Insulin stimulates the tyrosyl phosphorylation and activation of the 52 kDa peripheral plasma-membrane cyclic AMP phosphodiesterase in intact hepatocytes

Nigel J. PYNE,* William CUSHLEY, Hugh G. NIMMO and Miles D. HOUSLAY†

Molecular Pharmacology Group, Department of Biochemistry, University of Glasgow, Glasgow G12 8QQ, Scotland, U.K.

The 52 kDa subunit of the peripheral-plasma-membrane insulin-stimulated high-affinity cyclic AMP phosphodiesterase can be specifically detected by the antibody PM1 by Western-blotting procedures and also can be immunoprecipitated from a hepatocyte extract. PM1-mediated immunoprecipitation from hepatocyte extracts showed that insulin treatment of intact ³²P-labelled hepatocytes caused the rapid phosphorylation of the peripheral-plasma-membrane cyclic AMP phosphodiesterase. Phosphoamino acid analysis and the use of a phosphotyrosine-specific antibody indicated that phosphorylation occurred on tyrosyl residue(s) of this phosphodiesterase. Prior treatment of hepatocytes with glucagon (10 nM) completely blocked the insulin-mediated tyrosyl phosphorylation of this 52 kDa protein, as detected with both the PM1 and the anti-phosphotyrosine antibodies. Treatment of hepatocytes with glucagon alone did not increase the phosphorylation state of the peripheral-plasma-membrane cyclic AMP phosphodiesterase. The specific anti-phosphotyrosine antibody also detected the insulin-stimulated phosphorylation of proteins of 180 kDa, 95 kDa and 39 kDa. Prior treatment of hepatocytes with glucagon decreased the ability of insulin to phosphorylate the 180 kDa and 39 kDa species, but not the 95 kDa species.

INTRODUCTION

Insulin exerts potent effects on cyclic AMP metabolism in hepatocytes, where it can attenuate the accumulation of cyclic AMP both by inhibiting adenylate cyclase activity and by stimulating the degradation of cyclic AMP through the activation of specific cyclic AMP phosphodiesterase isoenzymes (for discussion see Houslay, 1985, 1986a; Houslay *et al.*, 1983).

Treatment of intact hepatocytes with insulin has been shown to lead to the activation of at least three 'high-affinity' cyclic AMP phosphodiesterases (Heyworth *et al.*, 1983a, 1984; Houslay, 1986a). These include a peripheral-plasma-membrane enzyme and an enzyme that we have called the 'dense-vesicle' phosphodiesterase (Heyworth *et al.*, 1983a), which is found associated with an intracellular vesicle fraction. Both of these enzymes have been purified to apparent homogeneity from rat liver, the plasma-membrane enzyme being a 52 kDa monomer and the 'dense-vesicle' enzyme a 126 kDa dimer (Marchmont *et al.*, 1981; Pyne *et al.*, 1987a). These cyclic AMP-specific phosphodiesterases have been shown to be isoenzymes on the basis of their characteristic kinetic properties, different inhibitor sensitivities, thermal-denaturation kinetics, lack of immunological cross-reactivity, unique iodinated two-dimensional peptide maps and distinct tissue-distribution pattern (Pyne *et al.*, 1987a,b). In addition to these two enzymes, evidence suggests that a third, less well-defined, activity must also be activated in response to insulin (Heyworth *et al.*, 1984). This species may be the so-called cyclic GMP-activated cyclic AMP phosphodiesterase, which we have suggested can be activated by an insulin-mediator preparation (Pyne & Houslay, 1988).

Insulin has been shown to activate the peripheral-plasma-membrane and 'dense-vesicle' enzymes through apparently different mechanisms (Wilson *et al.*, 1983). The activation of the 'dense-vesicle' phosphodiesterase, but not the peripheral-plasma-membrane enzyme, can be blocked by lysosomotropic agents and also by lowering the incubation temperature to 23 °C. Based on these observations, it has been suggested (Wilson *et al.*, 1983) that the 'dense-vesicle' enzyme is activated by insulin through a route which may involve a membrane traffic/processing step of either the receptor or the enzyme itself. Activation of the peripheral-plasma-membrane enzyme by insulin has been observed both in intact hepatocytes (Heyworth *et al.*, 1983a; Wilson *et al.*, 1983; Wallace *et al.*, 1984) and in isolated liver plasma membranes (Marchmont & Houslay, 1980b). In studies using isolated plasma-membrane fractions it was shown (Marchmont & Houslay, 1980b) that insulin's ability to activate this enzyme was dependent on the presence of ATP, and that insulin stimulated the incorporation of ³²P into this enzyme when membranes were incubated with [γ -³²P]ATP (Marchmont & Houslay, 1981). These observations indicated that insulin activated this phosphodiesterase by causing its rapid phosphorylation.

The only well-defined biological functions associated with the insulin receptor are its ability to bind insulin specifically, to exhibit a tyrosyl kinase activity and to undergo receptor-mediated endocytosis (see Houslay, 1981, 1985, 1986b; Denton, 1986; Houslay & Siddle, 1989). However, other than the insulin receptor itself, no intracellular targets for the receptor kinase activity have been identified where the functional consequences of the modification on cellular processes is fully appreciated.

In this study we use an antiserum (PM1) that

Abbreviations used: PAGE, polyacrylamide-gel electrophoresis; PBS, phosphate-buffered saline.

* Present address: Department of Pharmacology, University of Strathclyde, Glasgow G4 0NR, Scotland, U.K.

† To whom correspondence and reprint requests should be addressed.

specifically recognizes the peripheral-plasma-membrane phosphodiesterase (Pyne *et al.*, 1987a,b; Houslay *et al.*, 1987) to isolate the enzyme from intact hepatocytes labelled with ^{32}P . We show here that insulin elicits phosphorylation of the peripheral-plasma-membrane enzyme in intact hepatocytes. This insulin-dependent phosphorylation was identified by using both the phosphodiesterase-specific antiserum PM1 and an antiserum with specificity for phosphotyrosine.

MATERIALS AND METHODS

Materials

Collagenase type II was from Worthington Biochemical Co., Cambridge Bioscience, Cambridge, U.K. All radiochemicals, $^{32}\text{P}\text{P}_i$ and cyclic $^3\text{H}\text{AMP}$ were from Amersham International, Amersham, Bucks., U.K. Glucagon was kindly given by Dr. W. W. Bromer, Eli Lilly and Co., Indianapolis, IN, U.S.A. All biochemicals, unless stated otherwise, were from Sigma, Poole, Dorset, U.K. Cyclic AMP was from Boehringer Corp. (U.K.), Lewes, East Sussex, U.K. Protein A (immunoprecipitin) was from Gibco-BRL, Paisley, Scotland, U.K. Nitrocellulose was obtained from Schleicher and Schuell, Dassel, Germany.

Antibodies with specificity for phosphotyrosine were prepared by immunizing rabbits with the phosphotyrosine analogue benzyl phosphonate coupled to keyhole-limpet haemocyanin, as described previously (Jones & Cushley, 1985; Jones *et al.*, 1986). IgG was prepared from hyperimmune serum by $(\text{NH}_4)_2\text{SO}_4$ precipitation, and specific antibody was isolated by affinity chromatography on phosphotyrosine-Sepharose. The column was washed exhaustively with PBS-7.2 (10 mM- $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$, 0.9% NaCl, pH 7.2) and bound antibody was eluted with 50 mM-phenyl phosphate in PBS. The eluting hapten was removed from the affinity-purified antibody pool by dialysis against PBS-7.2. The peroxidase-linked antibody was kindly given by the Scottish Antibody Production Unit (Law Hospital, Carlisle, Lanarkshire, Scotland, U.K.). The antiserum against a '50 kDa' phosphotyrosine substrate was generously given by Dr. Cecil Yip (University of Toronto, Canada) and is described in full by Kwok & Yip (1987).

Preparation of hepatocytes

Isolated hepatocytes were prepared from 225–250 g Sprague-Dawley rats (Elliott *et al.*, 1976). Cells (5 mg dry wt.) were preincubated for 50 min in Krebs-Ringer buffer, pH 7.4 ('low phosphate', i.e. 50 μM), containing 0.5 mCi of $^{32}\text{P}\text{P}_i$ in a total volume of 5 ml before addition of the hormone. Over this period, cells were routinely gassed with O_2/CO_2 (19:1) every 10 min as described by Houslay & Elliott (1979). Incubation with the hormone was rapidly stopped by addition of an equal volume of ice-cold buffer A [containing final concentrations of 10 mM-Tris/HCl (final pH 7.4), 0.25 M-sucrose, 1 mM-EGTA, 10 mM- β -glycerophosphate, 50 mM-NaF, 2 mM-benzamidine, 0.1 mM-phenylmethanesulphonyl fluoride and 10 μM -leupeptin]. The cells were washed twice in buffer A and finally resuspended in 250 μl of the same buffer.

Incubation of hepatocytes with ^{32}P for the stated times under these conditions has been shown by others to result in equilibrium labelling of the ATP pool (Hopkirk & Denton, 1986).

Cell extracts and immunoprecipitation

A cell extract was made first. This involved taking a 250 μl sample of the resuspended hepatocytes, which was added to 135 μl of PBS (10 mM- $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$, pH 7.4, 0.9% NaCl) and 100 μl of buffer B to give final concentrations of 50 mM-Tris/HCl, pH 7.4, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM-EDTA and 0.15 M-NaCl. To this mixture, 12.5 μl of gelatin (2%, w/v) was added, and finally 2.5 μl of PM1 antiserum (1:200 dilution). This was left at 4 °C for 12 h. In other experiments 50 μl (10 μg) portions of the anti-phosphotyrosine antibodies were used similarly for immunoprecipitation. The mixture, at 4 °C, was then centrifuged at 14000 g_{av} for 10 min to remove any insoluble material. The supernatant obtained from this step was removed, and 25 μl of Protein A (10%, w/v) added to it. This mixture was vortex-mixed and then left at 4 °C for 1.5 h. Samples were diluted 1:1 in PBS, and then Protein A was collected by centrifugation at 14000 g_{av} for 2 min at 4 °C. The pellet was washed once in PBS, then once in buffer B and finally once more in PBS by an identical centrifugal procedure.

The antiserum PM1 was employed in these studies. This was raised against a homogeneous preparation of the peripheral-plasma-membrane cyclic AMP phosphodiesterase, and has been characterized and described in detail elsewhere (Houslay *et al.*, 1987; Pyne *et al.*, 1987a,b).

Electrophoresis and immunoblotting

A 50 μl portion of electrophoresis buffer (0.0625 M-Tris/HCl, final pH 6.7, 1.25% SDS, 1.25% glycerol, 0.4 M- β -mercaptoethanol, 0.01% Bromophenol Blue) was added to the Protein A pellet obtained from the immunoprecipitation study, and boiled for 2–5 min. The sample was centrifuged at 14000 g_{av} for 2 min and the supernatant taken for electrophoresis.

Electrophoresis was performed by using a 5%-polyacrylamide stacking gel and a 10% separating gel. The samples were subjected to electrophoresis for 2 h at 40 mA and 300 V. The gels were then fixed in 10% (w/v) trichloroacetic acid and dried before autoradiography at –70 °C for 72 h. In some instances the 52 kDa phosphorylated peptide was located by alignment with the autoradiograph, excised from the gel and counted for radioactivity for 10–15 min by using Čerenkov counting.

Western-blot analysis was performed as described by Milligan *et al.* (1986).

Phosphopeptide analysis was performed as described by Kwok & Yip (1987).

Phosphoamino acid analysis was performed on phosphorylated peptide that was obtained by cleaving the protein from the gel by using trypsin as described by Takemoto *et al.* (1982). Samples obtained were first freeze-dried and subsequently subjected to acid hydrolysis (5.7 M-HCl) for 2 h at 110 °C. The phosphoamino acids were separated as described by Cooper *et al.* (1983) in the presence of applied standards. The standards were made visible on the chromatograph by ninhydrin staining, and the location of radioactivity was detected by autoradiography. The autoradiograph is shown as a digitized image prepared by analysis using a Datacopy 830 scanner connected to a Macintosh II microcomputer running a Mac Image II software package.

Percoll-gradient analysis of hepatocyte homogenates

This was performed as described in detail previously (Heyworth *et al.*, 1983a; Wilson *et al.*, 1983). The resolution of the plasma-membrane fraction allowed for quantification of the activation of the peripheral-plasma-membrane enzyme by insulin as described in some detail by us previously (Heyworth *et al.*, 1983a).

Assay of cyclic AMP phosphodiesterase

This employed the two-step procedure of Thompson & Appleman (1971), as described previously (Marchmont & Houslay, 1980a). The peripheral-plasma-membrane cyclic AMP phosphodiesterase was purified as described previously by us (Houslay *et al.*, 1987).

RESULTS

Cell extracts were first subjected to immunoprecipitation with the antiserum PM1 raised against the purified peripheral-plasma-membrane cyclic AMP phosphodiesterase. After this, the immunoprecipitated material was subjected to SDS/PAGE and then probed by the Western-blotting procedure, again with the antiserum PM1. This identified a single 52 kDa species, which represented the peripheral-plasma-membrane enzyme and showed that the antiserum PM1 could

Fig. 1. Western-blot analysis using the antibody PM1 identifies a 52 kDa species in extracts of rat hepatocytes

An extract of rat hepatocytes was prepared as described in the Materials and methods section and an immunoprecipitate made with the antiserum PM1. This was subjected to SDS/PAGE before immunoblotting with antiserum PM1 (directed against the peripheral-plasma-membrane phosphodiesterase). Reaction was identified by peroxidase-linked IgG as described in the Materials and methods section. A typical experiment which was performed three times on different cell preparations is shown.

Fig. 2. Antibody PM1 immunoprecipitates a 52 kDa phosphoprotein from hepatocytes challenged with insulin

(a) Hepatocytes were pre-labelled with ^{32}P , challenged for 5 min at 37 °C with 10 nM-insulin, harvested in the cold, and an extract was made immediately for immunoprecipitation. The immunoprecipitate was subjected to SDS/PAGE and subsequent autoradiography. In track 1 no insulin was added to the cells, and in track 2 insulin (10 nM) was added to the cells. A typical experiment of one performed five times on different cell preparations is shown. (b) Experiments were done with the antibody PM1 as described in the legend to part (a). Additions to the cell incubations were: track 1, no ligand (control); track 2, insulin (10 nM) for 5 min; track 3, glucagon (10 nM) for 5 min, then insulin for 5 min; track 4, glucagon (10 nM) for 5 min, with similar results being obtained for 10 min. A typical experiment which was performed five times on different cell preparations is shown here.

indeed immunoprecipitate this enzyme (Fig. 1). Very occasionally a minor species (approx. 45 kDa) was also detected on Western blots. This species, however, always represented less than 10 % of the total immunoreactive staining, and peptide mapping has shown it to be a proteolysed fragment of the native 52 kDa species (N. J. Pyne, unpublished work; see Pyne *et al.*, 1987b).

Table 1. Insulin-stimulated phosphorylation and activation of the peripheral-plasma-membrane cyclic AMP phosphodiesterase in intact hepatocytes

Hepatocytes were preincubated with ^{32}P as described in the Materials and methods section. In some instances they were then challenged with hormones as indicated before being harvested for the preparation of extracts for immunoprecipitation with the antiserum PM1 directed against the peripheral-plasma-membrane enzyme. Data are shown as the fold increase in labelling compared with that in the basal state, i.e. when hepatocytes had not been challenged with hormone. In such instances 110 ± 29 ($n = 5$ separate experiments) counts/15 min were observed in the labelled band extracted. Activation of the peripheral cyclic AMP phosphodiesterase through challenge of intact hepatocytes by insulin was performed as described previously by us (Heyworth *et al.*, 1983a), by assessing activity in plasma membranes isolated by Percoll-gradient treatment of disrupted hepatocytes. Incubation with either insulin or glucagon alone was for 10 min. When glucagon and insulin were both used to challenge hepatocytes, then glucagon was added 5 min before challenge with insulin for 5 min. Results are shown here for three separate experiments with different cell preparations.

Ligand challenge	Labelling of 52 kDa species (control = 1.0)	Activity of plasma-membrane cyclic AMP phosphodiesterase activity (control = 1.0)
Insulin (10 nM)	8.75 ± 0.82	2.10 ± 0.22
Glucagon (10 nM)	1.16 ± 0.21	1.06 ± 0.05
Glucagon + insulin	1.32 ± 0.25	1.08 ± 0.08

Fig. 3. Analysis of the 52 kDa phosphoprotein immunoprecipitated from insulin-treated hepatocytes

(a) Phosphopeptide analysis. The immunoprecipitate, obtained by treating an hepatocyte extract with antiserum PM1, was treated with protease as described by Kwok & Yip (1987) before SDS/PAGE. The gel was sliced and sections were counted for radioactivity. The graph plots radioactivity against the length of the gel. Arrows indicate

Incubation of hepatocytes with $[^{32}\text{P}]\text{P}_i$ (0.5 mCi) for 50 min, followed by treatment with insulin (10 nM) for 5 min, led to the phosphorylation of the 52 kDa peripheral-plasma-membrane cyclic AMP phosphodiesterase (Fig. 2a, Table 1). This phosphorylated protein was identified after immunoprecipitation with the antiserum PM1.

The PM1 antiserum appeared also to immunoprecipitate a peptide of approx. 20 kDa. This, too, exhibited insulin-dependent phosphorylation. Since Western blots with the PM1 antiserum failed to detect this peptide, it most likely represents either a protein which adsorbs strongly to the immobilized Protein A or a tightly associated peptide which interacts with the 52 kDa enzyme.

When hepatocytes were incubated for 5 min with insulin (10 nM) and then membrane fractions were resolved by Percoll-gradient analysis (Heyworth *et al.*, 1983a), we observed (Table 1) an increase in the cyclic AMP phosphodiesterase activity of the plasma-membrane fraction. The magnitude of the increase was similar to that noted previously by us (Heyworth *et al.*, 1983a).

After immunoprecipitation with PM1 from insulin-treated cells labelled with ^{32}P , the 52 kDa plasma-membrane phosphodiesterase was subjected to proteolytic degradation, the peptides were separated by SDS/PAGE and the gel was sliced and counted for radioactivity. The phosphopeptide pattern is shown in Fig. 3(a).

Phosphoamino acid analyses of the phosphorylated 52 kDa species immunoprecipitated by antiserum PM1 revealed that labelling was associated with phospho-

peptide sizes in kDa. (b) Phosphoamino acid analysis. This was performed as described in the Materials and methods section on an immunoprecipitate made from an hepatocyte extract treated with antiserum PM1. The data are shown as a digitized image of the autoradiograph. The migration of standards for phosphotyrosine (a), phosphoserine (b) and phosphothreonine (c) are shown as outlines together with the designated letter. The migration of phosphopeptides (d) and free phosphate (e) is also shown, and the origin (O).

Fig. 4. Immunoprecipitation of hepatocyte phosphoproteins with a phosphotyrosine-specific antibody

Hepatocytes were pre-incubated with ^{32}P before challenge for 10 min with (a) glucagon (10 nM) for 5 min, with similar results being obtained after 10 min, (b) glucagon (10 nM) for 5 min and then insulin (10 nM) for 5 min, (c) insulin (10 nM) for 5 min, or (d) no ligands (control). After this time, cells were harvested in the cold and an extract was made for immunoprecipitation with a specific anti-phosphotyrosine antibody as described in the Materials and methods section. The immunoprecipitates were subjected to SDS/PAGE with subsequent autoradiography. A typical experiment of one performed three times on different cell preparations is shown here.

tyrosine (Fig. 3b). This was also implied in studies using a highly specific anti-phosphotyrosine antiserum which was also capable of immunoprecipitating a phosphorylated 52 kDa polypeptide from insulin-treated hepatocytes (Fig. 4).

The anti-phosphotyrosine antibody also immunoprecipitated a number of other phosphorylated species from the extracts of ^{32}P -labelled hepatocytes (Fig. 4). These proteins had molecular masses of approx. 180 kDa, 95 kDa and 39 kDa. Interestingly, prior exposure of the cells to glucagon decreased the insulin-stimulated labelling of the 180 kDa and 39 kDa species as well as that of the 52 kDa species, but not that of the 95 kDa species (Fig. 4).

Treatment of hepatocytes with glucagon (10 nM) did not lead to any change in either the phosphorylation state (Fig. 2b, Table 1) or activity of the peripheral-plasma-membrane enzyme (Table 1; Heyworth *et al.*, 1983a). However, exposure of the hepatocytes to glucagon before addition of insulin completely blocked the ability of insulin to elicit both the phosphorylation (Fig. 2b, Table 1) and the activation (Table 1; see also Heyworth *et al.*, 1983a) of the peripheral cyclic AMP phosphodiesterase. As found for the 52 kDa species which was immunoprecipitated with the antiserum PM1, glucagon pre-treatment of hepatocytes also blocked the ability of insulin to phosphorylate the 52 kDa species immunoprecipitated by the anti-phosphotyrosine antibody (Fig. 4).

Kwok & Yip (1987) have shown that a protein of approx. 50 kDa, from rat hepatocytes, could be phosphorylated on tyrosyl residues in response to insulin treatment. We employed their antibody to immunoprecipitate material from our cell extracts. There were then probed with the antibody PM1 by the Western-blotting procedure. This detected an immunoreactive species migrating at approx. 52 kDa in the immunoprecipitated material (Fig. 5a). In control experiments, mock immunoprecipitations were performed with the antiserum of Kwok & Yip (1987), but without any cell extract being present. When this mock preparation was used in immunoblotting studies, a very broad staining area appeared at the top of the gel. This appeared to be a non-specific interaction with the second antibody used in the immunoblotting procedure.

We were also able to confirm (Fig. 5b) the observations of Kwok & Yip (1987), which showed that their antiserum could immunoprecipitate a protein of approx. 50 kDa (52 kDa in our hands) whose tyrosyl phosphorylation has been shown (Kwok & Yip, 1987) to be stimulated by treatment of intact hepatocytes with insulin (10 nM, 5 min). Furthermore, if we added the purified peripheral cyclic AMP phosphodiesterase at the immunoprecipitation step, this clearly prevented their antiserum from immunoprecipitating a labelled species from insulin-treated cells (Fig. 5b).

DISCUSSION

We (Heyworth *et al.*, 1983a) have shown previously that treatment of intact hepatocytes with insulin elicits the rapid activation of two distinct membrane-bound cyclic AMP phosphodiesterases. One of these is a high-affinity species associated with the plasma membrane. We have previously purified this enzyme to homogeneity, characterized it (Marchmont *et al.*, 1981), and more recently have prepared and characterized a polyclonal antiserum (PM1) directed against it (Pyne *et al.*, 1987b; Houslay *et al.*, 1987). This recognizes the single 52 kDa subunit of the monomeric peripheral-plasma-membrane phosphodiesterase and does not cross-react with various other purified phosphodiesterases. In particular, it does not cross-react with the insulin- and glucagon-stimulated 'dense-vesicle' phosphodiesterase from hepatocytes (Pyne *et al.*, 1987a,b). Using this antiserum and a specific antiserum against the 'dense-vesicle' phosphodiesterase, we have been able to show that those two insulin-sensitive enzymes are selectively expressed in a variety of rat tissues (Pyne *et al.*, 1987b).

Here we show that the antiserum PM1 immunoprecipitates a single 52 kDa species, presumably the peripheral-plasma-membrane enzyme, from a rat hepatocyte extract (Fig. 1). Using this antiserum we can demonstrate that, after insulin treatment of intact hepatocytes which have been labelled with ^{32}P , this 52 kDa species becomes phosphorylated (Fig. 2a). This indicates that insulin can elicit the phosphorylation of the peripheral-plasma-membrane cyclic AMP phosphodiesterase in intact hepatocytes. Such an effect was specific for insulin and was not mimicked by treating cells with glucagon (Fig. 2b; Table 1). At the same time we showed that insulin enhanced the activity of this enzyme some 2-fold, whereas glucagon exerted no such effect (Table 1; Heyworth *et al.*, 1983a).

In our previous studies (Heyworth *et al.*, 1983a), which

Fig. 5. The antiserum of Kwok & Yip (1987) immunoprecipitates a protein from hepatocyte extracts which is recognized by the antiserum PM1 and displaced by the peripheral-plasma-membrane enzyme

(a) An extract was made from hepatocytes as described in the Materials and methods section, before immunoprecipitation with the antiserum prepared by Kwok & Yip (1987) against a species of approx. 50 kDa from rat liver. The immunoprecipitate was subjected to SDS/PAGE and subsequent immunoblotting with the antiserum PM1 against the peripheral-plasma-membrane phosphodiesterase. In track A hepatocyte extract was immunoprecipitated with the antiserum of Kwok & Yip (1987) and then probed with antiserum PM1 after SDS/PAGE. This identified a distinct band at 52 kDa and a broad staining

examined changes in phosphodiesterase activity in intact hepatocytes, we noted that, if hepatocytes were exposed to glucagon before their challenge with insulin, then such a procedure completely prevented the ability of insulin to activate the plasma-membrane enzyme. We are now able to demonstrate that the basis for this appears to be that glucagon pre-treatment prevents insulin from stimulating the phosphorylation of the peripheral-plasma-membrane enzyme in intact hepatocytes (Fig. 2b; Table 1). This would be consistent with our contention that the insulin-stimulated phosphorylation of the peripheral-plasma-membrane phosphodiesterase mediates the stimulating effect of insulin on the activity of this enzyme.

As observed with the 52 kDa species immunoprecipitated with the antiserum PM1, glucagon inhibited the ability of insulin to simulate the phosphorylation of the 52 kDa species immunoprecipitated by the anti-phosphotyrosine antibody (Fig. 2b). This suggests that the 52 kDa species detected by the anti-phosphotyrosine antibody may indeed be the peripheral-plasma-membrane phosphodiesterase. Such experiments indicate that insulin stimulates the phosphorylation of the peripheral-plasma-membrane phosphodiesterase on tyrosyl residues. This is consistent with the phosphoamino acid analysis of the 52 kDa species immunoprecipitated by the antiserum PM1, which showed the labelled species to be phosphotyrosine (Fig. 3b).

Kwok & Yip (1987) have shown that a protein of approx. 50 kDa provides a substrate for insulin-stimulated tyrosyl phosphorylation in hepatocytes. They prepared an antiserum to this protein, and here we show that the protein immunoprecipitated by this antiserum is recognized in immunoblotting studies with the antiserum PM1. This suggests that the species identified by Kwok & Yip (1987) is indeed the peripheral-plasma-membrane phosphodiesterase, a conclusion which is supported by two further pieces of evidence: firstly, the evident similarities between the phosphopeptide maps of the proteins immunoprecipitated by the antiserum PM1 (Fig. 3) and by the antiserum of Kwok & Yip (1987); secondly, that the ability of the antiserum of Kwok & Yip (1987) to immunoprecipitate a phosphorylated protein of approx. 52 kDa from insulin-treated hepatocytes was blocked by the inclusion of the purified peripheral-plasma-membrane cyclic AMP phosphodiesterase in the immunoprecipitation experiment (Fig. 5b). Such studies provide

area at much higher molecular size. In track B a control incubation was done with just the antiserum of Kwok & Yip (1987) and no hepatocyte extract. This was subjected to SDS/PAGE and probed with antiserum PM1, yielding a broad staining area at much higher molecular size only. (b) Cells were labelled with ^{32}P and immunoprecipitations performed as described in the experiments which used the anti-phosphotyrosine antiserum, except that the antiserum of Kwok & Yip (1987) was used at a 1:100 dilution. In tracks A and B immunoprecipitations were performed in the presence of 1 μg of the purified peripheral-plasma-membrane cyclic AMP phosphodiesterase, but in tracks C and D no such addition was made. In tracks B and D hepatocytes were challenged with 10 nM-insulin for 5 min before harvest, whereas no such hormonal challenge was made for cells analysed in tracks A and C. The experiment shows an autoradiograph of the immunoprecipitate resolved by SDS/PAGE. This is a typical experiment of one performed three times.

further support for the notion that insulin causes the tyrosyl phosphorylation of the peripheral-plasma-membrane phosphodiesterase in intact hepatocytes.

Kwok & Yip (1987), however, referred to the 50 kDa species that they examined as a cytosol protein, because it was found in the soluble extract from hepatocytes. However, the peripheral-plasma-membrane enzyme is easily displaced from the plasma membrane, especially when chelating agents, such as those used in the homogenization buffer employed by Kwok & Yip (1987), are present (see Houslay *et al.*, 1984). Thus under the homogenization conditions used by Kwok & Yip (1987) we would expect the peripheral-plasma-membrane enzyme to be released as a soluble species.

The anti-phosphotyrosine antiserum used here indicated that insulin elicited the tyrosyl phosphorylation of a number of substrates in intact hepatocytes. The 95 kDa species may be the β -subunit of the insulin receptor itself, which becomes autophosphorylated in pure receptor preparations and in intact cells (see Houslay, 1985, 1986a,b; Denton, 1986; Ballotti *et al.*, 1987). Such a species was also identified in the hepatocyte studies of Kwok & Yip (1987). The species of approx. 180 kDa, observed here, has been noted to occur in hepatocytes, in HEP-G2 cells and in a spectrum of other cell types (see White *et al.*, 1985, 1987; Shemer *et al.*, 1987), although its identity is as yet unknown.

We noted that challenge of hepatocytes with glucagon did not elicit any increase in the labelling of the 39, 52 or 180 kDa species. However, glucagon treatment did increase the labelling of the 95 kDa species. If the 95 kDa species is indeed the insulin receptor, this would be consistent with an increase in seryl phosphorylation effected by glucagon. Such seryl phosphorylation could be caused by either or both protein kinases A and C, as described by others (Takayama *et al.*, 1984; Stadtmauer & Rosen, 1986). As glucagon can stimulate two distinct signalling pathways, which leads to increases in the intracellular concentrations of both cyclic AMP and diacylglycerol (see Murphy *et al.*, 1987), then both kinases could possibly act on the insulin receptor. That the anti-phosphotyrosine antiserum detects an increase in labelling after challenge of hepatocytes with glucagon indicates that, under basal conditions, the insulin receptor must have phosphotyrosine associated with it which allows this 95 kDa species to be immunoprecipitated. Certainly a small degree of labelling of this species is evident from labelling studies (Fig. 4). Interestingly, however, pre-treatment of hepatocytes with glucagon attenuated the ability of insulin to enhance the phosphorylation of the 180 kDa and 39 kDa species as well as the 52 kDa species (Fig. 4). This suggests that the ability of glucagon to attenuate the tyrosyl phosphorylation of target proteins is not restricted to the peripheral-membrane phosphodiesterase. Indeed, such observations may reflect the fact that phosphorylation of the insulin receptor by protein kinase A or C leads to an attenuation of its tyrosyl kinase activity (Takayama *et al.*, 1984; Stadtmauer & Rosen, 1986; Häring *et al.*, 1986).

Our experiments indicate that the peripheral-plasma-membrane cyclic AMP phosphodiesterase may thus provide a direct substrate for the insulin-receptor kinase and that such phosphorylation may give rise to a functional change, namely enhanced activity of this enzyme. It is possible that this phosphodiesterase is

closely associated with the insulin receptor, as it has been shown to bind to a single class of protein sites which occur in similar numbers to the insulin receptor on the plasma membranes (Houslay & Marchmont, 1981). Furthermore, using radiation-inactivation studies we have shown (Houslay *et al.*, 1984) that this phosphodiesterase appears to interact with a species of similar size (approx. 90 kDa) to the β (tyrosyl kinase) subunit of the insulin receptor.

Previously we have examined the ability of insulin to activate this enzyme in isolated membrane preparations (Marchmont & Houslay, 1980b). In such studies we demonstrated that cyclic AMP, as well as ATP, was necessary for insulin to activate this enzyme and that the inhibitor protein of cyclic AMP-dependent protein kinase could block insulin's effect. Also, we found that the ^{32}P that could be incorporated into this enzyme in isolated membranes was predominantly alkali-labile, rather than in an alkali-stable form as would be expected for phosphotyrosine (Marchmont & Houslay, 1981). It would thus appear that the mechanism whereby insulin can lead to the phosphorylation and activation of this enzyme in broken membranes does not reflect that employed in intact cells. In broken membranes insulin can then apparently trigger a process whereby protein kinase A can phosphorylate and activate the peripheral-plasma-membrane phosphodiesterase. This is presumably as a result of the occupied insulin receptor eliciting an appropriate conformational change in the phosphodiesterase which allows it to become a substrate for protein kinase A, as the purified enzyme itself cannot be phosphorylated by protein kinase A (Marchmont & Houslay, 1981).

We therefore suggest that in intact hepatocytes the peripheral-plasma-membrane cyclic AMP phosphodiesterase can be activated by the insulin receptor through a mechanism which involves its phosphorylation by the receptor tyrosyl kinase. Thus the peripheral-plasma-membrane phosphodiesterase may provide a substrate for the insulin-receptor tyrosyl kinase, which has an identifiable function.

We thank the M.R.C. and California Metabolic Research Foundation for generous financial support.

REFERENCES

- Ballotti, R., Kowalski, A., White, M. F., Le Marchand-Brustel, Y. & Van Obberghen, E. (1987) *Biochem. J.* **241**, 99–104
- Cooper, J. A., Sefton, B. M. & Hunter, T. (1983) *Methods Enzymol.* **99**, 387–402
- Denton, R. M. (1986) *Adv. Cyclic Nucleotide Protein Phosphorylation Res.* **20**, 295–341
- Elliott, K. R. F., Ash, R., Pogson, C. I., Smith, S. A. & Crisp, D. M. (1976) in *Use of Isolated Liver Cells and Kidney Tubules in Metabolic Studies* (Tager, J. M., Söling, H. D. & Williamson, J. R., eds.), pp. 139–153, North-Holland, Amsterdam
- Häring, H., Kirsch, D., Obermeier, B., Ermel, B. & Machicao, F. (1986) *Biochem. J.* **234**, 59–66
- Heyworth, C. M., Wallace, A. V. & Houslay, M. D. (1983a) *Biochem. J.* **214**, 99–110
- Heyworth, C. M., Rawal, S. R. & Houslay, M. D. (1983b) *FEBS Lett.* **154**, 87–91
- Heyworth, C. M., Wallace, A. V., Wilson, S. R. & Houslay, M. D. (1984) *Biochem. J.* **222**, 183–187

- Hopkirk, T. J. & Denton, R. M. (1986) *Biochim. Biophys. Acta* **885**, 195–205
- Houslay, M. D. (1981) *Biosci. Rep.* **1**, 19–34
- Houslay, M. D. (1985) *Mol. Aspects Cell. Regul.* **4**, 279–333
- Houslay, M. D. (1986a) *Biochem. Soc. Trans.* **14**, 183–193
- Houslay, M. D. (1986b) in *Recent Advances in Diabetes* (Nattrass, M., ed.), vol. 2, pp. 35–53, Churchill Livingstone, Edinburgh
- Houslay, M. D. & Marchmont, R. J. (1981) *Biochem. J.* **198**, 703–706
- Houslay, M. D. & Elliott, K. R. F. (1979) *FEBS Lett.* **104**, 21–24
- Houslay, M. D. & Siddle, K. (1989) *Br. Med. Bull.* **45**, 264–284
- Houslay, M. D., Wallace, A. V., Wilson, S. R., Marchmont, R. J. & Heyworth, C. M. (1983) *Horm. Cell Regul.* **7**, 105–120
- Houslay, M. D., Wallace, A. V., Marchmont, R. J., Martin, B. R. & Heyworth, C. M. (1984) *Adv. Cyclic Nucleotide Res.* **16**, 159–176
- Houslay, M. D., Pyne, N. J. & Cooper, M. E. (1987) *Methods Enzymol.* **159**, 751–760
- Jones, J. A. & Cushley, W. (1985) *Biosci. Rep.* **5**, 707–714
- Jones, J. A., Wood, A. & Cushley, W. (1986) *Biosci. Rep.* **6**, 265–273
- Kwok, Y. C. & Yip, C. C. (1987) *Biochem. J.* **248**, 27–33
- Marchmont, R. J. & Houslay, M. D. (1980a) *Biochem. J.* **187**, 381–392
- Marchmont, R. J. & Houslay, M. D. (1980b) *Nature (London)* **286**, 904–906
- Marchmont, R. J. & Houslay, M. D. (1981) *Biochem. J.* **195**, 653–660
- Marchmont, R. J., Ayad, S. & Houslay, M. D. (1981) *Biochem. J.* **195**, 645–652
- Milligan, G., Gierschik, P., Spiegel, A. M. & Klee, W. A. (1986) *FEBS Lett.* **195**, 225–230
- Murphy, G. J., Hruby, V. J., Trivedi, D., Wakelam, M. J. O. & Houslay, M. D. (1987) *Biochem. J.* **243**, 39–46
- Pyne, N. J. & Houslay, M. D. (1988) *Biochem. Biophys. Res. Commun.* **156**, 290–296
- Pyne, N. J., Cooper, M. & Houslay, M. D. (1987a) *Biochem. J.* **242**, 33–42
- Pyne, N. J., Anderson, N., Lavan, B. E., Milligan, G., Nimmo, H. G. & Houslay, M. D. (1987b) *Biochem. J.* **248**, 897–901
- Shemer, J., Adamo, M., Wilson, G. C., Heffez, D., Zick, Y. & LeRoith, D. (1987) *J. Biol. Chem.* **262**, 15476–15482
- Stadtmauer, L. & Rosen, O. M. (1986) *J. Biol. Chem.* **261**, 3402–3407
- Takayama, S., White, M. F., Lauris, V. & Kahn, C. R. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 7790–7801
- Takemoto, D. J., Hansen, J., Takemoto, L. J. & Houslay, M. D. (1982) *J. Biol. Chem.* **257**, 14597–14599
- Thompson, W. J. & Appleman, M. M. (1971) *Biochemistry* **10**, 311–316
- Wallace, A. V., Heyworth, C. M. & Houslay, M. D. (1984) *Biochem. J.* **222**, 177–182
- White, M. F., Maron, F. & Kahn, C. R. (1985) *Nature (London)* **318**, 183–186
- White, M. F., Stegmann, E. W., Dull, T. J., Ullrich, A. & Kahn, C. R. (1987) *J. Biol. Chem.* **262**, 9769–9777
- Wilson, S. R., Wallace, A. V. & Houslay, M. D. (1983) *Biochem. J.* **216**, 245–248

Received 3 February 1989/29 March 1989; accepted 3 April 1989