Treatment of streptozotocin-diabetic rats with metformin restores the ability of insulin to inhibit adenylate cyclase activity and demonstrates that insulin does not exert this action through the inhibitory guanine nucleotide regulatory protein G_i

Debra GAWLER, Graeme MILLIGAN and Miles D. HOUSLAY

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

Insulin caused the inhibition of glucagon-stimulated adenylate cyclase activity in liver plasma membranes, but failed to inhibit this activity in liver membranes from rats made diabetic by treatment with either alloxan or streptozotocin. Treatment of streptozotocin-diabetic rats with insulin, to normalize their blood glucose concentrations, restored this action of insulin. Rats treated with the biguanide drug metformin exhibited a decreased content of the inhibitory guanine nucleotide regulatory protein G_i in liver plasma membranes assessed both structurally, by using a specific polyclonal antibody (AS7), and functionally. Treatment of normal rats with metformin did not alter insulin's ability to inhibit adenylate cyclase in liver plasma membranes; however, metformin treatment of streptozotocin-diabetic rats completely restored this inhibitory action of insulin. Liver plasma membranes from streptozotocin-diabetic animals which either had or had not been treated with metformin had contents of G_i which were less than 10% of those seen in control animals. We conclude that: (i) insulin does not inhibit adenylate cyclase activity through the inhibitory guanine nucleotide regulatory protein G_i ; (ii) streptozotocin- and alloxan-induced diabetes elicit a selective insulin-resistant state; and (iii) metformin can exert a post-receptor effect, at the level of the liver plasma membrane, which restores the ability of insulin to inhibit adenylate cyclase.

INTRODUCTION

Insulin can decrease the intracellular concentrations of cyclic AMP in a number of cell types (see, e.g., Butcher *et al.*, 1966; Jefferson *et al.*, 1968; Pilgis *et al.*, 1975; Blackmore *et al.*, 1979; Heyworth *et al.*, 1983). The mechanisms by which insulin elicits changes in cyclic AMP concentrations appear to be complex, and involve modulation of both the synthesis of cyclic AMP by adenylate cyclase and its degradation by specific cyclic AMP phosphodiesterases (see Houslay, 1986, for review; Lontos *et al.*, 1985; Heyworth *et al.*, 1984a).

In liver, as in other cells, adenylate cyclase activity has been shown to be under dual control, with stimulatory hormones acting through the guanine nucleotide regulatory protein G_s (Birnbauer *et al.*, 1985) and inhibitory action mediated via G_i (Heyworth *et al.*, 1984b). Thus treatment of hepatocytes with cholera toxin activates (Houslay & Elliott, 1979, 1981) and ribosylates (Heyworth *et al.*, 1985) G_s , and treatment with pertussis toxin ribosylates and inactivates G_i (Heyworth *et al.*, 1984b).

It has been suggested that insulin inhibits adenylate cyclase activity through interaction with the guanine nucleotide regulatory protein (G-protein) system in liver plasma membranes, as inhibition can be blocked by pre-treating intact hepatocytes with either cholera toxin or pertussis toxin (Heyworth & Houslay, 1983b; Houslay & Heyworth, 1983; Houslay, 1985, 1986; Heyworth *et al.*, 1986).

The receptor-mediated inhibition of adenylate cyclase is normally considered to be mediated by G_i (Houslay, 1983; Gilman, 1984; Birnbauer *et al.*, 1985; Northup, 1985). However, unlike inhibitory actions mediated via G_i (Aktories *et al.*, 1982; Murayama & Ui, 1983),

treatment of hepatocytes with cholera toxin also prevented insulin from inhibiting adenylate cyclase and, furthermore, high concentrations of the stimulatory hormone glucagon caused a dose-dependent obliteration of the inhibitory effect of insulin (Heyworth & Houslay, 1983b). Thus the role of G_i in this process is equivocal.

In this study we demonstrate that G_i is not required for insulin to exert its inhibitory action on adenylate cyclase. This was achieved with liver plasma membranes from streptozotocin- and alloxan-diabetic rats. These membranes did not exhibit any functional G_i activity (Gawler *et al.*, 1987), whether or not the diabetic animals were treated with the hypoglycaemic drug metformin (Hermann, 1979; Pagano *et al.*, 1983). However, the loss of insulin's inhibitory effect on adenylate cyclase in membranes from diabetic animals was fully restored by metformin treatment.

MATERIALS AND METHODS

Cholera toxin, streptozotocin, alloxan, *o*-dianisidine and phosphocreatine were from Sigma Chemical Co., Poole, Dorset, U.K. Glucagon and bovine insulin were kindly given by Dr. W. W. Bromer, Eli Lilly and Co., Indianapolis, IN, U.S.A. Forskolin was from Calbiochem, Cambridge, U.K. Purified homogeneous pertussis toxin was from Professor J. Freer, Department of Microbiology, University of Glasgow, U.K. Metformin (*N,N'*-dimethylbiguanide) (Fig. 1) was kindly given by Dr. Michel Noel, Aron S. A., Suresnes, France. All other biochemicals, including triethanolamine hydrochloride, were from Boehringer (U.K.), Lewes, East Sussex, U.K. All other general chemicals were of A.R. grade from BDH Chemicals, Poole, Dorset, U.K. Radiochemicals

Fig. 2. Inhibition of glucagon-stimulated adenylate cyclase by insulin

Inhibition is shown as a percentage of the activity expressed by adenylate cyclase in the presence of both GTP (0.1 mM) and a sub-saturating concentration of glucagon (0.1 nM). (a) Membranes were prepared from hepatocytes of control ($\diamond$), streptozotocin-diabetic ($\blacksquare$), insulin-treated streptozotocin-diabetic ($\square$) and alloxan-diabetic ($\blacktriangle$) rats. (b) Metformin-treated control ($\square$) and metformin-treated streptozotocin-diabetic ($\blacksquare$) rats. The specific activities

(Milligan & Klee, 1985), which yielded a linear plot as described by Gawler *et al.* (1987).

RESULTS

As demonstrated previously by us (Heyworth & Houslay, 1983b), in hepatocyte membranes insulin caused the dose-dependent inhibition of adenylate cyclase activity stimulated by physiological (0.1 nM) concentrations of glucagon (Fig. 2a). The K_i for this process was 89 ± 18 pM-insulin.

The ability of insulin to inhibit glucagon-stimulated adenylate cyclase activity was abolished when hepatocyte membranes were obtained from animals that had been made diabetic by treatment with either streptozotocin or alloxan (Fig. 2a). If, however, animals that had been made diabetic with streptozotocin were subsequently treated with insulin, then such an action completely restored the inhibitory effect of insulin on adenylate cyclase activity (Fig. 2a). With membranes from such insulin-treated animals, insulin inhibited adenylate cyclase activity, with a K_i value of 63 ± 15 pM-insulin (S.D., $n = 5$). Metformin treatment of streptozotocin-diabetic animals was equally as effective as insulin therapy at restoring insulin's ability to inhibit adenylate cyclase (Fig. 2b). Metformin treatment of normal animals did not, however, alter the ability of insulin to inhibit glucagon-stimulated adenylate cyclase activity compared with that observed with liver membranes from control animals (Fig. 2b). The K_i value observed for this inhibitory action of insulin in liver membranes from animals treated with metformin alone was 198 ± 42 pM-insulin, and for streptozotocin-diabetic animals treated with metformin it was 220 ± 36 pM-insulin (S.D., $n = 5$). Thus metformin treatment leads to an apparent increase in the K_i for insulin for this process.

We noted (Table 1) that diabetes induced by both streptozotocin and alloxan caused a fall in the specific activity of glucagon (+GTP)-stimulated adenylate cyclase in liver plasma membranes. This action was not appreciably reversed by insulin treatment of streptozotocin-diabetic animals. Metformin treatment of diabetic animals did not cause any change in the diminished glucagon-stimulated activity (Table 1) and, furthermore, metformin did not alter the activity seen with normal animals. Such a decrease in specific activity was observed with either liver or hepatocyte membranes, and was also observed for the forskolin-stimulated activity (Table 1). We have suggested elsewhere that diabetes might decrease the expression of the catalytic unit of adenylate cyclase (Gawler *et al.*, 1987).

The inhibition of forskolin-stimulated adenylate cyclase activity by guanine nucleotides has been used by us (Heyworth *et al.*, 1984b; Gawler *et al.*, 1987) and others (Hildebrandt *et al.*, 1982; Fain *et al.*, 1983; Katada

used to calculate these data are those shown for hepatocyte membranes with 'Glucagon + GTP' in Table 1. These percentage changes are based on observed activities without any subtraction of basal activity. All experiments were performed on hepatocyte membranes. Data are drawn from experiments performed on five animals, with adenylate cyclase assays done in triplicate; results are means $\pm$ S.D., $n = 5$. Data for inhibition performed at 0.1 μ M-insulin were obtained from three different animals (means $\pm$ S.D., $n = 3$).

Table 1. Adenylate cyclase activities in liver and hepatocyte membranes

Adenylate cyclase activity was monitored in liver plasma membranes and in a hepatocyte membrane preparation. Assays were performed in the presence of either a maximally stimulating concentration of forskolin (0.1 mM) or with GTP (0.1 mM) together with a sub-saturating concentration of glucagon (0.1 nM) (cf. K_s for glucagon of 1–2 nM; Heyworth *et al.*, 1983). Specific activities are in pmol/min per mg of membrane protein. Results are means $\pm$ s.d. for five animals, with triplicate assays of adenylate cyclase activity. Liver plasma membranes and hepatocyte membranes were prepared from different rats. The five experiments in the different batches were performed on separate days. Values in parentheses are the activity as a percentage of that for membranes from control untreated animals.

Stimulant	Liver plasma membranes		Hepatocyte membranes	
	Forskolin	Glucagon + GTP	Forskolin	Glucagon + GTP
None (control)	87.9 $\pm$ 7.6	15.7 $\pm$ 1.2	18.2 $\pm$ 2.0	10.5 $\pm$ 0.4
Streptozotocin	46.8 $\pm$ 3.6 (53)	7.7 $\pm$ 0.5 (49)	11.2 $\pm$ 1.0 (61)	5.2 $\pm$ 0.2 (50)
Streptozotocin + insulin	52.8 $\pm$ 3.4 (60)	9.8 $\pm$ 0.4 (62)	10.9 $\pm$ 0.9 (60)	6.6 $\pm$ 0.5 (63)
Streptozotocin + metformin	30.6 $\pm$ 3.4 (35)	5.1 $\pm$ 0.3 (32)	10.5 $\pm$ 0.3 (58)	3.4 $\pm$ 0.3 (32)
Alloxan	36.6 $\pm$ 4.3 (42)	6.1 $\pm$ 0.7 (39)	12.3 $\pm$ 1.5 (68)	4.3 $\pm$ 0.2 (41)
Metformin	85.5 $\pm$ 9.5 (97)	14.0 $\pm$ 1.7 (89)	17.3 $\pm$ 2.0 (95)	9.5 $\pm$ 1.0 (90)

& Ui, 1982) to detect functional G_i in isolated plasma membranes. This takes advantage of the fact (see Birnbaumer *et al.*, 1985) that G_s and G_i have very different affinities for guanine nucleotides, which allows them to be activated selectively. Treatment of control animals with metformin decreased the expression of functional G_i by about 50% (Fig. 3). We (Gawler *et al.*, 1987) have reported that streptozotocin-induced diabetes caused an abolition of functional G_i in liver membranes. This was not affected by treatment of streptozotocin-diabetic animals with metformin. Thus liver membranes from metformin-treated streptozotocin-diabetic animals exhibited no detectable functional G_i activity (Fig. 3).

The affinity-purified polyclonal antibody AS7 recognizes G_i and not G_o in rat liver plasma membranes as a single staining band migrating at M_r 40 000 on immunoblot analysis (Gawler *et al.*, 1987). This concurs with the presence of G_i as identified by us (Heyworth *et al.*, 1984b, 1985) and others (Itoh *et al.*, 1984), using pertussis-toxin-catalysed [32 P]ADP-ribosylation to identify a single labelled band at M_r 40 000. Quantitative immunoblotting (Gawler *et al.*, 1987) allowed us to demonstrate that treatment of animals with metformin decreased the amount of G_i in liver membranes from 7.2 ± 0.5 pmol/mg of plasma-membrane protein in control (untreated) animals to 1.6 ± 0.4 pmol/mg in those treated with metformin (s.d., $n = 4$ different animals). In streptozotocin-diabetic animals the amount of G_i in liver plasma membranes was undetectable (less than 0.2 pmol/mg of membrane protein) and in metformin-treated streptozotocin-diabetic animals G_i was also undetectable (less than 0.2 pmol/mg of membrane protein, $n = 4$ different animals). Thus, unlike treatment of diabetic animals with insulin (Gawler *et al.*, 1987), metformin failed to increase the concentration of G_i in plasma membranes from streptozotocin-diabetic rats.

The mechanism through which metformin decreased the G_i content of liver plasma membranes remains to be established. Interestingly, although metformin treatment of control animals led to a decrease in G_i content of approx. 78%, functional G_i activity fell by only approx. 50%. This must reflect the kinetics of the inhibitory mechanism employed.

Fig. 3. Inhibition of adenylate cyclase by G_i

G_i functioning was determined in hepatocyte membranes from the ability of low concentrations of guanosine 5'-[β -imido]triphosphate (p[NH]ppG) to inhibit basal adenylate cyclase activity that had been amplified with the diterpene forskolin (0.1 μ M). Specific activities for basal and forskolin-stimulated adenylate cyclase activities are given in Table 1. Inhibition is expressed as a percentage of the forskolin-stimulated activity, with no subtraction of basal activity. $\square$, Control animals; $\blacksquare$, control animals treated with metformin; $\blacklozenge$, streptozotocin-diabetic animals; $\blacktriangle$, streptozotocin-diabetic animals treated with metformin. Data are shown for experiments carried out on five animals, with adenylate cyclase activities measured in triplicate; results are means $\pm$ s.d. ($n = 5$).

DISCUSSION

Type I (insulin-dependent) diabetes is characterized by low circulating insulin concentrations (Himsworth, 1949). This state can be achieved in rats by treating them with chemical agents such as streptozotocin or alloxan, which destroy the insulin-producing pancreatic β -cells. Such treated animals become hyperglycaemic, a condition which can be normalized by treatment with insulin (see Montague, 1983). It has been noted, however, that in type I diabetes expressed in diabetic rats and also in human beings, various tissues can exhibit a decreased responsiveness to insulin as regards its ability to stimulate glucose utilization (Kobayashi & Olefsky, 1979; Karnieli *et al.*, 1981; Caro *et al.*, 1986; Tessari *et al.*, 1986) and to stimulate the activity and translocation of glucose transporters (Kobayashi & Olefsky, 1979; Karnieli *et al.*, 1981). Here we demonstrate that type I diabetes can lead to 'insulin-resistance', as identified by the complete loss of the ability of insulin to inhibit adenylate cyclase in liver membranes from diabetic rats (Fig. 2). This effect was not dependent on the chemical agent used, for it was observed when animals were made diabetic with either streptozotocin or alloxan. This action of insulin could be completely reversed by treating the diabetic animals with insulin (Fig. 2), as has been shown for a number of other acute actions of insulin which become impaired in insulin-deficient states (see Randle *et al.*, 1966).

The molecular mechanism of action of the biguanide hypoglycaemic agent metformin is unknown (see Hermann, 1979). However, it is believed to exert its action at a post-receptor level rather than by affecting the number of insulin receptors (Lord *et al.*, 1983, 1985). Certainly metformin treatment of normal and diabetic rats does not affect the number of high-affinity insulin-binding sites in hepatocytes (Lord *et al.*, 1983). Indeed, the maximal effects of insulin on processes such as inhibition of adenylate cyclase and stimulation of glucose transport are elicited at low levels of receptor occupancy and, as such, are unlikely to be influenced strongly by any small changes in receptor number (see, e.g., Houslay, 1985). We make here two novel observations with this agent. Firstly, treatment of normal rats with metformin led to a decrease in G_i assessed both functionally (Fig. 3) and structurally using a specific antibody. This effect was not accompanied by any decrease in the ability of insulin to inhibit adenylate cyclase. Secondly, metformin treatment of streptozotocin-diabetic rats completely restored the ability of insulin to inhibit glucagon-stimulated adenylate cyclase activity in hepatocyte membranes (Fig. 2), but did not restore G_i contents to their normal values, assessed either functionally (Fig. 3) or structurally with the antiserum AS7. These results indicate that insulin does not inhibit glucagon-stimulated adenylate cyclase activity by acting through the inhibitory G-protein G_i .

We (Gawler & Houslay, 1987) have shown that insulin can stimulate a novel GTPase activity in human platelets. This may represent the functioning of the putative G_{ins} (Heyworth & Houslay, 1983b; Houslay & Heyworth, 1983, 1986). We cannot, however, discount the possible involvement of a soluble 'mediator' as a means through which insulin exerts this action (see, e.g., Saltiel *et al.*, 1982).

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