Foulkes & Cohen, 1980; Aitken *et al.*, 1982), DARPP-32 from bovine brain (Hemmings *et al.*, 1984) and G-substrate from rabbit cerebellum (Aswad & Greengard, 1981a).

At the outset of these studies, we had planned to obtain *N*-terminal amino acid sequence information, but in the event all the components of the purified 22 kDa protein were found to be *N*-terminal-blocked. In preliminary experiments, we have been successful in obtaining some internal amino acid sequence which shows similarity to an internal sequence of G-substrate (Aswad & Greengard, 1981b) (5 residues in a sequence of 8 between the phosphorylation sites of G-substrate; T. A. Diggle & R. M. Denton, unpublished work).

Overall, these findings are compatible with the 22 kDa protein playing a general regulatory role, for example, in the regulation of protein phosphatase activity.

We have no explanation for the inability of Blackshear *et al.* (1982, 1983) to observe any increase in the phosphorylation of the 22 kDa protein with β -adrenergic agonists. In agreement with the original observations made in this laboratory (Belsham & Denton, 1980; Belsham *et al.*, 1980, 1982), we consistently found in the present study that β -adrenergic agonists increase the phosphorylation of the protein to about 60% of that observed with insulin. The effect is clearly exerted through β -adrenergic receptors (rather than α -adrenergic receptors) and is also observed with other agents which increase cell cAMP concentrations. Our results show that insulin and the β -adrenergic agonist isoprenaline probably cause increased phosphorylation

of the same sites within the protein (at least on one serine and two threonine residues). This similarity may appear surprising, since the metabolic effects of the two hormones are often in opposition. However, under appropriate circumstances, the hormones can have similar effects on fat-cell metabolism; examples include activation of the microsomal cAMP phosphodiesterase, glucose transport and glycolysis. Our previous studies (Diggle *et al.*, 1991) suggest that effects of insulin on the phosphorylation of the 22 kDa protein may partly be accounted for by the insulin activation of casein kinase 2. However, the mechanism underlying the increased phosphorylation by β -adrenergic agonists remains to be established. We have been unsuccessful in identifying any protein kinase (other than casein kinase 2) which is capable of phosphorylating the protein. In particular, a partially purified preparation of the protein is not a substrate for cAMP-dependent protein kinase (T. A. Diggle & R. M. Denton, unpublished work).

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