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Diabetes-induced alterations in the expression, functioning and phosphorylation state of the inhibitory guanine nucleotide regulatory protein G_i-2 in hepatocytes

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Levels of the G-protein α-subunits α-G_i-2, α-G_i-3 and the 42 kDa, but not the 45 kDa, form of α-G_s were markedly decreased in hepatocyte membranes from streptozotocin-diabetic animals as compared with normals. In contrast, no detectable changes in α-G_i subunits were seen in liver plasma membranes of streptozotocin-diabetic animals, although levels of the 45 kDa form of G_s were increased. G-protein β subunits in plasma membranes were unaffected by diabetes induction. Analysis of whole-liver RNA indicated that the induction of diabetes had little effect on transcript levels of G_i-3, caused an increase in G_s transcripts and decreased transcript number for G_i-2, albeit to a much lesser extent than was observed upon analysis of hepatocyte RNA. In both hepatocyte and liver plasma membranes, immunoblot analysis showed that levels of the catalytic unit of adenylate cyclase were increased upon induction of diabetes. Under basal conditions, α-G_i-2 from hepatocytes of diabetic animals was found to be both phosphorylated to a greater extent than α-G_i-2 isolated from hepatocytes of normal animals, and furthermore was resistant to any further phosphorylation upon challenge of hepatocytes with angiotensin, vasopressin or the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate. Treatment of isolated plasma membranes from normal, but not diabetic, animals with purified protein kinase C caused the phosphorylation of α-G_i-2. Treatment of membranes from diabetic animals with alkaline phosphatase caused the dephosphorylation of α-G_i-2 and rendered it susceptible to subsequent phosphorylation with protein kinase C. Low concentrations of the non-hydrolysable GTP analogue guanylyl 5′-imidodiphosphate inhibited adenylate cyclase activity in both hepatocyte and liver plasma membranes from normal, but not diabetic, animals.

INTRODUCTION

Guanine nucleotide regulatory proteins (G-proteins) play a pivotal role in transducing cellular signals between occupied receptors and appropriate signal-generation systems, such as adenylate cyclase [1–6]. Changes in the expression and/or functioning of receptors and G-proteins linked to adenylate cyclase can occur as a result of a number of different processes, such as desensitization [7,8], the phosphorylation of G_i-2 [9–11], the action of various bacterial toxins [1–6], and in a number of pathological conditions, including diabetes [12].

We have observed a marked enhancement in the ability of glucagon to activate adenylate cyclase in hepatocyte plasma membranes from diabetic rats [13]. This, we have suggested, was due to a decreased level of expression of the inhibitory G-protein G_i [13]. Here we extend our analysis to investigate the effect of streptozotocin-induced diabetes on the expression of the stimulatory G-protein, G_s, on the different forms of G_i and on G-protein β-subunits in both hepatocytes and liver, as well as analysing the phosphorylation state of α-G_i-2.

MATERIALS AND METHODS

[^{32}P]P_i was obtained from Amersham International, Amersham, Bucks., U.K. [^{32}P]NAD$^+$ was from New England Nuclear, Stevenage, Herts., U.K. Pertussis toxin was from PHLS Centre for Applied Microbiology, Porton Down, Wilts., U.K. 12-*O*-Tetradecanoylphorbol 13-acetate (TPA) was from Cambridge Bioscience, Cambridge, U.K. Hormones and Protein A–agarose were from Sigma, Poole, Dorset, U.K. All other biochemicals were from Boehringer, Lewes, East Sussex, U.K., and other chemicals were of A.R. grade, from BDH, Poole, Dorset, U.K.

Animals

Diabetes was induced in male Sprague–Dawley rats (225–250 g) by a single intraperitoneal injection of streptozotocin (50 mg/kg, 0.3 ml) in 0.1 M-sodium citrate, pH 4.5. Animals were killed 7 days after injection, and were judged to be diabetic when blood glucose levels exceeded 12 mM.

Hepatocyte preparation and harvesting and membrane preparation

Hepatocytes were prepared as described by Smith *et al.* [14], from 225–250 g fed male Sprague–Dawley rats as previously described by us [15]. For phosphorylation studies, cells (10^6 cells/ml) were preincubated for 50 min at 37 °C in Krebs–Henseleit buffer [16] containing 50 μM-potassium phosphate and 0.2–0.5 mCi of [^{32}P]P_i and supplemented with 2.5% BSA, 2.5 mM-$CaCl_2$ and 10 mM-glucose. Cells were gassed with O_2/CO_2

Abbreviations used: G-protein, guanine nucleotide regulatory protein; G_s, stimulatory G-protein acting on adenylate cyclase; G_i, so-called inhibitory G-protein acting on adenylate cyclase; IBMX, 3-isobutyl-1-methylxanthine; p[NH]ppG, guanylyl 5′-imidodiphosphate; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

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(19:1) for 30 s every 10 min. The ATP content of the isolated hepatocytes was determined by the luciferase method in a neutralized $HClO_4$ extract [15].

Ligands were added in less than 1% of the total incubation volume and, after an appropriate time, the reactions were stopped by addition of 4 vol. of ice-cold Krebs–Henseleit buffer [16]. The cells were harvested by centrifugation (1000 *g*, 3 min) and washed once in cold buffer before immunoprecipitation of specific G-proteins. For adenylate cyclase assays, cells were incubated in normal Krebs–Henseleit buffer [16], and the incubations were quenched by the addition of an equal volume of ice-cold 1 mM-$KHCO_3$, pH 7.2, and were kept on ice until further processed. A washed membrane fraction was obtained from the hepatocytes as described previously [17,18] and membranes were used within 2 h of preparation. Plasma membranes were also prepared from homogenates of whole rat liver as described by Exton and co-workers [19] or as reported previously by us [20,21] with similar results. Percoll-gradient analysis of hepatocyte homogenates was done as described in detail by us previously [22]. Percoll plasma-membrane fractions were prepared as described previously by us [13].

Assay of adenylate cyclase and G_i function

Adenylate cyclase was assayed as described previously [17,20]. Incubations contained 1.5 mM-ATP, 5 mM-$MgSO_4$, 10 mM-theophylline, 1 mM-EDTA, 7.5 mg of phosphocreatine/ml, 1 mg of creatine kinase/ml and 25 mM-triethanolamine/KOH, pH 7.4. The cyclic AMP produced was determined in a binding assay using the regulatory subunit of protein kinase A [23]. Assays were linear under all conditions, and initial rates were analysed.

G_i function was assessed by determining the ability of low concentrations of p[NH]ppG (1 nM) to inhibit adenylate cyclase activity in the presence of the diterpine forskolin (100 μM) as previously described [10,13,24,25].

Phosphorylation of α-G_i-2 in hepatocyte membranes by purified kinases

In order to examine the phosphorylation of α-G_i-2 by purified protein kinase C, hepatocyte membranes (100 μg of protein) were incubated (30 min; 30 °C) with 15 units of protein kinase C/ml (a mixture of isoenzymes purified from bovine brain as described in ref. [26]), 10 mM-$MgCl_2$, 100 μg of aprotinin/ml, 100 μg of leupeptin/ml, 10 μM-ATP, 5×10^6 c.p.m. of [γ-^{32}P]ATP, 0.5 mg of phosphatidylserine/ml, 1.5 mM-$CaCl_2$, 10 ng of TPA/ml and 50 mM-Hepes, pH 7. In some instances, phosphatidylserine, $CaCl_2$ and TPA were replaced by 1 mM-EGTA.

Immunoprecipitation of G_i and G_s

Cells or membranes were extracted by the addition of 1 ml of a buffer containing 1% Triton X-100, 0.1% SDS, 10 mM-NaH_2PO_4, 10 mM-NaF, 100 μM-Na_3VO_4, 10 mM-β-glycerophosphate, 1 mM-phosphoserine, 2 mM-phenylmethanesulphonyl fluoride, 10 μg of leupeptin/ml, 10 μg of aprotinin/ml and 50 mM-Hepes, pH 7.2. After 1 h at 4 °C, non-solubilized material was removed by centrifugation (14000 *g* for 10 min at 4 °C).

Labelled α-G_i-2 was immunoprecipitated with AS7 antiserum, α-G_i-3 was immunoprecipitated with 13B antiserum, and α-G_s was immunoprecipitated with CS1 antiserum. The production and characterization of these antisera have been described previously [13,27], as has their ability and efficiency at immunoprecipitation of these G-protein α subunits [10]. In each case, immunoprecipitation was critically dependent on the presence of the specific antisera in the incubation, and was not caused by either non-specific binding of phosphoproteins or the sole addition of immunoglobulins or Protein A–agarose [9,10]. Antiserum AS7 was raised in rabbits against the *C*-terminal decapeptide of the α subunit of transducin, and recognizes both α-G_i-1 and α-G_i-2 in addition to transducin. However, since hepatocytes do not express transducin and exhibit no detectable α-G_i-1, shown by either immunoblotting or transcript analysis (M. Bushfield, S. L. Griffiths & M. D. Houslay, unpublished work; [10]), AS7 can be used as a specific tool to immunoprecipitate α-G_i-2. I3B antiserum was raised in rabbits against the *C*-terminal decapeptide of α-G_i-3, and recognizes α-G_i-3 with slight cross-reactivity with α subunit of the related G-protein, α-G_o [27]. Hepatocytes, however, do not express G_o ([28]; M. Bushfield & M. D. Houslay, unpublished work), and thus we have used I3B to immunoprecipitate α-G_i-3 specifically. CS1 was raised in rabbits against the *C*-terminal decapeptide of α-G_s, and recognizes and immunoprecipitates both the 42 kDa and 45 kDa forms of α-G_s found in hepatocytes [10].

Antiserum (10 μl) was added to 1 ml of cell extract, and samples were incubated for 12 h at 4 °C. After this period, 50 μl of Protein A–agarose (25 μl of packed gel) was added and the incubation was continued for a further 2 h. Immune complexes were collected as Protein A–agarose pellets by centrifugation (14000 *g*; 2 min; 4 °C), and the pellets were washed three times in a buffer containing 1% Triton X-100, 0.1% SDS, 10 mM-NaCl, 100 mM-NaF, 50 mM-NaH_2PO_4 and 50 mM-Hepes, pH 7.2.

SDS/PAGE and autoradiography

Protein A–agarose pellets were resuspended in Laemmli sample buffer [29] and placed in a boiling-water bath for 3 min. Samples were then centrifuged (14000 *g*; 2 min) and the supernatants taken for SDS/PAGE. This was performed at 300 V and 60 mA for 2 h in 10%-acrylamide gels. After electrophoresis, gels were fixed in 10% trichloroacetic acid for 1 h before drying and were then subjected to autoradiography. Gels were scanned by multiple scanning of individual lanes and analysed quantitatively with a Bio-Rad Video densitometer connected to an Olivetti M21 computer driven by the Bio-Rad-1D analysis software package as detailed previously [10]. Labelled bands of interest were excised, and radioactivity was determined by Čerenkov counting. Under the conditions of our experiments we ensured a linear relationship between optical density of scanned gels and radioactivity of samples ensued.

Calculation of stoichiometry of phosphorylation

In order to determine the stoichiometry of labelling of α-G_i-2 in intact hepatocytes, we measured the amount of α-G_i-2 present in the incubation and the specific radioactivity of [γ-^{32}P]ATP in the cells. Cells were incubated for various times with [^{32}P]P_i, and samples were then divided into two halves: one was taken for immunoprecipitation of α-G_i-2, and the other was taken for the measurement of ATP content as described above. The specific radioactivity of the [γ-^{32}P]ATP was determined as described by Hawkins *et al.* [30]. The amount of α-G_i-2 protein present was determined by quantitative immunoblotting with purified bovine brain G_i as standard, as described previously by us [13,31].

Immunoblotting of hepatocyte and liver membranes

Immunoblotting of hepatocyte and liver membrane G-proteins was carried out as described previously by us [13], with an ^{125}I-labelled second antibody (Amersham). The α-subunit of G_i-2 was detected with the anti-peptide antiserum AS7, and the anti-peptide antisera I3B and CS1 were used to detect α-G_i-3 and α-G_s respectively. The antiserum BN1 was used to detect G-protein β subunits. The specificity and use of these antisera have been described previously [27]. We also performed studies with an ^{125}I-labelled monoclonal antibody (S. Mollner & T. Pfeuffer, unpublished work) raised against purified adenylate cyclase [32].

This detects (S. Mollner & T. Pfeuffer, unpublished work) the 130 kDa subunit of calmodulin-insensitive adenylate cyclase, which can be found in liver.

Generation of oligonucleotide probes

Synthetic 33-mer oligodeoxynucleotides generated on an Applied Biosystems DNA synthesizer were made so as to be complementary to bases of the mRNAs encoding amino acids 125–135 of the α-subunits of each of G_i-1, G_i-2, G_i-3, and 147–157 of G_s [33]. This region corresponds to one of the highly divergent areas of the coding sequence of these G-proteins as defined by analysis of rat cDNA clones [33,34]. A 29-mer probe for G-protein β-subunits was made and is complementary to mRNAs encoding amino acids 82–91 of the human and bovine β-1 and β-2 forms [35]. Probes were labelled at the 5′ end with ^{32}P (6000 Ci/mmol; Amersham International) and T4 polynucleotide kinase (Boehringer Mannheim) [36]. Incorporation of radioactivity averaged 7×10^8 d.p.m./μg of probe.

Detection of mRNA species corresponding to G-protein α and β subunits of G_i-1, G_i-2 and G_i-3

Liver was dissected from animals immediately after killing, ground to a fine powder in liquid N_2, and 1 g was homogenized in 6 ml of GIT buffer [4 M-guanidinium thiocyanate (Fluka) solution/25 mM-sodium acetate (pH 6.0)/120 mM-2-mercaptoethanol]. For hepatocytes, 1 ml of packed cells was homogenized in 6 ml of GIT buffer. Both homogenates were subsequently treated in an identical fashion. These were extruded through 18- and 23-gauge needles, centrifuged (10000 *g*, 10 min) to remove particulate material, and the supernatant was layered over 5.5 ml of CsCl buffer (5.7 M-CsCl/25 mM-sodium acetate, pH 6) in polyallomer ultracentrifuge tubes (Beckman). Samples were centrifuged overnight in a Sorvall TH641 rotor (170000 *g*, 20 h, 20 °C). Total RNA was recovered from the bottom of the tubes, resuspended in GIT buffer, extracted once against an equal volume of phenol/chloroform/3-methylbutan-1-ol (25:24:1, by vol.) and then twice against chloroform alone before being finally precipitated with ethanol. The concentration of precipitated RNA was determined from the A_{260}.

Northern blotting

Total cellular RNA was denatured by incubation at 65 °C with 2 M-formaldehyde and deionized 50% (v/v) formamide (Fluka). It was resolved in 1% agarose gels containing 2.2 M-formaldehyde before transfer to Hybond N nylon filters (Amersham) [37]. Filters were pre-hybridized at 37 °C for 4 h in hybridization solution [30% deionized formamide, 5 × SSPE (1 × SSPE = 150 mM-NaCl/10 mM-NaH_2PO_4/1 mM-EDTA, pH 7.4), 10 × Denhardt's solution (50 × Denhardt's solution = Ficoll 400, BSA and polyvinylpyrrolidone, each at 10 mg/ml), 0.1% SDS, 200 μg of denatured salmon testes DNA/ml] and were then hybridized (42 °C, 16 h) in hybridization solution containing the relevant oligodeoxynucleotide probes (5×10^6 c.p.m./ml).

The blots were washed to a final stringency of 0.5 × SSC (1 × SSC = 150 mM-NaCl/15 mM-trisodium citrate, pH 7.0)/0.1% SDS (55 °C, 30 min). Autoradiographic localization of the bound probes was performed by exposure to presensitized X-ray film at −80 °C. Quantification was done by densitometric scanning under conditions where it could be shown that a linear relationship between optical density and amount of probe present occurred. Alternatively, filters were stained with Methylene Blue to locate rRNA species.

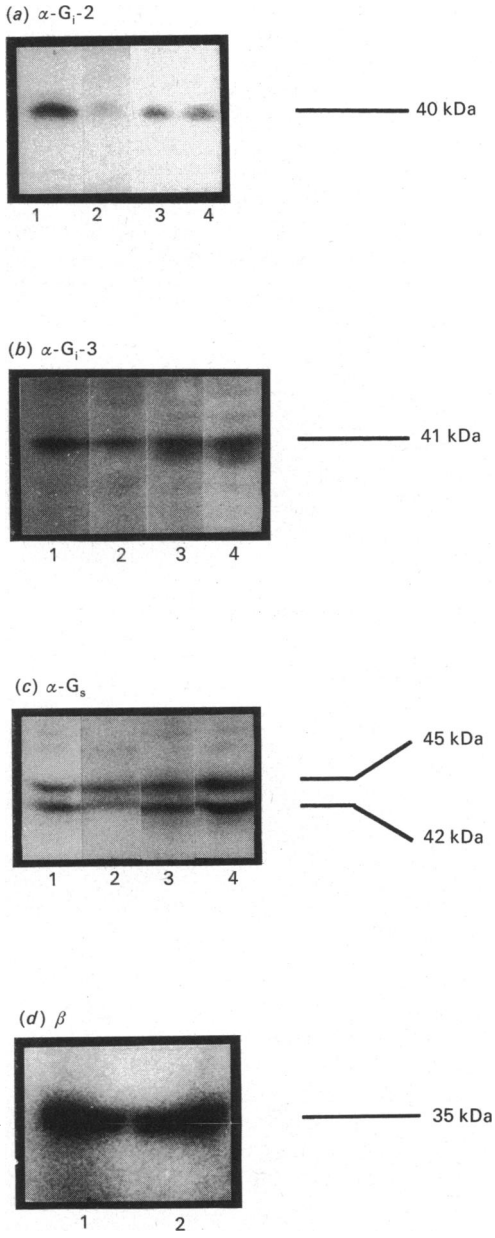

Fig. 1. Immunoblotting of hepatocyte and liver plasma membranes from normal and diabetic rats

Membranes were prepared and immunoblotted as described in the Materials and methods section. (*a*) Antiserum AS7 was used to detect levels of immunoreactive α-G_i-2, (*b*) immunoreactive α-G_i-3 was determined by using antiserum I3B, and (*c*) the α subunits of G_s were detected with antiserum CS1. In each case, we used 200 μg of plasma-membrane protein derived from normal-rat hepatocytes (track 1), diabetic-rat hepatocytes (track 2), normal-rat liver (track 3), or diabetic-rat liver (track 4). (*d*) G-protein β subunits were detected in hepatocytes from normal (track 1) and diabetic (track 2) animals.

RESULTS

Immunoblotting with G-protein-specific antibodies

Fig. 1 demonstrates that hepatocyte plasma membranes from diabetic animals expressed markedly decreased levels of α-G_i-2 (54 ± 6%; $P < 0.001$) and α-G_i-3 (52 ± 5%; $P < 0.001$) compared with membranes from cells of normal animals (Fig. 1). However, we observed no change (98 ± 4%) in the levels of G-protein β subunits (Fig. 1*d*).

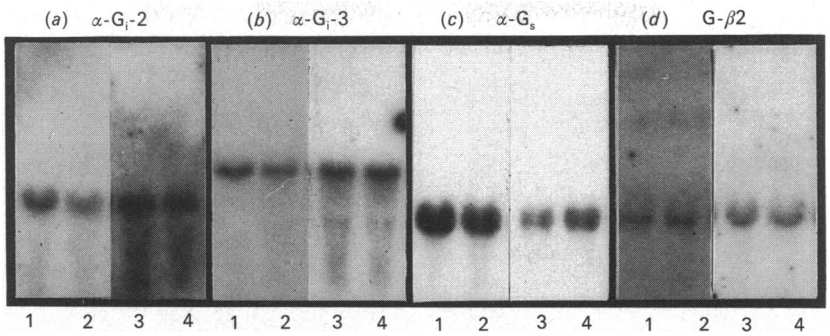

Fig. 2. Effect of streptozotocin-induced diabetes on mRNAs for G-proteins in hepatocytes and whole liver

mRNA for G-protein α and β subunits was detected in Northern blots of total cellular RNA (40 μg/lane), as described in the Materials and methods section. Lane 1 in each block (*a–d*), RNA isolated from control (untreated) hepatocytes; lane 2, that from diabetic hepatocytes; lane 3, that from control liver; lane 4, that from diabetic liver. Lanes 1–4 in (*a*) were hybridized with the α-G$_i$-2 oligonucleotide probe, those in (*b*) with the α-G$_i$-3 probe, those in (*c*) with the α-G$_s$ probe and those in (*d*) with the G-β probe. Autoradiographs were exposed for 1–3 days (α subunit) or 7 days (β-subunit). The Figure is representative of at least three independent experiments.

In contrast with such studies employing plasma membranes from isolated hepatocytes, when we analysed plasma membranes prepared from whole liver then we failed to detect (Fig. 1) any significant change ($P > 0.05$) in the levels of the α-subunits of either G$_i$-2 (97±7%) or G$_i$-3 (88±4%) upon induction of diabetes. Data are expressed relative to that found while immunoblotting equal amounts of membrane protein from normal animals (results are means±S.E.M. for three separate experiments using membrane preparations from different animals; control set at 100% in this and all other analyses).

We took care to establish that the plasma-membrane preparations from hepatocytes of both normal and diabetic animals migrated similarly on Percoll-density-gradient analysis (results not shown). Furthermore, Percoll-density-gradient analysis of hepatocytes, made under iso-osmotic conditions as described previously by us [22], showed that membrane markers migrated identically when hepatocytes from either normal or diabetic animals were used. Thus diabetes did not cause any change in the buoyant density of our membrane preparations which could have affected harvesting. Furthermore, immunoreactive forms of G$_i$ co-migrated with glucagon-stimulated adenylate cyclase activity and other plasma-membrane markers on Percoll-gradient analysis of hepatocyte plasma membranes (results not shown). We also subjected our Percoll-purified plasma membranes to repeated (×3) centrifugation (14000 *g* for 10 min) with resuspension in iso-osmotic buffer in order to observe if α-G$_i$ was released from its membrane environment by this procedure. However no specific losses of α-G$_i$ forms were observed, and similar results were obtained with membranes from either diabetic or normal animals. We also failed to find any immunoreactive material indicative of either α-G$_i$-2 or α-G$_i$-3 in cytosol (100000 *g* × 1 h supernatant) from normal and diabetic-rat hepatocytes (results not shown).

The levels of the 42 kDa and 45 kDa forms of α-G$_s$ in these membrane preparations were also assessed, by using the antiserum CS1 (Fig. 1). Such studies showed that diabetes elicited a fall in the 42 kDa form of G$_s$ (63±3%, $P < 0.001$; $n = 3$) found in hepatocyte membranes, with little effect on the 45 kDa species (95±3%; $n = 3$). In contrast with this, streptozotocin-induced diabetes elicited an increase in the amount of the 45 kDa form of G$_s$ (131±6%; $P < 0.01$; $n = 3$) found in liver membranes, while not affecting the amount (102±7%; $n = 3$) of the 42 kDa species (Fig. 1). These two forms of α-G$_s$ are believed to arise from alternative splicing [38], and it may be that diabetes elicits changes in mRNA processing in hepatocytes. The cellular consequences of altering the ratio of the 42 and 45 kDa forms of G$_s$ remain to be established. A recent report [39] suggests that the lower-molecular-mass form of G$_s$ may have greater functional activity, although an earlier study [40] indicated that preparations containing greater proportions of a higher-molecular-mass form was more effective at reconstituting the β-adrenoceptor-mediated stimulation of adenylate cyclase. This problem will need to be addressed in more detail in the future, as it is now apparent [38] that four distinct splice variants of α-G$_s$ are possible, and these might explain the different sizes of G$_s$ reported in the literature, from ~42 kDa to ~56 kDa [1,2].

Northern-blot analysis of G-protein transcripts

As reported by us [33], streptozotocin-induced diabetes elicited a decrease in the levels of transcripts for the α-subunits of all of these G-proteins in hepatocyte RNA preparations (Fig. 2). Thus transcript levels of α-G$_i$-2, α-G$_i$-3 and α-G$_s$ were decreased to some 53±7, 66±5 and 38±8% of control respectively, although levels of β-2 transcripts were unaltered at 97±6% of control (results are means±S.E.M. for $n = 6$ preparations; all significant at $P < 0.002$). In contrast with this, using RNA prepared from whole liver, here we show (Fig. 2) that streptozotocin-induced diabetes elicited a much smaller decrease in the number of transcripts for α-G$_i$-2 (74±4% of control; $P < 0.002$; means±S.E.M., $n = 6$ separate experiments); it had little effect on α-G$_i$-3 transcripts (91±6%) and even elicited an increase in the level of mRNA (123±7%; $P < 0.01$) encoding α-G$_s$, again with no change in β-2 (105±4%) (results are means±S.E.M. for $n = 6$ preparations from different animals; Fig. 2).

Phosphorylation of α-G$_i$-2 in intact hepatocytes

Challenge of intact [^{32}P]P$_i$-loaded hepatocytes with vasopressin (3 nM, 5 min), angiotensin (3 nM, 5 min) or TPA (10 ng/ml, 15 min) failed to elicit any increase in the degree of labelling of immunoprecipitated α-G$_i$-2 (Fig. 3*a*; Table 1) when cells from diabetic, but not normal, animals were used.

Equal quantities of [^{32}P]P$_i$-loaded hepatocytes, from either normal or diabetic animals, were solubilized and subjected to immunoprecipitation with the antiserum AS7. Analysis of the immunoprecipitated radioactivity, which migrated at 41 kDa on SDS/PAGE, and thus was due to α-G$_i$-2, showed a marked increase, of almost 2-fold, in the radioactivity obtained for hepatocytes from diabetic animals compared with hepatocytes from normal animals. Interestingly, this occurred despite the fact that in parallel immunoblotting studies the amount of α-G$_i$-2 present in hepatocytes from these diabetic animals was some 46% less than that in hepatocytes from normal animals (Fig. 1).

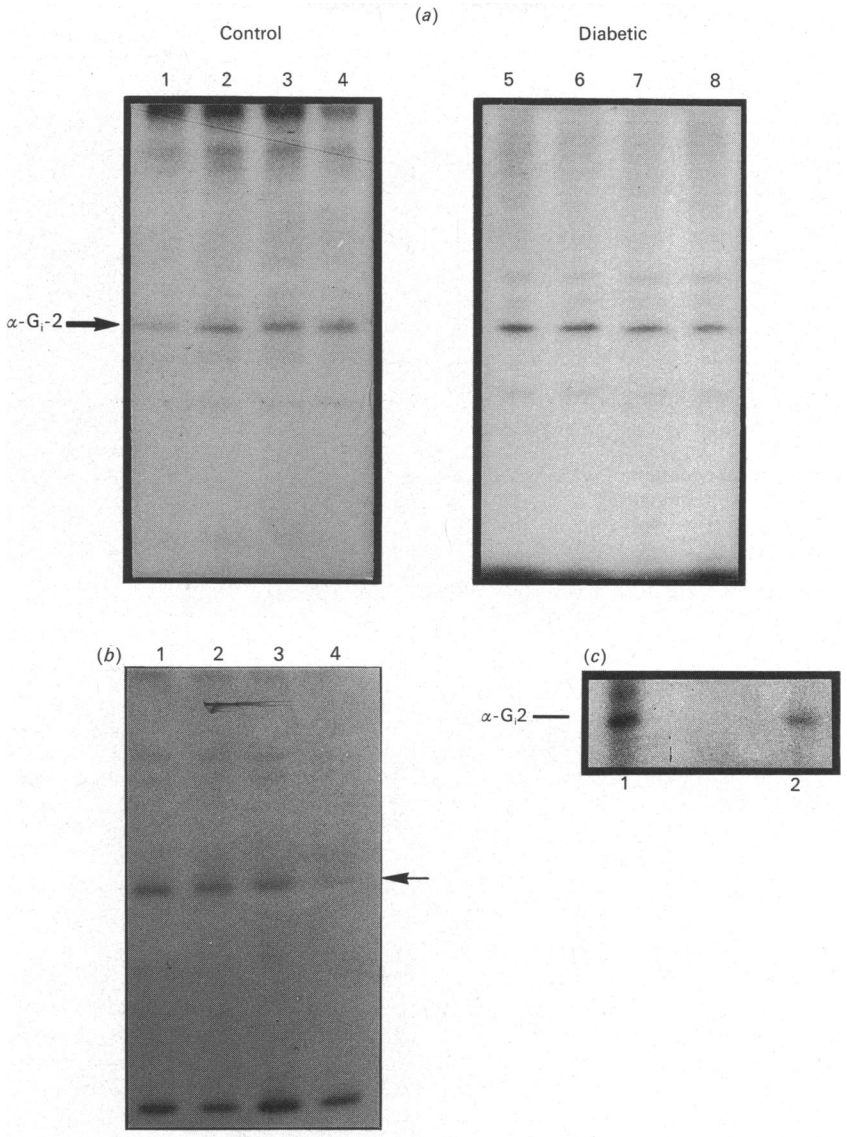

Fig. 3. Phosphorylation of α-G_i-2 both in [^{32}P]P_i-loaded intact rat hepatocytes and in membranes derived from normal and diabetic-rat hepatocytes

(*a*) Hepatocytes were prepared from normal rats (tracks 1–4) or streptozotocin-diabetic rats (tracks 5–8), labelled with [^{32}P]P_i, challenged with vehicle (tracks 1, 5), 10 ng of TPA/ml (tracks 2, 6), 3 nM-vasopressin (tracks 3, 7) or 3 nM-angiotensin (tracks 4, 8), and phosphorylation of α-G_i-2 was assessed as described in the Materials and methods section. The autoradiographs shown are from a single experiment which was representative of three, performed with different animals. (*b*) Hepatocyte membranes from normal rats (tracks 1, 2) or diabetic rats (tracks 3, 4) were subjected to phosphorylation by protein kinase C under conditions of maximal activation of the enzyme, i.e. in the presence of $CaCl_2$, phosphatidylserine and TPA, as described in the Materials and methods section. In some cases (tracks 1, 3) the membranes had been pre-treated (30 °C, 90 min) with alkaline phosphatase (600 units/ml) before incubation with protein kinase C. The experiment shown is representative of two similar experiments using membranes from different animals and was performed as described in the Materials and methods section. (*c*) Membranes from ^{32}P-loaded hepatocytes of diabetic animals were either not treated (track 1) or treated (track 2) with alkaline phosphatase (as above) before solubilization and immunoprecipitation of α-G_i-2. The autoradiograph shown is typical of three separate experiments.

We subsequently attempted to determine the stoichiometry of labelling of α-G_i-2 under steady-state conditions. The additional data gathered to make this estimation involved calculations of the specific radioactivity of the ATP pool, an evaluation of the amount of α-G_i-2 present, as determined by quantitative immunoblotting, and determination of the recovery of α-G_i-2 after solubilization and immunoprecipitation. As shown in Fig. 4, incorporation of radioactivity into both the γ-phosphate position of ATP and the α subunit of G_i-2 reached a steady-state level within 50–65 min of incubation of hepatocytes from normal or diabetic rats with [^{32}P]P_i. Addition of TPA (10 mg/ml; 15 min) after 50 min of incubation had little effect on the specific radioactivity of ATP, but caused a doubling in the labelling of G_i-2 in hepatocytes from normal, but not diabetic, rats (Fig. 4). From such analyses we were able to estimate that the stoichiometry of labelling of α-G_i-2 under basal conditions was 0.33 ± 0.06 mol of ^{32}P/mol of α-G_i-2 in hepatocytes from normal animals and 1.24 ± 0.18 mol of ^{32}P/mol of α-G_i-2 in hepatocytes from diabetic animals ($n = 3$ expts. with different cell preparations; means ± S.D.).

Phosphorylation of α-G_i-2 in isolated membranes

Treatment of membranes from hepatocytes of diabetic animals with pure protein kinase C and [γ-^{32}P]ATP failed to show any incorporation of label into the 40 kDa α-subunit of G_i-2 (Fig. 3*b*). In contrast, if these membranes were pretreated with alkaline

Table 1. Phosphorylation of α-G_i-2 in intact hepatocytes from normal and diabetic rats

Hepatocytes were labelled with [^{32}P]P_i and then challenged with ligands at the indicated concentrations for either 15 min (TPA) or 5 min (vasopressin and angiotensin). Detergent extraction and subsequent immunoprecipitation with antiserum AS7 were performed as described in the Materials and methods section, and the degree of labelling of α-G_i-2 was quantified by densitometry as described in the Materials and methods section. The results, expressed as a percentage of the basal phosphorylation in each instance, are means ± S.E.M. for three separate experiments on different animals. Under basal conditions we assessed that there was some 0.33 ± 0.06 mol of ^{32}P/mol of α-G_i-2 in hepatocytes from normal animals, and this was increased to some 1.24 ± 0.18 mol/mol in hepatocytes from diabetic animals ($n = 3$ separate experiments on different cell preparations): *significant at $P < 0.001$.

	Phosphorylation of α-G_i-2 (% of control)	
	Normal	Diabetic
Vehicle (basal)	(100)	(100)
TPA (10 ng/ml)	167 ± 4*	86 ± 14
Vasopressin (3 nM)	198 ± 17*	83 ± 7
Angiotensin (3 nM)	235 ± 30*	82 ± 9

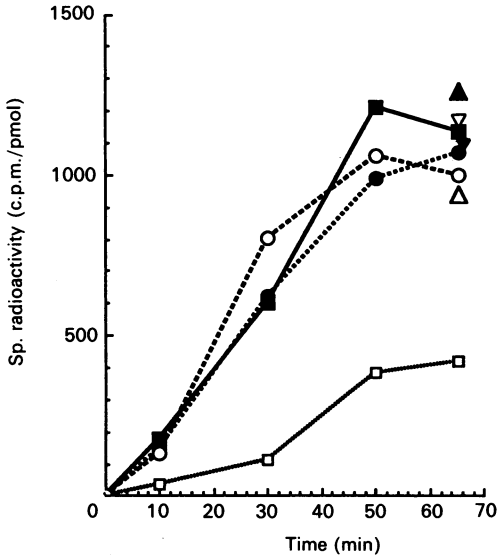

Fig. 4. Time course of labelling of α-G_i-2 and ATP in [^{32}P]P_i-loaded hepatocytes from normal and diabetic rats

Hepatocytes were prepared from normal (□, ○, △, ▽) or diabetic (■, ●, ▲, ▼) animals and then incubated at 37 °C with [^{32}P]P_i for the indicated times. Samples were taken for measurement of the specific radioactivity in both ATP (●, ○, ▽, ▼) and α-G_i-2 (■, □, ▲, △) as described in the Materials and methods section. In some instances, after 50 min of incubation TPA (10 ng/ml) was added (▲, △, ▽, ▼) and the incubation carried on for a further 15 min. The data shows a typical experiment of one done three times.

phosphatase (90 min, 600 units/ml at 30 °C) and then washed by centrifugation (twice at 14000 *g*, 10 min, 4 °C), before challenge with protein kinase C, then α-G_i-2 became labelled (Fig. 3*b*). We were also able to show that, if membranes made from [^{32}P]P_i-loaded hepatocytes from diabetic animals were treated with alkaline phosphatase, then such a treatment removed label from the α-subunit of G_i-2 (Fig. 3*c*).

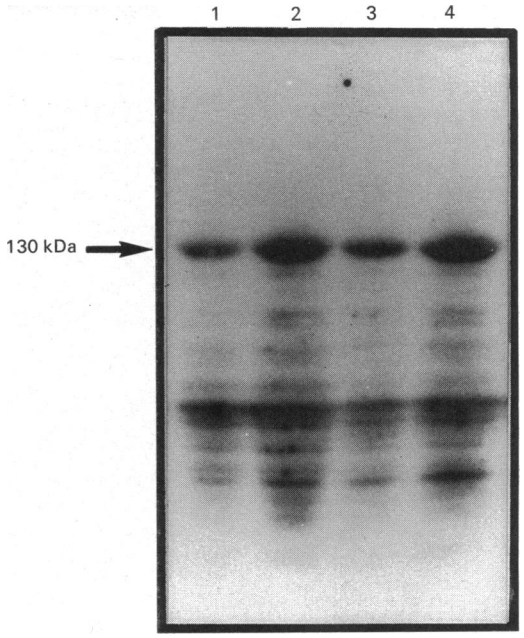

Fig. 5. Immunoblotting of adenylate cyclase from hepatocyte membranes

Hepatocyte membranes were prepared and immunoblotted as described in the Materials and methods section. An ^{125}I-labelled monoclonal antibody [32] was used to detect the catalytic subunit of adenylate cyclase, by using 150 μg (tracks 1, 3) or 300 μg (tracks 2, 4) of membrane protein from either normal rat hepatocytes (tracks 1, 2) or diabetic-rat hepatocytes (tracks 3, 4). A typical autoradiograph is shown.

Guanine-nucleotide-mediated inhibition of adenylate cyclase

We have previously shown [13], and confirm here, that the non-hydrolysable GTP analogue p[NH]ppG inhibited adenylate cyclase activity in isolated hepatocyte plasma membranes from normal, but not diabetic, animals. Here we noted that p[NH]ppG (1 nM) elicited a 38 ± 6% (S.E.M., $n = 4$ different cell preparations) inhibition of forskolin-stimulated adenylate cyclase activity in hepatocyte membranes from normal animals. This was abolished in hepatocyte membranes from diabetic animals, where the addition of p[NH]ppG (1 nM) to assays of forskolin-stimulated adenylate cyclase activity had no effect (less than 8% difference). This feature was not confined to hepatocyte membranes, as p[NH]ppG failed to inhibit adenylate cyclase in liver plasma membranes from diabetic animals also. In liver plasma membranes from normal animals, inhibition was some 30 ± 6%, and this was abolished (11 ± 1% stimulation) in liver membranes from diabetic animals (means ± S.E.M., $n = 3$ different preparations).

Immunoblotting with an antiserum to adenylate cyclase

Hepatocytes, as well as liver membranes, from normal and diabetic animals were immunoblotted with a monoclonal antibody prepared against adenylate cyclase from bovine brain and which has been shown to recognize the 130 kDa Ca^{2+}/calmodulin-insensitive adenylate cyclase in rat liver [32]. We found (Fig. 5) that membranes from hepatocytes exhibit immunoreactive material migrating at around 130 kDa, upon Western blotting, which we presume reflects adenylate cyclase. The amount of this enzyme appeared to increase by some 55–78% (range, $n = 2$ separate preparations) in diabetes. We have previously reported [13] that basal adenylate cyclase activity was actually decreased in hepatocyte plasma membranes from dia-

betic animals. This clearly cannot be attributed to any decrease in the catalytic unit of adenylate cyclase. One possible explanation for this functional change is that, although G-protein α subunits were decreased in hepatocyte plasma membranes of diabetic animals, we failed to note any corresponding decrease in G-protein β subunits. It is considered [2] that basal adenylate cyclase activity, assayed in the presence of Mg^{2+}, may be due to a residual input from G_s whose activity can be stimulated to a limited extent by endogenous GTP. The stimulatory input from this source is likely to be decreased in diabetes both through lowered levels of α-G_s and an attenuating action owing to a presumed increase in the levels of free G-protein β-subunits whose total levels are unchanged in hepatocytes of diabetic animals, whereas α-subunit numbers are decreased.

DISCUSSION

Here we confirm our previous work [13] which showed that streptozotocin-induced diabetes decreased the amount of α-G_i in hepatocyte plasma membranes. Our original study [13] was done before G_i multiplicity was established, and we now know that the AS7 antiserum used was capable of detecting only α-G_i-2 and α-G_i-1 forms. However, as G_i-1 is not expressed in hepatocytes [10,33], then the diabetes-induced fall in 'G_i' reported previously by us [13] can be taken to reflect a decrease in α-G_i-2.

We have since been able to establish that hepatocytes express α-G_i-3 [10], and show here that diabetes causes levels of this G-protein, as with α-G_i-2, to fall to about 50% of the values found in hepatocyte membranes from normal animals (Fig. 1). The diabetes-induced fall in the level of α-G_i-2 recorded here is somewhat less than that reported previously by us (approx. 90%; ref. [13]), but within the range (50–80% decrease) consistent with the effect of diabetes in diminishing the number of G_i α-subunits available for pertussis-toxin-catalysed ADP-ribosylation in hepatocyte membranes [31]. This latter study thus provides an independent method of assessment of the changes in the levels of these G-protein α subunits which occur in hepatocyte plasma membranes of diabetic animals.

Intriguingly, however, we obtained rather different results upon analysis of plasma membranes obtained from whole liver (Fig. 1). For, in this instance, the induction of diabetes did not appear to lead to any diminution in the amount of either G_i forms or the 42 kDa form of G_s, and even led to an increase in the 45 kDa form of G_s. We consider it unlikely that such differences were due to a particular susceptibility for the parenchymal-cell G-protein complement to alter specifically during the preparation of hepatocytes from diabetic animals. Indeed, diabetes appears to engender similar alterations in the amount of adenylate cyclase and the loss of guanine-nucleotide-mediated inhibition of adenylate cyclase (G_i function) in both parenchymal and non-parenchymal cells. Thus we consider it unlikely that preparation of the hepatocytes causes any gross fundamental changes in cell constituents. On this basis, we consider that the apparent differences in the effect of diabetes on G-protein expression when analysing membranes made from hepatocytes and whole liver homogenates may lie in the fact that parenchymal cells (hepatocytes) constitute only some 60% of cells in the liver, where they supply a proportional amount of the total liver plasma membrane [41]. Thus we believe that any diabetes-induced changes in parenchymal cells may be masked by the G-protein complement of the other cells found in liver, which perhaps were unaffected by diabetes. Indeed, there is no reason *a priori* to expect diabetes to decrease G_i expression in all cell types and, certainly, our studies on other tissues, employing both immunoblot [42] and mRNA analyses [33], indicate that hepatocytes actually appear to be rather unusual in this regard. Thus diabetes-induced falls in the transcript number for G-protein α-subunits do not occur in heart, kidney or skeletal muscle and diabetes even elevates levels of α-G_i-1 and -3 in adipocytes [33]. In this regard it is noteworthy that a recent investigation by another group [43] also did not observe any diabetes-induced changes in α-G_i-2 when they analysed a plasma membrane preparation obtained from homogenates of whole liver, but did note an increase in G_s. It therefore seems that 'whole liver' plasma-membrane preparations contain a sufficiently high enough contribution of G-protein α-subunits from non-parenchymal cells to mask any decrease in α-G_i-2 and -3 occurring in hepatocytes of diabetic animals. Certainly, the fact that levels of the 42 kDa form of α-G_s decreased in hepatocytes of diabetic animals, and yet appeared to increase in the whole liver preparation from diabetic animals (Fig. 1), would be consistent with this. Such observations at the protein level were confirmed by using transcript analysis where, in hepatocytes, diabetes causes a marked decrease in transcript number for G_i-2, G_i-3 and G_s, although such changes were severely blunted in analyses of whole-liver RNA preparations ([33]; Fig. 2). Indeed, even a small rise in the level of transcripts for α-G_s in whole liver was observed. This, again, is consistent with non-parenchymal cells contributing a significant amount to the total liver G-protein complement. Certainly, in studies [44] where parenchymal cells (hepatocytes) were separated from Kupffer and endothelial cells in liver, it was quite clear that plasma-membrane preparations from all of the cell fractions exhibited potent guanine-nucleotide-stimulated adenylate cyclase activity, but only in hepatocytes was this activity stimulated by glucagon. Such a comparative study of plasma membranes prepared from both hepatocytes and whole liver emphasizes the importance of analysing homogeneous cell populations, and indicates that organs consisting of many different kinds of cell types may show very different complements of cell-signalling components whose expression is controlled in distinct ways. In this study, then, we appear to have identified diabetes-induced cell-specific changes in G-protein mRNA levels occurring within one organ.

We suggest that the loss of guanine-nucleotide-mediated inhibition seen in hepatocyte and adipocyte membranes from animals in diabetic states has a biochemical explanation, namely that such a loss is related to the elevated level of phosphorylation of α-G_i-2 as well as any decrease in the levels of this G-protein. Phosphorylation, we suggest, might cause a conformational change in α-G_i-2 which attenuates the ability of guanine nucleotides to activate this G-protein. Our reason for suggesting this is based on our previous study [10], which showed that treatment of hepatocytes with agents which can activate protein kinase C and phosphorylate α-G_i-2 leads to the loss of guanine nucleotide inhibition of adenylate cyclase. We show here that, in hepatocytes from diabetic animals, the residual α-G_i-2 appears to be fully phosphorylated, at the putative 'C-kinase site' [10], under basal conditions. This is clearly evident from the facts that: (i) under basal conditions ^{32}P is incorporated into α-G_i-2 of hepatocytes from diabetic animals to a much greater extent than in cells from normal animals; (ii) α-G_i-2 in membranes from diabetic hepatocytes cannot be phosphorylated by purified protein kinase C unless membranes had been pre-treated with alkaline phosphatase in order to dephosphorylate this G-protein; and (iii) ligands known to increase the phosphorylation state of α-G_i-2 in hepatocytes from normal animals failed to do so in hepatocytes from diabetic animals. That ^{32}P can be incorporated into α-G_i-2 under basal conditions suggests that a dynamic phosphorylation/dephosphorylation system exists. Thus, changes in either kinase or phosphatase activities may lead to a new steady-state level of phosphorylation of α-G_i-2.

Lesions in G_i functioning have now been noted in hepatocytes

[13], adipocytes [41] and retina [45] of streptozotocin/alloxan-induced diabetic rats, both in congenitally diabetic (*db/db*) mice [46,47] and in insulin-resistant obese Zucker rats [31]. It is tempting to speculate that the increased phosphorylation of α-G_i-2 may characterize many insulin-resistant states. If so, it remains to be seen whether this occurs as a consequence of the mechanisms leading to insulin resistance, or whether it plays a part in the state of insulin resistance itself [48].

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REFERENCES

1. Gilman, A. G. (1987) Annu. Rev. Biochem. **56**, 615–649
2. Birnbaumer, L., Codina, J., Mattera, R., Cerione, R. A., Hildebrandt, J. D., Sunya, T., Rojas, F. J., Caron, M. J., Lefkowitz, R. J. & Iyengar, R. (1985) Mol. Aspects Cell. Regul. **4**, 131–182
3. Houslay, M. D. (1984) Trends Biochem. Sci. **9**, 39–40
4. Levitzki, A. (1990) Mol. Pharmacol. Cell Regul. **1**, 1–14
5. Gierschik, P. & Jakobs, K. H. (1990) Mol. Pharmacol. Cell Regul. **1**, 67–82
6. Spiegel, A. M. (1990) Mol. Pharmacol. Cell Regul. **1**, 15–30
7. Houslay, M. D., Wakelam, M. J. O., Murphy, G. J., Gawler, D. J. & Pyne, N. J. (1987) Biochem. Soc. Trans. **15**, 21–24
8. Lefkowitz, R. J., Stadel, J. M. & Caron, M. G. (1983) Annu. Rev. Biochem. **52**, 159–186
9. Pyne, N. J., Murphy, G. J., Milligan, G. & Houslay, M. D. (1989) FEBS Lett. **243**, 77–82
10. Bushfield, M., Murphy, G. J., Lavan, B. E., Parker, P. J., Hruby, V. J., Milligan, G. & Houslay, M. D. (1990) Biochem. J. **268**, 449–457
11. Bushfield, M., Griffiths, S. L., Strassheim, D., Tang, E., Shakur, Y., Lavan, B. & Houslay, M. D. (1990) Biochem. Soc. Symp. **56**, 1–18
12. Houslay, M. D. (1990) Mol. Pharmacol. Cell Regul. **1**, 197–230
13. Gawler, D., Milligan, G., Spiegel, A. M., Unson, C. G. & Houslay, M. D. (1987) Nature (London) **327**, 229–232
14. Smith, S. A., Elliott, K. R. F. & Pogson, C. I. (1978) Biochem. J. **176**, 817–825
15. Heyworth, C. M. & Houslay, M. D. (1983) Biochem. J. **214**, 93–98
16. Krebs, H. A. & Henseleit, K. (1932) Hoppe-Seyler's Z. Physiol. Chem. **210**, 33–36
17. Houslay, M. D. & Elliott, K. R. F. (1979) FEBS Lett. **104**, 359–363
18. Houslay, M. D. & Elliott, K. R. F. (1981) FEBS Lett. **128**, 289–292
19. Prpic, V., Green, K. C., Blackmore, P. F. & Exton, J. H. (1984) J. Biol. Chem. **259**, 1382–1385
20. Houslay, M. D., Metcalfe, J. C., Warren, G. B., Hesketh, T. R. & Smith, G. A. (1976) Biochim. Biophys. Acta **436**, 489–494
21. Marchmont, R. J., Ayad, S. & Houslay, M. D. (1981) Biochem. J. **195**, 645–652
22. Heyworth, C. M., Wallace, A. V. & Houslay, M. D. (1983) Biochem. J. **214**, 99–110
23. Whetton, A. D., Needham, L., Dodd, N. J. F., Heyworth, C. M. & Houslay, M. D. (1983) Biochem. Pharmacol. **32**, 1601–1608
24. Heyworth, C. M., Hanski, E. & Houslay, M. D. (1984) Biochem. J. **222**, 189–194
25. Hildebrandt, J. D., Hanoune, J. & Birnbaumer, L. (1982) J. Biol. Chem. **257**, 14723–14725
26. Parker, P. J., Stabel, S. & Waterfield, M. (1984) EMBO J. **3**, 953–959
27. Mitchell, F. M., Griffiths, S. L., Saggerson, E. D., Houslay, M. D., Knowler, J. T. & Milligan, G. (1989) Biochem. J. **262**, 403–408
28. Price, S. R., Tsai, S. C., Adamik, R., Angus, C. W., Serrenti, I. M., Tsuchia, M., Moss, J. & Vaughan, M. (1989) Biochemistry **28**, 3803–3807
29. Laemmli, U. K. (1970) Nature (London) **227**, 680–682
30. Hawkins, P. T., Michell, R. H. & Kirk, C. J. (1983) Biochem. J. **210**, 717–720
31. Houslay, M. D., Gawler, D. J., Milligan, G. & Wilson, A. (1989) Cell. Signalling **1**, 9–22
32. Mollner, S. & Pfeuffer, T. (1988) Eur. J. Biochem. **171**, 265–271
33. Griffiths, S. L., Knowler, J. T. & Houslay, M. D. (1990) Eur. J. Biochem., in the press
34. Jones, D. T. & Reed, R. R. (1987) J. Biol. Chem. **262**, 14241–14249
35. Fong, H. K. W., Amatruda, T. T., Birren, B. W. & Simon, M. I. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 3792–3796
36. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) Molecular Cloning, a Laboratory Manual, p. 122, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
37. Reed, K. C. & Mann, D. A. (1985) Nucleic Acids Res. **13**, 7202–7220
38. Kaziro, Y. (1990) Mol. Pharmacol. Cell Regul. **1**, 47–66
39. Walseth, T. F., Zhang, H. J., Olson, L. K., Schroeder, W. A. & Robertson, R. P. (1989) J. Biol. Chem. **264**, 21106–21111
40. Sternweis, P. C., Northup, J. K., Smigel, M. D. & Gilman, A. (1981) J. Biol. Chem. **256**, 11517–11526
41. Van Berkel, T. J. C. (1979) Trends Biochem. Sci. **4**, 202–205
42. Strassheim, D., Milligan, G. & Houslay, M. D. (1990) Biochem. J. **266**, 521–526
43. Lynch, C. J., Blackmore, P. F., Johnson, E. H., Wange, R. L., Krone, P. K. & Exton, J. H. (1989) J. Clin. Invest. **83**, 2050–2062
44. Wincek, T. J., Hupka, A. L. & Sweat, F. W. (1975) J. Biol. Chem. **250**, 8863–8873
45. Hadjiconstantinou, M., Qu, Z.-X., Moroi-Fetters, S. E. & Neff, N. H. (1988) Eur. J. Pharmacol. **149**, 193–194
46. Begin-Heick, N. (1985) J. Biol. Chem. **260**, 6187–6193
47. Begin-Heick, N. (1987) Mol. Cell. Endocrinol. **53**, 1–8
48. Houslay, M. D. (1989) Trends Endocrinol. Metab. **1**, 83–99

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