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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

Department of Biochemistry, University of
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ABBREVIATIONS

A; agonist

cAMP; cyclic adenosine monophosphate

AMP; adenosine monophosphate

$\alpha_s$ the GTP binding subunit of $G_s$

$\alpha_0^{lf}$ the GTP binding subunit of $G_0^{lf}$

$\alpha_1$ the GTP binding subunit of $G_1$

$\alpha_2$ the GTP binding subunit of $G_2$

$\alpha_3$ the GTP binding subunit of $G_3$

$\alpha_z$ the GTP binding subunit of $G_z$

$\alpha_o$ the GTP binding subunit of $G_o$

$\alpha_t$ the GTP binding subunit of $G_t$

β-AR; β-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

XIII
G₄: guanine nucleotide protein which stimulates the cGMP-phosphodiesterase
GTP₇S; guanosine 5'(3-o-thiotriphosphate)
GppNHp; guanylyl 5'-imidodiphosphate
GTP; guanosine triphosphate
IR; insulin receptor
hIR; human insulin receptor
NaF; sodium floride
M_r; molecular weight
m Ach-r; muscarinic acetylcholine receptor
mRNA; messenger ribonucleic acid
ob/ob; obese Obese mice as opposed to lean obese mice
PGE₁; prostaglandin E₁
PIA; N⁶-(phenylisopropyl) adenosine
PIP₂; phosphoinositol 4,5-bisphoshate
PKA; cAMP activated protein kinase
PKC; 1,2-sn-diacylglycerol activated protein kinase
p21ras; the product of the ras gene(s)
P.tox; Pertussis toxin which catalyses the ADP-ribosylation of G₄.
PLC; phospholipase C, which catalyses the hydrolysis of PIP₂ to 1,2-sn diacylglycerol and inositol 1,4,5 triphoshate.
PLA₂; Phospholipase A₂ which catalyses the hydrolysis of phosphatidyl choline to arachidonic acid and lysophosphatidyl choline
PTK; Protein tyrosine kinase
Rₛ; receptor linked to the stimulation of Gₛ
R; any cell surface receptor
R₁; receptor linked to the stimulation of G₁
TSH; thyroid stimulating hormone
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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 occurring 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\textsubscript{j}' 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 β-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 $\alpha$-subunit for $G_1$. It was concluded that a diminution 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 $\beta$-adrenergic receptor to $G_S$ coupling could be investigated by classical ligand binding studies: (ii) the possibility of alteration in the ratio of $\beta_1/\beta_2$-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 dibutylcAMP. 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 lipogenesis 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 prevalence 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 INFORMATION 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 phosphatidyl 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 $\beta$-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; $\alpha_S$ which possesses the GTP binding site and is the target for ADP-ribosylation by NAD+ catalysed by cholera toxin, and the $\beta$-subunit and the $\gamma$-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 $\beta$-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 $\beta\gamma$ subunits are either very similar or identical to those of $G_S$. The $\alpha_i$ subunit is a substrate for ADP-ribosylation catalysed by pertussis but not cholera toxin, except under special conditions in vitro.

The homology between $\alpha_i$ and $\alpha_S$ 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 $\beta\gamma$ are known to exist and therefore $R_i$-and $R_S$-type receptors may recognize particular $\alpha\beta\gamma$ complexes and not just simply the $\alpha$ subunit. Never the less it has been assumed that the $\beta\gamma$ 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 $\beta$-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_5) 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^{2+} 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 α_γ/βγ.

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) yielding of 394, 381, 380 and 395 amino acid variants of \(\alpha_S\) 1, 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 \(\alpha_I\), \(\alpha_S\) 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 \(\alpha\) from the \(\beta\gamma\) subunits.
and if the latter provide the only site of membrane attachment the activated \( \alpha_5 \) would leave the membrane.

A fifth \( \alpha_5 \) 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 \( \alpha_{\text{olf}} \). This is a 381 amino acid protein, sharing 90% homology with other \( \alpha_5 \) species and apparently stimulates adenylate cyclase in neuroepithelial cells. The expression of \( \alpha_{\text{olf}} \) in \( \text{S49 cyc}^- \text{kin}^- \) 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 \( G_i \). \( \alpha_1 \) consists of 355 amino acid, \( \alpha_2 \) of 354, and \( \alpha_3 \) of 354 amino acid and they exhibit between 87 and 93% sequence identity (Suki 1987). The molecular weights of \( \alpha_1 \), \( \alpha_2 \) and \( \alpha_3 \), found by SDS-PAGE, are 41kD, 40kD and 41kD (actual \( M_r \) for \( \alpha_1 \) 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 \( [^3\text{H}] \) myristate of monkey kidney COS cells transfected with cytomegalovirus-based expression vectors have shown that \( \alpha_1 \), \( \alpha_2 \), \( \alpha_3 \), \( \alpha_O \), \( \alpha_Z \), \( \alpha_1 \) but not \( \alpha_5 \) undergo myristoylation (Mumby et al, 1990). Myristoylation which appears to be important for the attachment of the protein to the membrane. Experiments on the distribution of \( [{}^{35}\text{S}] \) methionine labelled \( \alpha_1 \) and \( \alpha_5 \) show that the former immunoprecipitates from particulate fractions when myristoylated. However \( \alpha_1 \) 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 $\alpha_1$ 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
$\alpha_1$, is shifted to the right for the mutant $\alpha_1$.

$G_i$ apparently also undergoes phosphorylation on the $\alpha_i$ 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

$G_0$

$G_0$ 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 $G_0$. Two different cDNAs for $\alpha_0$
have been cloned from several mammalian tissues including rat brain (Itoh
et al, 1988; Van Meurs et al, 1987). Bovine $\alpha_0$ contains 354 amino acid,
the same number as $\alpha_0$, their sequences being identical up to position 246,
thereafter 26 amino acid are different. It has been proposed that $\alpha_0$ and $\alpha_0$
arise due to alternative splicing of a single transcript (Hsu et al, 1990).
The function of $\alpha_0$ is uncertain although a number of target effector units
have been implicated.

$G_z$

The cDNA for $\alpha_z$ was isolated from a bovine retinal library with $\alpha_1$
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 $\alpha_i$ proteins (66-67% identity in sequence with $\alpha_i$, $\alpha_i^2$
and $\alpha_3$) although not strong enough to be part of a separate subfamily ($\alpha_i$ subunits exhibit 85-94% identity between each other). Northern and western blotting analyses have shown that $\alpha_z$ 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 $\alpha_i$ 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 $\alpha_i$ 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.

**$\beta$ subunits**

Three $\beta$ subunit cDNAs are known, and these are derived from three non-allelic genes located on chromosomes 1 ($\beta_1$ encoding a 37.329kD), 7 ($\beta_2$ encodes a protein 90% identical to $\beta_2$), and 12 ($\beta_3$ encoding a 340 amino acid 37.221kD protein) (Blatt et al, 1988; Levine et al, 1990). $\beta_3$ mRNA may undergoe two alternative splices but its tissue distribution is not yet known (Levine 1990). Most tissues appear to contain two $\beta$ subunits of 35 and 36 kD on SDS-PAGE both 340 amino acid and differ by only 42 amino acid.

**$\gamma$ subunits**

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

A $\gamma$ specific to ROS membranes, $\gamma_t$, 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 βγ1 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, PLA2 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, Rgs Gs, activate by increasing Vmax without altering Km for ATP (Birnbaumer et al, 1980). Since Gs 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 effectors 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 Gs activation since a single β-AR molecule can activate several molecules of Gs 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-Rs to activate a number of Gs. The second stage relates to the relative turnover numbers of Gs and C. The overall
rate of Gs 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, i.e 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 1100 min\(^{-1}\). Thus since Gs has a \( K_{off} \) of 13 min\(^{-1}\) it is in its active form for \( \sim 100 \) times as long as the active form of C, thus allowing active Gs to activate further molecules of C and hence increase the amplification of the initial signal.

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

\[
K_{on} = k[R_t] \cdot [A]/(K_d + [A])
\]

= \( k[R_t] \times \text{fraction of active receptor population} \)

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 \textit{et al}, 1984) it has been shown that the \( K_{0.5} \) for isoproterenol stimulation of \( [^{35}S] \) GTP\( \gamma \)S to Gs is only 2\% of the \( K_d \) for the \( \beta \)-AR antagonist \( [^{125}I] \)-CYP binding to \( \beta \)-AR of the vesicles. The \( K_d \) of the \( \beta \)-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 GTP\( \gamma \)S binding to Gs. The amount of Gs greatly exceeds that of \( \beta \)-AR so that the explanation cannot be that their is a molar excess of \( \beta \)-AR. These results could be explained by the catalytic efficiency of \( \beta \)-AR, such that only a fraction of the total population of \( \beta \)-AR is required to be activated in order to achieve full activation of the entire population of Gs.

Reconstitution of purified \( \beta \)-AR, Gs, Gi, 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, \( \beta \)-AR, Gs, Gi and C,
cannot be altered in order to analyses the effects on rate constants.

1.3.6 Activation of \( G_\text{S} \) by \( R_\text{S} \):

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 \( G_\text{S} \), has been relatively well worked out in comparison with the activation of other G-proteins. Basically, activated agonist-receptor complex appears to activate \( G_\text{S} \) by increasing its affinity for \( Mg^{++} \) and thereby activating \( G_\text{S} \) (Iyengar & Birnbaum 1982). Activation of \( G_\text{S} \) by \( R_\text{S} \) is recognized to involve the formation of a ternary complex of agonist, receptor and G-protein (\( A-R_\text{S}*G_\text{S} \)) 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 \( R_\text{S}*G_\text{S} \). The existence of the ternary complex can be inferred by the observation that addition of purified \( G_\text{i} \) to purified and reconstituted mACh-receptor increases the affinity of mACh-receptor for agonists, thus implying that \( G_\text{i} \) 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 \( \alpha \) 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).

Radioactively labelled agonist and antagonist binding to β-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 β-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 β-AR forms a stable complex with Gs (β-AR*Gs) but only in the absence of GTP. If GTP is included in the pre-incubation then the β-AR*Gs 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 α + βγ 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 βγ are associated and the single guanine nucleotide binding site is empty (Wessling-Resnick et al, 1987; Chabre et al, 1988). If βγ are not present there is no activation of the G-protein. There is evidence to favor βγ having a role in receptor recognition/contact for the transducin cGMP-PDE system (Fung, 1987).

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

The binding of Mg++ to Gs is thought to promote dissociation to αs and βγ subunits, thus releasing the active form of αs. 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, Gs cannot bind Mg++ until its affinity is increased (Iyengar, 1981).

The activation of Gs by A-R is at least partially achieved by, stimulating the release of GDP from Gs since Murayama & Ui (1983) showed that adipocyte membranes labelled with [³H]GTP when stimulated with isoproterenol release [³H]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, αs has a single amino acid alteration which radically alters its activity (Miller et al, 1988). This mutant Gs interacts normally with β-AR but fails to dissociate to α+ βγ upon binding GTP and therefore fails to activate adenylate cyclase (Miller et al, 1988). The mutant αs evidently binds GTP and undergoes the conformational change which causes β-AR*Gs to dissociate but cannot undergo the next step which leads to dissociation of Gs. A similar situation can be achieved by removal of Mg++. Addition of isoproterenol+ GTP, GTP binds β-AR*Gs dissociates to β-AR + Gs, αsβγ does not dissociate until Mg++ is added (Higashima et al, 1987) thus showing the role of A-Rs is to promote the binding of Mg++ and thereby the activation of adenylate cyclase.

The kinetics of activation of Gs by isoproterenol in phospholipid vesicles have been studied by measuring the slowly-reversible (quasi-irreversible) binding of [35S]-GTPγS to Gs (Asano & Ross, 1984; Asano et al, 1984). Analysis shows that the apparent first order rate constant for binding of [35S]-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 pseudo-first order in the other reactant (Gs), but should be linearly proportional to the concentration of the reactant in excess and this is clearly not the case. Thus the activation of Gs and subsequent dissociation upon activation is not just a simple association of β-AR and Gs.

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 β-AR, α₁-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 Gᵢ by Rᵢ

The hormonal inhibition of adenylate cyclase by platelet α₂-AR has been shown to involve the release of guanine nucleotides from Gᵢ (Michel & Lefkowitz, 1981). It was shown that stimulation of α₂-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 Gᵢ has come from the demonstration of different sedimentation properties of agonist and antagonist labelled α₂-AR (Rᵢ) in the absence of GTP as separated on sucrose density gradient profiles (Michel et al., 1981).

Overall the activation of Gᵢ by Rᵢ is very similar to that for Gₛ by Rₛ, except for the notable difference that Rᵢ does not stimulate Gᵢ by increasing its affinity for Mg²⁺. The affinity of Gᵢ for Mg²⁺ is ~10μM ensures that at physiological Mg²⁺ concentrations Gᵢ is always saturated with this divalent cation (Hildebrandt & Birnbaumer, 1983).

1.3.8 Activation of C by Gₛ

15
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 Gs and Gi by virtue of their distinctive susceptibility to inactivation by various agents (Cooper et al, 1979). Low concentrations of GTP thus activate Gs while at higher concentrations Gi 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 Rj 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 Gs causing the activation rate of Gs to be slower than with GTP. These experiments also show the central role of Mg++ binding in the activation of Gs.

That Gs 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 αs, 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 αs+βγ from the 4S αsβγ species of Gs. This behavior of Gs was believed to demonstrate that Gs 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-Rs to activate Gs. 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 Gs which is active even in the presence of hormones and GTP (Levitzki et al, 1977). Concerning the activation of Gs, Levitzki has shown by kinetic analysis and physical co-purification of Gs with C (Arad et al, 1984) that Gs remains tightly bound to C and therefore to the membrane throughout the activation cycle. Also it appears that the β subunit remains bound to αs during the entire activation-deactivation cycle of Gs. This has been demonstrated by the stoichiometric co-purification
of GppNHp preactivated adenylate cyclase as a complex of $\alpha_S$GppNHp$^*\beta^*C$ 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 $\beta$ from $\alpha_S^*C$. It has been shown that the dissociation of $\alpha_S^*\gamma$ depends on the nucleotide bound (Huff & Neer, 1986), with GTP$\gamma$S 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 $G_i$ 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 $\alpha_S$ compared with $\alpha_i$ (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$\gamma$ 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 $\beta\gamma$ as the mediator of somatostatin action. However Hildebrandt & Kohnken (1990) have shown that while somatostatin and $\beta\gamma$ are equally effective in inhibiting forskolin-stimulated adenylate cyclase in wild type S49 cells as found by (Katada et al, 1983), $\beta\gamma$ are much less effective than somatostatin at inhibiting isoproterenol-stimulated adenylate cyclase. The $\beta\gamma$ 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 $\beta\gamma$ was a simple non-competitive inhibitor. Thus $\beta\gamma$ 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 $\alpha_i$ mediates $R_i$-stimulated inhibition of $R_s$-stimulated adenylate cyclase while $\beta\gamma$ probably function to inhibit basal activity of adenylate cyclase (Hildebrandt & Kohnken, 1990).

It is also relevant that in a reconstituted system of purified components $\beta$-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, eliciting a tonic inhibition of adenylate cyclase.

In cyc" membrane $G_i$ is a simple non-competitive inhibitor of $G_s$-GTP$\gamma$S added exogenously. However $G_s$ activated by GTP$\gamma$S, which is thought to be in the dissociated state of free $\alpha_s$, is probably quite resistant to inhibition by $\beta\gamma$ (Northup et al, 1983). Cholera toxin substantially reduces the
affinity of $\beta Y$ for $\alpha S$ (Kahn & Gilman, 1984) and cholera toxin-catalysed ADP-ribosylation of adipocyte membranes in presence of GTP reduces the effectiveness of $R_1$ linked inhibition mediated by PIA by some 35% (Owens et al, 1985). Under these conditions there is little ADP-ribosylation of $G_1$ (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 $R_1$ to generate the inhibitory species. Thus, in adipocytes, ADP-ribosylation of $G_S$ may well reduce its ability to be inhibited by $R_1$ receptor means. Since ADP-ribosylation is known to reduce the affinity of $G_S$ for $\beta Y$ then the reduction in effectiveness of $R_1$-mediated inhibition in this system may well be due a decreased capacity of $\beta Y$ to inhibit adenylate cyclase because of reduced affinity between $\alpha S$ and $\beta Y$. However others (Toro 1987) have claimed that $R_1$ mediated inhibition is not sensitive to cholera toxin mediated ADP-ribosylation of $G_S$. However one of the strongest arguments against $\beta Y$ playing a direct role in the $R_1$ mediated inhibition of adenylate cyclase is that $\beta Y$ seem to be functionally interchangeable 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 $G_1$ might inhibit adenylate cyclase is would involve $G_1$ interaction with $R_S$. Evidence shows that $G_1$ will interact with $\beta$-AR in reconstituted systems in which it has been shown (Asano et al, 1988) that $\beta$-AR will stimulate GTPase activity of $G_1$. However more direct evidence has recently been obtained since Marbach et al (1988) have demonstrated a direct interaction between $G_1$ and $\beta$-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 $G_1$. 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 $\beta$-AR with $G_i$ (Marbach et al, 1988).

Antagonists of the $\delta$-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 $\alpha_2$-AR with $G_i$ showing that even in absence of $\alpha$-adrenergic agonist the empty $\alpha_2$-AR will activate $G_i$ (Cerione et al, 1986). Similarly the same process has been shown for the $\beta$-AR, $G_s$ 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 adipocyte membranes (Begin-Heick, 1985), hepatocyte 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 Gj or by an intrinsic basal activity of Gj. However, since empty receptor activation of Gj 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 Rj 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 \(\beta\gamma\) added will inhibit GTP\(\gamma\)S-stimulated but not isoproterenol stimulated adenylate cyclase (Cerione et al., 1985). One possibility is that active \(\alpha_s\) remains associated with \(\beta\)-AR, however this is difficult to reconcile with the demonstrated ability of a single \(\beta\)-AR to cataractically activate many G-proteins (Pederson & Ross 1982). An alternative may be that \(\alpha_s\)-GppNHp and \(\beta\gamma\) are evenly distributed but that \(\alpha_s\)-GTP+\(\beta\gamma\) formed using stimulation by isoproterenol+GTP are locally concentrated around \(\beta\)-AR. In this case more free \(\alpha_s\) may be needed to activate \(\beta\)-AR because of the higher local concentration of \(\beta\gamma\) 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 \(\beta\)-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 B-AR can be removed by proteolysis without altering ligand binding, indicating that the ligand binding domain must lie within the core of the protein (Rubenstein et al, 1987).

Similarly retinal interacts with opsin through the formation of a Schiff base with Lys^{296} in the 7th 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 B-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 B-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 β-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 β-AR.

Molecular replacement analysis has been less successful in determining amino acid responsible for β_1 and β_2 receptor subtype selectivity. This is determined by contacts made to the substitution on the amine group if helix 4 of β_1-AR is changed to predominantly α, β_2-like form, then the affinities of the β_1-AR became more like those of β_2-AR (Frielle et al, 1988). Thus a β_1-AR can be turned effectively into a β_2-like AR by replacing helices 4 and 5 of β_1-AR with those of β_2-AR. In contrast smaller helix replacements or single amino acid replacements failed to affect subtype specificity. Thus it appears β_1-AR and β_2-AR differences cannot be attributed to a single amino acid contact which is any different between β_1-AR and β_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 helix of the human β-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 β-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 \( n_{\text{Hill}} \) was unchanged. Examination of the GppNHp dose response curve of \( ^{125}\text{I}-\text{CYP} \) bound in the presence of 35pM \( ^{125}\text{I}-\text{CYP} \) and 10µM isoproterenol showed that the \( K_{0.5} \) for the mutant \( \beta\)-AR was much higher 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 \( \beta\)-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 \( \beta\)-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 \( \beta\)-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 \( \beta\)-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 \( \beta\)-AR desensitization?

Another approach to the mapping of domains of the \( \beta\)-AR which interact with \( G_s \) is the use of peptides corresponding to portions of the known
sequence of $\beta_1$-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, $\alpha_3\gamma$ with no guanine nucleotide bound. If a guanine nucleotide is then added it binds to the vacant site on $\alpha_s$, 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 (Pflister 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 $\delta$-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 Gs from A-
Rs*Gs converts A*Rs to low affinity, therefore dissociation rate increases so
that A leaves Rs, then Rs 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 α1-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 Gs, when the α1 of
transducin binds GTP, but not GDP, its affinity for βγ is substantially reduced
and the α1βγ dissociates into βγ and α1-GTP (Navon & Fung 1987). A single
activated rhodopsin can activate a number of α1βγ, since individual βγ can
recycle between many α1 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 p21ras which cannot bind βγ subunits is itself
farnesylated (Hanock et al, 1989), (Casey et al, 1989), (Schafer et al,
1989).

1.5.3 Rp-Gp-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 $\alpha_1,2,3$ or $\alpha_01$ 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 $\alpha_0$ 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 $\beta$-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 α5-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).

G0 has been found to be capable of inhibiting the channel by direct and indirect means. In chick dorsal root ganglia G0 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 G0 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 p21ras (Downward et al, 1990). This is the first time that the activity of p21ras has been shown to be activated by a receptor. Activation of p21ras appears to be due to a decrease in the GTPase activity of the p21ras 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 p21ras is not known however PKC appears to be involved in the signal transduction mechanism. It is
suggested that activation of p21\textsuperscript{ras} involves inhibition of GAP thereby leading to decreased inhibition of p21\textsuperscript{ras}. GAP (Ellis \textit{et al}, 1990), or GTPase activating protein, stimulates the GTPase activity of p21\textsuperscript{ras} thereby increasing the number of p21\textsuperscript{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\textsubscript{0} (Strittmatter \textit{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 GTP\textsubscript{y}S to G\textsubscript{0} by some 2 fold (Strittmatter \textit{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\textsubscript{0}. 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 \textit{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\textsuperscript{c-H-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\textsuperscript{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 \textit{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 (\textit{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 \( \text{G}_s \) appears to be the product of the RAS2 gene which is highly homologous in terms of sequence similarity to mammalian \( \text{ras} \) proteins (Broek \textit{et al}, 1985). However the catalytic unit (Kataoka \textit{et al}, 1985), CDC35, at 220kD is somewhat larger than its mammalian counterpart of 120-150kD.

The functional equivalent of \( \text{R}_s \) 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 \( \text{R}_s \). 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 \( \text{R}_s \) upon \( \text{G}_s \) in that CDC25 catalyses guanine nucleotide exchange of RAS2. In membranes prepared from mutant yeast lacking RAS2 and RAS1, mammalian p21\(^{H-ras}\) can reconstitute adenylate cyclase stimulation (Toda \textit{et al}, 1985). Identification of the domain of CDC35 with which CDC25 interacts could help to identify the effector of p21\(^{R-ras}\) proteins. RAS1 gene can transform NIH 3T3 cells provided that the C-terminus is replaced with that from p21\(^{H-ras}\) (DeFeo-Jones \textit{et al}, 1985). The partially purified catalytic unit of adenylate cyclase can be stimulated by purified RAS2 (Field \textit{et al}, 1988) and also RAS1 but to a much lesser extent (Broek \textit{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), (Tona'\text{'}a \textit{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.cerevisiae, GP1α (encoded by GPA1) and GP2α (encoded by GPA2) of Mr 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 α1 and αo. 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 β 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 β-subunit and γ-subunit respectively. It has been suggested (Whiteaway et al, 1989) that STE4 and STE18 are the βγ subunits of GPα1. Expression of STE4 and STE18 is limited to haploid cells, suggesting the existence of another set of genes encoding potential βγ 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 \( \text{G}_i \) proteins in mammalian cells. Cells loaded with \(^{32}\text{P} \) 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 \textit{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 \textit{et al}, 1988) in a cell free system. Among the mammalian small molecular weight proteins p21\(^{c-K-ras}\) has been shown to be phosphorylated by PKC and PKA (Ballester \textit{et al}, 1987) and p21\(^{v-H-ras}\) by PKC (Jeng \textit{et al}, 1987).

At least 15 smg molecules have been separated from bovine brain membranes. The smg include \( \text{ras; rho;ral;R-ras;ypt1;rab2; \ and arf} \) gene products (Barbacid 1987; Maduale \& Axel 1985; Chardin \& Tavitian 1986; Lowe \textit{et al}, 1987; Haubruck \textit{et al}, 1987; Touchot \textit{et al}, 1987; Sewell \textit{et al}, 1988). However these proteins may not be involved in signal transduction. The \( \text{ypt1} \) gene product has been implicated in secretory phenomena (Segev \textit{et al}, 1988). The \( \text{rho} \) p20 is known to be ADP-ribosylated by botulinum toxin (Kikuchi \textit{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 \textit{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 \( \beta_2 \)-AR together with mammalian \( G_5 \) 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 \( G_0 \) or \( G_i \) such that muscarinic agonists will show guanine nucleotide sensitive binding like that of native membranes (Florio & Sternweiss, 1984). The \( \beta \)-AR appears to be able to interact with \( G_i \) as well \( G_5 \) (Marbach et al 1988).
G₅ is known to be able to stimulate both Ca²⁺ channels and the C subunit of adenylate cyclase (Yanati et al, 1987a). Similarly G₀ appears to be capable of stimulating both PLC and inhibiting Ca²⁺ channels directly (Holz et al, 1986; Rane et al, 1986).

1.7 DESENSITIZATION OF ADENYLYLATE 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/effecter.

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 β-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 β-AR agonists (Pike & Lefkowitz, 1980). Purified β-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 β-AR can still be phosphorylated and this led to the discovery of β-AR kinase, called β-ARK (Benovic et al, 1986). The β-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 β-ARK by screening of a bovine cDNA library. Northern blot analysis shows that β-ARK is apparently expressed most highly in brain, spleen, heart and lung which have a high sympathetic input suggesting β-ARK is involved in modulating activity of synaptic receptors. This may be an important fact since the substrate specificity of β-ARK is not resolved since it also acts upon muscarinic receptors and α₂-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 rhodopsin (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 β-AR (Sibley et al, 1986). The phosphatase associated with this compartment reversed the desensitization. Duck erythrocyte β-AR desensitization can be reversed in isolated desensitized membranes by alkaline phosphatase treatment (Stadel et al, 1988). A protein, termed β-arrestin, possessing 59% sequence homology to arrestin, the protein intimately involved in deactivation of transducin activation, has been identified in non-retinal tissue (Lohse et al, 1990). By Northern blotting β-arrestin demonstrates a similar tissue distribution to β-ARK, implying the possibility that this protein may be the functional analog of arrestin, in the β-AR system.

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In contrast to shorter term desensitization there is little evidence that phosphorylation by either β-ARK or PKA is important in the mechanism of long term desensitization. Mutations in the two sites of phosphorylation by PKA, of the human β2-AR, from Ser to Ala 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 β2-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 β-AR (Mahan et al, 1985). In addition mutant receptors that show impaired coupling to Gs also show impaired down-regulation. However in H21a S49 cells, which lack coupling between Gs and β-AR, there is still normal agonist induced internalization. Long term exposure to agonists seems to reduce the mRNA for β2-AR this being due to a decrease in the stability of the mRNA rather than an alteration in rate of transcription (Haddock 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 Gs activity into S49 cyc− cell membranes shows that Gs activity is reduced 25% following glucagon treatment (Premont & Iyengar 1989). Addition of purified Gs 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 Gs 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 βγ 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 diacylglycerol intracellularly. This causes uncoupling of glucagon receptors from $G_5$, 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 β-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 β-cells of the pancreas. STZ has an LD₅₀ of 138mg/kg when
administered to rats by intravenous injection. One theory is that DNA is
damaged by generation of carbonium ions (CH₃⁺) and subsequent repair by
poly-ribose synthetase to repair the damage leads to NAD depletion and hence
the 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 develop at any age
but is most likely to develop 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, Massachusetts, 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*) littermates.

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 hyperglycaemia 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 hyperglycemia, 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 ADENYLATED 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 β-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 β-AR receptor coupling to Gs or at the level of activation of adenylate cyclase by Gs. 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 Gi. However others (Greenberg et al., 1987) have shown that Rl mediated inhibition appears to be still perfectly intact in ob/ob membranes. Thus its seems that, in ob/ob adipocytes tonic inhibition by Gi 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 Gi activity might have on the regulation of adenylate cyclase by Rg linked agonists. To this end Begin-Heick proposed a theory to link the loss of tonic inhibition by Gi with the reduced stimulation of AC by β-AR agonists. Since it is believed that the amount of Gi greatly, 10-30 fold, exceeds that of Gs (Bokoch et al., 1984) then if there was some perturbation of Gi which weakened the association of αiβγ such that the free βγ concentration within the membrane increased. The increased free concentration of βγ would by mass action tend to inhibit the dissociation of αsβγ thereby decreasing the effectiveness with which β-AR can activate adenylate cyclase.
It has been shown that the β-AR response of lean mice is mostly due to β₁-ARs but that of obese is more typical of β₂-AR response (Begin-Heick, 1981). The significance of this may be that β₂-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 β₁-ARs in the more responsive cells to predominantly β₂-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 adipocytes of db/db mice, show that there 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\textsubscript{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\textsubscript{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\textsubscript{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\textsubscript{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\textsubscript{i} function was shown to be abolished but this was claimed on the basis that \alpha\textsubscript{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\textsubscript{s}\alpha and increased pertussis toxin-catalysed labelling of G\textsubscript{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 β-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 Gsα-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, [AlF₄]⁻ 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 Gᵢ 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, whereas Lynch et al (1989) studied 'whole liver' containing a mixture of cell types. Thus it was shown (Bushfield et al, 1990) that streptozotocin diabetes causes cell-specific changes in Gᵢ expression., a 70% reduction in the expression of Gᵢα-2 and Gᵢα-3 Associated with this was the a phosphorylation of Gᵢα-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 Gᵢ 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, P₂ᵧ 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 Gᵢα-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
concomitant 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 β-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_iα-3, although the authors claim that it is the phosphorylation and inactivation of G_iα-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_iα-2 to G_sα 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 β-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 α₂β₂. The α subunit is 719 or 731 amino acids the β is 620 amino acids. The α precursor is 1355 amino acids (Ebina et al., 1985). The α and β subunit are generated from the proteolytic cleavage of a single chain precursor 1355 amino acids, this occurs during transfer from ER to plasma membrane. After cleavage of the precursor the nascent α and β subunits are glycosylated on asparagine (N-linked) and possibly serine/threonine (O-linked) residue. Thus the α and β subunits
have apparent Mr of 135 and 95 KD respectively, i.e. greater than their weights predicted from cDNA. In the absence of α subunit the β 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 β 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; Standtmayer & 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 β 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 in part by controlling the phosphorylation state of the relevant pacemaker enzymes. This must be at least in part achieved by controlling the levels of metabolically important second messengers such as cAMP. Insulin stimulates the activity of glycogen synthase in hepatocytes while α₁-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 α₁-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\textsubscript{i} and G\textsubscript{o} (Krupinski et al, 1988). These experiments were performed in reconstituted phospholipid vesicles and it was also inferred that the presence of G\textsubscript{i} and G\textsubscript{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 \(\alpha\)-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 β units from the 1355 amino acid precursor. It also encodes N-terminus of β-subunit, 20% of amino acid in this region of β are either serine or threonine, suggesting the N-terminus of β represents a region of o-linked oligosaccharide modification.

Exon 15 codes for membrane spanning region of β.

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 β.

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 precursor is derived from a precursor containing 27 amino acid serving as the signal peptide there can be two precursors, 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 origin results in delayed transport to the cell surface.

(ii) A mutation in α, Phe^{382} to Val, exon 5, in a person of Venezuelan origin, 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.
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 acquires normal insulin affinity only after proteolytic separation of $\alpha$ and $\beta$ subunits.

Three mutations in the $\beta$ 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 an 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 $\beta$-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 β-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 Hypothyroidism

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 β-AR agonists (Kunos, 1981). β-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 β-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 β-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	extsuperscript{−} membranes treated with NEM (devoid of G\textsubscript{S} and functional C ) restore β-AR response to hypothyroid membranes (Malbon et al, 1985). This implies that the defective component is neither G\textsubscript{S}, nor the membrane environment but, most probably β-AR. Thus the defect could be that β-AR is inactivated by such as some covalent modification such as phosphorylation or a change in β-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\textsubscript{1}-adenosine receptors linked to G\textsubscript{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 β-ARs is increased by 25%, the number of A1-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 β-AR agonists could be due to the decreased number of A1-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 Gsα and also the expression of β-subunits, since adrenalectomy causes a reduction in the cholera toxin-catalysed labelling of the two forms of Gsα 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.,
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 $G_\beta\gamma$-2 and $G_s\alpha$ mRNA levels are regulated by glucocorticoids in brain, unlike adipocytes where expression of $G_\gamma$-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 $\beta_2$-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 $R_s\cdot G_s$ 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 face, 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 olfaction 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 pseudopseudohypoparathyroidism 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 rearrangements within the Gsα 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 Gsα mRNA synthesis or processing is defective in these individuals.

The nature of the defect in the three PHP-la patients in the study by Levine et al. (1988) with normal Gsα mRNA levels but reduced Gsα protein is also unknown.
CHAPTER 2

MATERIALS AND METHODS
2 MATERIALS & METHODS

2.1 CHEMICALS

Sigma Chemical Co.,
Poole, Dorset, U.K.

BSA
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.),
Lewes, East Sussex, U.K.

Creatine Kinase
Creatine Phosphate
Dithiothreitol
Triethanolamine-HCl
Tris
GTP
Gpp(NH)p

May & Baker Ltd.,
Dagenham, Essex, U.K.

Hydrochloric Acid

Calbiochem,
Cambridge, U.K.

Forskolin
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 MgSO₄, 1.2 mM KH₂PO₄, 5 mM KCl, 120 mM NaCl, 2 mM CaCl₂ 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 O₂:CO₂ (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 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 15,000$g_{max}$ 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, to a concentration of between 0.5 and 1 mg/ml. 100 µl aliquots were rapidly frozen, by placing metal rack carrying eppendorf tubes 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 15,000 g_{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 from 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 10,000 g_{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 10,000 g_{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 β-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 β-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 β-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 MgSO₄ 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 $[^3H]$ cyclic AMP and a suitable concentration of binding protein until equilibrium had been reached. The labelled and unlabelled $[^3H]$ 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 $[^3H]$ cyclic AMP bound to the protein.

Total cyclic AMP binding to the protein was estimated by incubation of $[^3H]$ cyclic AMP in the absence of unlabelled cyclic AMP, whilst non-specific binding was estimated by incubating $[^3H]$ 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 $[^3H]$ 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 $[^3H]$ 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 \(\mu\)l 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 14,000 \(g_{av}\) at 4 degrees celsius for 4 minutes. 300 \(\mu\)l 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 \([\alpha-^{32}P]\) ATP (2x10\(^6\) c.p.m. per tube) was also present. Reactions were terminated by the addition of 100 \(\mu\)l 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 \(\mu\)l \([\^3\)H] cyclic AMP (10x10\(^3\) c.p.m. per tube) and 750 \(\mu\)l 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\(\text{-H}^+\) 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 [3H] 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 computer program which converted the d.p.m. per sample into pmol cyclic AMP by using the specific activity of the [α-32P] 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 followed 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'-methylenediamine.

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 (50 mM 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 α1 and α2

Separation of the alpha-subunits of Gi-1 and Gi-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 x 20 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 ml 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), β-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 Na₂HPO₄ & KH₂PO₄) 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) thimerosal. The nitrocellulose paper was incubated overnight at 30 degrees 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; McKenzie & 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 Gtα-1. This antiserum recognizes both Gtα-1 and Gtα-2 as well as Gtα-1 and Gtα-2, but as the expression of the latter two species is restricted to retinal tissue, SG1 can be used as a specific probe for Gtα-1 and Gtα-2 in other tissues. This antiserum does not recognize Gtα-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 $\beta_1$-subunit, which differs by one amino acid substitution from $\beta_2$ but by three residues from $\beta_3$ (Levine et al., 1990). Hence BN1 could be used to probe for both $\beta_1$ and $\beta_2$ 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 prepared 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)

log[GppNHp] M

control

diabetic

none

-12 -11 -10 -9 -8 -7 -6

81
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 500\mu{}M isoproterenol alone which was 157\pm{}9 pmol/min/mg. The V_{max} activity, ie that activity in the presence of 500\mu{}M GTP and 500\mu{}M isoproterenol, was 220\pm{}28 pmol/min/mg giving a 1.40-fold stimulation of activity by GTP.
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^-4M). 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.
none

Log[GTP]  M

adenylate cyclase activity (% of control)
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.
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.
Inhibition of adenylate cyclase

% Inhibition of adenylate cyclase

log[PIA] M

89
Fig 3.6. Identification of $\alpha_1$ and $\alpha_2$ in adipocyte membranes.

Western blotting data are shown which were obtained using the antiserum AS7 which in adipocyte membranes specifically recognizes $\alpha_1$ and $\alpha_2$ 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 $\alpha_3$ in membranes from control and diabetic animals.

The G-protein subunit $\alpha_3$ was identified by immunoblotting with antiserum I3B which specifically recognizes $\alpha_3$. Lane 1 was loaded with 70$\mu$g of protein from membranes prepared from control animals. Lane 2 was loaded with 70$\mu$g 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 $\alpha_S$ in membranes from control and diabetic animals.

The G-protein subunit $\alpha_S$ was detected in adipocyte membranes by use of antiserum CS1 which specifically recognizes $\alpha_S$. Lane 1 was loaded with 70$\mu$g of protein from membranes prepared from control animals. Lane 2 was loaded with 70$\mu$g 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 $5 \times 10^{-4}$ 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 pmol/min/mg for membranes from diabetic animals.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Normal (%)</th>
<th>IC$_{50}$ (nM)</th>
<th>Diabetic (%)</th>
<th>IC$_{50}$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PIA</td>
<td>54±8</td>
<td>37±7</td>
<td>83±9</td>
<td>20±4</td>
</tr>
<tr>
<td>PGE$_1$</td>
<td>54±6</td>
<td>38±6</td>
<td>68±9</td>
<td>45±7</td>
</tr>
<tr>
<td>Nicotinate</td>
<td>51±11</td>
<td>708±48</td>
<td>67±13</td>
<td>753±46</td>
</tr>
</tbody>
</table>

* 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\textsubscript{i} (ch 1). The consequences of this alteration in G\textsubscript{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 \(\beta\)-AR stimulation of adenylate cyclase by \(\beta\)-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\textsubscript{S}. The activation of adenylate cyclase by G\textsubscript{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\textsubscript{2}, will stimulate the activity of the catalytic unit directly as mentioned in ch 1 with a \(K_{0.5}\) of \(\sim0.3\) mM compared with \(\sim3.0\) mM for Mg\textsuperscript{++} (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\textsubscript{S} whilst leaving stimulation by the Mg\textsuperscript{++} binding site unaltered. This must therefore be a consideration in the
interpretation of experiments employing MnCl₂.

NaF in the range 3-20 mM activates adenylate cyclase by virtue of the presence of AIF₄⁻ ions which are a tetrahedral species thought to mimic the phosphate ion PO₄³⁻. Thus, since GDP is bound to G-proteins in isolated membrane preparations the tetrahedral AIF₄⁻ ion has been suggested to occupy the site occupied normally by the γ-phosphate of GTP such that GDP*AIF₄⁻ 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 Rₛ linked agonist is biphasic in adipocyte membranes (Cooper 1982). At low concentrations one sees the activation of Gₛ while at higher concentrations, in the second phase, activation of Gᵢ ensues with resultant inhibition of adenylate cyclase. Thus a GTP dose response curve can be used to determine inputs into adenylate cyclase from both Gₛ and Gᵢ.

The GTP analogue GppNHp, unlike GTP, activates adenylate cyclase significantly even in the absence of an added stimulator of adenylate cyclase such as an Rₛ 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 Gᵢ and thus leading to inhibition of adenylate cyclase whilst at higher concentrations Gₛ is activated and stimulation is seen (Cooper et al., 1982). This type of assay can be used to measure the balance of inputs from Gₛ and Gᵢ. 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 A₁ 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 Gᵢ, in order to observe inhibition of adenylate cyclase. Similarly PGE₁ (prostaglandin E₁) and nicotinic acid are agonists which activate Rᵢ type receptors which couple to Gᵢ 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 streptozotocin 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 Gᵢ 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...
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 somewhat 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 5-10 min with 1 μM 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 $\alpha$-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 $\alpha_{i1}$ 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 $\alpha_{i2}$ differed only by some 8±7% in membranes from diabetic animals as compared with membranes from control animals. In contrast to this, using antiserum 13B to detect $\alpha_{i3}$ we observed that levels of this particular G-protein $\alpha$-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 $\alpha_3$ 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 $\alpha_s$ 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 $\alpha$-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 $\alpha_i$ 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 $\alpha_i$ 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 $\alpha$-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 Gi, 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 Gi in membranes from both types of animals.

The loss of the ability of Gi 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 Gi, 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 Rj 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 Gi.

It has been demonstrated recently that Gi 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 Gi is now known to be a substrate not only for PKC (Pyne et al, 1989; Katada et al, 1985) but also PKA (Wanatabe
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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

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. $V_{\text{max}}$ activities were 134±12.5 and 119±12 pmol/min/mg for lean and obese membranes respectively.
adenylate cyclase activity

![Graph showing adenylate cyclase activity.](image)

- Log [isoproterenol] vs. none
- Obese vs. Lean
- pmol/min/mg

106
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 $V_{\text{max}}$ 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 $V_{\text{max}}$ 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.
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.
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

27.5 - 22.5 - 17.5 - 12.5 - 7.5 - 2.5

None

log[secretin] M

pmol/min/mg
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 $V_{\text{max}}$ 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.
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 $V_{\text{max}}$ activity was 77±3 pmol/min/mg and the $K_{0.5}$ was 8.5±1.2nM. In membranes from obese animals the $V_{\text{max}}$ activity was 70±5 pmol/min/mg and the $K_{0.5}$ was 28±3nM.
Fig 4.8. Detection of $\alpha_S$-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 20$\mu$g (track 1, 4), 40$\mu$g (track 2, 5) and 60$\mu$g (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 β-subunit in adipocyte membranes from lean and obese animals.

Detection of β-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 $\alpha_1$1 and $\alpha_1$2 in adipocyte membranes from lean and obese animals.

Detection of the $\alpha$ subunits of G$\alpha$-1 and G$\alpha$-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 $\alpha_3$ in adipocyte membranes from lean and obese animals.

Detection of $\alpha_3$ using antiserum 13B for membranes of lean (tracks 1,2,3) and obese (tracks 4,5,6) animals using 50$\mu$g (tracks 1,4), 100$\mu$g (tracks 2,5) and 150$\mu$g (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.
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 $B_{\text{max}}$ 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 GTP$_{\gamma}$S.

<table>
<thead>
<tr>
<th></th>
<th>LEAN</th>
<th>OBESE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GTP</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control* (pmol/min/mg)</td>
<td>17.7±1.7</td>
<td>16±1.6</td>
</tr>
<tr>
<td>$V_{max}$ (pmol/min/mg)</td>
<td>262±26</td>
<td>100±9</td>
</tr>
<tr>
<td>fold activation</td>
<td>16.0</td>
<td>6.25</td>
</tr>
<tr>
<td>$K_{0.5}$ (µM)</td>
<td>6.3</td>
<td>8.9</td>
</tr>
</tbody>
</table>

|                  |               |               |
| **GTP$_{\gamma}$S** |             |               |
| 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           |

$\Delta K_{0.5}$  

<table>
<thead>
<tr>
<th></th>
<th>LEAN</th>
<th>OBESE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10.5</td>
<td>3.56</td>
</tr>
</tbody>
</table>

* (100µM GTP only)
** (100µM GTP$_{\gamma}$S only)
Table 4.2 Receptor-independent stimulation of adenylate cyclase and β-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.

<table>
<thead>
<tr>
<th>ADENYLATE CYCLASE ACTIVATOR</th>
<th>$^{125}$I-CYP BINDING</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forskolin</td>
<td>NaF</td>
</tr>
<tr>
<td>100μM</td>
<td>20mM</td>
</tr>
<tr>
<td>Lean</td>
<td>170±15</td>
</tr>
<tr>
<td>Obese</td>
<td>160±12</td>
</tr>
</tbody>
</table>
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, PGE₁, 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.

<table>
<thead>
<tr>
<th>Percentage inhibition of adenylate cyclase activity</th>
<th>Lean</th>
<th>Obese</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibitory ligand</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PIA (1µM)</td>
<td>65±8</td>
<td>63±8</td>
</tr>
<tr>
<td>nicotinate (10µM)</td>
<td>54±7</td>
<td>58±5</td>
</tr>
<tr>
<td>DDA (100µM)</td>
<td>85±9</td>
<td>85±6</td>
</tr>
<tr>
<td>PGE₁ (1µM)</td>
<td>55±12</td>
<td>58±9</td>
</tr>
</tbody>
</table>
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, PGE₁, 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.

<table>
<thead>
<tr>
<th>Inhibitory ligand</th>
<th>Lean</th>
<th>Obese</th>
</tr>
</thead>
<tbody>
<tr>
<td>PIA (1μM)</td>
<td>48±4</td>
<td>49±3</td>
</tr>
<tr>
<td>nicotinate (10μM)</td>
<td>45±3</td>
<td>41±4</td>
</tr>
<tr>
<td>DDA (100μM)</td>
<td>83±5</td>
<td>87±7</td>
</tr>
<tr>
<td>PGE₁ (1μM)</td>
<td>47±5</td>
<td>46±4</td>
</tr>
</tbody>
</table>
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%).

<table>
<thead>
<tr>
<th>G-PROTEIN SUBUNIT</th>
<th>OBESE (fa/fa)</th>
<th>ANTISERUM</th>
</tr>
</thead>
<tbody>
<tr>
<td>αs42</td>
<td>65±10%</td>
<td>CS1</td>
</tr>
<tr>
<td>αs45</td>
<td>58±10%</td>
<td>CS1</td>
</tr>
<tr>
<td>β35+36</td>
<td>105±8%</td>
<td>BN1</td>
</tr>
<tr>
<td>αi1</td>
<td>35±6%</td>
<td>AS7</td>
</tr>
<tr>
<td>αi2</td>
<td>84±9%(NS)</td>
<td>AS7</td>
</tr>
<tr>
<td>αi3</td>
<td>38±7%</td>
<td>I3B</td>
</tr>
</tbody>
</table>

*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 GTP_7S (Fig 4.2 & Table 4.1). The activities seen with GTP_7S alone were similar for membranes from both lean and obese
animals, as were the $V_{\text{max}}$ activities observed in the presence of saturating concentrations of isoproterenol (Table 4.1). The fact that $V_{\text{max}}$ activities were similar in Fig 4.2 is at first sight quite surprising given the large difference in $V_{\text{max}}$ values seen in Fig 4.1 for isoproterenol stimulation of adenylate cyclase. However it can be seen in Table 4.1 that the $V_{\text{max}}$ activity achieved with membranes from lean animals is much less when GTP$_y$S was utilized rather than GTP. The reason for the lower efficacy of GTP$_y$S +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 GTP$_y$S, 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 GTP$_y$S 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 GTP$_y$S was also investigated (Fig 4.3), showing that the $V_{\text{max}}$ 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 GTP$_y$S 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 GTP$_y$S 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 100 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 Gs-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 125I-cyanopindolol (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 125I-CYP, the Kd 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 β-AR are believed to occur in adipocytes. Thus, a quite substantial reduction in the number of β-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 R3Gs 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 Vmax of adenylate cyclase activity achieved through activation of the R3 in question will be unaffected although K0.5 is likely to be increased. Furthermore since β1, β2 and β3-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 R3 hormones was also reduced implies to me that a defect, at a level common to all R3 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 Gs, 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 MnCl₂. 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 Gₛ 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 Gₛ to stimulate it. This suggests that the defect is at the level of Rₛ or at the receptor coupling interface of Gₛ.

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 Rₛ 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 K₀.₅ 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 Gₛ functioning. Possible explanations for this are (i) adipocyte Gₛ molecules in membranes from lean and obese animals may be different and could actually exhibit different Kₒ for GTPγS, such that the affinity for GTPγS was decreased in the obese state; (ii) whilst, the actual affinity of Gₛ molecules for GTPγS in both instances might be identical, the amount of Gₛ which has to be activated to yield a particular degree of activation of adenylate cyclase might be different and (iii) the levels of free β-subunits may be different thus altering the ratio of Gₛ in the holomeric state/Gₛ in dissociated state and hence altering the K₀.₅ for Gₛ activation of adenylate cyclase.

The levels of α subunits and β subunit of Gₛ 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 \( \alpha_S \) 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 \( \alpha_S \) 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 \( K_{0.5} \) \( \text{GTP} \gamma S \) seen in membranes from obese animals in Fig 4.7.

Immunoblotting with antiserum BN1, which specifically recognizes G-protein \( \beta \) 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 \( \beta \) subunits, 36 KD \( \beta_1 \) and 35 KD \( \beta_2 \) are known to exist in adipocyte membranes and others have been recently identified from cloning studies (ch 1). The antiserum BN1 identifies both \( \beta_1 \) and \( \beta_2 \) 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 \( \beta \)-subunits. Neither can we rule out the possibility of an alteration in the \( \gamma \)-subunits associated with the \( \beta \) subunits of which their are believed to be multiple types (ch 1).

Increased activity of \( G_j \) 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 \( \alpha_{1,2} \) 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 \( \alpha_{1,1} \) and 40 KD \( \alpha_{1,2} \) proteins. We observed (Fig 4.10; Table 4.5) that there was a large reduction in the level of \( \alpha_{1,1} \) 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 \( \alpha_{1,2} \) in membranes from obese animals Fig 4.10 and
Table 4.5.

We examined the ability of the R1-linked receptor agonists PIA, PGE₁ 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 R1 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 G₁ 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 Rs receptor linked agonists. These include a reduced β-AR action without any reduction in β-AR number. However as we see here for membranes from obese animals, they exhibit no change in levels of αs and no apparent change in the functioning of the catalytic unit of adenylate cyclase (ch 1). A point of difference concerns G₁ expression where in membranes from hypothyroid animals there is an increase in α₁-1, no alteration in α₁-2 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 Rs-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 Rs-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 β-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 development 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.

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CHAPTER 5
REGULATION OF ADENYLATE CYCLASE ACTIVITY
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, $V_{\text{max}}$, 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 $V_{\text{max}}$ activity was 160±12 pmol/min/mg and the activity 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 activity

pmol/min/mg

300
250
200
150
100

+/+

-4.0
-8.0
-7.0
-6.0
-5.0
-4.0

db/db

log[isoproterenol] M

NONE

1 3 7
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 $V_{\text{max}}$ 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 $V_{\text{max}}$ activity was 16.1 pmol/min/mg, the activity 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.
adenylate cyclase specific activity

**D)**

\[ 7.5 - 6.0 - 7.0 - 8.0 \]

\[ \text{-log[glucagon]} \text{ M} \]

\[ 139 \]
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 $V_{\text{max}}$ activity was 18.2±1.8 pmol/min/mg, the activity 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 $V_{\text{max}}$ 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.
Fig 5.4 Dose response curve for secretin stimulation of adenylate cyclase activity 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) $V_{\text{max}}$ activity was $24\pm1$ pmol/min/mg, activity in the presence of GTP alone was $7.2\pm0.5$ pmol/min/mg and fold stimulation by secretin was 3.4-fold. In membranes from db/db mice (closed diamond) the $V_{\text{max}}$ activity was $13.5\pm1.7$ pmol/min/mg, the alone activity in the presence of GTP alone was $6.0\pm0.6$ 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

None

log[secretin] M

(+/+) [open square]

(db/db) [filled square]
Fig 5.5 Immunoblot detection of $\alpha_s$ in adipocyte membranes from $+/+$ and $db/db$ mice.

The antiserum CS1 was used to detect the $\alpha$-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 40$\mu$g 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.

Comparison of levels of $G_s\alpha$ in control and $db/db$ mouse adipocyte membranes
Fig 5.6 Immunodetection of G-protein β-subunit in adipocyte membranes from +/+ and db/db mice.

The antiserum BN1 was used to detect the β-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.

Comparison of levels of β-subunit in control and db/db mouse adipocyte membranes
Fig 5.7 Immunodetection of $\alpha_1$ and $\alpha_2$ in adipocyte membranes from $+/+$ and db/db mice

The antiserum AS7 was used to detect the $\alpha_1$ and $\alpha_2$ subunits of G$_i$. Data was obtained from four different $+/+$ and db/db membrane preparations with comparisons being made between 30$\mu$g 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.

Comparison of levels of G$_i$$\alpha_1$ and $\alpha_2$ in lean and db/db adipocyte membranes

![Diagram showing G$_i$$\alpha_1$ and G$_i$$\alpha_2$ with 40 KDa marker]
Fig 5.8 Immunodetection of $\alpha_3$ in adipocyte membranes from $+/+$ and $db/db$ mice.

The antiserum I3B was used to detect $\alpha_3$ in adipocyte membranes. Data were obtained from four different membrane preparations from $+/+$ and $db/db$ mice and comparisons of 100$\mu$g 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.

Comparison of levels of $G_1\alpha_3$ in control and $db/db$ mouse adipocyte membranes

Lane 1 was loaded with 100$\mu$g of membrane protein from $+/+$ animals. Lane 2 was loaded with 100$\mu$g membrane protein from $db/db$ animals. Lane 3 was loaded with 100$\mu$g membrane protein from $db/db$ animals.
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 ADENYLYLATE CYCLASE IN ADIPOCYTE MEMBRANES

% INHIBITION OF ISOPROTERENOL-STIMULATED ADENYLYLATE CYCLASE ACTIVITY

Log [PIA] M
Fig 5.10 Dose response curve to PGE\textsubscript{1} mediated inhibition of isoproterenol stimulated adenylate cyclase of adipocyte membranes from +/+ and \textit{db/db} mice.

The effect of increasing concentrations of PGE\textsubscript{1} upon isoproterenol (500\textmu M) stimulated adenylate cyclase were determined in the presence of 100\textmu M GTP. The results means and standard deviations of a typical result repeated three times in separate membrane preparations. The IC\textsubscript{50} for PGE\textsubscript{1} in membranes from both +/+ and \textit{db/db} mice were 15nM.
PROSTAGLANDIN E1-MEDIATED INHIBITION OF ADENYLATE CYCLASE IN ADIPOCYTE MEMBRANES

% INHIBITION OF ISOPROTERENOL-STIMULATED ADENYLATE CYCLASE ACTIVITY

log [PGE1] M
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 IC50s 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

% INHIBITION OF ISOPROTERENOL-STIMULATED ADENYLATE CYCLASE ACTIVITY

Log [NICOTINIC ACID] M
Fig 5.12 Dose response of forskolin stimulated adenylate cyclase 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.
Percentage stimulation of adenylate cyclase

\[
\begin{array}{cccc}
\text{log(GppNHp)(M)} & \text{None} & -12 & -10 \\
\end{array}
\]

+/

- 4

- 1

2

8

6

- 8

- 6

- 4

None

(db/db)

(+/+)

100

120

140

Percentage stimulation of adenylate cyclase

log(GppNHp)(M)
Fig 5.13 Scatchard representation of $^{125}\text{I}$-cyanopinidol binding to $+/-$ and $db/db$ mouse adipocyte membranes.

Binding of $^{125}\text{I}$-CYP to membranes was determined as described in Material and Methods. The parameters $B_{\text{max}}$ 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 β-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 $B_{\text{max}}$ (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.

<table>
<thead>
<tr>
<th>ACTIVATOR</th>
<th>125I-CYP BINDING</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$B_{\text{max}}$</td>
</tr>
<tr>
<td>100μM forskolin</td>
<td>20mM NaF</td>
</tr>
<tr>
<td>Control (+/+)</td>
<td>180±14</td>
</tr>
<tr>
<td>Diabetic (db/db)</td>
<td>175±12</td>
</tr>
</tbody>
</table>
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).

<table>
<thead>
<tr>
<th>DIABETIC (db/db)</th>
<th>ANTISERUM</th>
</tr>
</thead>
<tbody>
<tr>
<td>α₅45</td>
<td>108±7%</td>
</tr>
<tr>
<td>α₅42</td>
<td>111±7%</td>
</tr>
<tr>
<td>β₃5+36</td>
<td>105±2%</td>
</tr>
<tr>
<td>α₁1</td>
<td>192±8%</td>
</tr>
<tr>
<td>α₁2</td>
<td>115±12%</td>
</tr>
<tr>
<td>α₁3</td>
<td>115±10%</td>
</tr>
</tbody>
</table>

* 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 hereafter 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 100\( \mu \)M 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 \textit{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_{\text{max}} in membranes from +/+ and \textit{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 \textit{db/db} mice.

Thus it appears that the defect in catecholamine stimulation of adenylate cyclase in membranes from \textit{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_{\alpha} functioning, I determined the ability of NaF to stimulated adenylate cyclase activity (Table 5.1) which was similar in membranes from both +/+ and \textit{db/db} mice. Also, direct stimulation of adenylate cyclase activity activity by forskolin was similar in membranes from both +/+ and \textit{db/db} mice (Table 5.1).

The number and affinity of \beta-AR for ligand was determined using the high affinity antagonist $^{125}$I-cyanopindolol in membrane from +/+ and \textit{db/db} mice (Table 5.1). This showed that there was a very marked reduction in the maximal specific binding (B_{\text{max}}) in membranes from \textit{db/db} but essentially no change in K_{d} was observed. This large reduction in B_{\text{max}} of membranes from \textit{db/db} mice could, in principle, explain the reduced response to isoproterenol. The uncertainty arises because there may well be spare \beta-ARs such that a very substantial decrease in receptor number may have to occur before an alteration in the V_{\text{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 $\alpha_S$ subunit of $G_S$ (Fig 5.5) I found no difference in the levels of either 42kD or 45kD $\alpha_S$ 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 $\alpha_S$.

Immunobloting membranes from +/+ and $db/db$ mice with antiserum BN1, which recognizes the G-protein $\beta$-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 $\beta$-subunits which might reduce hormonal activation of adenylate cyclase by inhibiting $\alpha_S$ dissociation.

Reduced efficacy of $G_S$ linked hormones could also be due to increased levels of $\alpha_1$ 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 $\alpha_1$ and $\alpha_2$ (Fig 5.7 and 5.2). This showed that membranes from $db/db$ mice had nearly 2-fold greater levels of $\alpha_1$ compared with membranes from +/+ mice (Table 5.2). Immunobloting of membranes from +/+ and $db/db$ mice with antiserum I3B, which specifically recognizes $\alpha_3$ (Fig 5.8), showed no alteration in the levels of $\alpha_3$ between membranes from both animals. Since the levels of $\alpha_1$ 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 $G_1$ inhibition of adenylate cyclase
was determined (Fig 5.12) and from this data it was seen that GppNHp-activated G\textsubscript{i} can inhibit forskolin stimulated adenylate cyclase equally effectively in membranes from both animals. Thus the reason for the decreased response of membranes from \textit{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\textsubscript{j}-G\textsubscript{j} linked agonists to see if these responses were altered in membranes from \textit{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 \textit{db/db} mice (Fig 5.9) showed similar IC\textsubscript{50} values but the maximal degree of inhibition achieved was greater in membranes from \textit{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 \textit{in vivo}. The mechanism behind increased the inhibition seen with PIA in membranes from \textit{db/db} mice may relate to the large increase seen in the levels of \(\alpha_{1}1\). It could also be due to a change in the activity of A\textsubscript{1}-adenosine receptors. Using PGE\textsubscript{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\textsubscript{s} linked hormones, except that for TSH, remains unclear but several possibilities exist. It is possible that a selective reduction in receptor number in \textit{db/db} adipocyte membranes may occur, although nothing is known about the degree of spare \(\beta\)-ARs. Since the response to TSH is equivalent in adipocyte membranes from +/+ and \textit{db/db} animals one might conclude that the defect probably occurs at a level prior to the
activation of $G_S$ is 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 $\beta$-subunits occur in adipocytes, with an uncertain number of $\gamma$-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 $\beta$-AR desensitization. A change in receptor subtype might occur in the case of the $\beta$-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 pivotal role in determining obesity through controlling adipocyte triacylglycerol levels.
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APPENDIX
Appendix 1 Effects of increasing protein concentrations on the detection of $\alpha_S$ 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 $\alpha_S$ the amounts loaded were, from left to right, 80$\mu$g, 40$\mu$g and 20$\mu$g 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 $\alpha_1$ and $\alpha_2$ 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 $\alpha_1$ and $\alpha_2$ the amounts loaded were, from left to right, 15, 30, 60 and 120$\mu$g 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 $\alpha_1^3$ 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 $\alpha_1^3$ the amounts loaded were, from left to right 50, 100 and 150 $\mu$g 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 β-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.