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Quantification of the α and β subunits of the transducing elements (G_s and G_i) of adenylate cyclase in adipocyte membranes from lean and obese (*ob/ob*) mice

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The abundance of the α and β subunits of the GTP-binding proteins (G-proteins) that transduce hormonal messages to adenylate cyclase was assessed in adipocyte membranes from lean (+/+) and obese (*ob/ob*) mice, using ADP-ribosylation with bacterial toxin and immunodetection. Both methods revealed two $G_s\alpha$ species (48 and 42 kDa) in the membranes. Compared with those of lean mice, the membranes from obese mice contained substantially less of the 48 kDa species of $G_s\alpha$, as assessed by both methods. ADP-ribosylation by pertussis toxin showed that only half as much ADP-ribose was incorporated into $G_i\alpha$ in the membranes from obese as compared with lean mice. Immunodetection revealed two separate $G_i\alpha$ peptides (39 and 40 kDa) and showed that the 40 kDa species was less abundant in the membranes from obese mice, whereas the amount of the 39 kDa species was similar in membranes from both lean and obese animals. Based on ADP-ribosylation assays, in membranes from lean mice the ratio $G_s\alpha/G_i\alpha$ was 1:16, whereas in the membranes from obese mice it was 1:10. Similar amounts of immunodetectable β peptide were found in both types of membranes. On the basis of the currently accepted dissociation model of adenylate cyclase activation, the decrease in the abundance of the $G_i\alpha$ subunit in adipocyte membranes from obese mice could account for the abnormal kinetics of the enzyme in these membranes.

INTRODUCTION

It is generally accepted that the transducing element of adenylate cyclase (AC) is composed of two sets of GTP-binding proteins (G-proteins), i.e. G_s and G_i, each an oligomer of $\alpha\beta\gamma$ composition. One of the oligomers, G_s, mediates the activation of the catalytic unit of AC whereas the other, G_i, prevents it (see Gilman, 1987, and references therein). G_s is composed of two major groups of α subunits of different relative mobilities (approx. 48 kDa and 42 kDa) produced by differential splicing of a common mRNA (Robishaw *et al.*, 1986; Mattera *et al.*, 1986). These are substrates for ADP-ribosylation by cholera toxin and they have been found in most tissues. Several pertussis toxin substrates have also been identified, varying in molecular mass from 39 kDa to 41 kDa (Suki *et al.*, 1987; Jones & Reed, 1987); three of these have been classified as $G_i\alpha$ and one as G_o. The exact functions of all the pertussis toxin substrates are not yet known.

The current model proposed for the regulation of AC is concerned mostly with the interactions of the α and βγ components of G_s and G_i. On the basis of functional studies and of amino acid analysis of fragments obtained after proteolytic digestion, it is believed that the β subunits of G_s and G_i are interchangeable, although at least two forms of the β subunit (β_{35} and β_{36}) are known to occur (Evans *et al.*, 1986). Excess βγ subunit inhibited the stimulatory effect of $G_s\alpha$ in a reconstituted AC system and the $G_i\alpha$ subunit was able to relieve this inhibition. In certain cell types, an excess of βγ subunit apparently maintains the AC in a permanently inhibited state (Bokoch, 1987). These and other findings have led to the presently held view that the dual regulation of AC is the result of the mass action effect of the β subunit. According to the model, the main function of the α subunit of G_i is to act as a receptacle for the βγ subunits. Although the model does not explain all of the findings, it is widely used to explain cyclase regulation (Gilman, 1987). Many mutants of the AC system have been found (Haga *et al.*, 1977; Bourne *et al.*, 1982; Schimmer & Tsao, 1984) which alter the regulatory properties of AC. There are several examples showing that tissues differ in the proportion of regulatory components they contain and that the relative amounts of the subunits of the transducing G-proteins are subject to modulation (Bokoch, 1987; Carter *et al.*, 1987; Milligan *et al.*, 1987; Watkins *et al.*, 1987; Hinsch *et al.*, 1988; Houslay *et al.*, 1989; Garcia-Sainz *et al.*, 1989; Green & Johnson, 1989).

The purpose of the present work was to examine the likelihood that an imbalance in the G-protein subunits is responsible for the abnormal kinetics of AC seen in the adipocyte membranes of the *ob/ob* mouse (Bégin-Heick, 1985). We know that both G_s and G_i are present and are ADP-ribosylated in the adipose tissue and in other tissues of the obese mouse (Bégin-Heick, 1985; Greenberg *et al.*, 1987; Bégin-Heick & Welsh, 1988), but we do not know their relative abundance. The data presented in this paper show that compared with those from the lean mouse, adipocyte membranes from the *ob/ob* mouse have a lower abundance of $G_i\alpha$ and $G_s\alpha$ subunits.

These data were presented in part at the 48th meeting of the Federation of American Societies of Experimental Biology, New Orleans, LA, U.S.A., in March, 1989.

EXPERIMENTAL

Materials

[α-^{32}P]ATP, [^{3}H]cyclic AMP, [^{3}H]AMP and [^{32}P]NAD^+ were obtained from the New England Nuclear Corporation, Lachine, Quebec, Canada. [γ-^{32}P]ATP, ^{125}I-labelled goat anti-rabbit IgG

Abbreviations used: G-protein, GTP-binding protein, AC, adenylate cyclase; Gpp[NH]p, guanosine 5′-[βγ-imido]triphosphate; PIA, N^6-(L-2-phenylisopropyl)adenosine; Me_2SO, dimethyl sulphoxide.

and the biotin–streptavidin immunodetection system were from Amersham Canada Ltd., Oakville, Ontario, Canada. Caffeine, ATP (cat. no. A-2383, prepared by the phosphorylation of adenosine), cyclic AMP, phosphocreatine (Tris salt), creatine kinase, myokinase, (−)-isoprenaline and cholera toxin were from the Sigma Chemical Co., St. Louis, MO, U.S.A. GTP and guanosine 5′-[βγ-imido]triphosphate (Gpp[NH]p) were from PL Biochemicals, Milwaukee, WI, U.S.A. N^6-(L-2-Phenylisopropyl)-adenosine (PIA) was from Boehringer–Mannheim, Montreal, Canada. Forskolin was from Calbiochem–Behring (Terochem Laboratories, Rexdale, Ontario, Canada). Pertussis toxin was from List Biochemicals (Campbell, CA, U.S.A.). Universol was from ICN Biomedicals, Montreal, Quebec, Canada.

Animals

Male C57Bl/6J *ob/ob* mice and lean controls (+/+) were obtained from the Jackson Laboratories, Bar Harbor, ME, U.S.A., at 7–8 weeks of age. They were kept in a temperature-controlled room (23±1 °C) with 12 h light–dark cycles. The animals were fed on Purina chow and given water *ad libitum*; they were used in the experiments at the age of 9–12 weeks.

Preparation of membranes from adipocytes

The mice were killed by cervical dislocation and their white epididymal fat pads were removed. Adipocyte membranes were prepared from the white epididymal fat pad as described previously (Bégin-Heick, 1985) (Method A) or according to the method of Greenberg *et al.* (1987) with the exception that the sucrose-density-gradient centrifugation was omitted (Method B).

Enzyme assays

(a) Adenylate cyclase. This was carried out essentially as described by Salomon *et al.* (1974). The incubation mixture contained the following components in a total volume of 0.1 ml: 30 mM-Tris buffer containing 0.75 mM-EGTA adjusted to pH 7.4 with 0.2 mM-acetic acid, 20 mM-phosphocreatine, 1.75 units of creatine kinase, 1 unit of adenosine deaminase, 0.1 mM-cyclic AMP and 0.05 mM-[α-^{32}P]ATP (2.6×10^6 c.p.m./assay). [^{3}H]Cyclic AMP (12000–15000 c.p.m./assay) was added to monitor the recovery of [^{32}P]cyclic AMP. Other components of the reaction such as nucleotides, agonists and $MgSO_4$ were added at the concentrations specified in the legends to Tables and Figures. After adding the enzyme (10 μg of protein), the tubes were incubated with shaking at either 25 or 30 °C for 10 min. When PIA was present, it was added as a dimethyl sulphoxide (Me_2SO) solution. In these experiments, Me_2SO (10 μl) was also added to the control tubes. In the experiments with PIA, caffeine was omitted from the incubation medium. The incubation was terminated by the addition of 0.1 ml of a solution consisting of 0.2% SDS, 1.4 mM-cyclic AMP and 40 mM-ATP at pH 7.5, followed by 0.8 ml of water. [^{32}P]Cyclic AMP was separated from the [^{32}P]ATP essentially by the double column method of Salomon *et al.* (1974) with slight modifications (Bégin-Heick, 1985). The eluate from the alumina column was collected and counted for radioactivity in a Beckman LS-250 liquid scintillation counter after the addition of 10 ml of Universol.

(b) 5′-Nucleotidase (EC 3.1.3.5). This was done essentially by the method of Avruch & Wallach (1971), using concentrations of AMP varying from 5 to 100 μM. Membrane protein (2–5 μg) was added to start the reaction and the tubes were incubated for 30 min at 37 °C.

(c) ($Na^+ + K^+$)-ATPase (EC 3.6.1.3). This was done essentially according to the method of Esmann (1988), except that [γ-^{32}P]ATP was used. The concentrations of ATP ranged from 0.5 to 3 mM. The reaction was started by the addition of 10 μg of membrane protein and the tubes were incubated for 10 min at 37 °C. The liberated P_i was separated from the ATP by adsorption on charcoal as described by Avruch & Wallach (1971).

Activation of membranes with cholera toxin

This was done essentially according to the method of Ribeiro-Neto *et al.* (1987). Cholera toxin was preactivated by incubation at 37 °C for 10 min in a medium consisting of 0.01 M-Tris/HCl buffer, pH 7.5, containing 0.04 M-NaCl, 0.6 mM-NaN_3, 0.2 mM-Na_2EDTA, 50 mM-glycine and 20 mM-dithiothreitol. Adipocyte membranes (40–50 μg) were incubated at 30 °C for 30 min in 250 μl of a medium containing 10 μg of preactivated cholera toxin, 20 mM-Tris/HCl, 1 mM-GTP, 10 mM-$MgSO_4$, 1 mM-EDTA, 300 mM-P_i, 10 mM-thymidine, 1 mM-nicotinamde, 4 μM-[^{32}P]NAD^+ at a specific radioactivity of 15 μCi/mmol and a regeneration system consisting of 20 mM-phosphocreatine, 40 units of creatine kinase/ml and 40 units of myokinase/ml. The pH of the incubation mixture was 7.5. Preliminary experiments showed that the addition of SDS to the incubation mixture diminished rather than enhanced ADP-ribosylation in adipocyte membranes. The ribosylation of the 48 kDa moiety was diminished more than was that of the 42 kDa moiety. This is in contrast with brain membranes which, as reported by others (Neer *et al.*, 1987) and in our hands (N. Bégin-Heick, unpublished work), require the addition of detergent for complete ribosylation to occur. The reaction was stopped by the addition of 4 ml of ice-cold buffer followed by centrifugation at 15000 ***g*** to sediment the membranes. The membranes were washed twice with cold buffer and dissolved in electrophoresis buffer as described below.

Treatment with pertussis toxin

Pertussis toxin was preactivated for 20 min at 30 °C in a medium consisting of 0.05 M-Tris/HCl, 50 mM-glycine (pH 7.5) and 20 mM-dithiothreitol. The activated toxin (2 μg) was added to 250 μl of a medium consisting of 20 mM-Tris/HCl, pH 7.5, 10 mM-thymidine, 1 mM-nicotinamide, 1 mM-ATP, 1 mM-GTP, 1 mM-EDTA, 5 μM-[^{32}P]NAD^+ (15 μCi/mmol) and the ATP-regenerating system described for the cholera toxin activation. Prior to their addition to the incubation mixture, the membranes (150–200 μg of protein/ml) were incubated for 10 min on ice with 1% Lubrol. After adding the membranes to the incubation mixture, the ADP-ribosylation reaction was allowed to proceed for 30 min at 30 °C. The reaction was stopped by the addition of NaCl and acetone, and the membranes were sedimented and extracted as described by Ribeiro-Neto *et al.* (1987).

SDS/polyacrylamide-gel electrophoresis

This was carried out in slab gels using the discontinuous buffer system of Laemmli (1970) with the modifications of Newbold *et al.* (1982). Essentially, a 3% (w/v) acrylamide stacking gel and a 10% (w/v) acrylamide separating gel were used, with a ratio of acrylamide to bisacrylamide of 30/0.4. Membrane suspensions were dissolved in an equal volume of sample buffer containing 2% SDS, 10% 2-mercaptoethanol, 2 mM-EDTA, 4 mM-phenylmethanesulphonyl fluoride and 100 mM-Tris/HCl (pH 7.8), and boiled for 3 min before application to the gel. Following electrophoresis, the gels were dried and autoradiographed to assess the extent of ADP-ribose incorporation. The intensity of the bands was assessed with a laser densitometer (LKB Ultroscan).

Immunoblotting

Following electrophoresis, the proteins were transferred from the gels to nitrocellulose membranes using an electroblotting

apparatus (Bio-Rad). Before transfer, the gels and the nitrocellulose membranes were equilibrated for 30 min in the transfer buffer (25 mM-Tris and 192 mM-glycine adjusted to pH 8.3 with HCl and containing 20% methanol). The transfer was effected at 0.3 A for 2.5 h. The nitrocellulose membranes were then incubated for 50 min in 10 mM-Tris/HCl buffer, pH 7.5, containing 500 mM-NaCl and 3% skimmed-milk protein to quench the non-specific charges. Immunodetection was carried out according to a modification of the method of Gierschik *et al.* (1985) by incubation for 3 h at 37 °C with specific antibodies (1:100 dilution in the Tris/NaCl/skimmed-milk buffer) to G-protein components as detailed in the legends to Tables and Figures. The membranes were washed several times with Tris/NaCl/HCl buffer containing 0.2% Tween-20. They were then incubated with an ^{125}I-labelled goat anti-rabbit IgG diluted in the Tris/NaCl/skimmed-milk buffer for 2 h at room temperature. After drying, the membranes were autoradiographed and the intensity of the radioactive bands was assessed with a laser densitomer. In some cases, the antigen–antibody complex was revealed with a biotin–streptavidin system using horseradish peroxidase and 4-chloro-1-naphthol. The biotinylated antibody was used at 1:50 dilution and the streptavidin–peroxidase at 1:100 dilution.

Protein determinations

The protein content of the membrane fractions was determined by the method of Bradford (1976) using bovine serum albumin as the standard.

Statistical analyses

The differences between means were evaluated by Student's *t* test for matched pairs. Differences between means were considered to be statistically significant when the *P* value was less than 0.05.

RESULTS

Characterization of membranes

AC activity. The preparation and storage of the membranes in the presence of proteolytic inhibitors (Method B) led to a significant enhancement of the AC activity in adipocyte membranes from both the lean and the obese mice, compared with the

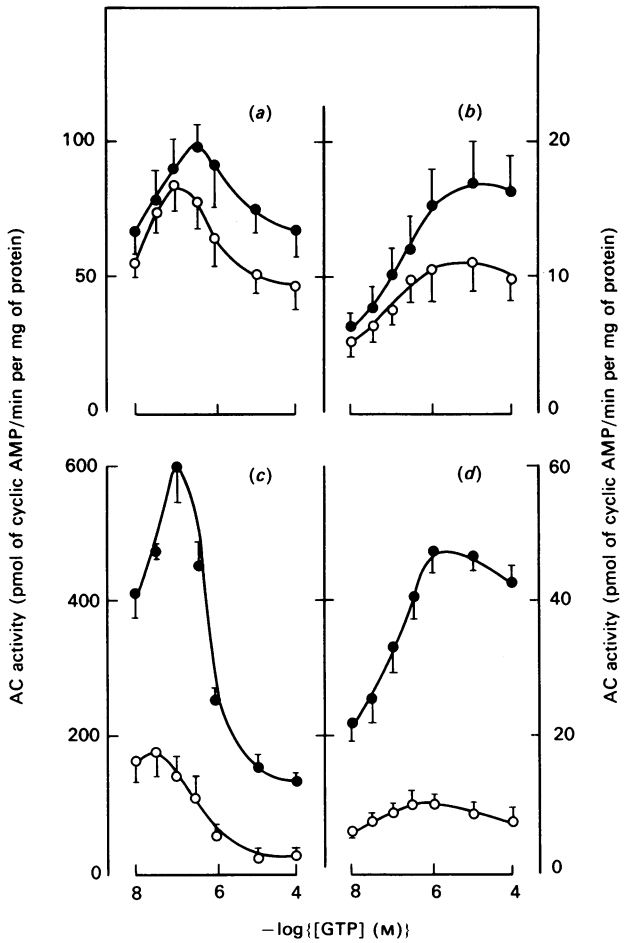

Fig. 1. Effect of assay conditions on AC activity in membranes prepared by two different methods

Membranes were prepared from adipose tissue of lean (+/+) (*a* and *c*) and obese (*ob/ob*) (*b* and *d*) mice according to Method A (*a* and *b*) or B (*c* and *d*) and assayed for AC activity with 500 μM-isoprenaline and the concentrations of GTP shown on the abscissa. The assays were done either at 25 °C with 1 mM-$MgSO_4$ (○) or at 30 °C with 5 mM-$MgSO_4$ (●). The data are expressed as pmol of cyclic AMP produced/min per mg of protein and are means ± S.E.M. for four observations with different membrane preparations.

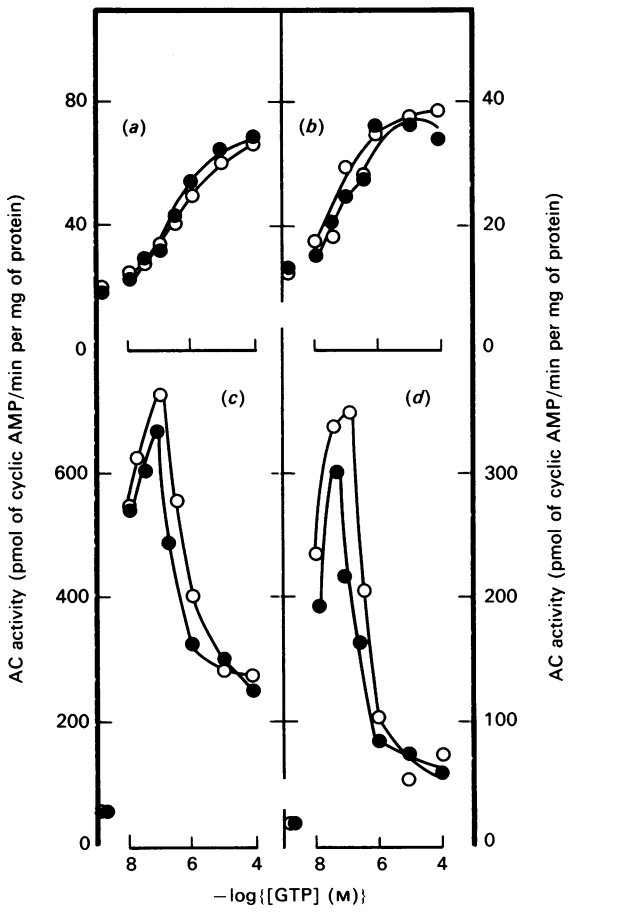

Fig. 2. Effect of PIA on AC activity

Membranes from lean (+/+) (*c* and *d*) and obese (*ob/ob*) (*a* and *b*) mice were incubated either at 30 °C with 5 mM-$MgSO_4$ (*a* and *c*) or at 25 °C with 1 mM-$MgSO_4$ (*b* and *d*) at the concentrations of GTP shown on the abscissa. The medium also contained 500 μM-isoprenaline. The membranes were incubated with PIA (10 μM) dissolved in Me_2SO (●) or with Me_2SO alone (○). The data shown are from one experiment, representative of six similar experiments at 30 °C and four at 25 °C using different membrane preparations.

membranes prepared without inhibitors that had been used in previous work (Method A). Membranes prepared by each method were also compared for their AC response to GTP under different conditions. The data shown in Fig. 1 for membranes incubated at 25 °C with 1 mM-$MgSO_4$ (cf. Bégin-Heick, 1985) or at 30 °C with 5 mM-$MgSO_4$ (cf. Greenberg *et al.*, 1987) indicate that, under both conditions, the inhibitory effect of GTP was evident in the membranes from lean mice. In contrast, in the membranes from obese mice, there was no evidence that GTP had an inhibitory effect under any of the conditions tested. In addition, the stimulatory response to GTP was greater in membranes from both lean and obese animals prepared by Method B compared with Method A. Because membranes prepared and stored in the presence of proteolytic inhibitors had a much higher specific activity and, in the case of the membranes from lean mice, showed a much more pronounced inhibitory effect of GTP, Method B was used for all further experiments.

The effect of PIA on AC activity was next examined in the presence of isoprenaline and various concentrations of GTP. The assays were done either at 30 °C and 5 mM-$MgSO_4$ or at 25 °C and 1 mM-$MgSO_4$ (Fig. 2). At 30 °C there was little effect of PIA on the dose–response curve to GTP in the membranes from both lean and obese mice (Figs. 2*a* and 2*c*). When the membranes were incubated at 25 °C, however, there was a significant inhibition of AC activity by PIA in the membranes from the lean but not the obese mice. The greatest effect of PIA in the membranes from lean mice was observed at concentrations of GTP that promoted maximal activity of the enzyme. An analysis of the effects of PIA in several different membrane preparations is shown in Fig. 3. It is evident from these data that PIA was able to inhibit AC activity significantly at GTP concentrations between 30 nM and 1 μM, the maximal inhibitory effect (34±2.2%) occurring at 0.1 μM-GTP. The inhibitory effect of PIA was largely attenuated by increasing the temperature to 30 °C and the concentration of $MgSO_4$ to 5 mM.

These data are therefore consistent with my previously published work (Bégin-Heick, 1985) and show that neither GTP nor PIA significantly inhibits AC activity in adipocyte membranes from obese mice, even under conditions where the inhibition is clearly demonstrable in membranes from lean mice.

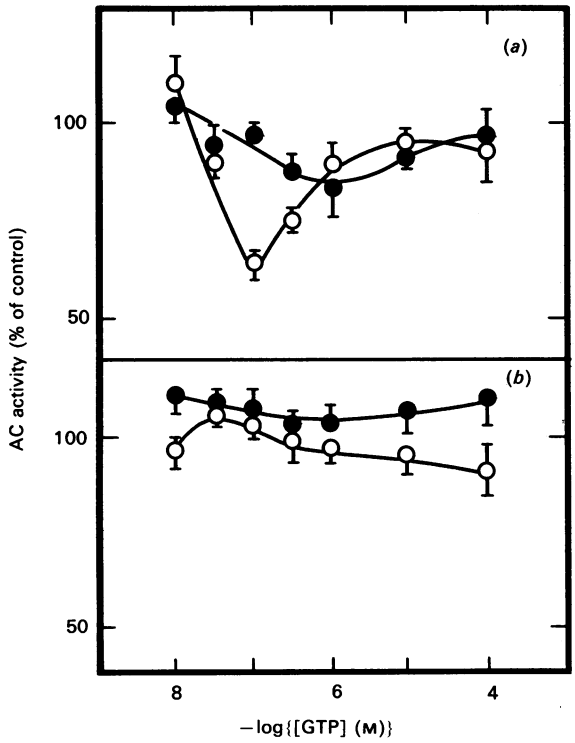

Fig. 3. Influence of GTP concentration on the inhibitory effect of PIA

At each GTP concentration, the AC activity in the presence of 10 μM-PIA was calculated as a percentage of control activity with Me_2SO alone. The data are means±S.E.M. for five experiments at 30 °C (●) and four experiments at 25 °C (○). (*a*) Lean (+/+), (*b*) obese (*ob/ob*).

Table 1. Activities of marker enzymes in white-adipocyte membranes of lean (+/+) and obese (*ob/ob*) mice

The enzyme assays were done as described in the Experimental section. The data shown are means±S.E.M. for five and seven separate experiments for $(Na^+ + K^+)$-ATPase and 5′-nucleotidase respectively. Each experiment was with a different membrane preparation. * Significantly different from control (+/+); $P < 0.01$.

	$(Na^+ + K^+)$-ATPase		5′-Nucleotidase	
	K_m (mM)	$V_{max.}$ (μmol/μg of protein)	K_m (μM)	$V_{max.}$ (nmol/μg of protein)
+/+	0.46±0.02	26.9±2.75	19.9±2.55	0.88±0.16
ob/ob	0.46±0.02	20.7±1.40	21.4±1.49	0.22±0.07*

Marker enzymes. To characterize the membranes further, the activities of the membrane enzymes $(Na^+ + K^+)$-ATPase and 5′-nucleotidase were monitored to determine whether each was proportional to the amount of protein in the membrane fraction. The results given in Table 1 show that the apparent K_m values [of $(Na^+ + K^+)$-ATPase for ATP and of 5′-nucleotidase for AMP] were similar in membranes from lean and obese mice. In contrast, although the $V_{max.}$ for $(Na^+ + K^+)$-ATPase was not significantly different in the membranes from the lean and obese mice, in the membranes from the obese mice, the $V_{max.}$ of 5′-nucleotidase was only 25% of that in the membranes from lean mice. These data demonstrate a profound alteration of the activity of 5′-nucleotidase in the adipocyte membranes of obese mice and show that the enzyme is not an appropriate marker in this tissue.

Table 2. Effect of cholera toxin on the incorporation of ADP-ribose in adipocyte membranes from lean (+/+) and obese (*ob/ob*) mice

Membranes were incubated with cholera toxin and [^{32}P]NAD^+ as described in the Experimental section. After protein separation by electrophoresis, the gels were autoradiographed for 35 h. The data are expressed in arbitrary units/mg of protein and are the means±S.E.M. for eight different determinations with different membrane preparations. Incorporation into 42 kDa and 48 kDa substrates is shown, along with total incorporation and the ratio of incorporation into the 42 and 48 kDa species. Significant differences from control (+/+) are indicated: * $P < 0.05$; ** $P < 0.01$.

	Incorporation of ADP-ribose (arbitrary units)			42 kDa/48 kDa ratio
	42 kDa	48 kDa	Total	
+/+	21±3.6	25±4.6	45±7.5	0.88±0.08
ob/ob	16±3.5	14±3.6*	29±7.1	1.18±0.09**

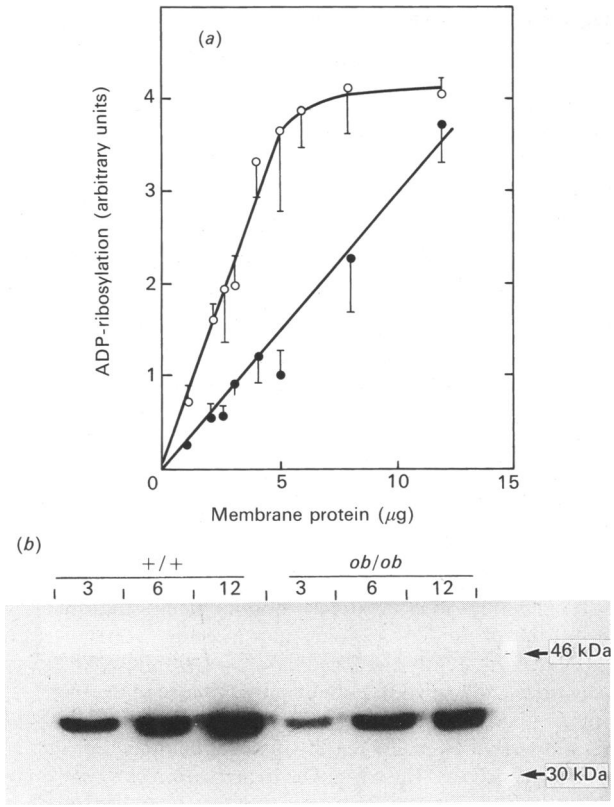

Fig. 4. ADP-ribosylation of pertussis toxin substrates in adipocyte membranes

Adipocyte membranes from lean (+/+) (○) and obese (*ob/ob*) (●) mice were ADP-ribosylated with activated pertussis toxin as described in the Experimental section. For each type of membrane, 1–12 μg of membrane protein was subjected to electrophoresis followed by autoradiography. The intensity of the radioactive spots was then quantified by densitometry. (*a*) Relationship between amount of membrane and incorporation of ADP-ribose. Each point represents the mean ± S.E.M. for three to five measurements. (*b*) Typical autoradiogram, with 3, 6 or 12 μg of protein from membranes of either +/+ or *ob/ob* mice.

Quantification of G-proteins

ADP-ribosylation with bacterial toxins. In order to determine the relative abundance of the regulatory elements of AC in the adipocyte membranes, the ability of cholera and pertussis toxins to ADP-ribosylate the α subunit of G_s and G_i respectively was evaluated. The conditions were chosen to maximize the ADP-ribosylation reaction with each toxin. The results obtained with cholera toxin are shown in Table 2. As expected, cholera toxin catalysed the ADP-ribosylation of two peptides with molecular masses of 42 and 48 kDa, corresponding to the two forms of $G_s\alpha$ present in most tissues. There was a lower amount of ADP-ribose incorporated in the membranes from obese mice. This was particularly evident for the 48 kDa peptide, leading to significantly different ratios of the 42/48 kDa peptides in the adipocyte membranes from lean and obese mice.

Fig. 4 shows the comparison between membranes from lean and obese mice for their ability to be ADP-ribosylated by pertussis toxin. The data demonstrate that the membranes from lean mice incorporated approx. twice as much ADP-ribose than did the membranes from obese mice, suggesting either a decreased ability of $G_i\alpha$ to be ADP-ribosylated or a lower amount of the peptide in the obese-mice membranes. The inclusion of Lubrol in the ADP-ribosylation mixture (see the Experimental section) was essential to maximize the activity of pertussis toxin. The ADP-ribosylating activities were considerably lower without Lubrol than in its presence (lean, 120 ± 21 units/mg of protein without versus 710 ± 41 units/mg with Lubrol; obese, 120 ± 10 units/mg of protein without versus 250 ± 10 units/mg with Lubrol). A comparison between the cholera toxin and pertussis toxin data also revealed that the ratio of ADP-ribose incorporation due to cholera toxin and to pertussis toxin was approx. 1:16 in the membranes from lean mice (45 ± 7.5 and 710 ± 41 units/mg of protein, respectively) and 1:10 in membranes from obese mice (36 ± 9.1 and 350 ± 12 units/mg of protein, respectively).

Since two $G_i\alpha$ species were identified by immunoblotting (see below), the ADP-ribosylated peptides were subjected to electrophoresis using high resolution urea-containing gels (Ribeiro-Neto & Rodbell, 1989). The results show that, under these conditions, two species of ADP-ribosylated peptides can also be identified. As is the case with immunodetection, the abundance of the 39 kDa peptide was similar in lean and obese tissue (50 and 52 units respectively in two experiments) but the amount of the 40 kDa subunit in the membranes from obese mice was only approx. half that in the membranes from lean mice (44 and 100 units respectively in two experiments).

Immunoblotting. In order to find out whether it was the abundance of the proteins or their ability to be ADP-ribosylated that is different in the membranes for lean and obese mice, the abundance of the G-protein components was measured by immunoblotting using specific antibodies. The antibody specific for $G_s\alpha$ revealed two species corresponding to the 42 and 48 kDa cholera toxin substrates (Table 3). The data obtained with immunoblotting supported those obtained with the ADP-ribosylation studies and again indicated a lower level of the 48 kDa species in the membranes from the obese mice, leading to a different ratio between the 42 and 48 kDa species in membranes from obese and lean animals, although these differences were not statistically significant.

The data obtained with an antibody specific for $G_i\alpha$ are given in Table 4. Two peptides were revealed by this antibody, one of 39 kDa and the second of 40 kDa. The abundance of the 39 kDa species was not significantly different in membranes from lean and obese mice, but the 40 kDa substrate was significantly more abundant in membranes from lean compared with obese mice.

The results obtained by immunoblotting therefore confirm the data obtained with ADP-ribosylation studies, although it is not possible to compare the abundance of $G_s\alpha$ and $G_i\alpha$ from the immunodetection experiments, since they were done by different techniques.

Table 3. Quantification of $G_s\alpha$ by immunodetection

Membranes (15–35 μg of protein) were separated by electrophoresis and transferred to nitrocellulose membranes as described in the Experimental section. Immunodetection was carried out as described in the Experimental section, using antibody 130/R at 1:100 dilution as the first antibody and ^{125}I-labelled goat anti-rabbit IgG as the second antibody. The data are expressed in arbitrary units/mg of membrane protein and are the means ± S.E.M. for five different determinations with different membrane preparations.

	Content (arbitrary units/mg of protein)			42 kDa/48 kDa ratio
	42 kDa	48 kDa	Total	
+/+	68 ± 17	84 ± 20	152 ± 38	0.82 ± 0.07
ob/ob	53 ± 18	54 ± 12	106 ± 29	1.03 ± 0.16

Table 4. Quantification of $G_i\alpha$ species by immunodetection

Membranes (15–35 μg of protein) from lean and obese mice were separated by electrophoresis as described in the Experimental section. After transfer to the nitrocellulose membranes, immunodetection was carried out using antibody AS/7 followed by a biotinylated IgG, as described in the Experimental section. The intensity of the coloured bands was assessed by laser densitometry of enlarged (4 ×) photographs of the blots. Amounts of 39 kDa and 40 kDa proteins are indicated in arbitrary units/mg of protein. In each experiment, the intensity generated by 0.5 μg of transducin was measured and used to normalize the intensity of colour. The data are means ± S.E.M. for four separate experiments with different membrane preparations; in each experiments, at least three membrane protein concentrations were included. Significant differences from control (+/+) are indicated: * $P < 0.05$; ** $P < 0.01$.

	Content (arbitrary units/mg)			39 kDa/40 kDa ratio
	39 kDa	40 kDa	Total	
+/+	16.1 ± 1.64	23.2 ± 2.10	39.4 ± 3.75	0.69 ± 0.01
ob/ob	14.0 ± 1.60	10.2 ± 0.70*	22.6 ± 0.93*	1.62 ± 0.19**

The abundance of the β subunits(s) was also assessed in both sets of membranes, using a specific antibody (J6) and ^{125}I-IgG. It was not significantly different in the membranes from lean and obese mice (14.0 ± 7.5 and 12.4 ± 6.0 units/mg of membrane protein respectively; $n = 3$).

DISCUSSIONS

The results presented above show the importance of selecting the appropriate marker enzyme when membranes from two different sources are compared. It has previously been noted by others (Green *et al.*, 1981) and confirmed in the present paper that the specific activity of 5′-nucleotidase is abnormally low in the adipose tissue of the *ob/ob* mouse compared with its lean counterpart, as well as in the adipose tissue of aged compared with young rats (Green & Johnson, 1989). In contrast, the specific activity of $(Na^+ + K^+)$-ATPase in membranes from obese mice was not significantly altered. The differences in the activity of membrane enzymes could mean either (1) that the membrane proteins are diluted by contaminating protein from other cell organelles or (2) that the activity of these enzymes is influenced by the state of obesity. If the first alternative were correct, all of the enzymes would be affected to the same extent. The fact that they are not indicates that the second alternative is more likely.

The preparation of membranes in the presence of proteolytic inhibitors greatly improves the specific activity of AC in membranes from both lean and obese mice and also increases the response to inhibitory ligands in the lean-mouse membranes. The presence of proteolytic inhibitors did not, however, improve the response to inhibitory ligands in obese-mouse membranes, contrary to the report by others (Greenberg *et al.*, 1987). The inhibitory effects of GTP are most evident at the high concentration of isoprenaline (500 μM) that is needed to stimulate the enzyme maximally *in vitro*. The report (Greenberg *et al.*, 1987) of a similar effect of inhibitory ligands in membranes from lean and obese mice may reflect a less than maximal activation of the enzyme in the lean-mouse membranes at the lower isoprenaline concentrations used (50 μM), since the inhibitory response obtained in the membranes from lean mice was small and inconsistent.

The results presented above show clearly that even under conditions that maximize the activation of AC and its response to inhibitory ligands in the membranes from lean mice, both responses were significantly abnormal in the membranes from obese mice.

When care was taken to employ conditions that favour maximum activation by bacterial toxins (Ribeiro-Neto *et al.*, 1987), the pattern of ADP-ribosylation of $G_s\alpha$ was different in membranes from lean and obese mice, with the amount of ADP-ribose incorporated into $G_i\alpha$ being significantly less in the obese. Immunodetection confirmed these findings and, in addition, showed the presence of two bands of immunoreactivity with the antibody specific for $G_i\alpha$, only one of which was altered in the obese-mouse membranes. As similar distributions of ADP-ribosylated and total immunodetectable peptides were found, it is concluded that, under the assay conditions used, the amounts of peptide AD-ribosylated by cholera and pertussis toxin represent the amounts of $G_s\alpha$ and $G_i\alpha$ respectively, since $G_o\alpha$ does not seem to be present in adipocyte membranes (Homburger *et al.*, 1987; Hinsch *et al.*, 1988).

In the membranes from obese mice, AC is resistant to activation by catecholamine plus GTP (Bégin-Heick, 1985). Increasing both the Mg^{2+} concentration in the medium and the temperature of incubation increased the maximum activity in the presence of isoprenaline plus GTP relatively more in membranes from obese (5-fold) than from lean (3-fold) mice (see Fig. 2). Cholera toxin treatment consistently enhanced basal and stimulated (by guanine nucleotides and/or isoprenaline) activity more in the membranes from the obese than the lean mice (Table 2 of Bégin-Heick, 1985). This shows that G_s is less readily dissociable in membranes from *ob/ob* mice, but that the resistance to activation can be overcome, at least partially, under conditions that favour the dissociation of G_s. The presence of a less readily dissociable G_s in the obese-mouse membranes would explain the further activation of forskolin-stimulated activity by high concentrations of Gpp[NH]p, but not the lack of inhibitory effect of low concentrations of Gpp[NH]p on forskolin-stimulated activity or the lack of inhibitory effect of GTP on isoprenaline-stimulated activity noted in the obese-mouse membranes (Bégin-Heick, 1985; Bégin-Heick & Coleman, 1988).

According to the currently held model of the role of G-proteins in AC activation, a decrease in the numbers of the $G_i\alpha$ subunit(s) such as that found in the adipocyte membranes from obese mice would lead to the availability of excess $\beta\gamma$ subunits to interact with both $G_s\alpha$ and $G_i\alpha$, leading to a repression of both the stimulatory and the inhibitory pathways. In most membranes, G_i is present at a large excess over G_s (Gilman, 1987). This is also true in mouse adipocyte membranes (lean, 16:1 and obese, 10:1 membranes). The lower abundance of $G_i\alpha$ in membranes from obese mice would thus explain the resistance of AC to activation by various ligands and the lack of an inhibitory effect of guanine nucleotides on the stimulated enzyme. On the other hand, we found a lower abundance of the 48 kDa form of $G_s\alpha$ and, since that form has a 2–3-fold faster rate of dissociation of GDP compared with the 42 kDa form (Graziano *et al.*, 1989), the altered proportions of the two forms in the membranes from obese mice could contribute to a decrease in the rate of activation of AC. It is difficult at this point to assess the precise role of the alterations in the α subunits of G_s and G_i on the kinetics of activation of AC in adipocytes from the obese mouse. Reconstitution studies with specific subunits will be necessary to clarify this point.

Most of the regulatory components of the AC system have already been ascribed to specific genes in the mouse (Ashley *et al.*, 1987; Blatt *et al.*, 1988). So far, none of these has been assigned to chromosome 6, which is the locus of the *ob/ob* gene. This shows that anomalies of the structure of the α or β subunits of transducing proteins are not a primary defect in the obese–hyperglycaemic syndrome in mice. Anomalies in G-proteins have

been identified in the *fa/fa* rat, another animal model of obesity (Houslay *et al.*, 1989), in aged rats (Green & Johnson, 1989) and in hypothyroid and obese human subjects (Ohisalo & Milligan, 1989). These and several other lines of evidence suggest that insulin or thyroid hormones may alter the activity of the G-proteins (Gawler *et al.*, 1987; Irvine & Houslay, 1988; Rothenburg & Kahn, 1988). It is therefore possible that the hyperinsulinaemia prevalent in the *ob/ob* mouse is responsible for the alterations in the AC transducing system.

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