THE REGULATION OF ADENYLATE CYCLASE IN ADIPOSE TISSUE FROM THE STREPTOZOTOCIN DIABETIC RAT, THE OBESE ZUCKER RAT AND THE DIABETIC MOUSE.

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This thesis is submitted for the degree of Doctor of Philosophy

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January 1991

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ABBREVIATIONS

A; agonist

cAMP; cyclic adenosine monophosphate

AMP; adenosine monophosphate

α_s the GTP binding subunit of G_s

α_{olf} the GTP binding subunit of G_{olf}

α_i1 the GTP binding subunit of G_i1

α_i2 the GTP binding subunit of G_i2

α_i3 the GTP binding subunit of G_i3

α_z the GTP binding subunit of G_z

α_o the GTP binding subunit of G_o

α_t the GTP binding subunit of G_t

ß-AR; ß-adrenergic receptor coupled to the stimulation of G_s

C; the catalytic unit of adenylate cyclase

cGMP; cyclic guanosine monophosphate

C.tox; Cholera toxin which catalyses ADP-ribosylation of G_s

db/db; obese diabetic mice

DDA; 2',5'-dideoxyadenosine

fa/fa; obese Zucker rats

Fa/Fa; lean Zucker rats

G; guanine nucleotide binding protein

G_s; guanine nucleotide binding protein which stimulates adenylate cyclase

G_i; guanine nucleotide binding protein which inhibits adenylate cyclase

G_p guanine nucleotide binding protein which stimulates PIP_2 specific phospholipase C

G_t; guanine nucleotide protein which stimulates the cGMP-phosphodiesterase

GTPγS; guanosine 5'(3-o-thiotriphoshate)

GppNHp; guanylyl 5'-imidodiphosphate

GTP; guanosine triphosphate

IR; insulin receptor

hIR; human insulin receptor

NaF; sodium floride

M_r; molecular weight

mAch-r; muscarinic acetylcholine receptor

mRNA; messenger ribonucleic acid

ob/ob; obese Obese mice as opposed to lean obese mice

PGE_1; prostaglandin E_1

PIA; N^6-(phenylisopropyl) adenosine

PIP_2 ; phosphoinositol 4,5-bisphoshate

PKA; cAMP activated protein kinase

PKC; 1,2-sn-diacylglycerol activated protein kinase

$p21^{ras}$; the product of the ras gene(s)

P.tox; Pertussis toxin which catalyses the ADP-ribosylation of G_i.

PLC; phospholipase C, which catalyses the hydrolysis of PIP_2 to 1;2-sn diacylglycerol and inositol 1,4,5 triphoshate.

PLA_2; Phospholipase A_2 which catalyses the hydrolysis of phosphatidyl choline to arachidonic acid and lysophosphatidyl choline

PTK; Protein tyrosine kinase

R_s; receptor linked to the stimulation of G_s

R; any cell surface receptor

R_i; receptor linked to the stimulation of G_i

TSH; thyroid stimulating hormone

ACKNOWLEDGEMENTS

I gratefully acknowledge the following people in the Department of Biochemistry.

My supervisor Professor Mi!es.D.Houslay for provision of experimental facilities, guidance, inspiration and for finding additional funding at the terminal stages of my research work for my Ph.D. Dr.Graeme.Milligan for the provision of polyclonal antisera, provision of funds to conduct post-doctoral research, and generally inspiring enthusiasm. Dr.R.Leake, for important assistance at particular times during the course of my research.

I also acknowledge the Science and Engineering Research Council for the provision of a Ph.D studentship award.

Conclusions

In this study I have investigated alterations in the regulation and hormonal responsiveness of adipocyte adenylate cyclase occurring in animal models showing insulin-resistance. Three models have been investigated, the streptozotocin diabetic rat, which approximates as a model of juvenile onset diabetes in humans, genetically obese Zucker fatty rat, approximating a model of human obesity and the genetically obese diabetic mouse, which also approximates to a model of human obesity. My reason for undertaking such a study was because therapeutic benefits may come from a greater understanding of alterations in signal transduction systems of hormones controlling metabolism in insulin-resistant pathological states. These are seen in diabetes and in obesity where profound alterations in metabolism have been noted. An important underlying facet in determining these alterations in metabolism may be that defects have occurred in signal transduction systems of various hormones which regulate metabolic events. Any such changes occuring in signal transduction systems associated with the liver, skeletal muscle and adipose tissue are likely to give rise to serious metabolic disturbances.

Previous studies on the streptozotocin diabetic rat have shown that alterations do occur in the signal transduction of the adenylate cyclase system in both hepatocytes and adipocytes. Alterations in other signal transduction systems have also been noted, most notably in the insulin transmembrane signal transduction system itself. Previous studies from Professor Houslays group in which this work was undertaken had shown that changes in G-protein regulation of adenylate cyclase had occurred in hepatocytes from streptozotocin diabetic rats. The changes appeared to be directed at a particular subclass of the G-protein family, that is the 'G_i' subgroup which are involved in the inhibition of adenylate cyclase activity. Alteration in the

regulation of adipose tissue hormone-stimulated lipolysis had already been documented in the same pathological state and these changes seemed to be due to alterations in the regulation of adipocyte adenylate cyclase activity. However very little was understood about the molecular mechanisms involved. I thus conducted an investigation into the regulation of adenylate cyclase with particular respect to the kind of molecular changes that may occur as a result of alterations in the G-protein system. My results indicated that reduced activity of G_i had a potentiating effect on the stimulation of adenylate cyclase by ß-adrenergic agonists. This appeared to relate to an abolition of the ability of G_i to exert a 'tonic' inhibitory effect upon adenylate cyclase. I concluded that the nature of the altered regulation of adenylate cyclase in adipocyte membranes from streptozotocin diabetic rats was essentially very similar to the changes seen in hepatocyte membranes from streptozotocin diabetic animals.

My investigation into the regulation of adenylate cyclase in adipocyte membranes from lean and obese Zucker rats was conducted, in particular, to see if there was a reduction in the ability of lipolytic hormones to stimulate adenylate cyclase. The reason for suspecting the latter scenario relates to the observations that such defects have been noted in the adipose tissue of the genetically obese, obese mouse and diabetic mouse. The defect in adenylate cyclase, and hence lipolysis, found in the latter two model systems was viewed to be capable, potentially, of explaining the hyperadiposity which is characteristic of obesity. A further reason for predicting that changes in the signal transduction system of such animals may occur relates to the fact that obese Zucker rats are hypothyroid. In this regard adipocytes from hypothyroid rat have been shown to be much less responsive to the stimulation of lipolysis by lipolytic hormones. This latter defect appears to be due to a parallel reduction in the ability of lipolytic hormones to stimulate

lipolysis.

My results have shown that there is, as I surmised, a large reduction in the ability of a variety of lipolytic hormones to stimulate adenylate cyclase in adipocyte membranes from obese Zucker rats. The molecular basis of the effect is still uncertain, however a number of strong possibilities have been proposed. These include: (i) reduced numbers of receptors, R_s type: (ii) reduced amounts of G_s: and (iii) reduced activity of G_s.

The genetically diabetic *db/db* mouse has been shown by others to exhibit a reduced responsiveness to adrenaline as regards stimulation of lipolysis and of adenylate cyclase activity in adipose tissue. Thus my studies were conducted in order to investigate the molecular basis observations. I found that the responsiveness of adenylate cyclase to most but not all lipolytic hormones was severely reduced in adipocyte membranes from these animals. In contrast to the changes found in obese Zucker rat adipocyte membranes, no alterations in G-protein subunit levels were found other than an increase in the amount of the α-subunit for G_i1. It was concluded that a diminuition in receptor number or activity was one of the most likely molecular explanations for reduced responsiveness of adenylate cyclase to stimulation by catecholamines and other hormones.

In my studies on the obese Zucker rat and diabetic mouse, although the alterations in the regulation of adenylate cyclase were well studied it was not possible to define the molecular cause of the alterations with a high degree of certainty. It was however, in each model system, possible to list a number of strong possibilities. For instance (i) regulation of ß-adrenergic receptor to G_s coupling could be investigated by classical ligand binding studies: (ii) the possibility of alteration in the ratio of $ß_1/ß_2$-adrenergic receptors: (iii) further investigations into the nature of the G_s directed defect in obese Zucker rat adipose tissue could be made. In addition, studies on hormone stimulated lipolysis could be conducted in order to ascertain how the alterations in the

regulation of adenylate cyclase express themselves further downstream, at the level of lipolysis. Similarly one, could determine whether there might be a reduction in the activity of lipolytic enzymes within the adipocyte. This could be simply addressed by measuring lipolysis stimulated by non-metabolizable analogues of cAMP, such as dibutyryl cAMP. The possible occurrence of reduced hormone stimulated adenylate cyclase and/or lipolysis in adipocytes from other obesity model systems, including humans, could be investigated. Such genetically obese insulin resistant animal syndromes include: the obese yellow mouse; the yellow-KK mouse: KK-mouse: New Zealand obese mouse and the DBM mouse.

Another possible locus of a metabolic defect in adipocytes, which may contribute to the hyperadiposity characteristic of obesity, may be that of lipogenesis. Increased hormone stimulated lipogenesis might well act in parallel with decreased hormone stimulated lipolysis to increase the size of the fat globule in the greatly enlarged adipocytes characteristic of obesity. Inositol polyphosphate mobilizing hormones regulate lipogensis in adipose tissue. Consequently one might consider looking at the ability of such hormones to stimulate phospholipase C in adipocytes from genetically obese animals, such as those listed above.

The heart tissue of obese Zucker rats shows impaired contractility and adenylate cyclase stimulation in response to inotropic cAMP mobilizing hormones such as secretin and adrenaline (Robberecht *et al*, 1983). Since the cAMP mobilizing hormones are inotropic agents, the pathological significance of the above defect(s) may relate to the tendency for impaired cardiac function to be associated with obesity (Crandall & DiGirolamo, 1990). Further investigations into the hormonal regulation of adenylate cyclase in heart tissue from obese Zucker rats could be conducted with a view to uncovering the molecular basis of the defect. In addition studies along the

same lines could be performed in heart tissue from other obesity model systems in order to establish the prevelevance of the effect.

CHAPTER 1

INTRODUCTION

1.1 THE cAMP SIGNALLING SYSTEM

The cAMP signalling system is probably present in all mammalian cells and is absolutely crucial for the correct regulation of a wide variety of intracellular processes by extracellular signals. In addition to generation of cAMP by adenylate cyclase, the production of cAMP is also regulated by cAMP-phosphodiesterases, cAMP-PDEs, which degrade cAMP to AMP.

It is now apparent that a number of other signal transduction systems are regulated in apparently similar fashions to that of adenylate cyclase. Consequently much can be learned about the regulation of adenylate cyclase by comparison with the regulation of other G-protein-regulated effector enzymes and *vice-versa*. Also the study of the regulation of adenylate cyclase in disease states, important in its own right for obvious reasons, significantly improves our understanding of the regulation of adenylate cyclase in the normal state.

1.2 FLOW OF IMFORMATION THROUGH G-PROTEIN COUPLED SIGNAL TRANSDUCTION SYSTEMS IN VERTEBRATES.

It has been estimated that 80% of all known hormones/autocrine factors and neurotransmitters (primary messengers) are known to elicit cellular responses by combining with receptors coupled to G-proteins which then activate effectors. To date approximately 80 receptors have been identified, while the total number of agonists known is approximately 40. Some investigations (Birnbaumer *et al,* 1990) have predicted that the total number of distinct receptors coupled to G-proteins will be in the region of 100 to 150. Thus the number of receptors (R) is greater than the number of primary messengers. In contrast the number of G-proteins is probably only in the region of 15 of which 12 have been identified to date, representing five different classes of G-protein. Similarly the number of effectors is small, 6

being known, with the possible total of around 12 suggested. Of the receptors, 18 have been purified and cloned. Of the other effectors, only two have been purified close to homogeneity in a G-protein-responsive state.

In contrast to the many conserved features amongst different G-protein signalling systems, the effector step is the least conserved as one might logically predict. The range of effectors regulated includes the following; adenylate cyclase (Levitski 1990); PIP_2-specific PLC which hydrolyses PIP_2 to diacylglycerol and inositol 1,4,5 triphosphate (Fain 1990); cGMP-phosphodiesterase of retina (Stryer 1988); PLA_2 which hydrolyses phophatidyl choline to arachidonic acid and lysophosphatidyl choline (Burch *et al*, 1986); K^+ channel (Yanati *et al*, 1987); Ca^{++} channel (Yanati *et al*, 1987) and the Na^+ channel (Cantiello *et al*, 1989; Krapisinky *et al*, 1989). Other potential candidates include a Mg^{++} transporter (Erdos *et al*, 1981) and the glucose transporter(s) (Kuroda *et al*, 1987).

Strictly speaking only adenylate cyclase, PLC and calcium channels are effector enzymes since they are hormonally regulated enzymes which catalyse the production of second messengers leading to changes in the activity of protein phosphorylation within the cell.

1.3.1 HORMONAL REGULATION OF ADENYLATE CYCLASE

Adenylate cyclase is a multicomponent system composed of five functional units.

(1) The stimulatory receptor R_S. This class of receptor appear to be transmembrane glycoproteins with seven transmembrane-spanning sequences. The ß-AR is the only member of this large family of receptors which has been purified and reconstituted with other pure components of the adenylate cyclase system (Feder *et al*, 1986).

(2) The G_S protein, which binds GTP and may be tightly associated

with the catalytic unit C. G_s (Arad *et al*, 1984) is composed of 3 subunits; α_s which possesses the GTP binding site and is the target for ADP-ribosylation by NAD+ catalysed by cholera toxin, and the β-subunit and the γ-subunit which are believed to be tightly associated with each other (Cassey & Gilman, 1988). The heterotrimeric G_s is localized on the inner leaflet of the plasma membranes and is much less hydrophobic than either the β-AR or the catalytic unit of adenylate cyclase.

(3) The catalytic unit (C) is a hydrophobic protein with 12 transmembrane-spanning domains carrying its catalytic site at the cytoplasmic side of the membrane (Krupinski *et al*, 1989).

(4) The inhibitory receptor, R_i, appears to be homologous in structure to R_s (Kubo *et al*, 1986), (Bonner *et al*, 1987), (Kobilka *et al*, 1987).

(5) The inhibitory GTP binding protein, G_i, is structurally homologous to G_s where βγ subunits are either very similar or identical to those of G_s. The α_i subunit is a substrate for ADP-ribosylation catalysed by pertussis but not cholera toxin, except under special conditions *in vitro*.

The homology between α_i and α_s is high in the GTP-binding domain, but lower at the C-terminal sequence which is involved in receptor recognition (Masters *et al*, 1989). It is expected that the effector interaction domain will be different for each G-protein. Different βγ are known to exist and therefore R_i-and R_s-type receptors may recognize particular αβγ complexes and not just simply the α subunit. Never the less it has been assumed that the βγ of G_s and G_i are identical and functionally interchangable (Levitzki, 1990).

1.3.2 Ubiquity of the adenylate cyclase system

The adenylate cyclase system is very similar across the evolutionary sphere. The β-adrenergic receptor from turkey erythrocytes and the glucagon

receptor from rat liver can be transferred into Friend erythroleukaemia cells and activate their adenylate cyclase (Schramm *et al*, 1977; Schramm *et al*, 1979). In rat brain it is known that β-AR (R_S) and A_2-adenosine receptor (R_S) couple to the same pool of adenylate cyclase catalytic units (Braun & Levitzki, 1979).

1.3.3 The adenylate cyclase catalytic unit (C subunit).

Both genetic and biochemical evidence indicate multiple forms of adenylate cyclase, with an estimated molecular weight range from 120-150kD (Smigel, 1986; Mollner & Pfeuffer, 1988).

Evidence suggests that neural tissue contains calmodulin-sensitive and insensitive adenylate cyclases (Westcott *et al*, 1979). The calmodulin-sensitive form is believed to bind calmodulin directly (Salter *et al*, 1981). Calmodulin-mediated stimulation of activity ranges from 1.4 to 5-fold depending on the region of the brain (Cooper *et al*, 1988). Stimulation is observed at Ca^{++} concentrations in the range 0.1-1.0μM, that can be achieved either upon neural depolarisation or activation of receptors linked to PLC (Cooper *et al*, 1988). Since calmodulin is present in high concentrations in the brain the stimulation of adenylate cyclase becomes linked with the stimulation of PLC.

The C unit must have the following domains.

(i) an active site which binds Mg^{2+}ATP.

(ii) a site for interaction with G_S.

(iii) a domain for P-site inhibition by adenosine.

(iv) a possible domain for the interaction with any of $\alpha_i/\beta\gamma$.

The P-site binds adenosine and thereby inhibits the activity of C (Johnson *et al*, 1989). Inhibition is characteristically non-competitive with respect to MgATP and is very potent; greater than 80% inhibition of hormone stimulated activity can be achieved. Stimulated adenylate cyclase is substantially more sensitive to P-site agonists than the unstimulated

adenylate cyclase (MacKenzie & Bar, 1973). The physiological significance of P-site is unknown.

There are four possible sites in the model of (Krupinski *et al*, 1989) for glycosylation (N-linked) but only one of these sites, Asn^{706}, would be exposed extracellularly. A 120kD form of C is phosphorylated by PKC in frog erythrocytes (Yoshimasa *et al*, 1987), a possible site for this being Ser^{481}. Similarly, a consensus site for phosphorylation by PKA is present at Ser^{1035}.

The most striking feature of C is the tertiary structure of the enzyme. The enzyme contains 12 transmembrane helices, this seems rather excessive in light of the fact that all of the regulatory domains are located in the cytoplasmic parts (Krupinski *et al*, 1989). The 12 transmembrane helices resemble the tertiary structure of certain G-protein linked ion channels, such as the dihydropyridine-sensitive Ca^{++}channel (Yanati *et al*, 1987).

1.3.4 Structural features of G_s and G_i of adenylate cyclase system

G_s

Multiple forms of G_s have been identified. These arise as four alternative splice variants of a single mRNA (Northup *et al*, 1980) yeilding of 394, 381, 380 and 395amino acid variants of α_s1, 2, 3 & 4. The actual M_r of the lower (381 & 380 amino acid) and higher (394 & 395 amino acid) M_r forms are 44kD and 46kD respectively (Kozasa *et al*, 1988).

The turnover number of the GTPase activity is quite low, as one might predict for a regulatory enzyme, at 13-15 min^{-1} (Arad & Levizki, 1979).

Unlike α_i, α_s does not undergo acylation so that the method of attachment to the membrane may be by association with adenylate cyclase (Levitski, 1987). The nature of the attachment is important because G-protein activation seems to involve dissociation of the α from the $\beta\gamma$ subunits

and if the latter provide the only site of membrane attachment the activated α_s would leave the membrane.

A fifth α_s like species is known but this is only expressed in olfactory neuroepithelial cells, as far as is known to date (Jones *et al*, 1988) and has been termed α_{olf}. This is a 381amino acid protein, sharing 90% homology with other α_s species and apparently stimulates adenylate cyclase in neuroepithelial cells. The expression of α_{olf} in S49 cyc$^-$ kin$^-$ lymphoma cells, which lack G_s, has been used to demonstrate its capacity to stimulate C in a non-olfactory system (Jones & Reed 1989).

G_i

Three distinct genes are known each coding for different forms of G_i. α_i1consists of 355amino acid, α_i2 of 354, and α_i3 of 354amino acid and they exhibit between 87 and 93% sequence identity (Suki 1987). The molecular weights of α_i1, α_i2 and α_i3, found by SDS-PAGE, are 41kD,40kD and 41kD (actual M_r for α_i1 is 40.4kD), respectively.

As regards covalent modification, G_i undergoes N-myristylation (Buss *et al*, 1987) at the N-terminal residue (glycine), the function of which is uncertain, although it could simply to be to anchor the protein in the membrane. Metabolic labelling studies with [^{3}H]myristate of monkey kidney COS cells transfected with cytomegalovirus-based expression vectors have shown that α_i1, α_i2, α_i3, α_o, α_z, α_t but not α_s undergo myristolyation (Mumby *et al*, 1990). Myristoylation which appears to be important for the attachment of the protein to the membrane. Experiments on the distribution of [^{35}S]methionine labelled α_i and α_s show that the former immunoprecipitates from particulate fractions when myristoylated. However α_i1 precipitates from soluble fractions when myristoylation is blocked by utilizing cDNAs in which the second residue, glycine, was converted to alanine (Jones *et al,* 1990). The lack of the myristoylation modification does not

prevent association with $\beta\gamma$ subunits since $\beta\gamma$ are required for Pertussis toxin catalysed ADP-ribosylation of α_i and this reaction is not impaired with non-myristoylated substrate (Jones *et al*,1990). However the dose response curve for the stimulatory effect of exogenous $\beta\gamma$ added to soluble α_i1, is shifted to the right for the mutant α_i1.

G_i apparently also undergoes phosphorylation on the α_i subunit which appears to inhibit its activity (Jakobs *et al*, 1985). Treatment of intact hepatocytes with the PKC activating phorbol ester TPA, elicits the phosphorylation and inactivation of G_i (Pyne *et al*,1989). This may account for the fact that phorbol esters can by a presumed loss of tonic G_i function augment the stimulation of adenylate cyclase by isoproterenol in S49 lymphoma cells (Bell *et al*, 1985).

Other G_i like proteins

G_o

G_o was first identified as a predominant Pertussis toxin substrate in bovine brain (Sternweiss & Robishaw, 1984). Several lines of evidence indicate the presence of multiple forms of G_o. Two different cDNAs for α_o have been cloned from several mammalian tissues including rat brain (Itoh *et al*, 1988; Van Meurs *et al*, 1987). Bovine α_o1 contains 354 amino acid, the same number as α_o2, their sequences being identical up to position 246, thereafter 26 amino acid are different. It has been proposed that α_o1 and α_o2 arise due to alternative splicing of a single transcript (Hsu *et al*, 1990). The function of α_o is uncertain although a number of target effector units have been implicated.

G_z

The cDNA for α_z was isolated from a bovine retinal library with α_t cDNA at low stringency (Fong *et al*, 1988). The deduced amino acid sequence consists of 355 amino acid (M_r =40,879) exhibiting a particularly strong homology with the α_i proteins (66-67% identity in sequence with α_i1, α_i2

and α_i3) although not strong enough to be part of a separate subfamily (α_i subunits exhibit 85-94% identity between each other). Northern and western blotting analyses have shown that α_z occurs in the retina, brain, adrenal gland, kidney and liver but not testis, lung or spleen (Fong *et al*,1988). One interesting deviation from classical α_i structure is the lack the cysteine residue, 4 amino acid from the C-terminus, which serves as the site for Pertussis toxin ADP-ribosylation of the α_i subunits. The intrinsic GTPase activity of G_z is very low, $0.05min^{-1}$ at 30 celsius,ie 200 times slower than for other G-proteins (Cassey *et al*, 1990). Thus G_z is very likely to have quite a different mode of regulation from the more classical G-proteins.

ß subunits

Three ß subunit cDNAs are known, and these are derived from three non-allelic genes located on chromosomes 1 ($ß_1$ encoding a 37.329kD), 7 ($ß_2$ encodes a protein 90% identical to $ß_2$), and 12 ($ß_3$ encoding a 340 amino acid 37.221kD protein) (Blatt *et al*, 1988; Levine *et al*, 1990). $ß_3$ mRNA may undergoe two alternative splices but its tissue distribution is not yet known (Levine 1990). Most tissues appear to contain two ß subunits of 35 and 36 kD on SDS-PAGE both 340 amino acid and differ by only 42 amino acid.

γ subunits

The γ subunit, is a hydrophobic protein, of 74 amino acid, and binds tightly to the ß subunit and consequently remains bound to ß subunit during purification (Marbach *et al*, 1990).

A γ specific to ROS membranes, γ_t, associated with the transducin system is also of 74 amino acids, and contains a large proportion of acidic residues while remaining a highly hydrophobic protein (Yatsunami *et al*, 1985; Ovchinnikov *et al*, 1985).

Functional differences may exist between these two γ subunits since βγ purified from bovine brain is much more effective at inhibiting adenylate cyclase than $\beta\gamma_t$ purified from ROS membranes (Cerione *et al*, 1987).

1.3.5 Mechanisms of hormonal regulation of Adenylate cyclase

The kinetics of adenylate cyclase regulation have been studied in great detail. The kinetics are very complicated but relatively easy to study experimentally since the adenylate cyclase system can be studied in isolated membranes where it retains generally excellent hormone responsiveness. Thus the vast differences in the understanding of the regulation of adenylate cyclase as compared with PLC, PLA_2 and ion channels, for example is mostly due to the ability to study the system as isolated membranes by classical biochemical means *in vitro*.

The kinetics of regulation of adenylate cyclase show that stimulators, R_S G_S, activate by increasing V_{max} without altering K_m for ATP (Birnbaumer *et al*, 1980). Since G_S is essentially regulating adenylate cyclase in an allosteric manner then the regulation of adenylate cyclase can be seen to of the V-system type as described by Monod *et al*, (1965), (Monod *et al*, 1963) as opposed to the much more common K-system in which allosteric efectors only alter affinity of substrate for the enzyme.

Amplification of the initial signal is a prominent feature of the adenylate cyclase system. This occurs firstly at the step of G_S activation since a single ß-AR molecule can activate several molecules of G_S in native membranes (Tolkovsky & Levitski 1978) and in reconstituted systems (Hekman *et al*, 1984). It has been estimated that amplification is of the order of 1000, that is one activated receptor results in the production of 1000 molecules of cAMP (Levitzki *et al*, 1988). The first step in the amplification process is the ability of 1 A-R_S to activate a number of G_S. The second stage relates to the relative turnover numbers of G_S and C. The overall

rate of G_s activation (K_{on}), *i.e* exchange of GDP for GTP, is 0.5-1.5 min^{-1} which is much slower than the rate of turn off, *ie* the GTPase reaction, at 13-15 min^{-1}(Arad & Levitzki 1979). Thus GTP hydrolysis occurs quickly in comparison to the rate of nucleotide exchange. The turnover number K_{cat} of C is 1100min^{-1}. Thus since G_s has a K_{off} of 13 min^{-1} it is in its active form for ~100 times as long as the active form of C, thus allowing active G_s to activate further molecules of C and hence increase the amplification of the initial signal.

The activation of adenylate cyclase by ß-AR is first order where the rate constant of activation K_{on} is linear with the concentration of A-R_s (Hekman *et al*, 1984). This can be expressed as

$$K_{on} = k[R_t].[A]/(K_d + [A])$$

$$= k[R_t] \text{ x fraction of active receptor population}$$

where $[R_t]$ is the total receptor concentration and K_d is the equilibrium dissociation constant for the A-R complex.

In reconstituted systems (Asano *et al*, 1984) it has been shown that the $K_{0.5}$ for isoproterenol stimulation of [^{35}S]GTPγS to G_s is only 2% of the K_d for the ß-AR antagonist [^{125}I]-CYP binding to ß-AR of the vesicles. The K_d of the ß-antagonist propanolol, at 2.3nM to compete off [^{125}I]-CYP is the same as the K_i for propanolol to inhibit isoproterenol stimulation of GTPγS binding to G_s. The amount of G_s greatly exceeds that of ß-AR so that the explanation cannot be that their is a molar excess of ß-AR. These results could be explained by the catalytic efficiency of ß-AR, such that only a fraction of the total population of ß-AR is required to be activated in order to achieve full activation of the entire population of G_s.

Reconstitution of purified ß-AR, G_s, G_i, and C (Citri & Schramm, 1980) has greatly aided the kinetic analysis of the adenylate cyclase system. This overcomes determinations done in native membranes which are subject to the major drawback that the amounts of each component, ß-AR,G_s,G_i and C,

cannot be altered in order to analyses the effects on rate constants.

1.3.6 Activation of G_S by R_S.

When an agonist has diffused within a few molecular diameters of the receptor the two begin to interact and part of the free energy of binding is used to deform the protein (Jencks, 1975; Burgen, 1981) and thereby switch the receptor to an active conformation.

R and G can easily be seen as E and S respectively. Both enzymes and A-R complexes (agonist bound receptor) bind tightly to reactive intermediate or transition state ($\alpha\beta\gamma$ with no nucleotide bound) but only weakly to the substrate or product ($\alpha\beta\gamma$-GTP).

The activation of G_S, has been relatively well worked out in comparison with the activation of other G-proteins. Basically, activated agonist-receptor complex appears to activate G_S by increasing its affinity for Mg^{++} and thereby activating G_S (Iyengar & Birnbaumer 1982). Activation of G_S by R_S is recognized to involve the formation of a ternary complex of agonist,receptor and G-protein ($A\text{-}R_S{}^*G_S$) but theoretically this does not have to exist since alternatively agonist-receptor complex, A-R, could dissociate to A+R and R could then interact with G to form a binary complex $R_S{}^*G_S$. The existence of the ternary complex can be inferred by the observation that addition of purified G_i to purified and reconstituted mAch-receptor increases the affinity of mAch-receptor for agonists, thus implying that G_i stabilizes the receptor (Florio & Sternweiss, 1985). The addition of GTP to the reconstituted components results in the loss of high affinity binding

The ternary complex of A-R*G (Delean *et al*, 1980) is normally transitory because guanine nucleotide binds to the α subunit within milliseconds (May & Ross 1988). The affinity of R for G is then decreased and the R*G complex dissociates. However under experimental conditions

where guanine nucleotides can be omitted, the association between the protein is long lived and can be measured (Delean *et al*, 1980).

Radiolabelled agonist and antagonist binding to ß-AR present in digitonin solubilised membranes, prepared in absence of GTP, binding separated into two clearly distinct peaks on an AcA-34 Ultrogel column (Limbird *et al*, 1980). Thus agonist and antagonist bound receptors form different sized complexes, that is antagonist bound receptor does not form a ternary complex unlike the agonist bound receptor. The antagonist and agonists peaks were eluted in same fractions when GTP was included in the solubilization buffer, thus showing that the A-R-G ternary complex is GTP labile. The same effects were exhibited by the bovine anterior pituitary dopamine receptor (Kilpatrick & Caron 1983).

If frog or turkey erythrocyte membranes are preincubated in the absence of GTP with isoproterenol or water, then subjected to washes by centrifugation in the presence of ß-AR antagonists and then assayed for their abilities to support stimulation of adenylate cyclase activity by GppNHp, then the membranes pretreated with isoproterenol show significantly greater stimulation of adenylate cyclase (Stadel *et al*, 1980). This effect comes about because the ability of activated receptors to form a ternary complexes with G-proteins. If GTP is included in the preincubation there is no difference between stimulations achieved by isoproterenol or water preincubated membranes. Since there is no isoproterenol present there must be a memory process. The molecular explanation offered (Stadel *et al*, 1980) is that activated ß-AR forms a stable complex with G_S (ß-AR*G_S) but only in the absence of GTP. If GTP is included in the pre-incubation then the ß-AR*G_S complex breaks down rapidly.

Whereas GTP promotes activation of a G-protein and therefore dissociation, GDP stabilizes the oligomeric form and thereby prevents

activation. When GDP binds, the G-protein leaves the receptor in the holomeric form, but when GTP binds G dissociates into $\alpha + \beta\gamma$ and can only reassociate after GTP has been hydrolyzed. Thus the dissociation of G that follows GTP binding effectively makes the process irreversible (Fung, 1983).

The activated receptor binary complex has a high affinity for a conformation of the G-protein in which α and $\beta\gamma$ are associated and the single guanine nucleotide binding site is empty (Wessling-Resnick *et al*, 1987; Chabre *et al*, 1988). If $\beta\gamma$ are not present there is no activation of the G-protein. There is evidence to favor $\beta\gamma$ having a role in receptor recognition/contact for the transducin cGMP-PDE system (Fung, 1987).

With a physiological Mg^{++} concentration of around 0.5mM, G_s cannot bind Mg^{++} until its affinity for this cation is increased. The K_m of G_s for GTP is not greater than 1μM and with intracellular GTP around 320μM and GDP around 90μM in hepatocytes (Kleineke, 1979), G_s is always saturated with GTP. Thus the binding of GTP to G_s cannot be the rate-determining step in the G-protein activation cycle.

The binding of Mg^{++} to G_s is thought to promote dissociation to α_s and $\beta\gamma$ subunits, thus releasing the active form of α_s. The affinity for Mg^{++} has been estimated at 25mM in basal state and is reduced upon activation to 10μM. Thus with a physiological Mg^{++} concentration of around 0.5mM, G_s cannot bind Mg^{++} until its affinity is increased (Iyengar, 1981).

The activation of G_s by A-R is at least partially achieved by, stimulating the release of GDP from G_s since Murayama & Ui (1983) showed that adipocyte membranes labelled with [^{3}H]GTP when stimulated with isoproterenol release [^{3}H]GDP. The activated R not only increases the rate of GDP release but (i) hold open the GTP binding site and (ii) they may even increase its affinity for GTP relative to GDP (Birnbaumer *et al*, 1980; May & Ross, 1988; Florio & Sternweiss, 1989).

In the mutant cell line H21a, α_s has a single amino acid alteration which radically alters its activity (Miller *et al*, 1988). This mutant G_s interacts normally with ß-AR but fails to dissociate to α+ $\beta\gamma$ upon binding GTP and therefore fails to activate adenylate cyclase (Miller *et al*, 1988). The mutant α_s evidently binds GTP and undergoes the comformational change which causes ß-AR*G_s to dissociate but cannot undergo the next step which leads to dissociation of G_s. A similar situation can be achieved by removal of Mg^{++}. Addition of isoproterenol+ GTP, GTP binds ß-AR*G_s dissociates to ß-AR + G_s, $\alpha_s\beta\gamma$ does not dissociate until Mg^{++} is added (Higashima *et al*, 1987) thus showing the role of A-R_s is to promote the binding of Mg^{++} and thereby the activation of adenylate cyclase.

The kinetics of activation of G_s by isoproterenol in phospholipid vesicles have been studied by measuring the slowly-reversible (quasi-irreversible) binding of [^{35}S]-GTPγS to G_s (Asano & Ross, 1984; Asano *et al*, 1984). Analysis shows that the apparent first order rate constant for binding of [^{35}S]-GTPγS, K_{app}, does not vary with increasing concentration of GTPγS but when 100μM isoproterenol is included K_{app} the relationship is nonlinear and as such indicates that binding of GTPγS is not a simple second order bimolecular reaction. For a bimolecular reaction in which one component, GTPγS is in excess, the rate should be psuedo-first order in the other reactant (G_s), but should be linearly proportional to the concentration of the reactant in excess and this is clearly not the case. Thus the activation of G_s and subsequent dissociation upon activation is not just a simple association of ß-AR and G_s.

An apparently ubiquitous idiosyncrasy of R*G interactions is the nucleotide sensitivity of agonist binding. Why should the ability of G to physically increase the affinity of R for A be ubiquitous amongst all known R*G species. Obviously the R*G binary complex should have a lower free

energy than R+G,*i.e* should have a greater stability so that the process of association is thermodynamically favourable. But on these grounds there seems to be no reason why this should always manifest itself as an increase in affinity between R and A. The real reason for this latter effect must be to meet the catalytic requirements of a later step in G-protein activation by R. The activation of G is driven by the transfer of the binding energy of A to R. Since this binding energy is of only a finite amount there must therefore be a limit to the number of G-proteins which can be activated.

For many receptors this increased rate of dissociation is substantial for full agonists. For example 50-100 fold ß-AR, α_1-AR or mAch-receptor (Kent *et al*, 1980; Goodhardt *et al*, 1986; Evans *et al*, 1985). Thus activation of G is effectively coupled to recycling of R.

1.3.7 Activation of G_i by R_i

The hormonal inhibition of adenylate cyclase by platelet α_2-AR has been shown to involve the release of guanine nucleotides from G_i (Michel & Lefkowitz, 1981). It was shown that stimulation of α_2-AR will stimulate the release of [^{3}-H]GppNHp from membranes pre-incubated with this nucleotide.

Direct evidence for the ternary complex in the activation of G_i has come from the demonstration of different sedimentation properties of agonist and antagonist labelled α_2-AR (R_i) in the absence of GTP as separated on sucrose density gradient profiles (Michel *et al*, 1981).

Overall the activation of G_i by R_i is very similar to that for G_S by R_S, except for the notable difference that R_i does not stimulate G_i by increasing its affinity for Mg^{++}. The affinity of G_i for Mg^{++} is $\sim$10µM ensures that at physiological Mg^{++} concentrations G_i is always saturated with this divalent cation (Hildebrandt & Birnbaumer, 1983).

1.3.8 Activation of C by G_s

The regulation of adipocyte adenylate cyclase by GTP was shown to be bimodal by Cooper *et al*, (1978). Thus low concentrations of GTP were stimulatory and high concentrations were inhibitory. These actions were then attributed to the function of G_s and G_i by virtue of their distinctive susceptibility to inactivation by various agents (Cooper *et al*, 1979). Low concentrations of GTP thus activate G_s while at higher concentrations G_i becomes activated and this leads to a net inhibition of activity (Cooper *et al*, 1979). For the stimulatory effect of GTP to be seen in adipocyte membranes, isoproterenol has to be included otherwise the response to GTP is very poor (Cooper *et al*, 1979). Similarly, inhibition of adenylate cyclase at high concentrations of GTP requires adenylate cyclase to be stimulated with isoproterenol in order to detect inhibition (Cooper *et al*, 1979). However it is significant that the presence of an R_i agonist is not required, thus implying the presence of tonic (receptor-independent) inhibition. The bimodal regulation undoubtly endows the system with a very high degree of hormonal regulation befitting its crucial role in cellular regulation.

The activation of adenylate cyclase by the non-hydrolysable GTP analog GppNHp was found to be hysteretic in contrast to that achieved using GTP itself (Iyengar & Birnbaumer, 1981). This could be interpreted as meaning that the pre-steady state lag phase, lasting minutes, found with GppNHp is due to its nonhydrolysable nature. However by reducing the $[Mg^{++}]$ to <1.0mM it was found that GTP and NaF both show lag phases in their stimulation of adenylate cyclase (Iyengar & Birnbaumer, 1981). Most enzymes that display long lag phases (ie minutes), in terms of ligand, binding show this effect because of the time required for the association of discrete subunits. Hence the lag phase in the case of adenylate cyclase is then probably related to the separate subunit composition of adenylate cyclase (Iyengar & Birnbaumer, 1981). The greater lag phase with GppNHp at $[Mg^{++}]$ >1.0mM is probably that the different contacts of GppNHp made in the guanine

nucleotide binding site of G_S causing the activation rate of G_S to be slower than with GTP. These experiments also show the central role of Mg^{++} binding in the activation of G_S.

That G_S exists in three quite different conformations during its activation cycle can be seen from the hydrodynamic behavior of the purified protein by density gradient centrifugation (Codina *et al*, 1984). The GppNHp bound form sediments as 4S, the GppNHp+Mg^{++} form at 3S, and the dissociated α_S, form as 2S. The 3S form cannot normally be detected since is rapidly converted to 2S, although it can be if the temperature is lowered to 4 degrees celsius (Codina *et al*, 1984). The 3S form rapidly breaks down to the 2S form, because the 3S form is essentially an unstable intermediate in the process of forming the 2S dissociated $\alpha_S+\beta\gamma$ from the 4S $\alpha_S\beta\gamma$ species of G_S. This behavior of G_S was believed to demonstrate that G_S undergoes dissociation as part of its route to the attainment of an activated state, at least as activated by GppNHp and Mg^{++}.

The catalytic unit C also contains a Mg^{++} binding site (Somkuti *et al*, 1981). According to this data, the allosteric binding site for Mg^{++} appears to be essential in that the site must be occupied in order for A-R_S to activate G_S. This allosteric site appears to be quite distinct from the MgATP binding site.

It has been estimated that since the deactivation rate is 15 to 20 times faster than the activation rate,ie K_{on} is 0.5-1.5min^{-1} while K_{off} is 13-15min^{-1}, there is only 5 to 7% of G_S which is active even in the presence of hormones and GTP (Levitzki *et al*, 1977). Concerning the activation of G_S, Levitzki has shown by kinetic analysis and physical co-purification of G_S with C (Arad *et al*, 1984) that G_S remains tightly bound to C and therefore to the membrane throughout the activation cycle. Also it appears that the ß subunit remains bound to α_S during the entire activation-deactivation cycle of G_S. This has been demonstrated by the stoichiometric co-purification

of GppNHp preactivated adenylate cyclase as a complex of α_sGppNHp*ß*C from turkey erythrocyte membranes (Bar-Sinia *et al*, 1990) and bovine brain membranes (Marbach *et al*, 1990). However high salt concentrations do promote the dissociation of ß from α_s*C. It has been shown that the dissociation of $\alpha\beta\gamma$ depends on the nucleotide bound (Huff & Neer, 1986), with GTPγS causing complete dissociation and GTP causing no dissociation at all. These results imply that the mechanism of G_s activation involving dissociation of $\alpha_s\beta\gamma$ may be incorrect and consequently the inhibition of G_s by G_i cannot be due to the release of free $\beta\gamma$.

1.3.9 Inhibition of adenylate cyclase by G_i and the role of G_i in regulation of adenylate cyclase.

The mechanism by which G inhibits adenylate cyclase is not yet resolved, partly because, in theory, there are a number of ways in which this can be achieved. In practice more than one way probably operates in any case. The potential mechanisms to inhibit adenylate cyclase include (i) direct action of α_i upon adenylate cyclase or its associated components necessary for activation and (ii) Involvement of $\beta\gamma$ exerting effects directly upon C subunit or associated components. This latter possibility is made more likely by the relatively high affinity of $\beta\gamma$ for α_s compared with α_i (Sternweis, 1986; Pang & Sternweis, 1989) and also the large excess of G_i over G_s, presumed by some, to occur in most membranes (Gilman, 1987).

It has been shown in S49 cyc$^-$ cells, which lack G_s and its mRNA, that there is direct effect of G_i upon the C subunit, since both R_i linked hormones and GTP still inhibit adenylate cyclase (Hildebrandt *et al*, 1982; Jacobs *et al*, 1983). Studies on the wild type S49 cell line show that in the case of forskolin-stimulated adenylate cyclase, $\beta\gamma$ inhibits adenylate cyclase activity to the same extent as somatostatin and such effects are non-additive when $\beta\gamma$ was added together with somatostatin (Katada *et al*, 1984). This would then

tend to support ßγ as the mediator of somatostatin action. However Hildebrandt & Kohnken (1990) have shown that while somatostatin and ßγ are equally effective in inhibiting forskolin-stimulated adenylate cyclase in wild type S49 cells as found by (Katada *et al*, 1983), ßγ are much less effective than somatostatin at inhibiting isoproterenol-stimulated adenylate cyclase. The ßγ complex only inhibited isoproterenol-stimulated adenylate cyclase at very high concentrations. In addition, somatostatin served as a mixed inhibitor of isoproterenol stimulated adenylate cyclase while ßγ was a simple non-competitve inhibitor. Thus ßγ and somatostatin appear to inhibit adenylate cyclase by different mechanisms, at least when stimulated by an R_S-linked hormone. Thus the authors propose that α_i mediates R_i-stimulated inhibition of R_S-stimulated adenylate cyclase while ßγ probably function to inhibit basal activity of adenylate cyclase (Hildebrandt & Kohnken, 1990).

It is also relevant that in a reconstituted system of purified components ß-AR, G_S, C and G_i the fold stimulation of adenylate cyclase activity by isoproterenol was only 1.3 fold over basal (in the absence of G_i) but when G_i was included this increased to 7-8 fold (Cerione *et al*, 1985). It was found that a ratio of 4-5 G_i molecules to every G_S was required to achieve an increase in the fold activation reported. This may well relate to an overall excess of G_i to G_S of 4-10 times, as found in purification studies (Codina *et al*, 1984). Thus it appears that G_i might serve more than one function if this phenomena was to occur *in vivo* since G_i would be required for maximal activation of activity apparently by suppressing the basal activity of adenylate cyclase, ie eliciting a tonic inhibition of adenylate cyclase.

In cyc$^-$ membranes G_i is a simple non-competitive inhibitor of G_S-GTPγS added exogenously. However G_S activated by GTPγS, which is thought to be in the dissociated state of free α_S, is probably quite resistant to inhibition by ßγ (Northup *et al*, 1983). Cholera toxin substantially reduces the

affinity of βγ for α_s (Kahn & Gilman, 1984) and cholera toxin-catalysed ADP-ribosylation of adipocyte membranes in presence of GTP reduces the effectiveness of R_i linked inhibition mediated by PIA by some 35% (Owens *et al*, 1985). Under these conditions there is little ADP-ribosylation of G_i (Milligan, 1987) therefore this reduction in PIA mediated inhibition probably reflects an alteration in the ability of adenylate cyclase system to be inhibited rather than any ability of R_i to generate the inhibitory species. Thus, in adipocytes, ADP-ribosylation of G_s may well reduce its ability to be inhibited by R_i receptor means. Since ADP-ribosylation is known to reduce the affinity of G_s for βγ then the reduction in effectiveness of R_i-mediated inhibition in this system may well be due a decreased capacity of βγ to inhibit adenylate cyclase because of reduced affinity between α_s and βγ. However others (Toro 1987) have claimed that R_i mediated inhibition is not sensitive to cholera toxin mediated ADP-ribosylation of G_s. However one of the strongest arguements against βγ playing a direct role in the R_i mediated inhibition of adenylate cyclase is that βγ seem to be functionally interchangable between different G-proteins. Thus any time a G-protein is activated, adenylate cyclase would be inhibited. This is then a rather unsatisfactory biological non-specificity.

Another mechanism by which G_i might inhibit adenylate cyclase is would involve G_i interaction with R_s. Evidence shows that G_i will interact with ß-AR in reconstituted systems in which it has been shown (Asano *et al*, 1988) that ß-AR will stimulate GTPase activity of G_i. However more direct evidence has recently been obtained since Marbach *et al* (1988) have demonstrated a direct interaction between G_i and ß-AR in S49 lymphoma cells. This interaction requires the presence of G_s since in does not occur in cyc^- S49 cells or in turkey erythrocyte membranes which lack G_i. The interaction was inferred by showing that Pertussis toxin catalysed ADP-

ribosylation of G_i in membranes results in a 3 fold reduction in affinity for isoproterenol. This interaction could only be demonstrated by utilising an improved method of ligand binding studies to detect the interaction of ß-AR with G_i (Marbach *et al*, 1988).

Antagonists of the ∂-opiate receptor have been found which exhibit negative intrinsic activity (Costa & Herz, 1989) and for this to occur it has been suggested receptor catalysed partial activation of G_i may occur due to unoccupied receptors, others have arrived at similar conclusions (Sunyer *et al*, 1989). Evidence for an activity of unoccupied receptors in stimulating G-proteins also comes from reconstitution of α_2-AR with G_i+C showing that even in absence of α-adrenergic agonist the empty α_2-AR will activate G_i (Cerione *et al*, 1986). Similarly the same process has been shown for the ß-AR, G_s,C reconstituted system (Cerione *et al*, 1985). Antagonist affinity has been found to be modulated by GTP or its analogs for mAch-receptor (Burgisser *et al*, 1982), D_2-dopamine receptor (De lean *et al*, 1982) and the A_1-adenosine receptor (Green *et al*, 1984). In these cases the antagonist must be active in promoting the dissociation of R*G which requires empty receptors to be able to activate G-proteins.

Tonic, receptor-independent, inhibition of adenylate cyclase by G_i has been observed in a number of systems including fat cell membranes (Cooper *et al*, 1979). This was determined by virtue of the inhibition of adenylate cyclase seen at high concentrations of GTP in the biphasic dose response curve of isoproterenol stimulated adenylate cyclase to GTP. The tonic inhibition of adenylate cyclase can also be seen by using low concentrations of GppNHp upon forskolin stimulated adenylate cyclase in adipoctye membranes (Begin-Heick, 1985), hepatoctye membranes (Houslay, *et al* 1988), and platelet membranes (Spence & Houslay, 1989). Tonic inhibition of adenylate cyclase by G_i could occur by at least two mechanisms: either by empty-receptor

catalysed activation of G_i or by an intrinsic basal activity of G_i. However, since empty receptor activation of G_i seems to be a real possibility, it is interesting to note that ∂-opiate antagonists which display negative intrinsic activity do so only at low [Na^+]. At high concentrations of Na^+ tonic inhibition is lost but that mediated by PIA acting upon R_i in adipocyte membranes is not (Cooper 1982). Thus it is possible to both divorce the two modes of inhibition by selectively inhibiting one and not the other. It has been noted that Na^+ promotes the activation of adipocyte adenylate cyclase by GTP (Katz *et al*, 1981). This latter effect could be because high Na^+ has abolished the tonic inhibition by GTP, resulting in greater stimulation by GTP.

In lipid vesicles exogenous βγ added will inhibit GTPγS-stimulated but not isoproterenol stimulated adenylate cyclase (Cerione *et al*, 1985). One possibility is that active α_s remains associated with ß-AR, however this is difficult to reconcile with the demonstrated ability of a single ß-AR to catarlytically activate many G-proteins (Pederson & Ross 1982). An alternative may be that α_s-GppNHp and βγ are evenly distributed but that α_s-GTP+βγ formed using stimulation by isoproterenol+GTP are locally concentrated around ß-AR. In this case more free α_s may be needed to activate ß-AR because of the higher local concentration of βγ and in consequently the susceptibility to inhibition is lost.

1.4 FEATURES OF R_s AND R_i OF THE ADENYLATE CYCLASE SYSTEM AND COMPARISON WITH RECEPTORS FROM OTHER SIMILAR SIGNAL TRANSDUCTION SYSTEMS.

1.4.1 General structural features.

Rhodopsin and ß-AR are the most well characterised G-protein linked receptors known. Thus far, all receptors coupled to G-proteins appear to share common tertiary structural features, most notably the seven transmembrane spanning helices. Each receptor is approximately 40-50KD, approx 350-500 amino acid and probably forms seven transmembrane

regions linked by 3 cytoplasmic and 3 extracellular loops (Wang *et al*, 1989). The extracellular N-terminal tail may contain one or more glycosylated residues and the intracellular C-terminal tail has several serine and threonine residues that are potential or demonstrated sites of phosphorylation. The transmembrane sequences are unusual in having many proline and glycine residues. These may form 'kinks' in the helices that help form the binding pocket buried deep within the transmembrane regions. They may also play a part in transmitting to the cytoplasmic surface of the receptor the conformational change induced by ligand binding (Dolhman *et al*, 1987; Chambre & Deterre 1989). The transmembrane residues, 20-25 amino acid for each helix, are the most conserved amino acid between different receptor molecules (Lefkowitz *et al*, 1989).

1.4.2 Ligand recognition

Ligand recognition also seems to show, albeit unexpectedly, some common structural features. Negatively charged acidic residues in the putative intramembrane binding pocket serve as counterions for positively charged retinal, acetylcholine, or adrenaline (Applebury & Hargrave 1986).

Most of the extramembrane regions of the ß-AR can be removed by proteolysis without altering ligand binding, indicating that the ligand binding domain must lie within the core of the protein (Rubstein *et al*, 1987).

Similarly retinal interacts with opsin through the formation of a Schiff base with Lys^{296} in the 7^{th} transmembrane helix. Biophysical studies suggest that the bound chromophore in rhodopsin is buried approximately 30% into the membrane bilayer (Chambre & Deterre, 1989).

In the case of the ß-AR, the protonated amine group of catecholamines is located to Asp^{113}, as substitution to Asn results in a 10,000 fold decrease in affinity for adrenergic agonists. In the ß-AR, Asp^{79} also seems to be involved in agonist binding, however unlike Asp^{113}, Asp^{79} is not involved

with antagonist binding. The residues in the m1 muscarinic acetylcholine receptor corresponding to Asp^{113} and Asp^{79} of ß-AR are Asp^{105} and Asp^{71} respectively (Strader et al, 1989) showing that some of the contacts are made in the binding pocket of the m1 muscarinic receptor are similar to those made in the ß-AR.

Molecular replacement analysis has been less succesful in determining amino acid responsible for $ß_1$ and $ß_2$ receptor subtype selectivity. This is determined by contacts made to the substitution on the amine group If helix 4 of $ß_1$-AR is changed to predominantly a, $ß_2$-like form, then the affinities of the $ß_1$-AR became more like those of $ß_2$-AR (Frielle *et al*, 1988). Thus a $ß_1$-AR can be turned effectively into a $ß_2$-like AR by replacing helices 4 and 5 of $ß_1$-AR with those of $ß_2$-AR. In contrast smaller helix replacements or single amino acid replacements failed to affect subtype specificity. Thus it appears $ß_1$-AR and $ß_2$-AR differences cannot be attributed to a single amino acid contact which is any different between $ß_1$-AR and $ß_2$-AR, rather this recognition difference arises from the conformation of the receptor helices which is controlled by sequences within the transmembrane helices 4 and 5 (Dixon *et al*,1989).

Mutagenesis of the highly conserved Asp^{130} in the putative third transmembrane helice of the human ß-AR to Asn results in a receptor which is completely unable to stimulate adenylate cyclase (Fraser *et al*, 1988). However, somewhat paradoxically, this receptor is still responsive to guanine nucleotide effects on agonist affinity in membranes. The quantitative effects were somewhat different in that whereas in wild type ß-AR containing membranes 100µM GppNHp caused 5 to 10 fold shifts to the right in competition studies of ^{125}I-CYP/isoproterenol, the shift was only 3-5 fold in the mutant receptor. In addition the Hill co-efficient n_{Hill} changed from 0.5-0.66 to 0.86-1.0 for the wild type receptor, but in the case of the

mutant the co-efficient n_{Hill} was unchanged. Examination of the GppNHp dose response curve of % ^{125}I-CYP bound in the presence of 35pM ^{125}I-CYP and 10μM isoproterenol showed that the $K_{0.5}$ for the mutant ß-AR was much higher than than that for wild type. These experiments thus separate the two effects of G_s activation and GTP effect on agonist binding. In cells the affinity of mutant receptors for agonist is much higher and they show only a single class of sites but the affinity for antagonist is unaltered.

1.4.3 G-protein recognition

Receptor recognition of G-protein seems to involve both the loop that links the 5 and 6 transmembrane domains, *i.e* the 3rd cytoplasmic loop, and part of the C-terminal domain (Kubo *et al*, 1988; Kobilka *et al*, 1988; O'Dowd *et al*, 1989) with positively charged residues possibly playing a major role (Ross, 1989). This agrees with the experiments showing that proteolytic cleavage within the 3rd cytoplasmic loop of rhodopsin disrupts its ability to couple to transducin (Findlay & Pappin, 1986). Mutations in the ß-AR which uncouple it from G_s activation only reduce the maximal stimulation of adenylate cyclase by isoproterenol and do not affect the $K_{0.5}$ (Strader *et al*, 1987). It seems that regions of the N- and C-terminal ends of the 3rd cytoplasmic loop of ß-AR are essential for coupling to G_s. However these are unlikely to be the only contacts between R_s and G_s.

However using proteolysis of purified ß-AR from erythrocytes, Rubenstein *et al*, (1987) showed that most of the 3rd cytoplasmic intracellular loop and the C-terminal tail are not required in any way for functional coupling to G_s. Also most of the putative extracellular hydrophilic domains of ß-AR can be deleted without affecting coupling to G_s. The question then posed is what is the purpose of these domains: for example do they have a role in ß-AR desensitization ?

Another approach to the mapping of domains of the ß-AR which interact with G_s is the use of peptides corresponding to portions of the known

sequence of β_1-AR to inhibit isoproterenol stimulated adenylate cyclase (Palm *et al*, 1989).

1.4.4 Antagonist binding.

An antagonist must bind more strongly to the active conformation of its receptor, otherwise many binding events would be unproductive. This state is the conformation of R which binds most tightly to the 'transition' state, $\alpha\beta\gamma$ with no guanine nucleotide bound. If a guanine nucleotide is then added it binds to the vacant site on α_s, the transition state is lost, the association R^*G_s is weakened and with it the high-affinity binding of A to R. The analogous phenomenon for photoreceptors is the decreased stability of the active form of rhodopsin (meta rhodopsin II) in presence of GTP (Pfister *et al*, 1983). Antagonist receptors cannot bind more tightly than empty receptors to the G transition state or they would activate G. An antagonist may simply sterically block the binding of agonist but it may also bind more tightly to the inactive form of G and will exhibit negative intrinsic activity and thus inhibit basal turnover of G (Costa & Herz 1989). When the antagonist occupied receptor binds more tightly to the inactive (non-transition state) forms of R, GTP then stabilizes this antagonist binding. This appears to be true for ∂-opoid, D_2-dopaminergic, A_1-adenosine and muscarinic receptors (Burgisser *et al*, 1982).

1.5 REGULATION OF OTHER G-PROTEIN COUPLED EFFECTORS AND COMPARISON TO ADENYLATE CYCLASE.

1.5.1 Why use G-proteins?

The types of events controlled by receptors and the time scale over which they elicit their actions vary enormously. Thus activation is seen within milliseconds in the case of acetylcholine's ability to cause postsynaptic depolarisation. In contrast the effects that a growth factor, involving protein tyrosine kinase activation dependent activation, are much slower to elicit an

end result. Slow dissociation of A from R would provide high affinity binding and consequently a sensitivity to low concentrations of A, but would inevitably leave R insensitive to rapid changes in concentration of A. For G-protein linked R there are additional problems stemming from the agonist being bound to the receptor for too long, thus the interaction with G becomes inefficient. Active R will initially collide with only inactive G but as more of G is activated with time, an increasing fraction of collisions will be with already active G.

The conflict between responding rapidly and still maintaining sensitivity to low concentrations of agonist can therefore be satisfied by having receptors of relatively low affinity, hence fast dissociation rates, but to have so many spare receptors that maximal response can be evoked when only a small fraction are occupied so that sensitivity to low agonist concentrations is retained. In guinea pig ileum occupancy of less than 0.25% of muscarinic cholinergic receptors by acetylcholine is sufficient to cause half maximal contraction (Kenakin 1984).

The discrepancy between curves for receptor occupancy and certain responses, arises whenever one saturatable event controls the next saturatable step in the sequence (Strickland & Loeb 1981; Kenakin 1984). Thus the inclusion of G-proteins into the signalling sequence exaggerates the discrepancy between occupancy and response curves. Spare receptors therefore allow cells to respond to low agonist concentrations without losing sensitivity to rapid changes in agonist concentration. A change in receptor number can thus substantially alter sensitivity of adenylate cyclase without preventing full response.

G-proteins provide a means to enhance temporal sensitivity without sacrificing sensitivity to low agonist concentrations. They essentially, speed up the dissociation of agonist from R by virtue that dissociation of G_s from A-

R_S*G_S converts A*R_S to low affinity, therefore dissociation rate increases so that A leaves R_S, then R_S is back in a high affinity form to seek out and bind new agonist. Thus the recycling of R is coupled to the activation of G.

1.5.2 Rhodopsin-transducin-cGMP-phoshodiesterase system.

As regards the particular functional roles within a G-protein of the individual α,β and γ subunits the best resolved case is that of transducin. In retinal rods free α_t-GTP activates cGMP-PDE by binding to the small inhibitory subunit and relieving the inhibition upon the cGMP-PDE (Fung & Griswald-Penner, 1989; Chabre & Deterre, 1989). In other signal transduction pathways the relative roles of α,β and γ are less clear.

In comparison with the dissociational activation of G_S, when the α_t of transducin binds GTP, but not GDP, its affinity for $\beta\gamma$ is substantially reduced and the $\alpha_t\beta\gamma$ dissociates into $\beta\gamma$ and α_t-GTP (Navon & Fung 1987). A single activated rhodopsin can activate a number of $\alpha_t\beta\gamma$, since individual $\beta\gamma$ can recycle between many α_t subunits. Thus the transducin-cGMP-PDE signal transduction system like that of the adenylate cyclase system is able to achieve signal amplification.

Recently the γ subunit of transducin has been found to be farnesylated (Fukada *et al*, 1990). This modification appears to be essential for full GTP binding activity of holomeric transducin. The more active, farnesylated, form runs at 6kD rather than 8kD for the less active non-farnesylated form since bovine brain γ subunit was found to exist as in two forms in native ROS membranes (Fukoda *et al*, 1989) which can be separated by electrophoresis and identified by a common antigen site. A similar covalent modification may occur in the other γ subunits of different G-proteins. It is of interest that the GTP binding proteins $p21^{ras}$ which cannot bind $\beta\gamma$ subunits is itself farnesylated (Hanock *et al*, 1989), (Casey *et al*, 1989), (Schafer *et al*, 1989).

1.5.3 R_p-G_p-phospholipase C system

There are multiple PLC isoenzymes some of which appear to be regulated in a G-protein dependent fashion, G_p, (Fain, 1990). In some systems G_p appears to be a Pertussis toxin sensitive protein (presumably one of α_i1,2,3 or α_o1 or 2) as in neutrophils stimulated by fMLP (Nakamura & Ui, 1985). In other systems such as hepatocytes stimulated by vasopressin G_p is Pertussis toxin insensitive (Martin *et al*, 1986). In *Xenopus* oocytes activated α_o appears to stimulate PLC (Moriarty *et al*, 1990).

In contrast to the regulation of adenylate cyclase there is no good evidence for the existence of an inhibitory G-protein for the PLC system. However a Pertussis toxin insensitive inhibition of pituitary cell PLC by dopamine receptors has been observed (Enjalbert *et al*, 1986). It is of note that turkey erythrocyte ß-AR*G*C system, contains no G_i and therefore has no means to achieve receptor-mediated inhibition of adenylate cyclase. Thus regulation of adenylate cyclase in turkey erythrocytes has some analogy to the regulation of PLC in mammalian systems.

There is a family of low molecular weight guanine nucleotide binding proteins, the p21 proteins, which are encoded by the genes H-ras, K-ras, N-ras and others (Barbacid, 1987). The function of these proteins is uncertain but they may be regulated by cell surface receptors and thereby control the activity of effector enzymes *vide infra*. It has been reported that $p21^{ras}$ proteins coupled bombesin receptors to PLC (Wakelam *et al*, 1987).

1.5.4 R-G-phospholipase A_2 system

Phospholipase A_2 is an effector enzyme, which when hormonally stimulated, hydrolyses phosphatidyl choline to arachidonic acid and lysophosphatidyl choline. In mast cells (Bokoch & Gilman, 1984) and neutrophils (Okajima & Ui, 1984) stimulation of PLA_2 is Pertussis toxin sensitive. The identity of the G-protein involved is unknown.

1.5.5 The dihydropyridine-sensitive, voltage gated Ca^{++}-channel(s)

For Ca^{++} channels α_s-GTPγS activates the channel protein in heart tissue membranes (Yanati *et al*, 1987). In addition PKA seems to activate the channel by phosphorylation (Yanati *et al*, 1987). The stimulation of L-type Ca^{++}channels in guinea pig ventricular myocytes occurs by potentiation of high activity gating modes (Yue *et al*, 1990).

G_o has been found to be capable of inhibiting the channel by direct and indirect means. In chick dorsal root ganglia G_o may directly inhibit the channel via α-adrenergic receptors and $GABA_B$-receptors (Holz *et al*, 1986; Rane *et al*, 1986). In these same cells the inhibitory effect can also be mimicked, in Pertussis toxin-treated cells, by the addition of phorbol esters (Rane *et al*, 1986). Thus activation of PKC via stimulation of G_o may be another route of inhibition of the Ca^{++} channel.

1.5.6 Signal transduction of growth factor receptors exhibiting tyrosine kinase activity

G-proteins are not generally believed to couple to growth factor receptors possessing protein tyrosine kinase activity. This has been in part due to the possibility that effector activation was mediated directly by the PTK activity however recent results may indicate otherwise.

Recently stimulation of T-cell activation by PHA, phytoheamagglutinin, has been shown to activate $p21^{ras}$ (Downward *et al*, 1990). This is the first time that the activity of $p21^{ras}$ has been shown to be activated by a receptor. Activation of $p21^{ras}$ appears to be due to a decrease in the GTPase activity of the $p21^{ras}$ such that a larger number of the molecules exist in the active GTP-bound form rather than the GDP-bound inactive form. The mechanism of activation of $p21^{ras}$ is not known however PKC appears to be involved in the signal transduction mechanism. It is

suggested that activation of p21^{ras} involves inhibition of GAP thereby leading to decreased inhibition of p21^{ras}. GAP (Ellis *et al*, 1990), or GTPase activating protein, stimulates the GTPase activity of p21^{ras} thereby increasing the number of p21^{ras} molecules in the GDP-bound inactive state.

It is interesting to note that a GTPase activating protein, called GAP-43, has been found to interact with G_o (Strittmatter et al, 1990). The protein GAP-43 has been shown to be a peripheral membrane protein. This protein is also known as B50 or neuromodulin. GAP-43 stimulates the binding of GTPγS to G_o by some 2 fold (Strittmatter *et al*, 1990). Other activities of GAP-43 include binding of calmodulin, inhibition of PIP kinase and ability to be a substrate for PKC. It is the N-terminal sequence of GAP-43 which stimulates binding of GTP to G_o. The N-terminus of GAP-43 contains a 10 amino acid sequence possessing a consensus sequence found in the cytoplasmic loop of G-protein linked receptors.

In light of the findings of Downward *et al* (1990) it is worthy of note that a somewhat similar story emerged sometime ago when Kamata & Feramisco, (1984) reported that they had found that EGF could stimulate the phosphorylation and GTP binding of p21$^{c\text{-}H\text{-}ras}$ in membranes from Ha-NRK cells (non-producer line of rat kidney cells transformed by Harvey murine sarcoma virus).

Concerning the involvement of p21 proteins in signal transduction, the p21^{ras} protein has been found to associate with aggregated receptors cell surface immunoglobulins, co-capp, in activation of B-cells as shown by immunoflorescence (Graziadei *et al*, 1990). This represents further evidence that the p21 proteins mediate in cell surface receptor transmembrane signal transduction processes.

1.5.7 Adenylate cyclase in yeast (*S.cerevisae*)

Studies on this system are important in that it bears a considerable degree of homology to the mammalian system.

As with mammalian adenylate cyclase, Mn^{++} stimulates the C subunit directly without any involvement of G-protein. In yeast the functional homologue of G_S appears to be the product of the RAS2 gene which is highly homologous in terms of sequence similarity to mammalian *ras* proteins (Broek *et al*, 1985). However the catalytic unit (Kataoka *et al*, 1985), CDC35, at 220kD is somewhat larger than its mammalian counterpart of 120-150kD.

The functional equivalent of R_S is probably CDC25, however this molecule does not appear to contain protein sequences which would cause it to possess transmembrane domains like those of classical R_S. However sequence homology with ligand binding domain of cytochrome P-450, suggests that CDC25 possess a binding domain for a yet unidentified ligand. CDC25 does act upon RAS2 in an analogous manner to R_S upon G_S in that CDC25 catalyses guanine nucleotide exchange of RAS2. In membranes prepared from mutant yeast lacking RAS2 and RAS1, mammalian $p21^{H\text{-}ras}$ can reconstitute adenylate cyclase stimulation (Toda *et al*, 1985). Identification of the domain of CDC35 with which CDC25 interacts could help to identify the effector of $p21^{ras}$ proteins. RAS1 gene can transform NIH 3T3 cells provided that the C-terminus is replaced with that from $p21^{H\text{-}ras}$ (DeFeo-Jones *et al*, 1985). The partially purified catalytic unit of adenylate cyclase can be stimulated by purified RAS2 (Field *et al*, 1988) and also RAS1 but to a much lesser extent (Broek *et al*, 1985).

Yeast adenylate cyclase is negatively regulated by IRA-1 and IRA-2 gene products and recently (Tanaka, 1990a) it has been shown that these two proteins have significant sequence homology to the product of the human neurofibromatosis type-1 (NF-1) gene. NF-1 encodes a tumor suppressor gene encoding a large protein as is the case for IRA-1 (2938 amino acid) and IRA-2 (3079 amino acid), (Tonaka *et al*, 1990b) Both IRA-1 and IRA-2

exhibit regionalised but significant homology to mammalian GAP and mammalian GAP can suppress the phenotype of IRA mutants (Tanaka 1990a) suggesting that IRA-1 and IRA-2 may be functionally analogous to GAP. The homology of GAP to IRA-1 and IRA-2 has been localized to a small region in the middle, 1451-1780 amino acid for IRA-1 and 1597-1925 amino acid for IRA-2, of these proteins (Tonaka 1990a).

Two mammalian G-protein homologs have been found in S.cerevisae, GP1α (encoded by GPA1) and GP2α (encoded by GPA2) of M_r 54075 and 50516 respectively (Nakafuku *et al*, 1987; Nakafuku et al, 1988). The deduced amino acid sequences of GP1α and GP2α are highly homologous with those of rat brain α_i and α_o. GPA1 is a haploid specific gene, only expressed when cells are involved in mating factor signal transduction. However GPA2 is expressed in both haploid and diploid cells, and may be involved in the regulation of cAMP levels in yeast (Nakafuku *et al*, 1988). The level of cAMP regulates progression through the cell cycle, acting as an indicator of nutritional status of environment.

Analogs of ß and γ of mammalian G-proteins may have been found (Whiteaway *et al*, 1989) since the STE4 and STE18 genes of yeast encode gene products with significant sequence homology to ß-subunit and γ-subunit respectively. It has been suggested (whiteaway *et al*, 1989) that STE4 and STE18 are the $\beta\gamma$ subunits of GPα1. Expression of STE4 and STE18 is limited to haploid cells, suggesting the existence of another set of genes encoding potential $\beta\gamma$ subunits of GPα2 which is present in both haploid and diploid cells. Thus STE4 and STE18 are probably specifically employed in the mating factor signalling system.

STE4 gene product has been found to be phosphorylated on serine residues (Cole & Reed, 1990). Thus phosphorylation of G-proteins occurs in yeast, holding the possibility that this is also used as a mechanism to regulate

their functioning as is the case for G_i proteins in mammalian cells. Cells loaded with ^{32}P show phosphorylation of RAS2 and RAS1 exclusively on serine residues. At least two tryptic phosphopeptides have been which can be dephosphorylated by alkaline phosphatase (Cobitz *et al*, 1989). However it appears that neither RAS proteins can serve as substrates for PKC but that RAS2 can be phosphorylated by PKA and that this modification decreases its ability to activate CDC35 (Resnick & Racker, 1988). Interestingly phosphorylation of a platelet 22kD GTP binding protein by PKA, smg p21, has been reported (Hoshijima *et al*, 1988) in a cell free system. Among the mammalian small molecular weight proteins $p21^{c\text{-}K\text{-}ras}$ has been shown to be phosphorylated by PKC and PKA (Ballester *et al*, 1987) and $p21^{v\text{-}H\text{-}ras}$ by PKC (Jeng *et al*, 1987).

At least 15 smg molecules have been separated from bovine brain membranes. The smg include *ras*; *rho*;*ral*;*R-ras*;*ypt1*;*rab2*; and *arf* gene products (Barbacid 1987; Maduale & Axel 1985; Chardin & Tavitian 1986; Lowe *et al*, 1987; Haubruck *et al*, 1987; Touchot *et al*, 1987; Sewell *et al*, 1988). However these proteins may not be involved in signal transduction. The *ypt1* gene product has been implicated in secretory phenomena (Segev *et al*, 1988). The *rho* p20 is known to be ADP-ribosylated by botulinum toxin (Kikuchi *et al*, 1988) and this toxin inhibits neurotransmitter release from cholinergic nerves. However smg21 has sequence similarity in its C-terminal tail to the corresponding region of $p21^{ras}$ proteins (Kawata *et al*, 1988) which is the putative effector domain as deduced by comparison with the effector domain of the GTP binding protein EF-Tu. It seems most likely that many of these smg are not involved in transmembrane signal transduction. However the fact that both $p21^{ras}$ proteins which may be involved in transmembrane signal transduction like their yeast counter parts are potentially subject to phosphorylation may have important clues to the elucidation of the role that phosphorylation plays in

the regulation of conventional G-proteins within mammalian systems such as adenylate cyclase system. Thus there may be important parallels to draw with mammalian systems.

The human $ß_2$-AR together with mammalian G_s can when expressed in yeast can stimulate the endogenous adenylate cyclase, ie CDC35 (King et al, 1990). This approach provides a valuable new method to assess signal transduction.

1.6 SPECIFICITY IN R-G INTERACTIONS & G-E INTERACTIONS.

1.6.1 General points.

Obviously one of the major problems of research in G-protein signal transduction systems is specificity of interaction between receptor and G-protein and similarly between G-protein and effector. In the first part there are 80 receptors but only 12 known G-proteins with not much more than 15 suspected G-proteins. In the second part it is now appreciated that the same G-protein can apparently couple to completely different effectors.

1.6.2 Fidelity of R-G interactions

The peptide hormone thrombin appears to stimulate two distinct G-protein GTPase activities in human platelet membranes (Houslay *et al*, 1986). It is suggested that thrombin couples through its receptor(s) to G_i and G_p. The novel activity of G_p was identified due to a lack of susceptibility to Pertussis toxin mediated inhibition. There are two possibilities: either there is one thrombin receptor coupling to two G-proteins or two receptors each coupling to different G-proteins. It has been shown that purified mAch-receptor can be reconstituted with purified G_o or G_i such that muscarinic agonists will show guanine nucleotide sensitive binding like that of native membranes (Florio & Sternweiss, 1984). The ß-AR appears to be able to interact with G_i as well G_s (Marbach et al 1988).

G_s is known to be able to stimulate both Ca^{++} channels and the C subunit of adenylate cyclase (Yanati *et al*, 1987a). Similarly G_o appears to be capable of stimulating both PLC and inhibiting Ca^{++} channels directly (Holz *et al*, 1986; Rane *et al*, 1986).

1.7 DESENSITIZATION OF ADENYLATE CYCLASE AND OTHER G-PROTEIN LINKED SYSTEMS.

1.7.1 General points.

Prolonged exposure of cells or tissues to drugs/hormones such as catecholamines leads to a state of refractoriness to further stimulation by that agent, known as homologous desensitization. However the desensitization can also become heterologous such that it affects signalling through any receptor linked to the same G-protein/effector.

1.7.2 Desensitization of the ß-AR system.

Adenylate cyclase can become desensitized to a wide variety of agonists in a number of cells, however probably the best characterised is that of the ß-AR. This process involves alterations at a number of points including an increase in the rate of transcription of cAMP-PDE (Swinnen *et al*, 1989). However this change in itself is not sufficient to reduce cAMP levels to the extent seen, since desensitization still occurs in presence of cAMP-PDE inhibitors, nor is it rapid enough. Two potential mechanisms to explain rapid effects of desensitization have been popular in the past: phosphorylation of ß-AR and secondly the internalisation of the ß-AR. However although internalization can be rapid, several lines of evidence suggest this is not important in achieving rapid desensitization. Firstly, internalisation can be blocked by the use of concanavalin A or phenylarsene oxide without disrupting rapid desensitization. (Waldo *et al*, 1983). Also the onset of desensitization has been detected before internalisation. Thus in frog and turkey erythrocytes

desensitized membranes can be isolated and ß-AR stimulated adenylate cyclase and GTPase activities determined. Using this approach it has been shown that the reduction in adenylate cyclase stimulation is paralleled by an equivalent reduction in GTPase stimulated by ß-AR agonists (Pike & Lefkowitz, 1980). Purified ß-AR, 64kD, can be phosphorylated in vitro by purified PKA to a stoichiometry of 2 mol phosphoserine/ mol of receptor and isoproterenol increases the rate of phosphorylation by 2-3 times (Benovic *et al*, 1985). In, S49 kin$^-$ cells which lack PKA activity, the ß-AR can still be phosphorylated and this led to the discovery of ß-AR kinase, called ß-ARK (Benovic *et al*, 1986).The ß-ARK gene has been cloned (Benovic *et al*, 1989) and from this a multigene family of similarly functioning receptor kinases has been predicted. The cDNA was expressed in COS cells, and shown to contain encode a 689 amino acid (80kD) protein which shows only 33% homology to catalytic domains of PKC and PKA this being restricted to the putative catalytic domain of 239 amino acid. The lack of homology to previously known protein kinases suggests this may be the first member of a distinct family of protein kinases and indeed a second member of this family has been found with 85% homology to ß-ARK by screening of a bovine cDNA library. Northern blot analysis shows that ß-ARK is apparently expressed most highly in brain, spleen, heart and lung which have a high sympathetic input suggesting ß-ARK is involved in modulating activity of synaptic receptors. This may be an important fact since the substrate specificity of ß-ARK is not resolved since it also acts upon muscarinic receptors and α_2-AR (Kwatra *et al*, 1989). In the rhodopsin system, phosphorylation of the visual receptor by purified rhodopsin kinase by itself resulted in little deactivation (Uhl *et al*, 1990). Phosphorylation allows the binding of arrestin, 48kD, to inhibit competitively the binding of transducin to rhodoposin (Wilden *et al*, 1986). A cDNA possessing 59% identity to retinal arrestin, the protein which is intimately involved with the deactivation of

transducin, has been found. High levels of phosphatase activity appear to be associated with subcellular vesicles containing internalized ß-AR (Sibley *et al*, 1986). The phosphatase associated with this compartment reversed the desensitization. Duck erythrocyte ß-AR desensitization can be reversed in isolated desensitized membranes by alkaline phosphatase treatment (Stadel *et al*, 1988). A protein, termed ß-arrestin, possessing 59% sequence homology to arrestin, the protein intimitly involved in deactivation of transducin activation, has been identified in non-retinal tissue (Lohse *et al*, 1990). By Northern bloting ß-arrestin demonstrates a similar tissue distribution to ß-ARK, implying the possibility that this protein may be the functional analog of arrestin, in the ß-AR system.

High levels of phosphatase activity appear to be associated with subcellular vesicles containing internalized ß-AR (Sibley *et al*, 1986). The phosphatase associated with this compartment reversed the desensitization. Duck erythrocyte ß-AR desensitization can be reversed in isolated desensitized membranes by alkaline phosphatase treatment (Stadel *et al*, 1988).

In contrast to shorter term desensitization there is little evidence that phosphorylation by either ß-ARK or PKA is important in the mechanism of long term desensitization. Mutations in the two sites of phosphorylation by PKA, of the human $ß_2$-AR, from Ser to Aln has been conducted (Hausdorff *et al*, 1989). The mutant receptor was stably expressed in a cell line and found to show no impairment to internalization, or its ability to couple to adenylate cyclase stimulation but was unable to undergo phosphorylation and desensitization.

Thus mutant $ß_2$-AR exposed to isoproterenol for several hours ultimately desensitize to an equivalent degree to normals (Strader *et al*, 1987). This data implies that phosphorylation is important for short term desensitization but is relatively unimportant in the mechanism of long term

desensitization. In S49 cyc$^-$ cells there is very little down-regulation of ß-AR (Mahan *et al*, 1985). In addition mutant receptors that show impaired coupling to G_s also show impaired down-regulation. However in H21a S49 cells, which lack coupling between G_s and ß-AR , there is still normal agonist induced internalization. Long term exposure to agonists seems to reduce the mRNA for $ß_2$-AR this being due to a decrease in the stability of the mRNA rather than an alteration in rate of transcription (Hadcock *et al*, 1989).

1.7.3 Glucagon desensitization of hepatocyte adenylate cyclase system in chick hepatocytes.

Exposure of chick hepatocytes to glucagon results in the rapid onset of heterologous desensitization as measured by decreased response of adenylate cyclase to NaF stimulation. An interesting feature of this heterologous desensitization is that it is rapidly reversible under conditions where homologous desensitization is retained (Premont & Iyengar 1989). Degradation and *de novo* synthesis would be too slow to account for this process. Reconstitution of G_s activity into S49 cyc$^-$ cell membranes shows that G_s activity is reduced 25% following glucagon treatment (Premont & Iyengar 1989). Addition of purified G_s to desensitized chick membranes results in partial restoration of glucagon responsiveness. The extent of this restoration of NaF stimulated adenylate cyclase activity was some 10-15%. Resorting to 8-Bromo-cAMP treatment of intact non-desensitized cells results in a 15-20% loss of NaF-stimulated activity which could not be restored by the addition of purified G_s to membranes. Some experiments appear to show that incubation of control membranes but not desensitized membranes with PKA, results in decreased activity of adenylate cyclase (Premont & Iyengar 1990). The effect seems to be more pronounced in S49 kin$^-$ cell membranes. Experiments indicate that ßγ are not phosphorylated (Premont & Iyengar 1990).

1.7.4 Glucagon desensitization in Rat hepatocytes.

Exposure of rat hepatocytes to glucagon results in desensitization, $t_{0.5}$ 1.5-2.0 min and $K_{0.5}$ glucagon 0.4nM (Houslay *et al*, 1990) with no change in receptor number or response of membranes to NaF. The addition of cAMP-PDE inhibitors has no affect on the $t_{0.5}$ for desensitization and stable analogs of cAMP such as dibutryl-cAMP and 8-bromo cAMP do not elicit desensitization. Therefore the process of desensitization appears to be cAMP independent. Desensitization appears to involve a different population of glucagon binding receptors to those which stimulate adenylate cyclase since the $K_{0.5}$ for desensitization is 0.4nM while the $K_{0.5}$ for stimulation of adenylate cyclase is 8nM. In support of this theory, TH-glucagon, an analogue of glucagon which does not stimulate adenylate cyclase, causes glucagon desensitization in hepatocytes. Apparently both glucagon and TH-glucagon can stimulate the production of inositol polyphosphates in hepatocytes. As the PKC activating phorbol ester TPA was found to be capable of eliciting glucagon desensitization and also hormones capable of producing diacyglycerol intracellularly. This causes uncoupling of glucagon receptors from G_s, probably through the phosphorylation of one of these components.

1.8 INSULIN RESISTANCE/DIABETES/OBESITY.

1.8.1 Type 1 (IDDM)

Human insulin dependent diabetes mellitus (IDDM) is clinically characterised by the abrupt onset of symptoms such as hyperinsulinaemia, ketosis, hyperglycaemia and glycosuria.In order to sustain life, daily injections of insulin are required. This class of diabetes mellitus was classically diagnosed in juveniles and is often therefore known as juvenile onset diabetes.This classification however is not strictly true because diagnosis of this disease has been made within a wide range of groups.

Factors associated with the onset of this form of diabetes include

abnormal immune response (islet cell antibodies are often present at diagnosis), genetic inheritance (genes on chromosome 6 suggested) and viral infection. Chemically induced destruction of ß-cells of pancreas has been demonstrated to mimic type 1 diabetes. Streptozotocin (2-deoxy-2-(3-methyl-3-nitrosoureido)-D-glucopyranose) and alloxan (2,4,5,6-tetra oxohexahydropyrimidine) have been used in rats. Streptozotocin accumulates in the ß-cells of the pancreas. STZ has an LD_{50} of 138mg/kg when administered to rats by intravenous injection. One theory is that DNA is damaged by generation of carbonuim ions ($CH_3^{\cdot}$) and subsequent repair by poly-ribose synthetase to repair the damage leads to NAD depletion and hence cell death (Uchigata *et al*, 1982).

1.8.2 Type 2 (NIDDM)

This type has been termed maturity onset diabetes but the method of classification is not strictly correct because NIDDM may develope at any age but is most likely to develope at 40 and over. Statistics show that 60-90% of all NIDDM patients are obese and treatment in many cases, is restricted to diet alone. In other cases, controlled diet together with sulphonylurea or biguanide therapy is administered. Clinical diagnosis of NIDDM in absence of readily observable symptoms is generally made by oral glucose tolerance test (OGTT).

1.8.3 The Zucker rat.

The obese Zucker rat appeared as a spontaneous genetic obese condition in the laboratory of Comparative Pathology, Stow,Massachusett,USA in 1961.(Zucker & Zucker 1961). Obese (*fa/fa*)progeny were observed at a 25% incidence/litter the condition being due to a single recessive gene.

Obesity is observable within 3 weeks and food intake is significantly increased in these animals. The fatty acid level is 10 times that found in lean (Fa/Fa) littermates and is milky in appearance. Even when food intake is

restricted *fa/fa* rats are still obese in comparison with their lean (Fa/Fa) litermates.

Hyperinsulinaemia is present in *fa/fa* animals before obesity is observed and plasma insulin concentration appears to be dependent on the age of the rat. In contrast circulating glucagon levels are decreased in obese animals (Eaton *et al*, 1976). Interestingly Clark *et al*,(1983) have identified the presence of insulin-resistance, as assessed by oral glucose tolerance test and the lowering of body fat & triglyceride when compared to obese normoglycaemic rats. This condition appeared after 6 months and surprisingly occurred more in males than females. It has been (Geurre-Milo *et al*, 1985) suggested that insulin stimulated glucose transport is enhanced in *fa/fa* animals.

1.8.4 The *db/db* diabetic mouse

The *db* gene is inherited as an autosomal recessive trait (Coleman & Hummel, 1967). The *db/db* animals exhibit abnormally high deposition of fat, by 4 weeks of age, followed by hyperglcaemia and polyuria. Blood sugar, non-fasting, from control mice did not exceed 200mg/100ml at any age but in contrast *db/db* mice at 10 weeks of age exhibit levels not less than 300mg/100ml.

1.8.5 The *ob/ob* obese mouse

The *ob/ob* syndrome is the result of a mutation in one gene located on chromosome 6. The consequences of this mutation are numerous. In addition to hyperglcemia, hyperinsulinemia, and extreme resistance to insulin (Herberg & Coleman, 1977; Bray & York, 1979) this animal model of obesity is characterised by the extreme resistance of its adipose tissue to the action of lipolytic hormones *vide infra.*

1.9.1 INSULIN RESISTANT SYSTEMS SHOWING IMPAIRED CATECHOLAMINE STIMULATION OF ADENYLATE CYCLASE IN

ADIPOSE TISSUE

A number of rodents which exhibit insulin resistance show impaired catecholamine stimulation of lipolysis and all of these are obese. There is a strong correlation between intracellular concentration of cAMP and the rate of lipolysis (Londos *et al*, 1984). The bodies of these animals show increased amounts of adipose tissue and in some this is extreme. These include the genetically obese *ob/ob* mouse (Begin-Heick 1985); the genetically obese diabetic mouse *db/db* (Levilliers *et al*, 1978), the Toronto-KK mouse (Kupiecki and Adams 1974), the New Zealand obese mouse (Kupiecki and Adams, 1974). The *ob/ob* mouse; the *db/db* mouse; Toronto-KK; New Zealand obese mouse; and the high fat diet fed rat (Gorman *et al*, 1973) all show decreased stimulation of adenylate cyclase by catecholamines.

1.9.2 Adenylate cyclase defect in *ob/ob* mouse adipocytes

The adenylate cyclase defect has been best characterised in the *ob/ob* mouse. From such studies there is no doubt that a severe defect in the stimulation of adenylate cyclase by catecholamines exists in the adipocytes of *ob/ob* mice but the presence of a corresponding defect in catecholamine-stimulated lipolysis has been refuted (Sheperd *et al*, 1977). However, more recent evidence suggests otherwise (Dehaye *et al*, 1985) as does earlier evidence (Dehaye *et al*, 1977; Jolly *et al*, 1978). It is also of note that lipogenesis is reported to be increased in *ob/ob* adipose tissue (Winand *et al*, 1973).

The cAMP-phosphodieterase activity has been shown to be elevated in *ob/ob* adipocytes (Begin-Heick 1977; Dehaye *et al*, 1977; Sheperd *et al*, 1977; Kaplan *et al*, 1973). Thus impaired stimulation of lipolysis in this system by catecholamines could be due to increased degradation of cAMP by the cAMP-PDEs. However the addition of methylxanthine cAMP-PDE inhibitors could not restore sensitivity or the capacity to respond to lipolytic hormones in adipose tissue of *ob/ob* mice. Thus there must be a significant

defect at the level of cAMP generation by adenylate cyclase.

Dibutryl cAMP is able to elicit similar lipolytic responses in lean and obese adipocytes (Herberg *et al*, 1970; Begin-Heick & Heick 1977). This implies that the capacity of TAG lipase, the pacemaker enzyme of triacylglycerol hydrolysis, is similar in lean and obese mice and hence the defect must lie at the steps controlling the production of cAMP.

It has however been reported (Kahn *et al*, 1973; Begin-Heick, 1980) that there is no decrease in the number of ß-AR receptors and that there is no apparent defect in the number of catalytic units. Thus the defect(s) in stimulation of adenylate cyclase probably lie at the level of ß-AR receptor coupling to G_s or at the level of activation of adenylate cyclase by G_s. It has (Begin-Heick, 1985) been shown that membranes from *ob/ob* mice, as opposed to their lean littermates (+/?), show tonic inhibition of adenylate cyclase as mediated by G_i. However others (Greenberg *et al*, 1987) have shown that R_i mediated inhibition appears to be still perfectly intact in *ob/ob* membranes. Thus its seems that, in *ob/ob* adipocytes tonic inhibition by G_i upon adenylate cyclase is abolished, whilst in contrast agonist occupied receptor mediated inhibition is still perfectly functional. A question arises as to what the consequences of the above alterations in G_i activity might have on the regulation of adenylate cyclase by R_s linked agonists. To this end Begin-Heick proposed a theory to link the loss of tonic inhibition by G_i with the reduced stimulation of AC by ß-AR agonists. Since it is believed that the amount of G_i greatly, 10-30 fold, exceeds that of G_s (Bokoch *et al*, 1984) then if there was some perturbation of G_i which weakened the association of $\alpha_i\beta\gamma$ such that the free $\beta\gamma$ concentration within the membrane increased. The increased free concentration of $\beta\gamma$ would by mass action tend to inhibit the dissociation of $\alpha_s\beta\gamma$ thereby decreasing the effectiveness with which ß-AR can activate adenylate cyclase.

It has been shown that the ß-AR response of lean mice is mostly due to $ß_1$-ARs but that of obese is more typical of $ß_2$-AR response (Begin-Heick, 1981). The significance of this may be that $ß_2$-receptors may be less effectively coupled to the activation of adenylate cyclase, hence helping to explain the loss of responsiveness to catecholamines. In support of this latter possibility are the observations made with differentiated 3T3-F442A adipocytes (Feve *et al*, 1990). These latter cells are responsive to catecholamines, however if these same cells are exposed for long periods of time to glucocorticoids then they subsequently exhibit a reduced responsiveness to stimulation of adenylate cyclase by isoproterenol. This latter effect is associated with the switch of predominantly $ß_1$-ARs in the more responsive cells to predominantly $ß_2$-ARs in the less responsive cells.

1.9.3 Adenylate cyclase defect in *db/db* adipocytes.

The only data on the regulation of adenylate cyclase in adipoctes of *db/db* mice, show that their is no apparent defect at the level of the catalytic unit of adenylate cyclase itself (Laudat & Pairault 1975; Levillier *et al*, 1978). The type of G-protein lesion seen the *ob/ob* (above) is not seen in the *db/db* mouse (Begin-Heick & Coleman, 1988).

1.9.4 Regulation of adenylate cyclase in hepatocytes from animal model systems of type II diabetes mellitus (NIDDM).

The main characteristics of hepatic metabolism in the Type II diabetic state are increased lipogenesis, leading to triglyceride accumulation, and an enhanced hepatic glucose output which contributes to the observed hyperglycaemia (DeFronzo, 1988). As for IDDM, several animal models of NIDDM have been investigated to determine whether these effects can be explained by alterations in the regulation of adenylate cyclase activity. This possibility has been studied in several animal models of obesity and NIDDM including the genetically obese (*fa/fa*) Zucker rat (Houslay *et al.*, 1989),

the genetically obese (*ob/ob*) mouse (Bégin-Heick & Welsh, 1988).

In hepatocyte membranes prepared from obese Zucker rats, there seem to be two defects affecting adenylate cyclase regulation (Houslay *et al.*, 1989). Firstly, there is an abolition of the ability of GppNHp to inhibit forskolin-stimulated adenylate cyclase activity in membranes from obese animals, although the amounts of $G_i\alpha$-2 are similar in lean and obese membranes as determined by immunoblotting and pertussis toxin-catalysed ADP-ribosylation studies (Houslay *et al.*, 1989). As with the stz-diabetic system, P_{2y} purinergic receptor-mediated inhibition of cyclic AMP accumulation persists in the obese state (Murphy & Houslay, unpublished data). There is also a defect in the ability of glucagon to stimulate adenylate cyclase activity, as hepatocyte membranes from obese animals exhibit a ten-fold higher $K_{0.5}$ value for glucagon activation of adenylate cyclase. However, the specific activities produced at maximal glucagon concentrations are the same in lean and obese rats (Houslay *et al.*, 1989). The reduced efficacy of hormonal activation was attributed to a 50% reduction in expression of the two forms of $G_s\alpha$ expressed in hepatocytes, as determined by cholera toxin-catalysed ADP-ribosylation (Houslay *et al.*, 1989).

Another study employing sucrose-purified liver plasma membranes from lean and genetically obese (*ob/ob*) mice gave similar but not identical findings to those in obese Zucker rats (Bégin-Heick & Welsh, 1988). G_i function was shown to be abolished but this was claimed on the basis that α_2-adrenergic receptor-mediated inhibition was lost unlike Zucker rat hepatocytes. This was associated with an enhanced ability of isoproterenol, but not glucagon, to stimulate adenylate cyclase activity in the presence of GTP (Bégin-Heick & Welsh, 1988). Whether these changes in regulation are related to the reduced cholera toxin-catalysed labelling of $G_s\alpha$ and increased pertussis toxin-catalysed labelling of $G_i\alpha$ observed in liver membranes from obese animals compared with leans is unknown (Bégin-Heick & Welsh,

1988).

1.9.5 Adenylate cyclase regulation in streptozotocin diabetes/type I diabetes mellitus (IDDM)

Type I diabetes mellitus (IDDM) arises due to the inability of the ß-cells within the islets of Langerhans in the pancreas to secrete insulin: the consequences of this state for whole body metabolism have been recently reviewed (Taylor & Agius, 1988).

1.9.5.1 Liver tissue

Evidence suggests that the hyperglucagonaemia associated with IDDM is responsible for the increased hepatic glycogenolysis, gluconeogenesis and ketogenesis in diabetic patients (Unger & Orci, 1981; Johnson *et al.*, 1982). In both genetic (BB/Wor rats) and chemically-induced (streptozotocin [stz] or alloxan) rat models of IDDM, these changes are associated with an increase in hepatic cyclic AMP concentrations (Pilkis *et al.*, 1974; Appel *et al.*, 1981). Several groups have attempted to ascertain whether this increase in cyclic AMP content is attributable to defects in the adenylate cyclase signalling complex, with variable results being obtained (Allgayer *et al.*, 1982; Dighe *et al.*, 1984; Gawler *et al.*, 1987; Lynch *et al.*, 1989).

Most groups have shown there to be an increase in the ability of glucagon to stimulate adenylate cyclase in plasma membranes prepared from either whole liver (Allgayer *et al.*, 1982; Lynch *et al.*, 1989) or hepatocytes (Gawler *et al.*, 1987). However the mechanisms by which this phenomenon occurs are still controversial. Lynch *et al.* (1989) claim that liver plasma membranes prepared from streptozotocin-induced diabetic animals and BB rats contain increased amounts of $G_s\alpha$-subunits compared with normal controls as assessed by immunoblotting and cholera toxin-catalysed ADP-ribosylation. This results in an enhanced activation of

adenylate cyclase activity by cholera toxin, $[AlF_4]^-$ and GTPγS as well as glucagon (Lynch *et al.*, 1989). Also, inhibition of adenylate cyclase by angiotensin II in isolated membranes and intact hepatocytes was unchanged in streptozotocin-induced diabetic animals and slightly increased in BB rats compared with normal controls (Lynch *et al.*, 1989). These results appeared contradict the earlier findings of Gawler *et al.* (1987) who attributed the enhanced activation of adenylate cyclase activity by glucagon in hepatocyte plasma membranes to a loss of functional G_i activity relieving a tonic inhibitory effect on stimulation, thereby sensitising the response to glucagon. However, Bushfield *et al* (1990), have now explained this discrepancy. The reason for this is that Gawler *et al* (1987) used a hepatocyte preparation consisting of pure parenchymal cells, wheras Lynch *et al* (1989) studied 'whole liver' containing a mixture of cell types. Thus it was shown (Bushfeild *et al*, 1990) that streptozotocin diabetes causes cell-specific changes in G_i expression., a 70% reduction in the expression of $G_i\alpha$-2 and $G_i\alpha$-3 Associated with this was the a phosphorylation of $G_i\alpha$-2 in streptozotocin-diabetic hepatocytes causing an abolition of the ability of GppNHp to inhibit forskolin-stimulated adenylate cyclase activity (Gawler *et al.*, 1987; Bushfield *et al.*, 1990). This loss of G_i function accounted for amplification of glucagon action, a fact confirmed by mimicking it in hepatocytes from normal animals treated with pertussis toxin. However, whilst guanine nucleotide-mediated inhibition was abolished, P_{2y} purinergic receptor-mediated inhibition of cyclic AMP accumulation in streptozotocin-diabetic hepatocytes persists, albeit to a lesser extent commensurate with the reduction in expression of $G_i\alpha$-subunits (Bushfield *et al.*, 1990c).

1.9.5.2 Adipose tissue

The reduction in plasma insulin concentrations associated with IDDM results in the mobilization of fatty acids from adipose tissue due to the

concommitant reduction of insulin's anti-lipolytic effect and the enhanced action of lipolytic hormones (Williamson *et al.*, 1989). The latter effect presumably accounts for the elevated cyclic AMP content of streptozotocin-diabetic rat adipocytes (Ciappe de Cingolani, 1983). As with hepatocytes, different groups have attempted to characterize possible defects in the regulation of adenylate cyclase which could explain the observed increase in lipolysis (Kissebah & Fraser, 1972; Zumstein *et al.*, 1980; Chiappe de Cingolini, 1986; Strassheim *et al.*, 1990).

Several investigators have noted either an increased sensitivity (La Casa *et al.*, 1983) or increased stimulation of adenylate cyclase in response to catecholamines (Chiappe de Cingolini, 1986; Strassheim *et al.*, 1990). Whereas Chiappe de Cingolini (1986) attributed the enhanced responsiveness to an increase in the number of ß-adrenergic receptors, Strassheim *et al.* (1990) demonstrated that the tonic inhibitory effect of G_i was abolished in adipocyte membranes from streptozotocin-diabetic animals, which in this respect resemble hepatocyte membranes (Gawler *et al.*, 1987). Similarly, receptor-mediated inhibition of adenylate cyclase by PIA, PGE_1 and nicotinic acid persists, and is even slightly enhanced in streptozotocin-diabetic animals (Strassheim *et al.*, 1990). These changes were also accompanied by a 2-fold increase in expression of $G_i\alpha$-3, although the authors claim that it is the phosphorylation and inactivation of $G_i\alpha$-2 which causes the abolition of guanine nucleotide-mediated inhibition (Strassheim *et al.*, 1990).

Human studies on IDDM patients failed to show any alteration in their ratios of expression $G_i\alpha$-2 to $G_s\alpha$ compared with non-diabetic controls as determined by Western blotting (Ohisalo *et al.*, 1988) but functional studies have not been carried out to ascertain whether there was any alteration in adenylate cyclase regulation. However the number of patients studied was really too few to draw any conclusions.

1.10 ALTERATION IN SIGNAL TRANSDUCTION ELEMENTS OTHER THAN ADENYLATE CYCLASE IN INSULIN RESISTANT STATES.

1.10.1 Human obesity

The rate at which insulin stimulates glucose disposal in human obese subject is much slower than in normal subjects (Molina *et al*, 1989). Insulin is thought to stimulate glucose transport by two mechanisms. Firstly by recruitment of transporters from an intracellular pool to the plasma membrane (Kono *et al*, 1982) and secondly by increasing the intrinsic activity of the recruited carriers (Simpson & Cushman, 1986). Human obesity is associated with a decrease in the number of low-density microsomal glucose transporters. However the decrease in basal and insulin stimulated glucose transport cannot entirely be explained by decreased number of glucose transporters, suggesting an impairment in intrinsic activity of the glucose transporters (Garvey *et al*, 1988). It appears that the defect in activation of intrinsic activity, the second stage, is not due to a binding defect of insulin to receptor but a post receptor defect.

1.10.2 Glucose transporters in obese and lean Zucker rats.

The skeletal muscle, the primary site of glucose disposal in normal rats, of 36 week old *fa/fa* rats shows impaired insulin stimulation of glucose transport (Crettaz *et al*, 1980). The level of the GLUT-4 glucose transporter is similar in lean and obese animals, however exercise of *fa/fa* animals increases the level of the GLUT-4 transporter. However in contrast, the white adipose tissue of *fa/fa* rats shows increased glucose transport and increased glucose oxidation (Ezaki 1989).

Insulin activates glucose transport in heart and brown adipose tissue in the same or very similar manner as in adipocytes. In these tissues, the second activation step involves an increase in affinity for glucose and

occurrence of positive co-operativity (Zaninetti *et al*, 1989). In *fa/fa* rat heart tissue insulin stimulation of the second step was decreased, while affinity for glucose was unaltered (Zaninetti *et al*, 1989).

1.10.3 Alteration in PKC signalling in insulin resistant animals.

The ability of phorbol esters, which activate PKC by mimicking the action of diacylglycerol, to inhibit glycogen synthase a in heart tissue of *fa/fa* rats is significantly impaired (Van de Werve *et al*, 1987).

The liver tissue of rats starved for 3 days shows impaired activity of the IR protein tyrosine kinase and hence insulin action (Karasik *et al*, 1990). The protein tyrosine kinase activity of IR, decreases by 45% and this decrease can be reversed by alkaline phosphatase treatment of membranes. Tryptic removal of the C-terminal region of IR ß-subunit, which functions to inhibit the kinase activity, similarly restored kinase activity of the IR from starved rats to normal. A two fold increase in PKC activity was found in cytosol and membrane extracts of liver from starved rats. A parallel increase in PKC was demonstrated by immunoblotting for PKC itself. Thus the decreased activity of IR in starved rats may be due to increased activity of PKC (Karasik *et al*, 1990).

1.11. INSULIN RECEPTOR .

1.11.1 General points.

The mature human IR is a heterotetramer $\alpha_2ß_2$. The α subunit is 719 or 731 amino acids the ß is 620 amino acids. The a precusor is 1355 amino acids (Ebina *et al*, 1985). The α and ß subunit are generated from the proteolytic cleavage of a single chain precusor 1355 amino acids, this occurs during transfer from ER to plasma membrane. After cleavage of the precusor the nascent α and ß subunits are glycosylated on asparagine (N-linked) and possibly serine/threonine (O-linked) residue. Thus the α and ß subunits

have apparent Mr of 135 and 95 KD respectively,ie greater than their weights predicted from cDNA. In the absence of α subunit the ß PTK is constitutively active, suggesting the α subunit inhibits the PTK and that the IR is activated by removing the negative affect of α upon ß PTK activity (Ellis *et al*, 1987).

The IR is a protein tyrosine kinase phosphorylating tyrosine residues on target proteins (Czech, 1985). Site directed mutagenesis studies have revealed a close relationship between PTK activity and insulin action.(Ellis *et al*, 1986; Ebina *et al*, 1987; Herra *et al*, 1985; Morgan & Roth 1987; Russell *et al*, 1987). A number of putative endogenous substrates have been found, mostly of high molecular weight (Rees-Jones *et al*, 1984; White *et al*, 1985; Kadowaki *et al*, 1985; Standtmauer & Rosen 1986; Accili *et al*, 1986; Sadoul *et al*, 1986). Some of these putative substrates are cytosolic, cytoskeletal and some are plasma membrane associated.

Despite a high degree of structural characterization of the IR virtually nothing at all is known about the next step in the signal transduction process after activation of the PTK of the ß subunit.

However the metabolic actions of insulin upon target enzymes is much better characterised. Insulin controls metabolism of skeletal muscle, cardiac muscle, hepatocytes and adipocytes essentially by stimulating anabolic metabolic pathways and inhibiting catabolic pathways (Czech, 1977; Denton *et al*; 1981). This is achieved intum by controlling the phosphorylation state of the relevant pacemaker enzymes. This must be at least impart, achieved by controlling the levels of metabolically important second messengers such as cAMP. Insulin stimulates the activity of glycogen synthase in hepatocytes while α_1-agonists inactivate the enzyme (Thomas *et al*, 1985). Insulin antagonizes the action of glucagon in hepatocytes by reducing the concentration of cAMP largely by activation of cAMP-PDE activity (Houslay, 1986). However insulin antagonism of α_1-agonists

inhibition of glycogen synthase appears to occur at the level of elevation of intracellular Ca^{++} (Thomas *et al*, 1985).

Previous reports of the purification of low molecular weight chemical entities capable of mimicking insulin action (Saltiel & Cuatrecasas, 1986) have not been verified.

A number of reports have hinted at the potential G-protein involvement in the signal transduction process. Insulin has been reported to phosphorylate G_i and G_o (Krupinski *et al*, 1988). These experiments were performed in reconstituted phospholipid vesicles and its was also inferred that the presence of G_i and G_o increased the proportion of IR incorporated into the vesicles implying the possibility of some stabilizing interaction. Insulin has been reported to be able to inhibit the Pertussis toxin catalysed ADP-ribosylation of G-proteins (Rothenberg & Kahn, 1988). Insulin, as well as glucagon, has been reported to be able to attenuate the ability of cholera toxin to activate adenylate cyclase in hepatocytes (Irvine & Houslay, 1988).

1.11.2 Exon-intron organization of hIR gene

The hIR gene is located on the distal, short arm of chromosome 19 in the region of bands p13.3-p13.2 (Yang-Feng *et al*, 1985). The LDL-receptor gene is also in this region. The approximately 13,000 bp of the hIR is composed of 22 exons and 21 introns. The exons range in size from 36bp to >2500bp . All of the introns interrupt protein coding regions. The organization appears to reflect the organization of the protein, since many of the exons code for structural or functional domains of the receptor.

Exon 1 signal peptide

Exon 2 insulin binding

Exon 3 highly cysteine rich domain, may bind insulin.

Exon 6 located at interface between adjacent α-subunits in heterotetrameric $\alpha_2\beta_2$ form of hIR and contribute to co-operative site-site

interactions (Kadowaki *et al*, 1989).

Exon 11 the smallest only 36bp in size, alternate splicing of this exon results in synthesis of hIR proteins having α-subunits with different C-terminal sequences (Seino & Bell, 1989).

Exon 12 codes the tetrabasic amino acid sequence Arg-Lys-Arg-Arg the cleavage site to generate separate α and ß units from the 1355 amino acid precusor. It also encodes N-terminus of ß-subunit, 20% of amino acid in this region of ß are either serine or threonine, suggesting the N-terminus of ß represents a region of o-linked oligosaccharide modification.

Exon 15 codes for membrane spanning region of ß.

Exon16 encodes 22 amino acid segment that separates transmembrane and PTK domains.

Exon 17-21 encode for PTK domain. the tyrosine residues that are phosphorylated upon insulin binding are encoded by exon 20 (Tyr1158,1162,1163) and exon 22 (Tyr1328 and 1334).

Exon 22 encodes for highly charged C-terminus of ß.

These data suggest that exons encode functional/structural domains of hIR proteins and that the hIR gene like the LDL-receptor gene (Russel *et al*, 1989) is a mosaic constructed from exons recruited from other sources. The 1355 amino acid precusor is derived from a precusor containing 27 amino acid serving as the signal peptide there can be two precusors, 1370 or 1382 these could be expressed tissue specifically and be possibly developementally regulated by alternative splicing of exon 11, which encodes for a 12 amino acid segment at the C-terminal end of the α-subunit. Brain and spleen almost exclusively express the 719 amino acid α subunit, whereas other tissues including placenta, liver, kidney and adipose tissue express α-subunits of both 719 aa and 731 amino acid α subunit.

1.12.1 GENETIC DEFECTS OF INSULIN RECEPTOR IN HUMAN

INSULIN RESISTANT PATIENTS.

Recent studies have identified more than 8 different mutations of the insulin receptor, in one or both of the IR alleles of individuals with the syndromes of acanthosis nigricans, leprechaunism and Rabson-Mendenhall syndrome (Taylor 1985).

In the α subunit alone 4 different defects alone are known.

(i) A mutation in α, exon 3, Leu^{233} to Pro,in a person of Dutch orgin results in delayed transport to the cell surface.

(ii) A mutation in α , Phe^{382} to Val, exon 5, a person of Venezuelan orgin, resulting in delayed transport to the membrane surface.

(iii) A mutation in α, Lys^{460} to Glu and Gln^{672} to amber (TAG translation termination codon), exons 6 & 10, in an American, exhibits a truncated α subunit and qualitative abnormalities in insulin binding. This person is a compound heterozygote expressing different α-subunit mutations. There is a nonsense mutation in the codon for amino acid 672 of the paternally derived allele, which results in the synthesis of a truncated 671 amino acid fragment of α such a molecule would be secreted from the cell but biologically inactive. The maternally derived hIR allele has a missense mutation Lys^{460} to Glu that results in the expression of a protein with qualitative abnormalities in insulin binding, including increased stability of the insulin-IR complex. Such a mutation might impair the release of insulin in the acidic endosome after internalization of the insulin-IR complex and thereby affect IR recycling. Cells from the patients mother which express both normal Lys^{460} and mutant Gly^{460} IR, also exhibit qualitative abnormalities in insulin binding similar to the patient, however the mother was neither insulin resistant nor diabetic. By contrast the father who expresses both the normal IR and the truncated molecule, was moderately insulin resistant with impaired glucose tolerance although not overtly diabetic.

(iv) A mutation in a Arg^{735} to Ser, exon 12, in a Japanese subject, shows a defect in the processing of the receptor since the mutant IR is fully glycosylated but exists as the uncleaved proreceptor on the cell surface (Yoshima *et al*, 1988). this mutant IR can bind insulin and undergo insulin stimulated autophosphorylation but only at high concentrations of insulin and only partially as well. Thus the IR aquires normal insulin affinity only after proteolytic separation of α and ß subunits.

Three mutations in the ß subunit that represent post-binding defect in insulin action have been found. All are associated with decreased PTK activity or decreased autophosphorylation.

(i) A mutation in Gly^{1008} Val, exon 12, of a Japanese subject, exhibits decreased PTK activity. This Gly^{1008} is a conserved glycine that is part of ATP binding site of all protein kinases. The dominant negative character of these mutations may be due to the fact the IR is a heterotetramer and that heterotetramers formed from 2 mutant subunits will result in 50% mixed(ie mutant with non-mutant) As a consequence only 25% will be fully active.

Along with nucleotide substitutions that result in the expression of an abnormal protein, silent substitutions in the coding region of the hIR gene and mRNA but not the protein have been described (Ebina *et al*, 1985), (Kadawaki *et al*, 1988), (Seino *et al*, 1989). A nonsense mutation causing reduced levels of hIR mRNA has been described (Kadowaki et al, 1990). The patient has a inherited form of insulin resistance, leprechaunism (leprechaunism/Minn-1) which is associated with extreme insulin resistance and growth retardation. A nonsense mutation in the paternal allele of patients insulin receptor gene. An opal chain termination codon (TGA) is substituted for the codon (CGA) for Arg^{897} located in the intracellular domain of the ß-subunit. This nonsense mutation causes a reduction in the

level of the mRNA derived from paternal allele.

Several restriction length polymorphisms have been reported at the hIR locus, these all appear to be located within introns (Seino *et al*, 1990).

1.12.2 Animal model systems

The liver tissue of starved rats show increased activity of PKC and this has been linked to decreased autophosphorylation of the IR (Karasik *et al*, 1990). The protein tyrosine kinase activity of the IR is decreased in adipocytes from rats fed on a high fat diet (Watari *et al*, 1988), streptozotocin diabetic rats (Kadowaki *et al*, 1984) and a number of other insulin resistant systems (Reddy & Kahn 1988). Therefore defective protein tyrosine kinase activity of the IR ß-subunit appears to be a common defect in tissues exhibiting insulin resistance.

1.13. REGULATION OF ADENYLATE CYCLASE IN PATHOLOGICAL STATES OTHER THAN CLASSICAL INSULIN RESISTANCE STATES.

1.13.1 Hypothroidism

The adipocytes of hypothyroid animals show impaired stimulation of adenylate cyclase in response to catecholamines and this is correlated to a decreased lipolytic response to catecholamines (Saggerson, 1986). Thyroid hormones exert a permissive effect on the responsiveness of heart, skeletal muscle and adipose tissue to ß-AR agonists (Kunos, 1981). ß-AR action in heart (McNiel & Brody, 1968) and adipose tissue (Malbon *et al*, 1978) is potentiated by hyperthyroidism and severely blunted by hypothyroidism. The number of ß-AR receptors in rat heart (Willaims *et al*, 1977), in liver (Malbon, 1980), skeletal muscle (Sharma & Banerjee, 1978) and submaxillary gland (Pointon & Banerjee, 1978) has been reported to be influenced by thyroid hormone status. These change in receptor number may, in part, explain the altered responsiveness to catecholamines. However

adipocytes from hypothyroid rats show no change ß-AR number (Malbon *et al*, 1978), (Goswami & Rosenberg 1978) and the amount of C subunit was also normal. Hybridization of hypothyroid membranes with S49 cyc$^-$ membranes treated with NEM (devoid of G_s and functional C) restore ß-AR response to hypothyroid membranes (Malbon *et al,* 1985). This implies that the defective component is neither G_s, nor the membrane environment but, most probably ß-AR. Thus the defect could be that ß-AR is inactivated by such as some covalent modification such as phosphorylation or a change in ß-AR subtype such that the is an increase in the proportion of the weakly coupled subtype and a corresponding decrease in the proportion of the strongly coupled subtype which accounts for the alteration.

The anti-lipolytic effect of adenosine acting through A_1-adenosine receptors linked to G_i is increased in hypothyroid adipocytes by virtue of both sensitivity and responsiveness (Woodward & Saggerson, 1986), (Malbon, 1985). However hyperthyroidism has exactly the opposite effect (Rapiejko & Malbon, 1987).

Thyroid hormones also alter the response of adipocytes to insulin as well as catecholamines. Incubation of differentiated 3T3-L1 adipocytes in hypothyroid medium reduces isoproterenol stimulated cAMP accumulation, lipolysis and adenylate cyclase activity while increasing in particulate cGMP-inhibited PDE activity and that of soluble cAMP-PDE activity (Elks & Manganiello, 1985). In adipocytes from hypothyroid animals, catecholamine stimulated lipolysis can be restored to normal by incubating adipocytes with cGMP (Goswami & Rosenberg, 1985).

1.13.2 Hyperthyroidism

Hyperthyroidism is associated with a enhanced stimulation of lipolysis and cAMP accumulation by adrenaline in adipose tissue (Deykin & Vaughan, 1963), (Ichiwara *et al*, 1971), (Malbon *et al*, 1978). However an apparent paradox presents itself in that the stimulation of adenylate

cyclase by isoproterenol, NaF or GppNHp is actually reduced in hyperthyroid membranes. The number of ß-ARs is increased by 25%, the number of A_1-adenosine receptors is decreased by 65% but there is no change in the levels of any of the G-protein subunits (Rapiejko & Malbon, 1987). Isolated adipocytes from hyperthyroid animals do show increased accumulation of cAMP (Rapiejko & Malbon, 1987). If adenosine deaminase was included in the incubation medium for measurement of cAMP accumulation in the intact adipocyte then the accumulation of cAMP is lower in hyperthyroid than euthyroid cells (Rapiejko & Malbon, 1987). Thus the increased response of intact cells to ß-AR agonists could be due to the decreased number of A_1-adenosine receptors.

1.13.3 Adrenalectomy

Steroid hormones can modulate the abilities of several tissues to respond to hormones which regulate adenylate cyclase activity (Davies & Lefkowitz, 1984), including liver and adipose tissues (Fain, 1968; Exton *et al.*, 1972; Allen & Beck, 1972). Hence, one might anticipate that adrenalectomy will perturb the permissive regulation of adenylate cyclase activity by steroid hormones in their target tissues. This is observed in adipocytes where adrenalectomy of rats impairs the catecholamine-stimulated lipolytic response in adipocytes (Thotakura *et al*, 1982; Ros *et al.*, 1989b). This study indicated that glucocorticoids regulate cyclic AMP production at the level of expression of $G_s\alpha$ and also the expression of ß-subunits, since adrenalectomy causes a reduction in the cholera toxin-catalysed labelling of the two forms of $G_s\alpha$ and a decrease in the expression of β-subunit mRNA species present in adipocytes (Ros *et al.*, 1989b). A similar reduction in hormone-stimulated adenylate cyclase activity was also observed in reticulocytes and hepatocytes, although it is not known whether a similar mechanism is involved (Stiles *et al.*, 1981; Garcia-Sainz *et al.*,

1989).

Consistent with adrenalectomy causing a reduction in expression of $G_s\alpha$, dexamethasone therapy of adrenalectomized animals enhances levels of $G_s\alpha$ in adipocytes and restores the catecholamine-stimulated lipolytic response to some extent, although the slightly reduced forskolin-stimulated adenylate cyclase activity observed might indicate a lower intrinsic activity of the catalytic unit (Malbon *et al.*, 1985). The fact that dexamethasone treatment of growth hormone-secreting cells causes increases in both cholera toxin-catalysed labelling of $G_s\alpha$ and forskolin-stimulated adenylate cyclase activity shows that the effects of steroids on the permissive regulation of adenylate cyclase activity are probably tissue-specific (Chang & Bourne, 1987). Consistent with this idea is the finding that both $G_i\alpha$-2 and $G_s\alpha$ mRNA levels are regulated by glucocorticoids in brain, unlike adipocytes where expression of $G_i\alpha$-subunits appears to be unaffected (Saito *et al.*, 1989; Ros *et al.*, 1989b).

Glucocorticoids also induce a state of insulin resistance. Incubation of differentiated 3T3-L1 adipocytes with the glucocorticoid analog dexamethasone, results in an increase in both the number of $ß_2$-AR and the sensitivity of adenylate cyclase to catecholamines (Lai *et al*, 1982). Incubation of the 3T3-L1 adipocytes with dexamethasone for 48-72 hours had no effect on basal cGMP inhibited-cAMP-PDE activity, but reduced both the maximal increase inactivity produced by insulin and isoproterenol and the sensitivity to these agents (Elks *et al*, 1983; Elks *et al*, 1984). It is now known that both insulin and isoproterenol activate the cGMP-inhibited cAMP-PDE in adipocytes by phosphorylation of the enzyme (Anderson *et al*, 1989; Degerman *et al*, 1990). These phosphorylations may be mediated by protein kinase A in the case of isoproterenol and an insulin activated serine kinase in the case of insulin

1.13.4 Alcoholism

Cultured lymphocytes from alcoholic subjects have altered cAMP signal transduction resulting in reduced stimulation of R_S-G_S route (Nagy *et al*, 1988). Decreased cAMP levels have been suggested to be of pathological significance in chronic alcoholism (Diamond *et al*, 1987). More recently studies of human platelets has confirmed that stimulated cAMP levels are significantly reduced in platelets from alcoholic subjects (Tabakoff *et al*, 1988).

1.13.5 Albright hereditary osteodystrophy (AHO)

Albright hereditary osteodystrophy is a disorder inherited as an autosomal dominant trait (Van Dop *et al.*, 1984), and is characterized by symptoms which include round fac short stature, subcutaneous ossification and obesity (Levine *et al.*, 1988). Most AHO sufferers also exhibit pseudohypoparathyroidism type Ia (PHP-Ia), a state characterized by mental retardation, impaired olefaction and a resistance to parathyroid hormone and other ligands capable of stimulating adenylate cyclase activity in many tissues (Farfel & Friedman, 1986; Weinstock *et al.*, 1986; Levine *et al.*, 1983a). Presumably this is due to the drastically reduced functioning of $G_S\alpha$ detected in all tissues thus far examined from PHP-Ia sufferers (Downs *et al.*, 1983; Farfel *et al.*, 1982; Levine *et al.*, 1983b). AHO patients with normal hormone responsiveness are termed pseudo-pseudohypoparathyroidism sufferers (PPHP) (Albright *et al.*, 1952).

The autosomal dominant nature of the PHP-Ia characteristics suggested that the molecular lesion is at the level of one of the $G_S\alpha$ genes in the human genome. Fibroblasts and erythrocytes from all AHO patients examined, whether PHP-Ia or PPHP sufferers, have reduced levels of $G_S\alpha$ protein compared with normal subjects and all but three of these also had correspondingly reduced levels of $G_S\alpha$ mRNA (Levine *et al.*, 1988). The lack of any differences in the genomic DNA restriction maps between normal and

AHO patients rules out the possibility of large deletions, substitutions or re-arrangements within the $G_s\alpha$ gene being responsible for the observed reduced mRNA levels, but it does not exclude the possibility of small changes within the gene or its promoter region (Levine *et al.*, 1988). At present it is not known whether $G_s\alpha$ mRNA synthesis or processing is defective in these individuals.

The nature of the defect in the three PHP-Ia patients in the study by Levine *et al.*(1988) with normal $G_s\alpha$ mRNA levels but reduced $G_s\alpha$ protein is also unknown.

CHAPTER 2

MATERIALS AND METHODS

2 MATERIALS & METHODS

2.1 CHEMICALS

Sigma Chemical Co.,	BSA
Poole, Dorset, U.K.	Cholera Toxin
	Theophylline
	PIA
	Nicotinic Acid
	Prostaglandin E_1
	Isoproterenol
	ATP
	Soya Bean Trypsin Inhibitor
	Non-ionic detergent NP 40
	Ortho-dianisidine
	Lubrol-PX
Boehringer (U. K.),	Creatine Kinase
Lewes, East Sussex, U.K.	Creatine Phosphate
	Dithiothreitol
	Triethanolamine-HCl
	Tris
	GTP
	Gpp(NH)p
May & Baker Ltd.,	Hydrochloric Acid
Dagenham, Essex, U.K.	
Calbiochem,	Forskolin
Cambridge, U.K.	

National Diagnostics, Aylesbury, Bucks, U.K.	"Ecoscint" Scintillation Fluid
Porton Research Products Ltd. Maidenhead, Berks, U.K.	Pertussis Toxin
Whatman Biosystems Ltd., Maidstone, Kent.	DE-50 Cellulose
Worthington Biochemical Corporation, Freehold, New Jersey, U.S.A.	Collagenase

Glucagon was donated by Dr. W. W. Brommer, Eli Lilly & Co., IN, U.S.A.

All radiochemicals were from Amersam International plc, Amersham,Bucks, U.K.

All other reagents were obtained from B.D.H. Ltd., Poole, Dorset, U.K.

2.2 Animals

Male genetically obese (fa/fa) and lean (Fa/Fa) Zucker rats were obtained from Olac Ltd, Shaws Farm, Bicester, UK & killed by cervical dislocation at 20-25 weeks of age for the immediate preparation of white adipocytes.

Male C57BL/KsJ heterozygous (+/+) lean and homozygous (*db/db*) obese diabetic animals aged between 6 and 15 weeks were obtained from Olac Ltd. Oxon, U.K.

Diabetes was induced, in Sprague-Dawley rats (220-280g fed *ad libitum*), by a single interperitoneal injection of streptozotocin (75mg/kg

of body weight; dissolved in 0.1M-citrate buffer, pH4.0). Animals were used within 3-4 days if the urinary glucose level reached 167-280nM, as deduced by the use of Boehringer Diabur Test 5000 strips or if blood glucose reached 175-250mg of glucose/dl of blood, as deduced by the use of Ames Dextrostix.

Animals were allowed *ad libitum* access to standard laboratory chow and water.

2.3 PREPARATION OF ISOLATED ADIPOCYTES

Adipocytes were prepared essentially as described by Rodbell (1964). Animals were killed by cervical dislocation before the white epididymal fat pads were removed and placed into pre-warmed (37 degrees celsius) Krebs-Ringer-Henseleit buffer, pH 7.4, containing 3% (w/v) BSA (KRH/BSA). The KRH buffer had the following composition:- 25 mM HEPES, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 5 mM KCl, 120 mM NaCl, 2 mM $CaCl_2$ and 20 mM glucose.

After fine chopping with scissors, the pieces of fat tissue were rinsed with KRH/BSA and placed into pre-warmed 100 ml glass conical flasks (pre-treated with dichlorosilane to prevent cell adhesion) containing 10 ml KRH/BSA supplemented with 2 mg/ml collagenase and 0.5 mg/ml soya bean trypsin inhibitor and placed in a shaking 37°C waterbath set at 120 cycles per minute. Each flask was then sealed with a "Suba-seal" (Gallenkamp & Co., Widnes, Cheshire, U.K.) and gassed continuously for between 45 and 60 minutes in an atmosphere of O_2:CO_2 (95%/5%).

After digestion the contents of the flasks were filtered through a plastic teastrainer and washed into a pre-warmed glass beaker. The contents of the beaker were then poured into prewarmed 50 ml plastic centrifuge tubes and centrifuged at 600 r.p.m. for 5 seconds in an MSE Centaur 2 bench centrifuge to float the adipocytes. Adipocytes were removed from the top of the solution using a plastic Pasteur pipette and allowed to settle in a glass

tube where the rest of the KRH/BSA was removed using a syringe with the attached needle placed below the floating cells. The cells were then added to 50 ml centrifuge tubes with 5 volumes of KRH/BSA and the procedure repeated twice more. Concentrated adipocytes from the last step were used for the subsequent membrane preparation .

2.4 Preparation of a crude membrane pellet from isolated adipocytes.

The preparation of membranes from adipocytes was performed by two different methods. Method 1 was used to prepare membranes used in all experiments described in chapter 3, while method 2 was used for preparation of membranes used in all experiments described in chapters 4 and 5. The reason being that at the time, I believed that method 2 yielded membranes in which adenylate cyclase was more more responsive to stimulation by glucagon and other peptide hormones. This latter reasoning was later found to be untrue, however I continued to use method 2 for the purposes of continuity in my studies described in chapters 4 and 5.

2.4.1 Method 1

Cells were added to a pre-warmed (37 degrees celsius) plastic 50 ml measuring cylinder and pre-warmed buffered sucrose (0.25 M sucrose, 50 mM Tris-HCl, 3 mM ATP, pH 7.4) added such that the volume ratio of cells to buffer was 1:3. This mixture was transferred to a 50 ml glass Potter-Elvejhem homogenizer and hand-homogenized at room temperature by 6 up-and-down strokes of a teflon pestle. The homogenate was then poured into chilled (4 degrees celsius) 50 ml plastic centrifuge tubes and centrifuged at 1500 g_{max} for 15 minutes at 4 degrees celsius in the 8x50 rotor of a MSE 21 centrifuge. After removal of the fat cake from the top of the tube, the supernatant was re-centrifuged at 15,000g_{max} for 15 minutes in the same

centrifuge at the same temperature. The resulting pellet was resuspended in chilled buffered sucrose by 5 up-and-down strokes of a teflon pestle in a 25 ml glass Potter-Elvejhem homogenizer at 4 degrees celsius and re-centrifuged at 15,000g_{max} at 4 degrees celsius for 15 minutes. The membrane pellet was resuspended in 1 mM Tris-HCl, 1 mM EDTA, pH 7.4, to a concentration of between 0.5 and 1 mg/ml. 100 µl aliquots were rapidly frozen, by placing metal rack carrying eppendorf tudes into refrigerator, for storage at -80 degrees celsius.

2.4.2 Method 2

Cells were added to a pre-warmed (37 degrees celsius) 50 ml measuring cylinder and pre-warmed buffered sucrose (0.25 M sucrose, 50 mM Tris-HCl, 3 mM ATP, pH 7.4) added such that the volume ratio of cells to buffer was 1:3. This mixture was transferred to a 50 ml glass Potter-Elvejhem homogenizer and hand-homogenized at room temperature by 6 up-and-down strokes of a teflon pestle. The homogenate was then poured into chilled (4 degrees celsius) 50 ml plastic centrifuge tubes and centrifuged at 1500 g_{max} for 15 minutes at 4 degrees celsius in the 8x50 rotor of a MSE 21 centrifuge. After removal of the fat cake from the top of the tube, the supernatant was re-centrifuged at 15,000g_{max} for 15 minutes in the same centrifuge at the same temperature. The resulting pellet was resuspended in chilled buffered sucrose by 5 up-and-down strokes of a teflon pestle in a 25 ml glass Potter-Elvejhem homogenizer at 4 degrees celsius and re-centrifuged at 15,000 g_{max} at 4 degrees celsius for 15 minutes. The membrane pellet was resuspended in 1 mM Tris-HCl, 1 mM EDTA, pH 7.4, homogenized as before, resuspended in the same buffer and centrifuged at 48,300 g_{ave}, for 10min. The resulting membrane pellet was resuspended in same buffer, to a concentration of between 0.5 and 1 mg/ml, and 100 µl aliquots rapidly frozen for storage at -80 degrees celsius.

2.5 Preparation of cyclic AMP binding protein fron bovine heart.

The protocol used was done as described by Rubin *et al.* (1974).

2.5.1 Homogenization of bovine heart tissue

After removal of pericardium and fat tissue, the heart was chopped into small (1 inch) cubes and minced using a Moulinex electric blender. The minced heart was then mixed with 4 l of 40 mM potassium phosphate, pH 6.1, containing 2 mM β-mercaptoethanol and homogenized in a Waring blender for 1 minute in small batches. After centrifugation at 10,000g_{max} for 10 minutes at 4 degrees celsius, the supernatant was retained and filtered under vacuum through Whatman No 54 filter paper using a Buchner funnel and flask. The remaining pellet from the centrifugation step was extracted twice more with one litre of the same buffer and the filtered supernatants pooled for the next stage.

2.5.2 Ammonium sulphate precipitation

The pooled supernatants were made up to 55% saturation by the addition of solid ammonium sulphate to a concentration of 320 g/l, the pH being maintained between 7 and 8 units by the addition of ammonium hydroxide. Protein precipitation was allowed to occur for 3 hours and precipitated material was collected by centrifugation at 10,000g_{max} for 10 minutes at 4 degrees celsius. The pellet was then dissolved in 500 ml 50 mM Tris-HCl, pH 7.6, containing 10 mM NaCl and 4 mM ß-mercaptoethanol. This solution was dialysed overnight against 5 l of 50 mM Tris-HCl, pH 7.6 containing 10 mM NaCl, overnight to remove the remaining ammonium sulphate.

2.5.3 Pretreatment of DE-50 Cellulose

50 g of DE-50 Cellulose was added to 2 l of 0.5 M HCl and the mixture stirred for 30 minutes. The resin was then washed with distilled water until

the pH rose to 4 units. The resin was stirred for 30 minutes with 2l of 0.5 M sodium hydroxide before washing with distilled water as before until the eluate was at neutral pH. The acid/alkali washing procedure was repeated once more and the resin finally equilibrated with 50 mM Tris-HCl, pH 7.6, containing 10 mM ß-mercaptoethanol and 10 mM NaCl, such that the final slurry gave a (wet) settled volume:final volume ratio of 2:1.

2.5.4 Absorption of cyclic AMP binding protein by DE-50 cellulose

The dialysed preparation was stirred for 1 hour with 800 ml equilibrated DE-50 Cellulose under conditions where cyclic AMP-dependent protein kinase activity should be absorbed. After filtration of the resin under vacuum with Whatman No. 54 filter paper on a Buchner funnel and flask, the resin was washed with 3 l of 50 mM Tris-HCl, pH 7.6, containing 10 mM NaCl and 4 mM β-mercaptoethanol, for 45 minutes. The resin was collected by filtration and washed with the 50 mM Tris-HCl buffer, pH 7.6, containing 10 mM NaCl and 4 mM β-mercaptoethanol, using a Buchner flask and funnel.

2.5.5 Isolation of the cyclic AMP binding protein

The combined filtrates were brought to 35% saturation with 119 g/l solid ammonium sulphate and the pH maintained between 7 and 8 units with ammonium hydroxide solution. After stirring for 1 hour, the precipitate was collected by centrifugation at 10,000g_{max} for 10 minutes at 4 degrees celsius and the resulting pellet discarded. The supernatant was brought to 75% saturation by adding 258 g/l solid ammonium sulphate and after stirring for 1 hour and the precipitate was collected by centrifugation at 10,000g_{max} as before. The pellet from this step was resuspended in a minimal volume of 50 mM potassium phosphate buffer containing 4 mM ß-mercaptoethanol, pH 7.0. This final suspension was dialysed against the same buffer overnight at 4 degrees. The purified protein kinase was stored in 250

µl aliquots at -80 degrees celsius and under these conditions was stable for between 6 and 9 months. Aliquots were never refrozen but could be used for up to 48 hours after thawing if stored between 0 and 4 degrees celsius.

2.6 Assay of adenylate cyclase activity.

This was done using either one of 2 methods:-

2.6.1. Determination of cyclic AMP formation using a binding protein assay for cyclic AMP.

This method was based on that described by Heyworth & Houslay (1983). The first part of the procedure involves the adenylate cyclase assay with subsequent analysis of cAMP formed using the binding protein. Incubations were carried out in a final volume of 100 µl containing 30 mM Tris-HCl and 0.75 mM EGTA, pH 7.4, with 10 mM theophylline (a non-selective cyclic AMP phosphodiesterase inhibitor), 22 mM creatine phosphate, 1.0 mM ATP, 2 mM $MgSO_4$ and 1 mg/ml creatine kinase. In experiments using inhibitory ligands, 1 U/ml adenosine deaminase was included and theophylline was omitted from the reaction medium and replaced with 100 µM Ro-20-1724, a non-methylxanthine cyclic AMP phosphodiesterase inhibitor. These alterations enhance inhibition by reducing the effects of adenosine accumulation in the reaction medium. Other components of the reaction mixture were added at the final concentrations specified in figure legends and tables.

Reactions were initiated by the addition of 2-10 µg of adipocyte membrane protein, followed by incubation or 30 minutes at 30 degrees celsius over which time periods cyclic AMP production was linear. Reactions were terminated by incubation in a boiling waterbath for 3 minutes. Samples were then centrifuged for 5 minutes at $14{,}000g_{ave}$ in a Hettich Micro Rapide centrifuge at 4 degrees celsius to sediment precipitated denatured protein. Aliquots were subsequently taken for assay of cyclic AMP using a

competitive binding method.

Determination of amounts of cyclic AMP in the samples assayed for adenylate cyclase activity was based on the saturation binding assay of Brown *et al.* (1972) as modified by Tovey *et al.* (1974) and described by Whetton *et al.* (1983). This assay depends upon the specific binding of cyclic AMP to the cyclic AMP binding protein purified from bovine heart as described above. Aliquots from experimental samples were incubated with [^{3}H] cyclic AMP and a suitable concentration of binding protein until equilibrium had been reached. The labelled and unlabelled [^{3}H] cyclic AMP compete for a finite number of binding sites, thus under suitable conditions increased concentrations of unlabelled cyclic AMP will lead to reduced binding of the labelled nucleotide. The bound cyclic AMP is then separated from unbound cyclic AMP by the addition of a charcoal/BSA slurry, in order to absorb free nucleotides. The bound cAMP is then separated by centrifugation, allowing estimation of the [^{3}H] cyclic AMP bound to the protein.

Total cyclic AMP binding to the protein was estimated by incubation of [^{3}H] cyclic AMP in the absence of unlabelled cyclic AMP, whilst non-specific binding was estimated by incubating [^{3}H] cyclic AMP in the absence of both unlabelled cyclic AMP and binding protein. By using unlabelled cyclic AMP over a certain range of known concentrations, one can sequentially reduce the proportion of [^{3}H] cyclic AMP bound to the protein. This allows a standard displacement curve to be constructed for the estimation of cyclic AMP content of unknown samples.

The cAMP assays were done by taking aliquots from the sample tubes were made up to a volume of 100 µl with binding buffer (50 mM Tris-HCl, 4 mM EDTA, pH 7.4) before 100 µl [^{3}H] cyclic AMP (stock solution of 5, 8 ^{3}H adenosine 3', 5'-monophosphate in 50% ethanol diluted in binding buffer to give 40-50,000 c.p.m. per 100 µl) and 100 µl of a suitable dilution of cyclic AMP binding protein in binding buffer were added. Also included in this

procedure were duplicate tubes containing known amounts of cyclic AMP (0.0625, 0.125, 0.25, 0.5, 1, 2, 4, 8 and 16 pmol per tube) with [^{3}H] cyclic AMP and binding protein added to the same concentrations as the sample tubes. After incubation for at least 2 hours at 4 degrees celsius, by which time binding of cyclic AMP to the binding protein had reached equilibrium, bound and free nucleotides were separated by the addition of 250 µl of a continuously stirred, chilled 2% (w/v) Norit GSX charcoal/1% (w/v) BSA slurry in assay buffer. This was vortexed and subsequently centrifugation at 14,000g_{av} at 4 degrees celsius for 4 minutes. 300 µl of the supernatant was extracted and added to 4 ml Ecoscint before being subject to scintillation counting. The inclusion of tubes with known amounts of cyclic AMP allowed for a standard curve of c.p.m. versus pmol cyclic AMP to be derived, from which amounts of cyclic AMP formed in the sample tubes could be deduced. The counter which I used had a computer attachment which allowed a curve of best-fit to be drawn for the standards used in each experiment, and printed out pmol cyclic AMP present in each sample.

2.6.2 Determination of cyclic AMP formation by purification using sequential column chromatography with dowex and alumina

Reaction conditions employed were identical to those above , except that [α-^{32}P] ATP (2x10^6 c.p.m. per tube) was also present. Reactions were terminated by the addition of 100 µl of a "stopping solution" consisting of 1.4 mM cyclic AMP, 40 mM ATP and 0.2% (w/v) SDS, pH 7.5. Following the addition of 100 µl [^{3}H] cyclic AMP (10x10^3 c.p.m. per tube) and 750 µl distilled water, [^{32}P] cyclic AMP was partially purified by the double column method of Salomon *et al.* (1974). This procedure involves separating cyclic AMP from other nucleotides by sequential column chromatography with Dowex and alumina.

Dowex-H$^+$ 50 x 4-400 was mixed with distilled water to form a

slurry (1:1 ratio, v/v). 2 ml of this was then added to each column and the water left to drain out: the columns were then ready for use. After use, the Dowex was washed with 2 ml 1 M hydrochloric acid until just before the next set of samples were applied, when the columns were washed with 10 ml distilled water.

The alumina columns were prepared with 0.6g of neutral alumina per column. These were washed with 8 ml 0.1 M imidazole, pH 7.4, before use and with 2 ml 0.1 M imidazole, pH 7.4, after each experiment. Before the next set of samples were loaded, the columns were washed with another 8 ml 0.1 M imidazole, pH 7.4.

Reaction tubes with added distilled water and [^{3}H] cyclic AMP were applied gently down the side of the Dowex columns using a Pasteur pipette. Once the columns had run dry, 1 ml distilled water was added slowly and the columns run dry once more. Another 1 ml distilled water was then added and the columns allowed to run dry again before being placed over the alumina columns. 3 ml distilled water were added to elute the Dowex columns and the system run until the alumina columns had run dry. Then the alumina columns were placed over scintillation vials and eluted with 4 ml 0.1 M imidazole, pH 7.4.

The final eluate from the alumina columns was collected and added to 12 ml Ecoscint before scintillation counting. Recovery of cyclic AMP in these experiments was routinely between 70 and 90%. The amount of cyclic AMP per sample was calculated by a computor program which converted the d.p.m. per sample into pmol cyclic AMP by using the specific activity of the [α-^{32}P] ATP used and by taking into account the recovery of cyclic AMP from the column steps.

Unless otherwise stated, formation of cyclic AMP was linear over the incubation periods and initial rates were taken for subsequent analysis.

2.7 Sodium dodecyl sulphate polyacrylamide electrophoresis (SDS-PAGE)

For these studies, a discontinuous SDS-PAGE system was used as originally described by Laemmli (1970), and subsequently modified by Milligan & Klee (1985). This involved concentrating samples in a 0.3% (w/v) acrylamide/0.08% (w/v) N, N'-methylenebisacrylamide stacking gel follwed by resolution in a 10% (w/v) acrylamide/0.25% (w/v) N, N'-methylenebisacrylamide 12 cm x 14 cm resolving gel using a LKB Bromma electrophoresis system.

2.7.1 Stock Solutions (all stored at 4 degrees celsius)

a. Buffer 1:- 1.5 M Tris, 0.4% (w/v) SDS adjusted to pH 8.8 with HCl.

b. Buffer 2:- 0.5 M Tris, 0.4% (w/v) SDS adjusted to pH 6.8 with HCl.

c. Acrylamide solution:-30% (w/v) acrylamide , 0.8% (w/v) N , N'-methylenebisacrylamide.

d. 50% (v/v) glycerol solution.

e. N, N, N', N'-Tetramethylenediamine (TEMED).

2.7.2 Gel Preparation

After assembly of the gel plates, a resolving gel solution was made consisting of 8.2 ml distilled water, 6 ml buffer 1, 8 ml acrylamide solution, 1.6 ml glycerol solution, 20 µl TEMED and 90 µl 10% (w/v) ammonium persulphate (prepared fresh). Once poured between the plates, 1 ml distilled water was added to cover the top of the solution thereby excluding air and enhancing polymerization. When set, the water was poured off and any unpolymerized acrylamide washed away with distilled water. A stacking gel mixture was then poured onto the resolving gel: this consisted of 9.75 ml distilled water, 3.75 ml buffer 2, 1.5 ml acrylamide solution, 20 µl TEMED and 150 µl 10% (w/v) ammonium persulphate. A gel comb was then inserted to facilitate loading of samples. Once set, the remainder of the kit was

assembled and the samples loaded using a Hamilton Microsyringe. The anode and cathode were immersed in running buffer (25 mM Tris , 200 mM glycine and 0.1% (w/v) SDS) and samples run overnight at 12 mA, 60 V.

2.7.3 Sample preparation

The required amount of membrane protein (10-300 µg) was sedimented by centrifugation at 14,000g_{av} for 10 minutes at 4°C. After removal of the supernatant the membrane pellet was resuspended in 40 µl electrophoresis sample buffer (50mM Tris, 4.5 M urea, 5% (w/v) SDS, 40 mM DTT and a grain of bromophenol blue dye). After solubilisation of samples by vortexing and incubation in a boiling water bath for 3 minutes, they were ready for electrophoresis.

2.7.4 electrophoretic separation of α_i1 and α_i2

Separation of the alpha-subunits of G_i-1 and G_i-2 was achieved by alkylating membrane samples before resolution of membrane proteins on a 12% (w/v) acrylamide/0.06% (w/v) N, N'-methylenebisacrylamide resolving gel, as described by Mitchell *et al.* (1989).

Before alkylation, the required amount of membrane protein (10-300 µg) was sedimented by centrifugation at 14,000g_{av} at 4 degrees celsius for 10 minutes. The supernatant was discarded and the pellet resuspended in 20 µl 10 mM Tris-HCl, 1 mM EDTA, pH 7.5. After the addition of 10 µl 5% (w/v) SDS in 50 mM DTT, the samples were boiled for 5-10 minutes before cooling on ice for 30 seconds (before the SDS precipitated out). 10 µl 100 mM N-ethylmaleimide (NEM) were added and the samples left at room temperature for at least 15 minutes to allow the alkylation reaction to occur. To stop the reaction, 20 µl electrophoresis sample buffer were added and the samples left overnight at 4 degrees celsius.

The method for electrophoretically separating the alkylated samples is very similar to the protocol described in section 2.8.2 using the solutions

described in section 2.8.1 apart from the following differences:-

a. A Bio-Rad Protean electrophoresis system was used to allow the use of a 20 cm x20 cm resolving gel.

b. For the resolving gel, a different stock acrylamide solution was used, consisting of 30% (w/v) acrylamide, 0.15% (w/v) N, N'-methylenebisacrylamide.

c. The resolving gel mixture consisted of the following:- 11.6 ml distilled water, 12 ml buffer 1, 20 ml acrylamide solution (described above), 4 m l

glycerol solution, 160 µl 10% (w/v) ammonium persulphate and 40 µl TEMED.

d. The stacking gel mixture consisted of the following:- 14.6 ml distilled water, 5.6 ml buffer 2, 2.3 ml "normal" acrylamide solution, 225 µl ammonium persulphate and 12 µl TEMED.

e. Once loaded, samples were run at 45 mA, 100 V overnight in the running buffer previously described.

2.7.5 Molecular weight markers

These were obtained from Gibco-BRL Research Products, Uxbridge, Middlesex, U.K. The biotinylated pre-stained proteins were myosin H-chain (200 KDa), phosphorylase *b* (97.4 KDa, BSA (68 KD), ovalbumin (43 KD), carbonic anhydrase (29 KD), ß-lactoglobulin (18.4 KD), and lysozyme (14.3 KD).

2.7.6 Staining of gels for protein

After electrophoresis, gels were stained for 1 hour in 200 ml of 0.1% (w/v) Coomassie Brilliant Blue R in 40% (v/v) methanol and 10% (v/v) acetic acid. Destaining was carried out overnight in the same solution minus the Coomassie Brilliant Blue R.

2.8 Immunoblotting of SDS-PAGE gels.

2.8.1 Transfer of proteins to nitrocellulose and detection

This was achieved using a modification of the method of Mitchell *et al.* (1989) which is derived from the original method of Towbin *et al.* (1979).

After separation of proteins by SDS-PAGE the stacking gel was removed and the resolving gel allowed to soak in transfer buffer (25 mM Tris, 200 mM glycine in 20 (v/v) methanol) for a few minutes. After soaking, a blotting sandwich was constructed which allowed the gel to press tightly against a piece of nitrocellulose paper (Schleicher & Schull, 0.45 μm). These were kept firmly in place by pressing a piece of chromatography paper (Whatman) and foam rubber either side of this sandwich and keeping them together with plastic holders. This sandwich was placed into a LKB Transblot tank and completely immersed in transfer buffer. A LKB Transphor powerpack was connected such that the gel side of the sandwich was connected to the negative electrode and the proteins transferred to the nitrocellulose paper for 2 hours at maximum voltage, producing a direct current of 1 mA.

After transfer, the blotting sandwich was removed, dismantled and the nitrocellulose paper incubated for 3 hours at 30 °C in a solution of 5% (w/v) gelatin in phosphate-buffered saline (PBS; 136 mM NaCl, 2.7 mM KCl, 80.5 mM Na_2HPO_4 & KH_2PO_4) to block non-specific sites on the nitrocellulose which the subsequently-used antibodies might bind to. Following blocking, the nitrocellulose paper was washed free of excess gelatin with PBS before the primary antibody was added as a 1 in 200 dilution in PBS containing 1% (w/v) gelatin, 0.2% (v/v) non-ionic detergent NP-40 and 0.05% (w/v) thimerasal. The nitrocellulose paper was incubated overnight at 30degrees celsius in this solution, by which time the binding of antibody had reached equilibrium. The antibody solution was then poured off and the nitrocellulose washed with PBS. The paper was then washed twice for 10 minutes with shaking in PBS containing 0.2% (w/v) NP-40 before the second antibody (horse radish peroxidase-conjugated goat anti-rabbit Ig G)

was added as a 1 in 200 dilution in PBS containing 1% (w/v) gelatin, 0.2% (v/v) non-ionic detergent NP-40 and 0.05% (w/v) thimerasal. After incubation for 3 hours at 30 degrees celsius, the antibody solution was poured off and the washing procedures outlined above were repeated. These were followed by a final wash for 10 minutes in PBS before this solution was removed and replaced by 40 ml 10 mM Tris-HCl, pH 7.5, and 1 ml of a 10% (w/v) orthodianisidine dihydrochloride solution dissolved in water. To develop the blot, 10 µl 30% (w/v) hydrogen peroxide solution were added and the mixture swirled over the nitrocellulose paper. Development was arrested by pouring off the developing solution and covering the blot with 50 ml 1% (w/v) sodium azide.

When stored at 4 degrees celsius before and after use, both the primary and secondary antisera could be stored as 1 in 200 dilutions in gelatin/PBS/NP-40 and used for up to five different experiments.

2.8.2 Description of antisera

The polyclonal antisera used for these studies were donated by Dr. Graeme Milligan, Institute of Biochemistry, University of Glasgow, and have been described in several publications (Mitchell *et al.*, 1989; M^{C}Kenzie & Milligan, 1990; Milligan & Unson, 1989). Briefly, antisera were raised in New Zealand White rabbits against glutaraldehyde conjugates of keyhole-limpet haemocyanin and synthetic peptides which represent sections of the deduced amino acid sequences of various cloned G-protein subunits (Table 2.1).

Antiserum SG1 was raised against the C-terminal decapeptide sequence of $G_t\alpha$-1. This antiserum recognizes both $G_i\alpha$-1 and $G_i\alpha$-2 as well as $G_t\alpha$-1 and $G_t\alpha$-2, but as the expression of the latter two species is restricted to retinal tissue, SG1 can be used as a specific probe for $G_i\alpha$-1 and $G_i\alpha$-2 in other tissues. This antiserum does not recognize $G_i\alpha$-3 (Mitchell

et al., 1989). Antiserum I3B was raised against a decapeptide which corresponds to the C-terminal sequence of $G_i\alpha$-3. It does not cross-react with $G_i\alpha$-1 or $G_i\alpha$-2 but does exhibit weak cross-reactivity with $G_o\alpha$ (Mitchell *et al.*, 1989). However, the expression of $G_o\alpha$ in both liver (Lynch *et al.*, 1989; Huff *et al.*, 1985) and adipocytes (Mitchell *et al.*, 1989) is below current levels of detection. Antiserum CS1 was generated against a decapeptide corresponding to the C-terminal sequence of $G_s\alpha$ which is present in all four of the $G_s\alpha$ cDNA sequences (Bray *et al.*, 1987). Antiserum BN1 was raised against the N-terminal sequence of the β_1-subunit, which differs by one amino acid substitution from β_2 but by three residues from β_3 (Levine *et al.*, 1990). Hence BN1 could be used to probe for both β_1 and β_2 under suitable conditions (Evans *et al.*, 1986), although we were unable to resolve these proteins under any of the electrophoretic conditions described above section.

2.9 Estimation of protein concentration.

This was carried out according to the method of Peterson (1977) which is a modification of the method of Lowry *et al.* (1951). The stock solutions were:

a 2% (w/v) sodium carbonate in 0.1M sodium hydroxide

b 1% (w/v) copper sulphate

c 2% (w/v) sodium potassium tartrate

Just before use the reagents are mixed in the following v/v ratio: a/b/c, 100/1/1. Protein standards were preparwed using bovine serum albumin. 1ml of the abc solution was added to each sample, mixed and left to stand for 10min. 100µl of Folin-Ciocalteau reagent, diluted 1:4 with water was added to each tube. The samples were mixed and left for 30 min. The absorbance of the samples was determined spectrophotometrically at 750nM in an LKB Ultrospec II spectrophotometer.

CHAPTER 3

THE REGULATION OF ADENYLATE CYCLASE IN THE ADIPOSE TISSUE FROM STREPTOZOTOCIN DIABETIC RATS

Fig 3.1 Dose-effect experiments for GppNHp on forskolin stimulated adenylate cyclase activity in adipocyte membranes.

Adenylate cyclase activity was monitored in the presence of forskolin (10^{-4}M). Increasing concentrations of GppNHp were added to assays employing adipocyte membranes from control (open square) and diabetic (closed square.) animals. Errors are S.D for six experiments (diabetic) and five experiments (control) using different membrane preparations each time. The reference activity of 100% is that activity obtained with forskolin (100μM) alone. In membranes from control rats the control activity was 335±40 pmol/min/mg, whilst in membranes from diabetic rats it was 139±22 pmol/min/mg.

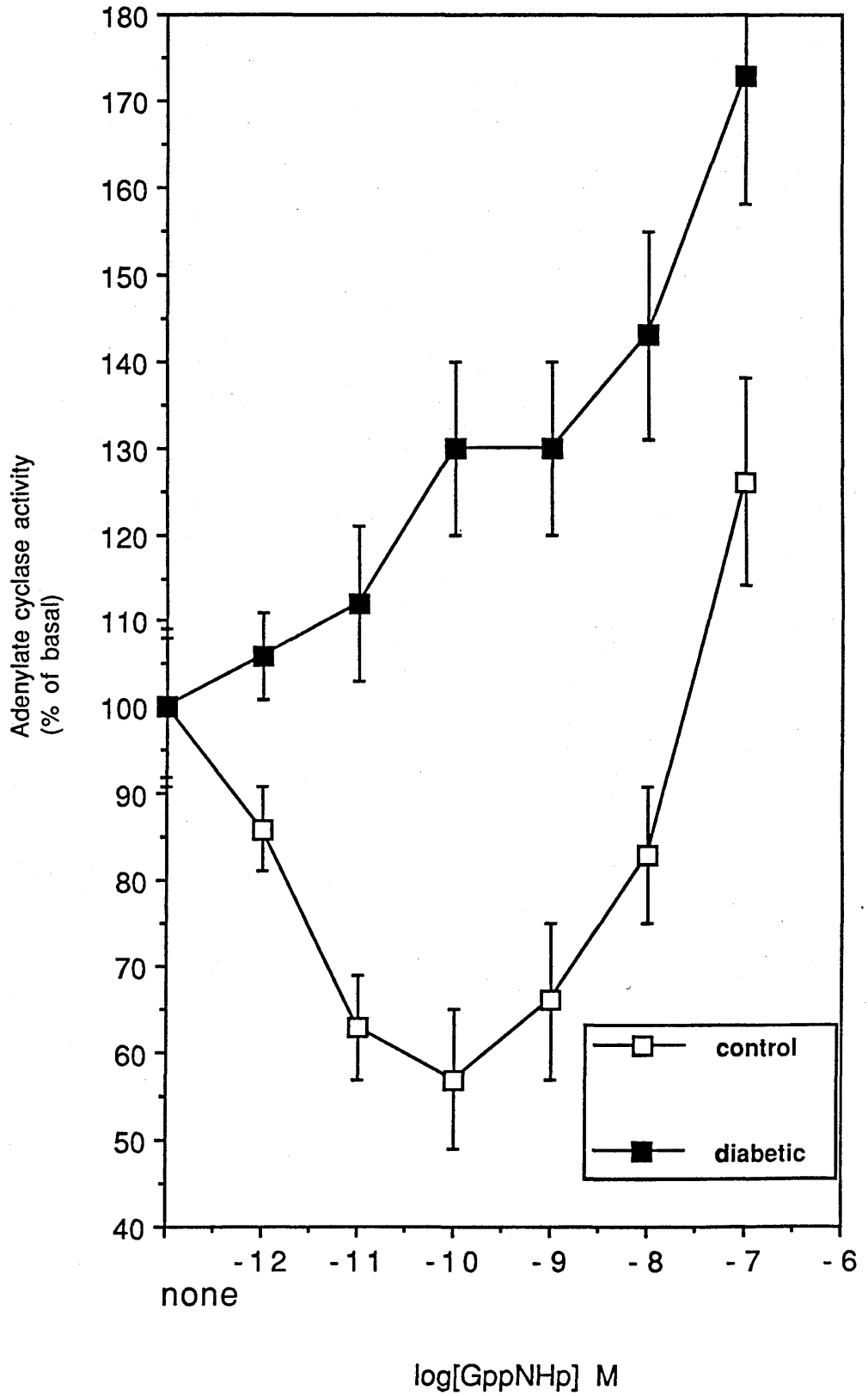

Adenylate cyclase activity
(% of basal)
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
control
diabetic
none
-12
-11
-10
-9
-8
-7
-6
log[GppNHp] M

Fig 3.2 Dose response experiment for GTP on hormone stimulated adenylate cyclase activity in adipocyte membranes from control animals.

Adenylate cyclase activity was monitored in the presence of isoproterenol ($5x10^{-4}M$). Errors are standard deviations for four experiments using different membrane preparations. The control, 100%, activity represents that activity with 500μM isoproterenol alone which was 157±9 pmol/min/mg. The V_{max} activity, ie that activity in the presence of 500μM GTP and 500μM isoproterenol, was 220±28 pmol/min/mg giving a 1.40-fold stimulation of activity by GTP.

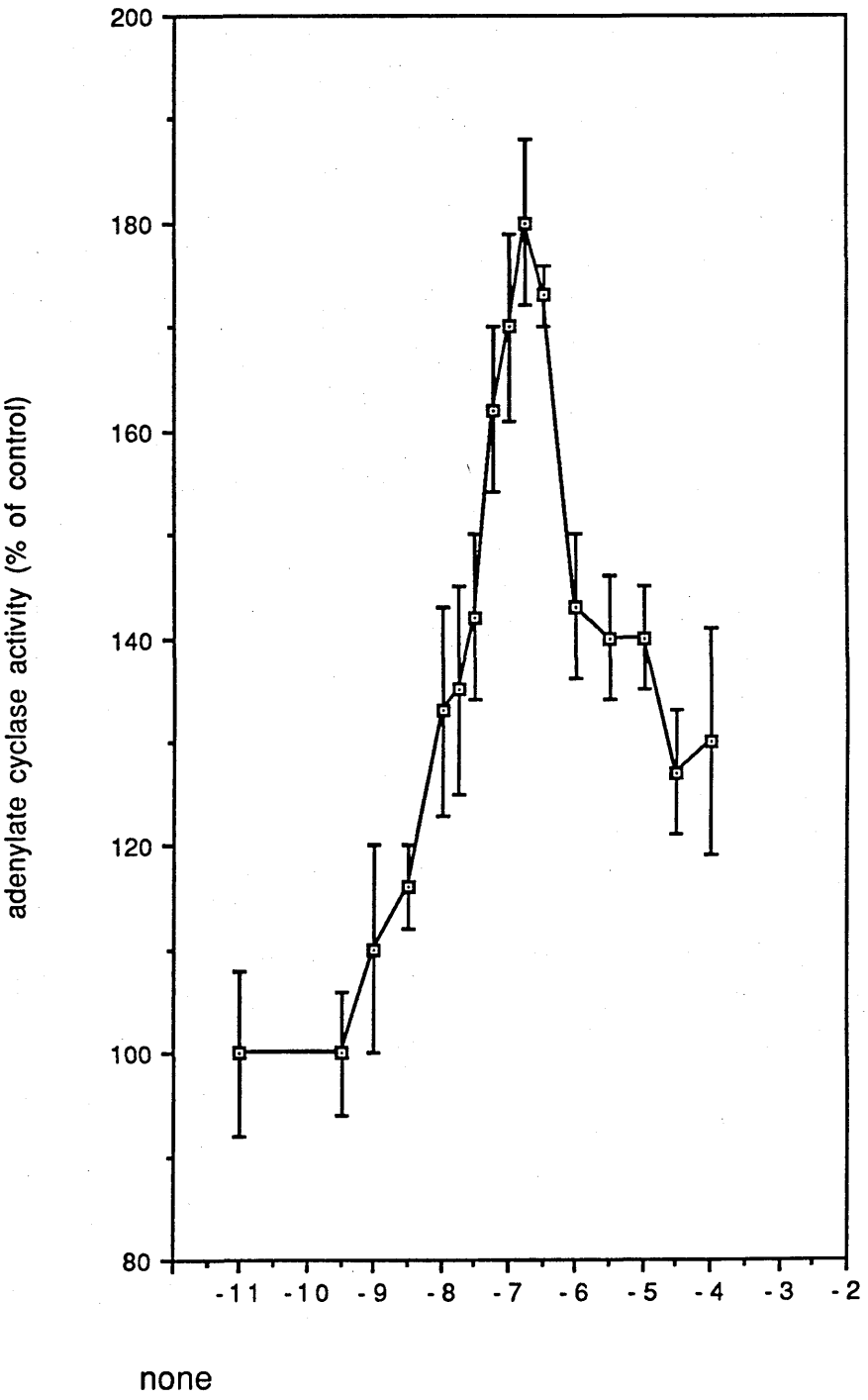

200
180
160
140
120
100
80
adenylate cyclase activity (% of control)
-11 -10 -9 -8 -7 -6 -5 -4 -3 -2
none
log[GTP] M

Fig 3.3 Dose-effect experiment for GTP on isoproterenol stimulated adenylate cyclase activity in adipocyte membranes from diabetic animals.

Adenylate cyclase activity was monitored in the presence of isoproterenol ($5x10^{-4}M$). Errors are standard deviations for six experiments using different membrane preparations. The reference activity of 100% refers to that activity in the presence of isoproterenol (500μM) alone, which was 56±8 pmol/min/mg. The V_{max} activity, ie that activity in the presence 500μM GTP and 500μM isoproterenol, was 250±49 pmol/min/mg giving a 4.5-fold activation of activity by GTP.

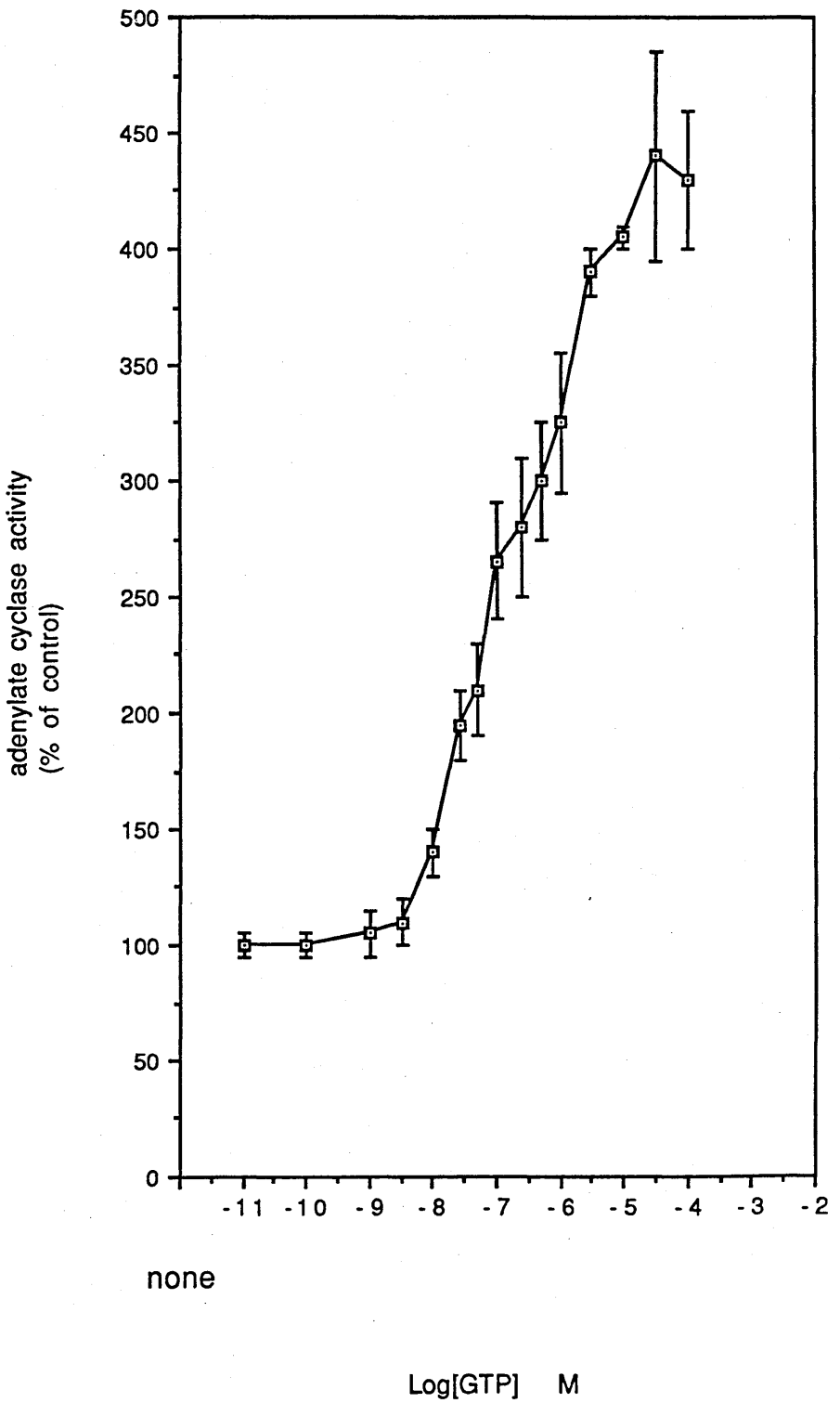

adenylate cyclase activity
(% of control)
500
450
400
350
300
250
200
150
100
50
0
-11 -10 -9 -8 -7 -6 -5 -4 -3 -2
none
Log[GTP] M

Fig 3.4 Dose response curve for inhibition of isoproterenol stimulated adenylate cyclase activity by GTP in presence of PIA for membranes from diabetic animals.

The experiment was carried out in the presence of 1μM PIA and 500μM isoproterenol, using membranes from diabetic animals. Experiment is typical of three similar experiments using different membrane preparations. The control activity of 100% represents that activity with 500μM isoproterenol and 500μM GTP which was 245±21 pmol/min/mg. The data shows means and standard deviations of a typical experiment.

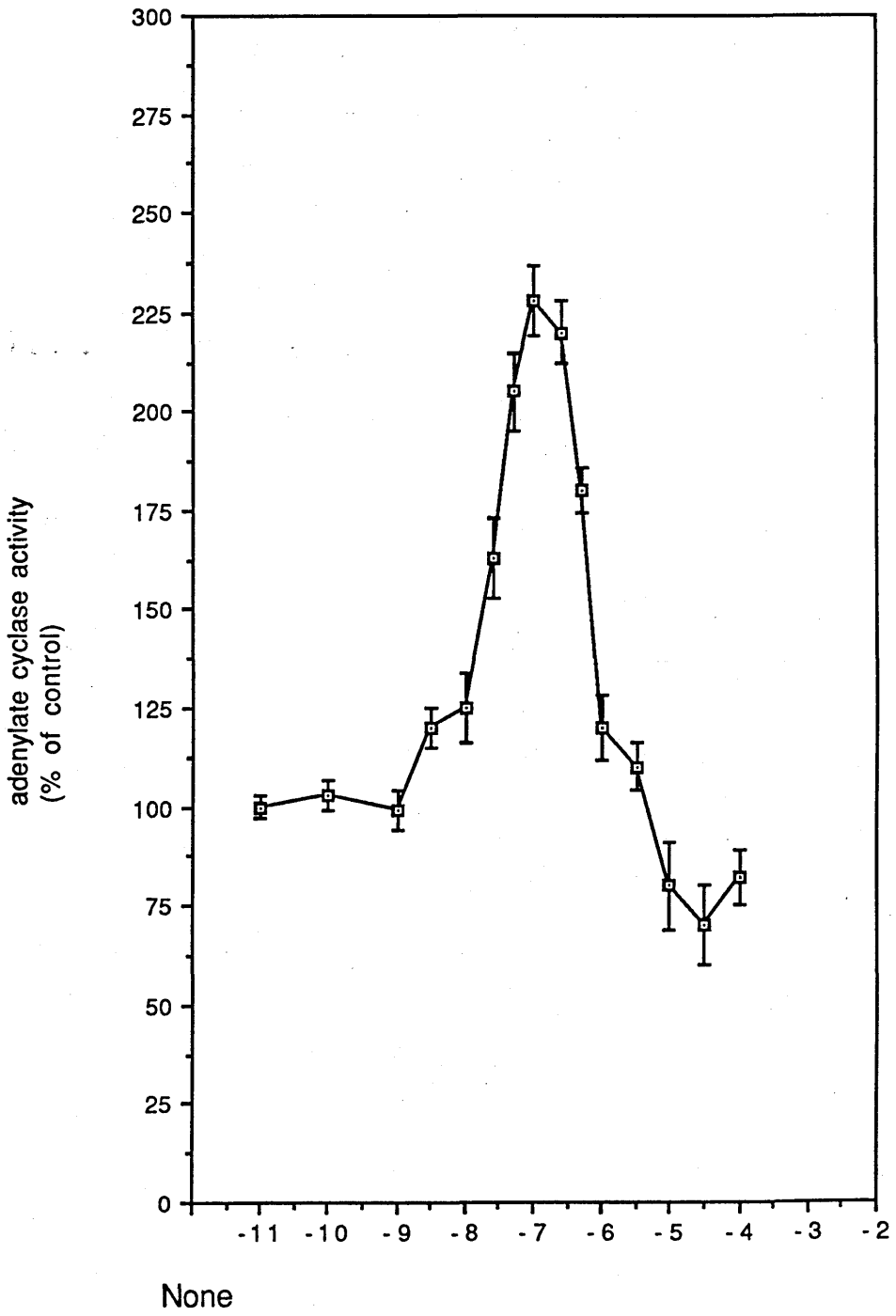

300
275
250
225
200
175
150
125
100
75
50
25
0
adenylate cyclase activity
(% of control)
-11 -10 -9 -8 -7 -6 -5 -4 -3 -2
None
log[GTP] M

Fig 3.5 PIA mediated effects on adenylate cyclase activity

Dose response curves for PIA were carried out in the presence of isoproterenol ($5x10^{-4}$ M) and GTP (10^{-5} M) using adipocyte membranes from control (open square) and diabetic (closed diamond) animals. This experiment is typical of three similar ones using different membrane preparations. The specific activity of adenylate cyclase in presence of 500μM GTP and 500μM isoproterenol only was 238±19 pmol/min/mg for membranes from control rats and 255±32 pmol/min/mg for membranes from diabetic rats. The data are means and standard deviations for a typical experiment.

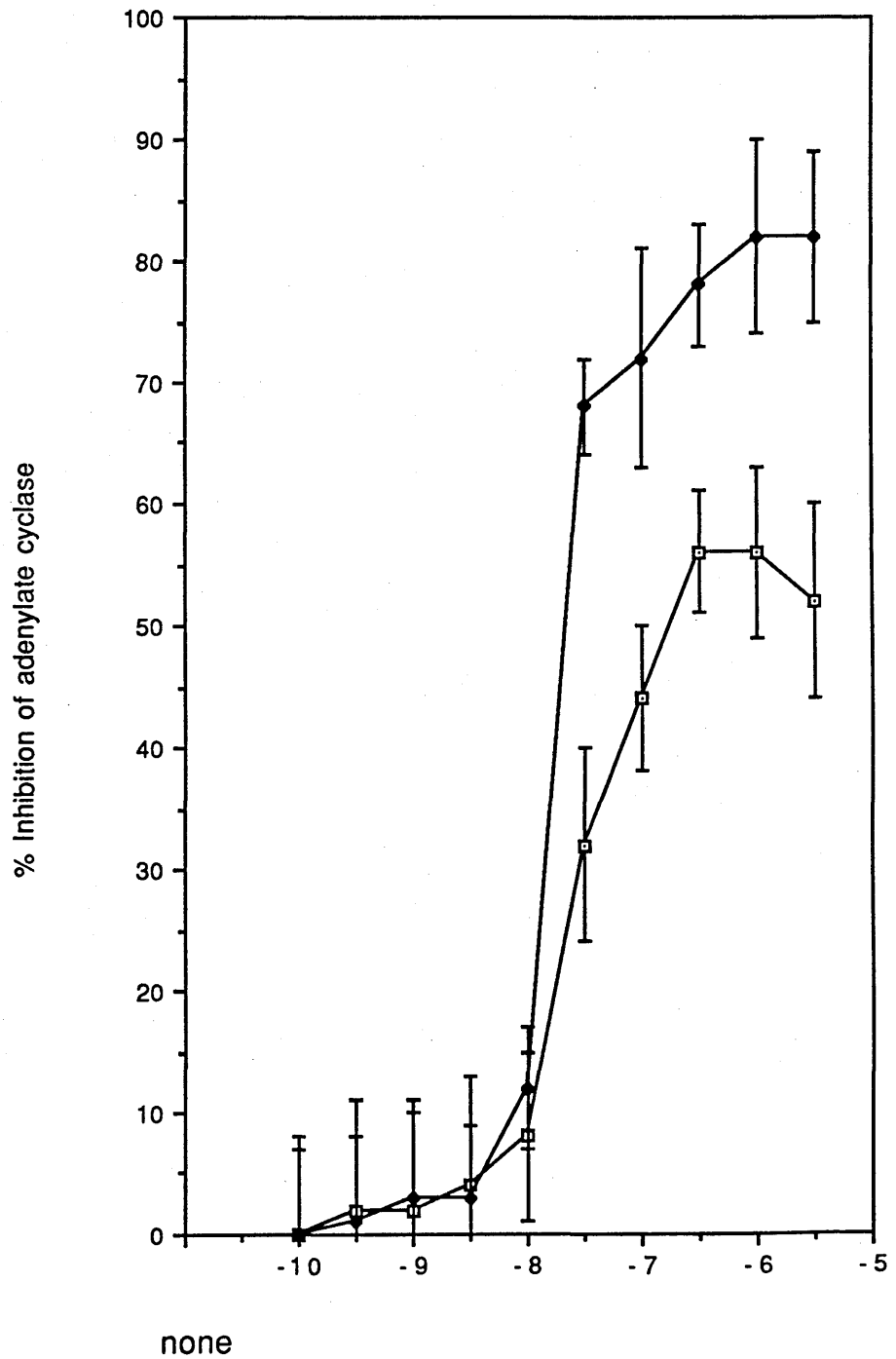

100
90
80
70
60
50
40
30
20
10
0
% Inhibition of adenylate cyclase
-10
-9
-8
-7
-6
-5
none
log[PIA] M

Fig 3.6. Identification of α_i1 and α_i2 in adipocyte membranes.

Western blotting data are shown which were obtained using the antiserum AS7 which in adipocyte membranes specifically recognizes α_i1 and α_i2 which can be resolved by virtue of the differences in molecular weight. Lane 1 contains 70μg of membranes from control animals. Lane 2 contains 70μg of membranes from diabetic animals. This result is representative of eight separate experiments using different membrane preparations from control and diabetic animals.

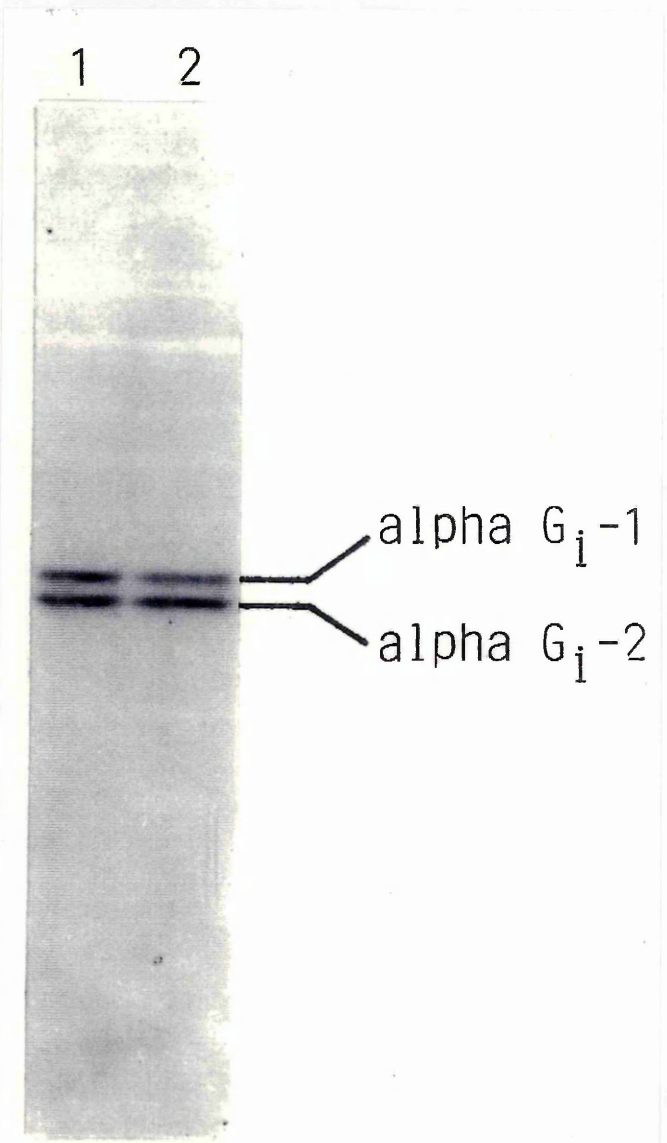


Fig 3.7 Detection of α_i3 in membranes from control and diabetic animals.

The G-protein subunit α_i3 was identified by immunoblotting with antiserum I3B which specifically recognizes α_i3. Lane 1 was loaded with 70μg of protein from membranes prepared from control animals. Lane 2 was loaded with 70μg of protein from membranes prepared from diabetic animals. This result is representative of eight separate experiments using different membrane preparations.

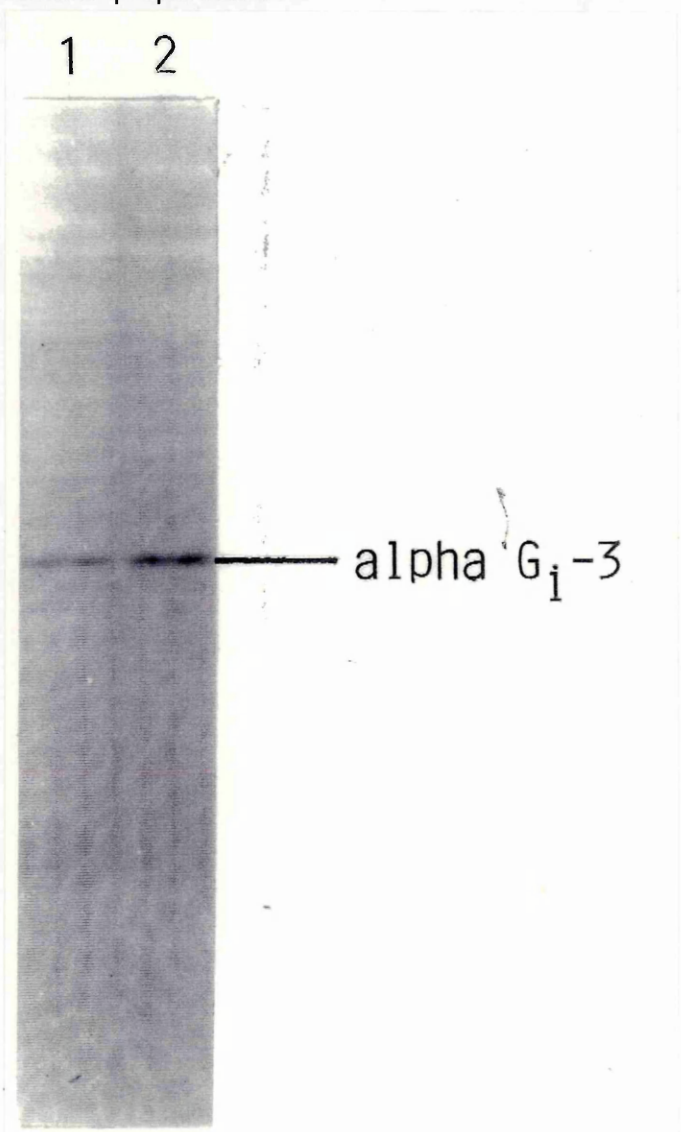


Fig 3.8 Detection of α_s in membranes from control and diabetic animals.

The G-protein subunit α_s was detected in adipocyte membranes by use of antiserum CS1 which specifically recognizes α_s. Lane 1 was loaded with 70μg of protein from membranes prepared from control animals. Lane 2 was loaded with 70μg of protein from membranes from diabetic animals. This experiment is representative of six different experiments using different membrane preparations each time.

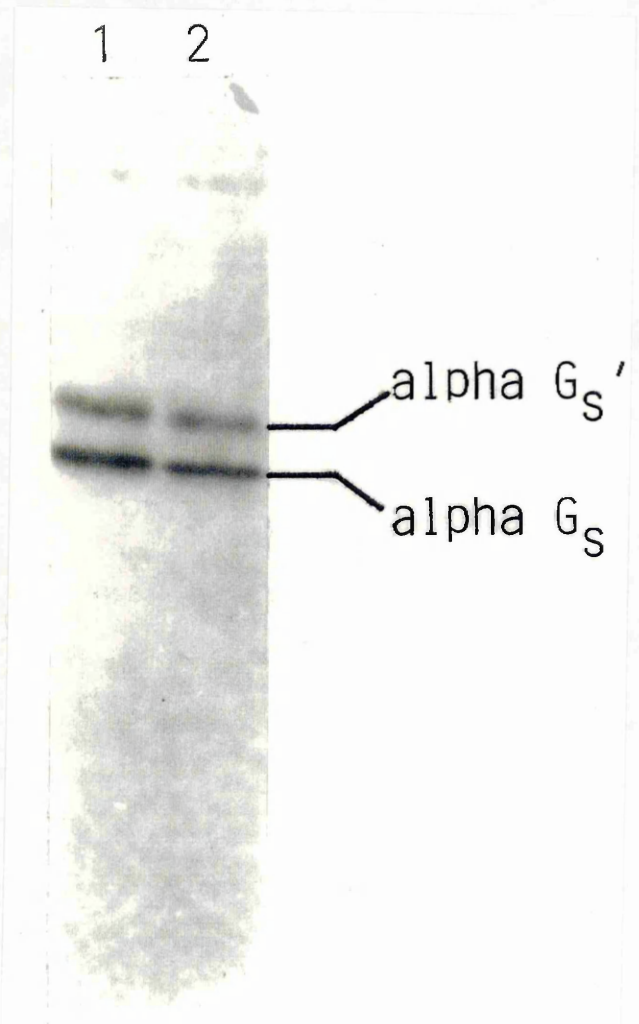


Table 3.1 Receptor mediated inhibition of adenylate cyclase.

Assays were performed in the presence of 10^{-5}M GTP and $5x10^{-4}$ M. The maximal inhibitory effect of PGE_2, nicotinate and PIA is shown together with the IC_{50} values*. Data were obtained from dose effect-curves done at eight different ligand concentrations, and errors are standard deviation for three separate experiments using different membrane preparations. The specific activity of adenylate cyclase in the absence of any inhibitory ligand was 220±18 pmol/min/mg for membranes from control animals and 248±15 pmo/min/mg for membranes from diabetic animals.

	Normal		Diabetic	
Ligand	Inhibition (%)	IC_{50} (nM)	Inhibition (%)	IC_{50} (nM)
PIA	54±8	37±7	83±9	20±4
PGE_1	54±6	38±6	68±9	45±7
Nicotinate	51±11	708±48	67±13	753±46

* The IC_{50} value is that concentration of the compound which causes half maximal inhibition of adenylate cyclase.

3.1 INTRODUCTION

Previous studies on the regulation of adenylate cyclase in hepatocyte membranes from rats made diabetic by injection of streptozotocin have shown that there is a defect in the regulation of adenylate cyclase by G_i (ch 1). The consequences of this alteration in G_i function are such that tonic inhibition of adenylate cyclase is lost and consequently the response to glucagon stimulation of adenylate cyclase is enhanced in hepatocyte membranes from diabetic animals (ch 1). Previous studies on adipocyte adenylate cyclase system of diabetic animals had reported an increased response to ß-AR stimulation of adenylate cyclase by ß-AR agonists (Zumstein *et al* 1980). However nothing was known about the molecular basis of this effect and thus I set out to identify possible underlying molecular mechanisms.

3.2 PHILOSOPHY OF ANALYSES EMPLOYED.

Forskolin is a diterpene which profoundly activates adenylate cyclase (Seamon & Daly, 1986). There appear to be two sites of action: firstly a direct activation of the catalytic unit (C) of adenylate cyclase, secondly an activation of adenylate cyclase involving the presence of functional G_S. The activation of adenylate cyclase by G_S and forskolin appears to be synergistic in nature. The degree of stimulation by forskolin is commonly used as a measure of the activity of the catalytic unit of adenylate cyclase itself, independent of any G-protein input, but this is not strictly true for the reason mentioned above. However $MnCl_2$, will stimulate the activity of the catalytic unit directly as mentioned in ch 1 with a $K_{0.5}$ of ~0.3mM compared with ~3.0mM for Mg^{++} (Somkuti *et al*, 1981). It is quite possible then to imagine that the response of the catalytic unit of adenylate cyclase can be altered with respect to stimulation by G_S whilst leaving stimulation by the Mg^{++} binding site unaltered. This must therefore be a consideration in the

interpretation of experiments employing $MnCl_2$.

NaF in the range 3-20mM activates adenylate cyclase by virtue of the presence of AlF_4^- ions which are a tetrahedral species thought to mimic the phosphate ion PO_4^{--}. Thus, since GDP is bound to G-proteins in isolated membrane preparations the tetrahedral AlF_4^- ion has been suggested to occupy the site occupied normally by the γ-phosphate of GTP such that $GDP^*AlF_4^-$ mimics the effect of non-hydrolysable GTP and thereby leads to activation.

GTP itself elicits a small stimulation of adenylate cyclase (Cooper *et al*, 1979). The dose response of adenylate cyclase to GTP, in the presence of an R_S linked agonist is biphasic in adipocyte membranes (Cooper 1982). At low concentrations one sees the activation of G_S while at higher concentrations, in the second phase, activation of G_i ensues with resultant inhibition of adenylate cyclase. Thus a GTP dose response curve can be used to determine inputs into adenylate cyclase from both G_S and G_i.

The GTP analogue GppNHp, unlike GTP, activates adenylate cyclase significantly even in the absence of an added stimulator of adenylate cyclase such as an R_S linked agonist or forskolin. If GppNHp alone is used to activate adenylate cyclase then the dose response curve to this agent is monophasic so that only a stimulatory action of GppNHp upon adenylate cyclase is seen (Cooper *et al*, 1982). However, when forskolin is used to activate the system then the GppNHp dose response curve is biphasic. This results from low concentrations of GppNHp activating G_i and thus leading to inhibition of adenylate cyclase whilst at higher concentrations G_S is activated and stimulation is seen (Cooper *et al*, 1982). This type of assay can be used to measure the balance of inputs from G_S and G_i. The reason why there is no inhibition of adenylate cyclase by low concentrations of GppNHp in the absence of forskolin is uncertain but it is probably due to the fact that

adenylate cyclase can only be inhibited while in a state of increased activity.

The adenosine A_1 receptor selective agonist PIA (N6-(phenylisopropyl)adenosine is has been used in this system to stimulate the activity of this receptor which is coupled to G_i, in order to observe inhibition of adenylate cyclase. Similarly PGE_1 (prostaglandin E_1) and nicotinic acid are agonists which activate R_i type receptors which couple to G_i in adipocytes.

Quantification of relative levels of G-protein subunits was done by immunoblotting using specific antipeptide antisera (chapter 2 & appendix)*.

3.3 RESULTS AND DISCUSSION

The dose response to GppNHp, in the presence of 100μM forskolin, was examined in adipocyte membranes membranes from control and strepotozotocin diabetic animals (Fig 3.1). Analysis of data in Fig 3.1 shows that, for membranes, from control animals the classical biphasic response to GppNHp was clearly evident with inhibition of adenylate cyclase activity occurring at low concentrations of GppNHp, ie 0.1-100nM, whilst activation occurs at concentrations greater than 100nM. Maximal inhibition achieved was 42±7% of the stimulated activity elicited in the presence of 100μM forskolin. In contrast, analysis of the response of membranes from diabetic animals to GppNHp (Fig 3.1) showed that there was no inhibition of adenylate cyclase at low concentrations of GppNHp. Thus it appears that the ability of G_i to exert a tonic inhibition on adenylate cyclase was lost in membranes from diabetic animals. The control activity of adenylate cyclase, obtained in the presence of 100μM forskolin only, was much lower in membranes from diabetic animals than in membranes from control animals (Fig 3.1). It is possible that, either the catalytic activity of individual adenylate cyclase molecules was reduced or the number of molecules was reduced.

The GTP dose response of membranes prepared from control animals was also determined (Fig 3.2) where a classical biphasic response to GTP was

*Densiometric scanning of immunoblots, using an Abaton-300 densiometric scanner driven by Apple MacIntosh 'C-Scan Software', was employed for the comparison of G-protein subunit levels between membranes from control and diabetic

demonstratable. Thus, high concentrations of GTP inhibit activity which, as described in ch 1, is due to the action of G_i. However when the same experiment was performed on membranes prepared from diabetic animals the classical biphasic response was completely lost (Fig 3.3). Analysis of Fig 3.3 shows that only a stimulatory phase was seen with GTP. Thus, as deduced from studies done with GppNHp, the ability of G_i to exert a tonic inhibitory effect on adenylate cyclase was abolished in membranes from isolated from diabetic animals. The control adenylate cyclase activity of membranes from diabetic animals was significantly lower than the corresponding activity in membranes from control animals (Figs 3.2 & 3.3).

A dose response curve to GTP was also conducted in the presence of PIA using membranes from diabetic animals (Fig 3.4). Clearly under the conditions employed in this experiment, a biphasic response to GTP was evident. This result was in marked contrast to that seen in Fig 3.3. Thus here we see that, using membranes from diabetic animals, as long as PIA is present then concentrations of GTP greater than 100nM leads to inhibition of adenylate cyclase. The dose response curve of membranes from control and diabetic animals to PIA is shown in Fig 3.5 which shows that inhibition by PIA was actually somewhat greater in membranes from diabetic animals than in membranes from control animals.

The fact that PIA appears to show enhanced inhibition of adenylate cyclase in membranes from diabetic animals (Fig 3.5; Table 3.1) is probably due to the fact that inhibition of adenylate cyclase, mediated by GTP alone, is abolished in the very same membranes and consequently PIA is able to achieve further inhibition because the system is more open to inhibition. On a some what similar note, it is well documented that pertussis toxin treatment of cells, thereby ADP-ribosylating and inactivating G_i, leads to enhanced stimulation of adenylate cyclase by glucagon in hepatocyte membranes

(Heyworth *et al*, 1984) and enhanced stimulation of adenylate cyclase by isoproterenol in adipocyte membranes (Katada *et al*, 1982).

In order for the inhibitory action of G_i to be seen in membranes from diabetic animals it was essential that PIA was present rather than membranes briefly subjected to its exposure. Indeed pre-incubation of membranes from diabetic animals in adenylate cyclase assay buffer for periods of 5-10min with 1μM PIA followed by harvesting of the membranes by centrifugation, and washing once with EDTA/Tris buffer, failed to expose any function of G_i when membranes were subsequently challenged for GTP dependent inhibition in the absence of PIA.

We sought to ascertain whether these changes in G_i-mediated responses could be attributed to any alteration in the expression of the three forms of G_i that have been shown to be expressed in adipocytes (Mitchell *et al*, 1989). Although the functional role of these various G-proteins has yet to be definitely ascertained it has been suggested that G_i2 might be the major species responsible for mediating the inhibition of adenylate cyclase (Bushfield *et al*, 1990; Codina *et al*, 1988). We found no evidence for differences in the apparent amounts of α-subunits for either of these two G-proteins in adipocyte membranes from normal and diabetic animals (Fig 3.6). In 8 separate experiments using different membrane preparations immunoblotted with antiserum AS7 we found that the level of α_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 the level of α_i2 differed only by some 8±7% in membranes from diabetic animals as compared with membranes from control animals. In contrast to this, using antiserum I3B to detect α_i3 we observed that levels of this particular G-protein α-subunit were actually increased by some 88±18% (8 separate experiments) in adipocytes 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, effect receptor independent (tonic) inhibition whereas other retained species might mediate receptor dependent inhibition. It is possible that the more pronounced inhibitory potency of PIA in membranes from diabetic animals could be due to elevated $\alpha_i 3$ levels or increased numbers of A_1-adenosine receptors. However I believe that it is equally possible that the effect is due to a loss of the tonic inhibition of adenylate cyclase by G_i.

The increased response employing membranes from diabetic animals was not due to any increase in the amount of the stimulatory G-protein G_S as the induction of diabetes did not affect the amount of the two forms of this G-protein that I detected here (45kD and 42kD). Fig 3.8 shows that the levels of 45 and 42 kD forms of α_S differed only by some 6±5% and -7±6% respectively from those seen using membranes from control animals.

Thus it appears somewhat paradoxical that R_i receptor mediated inhibition of adenylate cyclase was unimpaired in membranes from diabetic animals but that tonic inhibition mediated by G_i was abolished. However this latter effect is not completely unprecedented since the two functions can be divorced *in normal* adipocyte membranes such that inhibition by GTP or GppNHp is completely abolished but that by PIA is increased (Cooper 1982). This was shown in studies where the NaCl concentration in the adenylate cyclase assay buffer was increased to 150mM or greater. Under such conditions inhibition, achieved using low concentrations of GTP was abolished while inhibition by PIA was actually increased.

The tonic inhibition of adenylate cyclase could employ two potential mechanisms. Firstly the inhibition could be achieved by residual turnover of G_i without any requirement for input from agonist occupied R_i receptors. Alternatively it could be achieved by some input from unoccupied R_i

receptors activating G_i, as has been suggested for ∂-opiate (ch 1). The question of how 'tonic' inhibition, but not R_i mediated inhibition, is abolished in membranes from diabetic animals is made complex by the fact that the mechanism by which G_i inhibits adenylate cyclase is ill-understood. The inhibitory action of G_i has been suggested to be mediated by the release of $\beta\gamma$ subunits upon receptor activation of G_i (Katada *et al*, 1984). However various studies have implied that α_i also contributes to the inhibition of adenylate cyclase stimulated by activated R_s, but inhibition of basal adenylate cyclase activity is mediated by $\beta\gamma$ subunits (Hildebrandt & Kohnken, 1990). It has been demonstrated recently that α_i can be phosphorylated by challenging hepatocytes with TPA or PKC activating agents. This leads to the loss of the ability of GppNHp to inhibit forskolin stimulated adenylate cyclase activity. Indeed, the α-subunit of G_i is now known to be a substrate not only for PKC (Pyne *et al*, 1989; Katada *et al* ,1985) but also PKA (Wanatabe *et al*, 1988) and the insulin receptor kinase (O'Brien *et al*, 1987; Krupinski *et al*, 1988). It is therefore possible that the lesion which leads to a change in functioning of G_i is its phosphorylation state. In adipocyte membranes from diabetic animals only a stimulatory action of GTP was apparent (Fig 3.3). However, the net effect of any relative enhancement of catecholamine stimulated intracellular adenylate cyclase activity in the diabetic state will be attenuated by the fact that basal adenylate cyclase activity was itself reduced in diabetic adipocytes (Table 3.1). Thus, even though the response of adenylate cyclase to stimulation by isoproterenol in diabetic rats is increased, there is unlikely to be a large increase in the intracellular concentration of cAMP in response to stimulation by catecholamines in the intact adipocytes of these animals. Concerning the reduction in activity of the catalytic unit of adenylate cyclase itself, remains to be seen whether this is due to a reduction in the amount or activity of the

catalytic unit itself.

The situation found in streptozotocin diabetic rat adipocyte bears comparison with hepatocytes membranes of obese Zucker rats. In this case hepatocyte membranes from obese animals exhibit little functional G_i, as assessed by either GTP or GppNHp dependent inhibition, whereas an activity comparable with that seen in Sprague-Dawley rats was seen with the lean animals (Houslay *et al*, 1989). This occurred despite there being similar amounts of G_i in membranes from both types of animals.

The loss of the ability of G_i to to cause the inhibition of adenylate cyclase activity in adipocyte membranes from genetically obese and insulin resistant obese (*ob/ob*) mice has similarly been noted (Begin-Heick, 1985; Begin-Heick, 1988). Such experiments bear similarity to those described in this study and previous investigations (Houslay *et al*, 1989; Gawler *et al*, 1987) in highlighting a lesion in the inhibitory function of G_i, rather than any loss its of expression, in insulin-resistant states. However in the *ob/ob* mouse system, adipocyte membrane adenylate cyclase can still be inhibited by PIA (Greenberg *et al*, 1987). Thus again it appears that tonic inhibition of adenylate cyclase can be lost in a pathological state while adenylate cyclase is still fully responsive to inhibition by R_i receptor mediated mechanisms. Indeed, as reported here for adipocyte membranes from streptozotocin diabetic animals, Greenberg *et al*, (1987) also observed an enhanced ability of PIA to elicit inhibition in membrane preparations from obese animals which lack the tonic inhibitory capacity of G_i.

It has been demonstrated recently that α_i can be phosphorylated by challenging hepatocytes with TPA or PKC activating agents. This leads to the loss of the ability of GppNHp to inhibit forskolin stimulated adenylate cyclase activity. Indeed, the α-subunit of G_i is now known to be a substrate not only for PKC (Pyne *et al*, 1989; Katada *et al*, 1985) but also PKA (Wanatabe

et al, 1988) and the insulin receptor kinase (O'Brien *et al*, 1987; Krupinski *et al*, 1988). It is therefore possible that the lesion which leads to a change in functioning of G_i is its phosphorylation state.

CHAPTER 4

REGULATION OF ADENYLATE CYCLASE IN THE ADIPOSE TISSUE OF OBESE (*fa/fa*) ZUCKER RATS

Fig 4.1. Dose-response curves for stimulation of adenylate cyclase by isoproterenol in adipocyte membranes from lean and obese Zucker rats

The 100μM GTP stimulated activity was 16±1.6 pmol/min/mg and 17±1.7 pmol/min/mg for membranes from lean and obese animals respectively. The maximal specific activities attained when saturating doses of isoproterenol were used together with GTP were 262±26 pmol/min/mg and 100±9 pmol/min/mg for membranes from lean and obese animals respectively. The fold-stimulations by isoproterenol over GTP-stimulated activity were thus calculated to be 16 and 6 for membranes from lean and obese animals respectively. The $K_{0.5}$ values for isoproterenol-stimulation were deduced to be 6.3±0.8μM and 8.9±1.1μM for membranes from lean and obese animals respectively. Result shows mean and standard deviations for a typical experiment repeated six times with six different sets of lean (open square) and obese (filled diamond) Zucker rat membranes.

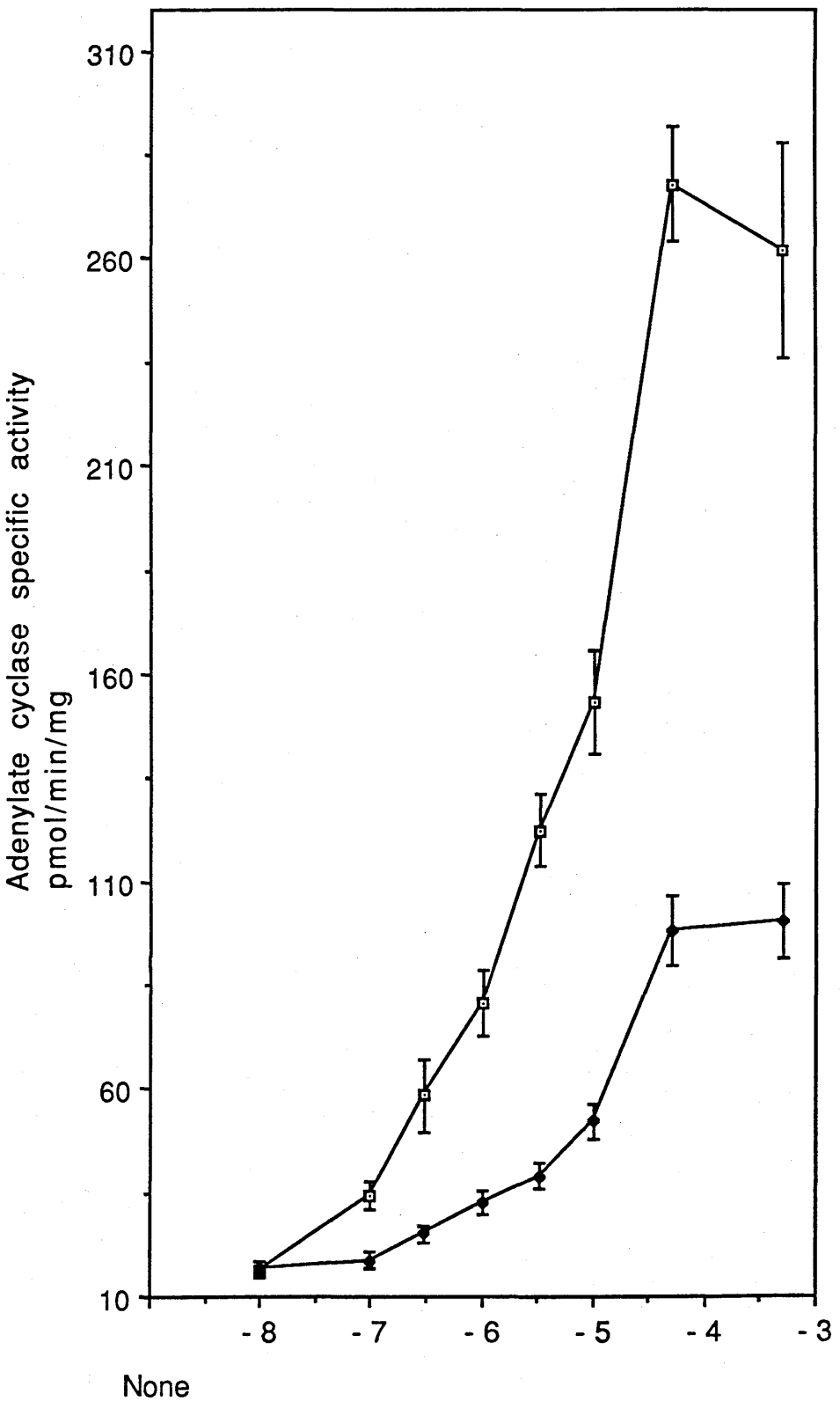

Adenylate cyclase specific activity
pmol/min/mg
310
260
210
160
110
60
10
- 8
- 7
- 6
- 5
- 4
- 3
None
log[isoproterenol] M

Fig 4.2 Dose response to isoproterenol stimulation of adenylate cyclase in the presence of GTPγS

The dose response to isoproterenol done in the presence of 100μM GTPγS was assessed for membranes from lean (closed square) and obese (open square) animals. The data shows mean and standard deviation for a typical result repeated three times in separate sets of membranes from lean and obese animals. The activities of adenylate cyclase with 100μM GTPγS alone were 83±7.5 and 70±9.1 pmol/min/mg for membranes from lean and obese animals respectively. V_{max} activities were 134±12.5 and 119±12 pmol/min/mg for lean and obese membranes respectively.

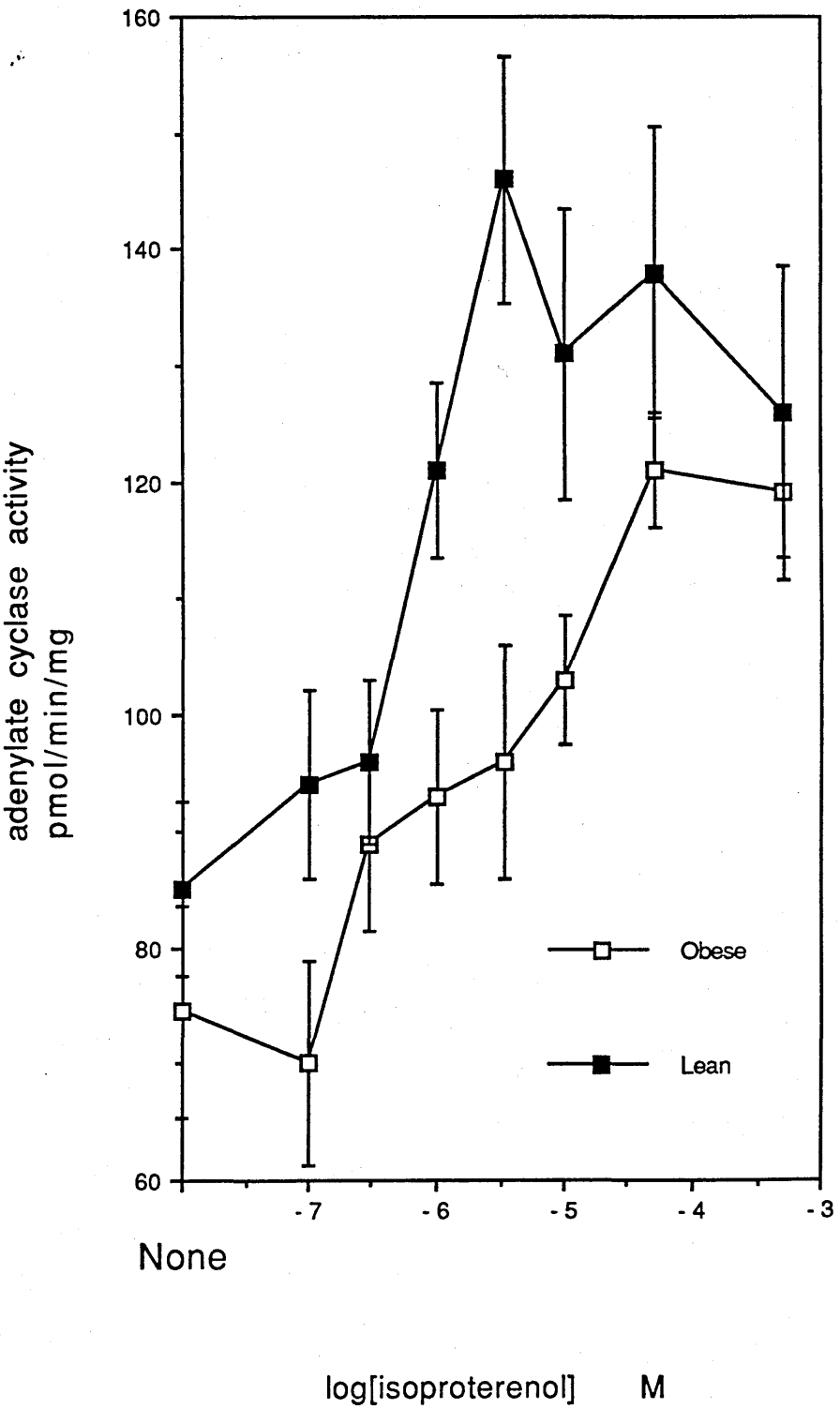

160
140
120
100
80
60
adenylate cyclase activity
pmol/min/mg
Obese
Lean
-7
-6
-5
-4
-3
None
log[isoproterenol] M

Fig 4.3 Dose response to GTPγS stimulation of adenylate cyclase in presence of 50μM isoproterenol

Data points show mean and standard deviations of a typical experiment repeated three times with different sets of lean (closed diamond) and obese (open square) membranes. Adenylate cyclase was stimulated with 50μM isoproterenol and the effect of increasing concentrations of GTPγS were determined. In membranes from lean animals the control activity was 46±5 pmol/min/mg, the V_{max} activity was 120±9 pmol/min/mg, the fold stimulation by GTPγS was calculated to be 2.6-fold and the $K_{0.5}$ for GTPγS was 0.45±0.1nM. In membranes from obese animals the control activity was 15.6 pmol/min/mg, the V_{max} activity was 125±11 pmol/min/mg, the fold stimulation by GTPγS was calculated to be 8.0 fold and the $K_{0.5}$ for GTPγS was 4.0±1nM.

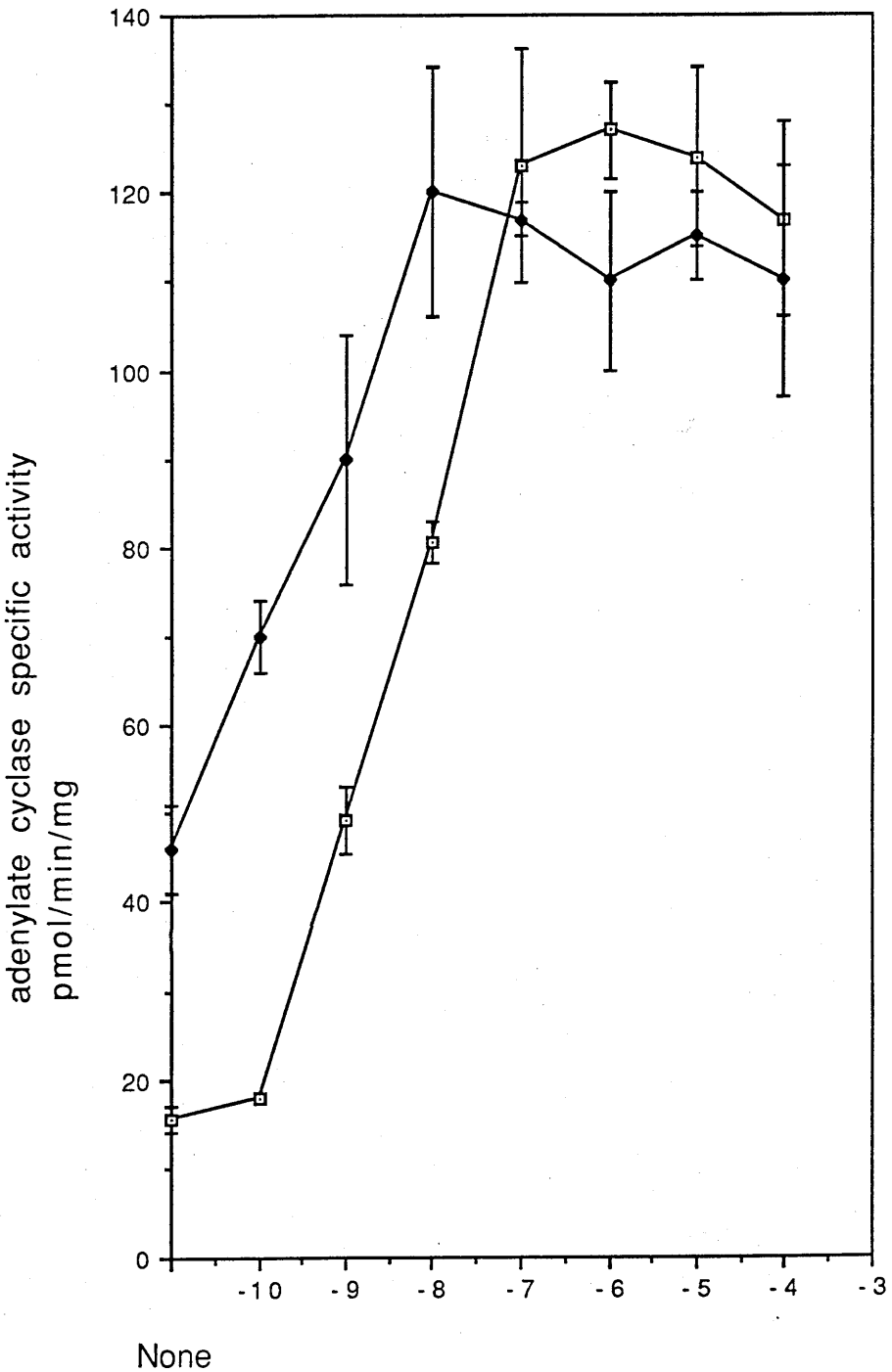

140
120
100
80
60
40
20
0
adenylate cyclase specific activity
pmol/min/mg
-10
-9
-8
-7
-6
-5
-4
-3
None
log[GTP(S)] M

Fig 4.4. Dose-response curves for stimulation of adenylate cyclase by glucagon in membranes from lean and obese Zucker rats.

In membranes from lean animals glucagon stimulated adenylate cyclase activity in the presence of 100μM GTP some 4.6-fold to a V_{max} of 55.4±4.3 pmol/min/mg with a $K_{0.5}$ of 501±100nM. In membranes from obese animals glucagon stimulated adenylate cyclase activity in the presence of 100μM GTP some 2.1-fold to a V_{max} of 25.7±3.0 pmol/min/mg with a $K_{0.5}$ of 1333±500nM. The data shows mean and standard deviations of a typical experiment repeated three times with different lean (open square) and obese (filled diamond) Zucker rat membranes.

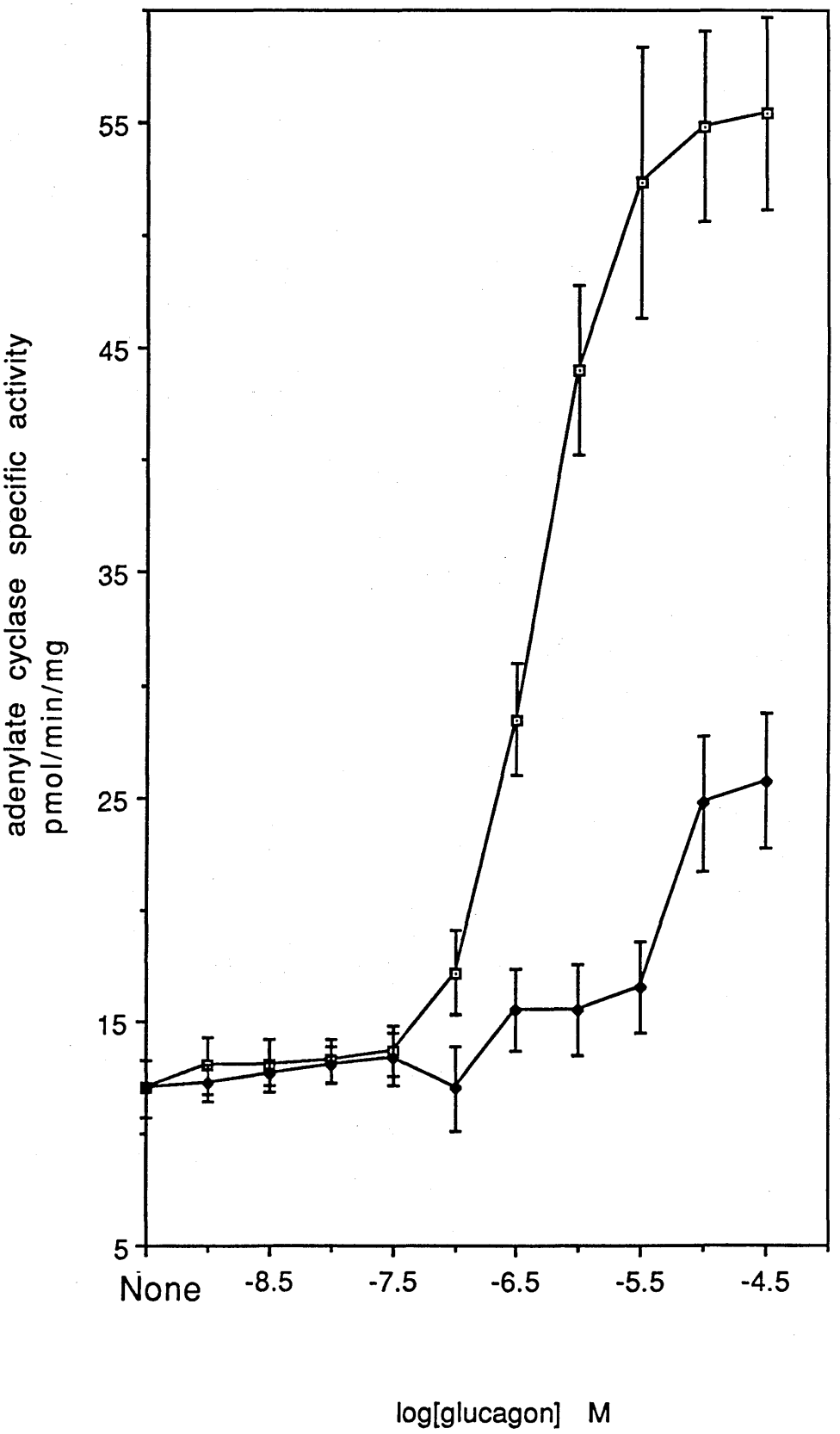

adenylate cyclase specific activity
pmol/min/mg
55
45
35
25
15
5
None
-8.5
-7.5
-6.5
-5.5
-4.5
log[glucagon] M

Fig 4.5. Dose-response curves for stimulation of adenylate cyclase by secretin in from membranes lean and obese Zucker rats.

In membranes from lean (open square) animals, secretin stimulated adenylate cyclase activity, in the presence of 100μM GTP, some 4.15-fold, to a V_{max} of 27±1.5 pmol/min/mg with a $K_{0.5}$ of 141±20nM. In membranes from obese animals (filled diamond) stimulation of adenylate cyclase (+ 100μM GTP) by secretin was negligible. The data shows mean and standard deviations for a typical experiment repeated three times with different lean and obese Zucker rat membrane preparations.

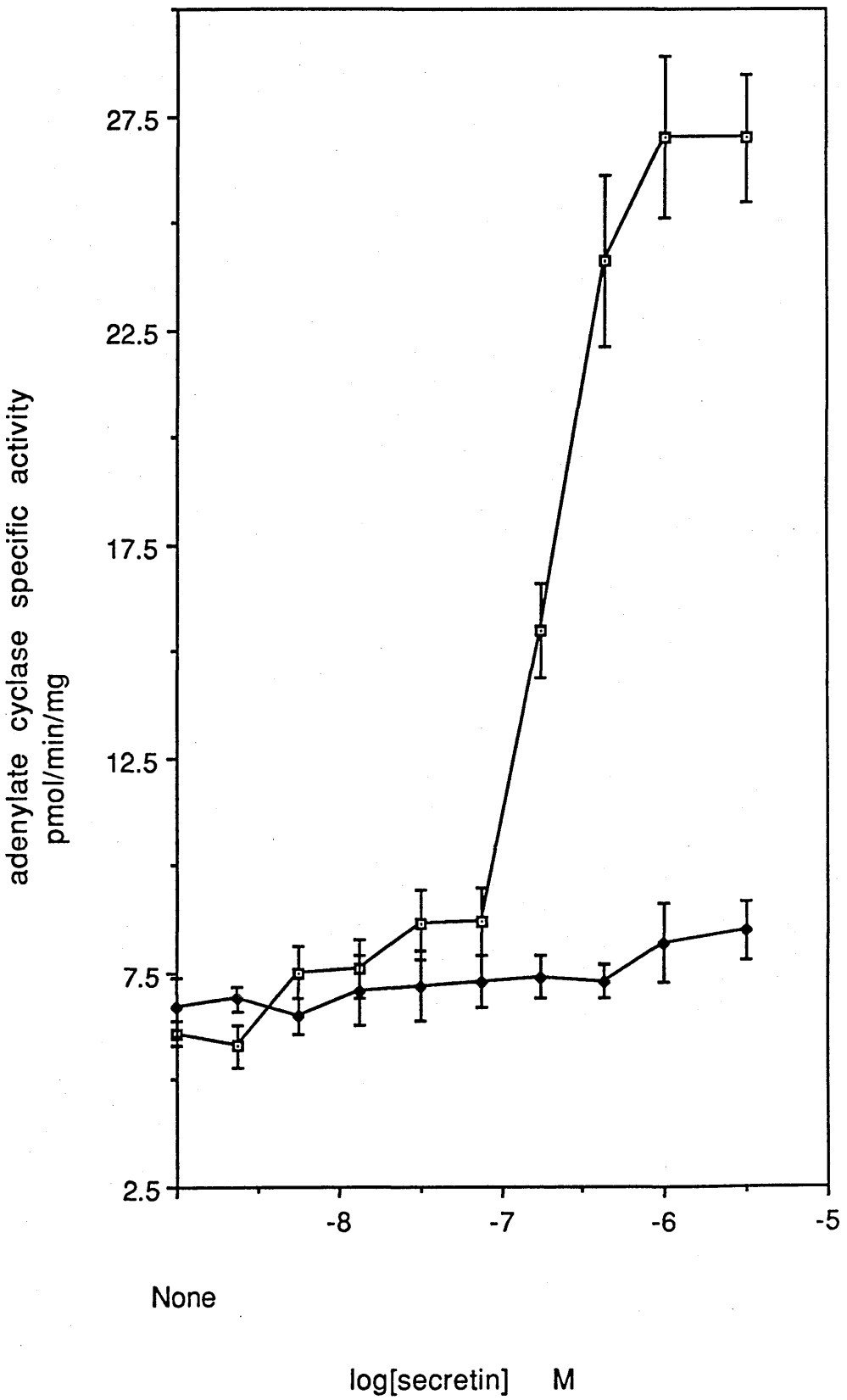

adenylate cyclase specific activity
pmol/min/mg
27.5
22.5
17.5
12.5
7.5
2.5
-8
-7
-6
-5
None
log[secretin] M

Fig 4.6 Dose-response curves for stimulation of adenylate cyclase by TSH in adipocyte membranes from lean and obese Zucker rats.

Basal activity is that with 100 μM GTP only. In membranes from lean animals (open square) TSH stimulated adenylate cyclase by some 3.75-fold reaching a V_{max} of some 30.4±3.5 pmol/min/mg with a $K_{0.5}$ of 266±50nM. In membranes from obese animals (filled diamond) TSH stimulation of adenylate cyclase was negligible. The data shows mean and standard deviation for a typical experiment repeated three times with different lean and obese Zucker rat membrane preparations.

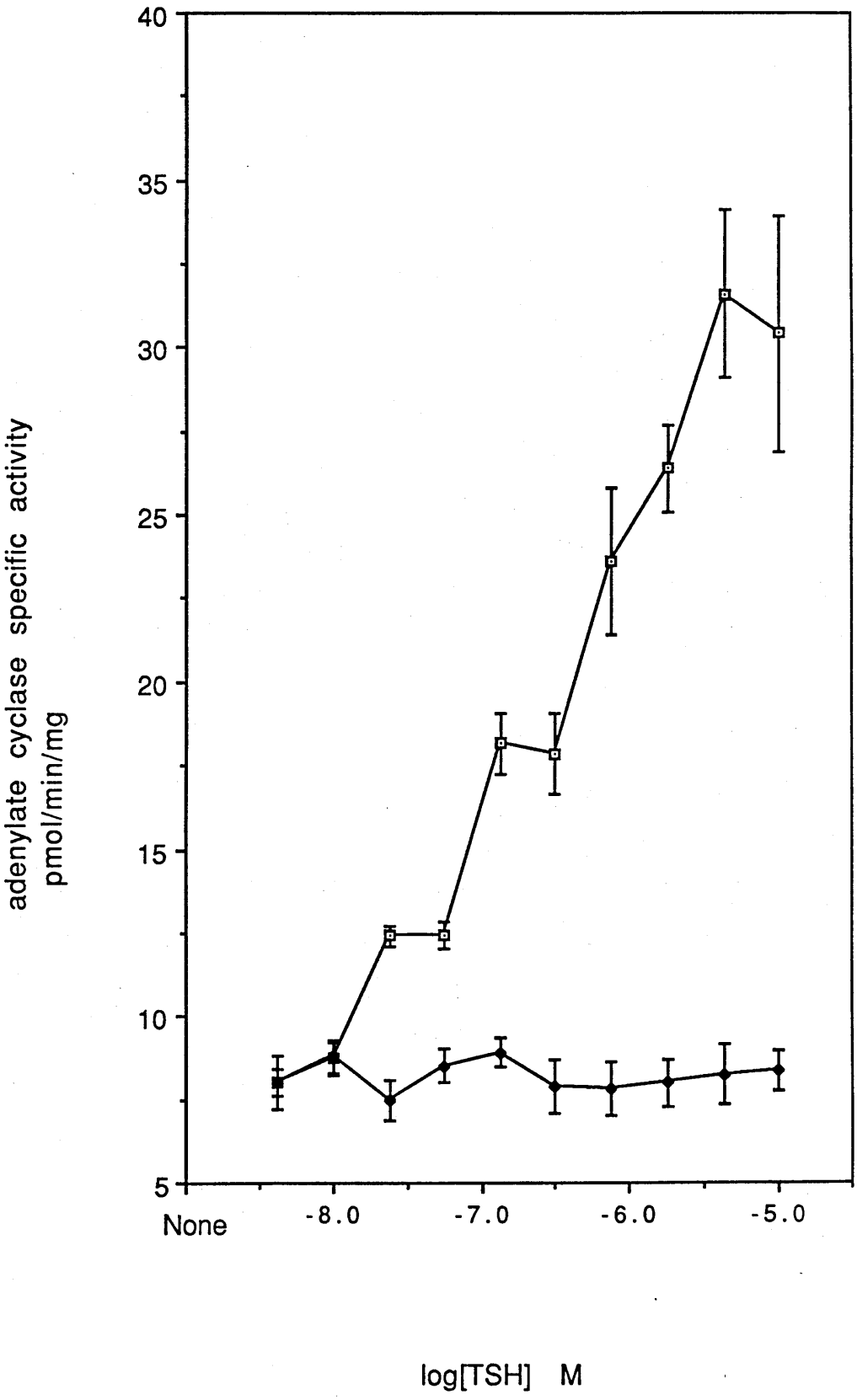

adenylate cyclase specific activity
pmol/min/mg
40
35
30
25
20
15
10
5
None
-8.0
-7.0
-6.0
-5.0
log[TSH] M

Fig 4.7 Dose response curves of adenylate cyclase stimulation to GTPγS in the absence of any hormones.

Adenylate cyclase activity was assayed with increasing concentrations of GTPγS with adipocyte membranes from lean (open square) and obese (closed diamond) Zucker rats. Results are mean±standard deviation for 3 different experiments using different membrane preparations each time. In membranes from lean animals the V_{max} activity was 77±3 pmol/min/mg and the $K_{0.5}$ was 8.5±1.2nM. In membranes from obese animals the V_{max} activity was 70±5 pmol/min/mg and the $K_{0.5}$ was 28±3nM.

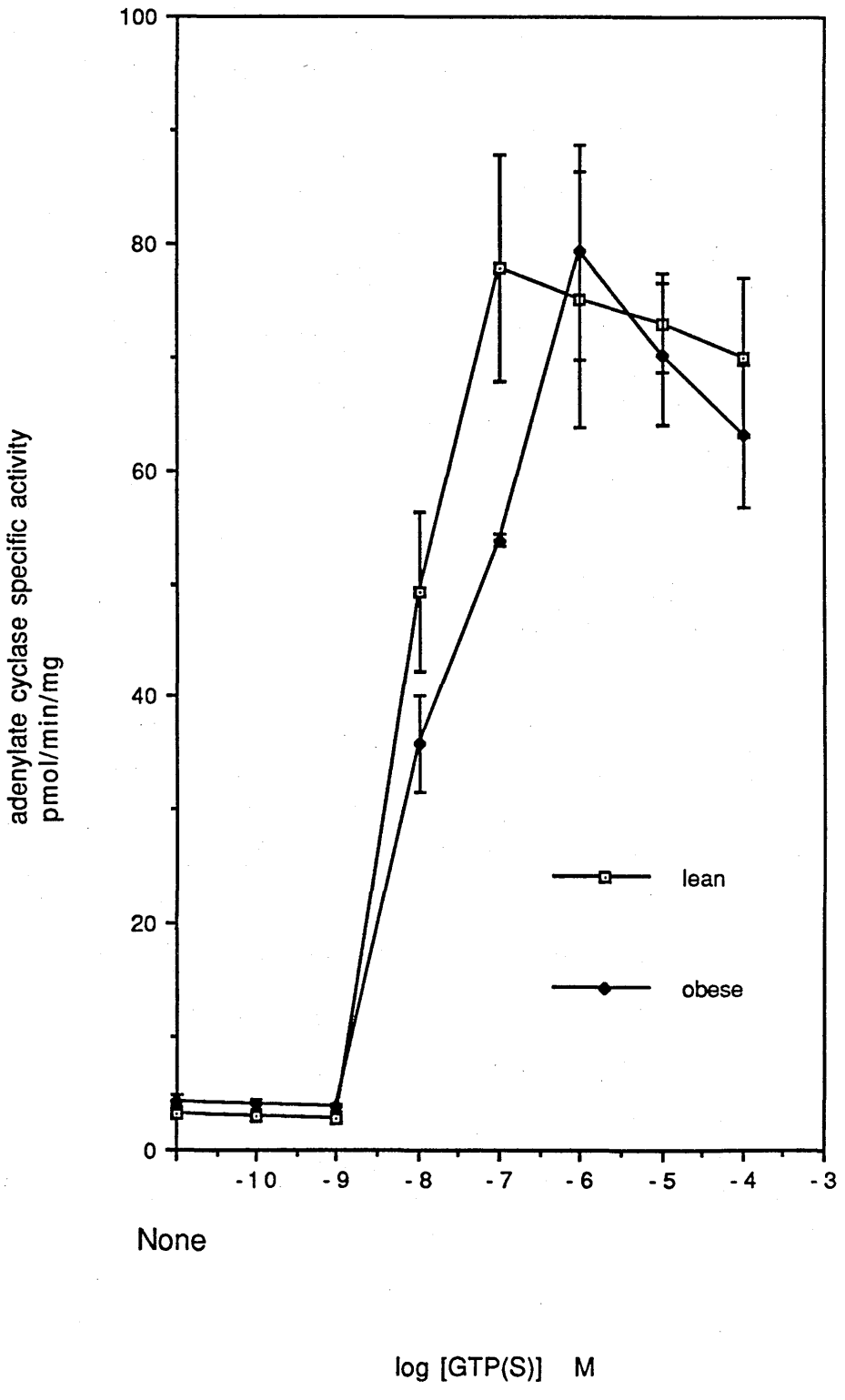

100
80
60
40
20
0
adenylate cyclase specific activity
pmol/min/mg
lean
obese
-10
-9
-8
-7
-6
-5
-4
-3
None
log [GTP(S)] M

Fig 4.8. Detection of α_s-subunits in adipocyte membranes from lean and obese animals.

Detection of the 42kD and 45kD forms of G_s using the antiserum CS1 with membranes from lean (tracks 1,2,3) and obese (tracks 4,5,6)animals at 20µg (track 1,4), 40µg (track 2,5) and 60µg(track 5,6) of membranes. Plasma membranes were subjected to SDS-PAGE and immunoblotted with antipeptide antisera as described in Materials and Methods. Data shown are typical experiments of those done three times using membranes from different lean and obese animals.

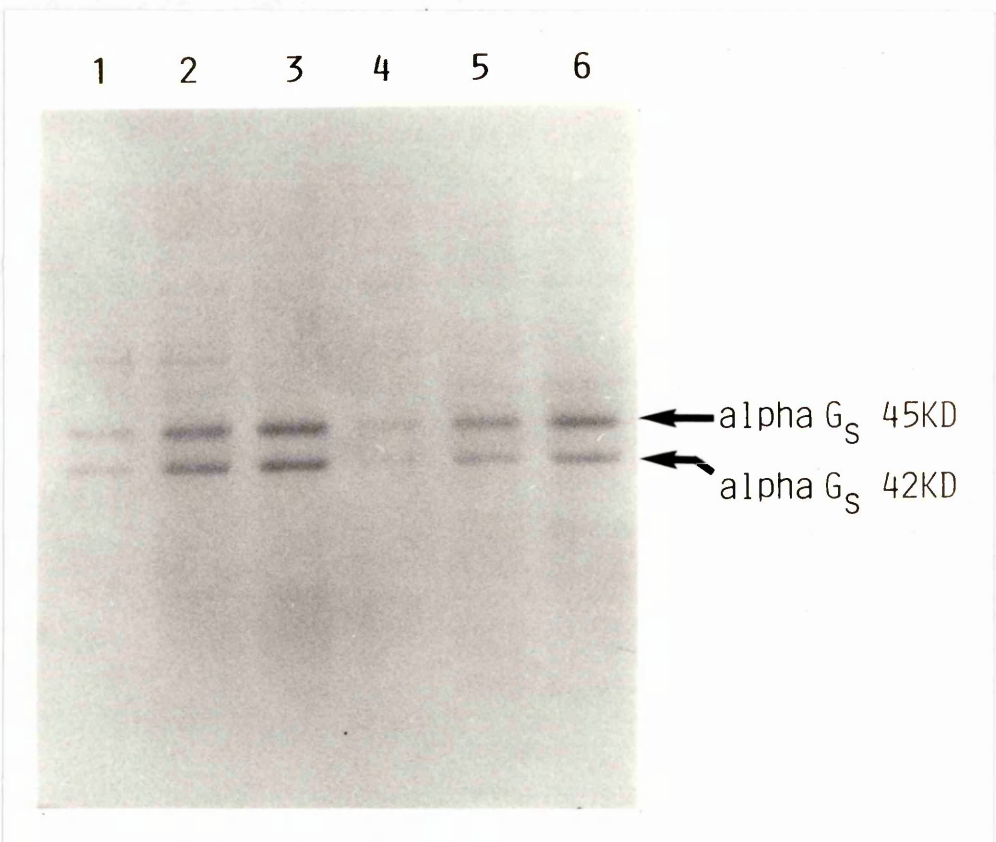


Fig 4.9 Detection of G-protein ß-subunit in adipocyte membranes from lean and obese animals.

Detection of ß-subunit by immunoblotting with antiserum BN1 with membranes of lean (tracks 4,5,6) and obese (tracks 1,2,3) animals at 40µg (tracks 3,6), 80µg (tracks 2,5) and 120µg (tracks 1,4) of membranes. Plasma membranes were subjected to SDS-PAGE and immunoblotted with antipeptide antisera as described in Materials and Methods. Data shown are typical experiments of those done three times using membranes from different lean and obese animals.

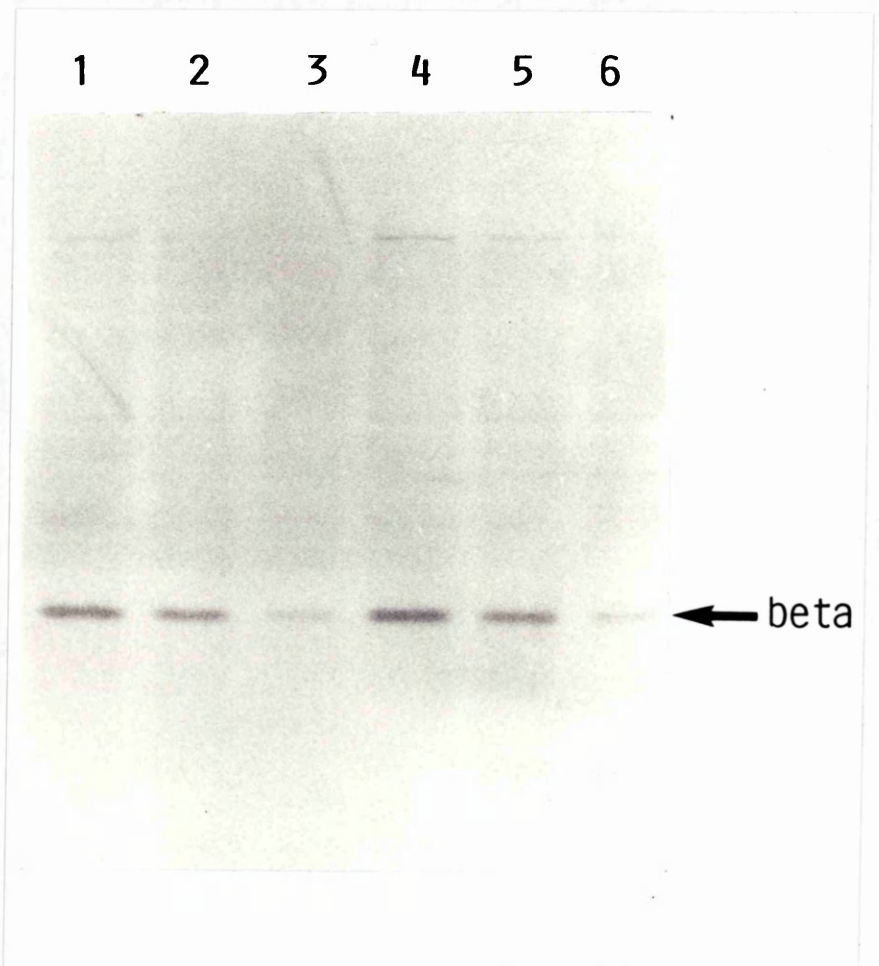


Fig 4.10. Detection of α_i1 and α_i2 in adipocyte membranes from lean and obese animals.

Detection of the α subunits of G_i-1 and G_i-2 using the antiserum AS7; track 1 80μg lean, track 2 obese 80μg and track 3 obese 160μg. Plasma membranes samples were subjected to SDS-PAGE and immunoblotted with antipeptide antisera as described in Materials and Methods. Data shown are typical experiments of those done three times using membranes from different lean and obese animals.

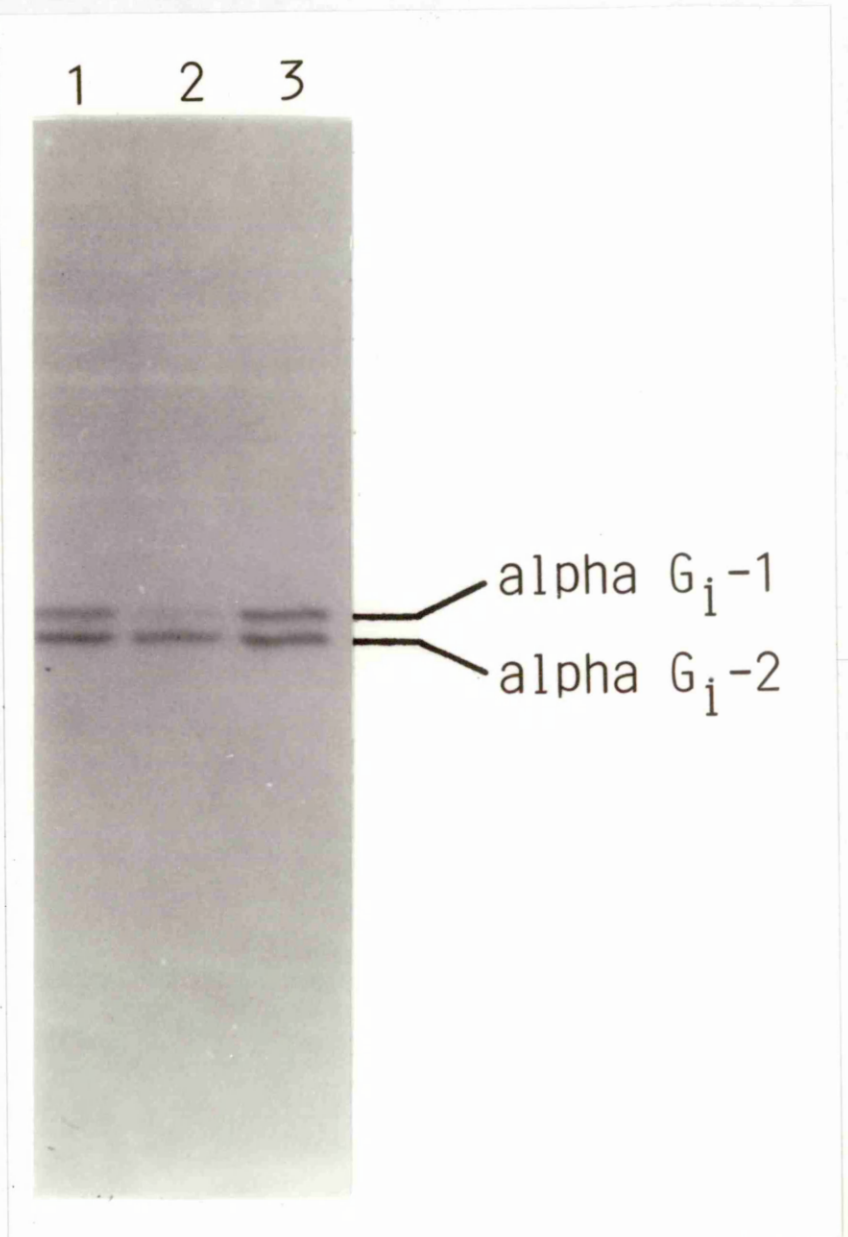


Fig 4.11. Detection of α_i3 in adipocyte membranes from lean and obese animals.

Detection of α_i3 using antiserum 13B for membranes of lean (tracks 1,2,3) and obese (tracks 4,5,6) animals using 50µg (tracks 1,4),100µg (tracks 2,5) and 150µg (tracks 3,6) of membranes. Plasma membranes were subject to SDS-PAGE and immunoblotted with antipeptide antisera as described in Materials and Methods. Data shown are typical experiments of those done three times using membranes from different lean and obese animals.

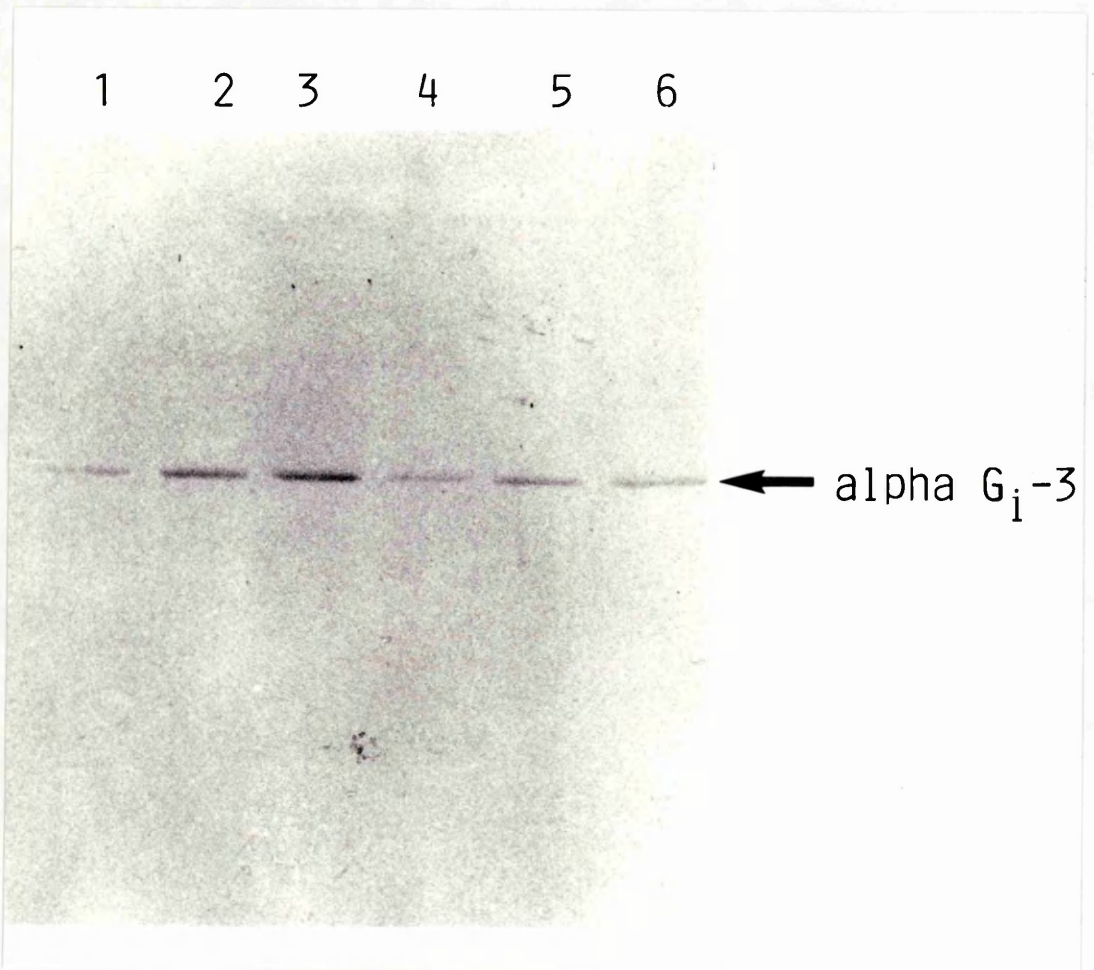


Fig 4.12 Dose response of forskolin stimulated adenylate cyclase curve to GppNHp in adipocyte membranes from lean and obese Zucker rats.

Adenylate cyclase activity was determined with 100μM forskolin and inhibition assessed by increasing concentrations of GppNHp. Membranes from lean (open square) and obese (filled diamond) were used. Results are means and standard deviations of three experiments using membranes from different animals. The value at 100% activity was taken to be that activity at 100μM forskolin alone this being 180±12 and 185±15 pmol/min/mg for membranes from lean and obese animals respectively.

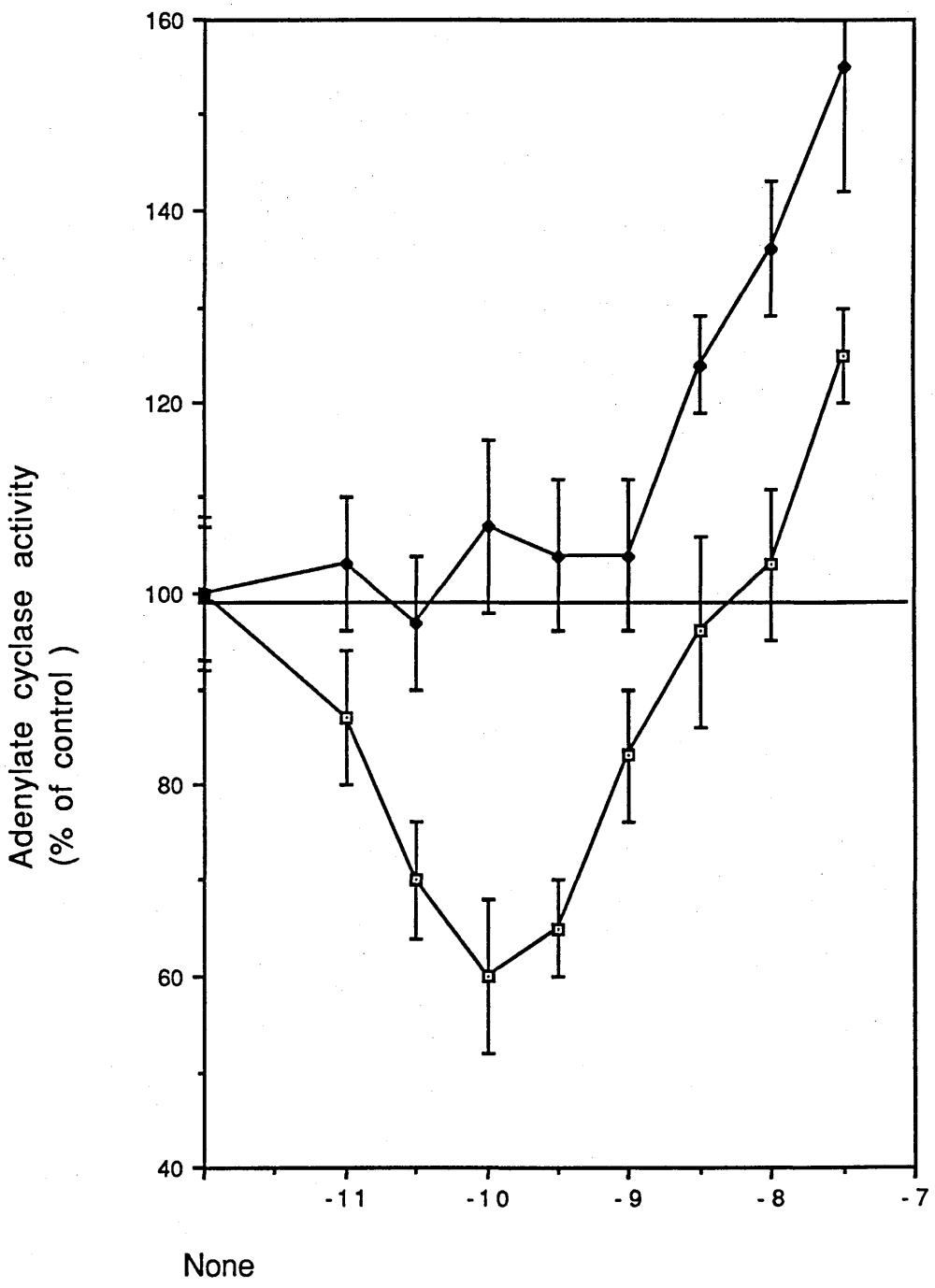
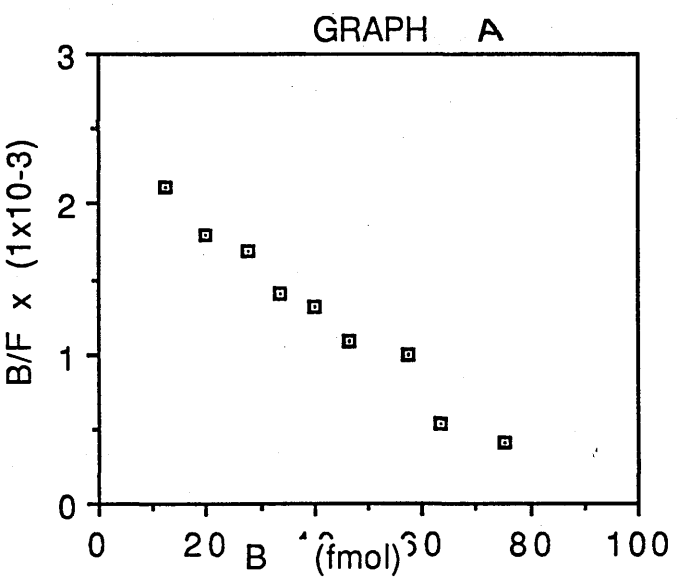

160
140
120
100
80
60
40
Adenylate cyclase activity
(% of control)
-11
-10
-9
-8
-7
None
log[GppNHp] M

Fig 4.13 Scatchard representation of ^{125}I-cyanopinidol binding to lean and obese Zucker rat adipocyte membranes.

Binding of ^{125}I-CYP to membranes was determined as described in Material and Methods. The parameters B_{max} and K_d are given in Table 4.2. Graphs A & B show the plots for membranes from lean and obese animals respectively.



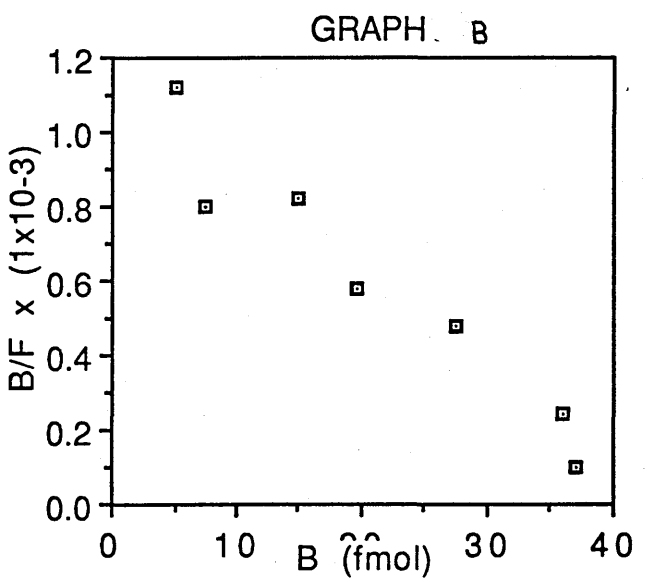


TABLE 4.1 The effect of guanine nucleotide upon the dose response curve to isoproterenol in membranes from lean and obese Zucker rats.

Data are obtained from Figs 4.1 and 4.2 respectively. $\Delta K_{0.5}$ is the ratio of $K_{0.5}$ for isoproterenol while utilizing GTP/$K_{0.5}$ for isoproterenol while utilizing GTPγS.

	LEAN	OBESE
GTP		
control* (pmol/min/mg)	17.7±1.7	16±1.6
V_{max} (pmol/min/mg)	262±26	100±9
fold activation	16.0	6.25
$K_{0.5}$ (μM)	6.3	8.9
GTPγS		
control** (pmol/min/mg)	83±7.5	70±9.1
V_{max} (pmol/min/mg)	134±12.5	119±12
fold activation	1.61	1.70
$K_{0.5}$ (μM)	0.6	2.5
$\Delta K_{0.5}$	10.5	3.56

* (100μM GTP only)

**(100μM GTPγS only)

Table 4.2 Receptor-independent stimulation of adenylate cyclase and ß-adrenergic receptor number in membranes from lean and obese Zucker rats.

Data relating to adenylate cyclase stimulation shows means and standard deviations of a typical experiment repeated five times with different lean and obese Zucker adipocyte membrane preparations. Data relating to ^{125}I-CYP binding shows typical result for binding to lean and obese membranes repeated six times with separate lean and obese membrane preparations each time. The parameters B_{max} and K_d were derived from Scatchard plot (Fig 4.13) of the data which gave a straight line.

	ADENYLATE CYCLASE ACTIVATOR			^{125}I-CYP BINDING	
	Forskolin 100µM	NaF 20mM	$MnCl_2$ 5mM	B_{max}	K_d
	(pmol/min/mg)			(fmol/mg)	(pM)
Lean	170±15	47±3	220±12	85±7	35
Obese	160±12	45±5	210±13	42±5	35

Table 4.3. Inhibition of isoproterenol-stimulated adenylate cyclase activity.

Assays were performed in the presence of 100 μM isoproterenol and 100 μM GTP. The maximal inhibitory effect of saturating doses of PIA, PGE_1, nicotinate and DDA are shown as percentage inhibitions of isoproterenol-stimulated adenylate cyclase which exhibited specific activities of 280±18 and 102±12 pmol/min/mg in membranes from lean and obese animals respectively. The data shows the mean and standard deviation of a typical experiment repeated four times with different lean and obese Zucker rat adipocyte membrane preparations.

	Percentage inhibition of adenylate cyclase activity	
	Lean	Obese
Inhibitory ligand		
PIA (1μM)	65±8	63±8
nicotinate (10μM)	54±7	58±5
DDA (100μM)	85±9	85±6
PGE_1 (1μM)	55±12	58±9

Table 4.4. Inhibition of forskolin-stimulated adenylate cyclase activity.

Assays were performed in the presence of 100 μM GTP and 100 μM forskolin. The maximal inhibitory effects of saturating doses of PIA, PGE_1, nicotinate and DDA are shown as percentage inhibitions of forskolin-stimulated adenylate cyclase activity. The data shows the mean and standard deviation of a typical experiment repeated four times with separate lean and obese Zucker rat adipocyte membrane preparations. The specific activities of forskolin-stimulated adenylate cyclase in lean and obese membranes were 180±12 and 185±15 pmol/min/mg respectively.

	Percentage inhibition of stimulated activity	
	Lean	Obese
Inhibitory ligand		
PIA (1μM)	48±4	49±3
nicotinate (10μM)	45±3	41±4
DDA (100μM)	83±5	87±7
PGE_1 (1μM)	47±5	46±4

TABLE 4.5 Changes in G-protein levels in adipocyte membranes from lean and obese Zucker rats.

Changes in levels of G-proteins were quantified by densiometric scanning using an Abaton-300 densiometric scanner*. The notation N.S in brackets means not statistically significant by student t-test. The relative amount of each G-protein subunit (under the column OBESE(fa/fa)) is expressed as a percentage of that in lean membranes (100%).

G-PROTEIN SUBUNIT	OBESE (fa/fa)	ANTISERUM
α_s42	65±10%	CS1
α_s45	58±10%	CS1
β_{35+36}	105±8%	BN1
α_i1	35±6%	AS7
α_i2	84±9%(NS)	AS7
α_i3	38±7%	I3B

*driven by Apple MacIntosh 'C-Scan Software'

4.1 RESULTS AND DISCUSSION

The regulation of adenylate cyclase by lipolytic hormones in lean (Fa/?) and obese (*fa/fa*) Zucker rat white adipocyte membranes was investigated in order to document any alteration in the hormonal responsiveness of adenylate cyclase in the obese state.

The dose-response to isoproterenol is shown in Fig 4.1 for membranes from lean and obese animals. Control activity was equivalent in membranes derived from both lean and obese animals. The maximal specific activity (V_{max}) stimulated by saturation concentrations of isoproterenol was markedly lower in membranes from obese animals than the corresponding activity in membranes from lean animals, yielding fold-stimulations of 16-fold and 6-fold for membranes derived from lean and obese animals respectively (Table 4.1& Fig 4.1). In contrast there was little change in $K_{0.5}$ values for isoproterenol stimulation, being between 6.3±0.8 μM and 8.9±1.1 μM respectively for membranes from lean and obese animals (Table 4.1 & Fig 4.1).

Thus the regulation of adenylate cyclase in adipocytes from the *fa/fa* rat shows similarities with other genetically obese rodents, which also exhibit impaired catecholamine stimulation of adenylate cyclase (ch 1). Indeed, one might predict that the obese Zucker rat is likely to show impaired catecholamine stimulation of adipocyte membrane adenylate cyclase as these animals are hypothyroid (ch 1), an alteration known to lead to impaired catecholamine stimulation of adipocyte adenylate cyclase activity (ch 1).

In order to explore the kinetics of the alterations further, the dose response to isoproterenol was performed in the presence of 100μM of the non-hydrolysable analog GTPγS (Fig 4.2 & Table 4.1). The activities seen with GTPγS alone were similar for membranes from both lean and obese

animals, as were the V_{max} activities observed in the presence of saturating concentrations of isoproterenol (Table 4.1). The fact that V_{max} activities were similar in Fig 4.2 is at first sight quite surprising given the large difference in V_{max} values seen in Fig 4.1 for isoproterenol stimulation of adenylate cyclase. However it can be seen in Table 4.1 that the V_{max} activity achieved with membranes from lean animals is much less when GTPγS was utilized rather than GTP. The reason for the lower efficacy of GTPγS +isoproterenol, rather than GTP+isoproterenol, is unknown. As a further point of interest, the concentration of isoproterenol which caused half maximal ($K_{0.5}$) activation of adenylate cyclase, done in the presence of GTPγS, was significantly lower than when GTP is used (Table 4.1). Indeed, such a decrease in the $K_{0.5}$ for isoproterenol activation of adenylate cyclase was much smaller when membranes from obese animals were used rather than those from lean: being only 3.6-fold compared with 10.5-fold in membranes from lean animals. Thus the ability of GTPγS to decrease the $K_{0.5}$ for isoproterenol stimulation of adenylate cyclase is severely impaired in membranes from obese animals. Seemingly then, the kinetic changes seen in membranes from obese animals do depend upon which guanyl nucleotide analogue was employed.

The effect of isoproterenol upon the dose response curve to GTPγS was also investigated (Fig 4.3), showing that the V_{max} values for adenylate cyclase in membranes from lean and obese were essentially equivalent as one would predict from Fig 4.2 However, in contrast, the $K_{0.5}$ for GTPγS was markedly lower in membranes from lean animals as opposed to those from obese animals (Fig 4.3). Thus it appears that isoproterenol is less able to bring about an increase in the 'affinity' of G_s for GTPγS in membranes from obese animals. Alternatively isoproterenol may simply be less able to decrease the amount of G_s which has to be activated in order to achieve half

maximal activation of adenylate cyclase in membranes from obese animals. The activity of adenylate cyclase determined with 50μM isoproterenol only is markedly lower in membranes from obese animals than in membranes from lean animals (Fig 4.3). Consequently, the fold stimulation of adenylate cyclase activity by GTPγS is significantly greater in membranes from obese animals than in membranes from lean animals (Fig 4.3).

The response to glucagon in lean and obese membranes, measured against the activity recorded with IOO μM GTP is given in Fig 4.4. Here it can be seen that glucagon achieves a much higher stimulation of adenylate cyclase activity in membranes from lean animals as opposed to membranes from obese animals. In addition, the sensitivity to glucagon stimulation of adenylate cyclase was reduced in membranes from obese animals.

Fig 4.5 details the response of membranes from both lean and obese animals to secretin stimulation of adenylate cyclase assayed in the presence of 100 μM GTP. In membranes from lean animals, secretin stimulated this activity some 4.2-fold however, in contrast, secretin failed to stimulate adenylate cyclase activity significantly in membranes from obese animals.

The response of adenylate cyclase in membranes from lean and obese animals to TSH-mediated stimulation of adenylate cyclase activity done in the presence of 100 μM GTP is detailed in Fig 4.6. Here we see membranes from lean animals the TSH stimulated activity some 3.8-fold, however in membranes from obese animals stimulation of activity by TSH was negligible.

It is thus apparent that the response of adenylate cyclase to all G_S-linked hormones is greatly reduced in membranes from obese animals. In particular, both secretin and TSH show negligible stimulation in membranes from obese animals. It is possible that a common defect at the G-protein level could contribute to all of this. However, the more marked effect seen with TSH and secretin could be due to a receptor specific action at the level of expression or post-translational modification. The number and affinity of β-

AR in membranes from lean and obese animals was determined using the high affinity β-AR antagonist ^{125}I-cyanopinidolol (Table 4.2). This revealed (Table 4.2) that there was a roughly half the number of β-AR in membranes from obese animals compared with that found using membranes from lean animals. There was no apparent change in the affinity for ^{125}I-CYP, the K_d being similar in both instances. The reduced response of adenylate cyclase to isoproterenol stimulation in membranes from obese animals could simply be due to the reduced number of β-AR. However reduced β-adrenergic receptor number does not necessarily lead to a reduction in catecholamine stimulated adenylate cyclase activity, as spare ß-AR are believed to occur in adipocytes. Thus, a quite substantial reduction in the number of ß-AR is believed to be needed to occur before any reduction in isoproterenol stimulated adenylate cyclase activity can ensue. It is also relevant that an inherent feature of the Collision Coupling Model, thought to represent the mode of R_sG_s activation of adenylate cyclase in a number of cells, buffers the adenylate cyclase system against any receptor loss (Houslay *et al*, 1980; Braun & Levitzki, 1979). Thus, unless the degree of receptor loss is very large, the V_{max} of adenylate cyclase activity achieved through activation of the R_s in question will be unaffected although $K_{0.5}$ is likely to be increased. Furthermore since $ß_1$, $ß_2$ and $ß_3$-AR are present in adipocytes (Bojanic & Nahorski, 1984; Emorine *et al*, 1989) a detailed analysis of changes of subtypes needs to be done in order to explore this further.

The fact that the response to other R_s hormones was also reduced implies to me that a defect, at a level common to all R_s linked hormones, may exist. Whilst this may be a general reduction in expression or inactivation of receptors by phosphorylation, it could also be at the level of G_s, or at the catalytic unit of adenylate cyclase itself. In this regard, we assessed the functional status of the catalytic unit of adenylate cyclase by using direct, G-

protein-independent activators. Table 4.2 records the stimulation of adenylate cyclase activity by the diterpene forskolin, NaF and $MnCl_2$. These showed little difference between adenylate cyclase in membranes from both lean and obese. Thus the functioning of the catalytic unit and its stimulation by G_S is, as far as we can detect, identical in membranes from lean and obese animals. Therefore it would appear that the defective hormonal stimulation of adenylate cyclase in membranes from obese animals is not due to a defect in the catalytic unit of adenylate cyclase or in the ability of G_S to stimulate it. This suggests that the defect is at the level of R_S or at the receptor coupling interface of G_S.

The receptor-independent G-protein stimulation of adenylate cyclase was also assessed by monitoring the stimulation of adenylate cyclase by GTPγS in the absence of any R_S linked agonist Fig 4.7. Fig 4.7 shows that the degree of activation was identical for membranes from both lean and obese animals. However, the $K_{0.5}$ GTPγS was higher in membranes from obese animals than those from lean animals (Fig 4.7). Thus the membranes from obese animals appear to show a defect at the level of G_S functioning. Possible explanations for this are (i) adipocyte G_S molecules in membranes from lean and obese animals may be different and could actually exhibit different K_ds values for GTPγS, such that the affinity for GTPγS was decreased in the obese state; (ii) whilst, the actual affinity of G_S molecules for GTPγS in both instances might be identical, the amount of G_S which has to be activated to yield a particular degree of activation of adenylate cyclase might be different and (iii) the levels of free ß-subunits may be different thus altering the ratio of G_S in the holomeric state/G_S in dissociated state and hence altering the $K_{0.5}$ for G_S activation of adenylate cyclase.

The levels of α subunits and β subunit of G_S in membranes from lean and obese animals were compared by immunoblotting with specific anti-

peptide antisera. The result of such studies, using the antiserum CS1 to detect α_s specifically, is shown in Fig 4.8 and the quantification of relative changes in levels is given in Table 4.5. It can be seen there is a reduction of the 42kD form of G_s species in membranes from obese animals, such that levels fall to some 50±6% of those in membranes from lean animals. Levels of the 45kD form of α_s were also reduced. Thus the reduction in the levels of G_s and changes in ratio of the forms may explain the increase in the $K_{0.5}GTP\gamma S$ seen in membranes from obese animals in Fig 4.7.

Immunoblotting with antiserum BN1, which specifically recognizes G-protein β subunits showed no significant change in the levels of this species between membranes from lean and obese animals (Fig 4.9 and Table 4.5). However at least two β subunits, 36 KD β1 and 35 KD β2 are known to exist in adipocyte membranes and others have been recently identified from cloning studies (ch 1). The antiserum BN1 identifies both ß1 and ß2 forms of subunit. Therefore, because we do not have the reagents to distinguish between various forms in adipocyte membranes, we cannot eliminate the possibility that there has been an alteration in the relative proportions of different ß-subunits. Neither can we rule out the possibility of an alteration in the γ-subunits associated with the β subunits of which their are believed to be multiple types (ch 1).

Increased activity of G_i could also impede hormonal stimulation of adenylate cyclase via increased tonic inhibition of adenylate cyclase (ch 1). To this end we determined the levels of α_i-1,2 and 3 in membranes from lean and obese animals. Fig 4.10 shows the result of an immunoblot with antiserum AS7, specific for the 41 KD α_i-1 and 40 KD α_i-2 proteins. We observed (Fig 4.10; Table 4.5) that there was a large reduction in the level of α_i1 in membranes from obese animals, such that its level is only some 35±6% of that found in membranes from lean animals. There was no obvious alteration in the level of α_i2 in membranes from obese animals Fig 4.10 and

Table 4.5.

We examined the ability of the R_i-linked receptor agonists PIA, PGE_1 and nicotinic acid to inhibit isoproterenol stimulated adenylate cyclase (Table 4.3). We noted that the maximum inhibition achieved, using membranes from lean and obese animals, was similar. We also tested the ability of the R_i linked agonists to inhibit forskolin stimulated adenylate cyclase (Table 4.4). The results showed that the maximum inhibition of forskolin stimulated adenylate cyclase achieved was similar for these ligands irrespective of the membranes used.

The ability of P-site agonists to inhibit adenylate cyclase activity was determined. This was done using a saturating concentration of 2',5'-dideoxyadenosine to inhibit isoproterenol (500 μM) and forskolin (100 μM) stimulated adenylate cyclase. The degree of inhibition was identical using membranes from both lean and obese animals. Thus the functional capacity of the P-site appears to be unaltered in the obese state.

The ability of G_i to exert 'tonic' inhibition on adenylate cyclase was determined by the ability of GppNHp to inhibit forskolin stimulated adenylate cyclase (Fig 4.12). This showed that GppNHp inhibited adenylate cyclase activity only in membranes from lean animals.

Obese Zucker rats are hypothyroid and in normal rats made hypothyroid a number of defects have been identified (ch 1) which are consistent with a reduced efficacy of R_S receptor linked agonists. These include a reduced ß-AR action without any reduction in ß-AR number. However as we see here for membranes from obese animals, they exhibit no change in levels of α_S and no apparent change in the functioning of the catalytic unit of adenylate cyclase (ch 1). A point of difference concerns G_i expression where in membranes from hypothyroid animals there is an increase in α_i-1, no alteration in α_i-2 but an increase in β-subunit levels.

Consistent with the changes I have observed in adipocyte membranes, membranes prepared from obese Zucker rat hearts also show reduced stimulation of adenylate cyclase by isoproterenol, secretin and other R_S-type agonists (chatelain *et al*, 1981). However, such changes are not uniform throughout the various tissues of the obese animals since membranes from both hepatocytes and anterior hypophysis (Robberecht *et al*, 1983) do not show the same decrease in the efficacy of R_S-linked hormones.

The brown adipose tissue of obese Zucker rats shows a 40% decrease in isoproterenol stimulated activity associated with a 38% decrease in NaF-stimulated adenylate cyclase activity (Muzzin *et al*, 1989). Therefore the changes in the ß-AR adenylate cyclase system of obese Zucker rat brown adipose tissue are distinct to those of white adipocytes reported here. Other obesity model systems such as the obese (*ob/ob*) mouse, the diabetic mouse (*db/db*), the high-fat diet-fed rat, the Toronto-KK mouse and the New Zealand obese mouse (ch 1) show the same decreased efficacy to catecholamines in adipocytes as we report here for obese Zucker rats. The pathological significance of this latter trend could relate to the developement of obesity itself. The *ob/ob* mouse, the *db/db* mouse, the Toronto-KK, and the New Zealand obese mouse (ch 1) all exhibit reduced catecholamine-stimulated lipolysis. Increased adipocyte volume, due to increased triglyceride globule mass, is a major feature of hyperadiposity of obesity. Since cAMP concentrations appear to be the major determinant of adipocyte lipolytic rates then the defect in adenylate cyclase in the obesity models mentioned above would certainly contribute to, and could in principle be the cause of (or significantly contribute to), the altered adipocyte morphology characteristic of obesity.

CHAPTER 5

REGULATION OF ADENYLATE CYCLASE ACTTIVITY IN THE ADIPOSE TISSUE FROM DIABETIC (*db/db*) MICE

Fig 5.1 Dose response curve for isoproterenol stimulation of adenylate cyclase in adipocyte membranes from +/+ and *db/db* mice.

Adenylate cyclase activity was assayed with increasing concentrations of isoproterenol in the presence of 100μM GTP. In membranes from +/+ mice the maximal specific activity, V_{max}, was 300 pmol/min/mg and the activity with GTP alone was 4.1±1.2 pmol/min/mg. In membranes from *db/db* mice the V_{max} activity was 160±12 pmol/min/mg and the activty in the presence of GTP alone was 4.2±0.5 pmol/min/mg. The data shows mean and standard deviations of a typical experiment repeated 3 times with different membrane preparations.

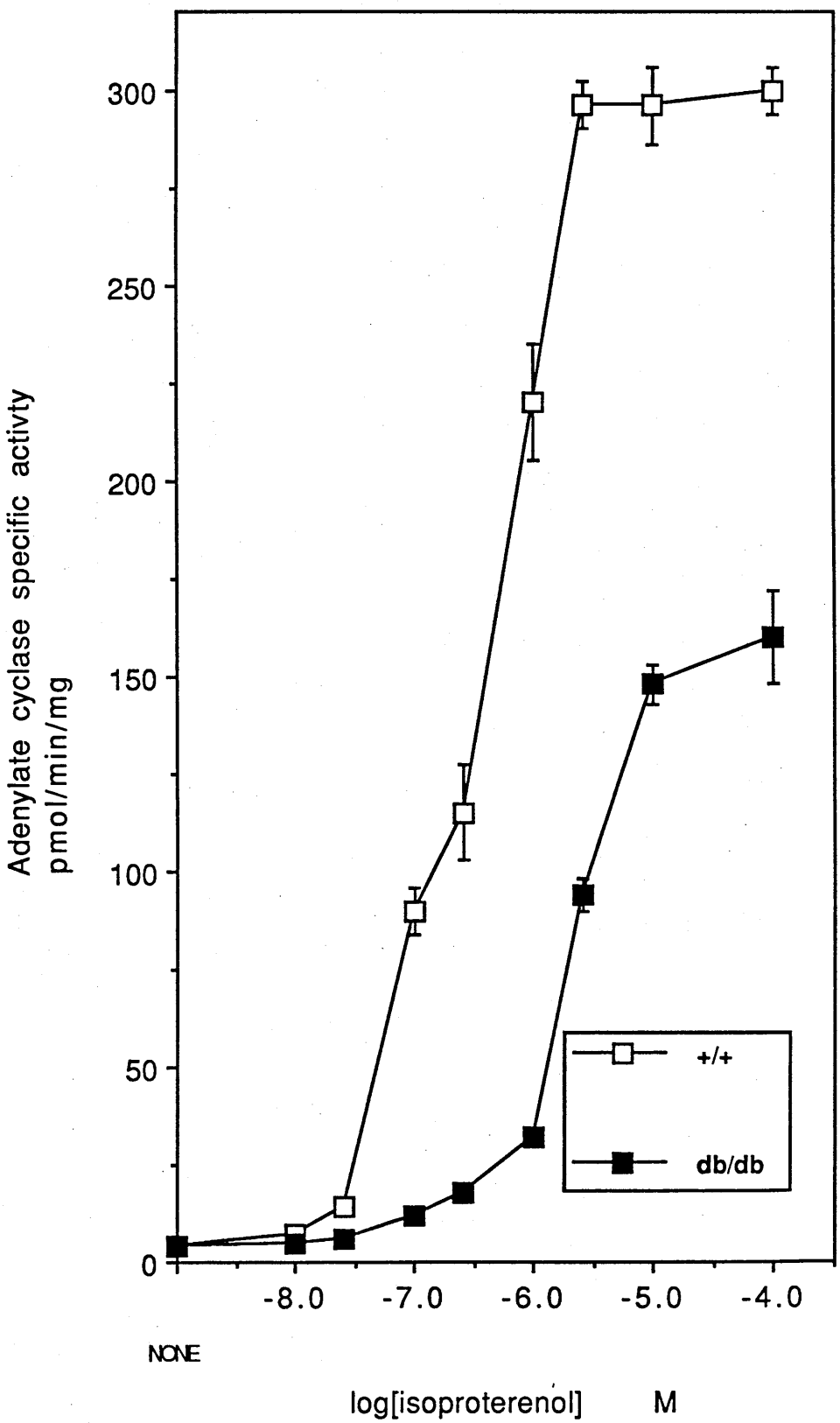

Adenylate cyclase specific activty
pmol/min/mg
300
250
200
150
100
50
0
-8.0
-7.0
-6.0
-5.0
-4.0
NONE
log[isoproterenol] M
+/+
db/db

Fig 5.2 Dose response curve for glucagon stimulation of adipocyte membrane adenylate cyclase from +/+ and *db/db* mice.

Adenylate cyclase was assayed with increasing concentrations of glucagon in the presence of 100μM GTP. In membranes from +/+ mice (closed diamond) the V_{max} activity was 33 pmol/min/mg, the activity in the presence of GTP alone was 5.9±0.3 pmol/min/mg and the fold-stimulation by glucagon was 5.5-fold. In membranes from *db/db* mice (open square) the V_{max} activity was 16.1 pmol/min/mg, the activty in the presence of GTP alone was 6.3±1.3 pmol/min/mg and the fold stimulation by glucagon was 2.7-fold. The result shows mean and standard deviation values for a typical experiment repeated three times with different membrane preparations each time.

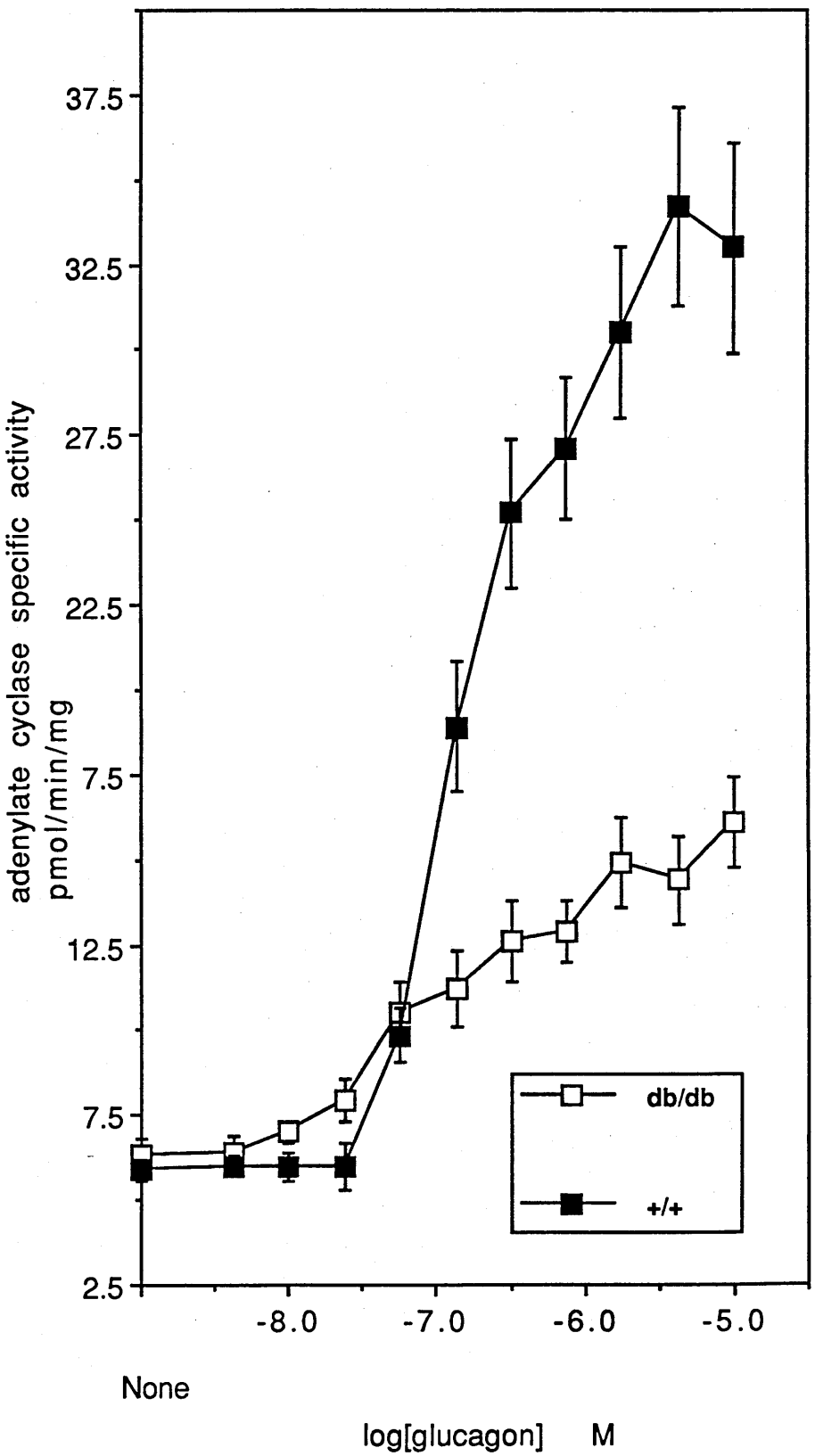

37.5
32.5
27.5
22.5
7.5
12.5
7.5
2.5
adenylate cyclase specific activity
pmol/min/mg
db/db
+/+
-8.0
-7.0
-6.0
-5.0
None
log[glucagon] M

Fig 5.3 Dose response curve for TSH stimulation of adenylate cyclase activity in membranes from +/+ and *db/db* mice

The effect of increasing concentrations of TSH in the presence of 100μM GTP were determined. In membranes from +/+ mice (open square) the V_{max} activity was 18.2±1.8 pmol/min/mg, the activty in the presence of GTP alone was 8.3±0.8 pmol/min/mg and the fold stimulation by TSH was 2.7-fold. In membranes from *db/db* mice (closed diamond) the V_{max} activity was 18.0±1.9 pmol/min/mg, the activity in the presence of GTP alone was 8.1±0.7 pmol/min/mg and the fold stimulation by TSH was 2.7-fold. The data shows a typical result repeated three times with separate membrane preparations of +/+ and *db/db* mice with data points showing means and standard deviations.

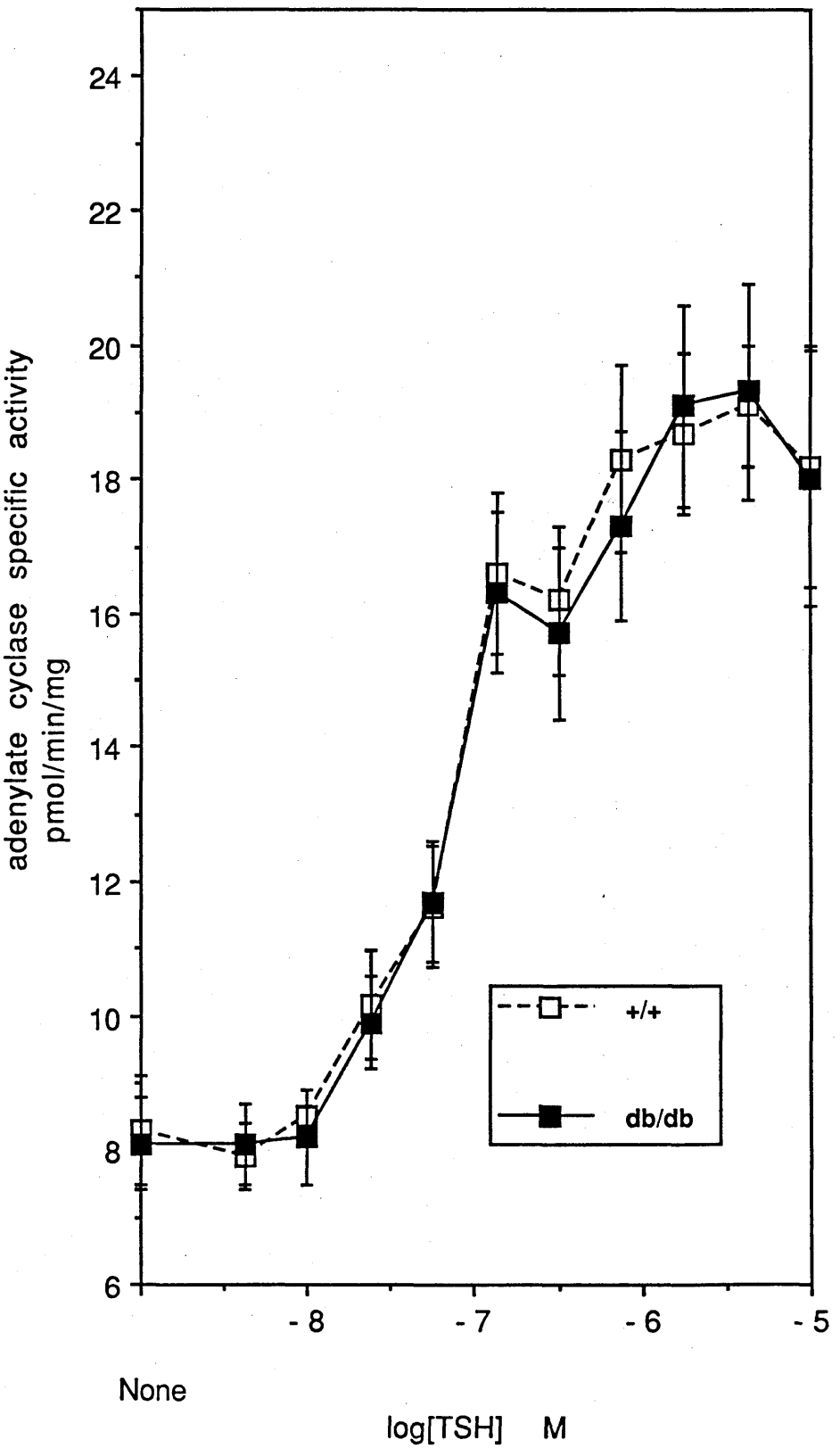

adenylate cyclase specific activity
pmol/min/mg
24
22
20
18
16
14
12
10
8
6
+/+
db/db
- 8
- 7
- 6
- 5
None
log[TSH] M

Fig 5.4 Dose response curve for secretin stimulation of adenylate cyclase activty in adipocyte membranes from +/+ and *db/db* mice.

Adenylate cyclase activity was assayed in the presence of increasing concentrations of secretin in the presence of 100μM GTP. In membranes from +/+ mice (open square) V_{max} activity was 24±1 pmol/min/mg, activity in the presence of GTP alone was 7.2±0.5 pmol/min/mg and fold stimulation by secretin was 3.4-fold. In membranes from *db/db* mice (closed diamond) the V_{max} activty was 13.5±1.7 pmol/min/mg, the alone activty in the presence of GTP alone was 6.0±0.6 pmol/min/mg. and the fold stimulation was 2.3-fold. The data shows means and standard deviations for a typical experiment repeated three times with different membrane preparations.

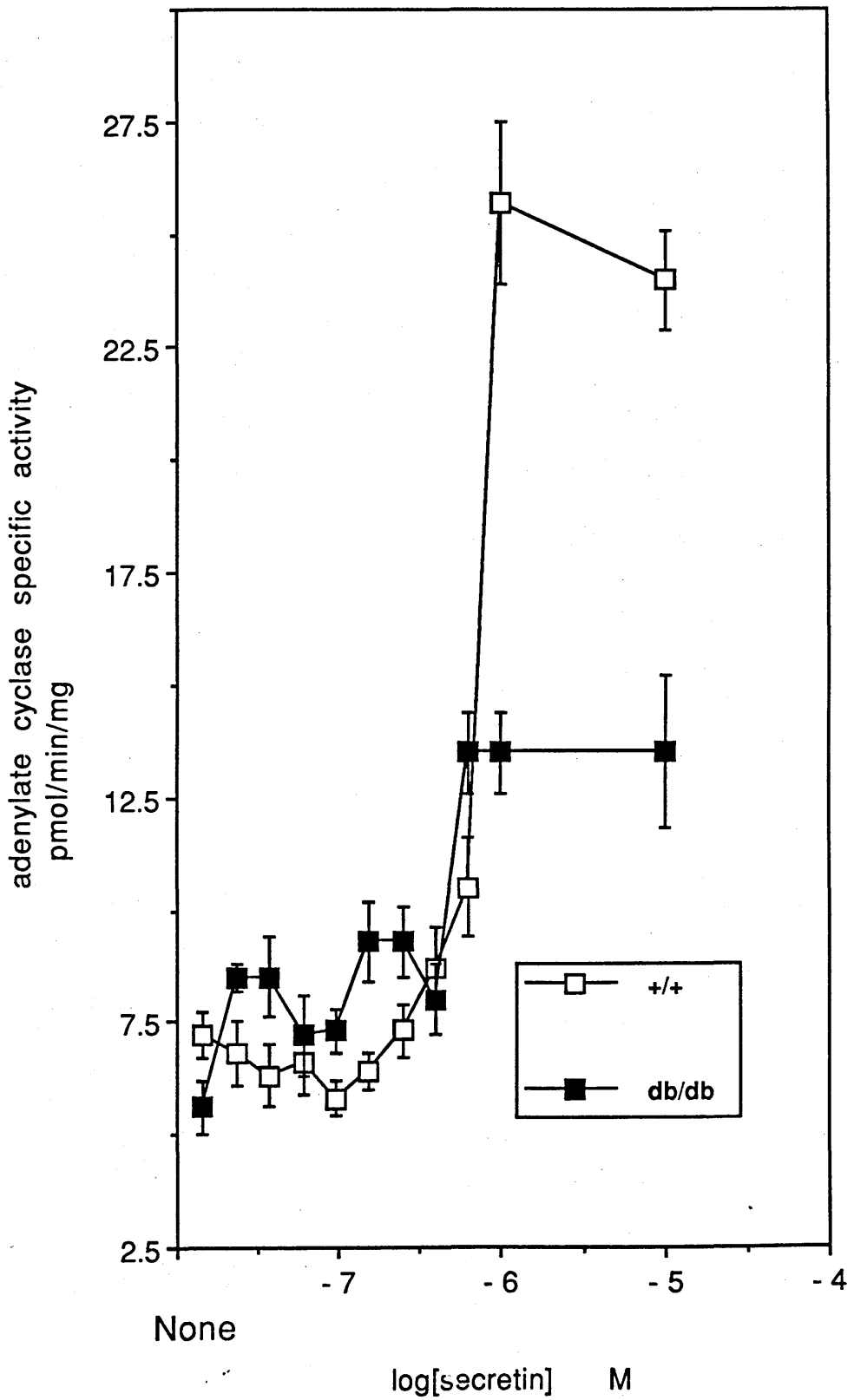

adenylate cyclase specific activity
pmol/min/mg
27.5
22.5
17.5
12.5
7.5
2.5
- 7
- 6
- 5
- 4
None
log[secretin] M
+/+
db/db

Fig 5.5 Immunoblot detection of α_s in adipocyte membranes from +/+ and *db/db* mice.

The antiserum CS1 was used to detect the α-subunit of G_s in adipocyte membranes. Data was determined from four different +/+ and four different *db/db* membrane preparations with comparisons being made of loadings of 40μg of membrane protein, over which linear increases in absorbance were obtained in quantitation studies as described in Materials and Methods Section. Quantification data is given in Table 5.2.

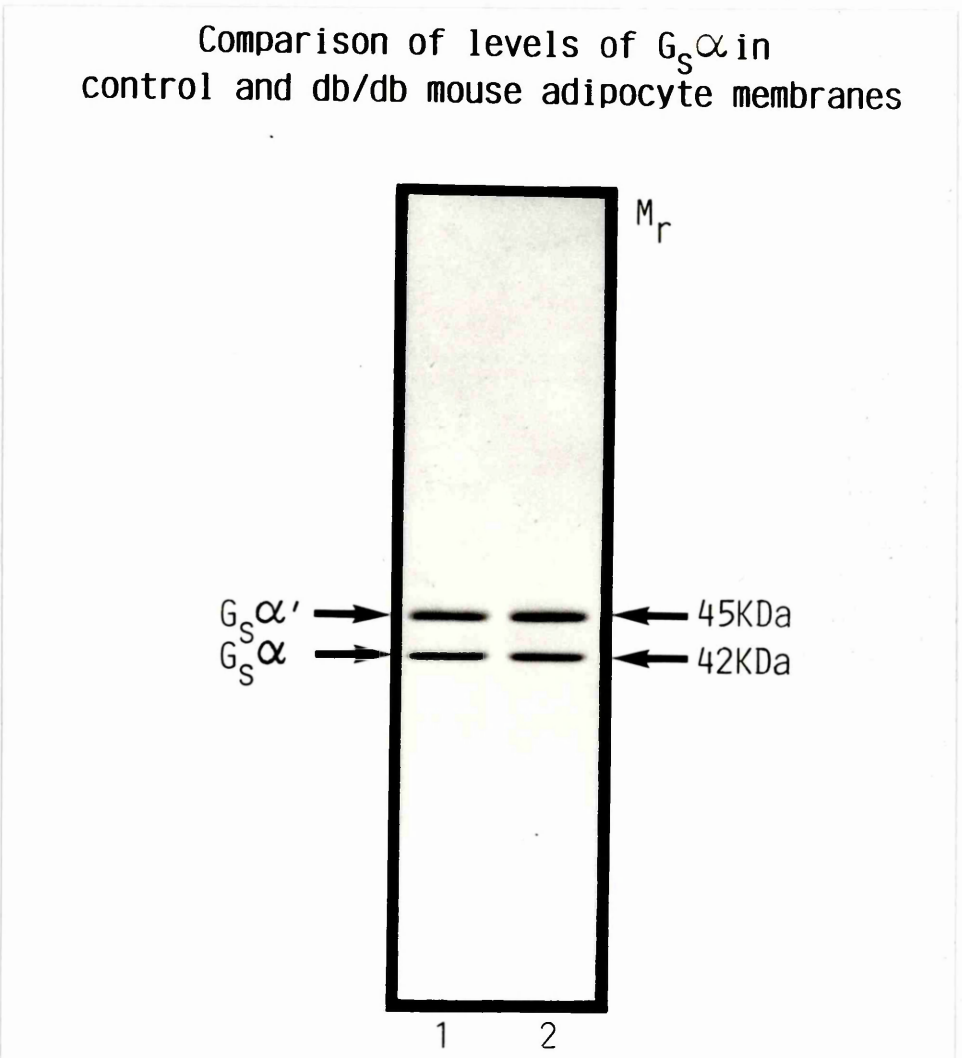


Fig 5.6 Immunodetection of G-protein ß-subunit in adipocyte membranes from +/+ and *db/db* mice.

The antiserum BN1 was used to detect the ß-subunit in adipocyte membranes. Data were obtained from four different +/+ and *db/db* mice with comparisons being made between 70µg of protein, over which linear increases in absorbance were obtained in quantitation studies as described in chapter 2. Quantification of this data is given in Table 5.2.

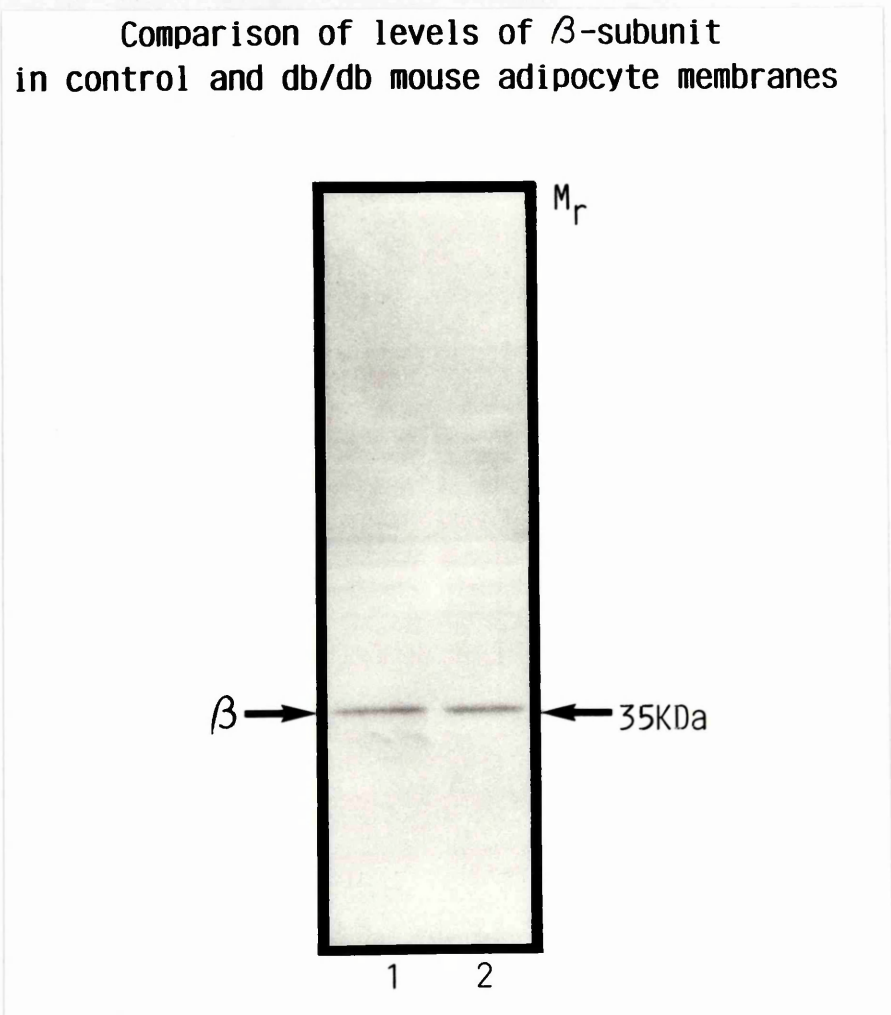


Fig 5.7 Immunodetection of α_i1 and α_i2 in adipocyte membranes from +/+ and db/db mice

The antiserum AS7 was used to detect the $\alpha_i1$and 2 subunits of G_i. Data was obtained from four different +/+ and *db/db* membrane preparations with comparisons being made between 30µg of membrane protein, over which linear increases in absorbance were obtained in quantitation studies as described in chapter 2. Quantification of this data is given in Table 5.2.

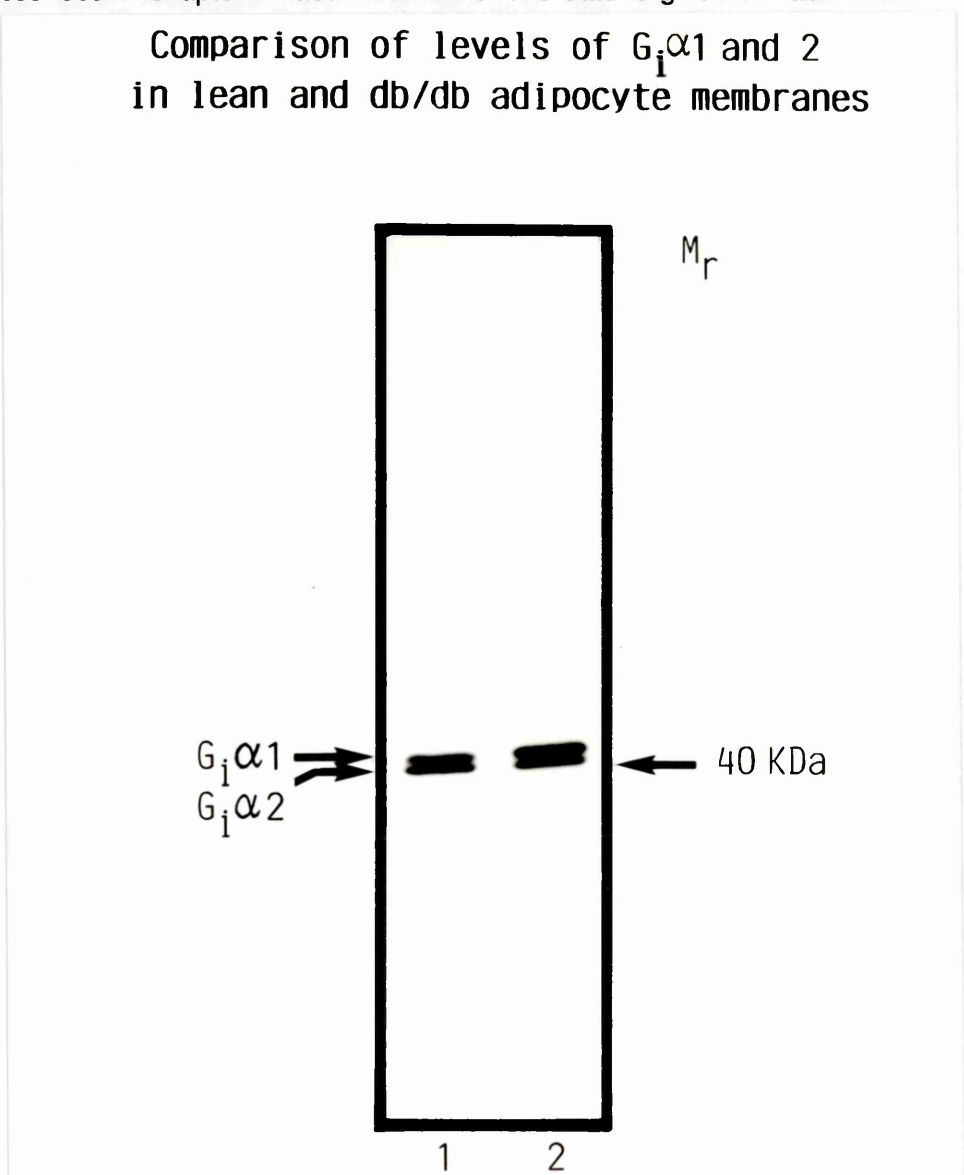


Fig 5.8 Immunodetection of α_i3 in adipocyte membranes from +/+ and *db/db* mice.

The antiserum I3B was used to detect α_i3 in adipocyte membranes. Data were obtained from four different membrane preparations from +/+ and *db/db* mice and comparisons of 100µg of membrane protein are shown, over which linear increases in absorbance were obtained in quantitation studies as described in chapter 2. Quantification data is given in Table 5.2.

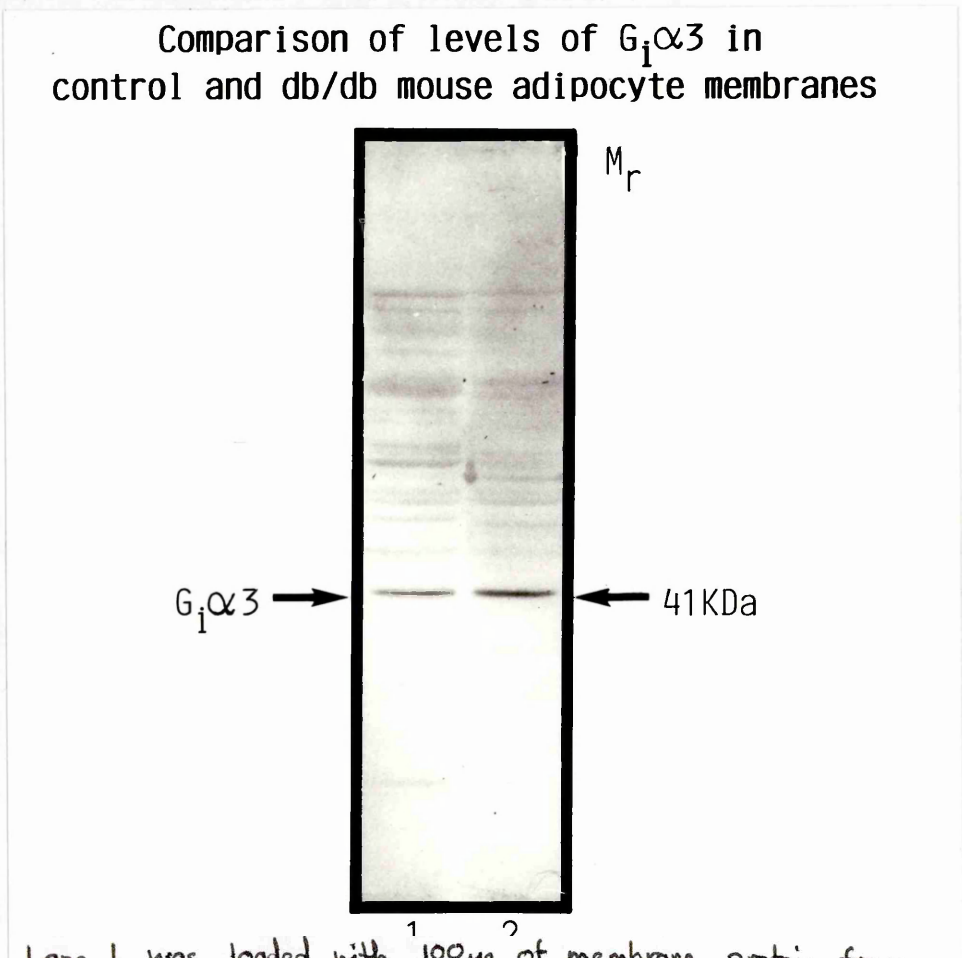


Fig 5.9 Dose response curve to PIA mediated inhibition of isoproterenol stimulated adenylate cyclase of adipocyte membranes from +/+ and *db/db* mice.

The effects of increasing concentrations of PIA upon the activity of isoproterenol (500μM) stimulated adenylate cyclase done in the presence of 100μM GTP were determined. The data shows means and standard deviations of a typical experiment repeated three times with different membrane preparations. In membranes from both *db/db* and +/+ mice the IC_{50} for PIA was 25±8nM. However in membranes from +/+ mice the maximal percentage inhibition was 60±5%, while in membranes from *db/db* mice it was 80±10%.

PIA-MEDIATED INHIBITION OF ADENYLATE CYCLASE IN ADIPOCYTE MEMBRANES

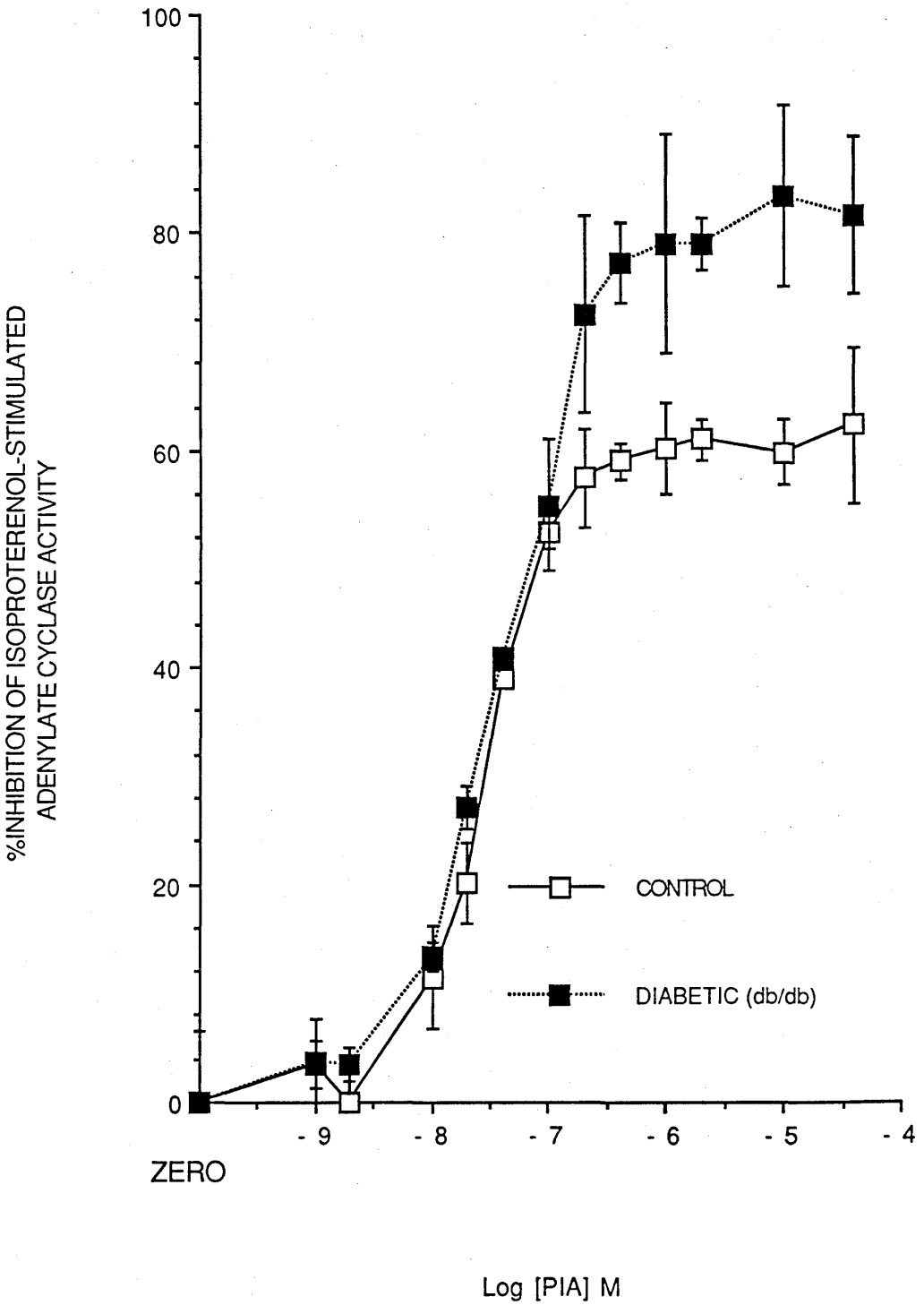


Fig 5.10 Dose response curve to PGE_1 mediated inhibition of isoproterenol stimulated adenylate cyclase of adipocyte membranes from +/+ and *db/db* mice.

The effect of increasing concentrations of PGE_1 upon isoproterenol (500μM) stimulated adenylate cyclase were determined in the presence of 100μM GTP. The results means and standard deviations of a typical result repeated three times in separate membrane preparations. The IC_{50} for PGE_1 in membranes from both +/+ and *db/db* mice were 15nM.

PROSTAGLANDIN E1-MEDIATED INHIBITION
OF ADENYLATE CYCLASE IN ADIPOCYTE MEMBRANES

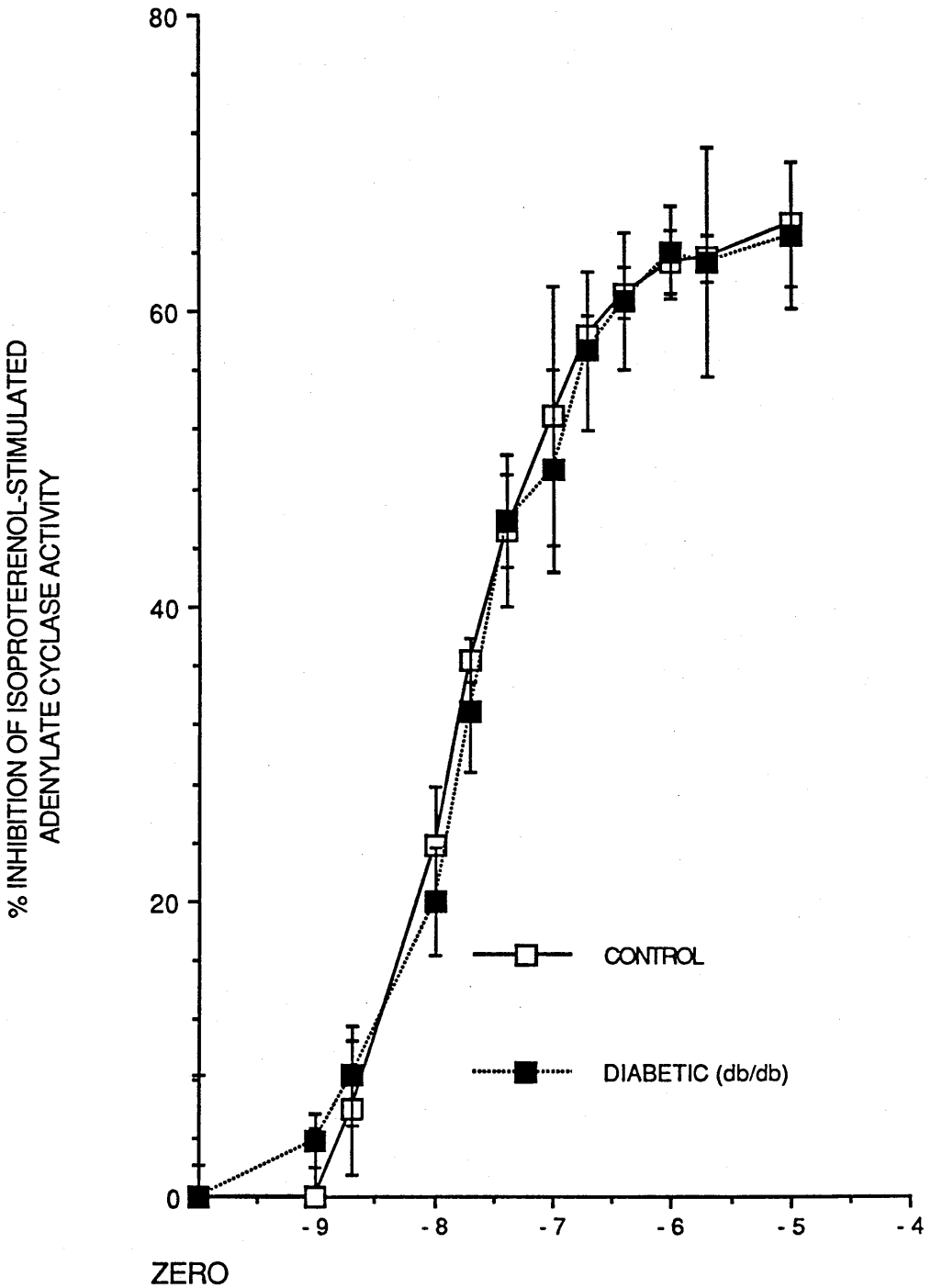


Fig 5.11 Dose response curve to nicotinic acid mediated inhibition of isoproterenol stimulated adenylate cyclase in adipocyte membranes from +/+ and *db/db* mice.

The effect of increasing concentrations of nicotinic acid upon adenylate cyclase in the presence of 100μM GTP and 500μM isoproterenol were determined. The data shows means and standard deviations for a typical experiment representative of three performed with different membrane preparations. In membranes from +/+ and *db/db* mice the IC_{50}s for nicotinic acid were 315nM and 562nM respectively. The maximal degree of inhibition by nicotinic acid was equivalent in membranes from both +/+ and *db/db* mice at 70±6%.

NICOTINIC ACID-MEDIATED INHIBITION OF
ADENYLATE CYCLASE IN ADIPOCYTE MEMBRANES

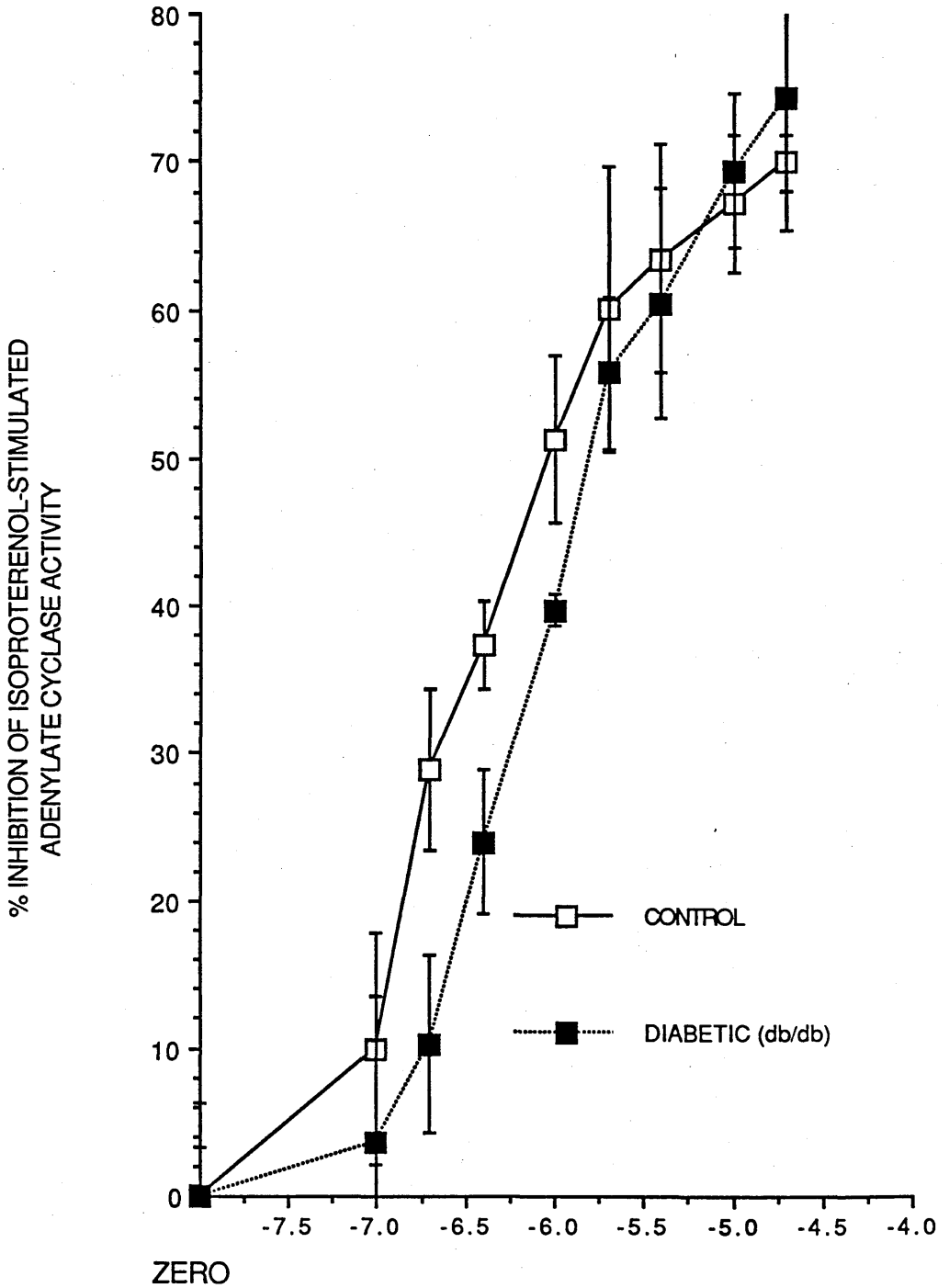


Fig 5.12 Dose response of forskolin stimulated adenylate cylcase to GppNHp.

The effect of increasing concentrations of GppNHp upon forskolin (100μM) stimulated adenylate cyclase was determined. The data shows means and standard deviations of a typical experiment representative of three performed with different membrane preparations. In membranes from +/+ mice (open square) the control activity, represented as 100% stimulation, with 100μM forskolin alone was 178±13 pmol/min/mg. In membranes from *db/db* mice (closed diamond) the control activity, represented as 100% stimulation, with 100μM forskolin alone was 183±15 pmol/min/mg.

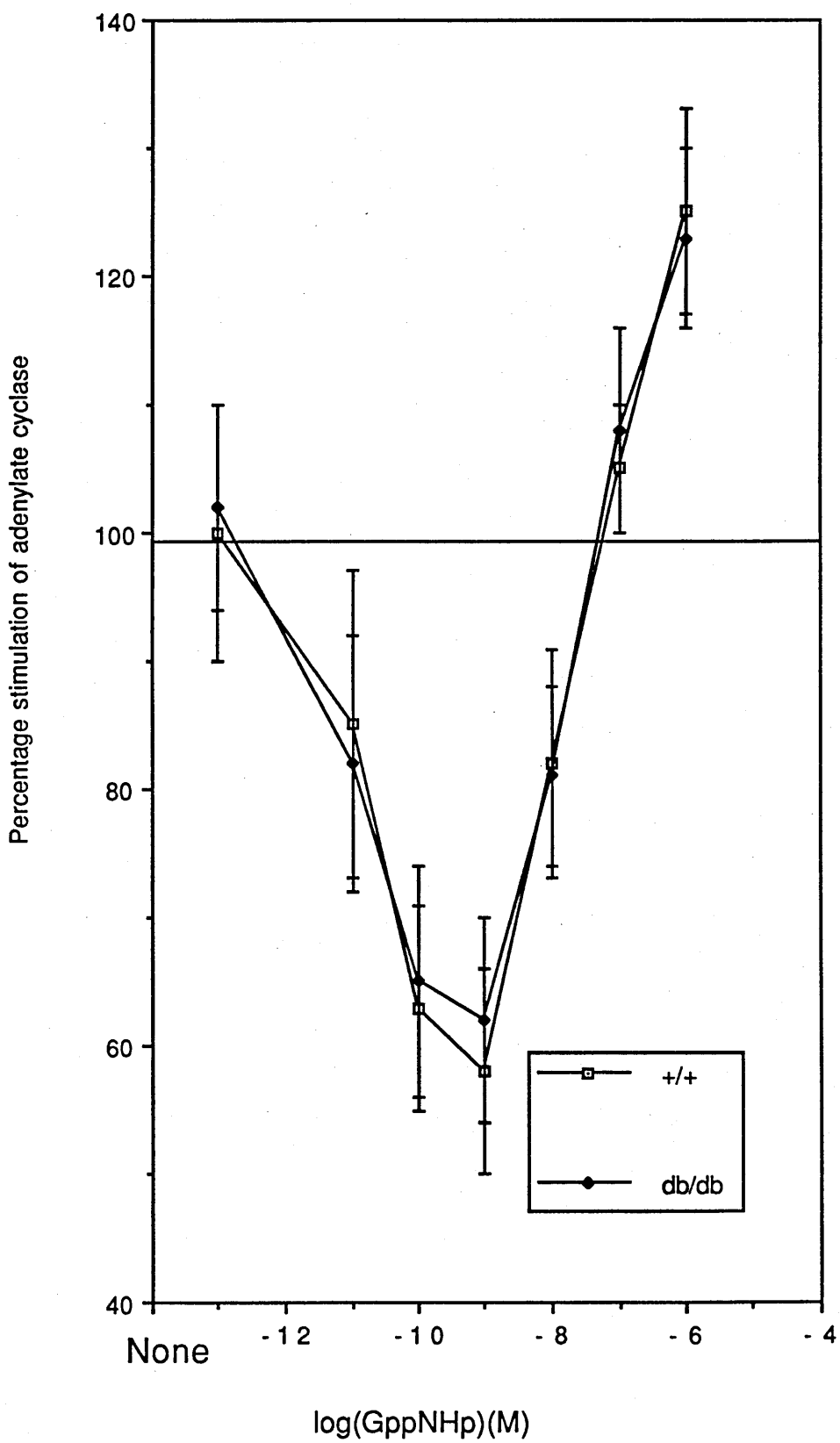
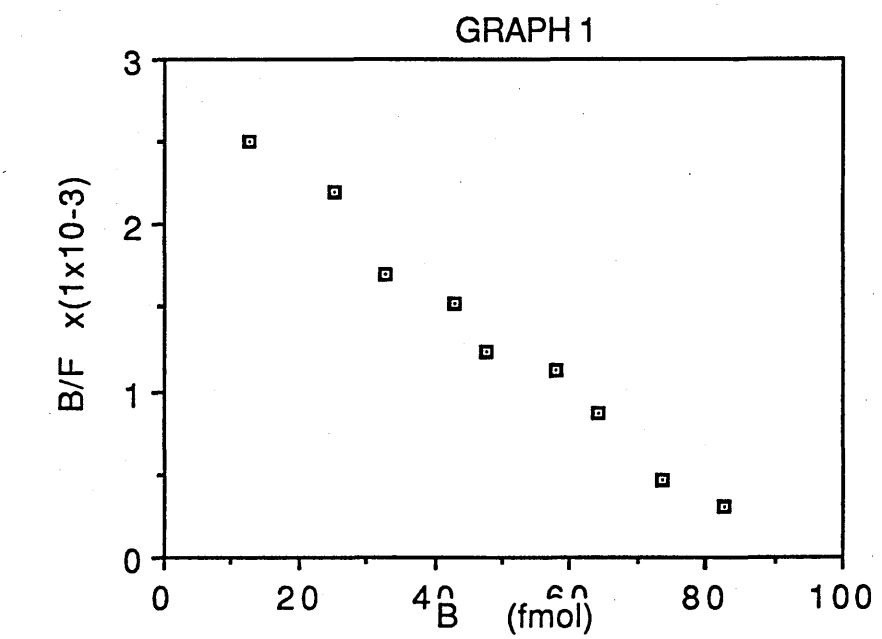

140
120
100
80
60
40
Percentage stimulation of adenylate cyclase
None
-12
-10
-8
-6
-4
log(GppNHp)(M)
+/+
db/db

Fig 5.13 Scatchard representation of ^{125}I-cyanopinidol binding to +/+ and *db/db* mouse adipocyte membranes.

Binding of ^{125}I-CYP to membranes was determined as described in Material and Methods. The parameters B_{max} and K_d are given in Table 5.1. Graphs 1 & 2 show the plots for membranes from +/+ and db/db animals respectively.



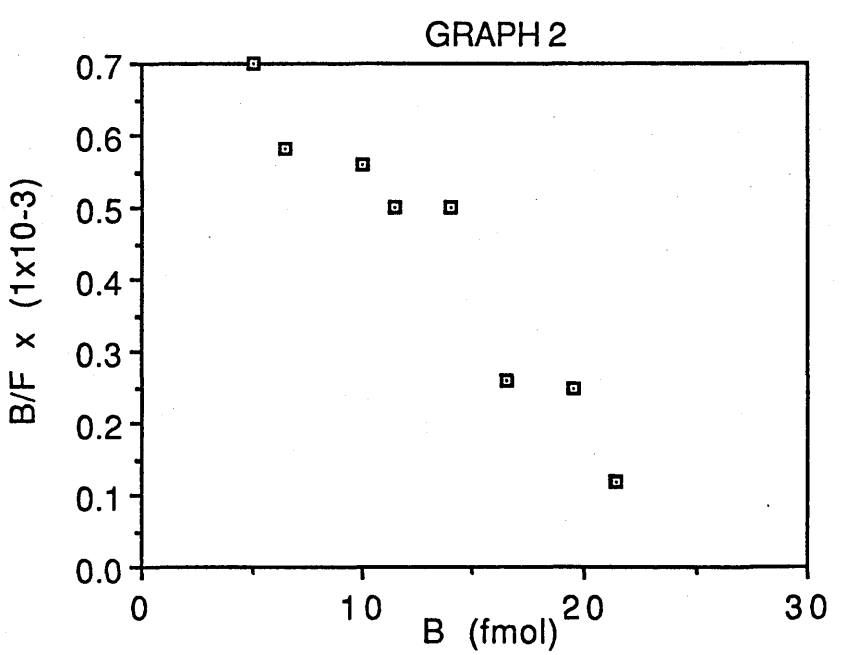


Table 5.1 Receptor-independent stimulation of adenylate cyclase and determination of ß-adrenergic receptor number in membranes from +/+ and *db/db* mice.

Adenylate cyclase was assayed in the presence of fixed concentrations of either forskolin or NaF. Binding of ^{125}I-CYP to membranes was determined as described in Materials and Methods. Binding parameters B_{max} (Total specific binding) and K_d (equilibrium dissociation constant) were determined by Scatchard analysis of the data which fitted a straight line on the Scatchard representation (Fig 5.13). The results for the adenylate cyclase determinations show means and standard deviations of an experiment typical of four, performed on different membrane preparations from +/+ and *db/db* mice.

	ADENYLATE CYCLASE ACTIVATOR		^{125}I-CYP BINDING	
	100µM forskolin (pmol/min/mg)	20mM NaF (pmol/min/mg)	B_{max} (fmol/mg)	K_d (pM)
Control (+/+)	180±14	52±3	90±5	30
Diabetic (*db/db*)	175±12	51±5	26±6	32

Table 5.2 Quantification of relative changes in G-protein levels between membranes from +/+ and *db/db* mice.

Immunoblotting was performed as outlined in chpt 2. Immunoblots were quantified by densometric scanning of blots employing non-saturating amounts of membrane protein (appendix).

	DIABETIC(*db/db*)	ANTISERUM
α_s45	108±7%	CS1
α_s42	111±7%	CS1
β_{35+36}	105±2%	BN1
α_i1	192±8%	AS7
α_i2	115±12%	AS7
α_i3	115±10%	I3B

* data shown as % (percentage) of that found using membranes from lean animals (100%).

An Abaton-300 densiometric scanner driven by Apple MacIntosh 'C-Scan Software' was employed for the purpose of densiometric scanning.

5.1 INTRODUCTION

Previous studies have shown stimulation of adenylate cyclase activity and lipolysis by catecholamines was significantly reduced in adipocytes from *db/db* mice in comparison with adipocytes from control animals (ch 1). The appropriate control animals are the C57BL/KsJ mice and are here after referred to as +/+ mice. As mentioned before (ch 1), *db/db* mice are obese with excessive deposition of adipose tissue. A number of other rodent obesity model systems, specifically the Toronto-KK mouse, the Zucker fatty rat and the New Zealand obese mouse, all exhibit reduced stimulation of adenylate cyclase by catecholamines (ch 1). Since this defect is potentially of great pathological significance in etiology of obesity, it was decided to investigate the defect in the *db/db* mouse system more fully. Thus a full characterization of the regulation of adenylate cyclase by all hormones and autocrine factors controlling lipolysis was conducted.

5.2 RESULTS AND DISCUSSION

In adipocyte membranes from +/+ mice, isoproterenol stimulated adenylate cyclase activity to a much greater degree (higher V_{max}) and with higher sensitivity (lower $K_{0.5}$ for isoproterenol)in membranes from +/+ mice than in membranes from *db/db* mice (fig 5.1). This result confirms previous studies (ch 1) that detail a large reduction in the efficacy of catecholamines to stimulate adenylate cyclase in membranes from *db/db* mice compared with their controls. I also confirmed that there is no alteration in the activity of adenylate cyclase in the presence of 100μM GTP alone, between membranes from +/+ or *db/db* mice.

The dose response curve for the stimulation of adenylate cyclase by glucagon (Fig 5.2) shows that in membranes from +/+ mice, glucagon stimulated adenylate cyclase to a much greater degree than in membranes

from *db/db* mice.

The dose response curve (Fig 5.3) for the stimulation of adenylate cyclase by TSH was found to be similar both in terms of $K_{0.5}$ and V_{max} in membranes from +/+ and *db/db* mice.

Analysis of dose response curve for the stimulation of adenylate cyclase by secretin (Fig 5.4) shows that in membranes from +/+ mice, secretin stimulated adenylate cyclase activity to a much greater extent than in membranes from *db/db* mice.

Thus it appears that the defect in catecholamine stimulation of adenylate cyclase in membranes from *db/db* mice extends to glucagon and secretin but not to TSH. Therefore, I submit that receptor specific defects may occur.

Nevertheless, to assay G_s functioning, I determined the ability of NaF to stimulated adenylate cyclase activity (Table 5.1) which was similar in membranes from both +/+ and *db/db* mice. Also, direct stimulation of adenylate cyclase activity activity by forskolin was similar in membranes from both +/+ and *db/db* mice (Table 5.1).

The number and affinity of ß-AR for ligand was determined using the high affinity antagonist ^{125}I-cyanopindolol in membrane from +/+ and *db/db* mice (Table 5.1). This showed that there was a very marked reduction in the maximal specific binding (B_{max}) in membranes from *db/db* but essentially no change in K_d was observed. This large reduction in B_{max} of membranes from *db/db* mice could, in principle, explain the reduced response to isoproterenol. The uncertainty arises because there may well be spare ß-ARs such that a very substantial decrease in receptor number may have to occur before an alteration in the V_{max} of adenylate cyclase stimulation by isoproterenol is reduced (ch 4).

I also conducted immunoblot analysis to determine the levels of G-

proteins in membranes from +/+ and *db/db* mice using anti-peptide antisera recognizing specific G-protein subunits. Using the antiserum CS1, which specifically recognizes α_s subunit of G_s (Fig 5.5) I found no difference in the levels of either 42kD or 45kD α_s subunits in adipocyte membranes from both types of animal (Table 5.2). Thus the reduced efficacy of isoproterenol, glucagon and secretin in adipocytes *db/db* mice cannot be due to a selective reduction in one form of α_s.

Immunobloting membranes from +/+ and *db/db* mice with antiserum BN1, which recognizes the G-protein ß-subunit, shows that there is no detectable difference between membranes from +/+ and *db/db* mice Fig 5.6 (Table 5.2 for quantification). Therefore the reduced efficacy of isoproterenol, secretin and glucagon in membranes from *db/db* mice cannot be due to an increase in the levels of ß-subunits which might reduce hormonal activation of adenylate cyclase by inhibiting α_s dissociation.

Reduced efficacy of G_s linked hormones could also be due to increased levels of α_i in membranes from *db/db* mice, a situation which occurs in hypothyroid rat adipocyte membranes (ch 1). In order to address this question membranes from +/+ and *db/db* animals were immunobloted with antiserum AS7 which specifically recognizes α_i1 and α_i2 (Fig 5.7 and 5.2). This showed that membranes from *db/db* mice had nearly 2-fold greater levels of α_i1 compared with membranes from +/+ mice (Table 5.2). Immunobloting of membranes from +/+ and *db/db* mice with antiserum I3B, which specifically recognizes α_i3 (Fig 5.8), showed no alteration in the levels of α_i3 between membranes from both animals. Since the levels of α_i1 do increase substantially, it remains possible that the decreased response of membranes from *db/db* mice to stimulation via certain R_s type receptors could be due to increased tonic inhibition of adenylate cyclase. However it is difficult to imagine how this could be selective and thus not affect stimulation via the TSH receptor. Nevertheless, tonic G_i inhibition of adenylate cyclase

was determined (Fig 5.12) and from this data it was seen that GppNHp-activated G_i can inhibit forskolin stimulated adenylate cyclase equally effectively in membranes from both animals. Thus the reason for the decreased response of membranes from *db/db* mice to isoproterenol, glucagon and secretin is unlikely to be due to increased tonic inhibition of adenylate cyclase in these membranes.

I also examined the response of adenylate cyclase to inhibitory, R_i-G_i linked agonists to see if these responses were altered in membranes from *db/db* mice, since this is a possible point of modulating the ability of adenylate cyclase to be regulated by lipolytic hormones. The dose response curve to PIA in membranes from +/+ and *db/db* mice (Fig 5.9) showed similar IC_{50} values but the maximal degree of inhibition achieved was greater in membranes from *db/db* mice than in membranes from +/+ mice. This difference might serve to magnify the effect of reduced stimulation of adenylate cyclase by catecholamines helping to reduce hormone-stimulated lipolysis *in vivo*. The mechanism behind increased the inhibition seen with PIA in membranes from *db/db* mice may relate to the large increase seen in the levels of α_i1. It could also be due to a change in the activity of A_1-adenosine receptors. Using PGE_1, the maximal degree of inhibition was similar for membranes from both animals (Fig 5.10) as was the case for nicotinic acid (Fig 5.11).

The mechanism(s) responsible for the decreased response of adenylate cyclase to R_s linked hormones, except that for TSH, remains unclear but several possibilities exist. It is possible that a selective reduction in receptor number in *db/db* adipocyte membranes may occur, although nothing is known about the degree of spare ß-ARs. Since the response to TSH is equivalent in adipocyte membranes from +/+ and *db/db* animals one might conclude that the defect probably occurs at a level prior to the

activation of G_S ie a selective loss or modification of these receptors. However this is not necessarily the case since there are two forms of G_S present in these membranes and very little is understood about their relative roles in the regulation of adenylate cyclase. In addition, two different ß-subunits occur in adipocytes, with an uncertain number of γ-subunits (ch 1), there could be anything up to eight or so species of G_S in normal adipocyte membranes. It is possible to envisage that these hypothetical multiple forms of G_S could recognize different R_S type receptors within the adipocyte plasma membrane. Indeed it is possible to conceive of a scenario in which a G-protein defect at the level of G_S has occurred in the adipocytes of *db/db* mice which could be selective for particular groups of receptors. Another possibility is selective uncoupling of R_S type receptors from the stimulation of G_S. This might be achieved by covalent modification of receptors by phosphorylation as occur in ß-AR desensitization. A change in receptor subtype might occur in the case of the ß-AR, such that the normal complement of receptors is replaced with a subtype which is less effectively coupled to activation of G_S.

The *db/db* mouse shows the same trend seen with other known obese rodents, specifically the Toronto-KK mouse; the Zucker fatty rat and the New Zealand obese mouse, in exhibiting decreased stimulation of adenylate cyclase by catecholamines. As pointed out before this defect may play a pivitol role in determining obesity through controlling adipocyte triacylglcerol levels.

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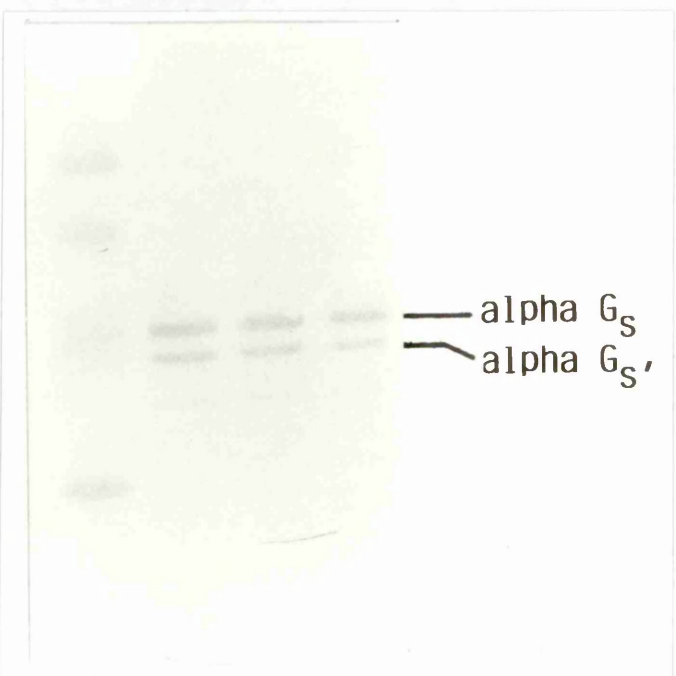
APPENDIX

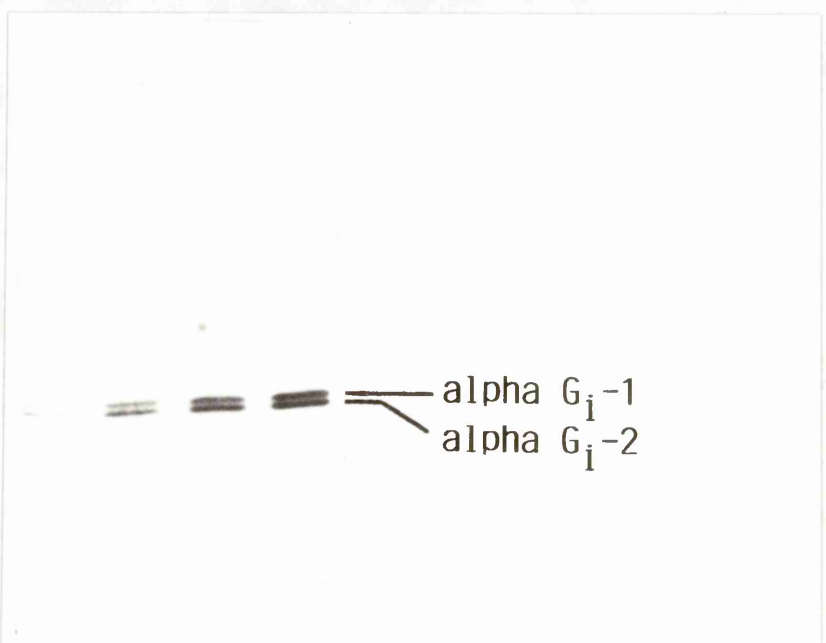
Appendix 1 Effects of increasing protein concentrations on the detection of α_s and in adipocyte membranes from +/+ mice and Sprague-Dawley rats.

Increasing amounts of adipocyte membrane protein prepared from +/+ animals were subjected to SDS-PAGE before transfer to nitrocellulose and immunoblotting with the appropriate antiserum. For the detection of α_s the amounts loaded were, from left to right, 80µg, 40µg and 20µg and the antiserum used was CS1. The result is representative of three performed with different membrane preparations. Identical results were obtained using membranes prepared from Sprague-Dawley rats used as controls in the studies described in chapter 3.



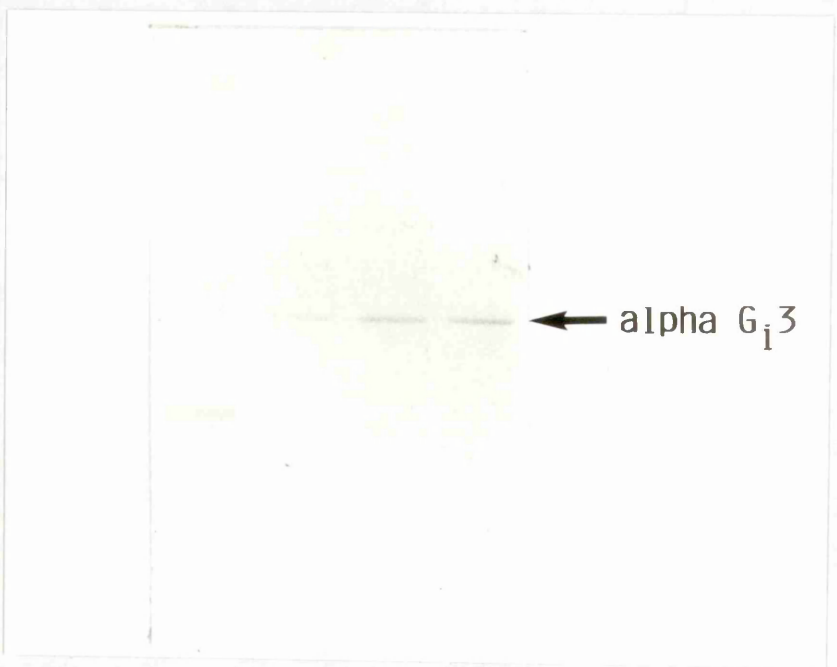
Appendix 2 Effects of increasing protein concentrations on the detection of α_i1 and α_i2 in adipocyte membranes from +/+ mice and Sprague-Dawley rats.

Increasing amounts of adipocyte membrane protein prepared from +/+ animals were subjected to SDS-PAGE before transfer to nitrocellulose and immunoblotting with the appropriate antiserum. For the detection of α_i1 and α_i2 the amounts loaded were, from left to right, 15, 30, 60 and 120µg and the antiserum used was AS7. The result is representative of three performed with different membrane preparations. Identical results were obtained using membranes prepared from Sprague-Dawley rats used as controls in the studies described in chapter 3.



Appendix 3 Effects of increasing protein concentrations on the detection of α_i3 in adipocyte membranes from +/+ mice and Sprague-Dawley rats.

Increasing amounts of adipocyte membrane protein prepared from +/+ animals were subjected to SDS-PAGE before transfer to nitrocellulose and immunoblotting with the appropriate antiserum. For the detection of α_i3 the amounts loaded were, from left to right 50, 100 and 150 µg and the antiserum used was I3B. The result is representative of three performed with different membrane preparations. Identical results were obtained using membranes prepared from Sprague-Dawley rats used as controls in the studies described in chapter 3.



Appendix 4 Effects of increasing protein concentrations on the detection of β-subunit in adipocyte membranes from +/+ mice.

Increasing amounts of adipocyte membrane protein prepared from +/+ animals were subjected to SDS-PAGE before transfer to nitrocellulose and immunoblotting with the appropriate antiserum. For the detection of G-protein ß-subunit the amounts loaded were, from left to right, 20, 40, 80, 120, 160 and 200μg the antiserum used was BN1. The result is representative of three performed with different membrane preparations.