Insulin inhibits the cholera-toxin-catalysed ribosylation of a M_r -25000 protein in rat liver plasma membranes

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A method is described for preparing a plasma-membrane fraction from hepatocytes by a rapid, gentle, Percoll fractionation procedure. Cholera toxin elicited the ribosylation of a number of proteins in these membranes, including the components of the stimulatory guanine nucleotide regulatory protein, N_s . Insulin, however, inhibited the ability of cholera toxin to ribosylate a protein of M_r 25000. The action was decreased in membranes from cells that had been pre-treated with glucagon. Ribosylation of both the components of N_s and the M_r -25000 species occurred in whole cells treated with cholera toxin, because membranes from such treated cells exhibited decreased labelling when incubated with [32 P]NAD⁺ and activated cholera toxin. The labelling of proteins, including the M_r -25000 species, with [32 P]NAD⁺ and cholera toxin in the plasma membranes was decreased by an inhibitor of ribosylation. Azido-GTP photoaffinity labelling identified several high-affinity GTP-binding proteins, including one of M_r 25000. Cholera toxin failed to ribosylate the M_r -25000 protein in membranes from cells that had been pre-treated with the tumour-promoting agent 12-*O*-tetradecanoylphorbol 13-acetate (TPA). In membranes from such treated cells, insulin actually allowed cholera toxin to label this species. As TPA activates protein kinase C, it is possible that the M_r -25000 protein, or a species that interacts with it, is a substrate for phosphorylation. These observations may offer an explanation for some of the perturbing effects that TPA exerts on insulin's action. It is suggested that (i) the insulin receptor interacts with the guanine nucleotide regulatory protein system in the liver, and (ii) that the M_r -25000 species may be a component of N_{in} , a specific guanine nucleotide regulatory protein that has been proposed to mediate certain of the actions of insulin on target cells [Houslay & Heyworth (1983) *Trends Biochem. Sci.* **8**, 449–452].

Insulin binds to specific receptors embedded within the cell plasma membrane. These are responsible for triggering a variety of responses within the cell. Although the receptors have been shown to exhibit a tyrosine kinase activity, the molecular mechanism whereby occupied insulin receptors elicit their effects has yet to be defined (Houslay, 1984*b*, 1985; Houslay & Heyworth,

1983; Denton *et al.*, 1981). Indeed, it has been suggested that multiple signalling mechanisms may exist (Houslay & Heyworth, 1983).

Insulin has, however, been demonstrated both to inhibit glucagon-stimulated adenylate cyclase activity (Heyworth & Houslay, 1983*b*) and to activate a peripheral liver plasma-membrane cyclic AMP phosphodiesterase (Marchmont & Houslay, 1980; Houslay *et al.*, 1983, 1984; Heyworth *et al.*, 1983*a,b*) both in intact hepatocytes and in broken membranes. These particular actions of insulin appear to be dependent on GTP, and it has been suggested that a unique guanine nucleotide regula-

Abbreviations used: SDS, sodium dodecyl sulphate; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

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tory protein, N_{in} , is responsible for mediating these effects of insulin (Heyworth & Houslay, 1983b; Houslay & Heyworth, 1983).

Many hormones exert their effects by altering the intracellular concentrations of cyclic AMP. They appear to do this by either activating or inhibiting adenylate cyclase (Ross & Gilman, 1980; Houslay, 1983, 1984a). This is achieved by the occupied hormone receptors interacting with specific species of guanine nucleotide regulatory proteins. Thus stimulatory effects are mediated through N_s (Northup *et al.*, 1983a,b; Hildebrandt *et al.*, 1983) and inhibitory effects through N_i (Katada & Ui, 1979, 1980, 1981; Hildebrandt *et al.*, 1983; Northup *et al.*, 1983b). Another guanine nucleotide regulatory protein, called transducin, has also been identified. This is responsible for mediating the rhodopsin-catalysed photoactivation of a cyclic GMP phosphodiesterase (Yamazaki *et al.*, 1983; Fung, 1983; Van Dop *et al.*, 1984). The identification of these distinct guanine nucleotide regulatory proteins has been greatly aided by specific toxins which catalyse the NAD^+ -dependent ADP-ribosylation of these proteins. Thus cholera toxin ribosylates and activates both N_s (Cassel & Pfeuffer, 1978) and transducin (Aboud *et al.*, 1982), whereas pertussis toxin (islet-activating protein) ribosylates and inhibits the functioning of both N_i (Katada & Ui, 1981; Hildebrandt *et al.*, 1983; Northup *et al.*, 1983b) and transducin (Van Dop *et al.*, 1984). These guanine nucleotide regulatory proteins all appear to be part of a family, as they consist of distinct species of α -subunits, which provide the site for ADP-ribosylation, and a common type of β -subunit (Manning & Gilman, 1983). These guanine nucleotide regulatory proteins are activated by interaction with the occupied receptor (or photoactivated rhodopsin), leading to a conformational change in the α -subunit, leading to its activation and dissociation from the β -subunit (Codina *et al.*, 1984b).

The putative N_{in} also appears to be acted on by cholera toxin. Evidence for this comes from the observation that cholera toxin activates the peripheral phosphodiesterase (Heyworth *et al.*, 1983b) and prevents insulin inhibiting adenylate cyclase (Heyworth & Houslay, 1983b), presumably by having activated N_{in} . Cholera toxin also obliterates insulin's GTP-dependent stimulatory effect on a cyclic AMP-independent protein kinase in sarcolemma membranes (Walaas *et al.*, 1981).

Unlike pertussis toxin, which only ribosylates a single protein species in hepatocytes (Heyworth *et al.*, 1984a) and other cell membranes (Katada *et al.*, 1982; Hanski & Gilman, 1982; Codina *et al.*, 1983; Bokoch *et al.*, 1983), cholera toxin catalyses the ribosylation of a number of plasma-membrane proteins (Cooper *et al.*, 1981; Gill, 1982). This

suggests that there may be a family of guanine nucleotide regulatory proteins. In this study we show that insulin inhibits the cholera-toxin-catalysed ribosylation of a single protein species of M_r 25000. The properties of this action of insulin are consistent with this target protein being the putative N_{in} that we (Houslay & Heyworth, 1983; Houslay *et al.*, 1984) have previously suggested mediates certain of the actions of insulin.

Materials and methods

Hepatocyte plasma membranes were prepared from male 200–250g Sprague–Dawley rats by a modification of the gentle disruption and Percoll fractionation procedure developed by us previously (Heyworth *et al.*, 1983b).

Preparation of Percoll gradient

A stock Percoll solution was made as a 90% (v/v) suspension in 0.25M-sucrose immediately before use. This stock solution was diluted to give final concentrations of 0.25M-sucrose, 3mM-imidazole and 15% (v/v) Percoll with respect to the 90% (v/v) stock Percoll solution. The pH of this suspension was adjusted to pH 7.4 with dilute HCl. The Percoll gradient was prepared by placing 30ml of this solution in 50ml centrifugation tubes and layering 2ml of 0.25M-sucrose/3mM-imidazole, pH 7.4, on top.

Preparation of hepatocytes

Hepatocytes were prepared from male Sprague–Dawley rats (225–250g body wt.) as described by Elliott *et al.* (1976). After the last wash with Krebs–Henseleit (1932) buffer, the cells (1g dry wt.) were washed finally with 0.25M-sucrose/3mM-imidazole, pH 7.4 (80ml), and sedimented in a MSE Minor bench centrifuge, at 500g_{max.} for 2.5 min. This and all further procedures were carried out at 4°C.

Homogenization of hepatocytes

The cells (1g dry wt.) were resuspended in 0.25M-sucrose/3mM-imidazole, pH 7.4 (40ml), and placed in a 50ml Amicon ultrafiltration device, which had the bottom filter plates removed and the gas outlet sealed with a gate clip. These cells were incubated for 10 min under N_2 (151b/in²). This system was depressurized rapidly by releasing the gate clip and allowing the cell suspension to be collected, through the bottom outlet valve of the Amicon ultrafiltration device, in a Camlab glass homogenization vessel (50ml capacity). With a Teflon pestle, the cells were homogenized with two gentle up-and-down strokes immediately after nitrogen cavitation (depressurization). This suspension was centrifuged at 500g_{max.} for 2.5 min. The supernatant was collected, and the pellet was

resuspended in 0.25M-sucrose/3mM-imidazole, pH 7.4 (20ml), and the whole of the above pressurization, depressurization and homogenization procedure was repeated once.

Preparation of plasma membranes from hepatocyte homogenates

The two supernatant fractions were combined and re-centrifuged at 27500g_{max} for 15min in a Beckman J2.21 centrifuge. The resulting pellet was resuspended in 0.25M-sucrose/3mM-imidazole, pH 7.4 (20ml), and 5ml of this was layered on top of each Percoll gradient. This gradient was centrifuged for 15min at 27500g_{max} in the Beckman J2.21 centrifuge. The plasma membranes appeared as a distinctive hazy band near the top of the Percoll gradient. The first 2ml of the gradient was removed and discarded. The plasma membrane fraction was collected in the next 5ml. This membrane fraction was diluted (1:2 in each instance) with 0.25M-sucrose/3mM-imidazole, pH 7.4. The collected diluted fractions when combined gave approx. 60ml of suspension from a liver. This was divided into two 30ml fractions, each of which was layered on a 10ml 'cushion' of 55% (w/v) sucrose/3mM-imidazole, pH 7.4. These gradients were centrifuged at 48000g_{max} for 15min. The membranes, which formed a layer at the bottom interface, were gently removed with a Pasteur pipette and washed once with 0.25M-sucrose/3mM-imidazole, pH 7.4, and collected by centrifugation at 48000g_{max} for 15min. In all cases care was taken to resuspend pellets by gentle agitation. They were never vortex-mixed. The loose pellet was stored in batches at -80°C until used. These batches were never used again after thawing.

Preparation of a crude washed plasma-membrane fraction

From cells which had been pre-treated with various agents, a crude washed plasma-membrane fraction was obtained as described in detail previously (Houslay & Elliott, 1979, 1981). In studies with these membranes and those prepared as above, essentially similar results were obtained.

Cholera-toxin-mediated ribosylation of hepatocyte plasma membranes

The cholera-toxin-catalysed ribosylation of these membranes, with [³²P]NAD⁺, was performed by a modification of the conditions used by Hildebrandt *et al.* (1983), except that ATP was not present in the incubation and Ca²⁺ (10μM) was added to the 'ribosylation mixture'. Briefly, membranes (50μg of protein) were incubated in a final volume of 50μl containing 15mM-thymidine, 100mM-potassium phosphate buffer (pH 7.5),

6mM-dithiothreitol and 20μM-[³²P]NAD⁺ (sp. radioactivity 500mCi/mmol). Incubations were performed for 10min at 30°C. In some experiments GTP or its analogue (0.1mM), insulin (1nM) and glucagon (0.1nM) were present. Ribosylation was elicited by the addition of cholera toxin (67.25μg/assay) to the assay. This was pre-activated (Johnson & Bourne, 1977) before its addition to the ribosylation assay. Briefly, this involved taking 50μl of stock cholera toxin (1mg/ml) and adding it to 50μl of 50mM-dithiothreitol. This was preincubated at 30°C for 20min before its addition (12.5μl) to the ribosylation mixture (total volume 50μl in all cases). After incubation of the membranes with cholera toxin, the samples were transferred to an ice bath and diluted 20-fold with ice-cold 50mM-Tris/HCl buffer, pH 7.4. The sample was centrifuged at 14000g for 10min at 4°C and the membrane pellet resuspended in Laemmli (1970) sample buffer. SDS/polyacrylamide-gel electrophoresis was performed as described by Laemmli (1970). In this instance 5%-acrylamide stacking gels were used, with a 12%-acrylamide running gel (see Heyworth *et al.*, 1984a). Autoradiography was performed with Kodak XAR-5 X-ray film by using Cronex-Dupont intensifying screens. Gels were scanned with a LKB laser densitometer with attached integrator.

Removal of the [³²P]ADP-ribose moiety from the protein was performed as described by Van Dop *et al.* (1984). The photoreactive GTP analogue GTP-γ-azidoanilide [guanosine 5'-O-triphospho-(5')-4-azidoanilide] was used to label GTP-binding proteins (Pfeuffer, 1977) as described previously for liver plasma membranes (Wong & Martin, 1983). ³²P-labelled GTP-γ-azidoanilide was synthesized from [γ-³²P]GTP prepared by the method of Johnson & Walseth (1979) and used to photo-label plasma membranes as described by Wong & Martin (1983). Guanosine 5'-[γ-thio]triphosphate (250μM) was used to identify high-affinity GTP-binding sites by inhibiting their labelling with GTP-γ-azidoanilide (Wong & Martin, 1983; Pfeuffer, 1977).

Insulin analysis

¹²⁵I-labelled bovine insulin (1nM) was incubated at 30°C for 10min in 100mM-potassium phosphate buffer, pH 7.5, containing 6mM-dithiothreitol. It was then either subjected to h.p.l.c. analysis or insulin radioimmunoassay analysis. H.p.l.c. was carried out on an LKB system with a reverse-phase column (Lichrosorb RP-18, 5μM) with isocratic elution with 0.1M-NaH₂PO₄/H₃PO₄, pH 3.1, 0.1% triethylamine and 36% acetonitrile. This allowed for the separation of insulin, its a-chain and its b-chain with retention times of approx. 5.5, 4.5 and

6.5 min respectively at a flow rate of 0.5 ml/min. Radioactivity was assessed by γ -emission. Insulin radioimmunoassay was performed in the clinical routine laboratory of the Department of Clinical Biochemistry, Royal Infirmary, Glasgow. In this assay, using a polyclonal anti-insulin antibody, the isolated a- and b-chains expressed 0.016% and 0.025% of the reactivity compared with native insulin (100%).

From both of these approaches it was shown that insulin did not (<5%) dissociate into its constituent chains under the assay conditions described.

Cholera toxin, pig and bovine insulin, bovine serum albumin, thymidine, TPA, snake venom phosphodiesterase (EC 3.1.4.1; *Crotalus atrox*) and alkaline phosphatase (EC 3.1.3.1; *Escherichia coli*) were from Sigma (London) Chemical Co., Poole, Dorset, U.K. Glaxo compound no. 117 (see Tait & Nassau, 1984) was kindly given by Dr. C. J. Coulson, Glaxo, Ware, Herts., U.K. Radiolabelled M_r marker proteins were from Amersham International, Amersham, Bucks., U.K. Nicotinamide-[adenylate- ^{32}P]adenine dinucleotide was from N.E.N., Southampton, U.K. Guanine nucleotides, dithiothreitol and all other enzymes were from Boehringer (U.K.), Lewes, East Sussex, U.K. ^{125}I -insulin was kindly given by Dr. D. O'Reilly, Glasgow Royal Infirmary, and glucagon by Dr. W. W. Bromer of Eli Lilly and Co., Indianapolis, IN, U.S.A. Gel-electrophoresis reagents were from Bio-Rad, Watford, Herts., U.K. All general chemicals were from BDH Chemicals, Poole, Dorset, U.K. Pure pertussis toxin (islet-activating protein) from *Bordetella pertussis* was obtained from the PHLS Centre for Applied Microbiology and Research, Porton Down, Wilts., U.K.

Results

Hepatocyte plasma membranes were isolated by a modification of the rapid, gentle, Percoll frac-

tionation procedure developed by us previously (Heyworth *et al.*, 1983b). The marker enzyme distribution for these is shown in Table 1. The plasma-membrane markers, leucine aminopeptidase (Peters *et al.*, 1972) and 5'-nucleotidase (Gurd & Evans, 1974; Reimer & Widnell, 1975), showed a pronounced purification over the homogenate. This fraction appeared to be relatively free of contamination from endoplasmic reticulum, mitochondria and lysosomes, as indicated by glucose-6-phosphatase, succinate dehydrogenase and acid β -galactosidase activities respectively. This fraction does, however, show contamination with Golgi, as indicated by the results with galactosyltransferase. This method provides a rapid, gentle, means of preparing a fraction enriched in plasma membranes.

Plasma membranes were incubated with cholera toxin (125 $\mu\text{g}/\text{ml}$) and [^{32}P]NAD $^{+}$ for 10 min at 30°C as detailed in the Materials and methods section. Such a treatment led to the labelling of a number of protein species, which were resolved by SDS/polyacrylamide-gel electrophoresis and identified by autoradiography. Densitometric scans of such gels are shown in Fig. 1. This labelling was believed to be due to the transfer of the ADP-ribose moiety of [^{32}P]NAD $^{+}$, as protein-bound label was stable to alkaline phosphate treatment but was removed by snake venom phosphodiesterase (Van Dop *et al.*, 1984; results not shown). Furthermore, washing the membrane samples by repeated centrifugation and resuspension (Houslay & Elliott, 1979, 1981) did not alter the pattern of labelling obtained and, indeed, no labelling was observed in the absence of cholera toxin (Fig. 1). We also found (results not shown, but see also Figs. 3 and 6) that similar labelling patterns were obtained by using either these purified plasma membranes or a crude plasma-membrane fraction prepared as described previously (Houslay & Elliott, 1979, 1981).

Table 1. Enzyme profile of Percoll-fractionated hepatocyte plasma membrane

Specific activities are shown as the averages ($\pm$ S.D.) of three separate experiments. Abbreviation: n.d., not determined.

Marker enzyme	Specific activity in isolated membranes	Purification over homogenate (-fold)	Yield (%)
Glucose-6-phosphatase	12 $\pm$ 5 nmol/min per mg of protein	0.75	1.3
Galactosyltransferase	5 $\pm$ 2 pmol/min per mg of protein	4	8
Succinate dehydrogenase	300 $\pm$ 15 pmol/min per mg of protein	0.22	0.4
Acid β -galactosidase	0.15 $\pm$ 0.005 pmol/min per mg of protein	0.25	0.2
Leucine aminopeptidase	503 $\pm$ 24 nmol/min per mg of protein	20	21
5'-Nucleotidase	298 $\pm$ 20 nmol/min per mg of protein	15	25
(Glucagon + GTP)-stimulated adenylate cyclase	73 $\pm$ 8 pmol/min per mg of protein	n.d.	n.d.
Cyclic AMP phosphodiesterase	30 $\pm$ 3 pmol/min per mg of protein	n.d.	n.d.

Fig. 1. Cholera toxin elicits the [32 P]NAD $^{+}$ -dependent labelling of a number of hepatocyte plasma-membrane proteins: inhibition of the labelling of a M_r -25000 species by insulin

Purified hepatocyte plasma membranes were incubated in a ribosylation mixture (see the Materials and methods section) containing [32 P]NAD $^{+}$ alone (a), plus cholera toxin (b), plus cholera toxin and GTP (c), plus cholera toxin, GTP and insulin (d) and plus cholera toxin, GTP and glucagon (e). The membranes were subjected to SDS/polyacrylamide-gel electrophoresis and autoradiography as described in the Materials and methods section. This Figure shows a densitometric scan of the autoradiography; this is a typical result of at least six experiments. M_r values of 25000 (i), 42000 (ii) and 45000 (iii). Insulin, glucagon and GTP were present at concentrations of 1 nM, 10 nM and 0.1 mM respectively.

There are two major labelled bands, of M_r 42000 and 45000 (Fig. 1), where cholera-toxin-mediated ribosylation was enhanced by GTP. These are presumably the α -subunit(s) of N_s . Whether one is

a modified form of the other remains to be seen. Various investigators have referred to the α -subunit of N_s as being either a M_r -42000 or M_r -45000 component, with purified preparations obtained from either erythrocytes (Pfeuffer *et al.*, 1983) or liver (Kahn & Gilman, 1984; Codina *et al.*, 1984a). Others (Malbon & Greenberg, 1982; Cooper *et al.*, 1981) have noted that two bands, of M_r 42000 and M_r 45000, appear to form the major cholera-toxin substrates in rat liver plasma membranes, although Doberska *et al.* (1980) noted only a M_r -42000 component. In the present study we observed the labelling of both the M_r -42000 and M_r -45000 species, although the intensity of labelling of the M_r -45000 species appeared to vary somewhat between experiments. Certainly, over this period of incubation with cholera toxin the adenylate cyclase activity has become fully activated (Martin *et al.*, 1977; C. M. Heyworth & M. D. Houslay, unpublished work). Our data (Fig. 1) also confirm other studies (Doberska *et al.*, 1980; Cooper *et al.*, 1981; Malbon & Greenberg, 1982) that purified liver plasma membranes contain a number of targets for cholera-toxin-catalysed ADP-ribosylation, other than just the components of N_s . One clearly resolved target, whose labelling by cholera toxin and [32 P]NAD $^{+}$ shows a marked GTP-dependent effect, has M_r 25000. Under the conditions used, labelling of the M_r -25000, -42000 and -45000 bands were maximal after 15 min incubation with pre-activated cholera toxin (125 μ g/ml). (In the studies described here, incubations were carried out for 10 min at 30°C.)

We note here (Fig. 1; Table 2) that, if insulin (1 nM) was included in the ribosylation assay together with GTP (0.1 mM), the labelling of the M_r -25000 subunit was inhibited. This effect of insulin occurred at half-maximal concentrations of 0.1–0.5 nM (range, $n = 2$). We also observed that, when high concentrations of insulin (greater than 10 nM) were used, the ability of insulin to block the cholera-toxin-mediated labelling of the M_r -25000 species was lost. Similarly, high concentrations of insulin (> 10 nM) have also been shown to lead to a loss in the ability of insulin to activate cyclic AMP phosphodiesterase (Elks *et al.*, 1983) and to inhibit adenylate cyclase (C. M. Heyworth & M. D. Houslay, unpublished work). Insulin did not, however, affect the degree of labelling of the M_r -42000 and -45000 bands (Table 2). If glucagon, rather than insulin, was included in the ribosylation mixture, no effect on the labelling of the M_r -25000 band was observed. However, we noted that glucagon, unlike insulin, allowed a small increase in the labelling of the M_r -42000 and -45000 bands during the experiment (Table 2).

Photoaffinity labelling of the purified plasma membranes with [32 P]GTP-azidoanilide allowed

Table 2. *Cholera-toxin-catalysed labelling of plasma-membrane proteins*

Results are expressed as means $\pm$ s.d. for n experiments using different cell preparations; data obtained for partially purified or purified plasma-membrane preparations are combined. Hormones were added to the ribosylation mixture as described in detail in the Materials and methods section. The degree of labelling was quantified by densitometric analysis of autoradiographs. In each instance comparison was made with appropriate controls with hormone present. All labelling reactions were performed in the presence of GTP (0.1 mM).

Hormone added	n	Band (M_r) ...	Labelling (% of control)		
			25000	42000	45000
Insulin (1 nM)	9		58 $\pm$ 11*	105 $\pm$ 9	102 $\pm$ 7
Glucagon (1 nM)	5		95 $\pm$ 12	116 $\pm$ 4	115 $\pm$ 8

* Range 37–71% of control.

several labelled species to be identified after SDS/polyacrylamide-gel electrophoresis and autoradiography (Fig. 2). The addition of guanosine 5'-[γ -thio]triphosphate (250 μ M) to the photolabelling assays decreased the labelling of these species, indicating that these might exhibit high-affinity GTP-binding sites (Fig. 2). There were differences in the degree of effectiveness of guanosine 5'-[γ -thio]triphosphate as regards particular bands (Fig. 2), no doubt reflecting the different affinities of these species for both GTP and guanosine 5'-[γ -thio]triphosphate, as has been shown for N_s and N_i (see Hildebrandt *et al.*, 1982). In particular, we noted that a component of M_r 25000 was labelled (Fig. 1). This presumed high-affinity GTP-binding protein co-migrated with the insulin-sensitive ribosylated component, of the same M_r , when run on the same gel.

If intact hepatocytes are preincubated with cholera toxin (1 μ g/ml) for 45 min at 37°C, it is known that adenylate cyclase has already become fully activated after 30 min incubation (Houslay & Elliott, 1979, 1981). When membranes from hepatocytes treated in such a fashion were incubated in the ribosylation assay, no labelling of the M_r -25000 band was seen and labelling of the M_r -42000 and -45000 components of N_s was decreased markedly (Fig. 3). Pre-treatment of the intact cells with islet-activating protein (pertussis toxin) at 100 ng/ml for 1 h, however, actually enhanced the ability of cholera toxin to ribosylate the components (M_r -42000 and -45000 bands) of N_s , in isolated membranes under the conditions specified (Fig. 3). It did not, however, enhance the ability of cholera toxin to ribosylate the M_r -25000 species. Indeed, the labelling of this species by cholera toxin was decreased by about 19–33%.

When thiol-pre-activated pertussis toxin was used to label plasma membranes (Fig. 3) with [32 P]NAD $^{+}$, a major ribosylated band of M_r -40000 was observed (Heyworth *et al.*, 1984a). The labelling of this band appeared to be enhanced in cells that had been pre-treated with cholera toxin,

Fig. 2. [32 P]GTP-azidoanilide causes the photolabelling of a series of plasma-membrane proteins

The experiment was performed as described in the Materials and methods section, using purified hepatocyte plasma membranes. Labelling was performed with [32 P]GTP-azidoanilide in either the absence (tracks *a* and *c*) or presence (track *b*) of guanosine 5'-[γ -thio]triphosphate (0.25 mM). Tracks (*a*) and (*c*) are different experiments. This shows a typical result of at least four experiments.

and decreased in those pre-treated with pertussis toxin (Fig. 3).

Glaxo compound no. 117 inhibits the ability of cholera toxin both to elicit NAD $^{+}$ -dependent ADP-ribosylation and to activate adenylate cyclase (Coulson *et al.*, 1983; Tait & Nassau, 1984). When the ribosylation inhibitor compound 117 (0.5 mM) was added to the ribosylation assays containing GTP (0.1 mM), the cholera-toxin-induced labelling of the M_r -25000, -42000 and -45000 protein species was inhibited (Fig. 4). This provides further support to the contention that the labelling of these bands reflects their ADP-ribosylation by cholera toxin.

Fig. 3. Cholera and pertussis toxins modify their plasma-membrane target proteins in intact hepatocytes

Hepatocytes were preincubated (i) with cholera toxin (1 ng/ml) for 45 min at 37°C (tracks d, e, f), (ii) with pertussis toxin (100 ng/ml) for 60 min at 37°C (tracks g, h, i) or (iii) without such toxin (tracks a, b, c) for either period of time (with similar results), before a crude plasma-membrane preparation was made. These isolated plasma membranes were then incubated in a 'ribosylation mixture' containing 0.1 mM-GTP, as described in the Materials and methods section, with no other addition (tracks a, d, g), with cholera toxin (b, e, h), or with pertussis toxin (c, f, i). The autoradiograph of a typical experiment is shown here. Track (j) shows the M_r markers, of 200 000, 92 500, 69 000, 46 000, 30 000 and 14 300. This shows a typical result of three experiments.

Fig. 4. Glaxo compound 117 prevents cholera toxin from labelling plasma-membrane proteins

Membranes were incubated in the ribosylation mixture together with GTP (0.01 mM) in either the absence (track a) or presence (track b) of Glaxo compound 117 (0.5 mM). This shows a typical result of three experiments.

If hepatocytes were preincubated for 5 min with insulin (1 nM) at 30°C, before preparation of membranes for use in the ribosylation assay, we observed that cholera toxin still affected the ribosylation of the M_r -25 000 species. Furthermore, when insulin (1 nM) was added to the ribosylation assay containing such pre-treated membranes, the addition of insulin to the membrane ribosylation assay still led to the inhibition of the ribosylation of the M_r -25 000 component (results not shown). However, if the hepatocytes were pre-treated for 5 min with glucagon (10 nM) at 37°C, before preparation of membranes, then, although cholera-toxin treatment of the isolated membranes led to the ribosylation of the M_r -25 000 species (Fig. 5), such a pre-treatment decreased the ability of insulin (1 nM) to inhibit the cholera-toxin-mediated ribosylation of this species in isolated membranes (Fig. 5). In such instances insulin decreased the labelling of the M_r -25 000 band to 75–106% of the control (GTP + cholera toxin; given as range for three separate experiments).

In membranes from hepatocytes pre-treated for 15 min at 37°C with TPA (10 ng/ml), cholera toxin failed to label the M_r -25 000 band, even though labelling of the M_r -42 000 and -45 000 bands ensued (Fig. 6), with a similar intensity to that of control

cells. However, if insulin (1 nM) was added to the ribosylation mixture, cholera toxin then appeared able to elicit the labelling of the M_r -25 000 band. The presence of insulin did not affect the degree of labelling of any other band (Fig. 6).

The M_r -25 000 band appeared to be localized in the plasma-membrane fraction, as it was not observed when various other subcellular membrane fractions from a Percoll fractionation of the cell homogenate (Heyworth *et al.*, 1983a) were treated with [32 P]NAD⁺ and cholera toxin (results not shown).

Discussion

Cholera toxin not only activates adenylate cyclase in whole cells (see Gill, 1982; Houslay & Elliott, 1979, 1981) but will also elicit activation in broken membranes if it is first pre-activated by exposure to thiol reagents (Johnson & Bourne, 1977; Gill, 1977) and then incubated with the

membranes together with NAD^+ and GTP. It elicits this activation by triggering the ADP-ribosylation of the α -subunit(s) of N_s (Cassel & Pfeuffer, 1978), M_r -42000 and for M_r 45000 (Pfeuffer *et al.*, 1983; Kahn & Gilman, 1984; Codina *et al.*, 1984a).

In purified liver (Doberska *et al.*, 1980; Cooper *et al.*, 1981; Malbon & Greenberg, 1982; Gordon & Blecher, 1984) and other (Cooper *et al.*, 1981) plasma membranes a number of proteins can be ADP-ribosylated by cholera toxin in an NAD^+ -dependent fashion. In the present study we confirm those observations and show that the effects are enhanced by GTP (Fig. 1).

The enhanced labelling by cholera toxin of the components of N_s , which we observed in membranes from pertussis-toxin pre-treated cells, might offer a molecular explanation for the observations by Hsia *et al.* (1984), who noted that such a pre-treatment enhanced the ability of cholera toxin to activate adenylate cyclase. We also demonstrate here that a number of proteins that are ADP-ribosylated by cholera toxin co-migrate with species that apparently exhibit specific GTP-binding sites as identified by their labelling with azido-GTP (Fig. 2).

We have proposed that insulin exerts certain of its actions through a specific species of guanine nucleotide regulatory protein called N_{in} , which should be a substrate for the action of cholera toxin (Houslay & Heyworth, 1983; Houslay *et al.*, 1984). The activation of N_s by cholera toxin and guanine nucleotides causes the dissociation of the N_s complex to yield the free β -subunit and an activated α -subunit (see Houslay, 1984a, for review). The conformation of the released activated α -subunit has been shown (Hudson *et al.*, 1981) to differ from the inactive α -subunit, which is complexed with the β -subunit (N_s complex). So far, all of the guanine nucleotide regulatory proteins investigated to date have a similar structure, with an α - (GTP-binding) subunit which dissociates from a (common) β -subunit (Manning & Gilman,

Fig. 5. Insulin decreases the cholera-toxin-mediated labelling of a M_r -25000 protein in liver plasma membranes. Hepatocytes were preincubated for 5 min at 37°C in either the absence (tracks a, b, c) or presence (tracks d, e) of glucagon (1 nM). A crude plasma-membrane fraction was prepared from them and used in the ribosylation assays. Incubations were carried out either in the absence (track a) or presence (tracks b, c, d, e) of cholera toxin plus GTP (0.1 mM). In tracks (c) and (e), insulin (1 mM) was also present. This shows a typical result of at least three experiments. (A) Autoradiography; (B) densitometric scans of autoradiography.

1983). It is not too unreasonable to suggest that the putative N_{in} has a similar structure. If so, one might expect that, at the very least, insulin should cause the activation of the GTP-binding (α -subunit) of the putative N_{in} . In doing so the conformation of ' N_{in}^{α} ' would be expected to be altered. Such a change might possibly affect the sensitivity of this protein to ribosylation by cholera toxin. Indeed, we show here that the cholera-toxin-mediated ribosylation of an M_r -25000 protein was either decreased or blocked if insulin was present in the ribosylation assay. This suggests that the occupied insulin receptor might interact with this species. The labelling of this species was only seen if GTP was also present in the ribosylation mixture. This is consistent with the requirement for GTP in order for insulin to inhibit adenylate cyclase (Heyworth & Houslay, 1983b) and to activate the cyclic AMP phosphodiesterase from liver plasma membranes (M. D. Houslay, unpublished work). Furthermore, we also observed (Fig. 2) that a high-affinity GTP-binding protein appears to migrate with a similar M_r of 25000. We therefore suggest that N_{in} has a M_r -25000 protein component which provides a GTP-binding site, a site for cholera-toxin-mediated ADP-ribosylation and a site for interaction with the insulin receptor.

Such a M_r -25000 species has also been observed by Gordon & Blecher (1984) to be ADP-ribosylated by cholera toxin. Indeed, they demonstrated that a cytosol factor enhanced the ability of cholera toxin to cause the ribosylation of both this species and N_s .

N_{in} is a substrate for cholera toxin not only in isolated membranes but also in whole cells. This is clearly seen in experiments where hepatocytes were preincubated with cholera toxin before

Fig. 6. TPA blocks the cholera-toxin-mediated labelling of the M_r -25000 protein: an effect that is overcome by insulin

Hepatocytes were preincubated for 15 min at 37°C with TPA (10 ng/ml), after which a crude plasma-membrane fraction was prepared and used in the ribosylation assay (see the Materials and methods section). Incubations were carried out with cholera toxin and GTP (0.1 mM) in either the absence (track a) or presence (track b) of insulin (1 nM). Membranes from control cells, preincubated in the absence of TPA, were subsequently used in the ribosylation assay and treated with cholera toxin plus GTP (0.1 mM) in either the absence (tracks c and e) or presence (tracks d and f) of insulin (1 nM). Tracks (a-d) were run on the same gel, whereas tracks (e) and (f) were run on a different gel but with membranes from the same experiment. (A) autoradiography; (B) densitometric scan of autoradiography. This shows a typical result of three experiments.

membrane preparation. In such instances no labelling of the M_r -25000 species and little labelling of N_s (M_r -42000 and -45000) occurred when membranes from cholera-toxin-pre-treated cells were incubated with [32 P]NAD $^{+}$ together with cholera toxin.

The identification of this M_r -25000 component with N_{in} is further supported by the observation that the ability of insulin to inhibit the cholera-toxin-mediated ribosylation of N_{in} was decreased in membranes from glucagon-pre-treated (desensitized) hepatocytes (Fig. 5). This is consistent with our observations that glucagon pre-treatment of hepatocytes obliterated the ability of insulin both to activate the peripheral cyclic AMP phosphodiesterase (Heyworth *et al.*, 1983b) and to inhibit hormone-stimulated adenylate cyclase (Heyworth & Houslay, 1983b). There we suggested that treatment of intact hepatocytes with glucagon led to a modification of N_{in} which prevented its activation by insulin. The nature of this cyclic-AMP-independent modification (Heyworth & Houslay, 1983a) remains to be determined.

TPA is a potent tumour-promoting agent (Weinstein *et al.*, 1978), which appears to be able to activate protein kinase C (Nishizuka, 1983) and can also enhance tyrosine phosphorylation in intact cells (Gilmore & Martin, 1983; Grunberger *et al.*, 1984), including that of the insulin receptor (Jacobs *et al.*, 1983) in intact cells. Here we see that, in membranes from cells pre-treated with TPA, cholera toxin then failed to elicit the ribosylation of the M_r -25000 protein (Fig. 6). Indeed, with such membranes, insulin now actually allowed cholera toxin to ribosylate this M_r -25000 species (Fig. 6). Clearly, in the present, as in all of these ribosylation studies, we can make no functional extrapolations from the degree, or not, of labelling that occurred. However, phorbol esters exert insulin-like actions in stimulating both lipogenesis (van de Werve *et al.*, 1985) and lactate production (Brown *et al.*, 1979), as well as inhibiting glucagon-stimulated adenylate cyclase at its point of regulation by guanine nucleotide (Heyworth *et al.*, 1984b). They also modulate the stimulatory effects of submaximal insulin concentrations on lipolysis (van de Werve *et al.*, 1985), implying that insulin and TPA can exert actions on a common target, perhaps N_{in} . The changes that are elicited nevertheless provide a strong indication for functional interactions between components, in particular the possibility that the insulin receptor interacts with a species of M_r 25000, tentatively identified as N_{in} .

The epidermal-growth-factor receptor, which bears many similarities to that for insulin (see Houslay, 1981, 1984b), has been shown to interact with H-ras-p21, the oncogene product of Harvey

murine sarcoma virus (Kamata & Feramisco, 1984). Preliminary experiments by the same authors have indicated that this is also true of the insulin receptor (Kamata & Feramisco, 1984). This oncogenic product, of M_r 21000–24000, associates with the plasma membrane and exhibits a high-affinity GTP-binding site (Cooper & Lane, 1984; McGrath *et al.*, 1984; Shih *et al.*, 1980; Willingham *et al.*, 1980). It is possible that N_{in} may be a cellular variant of p21 or a related protein, as a cellular homologue of viral p21 is expressed in small amounts in normal cells (Langbeheim *et al.*, 1980; Cooper & Lane, 1984). N_{in} might thus have importance in regulating a number of cellular processes, including exerting an influence on cell growth control (see Houslay & Heyworth, 1983).

At a minimum, our results suggest that the insulin receptor interacts with the guanine nucleotide regulatory system in liver plasma membranes. The resolution and reconstitution of the putative N_{in} with the insulin receptor and other components is now required.

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