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Diabetes abolishes the GTP-dependent, but not the receptor-dependent inhibitory function of the inhibitory guanine-nucleotide-binding regulatory protein (G_i) on adipocyte adenylate cyclase activity

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Adipocyte membranes from control rats exhibited a functional G_i (inhibitory guanine-nucleotide-binding protein) activity which could be assessed either by the inhibitory action of low concentrations of guanosine 5-[$\beta\gamma$-imido]triphosphate (p[NH]ppG) upon forskolin-stimulated adenylate cyclase activity or by the inhibitory action of high concentrations of GTP upon isoprenaline-stimulated adenylate cyclase activity. When membranes from animals made diabetic with streptozotocin were used, then both such inhibitory functions of G_i were abolished. In contrast, receptor-mediated inhibitory responses of G_i, effected by N^6-phenylisopropyl (adenosine), prostaglandin E_2 or nicotinate, were either unchanged or even apparently more effective in membranes from diabetic animals. Induction of diabetes did not cause any change in the adipocyte plasma membrane levels of the α, GTP-binding subunits of either G_i1 or G_i2 or of G_s (stimulatory guanine-nucleotide-binding protein), but elicited an increase in the level of α-G_i3. The induction of diabetes reduced the specific activity of adenylate cyclase in adipocyte membranes and enhanced the stimulatory effect of isoprenaline. It is suggested that diabetes causes selective changes in the functioning of G_i in adipocyte membranes which removes the tonic GTP-dependent inhibitory function of this G-protein.

INTRODUCTION

Adenylate cyclase is a key regulatory enzyme which transduces the action of many hormones in a variety of target cells. The receptors which determine its function can exert stimulatory or inhibitory effects on this enzyme depending upon whether they are coupled to stimulatory (G_s) or inhibitory (G_i) guanine-nucleotide-binding regulatory proteins [1–4].

We have proposed [5,6] that changes in G_i functioning may occur in insulin-resistant states that characterize some forms of diabetes and obesity. This was based upon our observations that in rats made acutely diabetic by treatment with streptozotocin, there followed a loss of functional G_i activity, as assessed by using the non-hydrolysable GTP analogue p[NH]ppG (guanosine 5'-[$\beta\gamma$-imido]triphosphate), both in purified liver plasma membranes and in hepatocyte membranes [6]. Such a loss of functional G_i was originally attributed entirely to a loss of protein [6], although recent evidence has suggested to us that any residual G_i is inactivated [7]. Indeed, we have shown [5] that whereas G_i is expressed in hepatocyte membranes from both lean and obese Zucker rats, only in the lean animals is it functional.

Hepatocytes have a P_2-purinergic receptor which couples to G_i [8]. As such, receptor-coupled G_i functioning cannot be assessed in isolated hepatocyte membrane systems, as adenylate cyclase depends upon the presence of ATP as a substrate which will also serve as an agonist at P_2-purinergic receptors. In contrast with this system, adipocyte membranes provide the possibility of analysing G_i function through both receptor activation and direct guanine nucleotide activation.

Here we determine the effect of diabetes on G_i function in rat white adipocytes. In membranes from these cells, G_i function is thus assessed either directly, using GTP and p[NH]ppG to activate it, or via receptors for PIA [N^6-(phenylisopropyl)adenosine], PGE_2 (prostaglandin E_2) and nicotinate [9].

MATERIALS AND METHODS

All biochemicals were obtained from Sigma, except for guanosine 5-triphosphate, creatine kinase and p[NH[ppG, which were obtained from Calbiochem. Radiochemicals were from Amersham International, Amersham, Bucks, U.K.

Induction of diabetes

Epididymal fat pads from Sprague–Dawley rats (220–280 g; fed *ad libitum*) were used for these studies. Diabetes was induced, as before [6], by a single interperitoneal injection of streptozotocin (75 mg/kg of body weight; dissolved in 0.1 M-citrate buffer, pH 4.0). Animals were used within 3–4 days if the urinary glucose level reached 167–280 mM, as deduced by the use of Boehringer Diabur Test 5000 strips, or if blood glucose reached 175–250 mg of glucose/dl of blood, as deduced by use of Ames Dextrostix [6].

Abbreviations used: p[NH]ppG, guanosine 5'-[$\beta\gamma$-imido]triphosphate; PIA, N^6-(phenylisopropyl)adenosine; PGE_2, prostaglandin E_2; G_i, inhibitory guanine-nucleotide-binding regulatory protein; G_s, stimulatory guanine-nucleotide-binding regulatory protein.

Preparation of membranes

Rats were killed by cervical dislocation and white epididymal fat pads removed. Adipocytes were prepared according to the method of Rodbell [10]. Isolated adipocytes were suspended in 4 ml of buffered sucrose (0.25 M-sucrose/5 mM-Tris/HCl/3 mM-ATP, pH 7.4)/g of original tissue, at a temperature of 20–25 °C, and hand homogenized in a Potter–Elvejhem homogeniser (10 strokes). The resulting homogenate was then centrifuged at 1500 *g* for 5 min at 4 °C, the fat cake was removed and the supernatant was subjected to centrifugation at 15000 *g* for 15 min at 4 °C. The pellet was resuspended in buffered sucrose, by five strokes of a hand-operated Potter–Elvejhem homogenizer, and centrifuged at 15000 *g* for 15 min at 4 °C. The resulting pellet was resuspended in 1 mM-EDTA/1 mM-Tris/HCl, pH 7.4, to concentrations of approx 1–2 mg/ml and frozen rapidly to −80 °C. Membranes stored at this temperature retained full activity for at least 8 weeks.

Assay of adenylate cyclase

This was done essentially as described by Heyworth & Houslay [11]. Briefly, the reaction conditions employed the use of a medium containing 5 mM-$MgSO_4$, 10 mM-theophylline, 1 mM-EDTA, 22 mM-disodium phosphocreatine, 1.0 mM-ATP, 1 mM-dithiothreitol, 1 mg of creatine kinase/ml and 25 mM-triethanolamine/KOH, pH 7.4. Incubations were carried out over 30 min, using a final volume of 0.1 ml with 3–7 μg of protein at a temperature of 24 °C or 30 °C as indicated in the Results section. In experiments where PIA was employed, theophylline was omitted from the assays and the non-methylxanthine cyclic AMP phosphodiesterase inhibitor Ro-20-1724 was added at 0.1 mM, together with 1 unit of adenosine deaminase/ml. During this time period, reactions were linear and initial rates were measured.

Immunoblotting of membranes

The specific antisera to the gene products of the α-subunits of the 'G_i'-like genes and the procedures employed to analyse adipocyte membranes have been detailed before by us. [12]. Antiserum AS7 was used to detect the α-subunits of G_i1 and G_i2, and antiserum I3B was used to detect the α-subunit of G_i3. Antiserum CS1, which was generated against the *C*-terminal decapeptide of α-G_s, was used to detect the 42 kDa and 45 kDa forms of α-G_s.

Briefly, membrane samples were resolved by Nadodecyl sulphate electrophoresis [12.5% acrylamide/0.15% bisacrylamide (w/v) on 20 cm × 20 cm slab gels overnight at 100 V]. Proteins were transferred to nitrocellulose (Schleicher & Schuell) and blocked for 2 h at 25 °C with 3% dried skimmed milk (Marvel) in Tris-buffered saline (TBS; 20 mM-Tris/HCl/500 mM-NaCl, pH 7.5) or with 3% gelatin in TBS under similar conditions, except that the incubation was done at 37 °C. The primary antiserum was used at a 1:200 dilution in 1% dried skimmed milk (or 1% gelatin)/TBS and left overnight on the nitrocellulose before removal. The blot was then washed extensively with distilled water followed by washes using TBS containing 0.1% (v/v) Tween 20 and then TBS. Detection used secondary antisera which were donkey anti-rabbit IgG coupled to horseradish peroxidase (1:200 dilution) in 1% dried skimmed milk (or 1% gelatin), left for 3 h and then removed and washed as above before the addition of *O*-dianisidine hydrochloride as the detection system. In some experiments ^{125}I-labelled second antisera were used. Detection of the coloured product of the *O*-dianisidine was done as described before [6] and for the ^{125}I second antibody it was achieved by locating labelled species with autoradiography followed either by densitometric scanning or by excising the radioactive bands from the nitrocellulose and counting the radioactivity in a gamma counter. We estimated that our minimum level of detection of G-protein α-subunits was 0.25 pmol per track, and that linear relationships held for additions of adipocyte membranes to gel tracks of between 40 and 200 μg/track.

RESULTS AND DISCUSSION

It is now well established that, in membranes from adipocytes, hepatocytes and various other tissues, the G-proteins G_s and G_i exhibit very different affinities for guanine nucleotides [1–4]. This has allowed the experimental detection of G_i function by selective activation using appropriate concentrations of either GTP or p[NH]ppG [13–15]. Thus, after amplifying basal adenylate cyclase activity using the diterpene forskolin, low concentrations of p[NH]ppG cause the dose-dependent inhibition of adenylate cyclase activity through

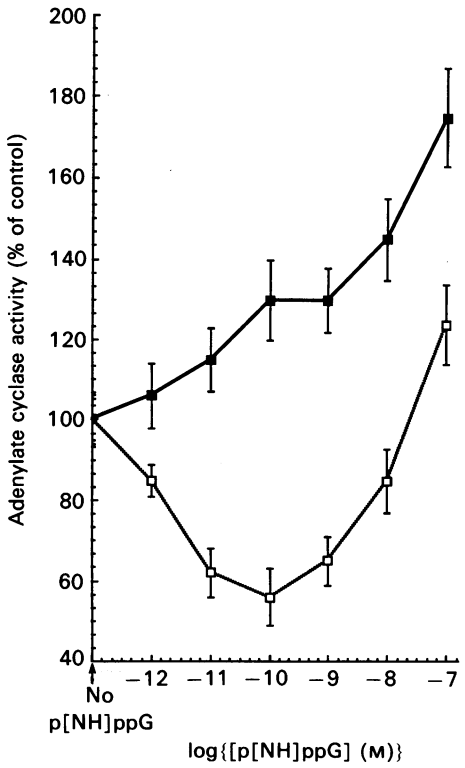

Fig. 1. Dose–effect experiments for p[NH]ppG on forskolin-stimulated adenylated cyclase activity in adipocyte membranes

Adenylate cyclase activity was monitored in the presence of forskolin (10^{-4} M). Specific activities are as in Table 1. Increasing concentrations of p[NH]ppG were added to assays employing adipocyte membranes from normal (□) and streptozotocin-diabetic (■) animals. Errors are S.D. for six experiments (diabetic) and five experiments (control) using different membrane preparations.

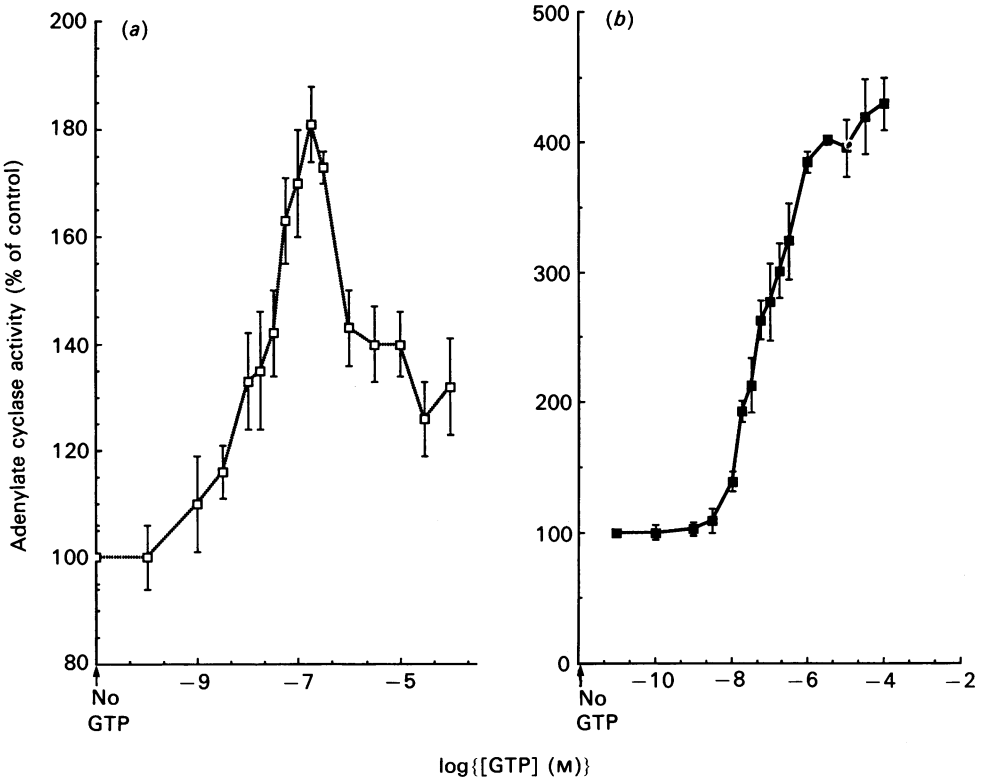

Fig. 2. Dose–effect experiments for GTP on isoprenaline-stimulated adenylate cyclase activity in adipocyte membranes

Adenylate cyclase activity was monitored in the presence of isoprenaline (5×10^{-4} M). Specific activities are as in Table 1. Experiments were done on (*a*) control and (*b*) streptozotocin-induced diabetic animals. Errors are S.D. for four (control) and six (diabetic) experiments using different membrane preparations.

a G_i-mediated response (Fig. 1) [6,14–16]. Alternatively, G_i function can be demonstrated by showing biphasic effects of GTP on isoprenaline-stimulated adenylate cyclase activity (Fig. 2*a*). In this instance, low concentrations of GTP promote activation by stimulating the coupling of the β-adrenoceptor to G_s, whereas high concentrations cause inhibition due to G_i, as reported by others in adipocytes [13] and hepatocytes [15].

Here we demonstrate that both of these G_i-mediated effects were abolished completely when adipocyte membranes were prepared from diabetic rats (Figs. 1 and 2*b*). Such an action may have physiological consequences because intracellular GTP concentrations reach approx. 500–700 μM [1] and can thus be expected to exert a tonic inhibitory effect on 'resting' (basal) adenylate cyclase activity. This tonic inhibitory action upon adenylate cyclase was abolished in adipocytes from diabetic animals, and only a stimulatory action of GTP was apparent (Fig. 2). This led to an elevation in adenylate cyclase activity under 'resting' conditions, i.e. in the absence of hormonal stimulation. The net effect of any such relative enhancement of 'resting' intracellular adenylate cyclase activity in the diabetic state would, however, be attenuated by the fact that basal adenylate cyclase activity was itself reduced in both adipocytes (Table 1) and in hepatocytes [6] from diabetic animals. It remains to be seen whether this is due to a reduced amount of adenylate cyclase itself or to some modification of the catalytic unit of this enzyme.

In contrast to the loss of functional G_i, as assessed by direct activation, in adipocyte membranes from diabetic animals, we found that the non-metabolizable adenosine analogue PIA was still able to inhibit isoprenaline-stimulated adenylate cyclase in these membranes (Table 2, Fig. 3*a*). Indeed, continued receptor-mediated inhibition was not restricted to adenosine receptors, as both nicotinate and PGE_2 were also able to elicit inhibitory responses in adipocyte membranes from both normal and diabetic animals (Table 2). It thus seems that the receptor-mediated inhibitory control of G_i is not lost in diabetic states. This is also consistent with our re-evaluation of G_i function in hepatocytes from diabetic animals (M. Bushfield & M. D. Houslay, unpublished work). In this state we can now show that whereas G_i function, as assessed by p[NH]ppG-mediated inhibition, is lost, P_2-purinergic-receptor-mediated inhibition still occurs, albeit in a reduced state which is commensurate with the degree of reduction in α-G_i2.

In order for an inhibitory action of G_i to be observed in membranes from diabetic animals, it was essential that PIA be present in the assay. Thus preincubation of membranes from diabetic animals in adenylate cyclase assay buffer for periods of 5–10 min with PIA (1 μM), followed by harvesting of the membranes by centrifugation and washing once with EDTA/Tris buffer, failed to expose any function of G_i when these membranes were assayed subsequently for GTP-dependent inhibition in the absence of PIA.

Table 1. Adenylate cyclase activity in adipocyte membranes from normal and diabetic animals

Adipocyte membranes from control and diabetic animals were assayed for adenylate cyclase activity in the absence of any added ligand (basal) and in the presence of either forskolin (10^{-4} M) or isoprenaline (5×10^{-4} M). Data are given for five separate experiments using different animals, with errors as S.D. Figures in parentheses indicated activation (-fold) in the presence of added ligand compared with basal activity (*$P < 0.001$).

	Specific activity (pmol/min per mg)		
	Basal	+Forskolin	+Isoprenaline
Control	157±9	335±40 (2.1)	22.0±28 (1.4)*
Diabetic	56±8	139±22 (2.5)	250±49 (4.5)*

The fact that PIA appears to show an enhanced inhibitory effect in membranes from diabetic animals (Table 2) is probably due to the fact that inhibition of adenylate cyclase mediated by GTP alone is abolished in diabetic membranes, whereas this is not the case in membranes from normal animals. Thus PIA, in the presence of GTP, has a greater apparent inhibitory effect. Indeed, the GTP-dependence of the inhibitory action of PIA can be seen clearly in membranes from diabetic animals (Fig. 3*b*). Thus we see that, in the presence of PIA (Fig. 3*b*), but not in its absence (Fig. 2*b*), elevated GTP concentrations do indeed lead to PIA-mediated inhibition of adenylate cyclase activity.

We also noted that the ability of isoprenaline to stimulate adenylate cyclase activity in membranes from diabetic animals was enhanced in comparison with that found in membranes from non-diabetic controls (Table 1). Such an observation has been made for both the isoprenaline- [17] and glucagon- [15] mediated stimulation of adenylate cyclase activity in membranes from normal animals whose cells were pretreated with pertussis toxin in order to inactivate G_i before assay. The heightened response employing diabetic membranes was not due to any increase in the amount of the stimulatory G-protein, G_s, as the induction of the diabetes did not affect the amount of the two forms [45 kDa (G_s') and 42 kDa (G_s); [1] of α-G_s subunits (Fig. 4). Thus levels of α-G_s' and α-G_s in membranes from diabetic animals differed by some 6 ± 5% and -7 ± 6% respectively from those seen using membranes from control animals (six different animals). We believe, then, that inactivation of the GTP-stimulation functioning of G_i in diabetic states removes a tonic inhibitory effect on receptor–G_s-mediated activation of adenylate cyclase, which leads to enhanced stimulatory receptor responses.

We sought to ascertain whether these changes in G_i-

Table 2. Receptor-mediated inhibition of adenylate cyclase

Assays were performed in the presence of 10^{-5} M-GTP and 5×10^{-4} M-isoprenaline. The maximal inhibitory effect of PGE_2, nicotinate and PIA is shown together with the IC_{50} values (concns. causing 50% inhibition). Data were obtained from dose–effect curves done at eight different ligand concentrations, and errors are S.D. for three separate experiments using different membrane preparations.

	Normal		Diabetic	
Ligand	Inhibition (%)	IC_{50} (nM)	Inhibition (%)	IC_{50} (nM)
PIA	54±8	37±7	83±9	20±4
PGE_2	54±6	38±6	68±9	45±7
Nicotinate	51±11	708±48	67±13	753±46

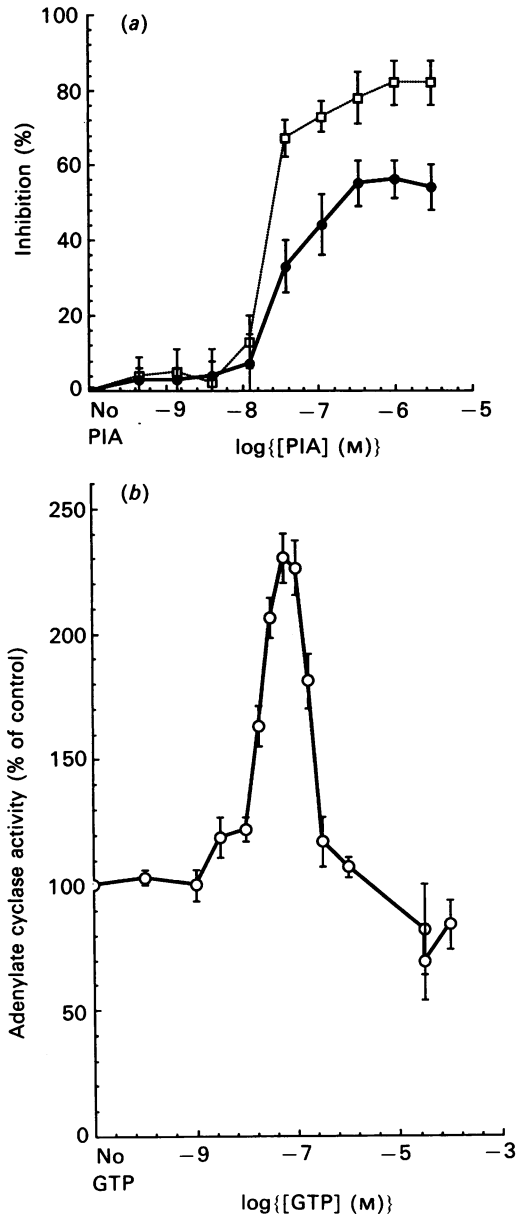

Fig. 3. PIA-mediated effects on adenylate cyclase activity.

(*a*) Dose–effect curve for PIA carried out in the presence of isoprenaline (5×10^{-4} M) and GTP (10^{-5} M) using adipocyte membranes from normal (●) and diabetic (□) animals. (*b*) Dose–effect curve for GTP carried out in the presence of isoprenaline (5×10^{-4} M) and PIA (1 μM) using membranes from diabetic animals. Experiments typical of three similar experiments using different membrane preparations are shown.

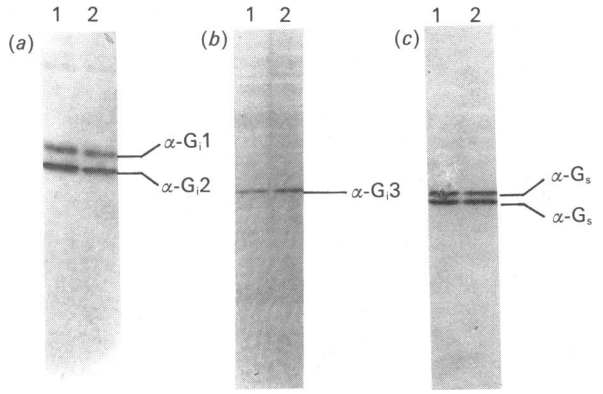

Fig. 4. Identification of α-G_i in adipocyte membranes

Western blotting data are shown which were obtained using specific antisera to identify the α-subunits of forms of G_i and of G_s in adipocyte membranes as described before by us [12]. Track 1 contains membranes from control animals and track 2 from diabetic animals. In each instance identical amounts of membrane protein, from control and diabetic animals, were loaded on to the gels. (*a*) Antiserum AS7 was used to detect α-G_i1 (41 kDa) and α-G_i2 (40 kDa) using 70 μg of membrane protein. (*b*) Antiserum I3B was used to detect α-G_i3 (41 kDa) using 70 μg of membrane protein. (*c*) Antiserum CS1 was used to identify the 45 kDa (G_s1) and 42 kDa (G_s) forms of α-G_s using 70 μg of membrane protein.

mediated responses could be attributed to any alterations in the expression of the three forms of G_i that are found in adipocytes [12]. Although the functional role of these various G-proteins has yet to be definitively ascertained, it has been suggested that G_i2 might be the major species responsible for determining the inhibition of adenylate cyclase [18]. Certainly this would be consistent with our observations that G_i-mediated inhibition of adenylate cyclase is seen both in adipocytes, which express little G_i3 [12], and in hepatocytes [6,16,17], which do not appear to express detectable levels of G_i1 (S. Griffiths, M. Bushfield & M. D. Houslay, unpublished work), whereas both cells express G_i2. In this study, we used the antiserum AS7 to detect both α-G_i1 (41 kDa) and α-G_i2 (40 kDa) (Fig. 4), which can be resolved by polyacrylamide-gel electrophoresis under appropriate conditions [12]. However, we found no evidence for differences in the apparent amounts of either of these two G-protein α-subunits in adipocyte membranes from normal and diabetic animals (Fig. 4). In eight separate experiments using different animals in which adipocyte membranes were immunoblotted with the antiserum AS7, we found that the level of α-G_i1 in membranes from diabetic animals differed by only some -5.5 ± 8 % compared with that in membranes from control animals. The same experiments showed that levels of α-G_i2 differed by some 8 ± 7 % in membranes from diabetic animals compared with membranes from control animals. In contrast with this, using the antiserum I3B to detect α-G_i3, we observed that levels of this particular G-protein α-subunit were increased by some 88 ± 18 % (eight separate experiments using different animals) in adipocyte membranes from diabetic animals compared with those from control animals. These results suggest that the loss of guanine-nucleotide-elicited G_i activity in adipocyte membranes of diabetic animals is not due to any selective loss of one form of G_i which might, for example, mediate GTP-dependent inhibition whereas other retained species mediate receptor-dependent inhibition. The more pronounced inhibitory potency of PIA in membrane from diabetic animals may be due to elevated α-G_i3 levels; however, we suggest that the explanation mooted above would serve equally well.

This situation bears comparison with hepatocyte membranes of Zucker rats. In this case, membranes from obese animals exhibited little functional G_i, as assessed by either GTP- or p[NH]ppG-dependent inhibition, whereas an activity comparable with that seen with normal Sprague–Dawley rats was seen with the lean animals [5], despite quantitative immunoblotting identifying similar amounts of α-G_i in membranes from both types of animals.

Begin-Heick and her collaborators have demonstrated a loss of functional G_i activity in adipocyte membranes from genetically obese (*ob/ob*) rats [19–21]. In such experiments, attempts to quantify α-G_i, using pertussis-toxin-catalysed ADP-ribosylation, indicated similar levels of this G-protein in membranes from both the normal and diabetic littermates. Such experiments bear similarity to those described in this study and our previous investigations [5,6] in highlighting a lesion in the inhibitory function of G_i, rather than any loss of expression, in insulin-resistant states. Interestingly, however, Londos and co-workers [9] have refuted the work of Begin-Heick [20], as they were unable to observe a loss of GTP-mediated inhibition of adenylate cyclase in adipocyte membranes from obese (*ob/ob*) mice. We believe that our study, however, offers an explanation for this discrepancy. This is because Londos and co-workers [9] performed their GTP dose-effect experiments in the presence of the adenosine analogue PIA, whereas Begin-Heick [20] did not. Thus if the situation is analogous to that which we describe here, both investigators are correct. Begin-Heick [20] will have monitored the loss in the ability of 'GTP-alone' to cause inhibition of adenylate cyclase, whereas Londos *et al.* [9] employed GTP to expose PIA-mediated inhibition. By analogy with what we describe here, it is possible to lose the first effect and not the second. Indeed, as here (Table 1) Londos and co-workers [9] also observed an enhanced ability of PIA to elicit inhibition in membranes from obese animals.

Our observation that GTP- and receptor-mediated inhibitory functions of G_i can be separated is not restricted to the modification of G_i seen in membranes from insulin-resistant states. Indeed, it has been shown [22] that the addition of high concentrations of NaCl (greater than 150 mM) can obliterate the inhibitory action of GTP while retaining and even amplifying that seen using PIA. It is possible, then, that both high NaCl concentrations and the lesion of G_i occurring in insulin-resistant states alter the conformation of G_i in such a way as to attenuate severely its ability to inhibit adenylate cyclase. However, functional coupling of G_i to an inhibitory receptor presumably elicits a sufficiently powerful effect on the conformation of this G-protein so as to overcome the attenuating action. Thus the inhibitory G-protein G_i may serve two functions in the cell, one of which involves mediating a tonic inhibitory effect on adenylate cyclase and the other the coupling to inhibitory receptors.

We have demonstrated recently [23] that the α-subunit of G_i can be phosphorylated by challenge of hepatocytes with hormones or agents that activate protein kinase C. This leads to the loss of ability of low concentrations of p[NH]ppG to inhibit forskolin-stimulated adenylate cyclase activity. Indeed, the α-subunit of G_i is now known to be a substrate not only for protein kinase C [23,24] but also for protein kinase A [25] and the insulin receptor kinase [26,27]. It is possible, therefore, that the lesion which leads to a change in functioning of G_i is its phosphorylation. We will show elsewhere (M. Bushfield & M. D. Houslay, unpublished work) that this occurs on α-G_i2 and that such a modification does not affect the ability of AS7 to either recognize or immunoprecipitate this G-protein. Indeed, one of us has suggested [28] that the protein kinase C-mediated phosphorylation of the insulin receptor and α-G_i may be key features of insulin-resistant states [28,29].

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