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RESEARCH COMMUNICATION

Demonstration of inhibitory guanine nucleotide regulatory protein (G_i) function in liver and hepatocyte membranes from streptozotocin-treated rats

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By using a defined plasma-membrane preparation, functional inhibition of adenylate cyclase activity by the inhibitory G-protein (G_i) was observed in liver and hepatocyte membranes from rats made diabetic by streptozotocin. These observations contrast with previous reports which have shown a defect in G_i in this diabetic animal model. These results suggest that G_i function is not impaired in the livers of streptozotocin-treated rats and that plasma-membrane preparation procedures should be clearly defined before ascribing G_i defects to a pathological state such as diabetes.

INTRODUCTION

The streptozotocin-treated adult rat is commonly used as a model of type 1 (insulin-dependent) diabetes [1]. This animal is hyperglycaemic, which in part is due to increased hepatic glucose output. This in turn has been attributed to increased cyclic AMP content within the liver [2].

Recently, loss of functional inhibitory G-protein (G_i), which could account for the increased cyclic AMP content of diabetic-rat livers, has been reported in hepatocyte membranes [3–5], but not whole liver membranes [6], isolated from streptozotocin-diabetic rats.

In a previous report we have shown, using a membrane preparation procedure which uses high dilution volumes after homogenization [7], that G_i function is retained in hepatocyte (parenchymal) and whole liver (parenchymal + non-parenchymal) membranes derived from fatty Zucker (*fa/fa*) rats. In contrast, using a different protocol others have reported that G_i activity is lost in hepatocyte membranes from this model of type II non-insulin-dependent diabetes [8].

In the present report the same membrane preparation procedure [7] has been used to investigate the role of G_i function in liver and hepatocyte membranes from streptozotocin-treated rats, in order to address some of the contradictions reported within the literature by groups using hepatocyte (parenchymal) or liver (parenchymal + non-parenchymal) membranes from this model of type I diabetes. The need for carefully defined membrane preparation procedures when correlating G-protein activity to a pathological condition such as diabetes is also discussed.

EXPERIMENTAL

Materials

Creatine kinase (EC 2.7.3.2), streptozotocin, theophylline, GTP and forskolin were all from Sigma Chemical Co., Poole, Dorset, U.K. BSA (fraction V), phosphocreatine, ATP and guanosine 5′-[$\beta\gamma$-imido]triphosphate (p[NH]ppG) were obtained from Boehringer Mannheim U.K., Lewes, East Sussex, U.K. Pertussis toxin was purchased from Porton Products, Maidenhead, Berks., U.K. [α-^{32}P]ATP and [2,3-^{3}H]adenosine 3′,5′-cyclic monophosphate (cyclic [^{3}H]AMP) were purchased from Amersham International, Amersham, Bucks, U.K. All other chemicals were A.R. grade from BDH Chemicals, Poole, Dorset, U.K.

Animals

Male Sprague–Dawley rats (180–200 g) were obtained from Interfauna, Huntingdon, U.K. They were housed at 22 ± 1 °C on a 12 h-light/12 h-dark cycle (lights on 06:00–18:00 h), with food (Oxoid Rat and Mouse Breeders Diet; H. C. Styles, Bewdley, Worcs., U.K.) and water available *ad libitum*. Rats were injected intravenously with streptozotocin (80 mg/kg) or vehicle (50 mM-sodium citrate, pH 4.5). Blood glucose was assayed on a 550 Express Ciba Corning autoanalyser by the method of Schmidt [9]. Only animals with blood glucose > 20 mM 3 days after streptozotocin treatment were defined as diabetic. Liver and hepatocyte membranes were prepared from control and diabetic rats within 1 week of streptozotocin treatment.

Membrane preparation

Liver and hepatocyte plasma membranes were prepared from non-fasted rats as described previously [7]. Briefly, individual livers were finely chopped and homogenized in approx. 6 vol. of 1 mM-$NaHCO_3$ (pH 7.4) per g wet wt. of tissue at 4 °C, by using no more than five strokes of a hand-held Teflon/glass homogenizer. The homogenate was immediately diluted to 30 vol. of 1 mM-$NaHCO_3$/g wet wt. of tissue and then strained through two layers of nylon mesh (150 μm) [10]. The filtrate was centrifuged at 1500 *g* for 15 min at 4 °C. The pellet was washed once and resuspended in the $NaHCO_3$ medium to a protein concentration of 5–10 mg/ml for immediate use in adenylate cyclase assays.

Hepatocytes were prepared as in [11] and lysed by drawing them through a needle (21-gauge) and expelled into 30 ml of 1 mM-$NaHCO_3$/g wet wt. of cells. Crude hepatocyte (parenchy-

Abbreviations used: G_i, inhibitory G-protein controlling adenylate cyclase activity; p[NH]ppG, guanosine 5′-[$\beta\gamma$-imido]triphosphate.

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mal) membranes were prepared by the same centrifugation protocol described for liver membranes.

Pertussis-toxin treatment of membranes

Membranes were treated with pertussis toxin by a modification of the method of Itoh *et al.* [12]. Briefly, membranes were incubated for 15 min at 37 °C in the absence or presence of thiol-preactivated pertussis toxin (10 μg/ml final concn.) in a ribosylation mixture containing 1 mM-ATP, 20 mM-thymidine, 1 mM-NAD^+, 5 mM-$MgCl_2$ and 100 mM-KH_2PO_4, pH 7.4. Membranes were rapidly centrifuged (12000 *g* for 5 min at 4 °C) and resuspended in $NaHCO_3$ medium for immediate use in the adenylate cyclase assay.

Adenylate cyclase assays

Adenylate cyclase was assayed essentially as described by Houslay *et al.* [13]. Briefly, 100–200 μg of membrane protein was incubated in a final volume of 100 μl with 1 mM-[α-^{32}P]ATP (0.2–1.8 μCi/assay), 10 mM-theophylline, 7.4 mg of phosphocreatine/ml, creatine kinase (240 units/ml), 5 mM-$MgSO_4$ and 25 mM-triethanolamine/HCl, pH 7.4. The cyclic AMP formed over 10 min was isolated by the method of Salomon *et al.* [14]. Reaction rates were linear over the assay period. Total protein concentrations were determined by the method of Pederson [15].

Statistics

Results are expressed as means ± S.E.M., and significance was determined by Student's *t* tests for non-paired data.

RESULTS

G_i function in liver and hepatocyte membranes

Concentration-dependent p[NH]ppG or GTP inhibition of forskolin-stimulated cyclic AMP production is well established as an assay of G_i activity [3,16]. G_i function was assessed by this method in liver membranes from control and streptozotocin-treated rats. Hepatocyte membranes were also prepared from diabetic rats to examine the possibility that a G_i defect might be restricted to the parenchymal cells.

Typical biphasic response curves to p[NH]ppG for liver and hepatocyte membranes were obtained, with a maximal inhibition at 0.1 μM (Fig. 1*a*). Liver membranes from control rats and liver and hepatocyte membranes from diabetic rats showed statistically significant inhibition ($P < 0.01$) of forskolin-stimulated adenylate cyclase. Similar p[NH]ppG-inhibition curves were observed in all preparations, irrespective of both the diabetic state of the animal, and whether liver or hepatocyte membranes were used. This contrasts with previous reports showing defective p[NH]ppG-mediated inhibition of forskolin-activated adenylate cyclase in hepatocyte membranes from diabetic animals [3–5].

G_i function was also demonstrated by using GTP-dependent inhibition of forskolin-stimulated adenylate cyclase (Fig. 1*b*). Significant inhibition ($P < 0.01$) for forskolin-stimulated adenylate cyclase was observed for liver membranes from both control and diabetic rats as well as for hepatocyte membranes from diabetic rats, which further supports the observation that no G_i defect occurred in any plasma-membrane preparation, irrespective of the diabetic state.

Pertussis-toxin abolition of G_i function in hepatocyte membranes from diabetic rats

Ribosylation and inactivation of G_i by pertussis toxin has been used as a tool to show that inhibition of adenylate cyclase is mediated by G_i [3,7,17]. The data in Table 1 demonstrate that the inhibition of forskolin-stimulated adenylate cyclase by both p[NH]ppG and GTP was abolished by pertussis toxin.

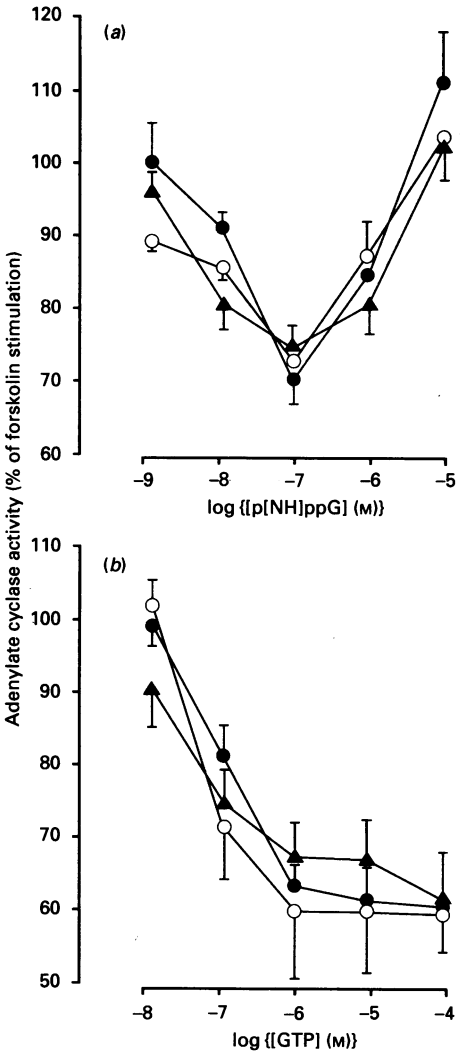

Fig. 1. Concentration–response curves for the effects of (*a*) p[NH]ppG and (*b*) GTP on forskolin-stimulated adenylate cyclase activity in plasma membranes

Membranes were assayed for adenylate cyclase in the presence of 100 μM-forskolin and (*a*) p[NH]ppG [○, control liver membranes ($n = 5$); ●, diabetic liver membranes ($n = 5$); ▲, diabetic hepatocyte membranes ($n = 6$)] or (*b*) GTP [○, control liver membranes ($n = 3$); ●, diabetic liver membranes ($n = 4$); ▲, diabetic hepatocyte membranes ($n = 6$)]. Results are expressed as percentages of the forskolin-stimulated adenylate cyclase rates (control liver membranes 111 ± 24 pmol of cyclic AMP produced/min per mg of protein, diabetic liver membranes 95 ± 15 pmol/min per mg and diabetic hepatocyte membranes 13 ± 2 pmol/min per mg). All data are means ± S.E.M. Maximal inhibitions by all guanine nucleotides were significant ($P < 0.01$).

DISCUSSION

Loss of G_i function in hepatocytes of streptozotocin-treated rats was identified originally as a defect in type I diabetes [3,4]. We show here that guanine nucleotide inhibition of forskolin-stimulated adenylate cyclase can be observed in the liver and hepatocyte membranes from rats made diabetic by streptozotocin and that this can be blocked by pertussis toxin. We therefore conclude that G_i function can be observed in membranes from diabetic rats. This report is therefore the first to demonstrate

Table 1. Pertussis-toxin sensitivity of G_i function in hepatocyte plasma membranes from diabetic rats

Hepatocyte membranes derived from diabetic animals were treated with ribosylation mixture in the absence or presence of pertussis toxin (PTX) and assayed for adenylate cyclase activity in the presence of forskolin (100 μM) and p[NH]ppG or GTP as shown. Results are expressed as percentages of the forskolin-stimulated rate. Data shown are means ± S.E.M. for 4 preparations: $*P < 0.05$ compared with incubations performed without pertussis toxin.

	Adenylate cyclase activity (% of forskolin-stimulated rate)	
Guanine nucleotide added	No PTX	+PTX
p[NH]ppG (0.1 μM)	72.5 ± 9.2	100.7 ± 2.7*
GTP (10 μM)	70.5 ± 12.5	105.8 ± 1.4*

clearly a receptor-independent inhibition of adenylate cyclase in liver and hepatocyte membranes from streptozotocin-treated rats by using a stringent membrane preparation protocol.

Previous contradictory reports have used hepatocyte (parenchymal) or liver (parenchymal + non-parenchymal) membranes [3,4,6]. Here, G_i function is clearly demonstrated in both types of membranes isolated from diabetic animals. This shows that a potential defect in hepatocyte membranes is not masked by functional G_i in non-hepatocyte material. Our results from both liver and hepatocyte membranes therefore differ from those of Gawler *et al.* [3,4], who were unable to show G_i function in this diabetic model, although, in agreement with those workers [4], we found a significant increase in forskolin-stimulated adenylate cyclase in liver membranes compared with that in membranes derived from hepatocytes (legend to Fig. 1).

In a previous report we have evaluated G_i function in hepatocyte membranes from type II diabetic animals [7]. We found that the degree of dilution of the initial tissue homogenate is an important factor in determining the level of G_i function in membranes [7]. In our hands, low dilution volume during homogenization leads to lowering of the percentage inhibition of stimulated adenylate cyclase, which could represent either a loss of G_i protein or a functional modification. Therefore, during the current experiments we have used the homogenization procedures described previously [7] which are those described to step 4 of the Neville procedure [10]. This method involves a dilution of 30 ml of buffer/g wet wt. of tissue and produces a significant dose-dependent guanine nucleotide inhibition of forskolin-stimulated adenylate cyclase. We consider such a homogenization procedure to be critical in the preparation of membranes for functional studies of G_i. Using this procedure we have been unable to reproduce the results of Gawler *et al.* [3,4], who employed similar hypo-osmotic $NaHCO_3$ medium at low dilution volume after homogenization and showed that G_i function is lost selectively in hepatocyte membranes from streptozotocin-treated animals.

G_i function has also been reported to be defective in adipocyte membranes prepared from streptozotocin-treated diabetic rats, although this can be over-ridden by activation of inhibitory receptors [18]. In hepatocyte membranes, our studies have focused on receptor-independent inhibition of adenylate cyclase by G_i. P_{2y}-purinergic receptors are the only well-defined inhibitory receptors coupled to G_i in this system [19]. However, activation of these receptors in membrane assays leads to difficulty in determining adenylate cyclase activity, since ATP serves as both substrate and agonist. Some workers have suggested that angiotensin II may couple to G_i in hepatocytes, and have argued that G_i function in liver membranes from streptozotocin-treated rats is functionally intact when assessed by angiotensin II inhibition of GTP-stimulated adenylate cyclase [6]. However, angiotensin II has also been demonstrated to stimulate phospholipase C [20] and diacylglycerol production [21] in intact hepatocytes, therefore making it difficult to attribute unambiguously an effect of angiotensin II to direct inhibition of adenylate cyclase through G_i.

Decreases in the levels of the mRNA encoding α-G_i-2 and α-G_i-3 have been reported in hepatocytes derived from streptozotocin-treated rats [22]. However, as discussed by these workers, attribution of changes in expression to consequent changes in function is problematical, particularly as it is still unclear which of these pertussis-toxin substrates is functional G_i [23]. Our present observations would suggest that, even if levels of G_i mRNA are decreased in this model system, G_i function, as assessed by inhibition of adenylate cyclases, appears little perturbed when assessed by the methodology described above.

We have observed normal G_i function in diabetic rats that have significantly raised blood glucose, and so conclude that impaired G_i function is unlikely to be responsible for the increased hepatic cyclic AMP concentration seen in livers from diabetic animals [2]. Indeed, earlier reports of hyperglucagonaemia and decreased cyclic AMP phosphodiesterase activity in the liver of streptozotocin-treated rats seem sufficient to explain the raised hepatic cyclic AMP concentration [24,25]. It is clear from the present observations and those reported elsewhere [7] that the level of G_i activity in membranes from normal and diabetic rats depends critically on the method of membrane preparation. Stringent description and control of preparative methods would seem a necessary part of any study which addresses the role of G_i in pathological states.

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