Biochem. J. (1989) 259, 191–197 (Printed in Great Britain)

Glucagon desensitization of adenylate cyclase and stimulation of inositol phospholipid metabolism does not involve the inhibitory guanine nucleotide regulatory protein G_i, which is inactivated upon challenge of hepatocytes with glucagon

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Brief exposure of hepatocytes to glucagon, angiotensin or the protein kinase C activator TPA (12-*O*-tetradecanoylphorbol 13-acetate) caused the inactivation of the inhibitory guanine nucleotide regulatory protein G_i. Glucagon-mediated desensitization of glucagon-stimulated adenylate cyclase activity was seen in hepatocytes from both normal rats and those made diabetic with streptozotocin, where G_i is not functionally expressed. Normal glucagon desensitization was seen in hepatocytes from young animals, 6 weeks of age, which had amounts of G_i in their hepatocyte membranes which were some 45 % of that seen in mature animals (3.4 pmol/mg of plasma-membrane protein). Streptozotocin-induced diabetes in young animals abolished the appearance of functional G_i in hepatocyte plasma membranes. Pertussis-toxin treatment of hepatocytes from both normal mature animals and those made diabetic, with streptozotocin, blocked the ability of glucagon or angiotensin or TPA to elicit desensitization of adenylate cyclase. The isolated B (binding)-subunit of pertussis toxin was ineffective in blocking desensitization. Neither induction of diabetes nor treatment of hepatocytes with pertussis toxin inhibited the ability of glucagon and angiotensin to stimulate the production of inositol phosphates in intact hepatocytes. Thus (i) G_i does not appear to play a role in the molecular mechanism of glucagon desensitization in hepatocytes, (ii) the G-protein concerned with receptor-stimulated inositol phospholipid metabolism in hepatocytes appears not to be a substrate for the action of pertussis toxin, (iii) in intact hepatocytes, treatment with glucagon and/or angiotensin can elicit the inactivation of the inhibitory G-protein G_i, and (iv) pertussis toxin blocks desensitization by a process which does not involve G_i.

INTRODUCTION

In many instances, challenge of cells with hormones leads to a rapid stimulatory response, followed by its attenuation through a desensitization process. In hepatocytes, glucagon causes a transient rise in intracellular cyclic AMP concentrations. This is due in part to a rapid desensitization process and in part to the activation of specific high-affinity cyclic AMP phosphodiesterases (Heyworth *et al.*, 1983; Heyworth & Houslay, 1983; Houslay, 1986). We have demonstrated that the glucagon-elicited desensitization of glucagon-stimulated adenylate cyclase activity in hepatocytes is not mediated by increased concentrations of cyclic AMP (Heyworth & Houslay, 1983). Rather, it appears to occur as a consequence of activation of inositol phospholipid metabolism by glucagon (Wakelam *et al.*, 1986; Murphy *et al.*, 1987). This is compatible with observations that such a desensitization process can also be elicited by hormones such as angiotensin and vasopressin, which also stimulate inositol phospholipid metabolism (Murphy *et al.*, 1987). That desensitization can be mimicked by the tumour-promoting agent TPA suggests that activation of protein kinase C may play a pivotal role in mediating the desensitization process (Heyworth *et al.*, 1984*b*, 1985*a*). Any role of increased cyclic AMP appears to be restricted to attenuating the resensitization process (Murphy & Houslay, 1988).

The site of the lesion of desensitization in hepatocytes has been shown (Heyworth & Houslay, 1983; Murphy *et al.*, 1987) to be at the level of coupling between the glucagon receptor and the stimulatory guanine nucleotide regulatory protein (G-protein) G_s. Indeed, as the functioning of G_s appears to be modified as a consequence of desensitization, we have therefore suggested (Heyworth & Houslay, 1983; Houslay, 1986) that G_s is a primary target for the desensitization process, although a modification of the glucagon receptor itself cannot be excluded. However, we (Heyworth *et al.*, 1984*a*), in hepatocytes, and others (Wilson *et al.*, 1986), in renal epithelial cells, have shown that pre-treatment of cells with pertussis toxin can completely obliterate the desensitization process. Now, a well-defined action of pertussis toxin is to cause the NAD^+-dependent ADP-ribosylation of the α subunit of the inhibitory G-protein G_i as well as that of a distinct G-protein called G_o, whose

Abbreviations used: G_i, guanine nucleotide regulatory protein controlling inhibition of adenylate cyclase; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; G_s, guanine nucleotide regulatory protein controlling activation of adenylate cyclase; p[NH]ppG, guanylyl 5′-imidodiphosphate; TH-glucagon, [1-*N*-α-trinitrophenylhistidine,12-homoarginine]glucagon.

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function has yet to be unequivocally elucidated (Gilman, 1987). Rat hepatocytes appear to express a single pertussis-toxin substrate, namely G_i (Heyworth *et al.*, 1984*a*; Itoh *et al.*, 1984). This G-protein can be triggered to exert an inhibitory effect on adenylate cyclase activity, and such an action can be completely blocked by prior treatment of hepatocytes with pertussis toxin (Heyworth *et al.*, 1984*a*; Gawler *et al.*, 1987). This observation might be taken to imply that desensitization could be mediated by the constitutive activation of G_i. Furthermore, in kidney MDCK cells it has been suggested (Rich *et al.*, 1984) that an increased expression of G_i might account for glucagon desensitization there.

In the present study, however, we demonstrate that the glucagon-mediated desensitization of adenylate cyclase occurs in hepatocytes from both young animals, which show marked decreases in G_i, compared with amounts expressed in mature animals, and also in hepatocytes from streptozotocin-diabetic animals, which fail to express functional G_i. Furthermore, we show that glucagon-induced desensitization leads to the functional inactivation of G_i rather than its constitutive activation. We thus demonstrate that the constitutive activation of G_i is not the molecular event which underlies the desensitization process in hepatocytes.

METHODS

Preparation and incubation of hepatocytes

Hepatocytes were prepared from fed 225–250 g male Sprague–Dawley rats by the method of Elliott *et al.* (1976). In instances where mature animals were used, these were 225–250 g in size. Young rats were 6 weeks old, i.e. 3 weeks post-weaning, and were 100–120 g in size. The hepatocytes were incubated as described in some detail by Heyworth & Houslay (1983). Briefly, cells (3–5 mg dry wt/ml) were preincubated at 37 °C for at least 20 min before use in Krebs–Henseleit (1932) buffer containing 2.5% (w/v) bovine serum albumin, 2.5 mM-$CaCl_2$ and 10 mM-glucose, and gassed (O_2/CO_2, 19:1) for 30 s every 10 min. Ligands were added to the reaction vessel in a volume which was less than 1% of the total incubation volume. After the appropriate time interval, samples were removed and the cells quenched by adding an equal volume of ice-cold 1 mM-$KHCO_3$, pH 7.2, and then placing them on ice. All further procedures were performed at 4 °C.

The ATP content of the isolated hepatocytes was determined by the luciferase method on a neutralized $HClO_4$ extract (Stanley & Williams, 1969). As done previously, cells with an ATP concentration of 8.6 nmol/mg dry wt. were judged to be viable, and were therefore used (Smith *et al.*, 1978; Heyworth & Houslay, 1983).

Preparation of liver and hepatocyte membranes

A washed membrane fraction was obtained as previously described (Houslay & Elliott, 1979). In all cases, membranes were used within 2 h of preparation. In some experiments, membranes were also made from whole liver, as described previously by us (Marchmont *et al.*, 1981).

Assay of adenylate cyclase

Adenylate cyclase was assayed as described previously (Houslay *et al.*, 1976) in a mixture containing (final concns.) 1.5 mM-ATP, 5 mM-$MgSO_4$, 10 mM-theophylline, 1 mM-EDTA, 7.4 mg of phosphocreatine/ml, 1 mg of creatine kinase/ml and 24 mM-triethanolamine/KOH buffer, pH 7.4. The cyclic AMP produced was measured in a binding assay using the cyclic AMP-binding subunit of protein kinase prepared from bovine heart (Whetton *et al.*, 1983).

Labelling and assay of G_i

Islet-activating-protein-catalysed ADP-ribosylation of hepatocyte membranes with [^{32}P]NAD^+ was performed essentially as described by Heyworth *et al.* (1985*a*). Detection of functional G_i was assessed by demonstrating the ability of low concentrations of the non-hydrolysable GTP analogue, p[NH]ppG, to inhibit adenylate cyclase activity which had been amplified by the diterpene forskolin (Gawler *et al.*, 1987). Sucrose-density-gradient-purified membranes from whole liver (Marchmont *et al.*, 1981) were used for the quantification of G_i by the Western-blotting procedure, as described in detail in Gawler *et al.* (1987).

Induction of diabetes

Diabetes was induced in male Sprague–Dawley rats weighing 200–220 g by one intraperitoneal injection of streptozotocin (50 mg/kg; 0.3 ml/animal) in sterile citrate buffer, pH 4.0, as described by Sauerheber *et al.* (1984), and in some detail by us previously (Gawler *et al.*, 1987). Animals were used when they were clearly diabetic (as judged by blood and urine glucose concentrations) which usually occurred 7 days after injection (for details see Gawler *et al.*, 1987, 1988).

Inositol phosphate production

The production of total inositol phosphates was measured as described previously (Murphy *et al.*, 1987) in Li^+-treated hepatocytes, by using the methodology of Berridge *et al.* (1982).

Other methods

Protein was determined by the method of Petersen (1977), with bovine serum albumin as a standard.

Materials

Collagenase type II was from Worthington Biochemical Co., Cambridge Bioscience, Cambridge, U.K. All other materials were as specified in the publications cited in this section. All radiochemicals were from Amersham International, Amersham, Bucks., U.K., except for [^{32}P]NAD^+, which was from New England Nuclear. Glucagon was kindly given by Dr. W. W. Bromer, Eli Lilly and Co., Indianapolis, IN, U.S.A. Pure (homogeneous) islet-activating protein, from *Bordetella pertussis*, was obtained from the PHLS Centre for Applied Microbiology and Research, Porton Down, Wilts., U.K. A sample of the purified B-oligomer subunit of islet-activating protein was generously given by Dr. D. L. Burns, Division of Bacterial Products, Food and Drug Administration, Bethesda, MD, U.S.A. This was purified to homogeneity as detailed in Burns & Manclark (1986).

RESULTS

Treatment of intact hepatocytes from mature rats with either glucagon (10 nM) or angiotensin (10 nM) led to the rapid, transient, desensitization of the glucagon-

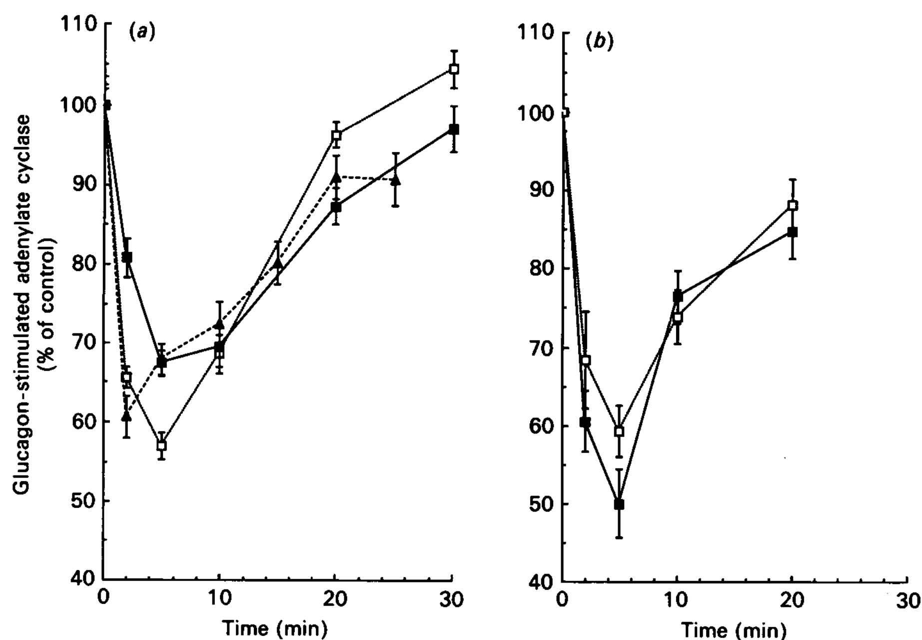

Fig. 1. Desensitization of glucagon-stimulated adenylate cyclase in intact hepatocytes of normal and diabetic rats

(*a*) Desensitization was elicited by glucagon (10 nM) in hepatocytes from normal mature (□), normal young (▲) and mature diabetic (■) rats. (*b*) Densensitization was elicited by angiotensin (10 nM) in hepatocytes from normal mature (■) and mature diabetic (□) rats. Densensitization was elicited by treating intact hepatocytes with either glucagon or angiotensin for the indicated times at 37 °C. After this time, cells were harvested and homogenized, and a washed membrane preparation was isolated. Glucagon (10 nM) was then re-added to the membranes in the assay to determine their glucagon-stimulated adenylate cyclase activity. Thus the change in this activity shown is that observed in broken membranes isolated from intact cells which had been pre-treated for the indicated period with glucagon. The apparent loss of this activity is called desensitization. Results are means ± S.D. for $n = 3$ experiments with different animals. Assays were done in triplicate. Specific activities are given in Table 1.

Table 1. Adenylate cyclase activity in cell preparations

Adenylate cyclase activity stimulated by either forskolin (0.1 mM) or by glucagon (1 μM) plus GTP (0.1 mM) was assayed in hepatocyte membrane fractions from normal mature, mature streptozotocin-diabetic and young rats. In some instances hepatocytes had been pre-treated with pertussis toxin (100 ng/ml) for 1 h, with glucagon (10 nM) for 5 min, or with pertussis toxin for 1 h and then with glucagon for 5 min, before being harvested for membrane preparation. Adenylate cyclase assays were done in triplicate, and the experiment was repeated three times with different cell preparations (results are means ± S.D. for $n = 3$).

		Adenylate cyclase activity			
Animals	Cell pre-treated...	Control	Pertussis toxin	Glucagon	Glucagon + pertussis toxin
Mature controls	Glucagon + GTP	27.7 ± 2.9	40.5 ± 4.6	16.1 ± 1.7	43.5 ± 4.5
	Forskolin	62.3 ± 4.5	72.4 ± 7.0	63.5 ± 5.8	67.2 ± 6.6
Streptozotocin-diabetic mature	Glucagon + GTP	23.1 ± 1.4	25.2 ± 1.9	16.8 ± 0.4	28.2 ± 4.6
	Forskolin	29.8 ± 2.1	29.8 ± 3.9	31.0 ± 0.7	30.7 ± 4.6
Young	Glucagon + GTP	23.5 ± 2.9	54.9 ± 3.3	16.6 ± 0.6	59.4 ± 2.3
	Forskolin	53.2 ± 1.5	57.7 ± 4.3	57.4 ± 4.9	54.8 ± 3.8

stimulated adenylate cyclase activity expressed in an isolated washed membrane fraction prepared from these cells (Figs. 1*a* and 1*b*; Table 1). Similar extents of glucagon-mediated desensitization, expressed as a percentage decrease in glucagon-stimulated adenylate cyclase activity, were evident with hepatocyte membranes from either young rats or mature rats that had been made diabetic with streptozotocin (Fig. 1*a*; Table 1).

Treatment of hepatocytes from control mature animals for 60 min at 37 °C with pertussis toxin (100 ng/ml) before exposure to glucagon (10 nM) for 5 min completely blocked the desensitization process elicited by the action of glucagon on mature (control) rats (Fig. 2a; Table 1). This action of pertussis toxin was also evident when desensitization of glucagon-stimulated adenylate cyclase activity was elicited by prior challenging of hepatocytes

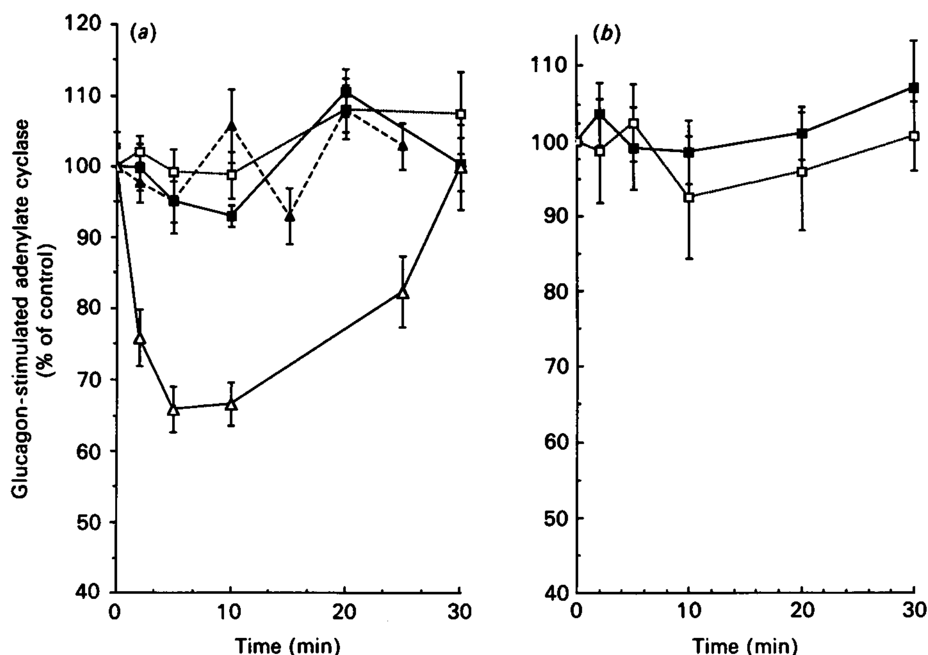

Fig. 2. Pertussis toxin blocks glucagon desensitization in hepatocytes from mature, young and streptozotocin-diabetic animals

(*a*) Hepatocytes (■, □, ▲) were pre-treated with pertussis toxin (100 ng/ml for 1 h) before challenge with glucagon (10 nM) for various times, as indicated. They were then harvested for assay of glucagon-stimulated adenylate cyclase activity in a washed broken membrane system as detailed in the legend to Fig. 1. Hepatocytes from mature (□), young (▲) and streptozotocin-diabetic mature animals (■) were used. In one instance, hepatocytes from mature animals were pre-treated with the isolated B-subunit of pertussis toxin (△) (100 ng/ml for 1 h), rather than the holotoxin, before challenge with glucagon. Specific activities are given in Table 2. (*b*) In this instance, pertussis-toxin-treated (as above) hepatocytes from mature (□) and streptozotocin-diabetic (■) animals were pre-treated with angiotensin (10 nM) for the various times shown. The glucagon-stimulated adenylate cyclase activity of a washed membrane fraction from hormone-pre-treated hepatocytes is shown. Assays are of glucagon-stimulated adenylate cyclase activity in a washed isolated membrane fraction from such treated cells. Results are means ± S.D. for three separate cell preparations ($n = 3$) with adenylate cyclase assays in triplicate. Specific activities are given in Table 2.

Table 2. Holomeric pertussis toxin, but not its isolated B-subunit, blocks the ability of glucagon and TPA to elicit desensitization of adenylate cyclase

Cells were preincubated for 1 h at 37 °C either in the absence of toxin or in the presence of either holomeric pertussis toxin (100 ng/ml) or its isolated B-subunit (100 ng/ml). In some instances either glucagon (10 nM) was added to the cells for a further 5 min before harvest, or TPA (10 ng/ml) was added to the cells for 15 min before harvest. Cells without ligand pre-treatment were incubated for either 5 or 15 min before harvest (similar results were obtained with either time period). The membrane fractions were assayed for glucagon (10 nM)- plus GTP (0.1 mM)-stimulated adenylate cyclase activity. Assays were done in triplicate for three separate experiments with different cell preparations (results are means ± S.D., $n = 3$). Adenylate cyclase activity is given in pmol/mol per mg of plasma-membrane protein. Values in parentheses are activities as a percentage of that seen with no ligand pre-treatment, i.e. no pre-treatment with either glucagon or TPA.

Ligand pre-treatment	Toxin treatment...	Adenylate cyclase activity (pmol/min per mg)		
		None	Pertussis toxin	B-subunit
None		24.9 ± 1.1 (100)	37.8 ± 1.5 (100)	23.1 ± 1.5 (100)
Glucagon		14.8 ± 1.9 (59.4)	35.6 ± 3.6 (94.2)	14.9 ± 1.7 (64.5)
TPA		16.7 ± 1.4 (67.1)	34.2 ± 2.3 (90.5)	15.1 ± 1.0 (65.4)

with angiotensin (Fig. 2*b*). However, treatment of hepatocytes from diabetic animals with pertussis toxin also abolished the ability of glucagon to cause desensitization (Fig. 2*a*; Tables 1 and 2). This action of pertussis toxin was not mimicked by using the isolated B-subunit of the toxin at concentrations comparable with that of the holotoxin (Table 2).

Indeed, although the kinetics of densensitization were unaffected by the presence of the B-subunit (Fig. 2*a*), the resensitization of adenylate cyclase was considerably slowed down (cf. Fig. 1*a*) in the presence of pertussis-toxin B-subunit. We do not know the underlying cause of this, but from earlier studies (Murphy & Houslay, 1988) we have noted that it is possible to influence the rate of resensitization, implying that this site can be independently controlled.

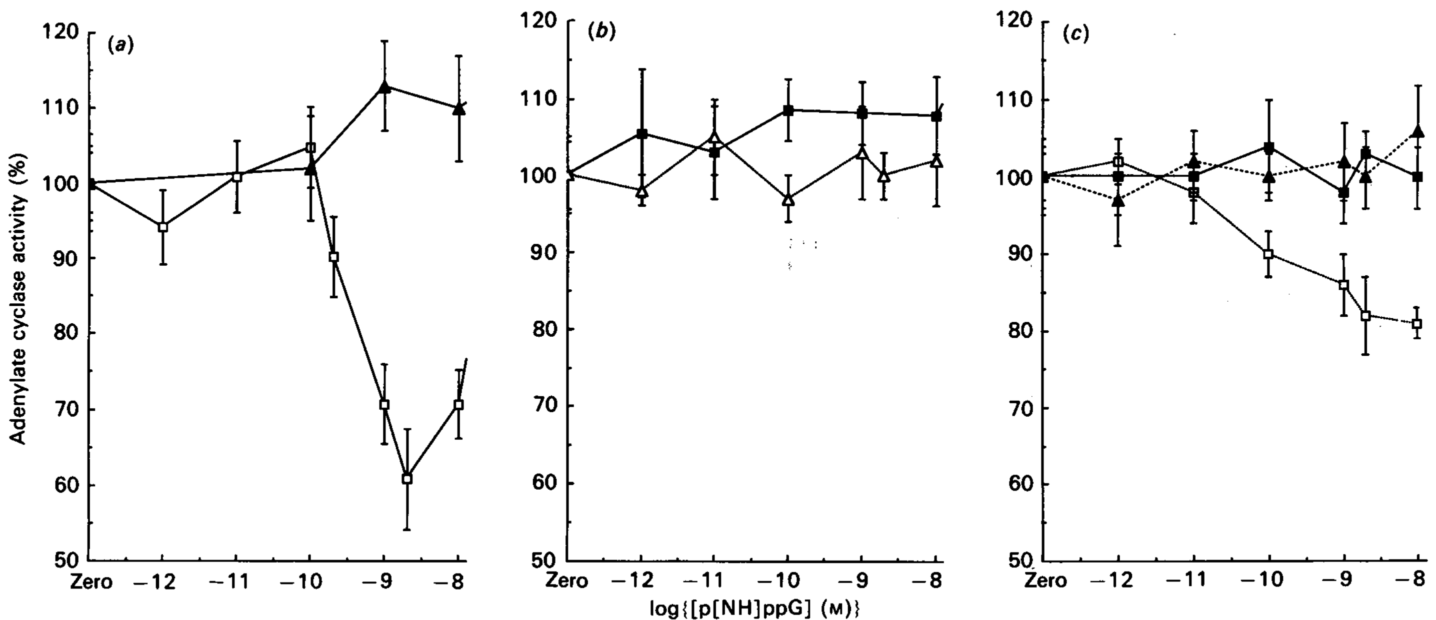

Fig. 3. Functional assessment of G_i in hepatocyte membranes

Functional G_i was assessed by amplifying basal adenylate cyclase activity with the diterpene forskolin (0.1 mM) and then selectively turning on the inhibitory G-protein G_i by using nanomolar concentrations of the non-hydrolysable GTP analogue p[NH]ppG. The dose–effect curves for such experiments are shown here. (*a*) Membranes from hepatocytes which had been pre-treated for 5 min at 37 °C with glucagon (10 nM) show no functional G_i (▲), in contrast with the functional G_i apparent in membranes from untreated hepatocytes (□). (*b*) Treatment of hepatocytes with pertussis toxin (100 ng/ml for 1 h) blocked the expression of functional G_i (■). Pre-treatment of cells with TPA (10 ng/ml for 15 min) similarly obliterated functional G_i activity (△). (*c*) Assessment of functional G_i in membranes from young rats (□), young streptozotocin-diabetic rats (■) and mature streptozotocin-diabetic rats (▲). Results are means ± S.D. for $n = 3$ experiments using different cell preparations. Adenylate cyclase assays were done in triplicate. Specific activities are given in Table 1.

We have shown previously (Heyworth *et al.*, 1984*b*, 1985*b*) that desensitization of adenylate cyclase can also be elicited by treatment of intact hepatocytes with the tumour-promoting phorbol ester TPA. Here we show that in pertussis-toxin-treated cells TPA (10 ng/ml for 15 min) was unable to mimic the desensitization of adenylate cyclase activity (Table 2).

Desensitization, elicited by glucagon, did not alter the forskolin-stimulated adenylate cyclase activities observed in hepatocyte membranes from mature, young or mature streptozotocin-diabetic animals (Table 1).

Functional G_i can be assessed (Hildebrandt *et al.*, 1982; Heyworth *et al.*, 1984*a*) by determining the degree of inhibition of adenylate cyclase activity elicited by low concentrations of p[NH]ppG, which selectively activates G_i rather than the stimulatory G-protein G_s (Fig. 3*a*). In practice, evaluation of G_i is performed in the presence of the diterpene forskolin, which serves to amplify the activity of adenylate cyclase. Here we confirm previous studies (Heyworth *et al.*, 1984*a*) that, with hepatocytes from normal animals, pertussis-toxin treatment of hepatocytes completely blocked the functioning of G_i (Fig. 3*b*). Intriguingly, however, in membranes both from glucagon-desensitized hepatocytes (Fig. 3*a*) and from TPA-treated hepatocytes (Fig. 3*b*), there was no evidence of any functional G_i. Similar results (not shown) were obtained for cells which had been pre-treated with either angiotensin (10 nM) or TH-glucagon (10 nM).

Using hepatocytes from immature animals (3 weeks post-weaning, i.e. 6 weeks old) we noted that the extent of inhibitory activity that could be attributed to G_i, as assessed by the ability of low p[NH]ppG concentrations to inhibit adenylate cyclase, was markedly decreased (by approx. 50%) compared with that observed with membranes from control animals (Fig. 3*c*). However, glucagon was equally efficacious at eliciting desensitization of adenylate cyclase in hepatocytes from these young animals (Fig. 1*a*; Table 1). As seen with mature animals, the induction of diabetes in young animals by using streptozotocin completely abolished the expression of functional G_i in hepatocyte membranes (Fig. 3*c*).

Using the polyclonal antibody AS7 to detect the α subunit of G_i we observed a single band migrating at 40 kDa in membranes from young animals. Treatment of young animals with streptozotocin, to make them diabetic, abolished the expression of this band. Quantitative immunoblotting indicated that hepatocyte plasma membranes from young animals contained 3.4 ± 1.2 pmol of α-G_i/mg of membrane protein (means ± S.D., for three different animals). This compares with a value of around 7.4 pmol/mg of membrane protein for membranes from mature animals. No detectable G_i (less than 0.5 pmol/mg of membrane protein) was observed in membranes from young animals which had been made diabetic with streptozotocin, which was similar to that found for membranes of mature streptozotocin-diabetic animals. Hepatocytes appear to exhibit a single 40 kDa substrate for ribosylation by pertussis toxin (Heyworth *et al.*, 1984*a*; Itoh *et al.*, 1984). We have recently shown (Houslay *et al.*, 1989) that, in liver membranes from streptozotocin-diabetic rats, the expres-

Table 3. Effect of pertussis toxin on the ligand-stimulated inositol phosphate production in hepatocytes from normal and diabetic rats

The percentage stimulation, over control, of production of total inositol phosphates in response to various hormones was measured as described in the Methods section. Experiments were performed at 1 nM-glucagon and -TH-glucagon and at 230 nM-vasopressin. Results are means ± S.D. for $n = 3$ separate experiments with different cell preparations. In some instances cells were pre-treated with pertussis toxin (100 ng/ml for 60 min at 37 °C) before their use.

Animals	Ligand challenge	Cell pre-treatment...	Stimulation of production of inositol phosphates (%) None	Pertussis toxin
Normal	Vasopressin		154 ± 15	129 ± 14
	Glucagon		19 ± 4	12 ± 3
	TH-glucagon		18 ± 3	17 ± 5
Streptozotocin-diabetic	Vasopressin		158 ± 22	176 ± 20
	Glucagon		16 ± 4	15 ± 6
	TH-glucagon		15 ± 2	15 ± 3

sion of the 40 kDa α subunit of G_i, as detected by using thiol-pre-activated pertussis toxin to catalyse its ADP-ribosylation, was almost abolished.

The ability of glucagon and vasopressin to stimulate the production of inositol phosphates was studied in Li^+-treated hepatocytes which had been labelled with [^3H]inositol. The extents of stimulation of the production of inositol phosphates by these two hormones were found to be unchanged whether the hepatocytes were obtained from mature animals or from mature animals which had been made diabetic with streptozotocin (Table 3). Furthermore, the ability of either glucagon or angiotensin to stimulate the production of inositol phosphates was unaffected (Table 3) by treating hepatocytes with pertussis toxin under conditions which obliterated functional G_i.

DISCUSSION

We (Heyworth *et al.*, 1984*a*) have shown that treatment of hepatocytes with pertussis toxin prevents glucagon desensitization, and Rich *et al.* (1984) have shown that glucagon treatment of MDCK cells appears to lead both to desensitization and to increased G_i expression. From such studies it was suggested that either an enhanced function or expression of G_i may provide the molecular basis for the desensitization process. In hepatocytes, then, desensitization might ensue from the constitutive activation of G_i, although we (Heyworth *et al.*, 1984*a*) considered this unlikely, as such a constitutive activation would be expected to decrease the functioning of adenylate cyclase itself: this was not observed (Table 2; Heyworth & Houslay, 1983; Murphy *et al.*, 1987). Here, however, we make the novel observation that low concentrations of the non-hydrolysable GTP analogue p[NH]ppG failed to elicit inhibition of adenylate cyclase through activating G_i in membranes from glucagon-desensitized cells (Fig. 3). The loss of p[NH]ppG-sensitivity cannot be due to the constitutive activation of G_i, as forskolin-stimulated adenylate cyclase activity was unchanged (Table 1). Thus apparent loss of detectable G_i activity appears to be due to its functional inactivation. Indeed, that such a process could be mimicked by treatment of hepatocytes with the phorbol ester TPA (Fig. 3), with angiotensin and with TH-glucagon, which causes desensitization, but does not activate adenylate cyclase (Murphy *et al.*, 1987), suggests that the functional inactivation of G_i was mediated through the activation of protein kinase C. This would be in accord with studies showing that purified G_i can be phosphorylated and inactivated by purified preparations of protein kinase C (Katada *et al.*, 1985) and that treatment of platelets with phorbol esters negates the receptor-coupled G_i-mediated inhibition of adenylate cyclase (Jakobs *et al.*, 1985).

Further evidence that G_i is unlikely to be involved in the desensitization process also comes from our observations using hepatocytes from either diabetic rats or young rats. In both instances, glucagon was able to elicit the rapid desensitization of adenylate cyclase activity in a manner indistinguishable from that seen with hepatocytes from normal mature animals. This was despite the facts that (i) there is no detectable G_i present in a hepatocyte membrane fraction prepared from diabetic animals, on the basis of either activity or immunological identity (Gawler *et al.*, 1987), (ii) there is no evidence for a 40 kDa pertussis-toxin substrate in the membranes of such diabetic animals (Houslay *et al.*, 1989), and (iii) hepatocyte membranes from young animals express markedly lower amounts of G_i compared with those of adult animals.

Nevertheless, pertussis-toxin treatment remained capable of blocking the desensitization process in hepatocytes from streptozotocin-diabetic animals, despite the absence of both functional G_i or any other plasma-membrane substrate of approx. 40 kDa. It has, however, been shown in some cells, but not in others (see Cockcroft & Gomperts, 1985; Houslay, 1987; Gilman, 1987), that receptor-stimulated inositol phospholipid metabolism is mediated by a pertussis-toxin-inhibitable G-protein. We demonstrate here that treatment of hepatocytes with pertussis toxin, under conditions which completely inactivated functional G_i activity (Fig. 3) and caused the ADP-ribosylation of a 40 kDa species (Heyworth *et al.*, 1985*a*), failed to alter the ability of both glucagon and angiotensin to stimulate inositol phospholipid metabolism. Similarly, glucagon and angiotensin were capable of exerting stimulatory effects on inositol phospholipid metabolism in the hepatocytes from diabetic rats, despite their lack of expression of G_i. These imply, as has been suggested for a number of other tissues (see Gilman, 1987), that the G-protein in liver plasma membranes which mediates receptor-stimulated inositol phospholipid metabolism is not a substrate for the action of pertussis toxin.

It has been shown (Tamura *et al.*, 1983; Vistica *et al.*, 1986; Banga *et al.*, 1987; Strnad & Carchman, 1987) that pertussis toxin can elicit cellular effects which do not appear to be mediated by the ADP-ribosylation of either G_i or G_o. In some instances (Strnad & Carchman, 1987) these actions can be attributed to the B-(binding) subunit of the toxin. However, in the present study we show that the isolated B-subunit of pertussis toxin was completely incapable of blocking the desensitization process. The

molecular basis of pertussis toxin's action remains to be defined. It could be that the holotoxin has a biological effect other than ADP-ribosylation. Alternatively, proteins in subcellular domains of the hepatocyte other than the plasma membrane, and which are involved in the desensitization process, can be inactivated by this toxin. Certainly, that the phorbol ester-mediated desensitization or 'uncoupling' of adenylate cyclase could be blocked by pertussis toxin might imply that a form of protein kinase C might be inactivated by pertussis toxin.

Further to this study, we have been able to demonstrate that the functional inactivation of G_i, in intact hepatocytes, is coupled to the phosphorylation of its α-subunit (Pyne *et al.*, 1989).

This work was supported by grants from the Medical Research Council and the California Metabolic Research Foundation. We thank Dr. D. L. Burns of the Division of Bacterial Products, Food and Drug Administration, U.S.A., for her generous gift of the purified B-subunit of pertussis toxin.

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Received 22 August 1988/23 November 1988; accepted 30 November 1988