Analysis of the adenylate cyclase signalling system, and alterations induced by culture with insulin, in a novel SV40-DNA-immortalized hepatocyte cell line (P9 cells)

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An immortalized cell line, called P9, was derived from hepatocytes by transfection with SV40 DNA. These cells expressed enzyme activities characteristic of hepatocytes, namely glucose-6-phosphatase, glycogen phosphorylase, bilirubin glucuronyltransferase and both glucagon- and prostaglandin E1 (PGE1)-stimulated adenylate cyclase activities, albeit at decreased levels compared with native hepatocytes. Levels of the G-protein subunits α -G_i-2, α -G_i-3, G β and the 'long' form of α -G_s (45 kDa) were ~4-fold higher relative to native hepatocytes, whereas those of the 'short' form of α -G_s (42 kDa) were lower by ~40%. Associated with this were marked alterations in the guanine nucleotide regulation of adenylate cyclase. Receptor-mediated stimulation, achieved by either PGE1 or glucagon, was apparent in P9 cells, although the latter was only evident upon

amplification with forskolin. Glucagon-stimulated cyclic AMP accumulation in P9 cells did not exhibit desensitization, as in hepatocytes, nor was the phosphorylation of α -G_i-2 evident. Culture of P9 cells with insulin led to a dose-dependent decrease (EC_{50} 0.2 $\pm$ 0.1 nM) in the ability of PGE1 to stimulate adenylate cyclase activity, with the maximum effect attained after ~6 h. A comparable attenuation of stimulation was seen for glucagon- and guanine-nucleotide-stimulated adenylate cyclase activities. In cells cultured with insulin, lower levels of GTP were required to stimulate adenylate cyclase, ADP-ribosylation of the 45 kDa form of α -G_s with cholera toxin was attenuated, and the expression of both α -G_i-2 and α -G_i-3 was increased. It is suggested that the expression of α -G_i-2 and α -G_i-3 may be directly regulated by the action of insulin in hepatocytes and P9 cells.

INTRODUCTION

Insulin exerts its effects on target cells by binding to specific receptors at the plasma membrane. The subsequent activation of receptor tyrosine kinase activity is then believed to set in motion a chain of events within the cell, which include alterations in both the tyrosyl and seryl phosphorylation of proteins. These lead to rapid changes in functioning of key proteins, as well as longer-term actions on cell function. Although the precise mechanisms whereby the pleiotropic actions of insulin are exerted remain to be defined, receptor autophosphorylation appears to play a crucial role (see Houslay, 1989, 1990; Houslay and Siddle, 1989), perhaps in triggering a kinase cascade (Nishida and Gotsh, 1993) and also in eliciting the phosphorylation of insulin-receptor substrate 1 (Sun et al., 1992).

We, and others, have shown that in various models of diabetes and insulin-resistant states, as well as in non-insulin-dependent diabetes mellitus in human beings, marked alterations in adenylate cyclase control occur, and that these are associated with changes in the regulation and expression of G-proteins, particularly as regards inhibitory effects exerted through the 'G_i' system (Begin-Heick, 1985; Bushfield et al., 1990b,c; Green and Johnson, 1991; Strassheim et al., 1991a,b; Kowluru et al., 1992; Palmer et al., 1992). This effect characterizes various cell types, including hepatocytes, adipocytes, platelets and cells within the retina. In this regard, we have identified decreases in the levels of both the α -subunits α -G_i-2 and α -G_i-3, together with a marked augmentation of glucagon-stimulated adenylate cyclase activity (Gawler et al., 1987; Bushfield et al., 1990b,c) in hepatocytes

from hypoinsulinaemic animals where diabetes was induced with either streptozotocin or alloxan. It is thus possible that insulin could act directly on hepatocytes to alter the expression of these G-proteins and the regulation of adenylate cyclase. However, both the inability to keep hepatocytes in long-term primary culture, and the fact that marked time-dependent changes in the functioning of signalling systems occur in short-term primary culture (see, e.g., Guguen-Guillouzo and Guillouzo, 1983; Schuetz et al., 1988), militate against the use of such cells in experiments aimed at determining the effect of culture with insulin on both the expression of G-proteins and the functioning of adenylate cyclase. In an attempt to address this problem, we have established an immortalized hepatocyte-derived cell line, called P9. These cells express a number of hepatocyte-like characteristics, including an adenylate cyclase activity which can be stimulated through G_s-coupled receptors. Using these cells, we have shown that signalling through high-affinity insulin receptors can attenuate the G_s-coupled-receptor stimulation of adenylate cyclase activity and increase the expression of the inhibitory G-proteins α -G_i-2 and α -G_i-3, the converse of which is seen in hepatocytes from hypoinsulinaemic animals.

MATERIALS AND METHODS

Materials

BSA, trypsin, collagen (type 1), ATP, cyclic AMP, GTP, guanosine 5'-[β -imidazo]triphosphate (p[NH]ppG), cholera toxin and hormones were from Sigma (Poole, Dorset, U.K.). Creatine kinase and phosphocreatine were from Boehringer Mannheim.

Abbreviations used: G_s, G-protein coupled to stimulation of adenylate cyclase; G_i, G-protein coupled to inhibition of adenylate cyclase; p[NH]ppG, guanosine 5'-[β -imidazo]triphosphate; V_{max}, maximum response; IBMX, 3-isobutyl-1-methylxanthine; PDE, cyclic AMP phosphodiesterase; PGE1, prostaglandin E1.

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Forskolin was from Calbiochem (Cambridge, U.K.). Pertussis toxin was from Porton Products (Maidenhead, Berks., U.K.). [32 P]NAD $^{+}$ was from Du Pont. Other radiochemicals were from Amersham International. Culture media and foetal-calf serum were from Gibco BRL. All other chemicals were of A.R. grade from BDH Chemicals.

Hepatocyte isolation and immortalization

Hepatocytes were prepared by collagenase digestion of livers from 220–250 g fed male Sprague–Dawley rats as described previously (Heyworth and Houslay, 1983). As a prelude to immortalization, they were further purified on a Percoll gradient by the method of Williams et al. (1977), and then transfected by the method described by Woodworth et al. (1986), which gives explicit details of the constructs, quantities and the strategies used by us to transfect hepatocytes and then to isolate the immortalized P9 cell line. In such experiments, hepatocytes were plated out in a 1:1 (v/v) mixture of Williams E medium and Ham's F12 with 5% foetal-calf serum, allowed to recover and then transfected, for 5 h, with a precipitate of p6-1 SV-40 DNA, prepared as described by Gluzman et al. (1980), followed by the selection strategy described by Woodworth et al. (1986). One of the isolated colonies was cloned to produce the P9 cell line.

Culture of P9 cells

P9 cells were cultured in Dulbecco's modification of Eagle's medium, containing 10% foetal-calf serum, with glutamine (2 mM) and penicillin (100 i.u./ml)/streptomycin (100 μ g/ml) at 37 °C in O $_2$ /CO $_2$ (9:1). All plasticware was collagen-coated and u.v.-irradiated for 16 h before use. Cells were subcultured by trypsin treatment, routinely at a split ratio of 1:10. Monolayers were grown to confluence, and before agonist challenge were exposed to serum-free medium for 16 h. Cells were harvested by scraping off plates into an isotonic Hepes buffer, pH 7.4, containing 145 mM NaCl, 6 mM KCl and 10 mM glucose. Cells were pelleted and membranes prepared. Cell passages over the range 5–22 times were analysed in this study, with similar results being obtained.

Preparation of a membrane fraction

Cells were suspended in ice-cold lysing buffer (10 mM Tris/HCl, 1 mM EDTA, pH 7.4) containing 1 mM phenylmethanesulphonyl fluoride, 3 mM benzamidine, 1 μ M leupeptin, 2 μ g/ml aprotinin and 0.5 μ M pepstatin A. They were homogenized by hand on ice in a Potter–Elvehjem homogenizer (10 strokes), and the resulting homogenate was centrifuged at 1000 g_{av} for 10 min. The supernatant was centrifuged at 20000 g_{av} for 10 min to sediment a fraction enriched in plasma membranes, and the pellet was washed and re-centrifuged. The final pellet was resuspended in buffer to a concentration of 1–2 mg/ml and rapidly frozen to –80 °C until use. This procedure was based upon that described by us previously (Houslay and Elliott, 1979).

Rat brain plasma membranes were prepared as described by Milligan et al. (1987) and stored at –80 °C until use.

Toxin labelling of G $_s$ and G $_i$

Pertussis-toxin- and cholera-toxin-catalysed ADP-ribosylation of isolated membranes was performed as described previously (Heyworth et al., 1985), by using [32 P]NAD $^{+}$ and thiol-preactivated toxins. Briefly, membranes were diluted in 1 mM

EDTA/10 mM Tris/HCl, pH 7.4, to a protein concentration of ~2 mg/ml. Samples (20 μ l) were then added to a 50 μ l reaction sample containing [32 P]NAD $^{+}$ (2 μ Ci/assay), 20 mM thymidine, 1 mM ATP, 100 μ M GTP, 20 mM arginine/HCl and 0.25 M potassium phosphate buffer, pH 7.0. Reactions (37 °C, 60 min) were terminated by addition of ice-cold 2% sodium deoxycholate and 24% trichloroacetic acid. Labelled membranes were pelleted by centrifugation (14000 g , 20 min at 4 °C) and subjected to SDS/PAGE and autoradiography.

Pertussis-toxin treatment of cells

Cells were incubated with pertussis toxin at a concentration of 100 ng/ml for 2 h in serum-free medium before agonist stimulation.

Assay of adenylate cyclase activity

This was done by the method of Salomon et al. (1974). Briefly, reactions were carried out in 50 mM triethanolamine buffer, pH 7.4, containing final concentrations of 50 μ M ATP containing [α - 32 P]ATP (2 μ Ci/tube), 2 mM MgSO $_4$, 0.1 mM EGTA, 1 mM 3-isobutyl-1-methylxanthine (IBMX), 1 mM dithiothreitol, 5 mM phosphocreatine and 0.4 mg/ml creatine kinase. Incubations were carried out over 10 min at 30 °C in a final volume of 0.1 ml and started by addition of membranes at a concentration of 5–10 μ g of protein per assay.

Assay of intracellular cyclic AMP

This was done as described previously by us in some detail for native hepatocytes (Heyworth and Houslay, 1983), with addition of ligands as stated in the text. With P9 cells, cells were routinely washed twice with serum-free medium and then incubated for 16 h in such medium before agonist stimulation. Where indicated, insulin at the designated concentrations was added to this serum-free medium. For determination of changes in intracellular cyclic AMP content, then, as a routine, cells were exposed to agonists for 2 min before removal of the medium by aspiration. The cyclic AMP was then extracted in ice-cold 2% (w/v) HClO $_4$, with cells being scraped off the plates and debris removed by centrifugation (14000 g , 2 min). The supernatant was neutralized with 0.5 M triethanolamine in 2 M KOH and the precipitate was pelleted by centrifugation as above. A sample was then taken for estimation of cyclic AMP by a binding-protein assay as described previously by us (Heyworth and Houslay, 1983).

Other enzyme assays

Glucose-6-phosphatase was assayed by the method described by Houslay and Palmer (1978), and glycogen phosphorylase was assayed as described by Bushfield et al. (1993). Bilirubin glucuronyltransferase was measured in the presence of 0.4 mM bilirubin, 4 mM UDP-glucuronic acid and 10 mM MgCl $_2$ by the diazotization procedure (Dutton, 1980).

125 I-glucagon-binding studies

The specific binding of radio-iodinated glucagon to P9 cell membranes and to hepatocyte membranes was done as described in some detail previously by us (Houslay et al., 1977, 1980).

Immunoblotting of membranes

This was carried out as detailed previously by us (Bushfield et al.,

1990c). Briefly, as a routine 100 μ g of protein was applied to each lane for SDS/PAGE on 10 % gels before transfer to nitrocellulose electrophoresis membranes for immunoblot analyses. The anti-serum SG1 was used to detect α -G_i-2 and α -G_i-1. The antiserum CS1 was used to detect the 42 and 45 kDa forms of G_s α . Antiserum I3C was used to detect the α -subunit of G_i-3 and the antiserum BN3 was used to detect G-protein β subunits. Horseradish-peroxidase-conjugated sheep anti-rabbit IgG was used to detect the complexes. Blots were then scanned with a Shimadzu CS-9000 densitometer.

Protein assays

These were carried out by the method of Lowry et al. (1951), with BSA as the standard.

RESULTS AND DISCUSSION

Cell lines have been established by expressing various viral oncogenes, of which SV40 large T is but one example, in primary cells (for review see Hunter, 1993). Such genes encode proteins which interact with normal cellular proteins to over-ride the control of progression through the cell cycle, thus enabling cells to proliferate indefinitely. These cell lines often continue to express tissue-specific characteristics and thus provide a convenient and reproducible alternative to freshly isolated cells or to primary cells in culture, when complex time-dependent changes in properties can occur, as indeed has been reported for hepatocytes (see, e.g., Guguen-Guillouzo and Guillouzo, 1983; Schuetz et al., 1988). Here we describe investigations done on a novel immortalized hepatocyte cell line.

Table 1 Comparison of some properties of P9 cells and native hepatocytes, including adenylate cyclase regulation and levels of G-protein expression

Data are given for at least three separate experiments using different hepatocyte preparations and P9 cultures as means $\pm$ S.D. Adenylate cyclase activity was determined in membranes isolated from these cells (pmol of cyclic AMP produced/min per mg of protein); values in parentheses indicate the fold activation of adenylate cyclase activity over that activity observed under either basal conditions (*) or in the presence of 0.1 mM GTP. The data for adenylate cyclase activity reflect the basal and indicated ligand-stimulated states. Levels of G-protein subunits are shown, as a percentage, relative to those levels found in normal hepatocytes (100%) as assessed by densitometric scanning of the immunoblots (see the Materials and methods section). Glucose-6-phosphatase and phosphorylase *a* activities are expressed as μ g of P_i produced/min per mg dry wt. of cells; bilirubin glucuronyltransferase is expressed as mol/min per mg of protein.

	Hepatocytes	P9 cells
Glucose-6-phosphatase	9.4 $\pm$ 0.1	1.7 $\pm$ 0.1
Phosphorylase <i>a</i>	3.7 $\pm$ 0.7	0.35 $\pm$ 0.05
Bilirubin glucuronyltransferase	8.4 $\pm$ 0.6	1.4 $\pm$ 0.2
Adenylate cyclase		
Basal activity	38 $\pm$ 3	20 $\pm$ 2
GTP (0.1 mM)	105 $\pm$ 3 (2.8)*	38 $\pm$ 6 (2)*
PGE1 (0.1 mM) + GTP	341 $\pm$ 14 (3.3)	95 $\pm$ 4 (2.6)
p[NH]ppG (0.1 mM)	330 $\pm$ 3 (8.7)*	130 $\pm$ 7 (6.8)*
Forskolin (0.1 mM)	870 $\pm$ 8 (22.9)*	611 $\pm$ 26 (32.1)*
NaF (15 mM)	620 $\pm$ 8 (16.3)	653 $\pm$ 24 (27.2)*
Glucagon (10 mM) + GTP	367 $\pm$ 26 (3.5)	41 $\pm$ 3.5 (1.1)
α -G _i -2	(100%)	422 $\pm$ 63
α -G _i -3	(100%)	449 $\pm$ 21
α -G _s (42 kDa)	(100%)	60 $\pm$ 5
α -G _s (45 kDa)	(100%)	393 $\pm$ 40
G-protein β subunits	(100%)	454 $\pm$ 62

Figure 1 Comparative morphology of P9 cells and native hepatocytes

These show phase-contrast photomicrographs (magnification $\times$ 45) of confluent P9 cells (a) and isolated hepatocytes (b).

P9 cells expressed enzyme activities (Table 1) which are typical of differentiated hepatocytes, namely that of glucose-6-phosphatase, glycogen phosphorylase *a* and bilirubin glucuronyltransferase, as well as an adenylate cyclase activity which could be stimulated by both glucagon (in the presence of forskolin; see below) and prostaglandin E1 (PGE1), albeit at lower levels compared with those found in native hepatocytes (see, e.g., Heyworth and Houslay, 1983; Zenser et al., 1974). Indeed, despite the activity of phosphorylase *a* being lower in P9 cells than in hepatocytes, such activity could be stimulated some 2.3 $\pm$ 0.3-fold in 3 min by addition of forskolin (10 μ M), which compares well with a value of 2.1 $\pm$ 0.2-fold for stimulation seen in native hepatocytes (n = 3 different experiments). These observations are consistent with the P9 cell clone being derived from hepatocytes rather than from a possible contaminant of the hepatocyte preparation used for immortalization.

P9 cells divide rapidly, with a doubling time of 18–20 h, and adopt a morphology in culture which, although distinct from that of freshly isolated hepatocytes (Figure 1), was similar to that reported by others (Woodworth et al., 1986) for SV40-immortalized hepatocyte cell lines. We were unable to elicit any noticeable change in the morphology of P9 cells by treatment with either dexamethasone (0.25 μ M, 16 h) or insulin (concentrations up to 0.5 μ M, for periods up to 16 h) or on their attaining confluency (results not shown).

The activity of the catalytic unit of adenylate cyclase activity

Figure 2 Regulation of P9-cell adenylate cyclase activity by GTP and p[NH]ppG

In (a) P9 cells were cultured in the absence ($\blacktriangle$) or presence ($\bullet$) of 1 nM insulin for 16 h before preparation of a membrane fraction for assay of adenylate cyclase activity. In (b) is shown the effect of increasing p[NH]ppG concentrations on adenylate cyclase activity in P9 cells. These experiments were done using three different cell preparations, with assays done in triplicate ($n = 3$; errors are S.D., and were $< 10\%$ in all instances). Activity is expressed here as a percentage of control (100%), i.e. basal activity; data in Table 1 indicate the range of levels of activities shown in various experiments for these various agents.

in P9 cells was lower than that seen in native hepatocytes (Table 1) when assessed either by analysing basal activity ($\sim 47\%$ lower) or with forskolin ($\sim 30\%$ lower), a diterpene which directly activates the catalytic unit of this enzyme (Birnbaumer et al., 1990). Using either GTP or its non-hydrolysable analogue p[NH]ppG, which both achieve stimulation of adenylate cyclase activity by a direct action on G_s (Birnbaumer et al., 1990), we observed a specific activity in P9 cells which was $\sim 60\%$ below that found with native hepatocytes (Table 1). Thus the level of decrease in guanine-nucleotide-stimulated adenylate cyclase activity was greater than one might have expected if just the functioning of the catalytic unit had been attenuated. This is exemplified when the degree of maximal stimulation affected by these guanine nucleotides was expressed relative to basal activity, whereupon a decrease in fold activation was evident, this being some 22–29% lower in P9 cells (Table 1; range $n = 5$). Such a difference indicates altered guanine nucleotide regulation in P9 cells which may be attributable to either an action at the level of

G_2 or an enhanced inhibitory input, through G_i . The role of specific forms of G_i in inhibiting adenylate cyclase remains to be defined (see, e.g., Birnbaumer et al., 1990). However, a growing body of evidence indicates that at least $\alpha\text{-G}_i\text{-2}$ can achieve this in a variety of cells (Milligan and McKenzie, 1988; Houslay, 1991a,b; Remaury et al., 1993), including hepatocytes (Bushfield et al., 1990a). In this regard, whereas P9 cells, like native hepatocytes (Bushfield et al., 1990c; Griffiths et al., 1990), did not express $\alpha\text{-G}_i\text{-1}$, the other two forms of G_i , $\alpha\text{-G}_i\text{-2}$ and $\alpha\text{-G}_i\text{-3}$, were expressed at dramatically (~ 4 -fold) higher levels (Table 1), which, by increasing the inhibitory input into adenylate cyclase, might attenuate the degree of guanine-nucleotide-mediated stimulation. This may explain why much higher concentrations of guanine nucleotides were required (Figure 2) to elicit both the stimulation and the inhibition of this enzyme in P9 cells compared with those needed in native hepatocytes (Heyworth et al., 1984). Thus concentrations in excess of $100\text{ }\mu\text{M}$ of either GTP or p[NH]ppG were needed to elicit inhibition of adenylate cyclase activity in P9 cells, whereas half-maximal inhibition was seen with $1\text{ }\mu\text{M}$ GTP and 0.1 nM p[NH]ppG in native hepatocytes (Heyworth et al., 1984; Gawler et al., 1987). In addition to such changes in G_i , we noted profound differences in the expression of $\alpha\text{-G}_s$ (Table 1; Figure 3), such that ~ 4 -fold greater levels of the 'long' 45 kDa form of G_s were seen in P9 cells and $\sim 40\%$ lower concentrations of the 'short' 42 kDa form (Table 1; Figure 3). This may also contribute to the alterations in guanine nucleotide regulation seen in P9 cells.

The fold stimulation of adenylate cyclase by PGE1 in P9 cells showed a comparable decrease, compared with native hepatocytes, with that seen for stimulation using guanine nucleotides, being $\sim 21\%$ (Table 1). This suggests that the underlying change may lie primarily at the level of regulation by G-proteins rather than at the level of the PGE-1 receptor. Nevertheless, receptor-specific alterations do appear to characterize P9 cells, in that glucagon, which is equally as good as PGE1 in stimulating adenylate cyclase activity in hepatocyte membranes, was unable to elicit such a response when added alone to P9 cells (Table 1). We believe that the inability of glucagon to elicit activation of adenylate cyclase in P9 cells was primarily due, however, to a drastic decrease ($> 90\%$) in the expression of glucagon receptors in these cells ($0.1 \pm 0.03\text{ pmol}$ of glucagon bound/mg of membrane protein; $n = 3$) compared with hepatocytes ($1.5 \pm 0.3\text{ pmol}$ of glucagon bound/mg of membrane protein; $n = 3$). Nevertheless, there clearly were functional glucagon receptors present on P9 cells, as glucagon was able to stimulate adenylate cyclase activity in P9 cell membranes (Figure 4) when the activity of the catalytic unit was amplified by using the diterpene forskolin ($10\text{ }\mu\text{M}$). Glucagon did this in a manner whose dose-dependency (Figure 4; $\text{EC}_{50} = 2.9 \pm 0.7\text{ nM}$; $n = 3$ separate experiments) reflects that seen with native hepatocytes (Heyworth and Houslay, 1983). Indeed, even when added alone to intact hepatocytes, 10 nM glucagon was able to exert a noticeable increase in phosphorylase *a* activity ($50 \pm 6\%$; $n = 3$ separate experiments). Although this was very small compared with the 3.9 ± 0.1 -fold increase that glucagon achieved in native hepatocytes ($n = 3$), it undoubtedly reflects the fact that glucagon could increase the intracellular cyclic AMP concentrations in P9 cells to a sufficient extent to activate protein kinase A. The increase in cyclic AMP levels was, however, below the limits that we could detect by our binding assay using a range of glucagon concentrations of 0.1 nM to $10\text{ }\mu\text{M}$ ($< 0.5\text{ }\mu\text{M}$ increase), even when P9 cells were challenged in the presence of the cyclic AMP phosphodiesterase (PDE) inhibitor IBMX. It is thus possible that some compartmentalization of the production of cyclic AMP occurs in P9 cells, such that localized levels of this second messenger reached a high

Figure 3 Analysis of G-protein subunits in P9 cells by immunoblotting and toxin-catalysed ADP-ribosylation

Immunoblot data were obtained by using specific anti-peptide antisera for the various G-protein subunits. This was done for native hepatocytes (track 3) and for P9 cells which either had (track 2) or had not (track 1) been cultured with 1 nM insulin for 16 h. Data are shown for experiments typical of ones done at least three times (see Tables 1 and 2). (a) Data for the 42 and 45 kDa splice variants of α -G_s; (b) data for α -G_i-2, except that in track 4 a sample of rat brain membranes was also analysed, because this, unlike P9 cells and hepatocytes, contains α -G_i-1 which can also be recognized by the antiserum used here. α -G_i-1 has a higher molecular mass than α -G_i-2 (Birnbauer et al., 1990) and is seen to behave as such in this experiment. (c) Data for α -G_i-3 (d) data for β / γ subunits. Membranes from P9 cells were also treated with thiol-pre-activated bacterial toxins and [³²P]NAD⁺ to achieve labelling of G-protein α -subunits as described in the Materials and methods section. This was done for cells which either had (track 2) or had not (track 1) been cultured with 1 nM insulin for 16 h. The labelling of α -G_s subunits was performed with pertussis toxin (e) and that of α -G_s with cholera toxin (f). Two splice variants of α -G_s were recognized, as with the antisera, namely the 42 and 45 kDa splice

enough value to activate a fraction of protein kinase A. In the presence of forskolin (10 μ M), however, dose-dependent increases in hepatocyte intracellular cyclic AMP concentrations were seen in response to challenge with glucagon (EC₅₀ 1 $\pm$ 0.5 nM; results not shown; n = 3), which reflects a similar action in membranes (Figure 4; see above). We were, however, unable to increase the ability of glucagon to stimulate adenylate cyclase activity in P9 cells by growing them in 0.25 μ M dexamethasone for various periods of up to 16 h (< 5% change; results not shown), indicating that glucocorticoids could not induce this response.

Glucagon's ability to increase the intracellular concentration of cyclic AMP (Figure 4b) in P9 cells was strikingly different from that seen in native hepatocytes. Thus, in P9 cells challenged with glucagon (+ forskolin), cyclic AMP levels rapidly rose to reach a plateau value, reflecting a new steady-state level of synthesis and degradation (Figure 4). However, in hepatocytes, glucagon elicits a transient increase in intracellular cyclic AMP levels, which reach a maximum after $\sim$ 5 min and decline rapidly thereafter (Johnson et al., 1972; Sonne et al., 1978; Heyworth and Houslay, 1983; Murphy et al., 1987), due to the desensitization of adenylate cyclase (Heyworth and Houslay, 1983; Murphy et al., 1987; Refnes et al., 1989; Bushfield et al., 1990b,c). That such desensitization did not occur in P9 cells was further corroborated from analyses of glucagon (+ forskolin)-stimulated membrane adenylate cyclase activity from cells treated with glucagon (10 nM for 2 or 5 min), which showed no alteration in activity (< 5%; results not shown), in marked contrast with the decrease seen for native hepatocytes (Heyworth and Houslay, 1983). Thus desensitization is not an inherent property of glucagon-stimulated adenylate cyclase system itself, but is rather an identifiably distinct process involving other components. A process which appears to occur in parallel with the desensitization of glucagon-stimulated adenylate cyclase (Murphy et al., 1987) is the phosphorylation of α -G_i-2, coupled to the subsequent ablation of the GTP-elicited 'tonic' inhibitory action of G_i on adenylate cyclase (Bushfield et al., 1990b,c). However, we were unable to identify phosphorylated α -G_i-2 in these cells (results not shown) or to elicit such a modification upon challenge of P9 cells with the phorbol ester phorbol 12-myristate 13-acetate (PMA; 'TPA'), glucagon or the protein phosphatase inhibitor okadaic acid (results not shown) under conditions which, in hepatocytes, have been shown to achieve the phosphorylation of this G-protein (Bushfield et al., 1991). As both of these processes are believed to be effected by protein kinase C (Houslay, 1991b), it could be that P9 cells have a specific lesion at this level.

In hepatocytes from both streptozotocin- and alloxan-induced diabetic animals (Gawler et al., 1987; Bushfield et al., 1990c; Griffiths et al., 1990) as well as genetically diabetic (*db/db*) mice (Palmer and Houslay, 1991), we have observed a marked decrease in the expression of G_i forms, a loss of GTP-elicited G_i function and an augmentation of the stimulatory action of glucagon. As such animals were hypoinsulinaemic, the possibility arises that alterations in circulating insulin levels may determine the expression of G-proteins in hepatocytes from such animals and affect signalling through the adenylate cyclase system. In order to evaluate this hypothesis, we determined whether culture of the immortalized hepatocyte P9 cell line with insulin would affect these regulatory systems. Indeed, we found that culture of P9 cells with low (1 nM) concentrations of insulin led to a progressive

variants. A single labelled band was observed for labelling with pertussis toxin, due to the co-migration of both α -G_i-2 and α -G_i-3, in membranes from both treated and untreated cells. Data are for experiments done at least three times using different cell preparations. Positions of pre-stained molecular-mass markers (Sigma) are indicated in kDa.

Figure 4 Glucagon activates P9-cell adenylate cyclase

In (a) the affect of increasing concentrations of glucagon was examined on the activity of adenylate cyclase in membranes from P9 cells. This was done in the presence of 10 μ M forskolin, with data shown as the percentage increase in forskolin-stimulated activity elicited by glucagon. In (b) is shown the incremental time-dependent effect of 10 nM glucagon in elevating intracellular cyclic AMP levels in hepatocytes over and above that achieved by 10 μ M forskolin alone, and whose presence was essential to amplify the activity of the catalytic unit of adenylate cyclase. Data show the cyclic AMP accumulation (pmol/10⁶ cells) found with forskolin alone and in the presence of glucagon. The action of forskolin was subtracted from that found with glucagon added together with forskolin, in order to identify the glucagon-stimulated component. Data show an experiment typical of one done three times, with errors as S.D. of triplicate determinations of cyclic AMP.

time-dependent decrease in the ability of PGE1 to stimulate adenylate cyclase activity (Figure 5a). This PGE1 response was attenuated by $\sim 50\%$ some 6 h after challenge of the cells with insulin and was still apparent after 16 h. This effect was dose-dependent for insulin, with an EC_{50} of 12 ± 4 pM ($n = 3$; mean $\pm$ S.D.), indicating that it was mediated by high-affinity insulin receptors (Figure 5b). Similarly, the ability of PGE1 to increase cyclic AMP levels in intact cells was also ablated after their culture with insulin. Such an effect was clearly due to an attenuated adenylate cyclase-coupled response rather than any enhanced PDE activity, as it was still apparent when the non-specific PDE inhibitor IBMX was added either together with PGE1 or with a 15 min preincubation. Although culture of P9

cells with low concentrations of insulin attenuated the degree of stimulation of adenylate cyclase achieved by PGE1, it did not affect the dose-dependency of this reaction, with similar EC_{50} values for activation being observed, namely 2.2 ± 0.9 μ M and 2.9 ± 0.8 μ M for untreated and insulin-treated P9 cells respectively (Figure 5c).

Attenuation of receptor-stimulated adenylate cyclase activity was not restricted to the action of PGE1, as, in P9 cells which had been treated with 1 nM insulin for 16 h, we also observed a similar decrease ($37 \pm 8\%$; $P = 0.001$) in the ability of 10 nM glucagon to stimulate this enzyme (done in the presence of 10 μ M forskolin; $n = 3$). It is possible that such similar changes affecting both stimulatory receptors might be mediated through a common alteration in their G_s -mediated coupling to adenylate cyclase. This was addressed by determining the degree of G_s -mediated activation that could be achieved by using the physiological effector GTP. Its action was characterized by a biphasic dose-effect curve, with G_s -mediated activation occurring at low concentrations and G_i -mediated inhibition occurring at higher concentrations. In membranes from insulin-treated cells we noted a decrease in the degree of GTP-mediated G_s stimulation, from 3.5 ± 0.3 -fold in membranes from untreated cells to 2.1 ± 0.2 -fold for membranes from insulin-treated cells ($n = 4$; $P = 0.005$). This equates to a reduction of $\sim 43\%$, which is similar to the level of attenuation that culture of P9 cells with insulin exerted on both PGE1- and glucagon-stimulated adenylate cyclase activity (see above). We also noted a similar ($33 \pm 7\%$; $n = 3$; $P = 0.001$) reduction, occurring as a result of culturing P9 cells with insulin (1 nM; 16 h) in the maximal stimulation of membrane adenylate cyclase that could be achieved through the activation of G_s by the non-hydrolysable GTP analogue p[NH]ppG. Such similarities in the attenuation of adenylate cyclase activation by both the PGE1 and glucagon receptors and also for G_s , when activated directly by either GTP or p[NH]ppG, might imply a common cause, namely that culture of P9 cells with low concentrations of insulin affects the guanine nucleotide regulation of adenylate cyclase. This might involve actions at the level of either or both of G_s and G_i . Analysis of G_s expression in cells cultured with insulin showed that little change resulted from culture of cells with insulin (Table 2). Intriguingly, however, when we analysed the ability of G_s to serve as a substrate for cholera-toxin-mediated ADP-ribosylation, this identified a small decrease in the labelling of the 42 kDa form, which may be attributable to decreased expression, but, more importantly, a marked decrease in the labelling of the 45 kDa form which could not be attributed to any change in the expression of this species (Table 2). The opposite effect to this was seen in hepatocytes from hypo-insulinaemic animals (Palmer and Houslay, 1991), implying that circulating insulin levels may have a profound effect on G_s functioning. Certainly, altered susceptibility of G_s to cholera-toxin-catalysed ADP-ribosylation can be achieved through changes in its dissociation state (Ribeiro-Neto et al., 1987), implying that the functioning of at least the 45 kDa species of G_s may be altered upon culture of P9 cells with insulin. If this form of G_s is the species through which the major stimulatory input into adenylate cyclase is routed, then we might predict a change in the dose-dependent action of GTP on adenylate cyclase in P9 cells cultured with insulin. Indeed, we noted a dramatic decrease in the EC_{50} value for the stimulatory action of GTP, this being 316 ± 25 nM in untreated cells and only 71 ± 8 nM in insulin-treated cells (Figure 2a). Thus culture of P9 cells with insulin profoundly affects the stimulatory regulation of adenylate cyclase that is mediated by G_s . Guanine-nucleotide-mediated regulation may also be affected by alterations in G_i input, and in this regard we found that culture of P9 cells with low (1 nM) concentrations

Figure 5 Culture of P9 cells with insulin attenuates the stimulatory action of PGE1 on adenylate cyclase

In (a) P9 cells were cultured in the presence of 1 nM insulin for various periods of time and then challenged with 10 μ M PGE1 for 2 min; the increase in intracellular cyclic AMP levels was then determined. Data are shown as a percentage of the response seen for cells incubated for equivalent periods in the absence of added insulin. For cells cultured in the absence of insulin, the PGE1-stimulated activity did not change ($< 5\%$) over the 16 h period. The data show a composite result of three experiments done using different cells. Errors are $\pm$ S.D. ($n = 3$). In (b) is shown the dose-dependent action on insulin for cultures done over a 16 h period with insulin present. Data are shown as a percentage of the response seen for cells incubated for 16 h in the absence of added insulin. The data show a composite result of three experiments done using different cells. Errors are $\pm$ S.D. ($n = 3$). In (c) is shown the dose-dependent action of PGE1 in cells which either had not ($\blacktriangle$) or had ($\bullet$) been treated for 16 h with 1 nM insulin. The data are for a representative experiment of one done three times with triplicate assays of cyclic AMP accumulation, expressed as pmol of cyclic AMP produced per 10^6 cells.

Table 2 Alterations in G-protein expression and in toxin-catalysed ADP-ribosylation in P9 cells cultured with insulin

Cells were treated with 1 nM insulin for 16 h before harvesting. G-protein levels in membranes were assessed by immunoblotting with specific antisera, and the results are expressed here as a percentage change relative to levels in untreated cells (see the Materials and methods section). Membranes from both untreated and insulin-treated cells were ADP-ribosylated by using thiol-pre-activated toxins as detailed in the Materials and methods section. The data here indicated the percentage change seen with membranes from insulin-treated cells relative to untreated controls. Cholera toxin was used to modify G_s forms, and pertussis toxin to modify G_i forms. For ADP-ribosylation with pertussis toxin it is impossible, due to the similar sizes of α -G_i-2 and α -G_i-3, to resolve these by SDS/PAGE, and hence data for the combined change due to these two forms is given. Results are means $\pm$ S.D. for three separate experiments: n/a, not applicable, for reasons indicated above. *Significant at $P < 0.01$, **significant at $P < 0.005$, ^{ns} not significant (Student's t test).

	Change in expression (%)	Change in ADP-ribosylation (%)
α -G _i -2	$20 \pm 6^{**}$	n/a
α -G _i -3	$31 \pm 9^{**}$	n/a
α -G _s (42 kDa)	-10 ± 4^{ns}	$-13 \pm 5^*$
α -G _s (45 kDa)	$10 \pm 2^*$	$-21 \pm 3^{**}$
G-protein β subunits	7 ± 3^{ns}	n/a
α -G _i + α -G _i -3	n/a	$18 \pm 3^*$

of insulin increased the expression of both forms of G_i (Table 2). However, P9 cells have much greater levels of G_i forms compared with hepatocytes, suggesting that any further increase may have little effect on the degree of G_i function. Indeed, this would seem to be the case, as the inhibitory phase of GTP action, due to G_i action and occurring at high concentrations (1 mM) of GTP, similarly decreased the level of adenylate cyclase activity in membranes from both untreated ($62 \pm 6\%$) and treated ($74 \pm 7\%$) cells [$n = 3$; decrease in activity seen with low concentrations (100 μ M) of GTP; see also Figure 2(a)]. Indeed, in P9 cells, as

compared with hepatocytes, higher, not lower, concentrations of GTP were required to activate adenylate cyclase. This suggests then that the prime mechanism through which culture of P9 cells with insulin influences regulation of adenylate cyclase at the G-protein level is by altering the functioning of G_s rather than any major effect caused by the increase in expression of G_i forms.

Changes in the guanine nucleotide regulation of adenylate cyclase might also occur as a result of alterations in the expression of one or more of the isoforms of this enzyme. Indeed, culture with insulin did affect adenylate cyclase itself, in that a small increase in the activity of the catalytic unit of this enzyme was detected by using forskolin (Figure 6a). This change was mediated by high-affinity insulin receptors, with an EC_{50} value of 23 ± 8 pM ($n = 3$). It did not, however, affect the dose-dependency of forskolin's stimulation of adenylate cyclase (EC_{50} values of 24 ± 6 μ M and 25 ± 4 μ M for cells cultured in the absence and presence of insulin respectively; Figure 6b; means $\pm$ S.D., $n = 3$). Indeed these values were very similar to those of ~ 10 – 20 μ M seen for native hepatocytes (Whetton et al., 1983). It remains to be seen whether the various isoforms of adenylate cyclase show differences in their susceptibility to activation by forskolin. However, our prejudice would be that the primary reason for the altered guanine nucleotide regulation of adenylate cyclase, caused by culture of P9 cells with insulin, is at the G-protein level.

To our knowledge, P9 cells provide the first example of an immortalized hepatocyte cell line which exhibits a functional glucagon response together with a high-affinity insulin-signalling system. The use of this cell line in the present study, which shows that culture of P9 cells with low, physiological, concentrations of insulin increases the expression of both α -G_i-2 and α -G_i-3, coupled with our previous observations that hypo-insulinaemia, seen in streptozotocin- and alloxan-induced diabetes (Gawler et al., 1987; Bushfield et al., 1990c; Griffiths et al., 1990) and in diabetic (*db/db*) mice (Palmer and Houslay, 1991), has the opposite effect, leads us to suggest that the expression of these

Figure 6 Insulin modulates the action of forskolin on adenylate cyclase in P9 cells

In (a) the effect of various insulin concentrations added to the culture medium was assessed on the ability of forskolin to increase cyclic AMP levels in P9 cells cultured for 16 h. Data are given as a percentage relative to the action seen in untreated cells incubated for the same period of time. Errors are S.D. for three separate experiments. In (b) is shown the dose-dependent ability of forskolin to increase intracellular AMP levels in P9 cells, expressed as pmol of cyclic AMP produced per 10⁶ cells, which either had (●) or had not (▲) been cultured for 16 h with 10 nM insulin. An experiment typical of one done three times is shown here, with assays done in triplicate.

two inhibitory G-proteins may be directly regulated by insulin action in both hepatocytes and P9 cells. Similarly, insulin would appear to be capable of regulating the ability of glucagon to activate adenylate cyclase through an action which is again centred at regulation by guanine nucleotides. Thus P9 cells, which express a number of the enzyme activities and regulatory signalling systems which are characteristic of fully differentiated hepatocytes, may provide a useful model system for analysing aspects of hepatocyte function and the cross-talk between different signalling systems.

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