Evaluation of inhibitory guanine nucleotide regulatory protein G_i function in hepatocyte and liver membranes from obese Zucker (fa/fa) rats and their lean (Fa/?) littermates

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Summary. Previous studies have shown that hepatocyte and liver membranes from insulin resistant animals exhibit an impairment of inhibitory guanine nucleotide binding regulatory protein, G_i function, such that a G_i defect may contribute towards the diabetic syndrome. In the current studies, it is shown that the demonstration of G_i activity in liver and hepatocyte membranes is dependent critically on the membrane preparation technique. A technique is defined that

allows functional G_i activity to be demonstrated in liver and hepatocyte membranes from both lean (Fa/?) and obese (fa/fa) Zucker rats. Consequently, previous reports on the loss of G_i function in insulin resistant states require reevaluation.

Key words: Insulin resistance, G_i protein function.

Hyperglycaemia associated with the diabetic state has been attributed to marked insulin resistance resulting in decreased glucose utilisation in peripheral tissues and increased hepatic glucose output [1]. The increase in hepatic glucose output has been linked to an increased cyclic AMP content in the livers of diabetic animals [2]. The concept that increased stimulation of cyclic AMP synthesis within the liver contributes to the diabetic state has gained favour in recent years from observations that plasma membranes from livers of diabetic animals possess abnormalities in the regulatory GTP-binding proteins that couple receptors to the stimulation (G_s) or inhibition (G_i) of adenylyl cyclase. It has been argued that animal models of both insulin-dependent and non-insulin dependent diabetes show impairments of G_i function in liver and hepatocyte plasma membranes [3–6]. These reports, however, have been challenged by others, who are unable to show changes in G_i function and expression in the diabetic state, but instead attribute increased cyclic AMP synthesis to the overexpression and stimulation of G_s protein function [7].

In this report we have investigated the role of G_i function in liver and hepatocyte membranes from Zucker (fa/fa) rats, a model of non-insulin dependent diabetes, and their lean (Fa/?) littermates. We show that normal G_i function is present in this model and that evaluation of G_i function depends critically on the methodology used for membrane preparation.

Materials and methods

Materials

Forskolin, creatine phosphokinase, pertussis toxin and theophylline were purchased from Sigma Chemical Co. Ltd., Poole, Dorset, UK. Guanylyl 5'-imidodiphosphate (Gpp(NH)p), creatine phosphate and ATP were from Boehringer UK Ltd., Lewes, East Sussex, UK. Radiochemicals were obtained from Amersham International, Amersham, UK. All other chemicals were of analytical reagent grade from BDH chemicals, Poole, Dorset, UK.

Animals. Male Zucker (fa/fa) rats and their lean littermates (Fa/?) aged 20–24 weeks were obtained from OLAC Harlan Ltd., Bicester, UK. Rats were housed at $22 \pm 1^\circ\text{C}$ on a 12-h light/dark cycle (lights on 06.00 h, off 18.00 h) with food (Oxoid rat and mouse breeders diet; H. C. Styles, Bewdley, Worcs, UK) and water available *ad libitum*.

Methods

Hepatocyte preparation. Hepatocytes were prepared by the method of Elliot et al. [8] and incubated as described by Smith et al. [9]. The cells were resuspended in Krebs-Henseleit incubation medium containing 2.5% weight/volume bovine serum albumin (Boehringer fraction V) and 2.5 mmol/l CaCl_2 .

Preparation of hepatocyte plasma membranes. Crude hepatocyte (parenchymal) plasma membranes were prepared by resuspending a hepatocyte pellet (sedimented at 50 g) in 60 volumes of ice cold NaHCO_3 (1 mmol/l) buffer. The hepatocytes were disrupted by a

Fig. 1. Dose-effect curves for the effect of guanylyl 5'imidodiphosphate (Gpp(NH)p) on forskolin-stimulated adenylyl cyclase activity in plasma membranes obtained from the livers of lean (Fa/?) and obese (fa/fa) Zucker rats. Adenylyl cyclase activity was measured in the presence of forskolin (100 $\mu\text{mol/l}$) and the indicated concentrations of Gpp(NH)p, as described, in liver plasma membranes obtained from lean (○) and obese (●) Zucker rats. The activity is expressed as a percentage of the forskolin-stimulated activity (100%), assessed in the absence of Gpp(NH)p. Values are given as the mean $\pm$ SD, $n = 5$ preparations

combination of this hypotonic buffer and drawing the cells through a syringe needle (21 gauge). Crude plasma membranes were sedimented at 1500 g for 20 min at 4°C. This pellet (P_1) was rinsed once in 60 volumes of ice cold NaHCO_3 (1 mmol/l) buffer before sedimenting at 1500 g for 20 min at 4°C to yield the final rinsed pellet (P_2). This was resuspended and assayed for adenylyl cyclase activity immediately.

Preparation of plasma membranes from liver. For comparison with hepatocyte (parenchymal cell) membranes, liver plasma membranes were prepared from homogenized chopped whole livers. Small amounts of tissue were homogenized at one time in 30 volumes of ice cold NaHCO_3 (1 mmol/l) buffer using no more than five strokes of a hand held teflon-glass homogeniser. The homogenate was then added to a large excess of ice cold NaHCO_3 (1 mmol/l) buffer to ensure rapid dilution of the membranes. The homogenate was strained through two layers of mesh (150 micron) before sedimentation (1500 g for 20 min at 4°C). The pellet was washed once with 30 volumes of ice cold buffer followed by re-pelleting (1500 g for 20 min) before immediate use in the adenylyl cyclase assay.

Pertussis toxin treatment. Plasma membranes were treated with pertussis toxin by modification of the method of Itoh et al. [10]. Briefly, plasma membranes (0.6–1.4 mg/ml) were incubated in a ribosylation mixture containing ATP (1 mmol/l), thymidine (20 mmol/l), NAD (1 mmol/l), MgCl_2 (5 mmol/l) and potassium phosphate (100 mmol/l), pH 7.4 in the presence or absence of preactivated per-

tussis toxin (10 $\mu\text{g/ml}$) for 15 min at 37°C. All other procedures are as described previously [10].

Adenylyl cyclase assay. Adenylyl cyclase was assayed in quadruplicate [11] by addition of plasma membranes (60–120 μg protein) to a final volume of 0.1 ml incubation cocktail containing [$\alpha^{32}\text{P}$]-ATP (0.9 mmol/l; 4×10^5 dpm/assay). Cyclic AMP was separated from ATP by the method of Salomon et al. [12].

Statistical analysis

Results are expressed as the mean $\pm$ SD or SEM (see legends to figures). Significance was assessed using an unpaired Student's t -test.

Results

Inhibition of forskolin-stimulated adenylyl cyclase at low concentrations of Gpp(NH)p is well accepted as an index of G_i function [3, 4, 6, 13, 14]. Using this assay, G_i function is readily observed in crude plasma membranes derived from livers of lean (Fa/?) and obese, insulin-resistant

Fig. 2a, b. Pertussis toxin sensitivity of G_i function in liver plasma membranes from (a) lean (Fa/?) and (b) obese (fa/fa) Zucker rats. Adenylyl cyclase activity was measured in the presence of forskolin (100 $\mu\text{mol/l}$), and in the presence or absence of 10^{-7} mol/l guanylyl 5'imidodiphosphate (Gpp(NH)p), as described. Membranes were prepared from the livers of lean (a) or obese (b) Zucker rats, and incubated as described in ribosylation buffer, with or without pertussis toxin (PTX, 10 $\mu\text{g/ml}$, 15 min) as indicated. Data are expressed as a percentage of forskolin-stimulated activity (100%) assessed in the absence of Gpp(NH)p. Values are given as the mean $\pm$ SD, $n = 3$ preparations

Fig. 3. Dose-effect curves for guanylyl 5'imidodiphosphate (Gpp(NH)p) on forskolin-stimulated adenylyl cyclase activity in hepatocyte membranes obtained from lean (Fa/?) and obese (fa/fa) Zucker rats. Adenylyl cyclase activity was measured in hepatocyte membranes from lean (○) or obese (●) Zucker rats in the presence of forskolin (100 $\mu\text{mol/l}$) and the indicated concentrations of Gpp(NH)p, as described. Data are expressed as a percentage of forskolin-stimulated activity (100%) in the absence of Gpp(NH)p. Values are given as the mean $\pm$ SD, $n = 5$ preparations

(fa/fa) rats. The specific activity of adenylyl cyclase was found to be increased by addition of forskolin (100 $\mu\text{mol/l}$) from 7.9 ± 2.3 to 268.4 ± 29.5 pmol cyclic AMP $\cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ in liver plasma membranes from lean (Fa/?); and from 9.3 ± 2.3 to 361.8 ± 62.2 pmol cyclic AMP $\cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ in liver plasma membranes from obese (fa/fa) Zucker rats (mean $\pm$ SEM, $n = 5$ preparations).

In the presence of Gpp(NH)p (10^{-7} mol/l), which is sufficient to give maximal inhibition (Fig. 1), the forskolin-stimulated adenylyl cyclase activity was decreased by 25% and 40% in liver plasma membranes from lean and obese Zucker rats respectively. The concentration of Gpp(NH)p required to produce maximal activation of G_i (10^{-7} mol/l) is consistent with that reported by others [14]. Furthermore, treatment of membranes with pertussis toxin, which inactivates G_i , blocked the Gpp(NH)p-dependent inhibition of forskolin-stimulated adenylyl cyclase activity in liver membranes from both lean (Fa/?) and insulin resistant (fa/fa) rats (Fig. 2).

At higher concentrations of Gpp(NH)p ($> 10^{-7}$ mol/l), G_s function is stimulated to the same degree in membranes derived from livers of both lean (Fa/?) and insulin resistant (fa/fa) rats. Both membrane preparations yield

the characteristic biphasic response curves shown in Figure 1 and Figure 3 [6].

The present results, using liver plasma membranes, are in contrast to the results of Houslay et al. [6], who found an impairment of G_i function in hepatocyte membranes from Zucker (fa/fa) rats. Hepatocyte (parenchymal) cells constitute approximately 60% of cells within the liver [15] and it was possible that the G_i impairment seen in the insulin-resistant hepatocyte could be restricted to that cell type only; leading to the observed discrepancies in G_i activity between hepatocyte membrane data and membrane data obtained from whole liver. To examine this possibility, hepatocyte plasma membranes were prepared from both Zucker (fa/fa) rats and lean littermates. Both the basal and the forskolin-stimulated adenylyl cyclase specific activity were reduced in comparison with the activities in whole liver membrane in both Zucker (fa/fa) rats (basal 2.3 ± 0.2 ; forskolin 86.5 ± 13.6 pmol cyclic AMP $\cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$) and lean littermates (basal 2.8 ± 0.4 ; forskolin 103.1 ± 11.1 pmol cyclic AMP $\cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$). A similar reduction in adenylyl cyclase activity in hepatocyte membranes compared to liver membranes has been noted by others [4]. Gpp(NH)p (10^{-8} and 10^{-7} mol/l) had a similar inhibitory effect on forskolin-stimulated adenylyl cyclase activity in hepatocytes from lean and Zucker (fa/fa) rats, demonstrating the presence of a functional G_i protein (Fig. 3). Thus, the difference in our data and that of Houslay et al. [6] is not due to the source of the plasma membranes.

In preliminary experiments, we found that the degree of dilution of membranes during the preparative procedure affected subsequent measurements of G_i function. To evaluate this further, membranes were prepared using the dilution protocol detailed in Figure 4, which was designed to compare our membrane preparation procedure (P_2 60 volume) with a protocol (P_2 5 volume) similar to that employed by other workers [3–6]. The specific activ-

Fig. 4. Flow-chart of the production of high- and low-volume rinsed hepatocyte membranes (see Methods for further details)

Table 1. G_i function in hepatocyte membranes prepared from high and low volume rinsed membranes

Hepatocyte pellet	Forskolin-stimulated adenylyl cyclase activity			
	Fa/? membranes		fa/fa membranes	
	p mol cyclic AMP $\cdot$ min ⁻¹ · mg protein ⁻¹ - Gpp (NH) p	% Inhibition + Gpp (NH) p	p mol cyclic AMP $\cdot$ min ⁻¹ · mg protein ⁻¹ - Gpp (NH) p	% Inhibition + Gpp (NH) p
P ₁ 5 volume	54.3 $\pm$ 6.7	13.7 $\pm$ 2.2	34.5 $\pm$ 3.6 ^d	14.3 $\pm$ 4.8
P ₂ 5 volume	59.0 $\pm$ 6.6	20.2 $\pm$ 4.6	44.8 $\pm$ 4.4	22.6 $\pm$ 2.7
P ₁ 60 volume	72.4 $\pm$ 8.8	21.8 $\pm$ 3.2	72.6 $\pm$ 13.1 ^a	30.3 $\pm$ 2.3 ^a
P ₂ 60 volume	103.1 $\pm$ 11.1 ^b	34.9 $\pm$ 1.6 ^c	86.5 $\pm$ 13.6 ^b	30.4 $\pm$ 2.8 ^a

Percentage inhibition of forskolin-stimulated (100 μ mol/l) adenylyl cyclase was obtained by addition of guanylyl 5'-imidodiphosphate (Gpp(NH)p), (10⁻⁷ mol/l).

Values expressed are the mean $\pm$ SEM, $n = 5$ preparations

^a $p < 0.05$ ^b $p < 0.01$ ^c $p < 0.001$ compared to the low-volume

(P₁ 5 volume) pellet. ^d $p < 0.05$ compared to the corresponding P₁ 5 volume pellet obtained from Fa/? lean littermates. (All statistics were derived using the unpaired Student's t -test)

ity of forskolin-stimulated adenylyl cyclase was then determined for each membrane preparation, together with the degree of inhibition of that activity by Gpp(NH)p (Table 1). The results show that the extent of Gpp(NH)p-mediated inhibition of forskolin-stimulated adenylyl cyclase is significantly decreased, although not lost, in rinsed membranes obtained using a low homogenisation volume (P₁ 5 volume) compared to similar membranes obtained using a high homogenisation volume (P₂ 60 volume). This difference was apparent whether membranes were prepared from normal (Fa/?) or insulin-resistant (fa/fa) Zucker rats. Also, in P₂ 60 volume membranes it was noted that the increased Gpp(NH)p-mediated inhibition paralleled the increased forskolin-stimulated adenylyl cyclase activity (Table 1), indicating that the high volume rinse removed an inhibitory constraint from the cyclase itself, as well as potentiating G_i function.

Discussion

There is considerable controversy concerning the impairment of G_i function in the liver of diabetic animals. In hepatocyte membranes from streptozotocin-treated diabetic rats, a model of insulin-dependent diabetes, Gawler et al. [3] showed a loss of G_i function, which was due to a quantitative loss of G_i protein. Other workers have failed to demonstrate this loss using either blotting techniques or functional assays for G_i [7].

In livers from rodent models of non-insulin dependent diabetes, G_i function has been shown to be impaired in the Zucker (fa/fa) rat [6] and the obese (ob/ob) mouse [5]. The impairment of G_i function obtained in isolated membrane studies has been supported by studies in vivo in the Zucker (fa/fa) rat [16]. Unlike the streptozotocin-treated rat, the loss of G_i function in Zucker rats is not associated with a reduction of G_i protein as indicated by Western blotting [6].

The present data show that careful rinsing of plasma membranes prepared from livers and hepatocytes leads to a preservation of G_i activity in membranes prepared from Zucker (fa/fa) rats and their lean littermates. This indicates that the tissue source (parenchymal vs non-parenchymal cells) and the insulin resistant state of the animals

cannot account for discrepancies between our results and those obtained by others [6].

The data also show that inadequate rinsing of plasma membranes can reduce the apparent G_i function, although this loss is not complete, and not limited to plasma membranes derived from insulin-resistant animals.

When impaired G_i function is observed in the diabetic state [3–6, 13, 17, 18], the impairment has been argued to represent a lesion in the coupling of G_i to adenylyl cyclase, perhaps associated with the physical loss of G_i from the plasma membrane. The mechanisms for this phenomenon are at present unclear, but it has been shown that G_i may be phosphorylated and inactivated by protein kinase A and protein kinase C in normal rats [19]. It is also possible that factors liberated during homogenisation of cells and tissues may modulate G_i activity independently of the phosphorylation state, especially as we have shown that G_i function may be conserved by certain homogenisation procedures.

With respect to the above hypotheses, it is not clear whether the conditions that we employ are stabilising a state of dephosphorylated G_i which retains its activity, or whether cellular factors which mask G_i activity have been washed away. The data presented in Table 1 suggest that low volume homogenates contain cellular factors which inhibit adenylyl cyclase activity, accompanied by a loss of guanine nucleotide inhibition of the enzyme.

Conversely the results presented in Table 1 could be due to cellular factors activating G_i in membrane fractions derived from low volume homogenates, resulting in both the lower forskolin-stimulated adenylyl cyclase activity, and the accompanying loss of G_i activation by added Gpp(NH)p. In support of this hypothesis, we have observed that preincubation of liver membranes with low concentrations of ATP (0.1 mmol/l) inhibits adenylyl cyclase as assessed by a decrease in forskolin-stimulated adenylyl cyclase activity in the absence of added guanine nucleotides. This effect is unlikely to be due to a kinase-dependent phosphorylation of part of the adenylyl cyclase complex, as similar results were obtained using adenylyl-5'-imidodiphosphate, an ATP analogue which is not a substrate for protein kinases (D.M. Kirkham and P. Young, unpublished observations). One possibility is that loss of G_i function may be due to an ATP-dependent activation

of P_{2y}-purinergic receptors, which are known to couple to and activate to G_i in rat liver [20].

The presence of ATP in plasma membrane preparations derived from low volume homogenates might explain the apparent loss of G_i function, and it should be noted that GTP can produce the same effect by direct activation of G_i. However, these contaminants do not explain the comparative (i.e. normal vs diabetic) differences in G_i function seen by others; as we are able to show loss of G_i function in membranes derived from low volume homogenates from both normal (Fa/?) and insulin-resistant (fa/fa) Zucker rats.

The Zucker (fa/fa) rat and obese (ob/ob) mouse represent two genetic models of non-insulin dependent diabetes with many metabolic abnormalities of which obesity and diabetes are but two. Care must therefore be taken in ascribing a particular lesion in membrane bound enzyme systems (such as G_i activity) to a pathological syndrome such as diabetes. For example, both the Zucker (fa/fa) rat and obese (ob/ob) mouse possess fatty livers which, perhaps as a consequence of the altered composition and hence physical properties of the bilayer, may affect the function of plasma membrane-bound enzyme preparations independently of the diabetic state.

The data presented here suggests therefore, that careful consideration needs to be given to the appropriateness of the plasma membrane preparation before attempting to assign a role for G-proteins in any pathological condition.

Acknowledgements. We are grateful to Prof. M. D. Houslay (University of Glasgow) for very constructive discussion during the course of these studies.

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Received: 13 February 1991
and in revised form: 29 April 1991

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