Diabetes-Induced Changes In Hormonal And G Protein Regulation Of Adenylate Cyclase Activity

Debra Jayne Gawler

Thesis submitted to the University of Glasgow for the degree of doctor of philosophy.

Department of Biochemistry.

August 1987.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Acknowledgements

I would like to thank Professor M. Houslay and Dr. H. Nimmo for their help throughout the undertaking of this project, Professor Smellie for the use of the facilities within the department and Mr. A. Wilson for his help with crucial "proof reading" and reference sorting.

I would also like to thank staff in the Medical Illustration Unit for preparing figures for the introduction.

I particularly would like to thank Mrs. S. Brown for her invaluable help with photocopying , printing and moral support in what seemed like the most stressful time in my life.

Finally, I would like to thank the warden and bursorial staff of Maclay Hall ; namely Dr. I. Logan, Miss A. Hood and Miss B. Leyden for the use of the hall computer , on which this thesis was written and compiled.

Abbreviations Used

APP(NH)P	Adenyly 5'-imidodiphosphate
ATP	Adenosine 5'-triphosphate
BSA	Bovine serum albumin
cAMP	Adenosine 3'-5' cyclic monophosphate
EDTA	Ethylendiaminetetra-acetic acid
EGTA	Ethyleneglycolbis(aminoethylether)-tetra-acetic acid
GAP-DH	Glyceraldehyde 3-phosphate dehydrogenase
GPP(NH)P	Guanylyl 5'-imidodiphosphate
GTP	Guanosine 5'-triphosphate
I.D.D.M.	Insulin-dependent diabetes mellitus
NAD	Nicotinamide adenine dinucleotide
N.I.D.D.M.	Non-insulin dependent diabetes mellitus
PGK	3-phosphoglycerate kinase
TPA	12-O-tetradecanoylphorbol 13-acetate
Tris	Tris(hydroxymethyl)aminomethane
G Protein	Guanine Nucleotide Regulatory Protein

CONTENTS

List of Figures

Illustrations :

All illustrations are numbered sequentially and are located in the introductory chapter.

Graphical Representation of Data:

All figures which are graphical representations of data are numbered with respect to the chapter and subsection in which they are presented.

Structural Formulae :

All structural formulae are presented in appendix II.

List of Tables

Summary

Using animal models of diabetes, hepatic guanine nucleotide regulatory protein (G protein) function has been investigated. Diabetes mellitus can be classified into two major catagories; type I (I.D.D.M.) an insulin - dependent form of the disease and type II (N.I.D.D.M.) a non- insulin dependent form of the disease often associated with obesity.

Guanine nucleotide regulatory proteins (G proteins) are membrane associated components which are involved in the transduction of chemical signals across cell plasma membranes. The G protein Gi, inhibits adenylate cyclase activity and the G protein Gs, stimulates adenylate cyclase activity. Adenylate cyclase is an integral membrane protein which catalyzes the formation of cAMP from ATP. cAMP is an important cellular signal for the control of metabolism.

In both type I and type II diabetic rats, activity of the inhibitory G protein, Gi, is absent in hepatocytes . It is proposed that in type I diabetic animals, hepatic Gi expression is abolished. In type II diabetic rats, hepatic Gi is modified, but present in a non-functional state.

Insulin has the ability to inhibit glucagon-stimulated adenylate cyclase activity. This action of insulin is reduced in liver plasma membranes prepared from type II diabetic rats and is absent in liver plasma membranes prepared from type I diabetic rats. Insulin administration to type I diabetic animals resulted in the return Gi function and insulin's ability to inhibit

adenylate cyclase activity. It is therefore proposed that the loss of Gi function and insulin mediated inhibition of adenylate cyclase activity is a result of insulin deficiency in this type I diabetic condition.

The mechanism by which insulin mediates inhibition of adenylate cyclase activity was investigated. It is proposed that insulin activates a G protein which is distinct from Gi, and inhibits glucagon-stimulated adenylate cyclase activity by the release of Bγ subunits from a distinct G protein subunit (αBγ) complex. These Bγ subunits inhibit the stimulatory G protein , Gs, from interacting with and thereby stimulating the activity of the adenylate cyclase catalytic unit.

In addition, the effect of the hypoglycaemic drug, metformin, was assessed. This drug has been used in human diabetic therapy and is reported to have post - receptor effects. This drug was found to restore insulin's ability to inhibit hepatic adenylate cyclase activity in type I diabetic animals and enhance this insulin action in type II diabetic animals.

Finally, insulin has the ability to stimulate a high affinity GTPase activity associated with a G protein which is distinct from the G proteins Gi, Gs and a putative G protein Gp. This insulin activated G protein may be the G protein through which insulin is able to regulate the activity of adenylate cyclase . The activity of this G protein may be altered in diabetic states.

It is proposed that alterations in G protein function may reflect post-receptor defects in the diabetic state. Transmembrane signal transduction may resultantly be suppressed and therefore the ability of hormones to control metabolism would be reduced.

1. INTRODUCTION

1.1 Insulin & Diabetes Mellitus

1.1.1 The Hormone ; Insulin

Insulin is a polypeptide hormone with a molecular weight of about 6 K Da. Structurally it consists of two peptide chains denoted A and B which are linked together by two interchain disulphide bridges. Between animal species, insulin displays highly conserved sequence homology (see figure 1). The amino acid sequence of the A and B chains was determined by Sanger and colleagues in the early nineteen fifties (Sanger & Tuppy 1951;Sanger & Thompson 1953).

The hormone is synthesized in and secreted from the beta cells of the islets of Langerhans in the pancreas. Studies on the biosynthesis of insulin have revealed the presence of two precursor forms of the hormone; proinsulin and preproinsulin (Steiner et al 1974; Steiner 1977).

Preproinsulin, the precursor of proinsulin, is believed to contain an additional N - terminal peptide sequence of about 20 amino acids which acts as a "signal" to allow binding to and insertion through endoplasmic reticulum membranes. Subsequent intralumenal molecular processing occurs rapidly (within minutes) to produce the insulin precursor molecule proinsulin.

Figure 1 Insulin Sequence Homology Between Animal Species

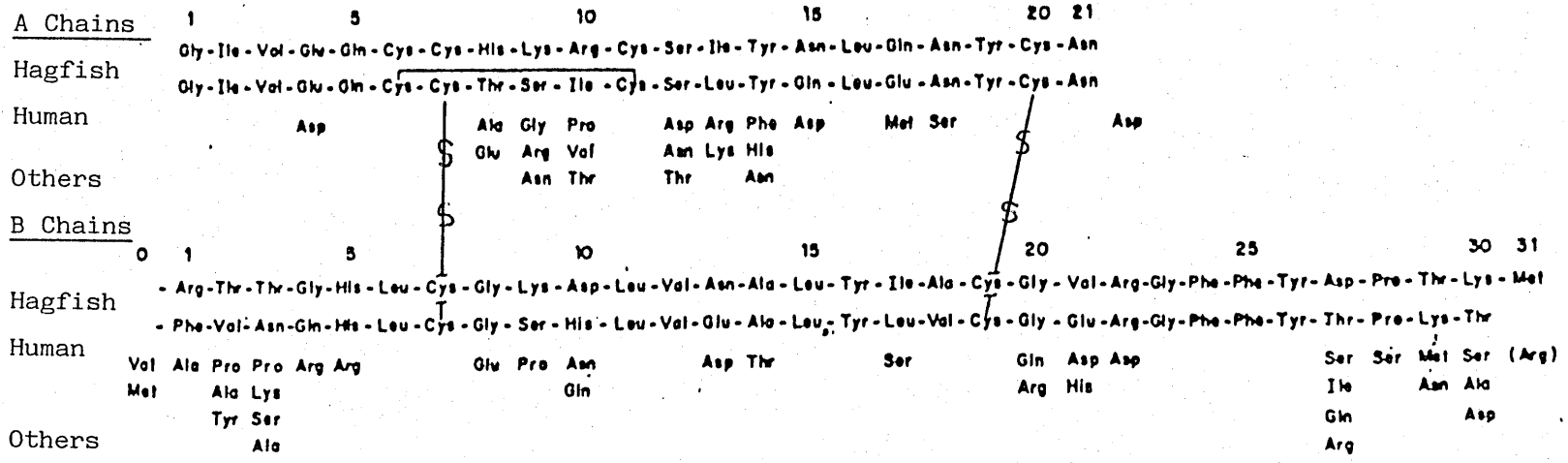
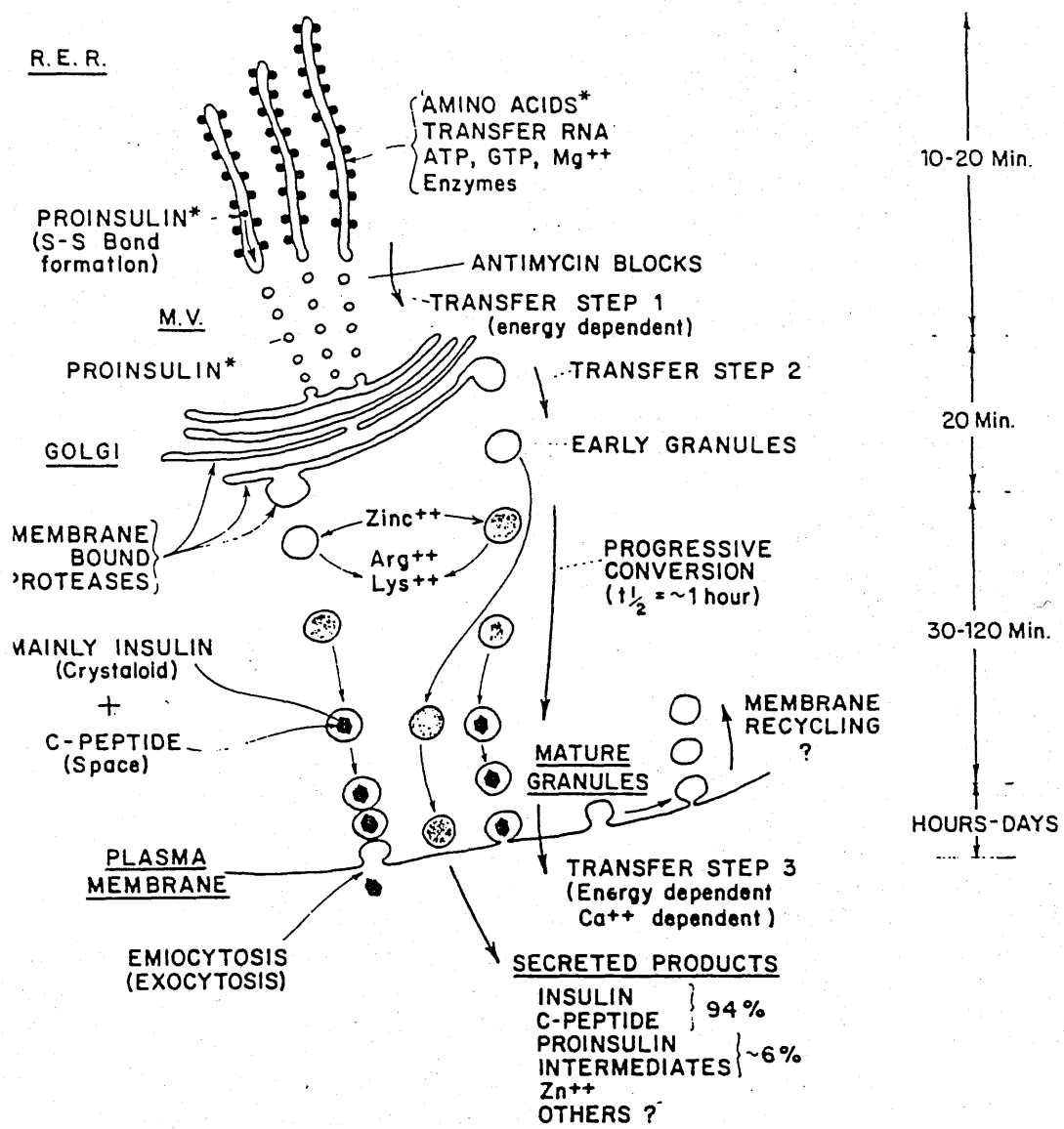


Figure illustrates the primary structures of insulin A and B chains in the hagfish and humans. "Others" denotes amino acid substitutions commomnly occurring at respective positions in a wide range of animal species which have been determined.
Figure is reproduced from Steiner (1977).

Studies using electron microscopy and pulse-chase radiolabelling of proinsulin have indicated the site of synthesis of this insulin precursor molecule to be associated with ribosomes which themselves are associated with rough endoplasmic reticulum fractions. Proinsulin molecules are released from the endoplasmic reticulum in membrane bound vesicles termed "micro vesicles" and within 10-30 min after their synthesis (see figure 2) they are transferred to the Golgi apparatus. The transfer of of proinsulin from the endoplasmic reticulum to the Golgi has been shown to be an energy dependent process and a necessary requirement for further proinsulin processing (Jamieson & Pallade 1968).

Proinsulin is a single polypeptide chain of between 81-86 amino acid residues with three distinct peptide regions; the B chain, a " C peptide " and the A chain (see figure 3). The C peptide acts as a connecting segment between the A and B chains. Formation of interchain disulphide bonds and proteolytic cleavage then occurs to yield a free C peptide and the disulphide bridge linked A and B chain insulin molecule. This proinsulin processing is believed to take place in the Golgi apparatus of the beta cell and has an estimated $t_{0.5}$ value of about 1h.

Figure 2 Proinsulin Processing In The Pancreatic Beta Cell



Schematic Summary of The Insulin Biosynthetic "pathway".

R.E.R. = rough endoplasmic reticulum; M.V. = micro vesicles.

The time scale for each stage of the process is given on the right side of the figure.

Figure is reproduced from Steiner et al 1974.

Figure 3 Amino Acid Sequence of Human Proinsulin

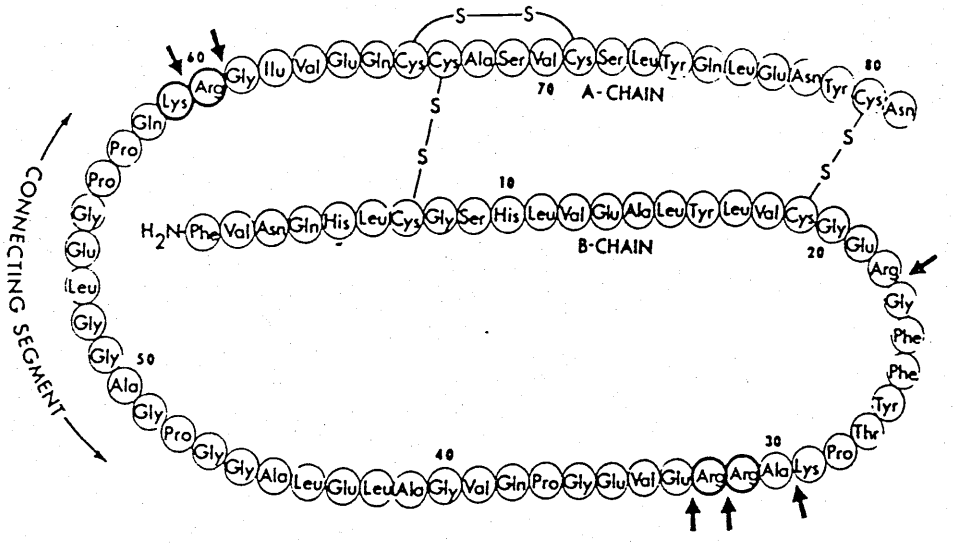


Figure Illustrates the A, B and C chain composition of human proinsulin.Arrows indicate where proteolytic cleavage occurs. Also, inter and intra chain disulphide bonds are indicated.

Insulin molecules crystallize as hexamers which are stabilized by two zinc atoms coordinated with the B10 Histidine residues and form particulate species termed secretory granules. There is also evidence that proinsulin and insulin molecules in the presence of zinc can form mixed dimeric and hexameric crystalline structures within the Golgi (Steiner 1973). The significance of this is not known, but insulinomas are known to secrete high levels of proinsulin molecules into the blood stream.

Secretory granules in the Golgi fuse with the plasma membrane of the beta cell. Their contents are expelled in response to appropriate stimuli such as high plasma glucose concentration, Ca^{2+} and in the case of diabetic therapy, hypoglycaemic drugs classified in the group "sulphonylureas" (Feinglos & Lebovitz 1978).

1.1.2 Diabetes Mellitus

The "normal" human fasting plasma insulin concentration is 5-24 u Units ml^{-1} which is approximately 4-6nM (Tager et al 1979).

The condition of diabetes mellitus is symptomised by fasting venous plasma glucose levels of greater or equal to 200mg dl^{-1} (which is approximately 11mM (Tager et al1979)). Hyperglycaemia and glycosuria are also often present. Clinical diagnosis of the disease is often by administration of an oral glucose

tolerance test (OGTT) which basically involves the ingestion of a given amount of glucose and venous plasma glucose concentration determinations are made at time points between 0 and 2h.

Diabetes is a heterogeneous disease and because of this a defined system of classification is used (National Diabetic Data Group 1979). Basically the disease can be considered as being of three distinct subclasses:-

1) The insulin dependent, ketosis prone type which has often been termed "Juvenile" or "Insulin Dependent Diabetes Mellitus" (IDDM). This subclass is also known as Type I diabetes.

2) Non - insulin - dependent - diabetes Mellitus (NIDDM) , a non ketosis prone type of diabetes which may be further subclassified with respect to the presence or absence of obesity. Often hyperinsulinaemia is present. This type of diabetes is also known as Type II diabetes.

3) Secondary diabetes mellitus, a form of diabetes associated with other conditions such as pancreatic disease or genetic syndromes.

High glucose intolerance accentuates the existance of insulin resistance because one of the primary actions of insulin is the stimulation of hexose transport into muscle, erythrocytes and adipose tissue. Over the last ten years or so, insulin stimulated glucose transport has been examined very closely in order to gain an insight into the possible molecular mechanism wherby

insulin exerts its effects upon this system.

Glucose Transport and Insulin Resistance

Glucose enters the cell by facilitated diffusion; under normal physiological conditions basal D-glucose is at a fairly constant concentration of 5mM in blood plasma (Lehninger 1977) . Thus, there is normally a glucose concentration gradient which acts as the driving force for a net influx of the sugar. However, as glucose is a polar compound and thus cannot passively diffuse into cells across the lipid bilayer, its translocation requires a specific carrier system located within the plasma membrane.

The hexose carrier is believed to be an intrinsic plasma membrane glycoprotein with a monomeric molecular weight of 55 K Da (Czech 1980; Shroer 1986). Experiments performed with less rigorous purification techniques suggest the possibility of oligomeric transporter complexes existing with a molecular weight of approximately 200 K Da (Baldwin & Lienhard 1981). As the molecular motion in the membrane appears to be important for the expression of the transporter's activity; it cannot operate as a rigid fixed channel or "pore"(Czech 1980).

Cytochalasin B is a reversible competitive inhibitor of the glucose transporter. This compound and immunoblotting techniques have been used to provide measurements of the amount of transporter in cell membranes. In addition, non -metabolizable

glucose analogues such as 2-deoxyglucose and 3,O-methylglucose or radiolabelled (U^{14}-C) glucose have been used to quantitate specific cellular uptake . In addition, kinetic parameters of the system in adipocytes and erythrocytes have been made. Whitesell & Gliemann (1979) demonstrated in rat adipocytes that insulin increases the Vmax value for glucose transport with no apparant change in the Km value. This observation therefore led to the suggestion that insulin increased the number of glucose carriers rather than changing the carrier's affinity to bind glucose molecules.

This observation was proven to be correct when Cushman & Wardzala (1979) used cytochalasin B to estimate the number of glucose carrier molecules both in the plasma membrane and intracellular locations. They found that insulin induced the "recruitment" of carrier molecules from a microsomal cell fraction to the plasma membrane and thereby increased the maximum capacity of the transport process. This insulin mediated stimulation of glucose carrier recruitment was rapidly initiated and was not a result of *de novo* synthesis of new carrier molecules.

Karnieli *et al* (1981) demonstrated that this recruitment process was reversible. Using anti-insulin antibody, after incubation of rat adipocytes with insulin, they observed the movement of carrier molecules from the plasma membrane to a microsomal fraction. They confirmed the

results found in these cytochalasin binding studies by using 3,0-methylglucose transport measurements and found the Vmax value for the transport of this compound simultaneously decreased to its original value as the carrier molecules were removed from the plasma membrane. Thus the molecular mechanism by which insulin enhances glucose influx in rat adipocytes has been elucidated. However, the human erythrocyte glucose transport system does not appear to respond to insulin in the same way as the rat adipocyte.

Work performed by Dustin & colleagues (1984) has demonstrated that human erythrocytes possess a rapid stereospecific D-hexose transport system. Incubation of cells with insulin resulted in a decreased Km value for glucose influx to the cell, but no Vmax change was observed. However, if the cells were subjected to two incubation periods with insulin not only did the Km value for the process decrease,but also 60 % of the insulin receptors were lost; a process known as downregulation. It was therefore suggested that the mechanism by which insulin stimulates glucose transport in erythrocytes may be different from that in adipocytes. The significance of this is unknown .Also, there is no evidence that insulin has the ability to stimulate glucose transport in liver. However, this tissue is a major target for insulin action and metabolic control.

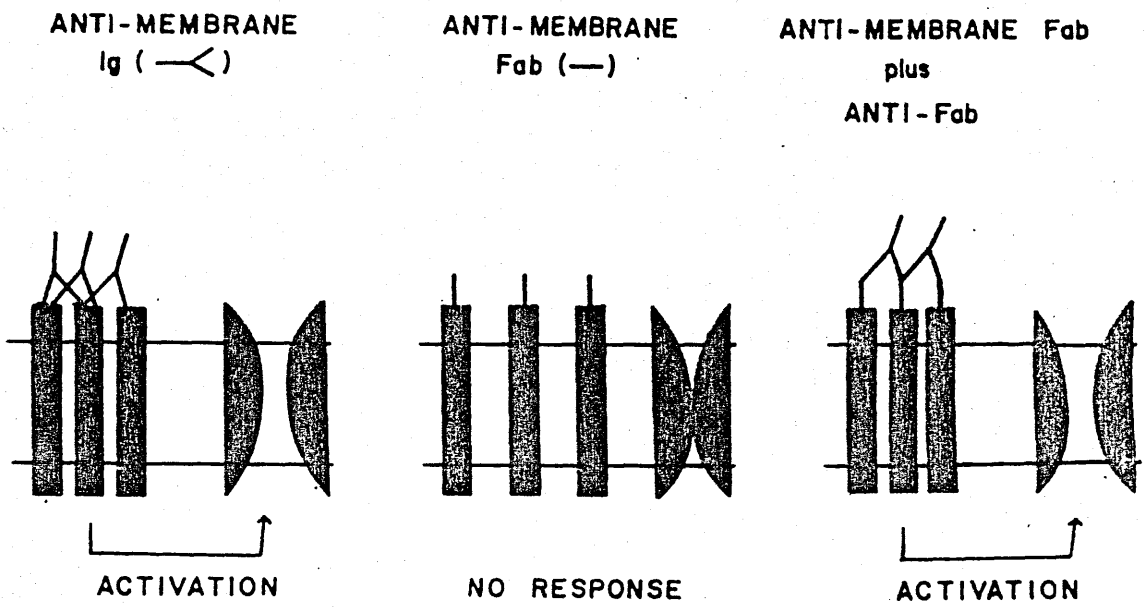
Also, the mechanism by which insulin has the ability to

stimulate the recruitment of glucose carriers from the microsomal fraction is still unknown. However, an important set of experiments performed by Kahnet al (1978); Pillion etal (1978a) and Pillion et al(1978b) using monovalent Fab and anti Fab antibodies demonstrated the possibility that lateral aggregation of the insulin receptor may be an obligatory requisite for carrier "activation" (see figure 4). Insulin receptor phosphorylation has also been linked with carrier "activation", but this will be discussed in section 1.2.4 which is entitled "Tyrosine Kinase Activity".

Diabetes and insulin resistance are clinically diagnosed on the basis of insulin's ability to activate glucose uptake from blood plasma. Understandably much work has been performed to investigate the relationship between insulin binding to its receptor (see section 1.2.2) and the ability of this action to stimulate glucose transport.Due to glucose transport being studied so extensively in adipose tissue and the relative ease of removing adipose tissue from diabetic patients during surgery, this is the tissue which has been most studied to determine a receptor-resistance relationship.

Experimentation suggests that certain obese patients (Ciciraldi et al 1981) and NIDDM patients (Kolterman et al 1981) may display both receptor and post - receptor defects. However, adipocytes isolated from IDDM patients (Jarvinen et al 1984) and aged subjects displaying glucose intolerance (Pagano et al 1982)

Figure 4 Requirement of Multivalency For Activation of Hexose Transport By Anti-Membrane Protein Antibodies In Intact Fat Cells



Outline of results obtained from antibody Fab fragment experiments described in text. In brief, multivalent antibody or antibody Fab fragments are required for activation of the hexose transport system in fat cells.

Figure is reproduced from Czech 1980.

indicated only a reduced number of insulin receptors on the cell surface, but no apparent post - receptor defect.

It has been demonstrated that occupancy of all the insulin receptors on the cell surface is not necessary for a maximal insulin response; a concept known as "receptor reserve". For example, Pedersen & Gliemann (1981) suggested that adult human adipocytes show maximum stimulation of hexose transport by insulin (1nM) during 25 % occupancy of receptors. Gammeltoft (1984) suggested that rat adipocytes require only 4 % receptor occupancy for maximal stimulation of hexose transport. Thus , it appears that insulin responsiveness for a given insulin concentration would be affected only if receptor number was significantly reduced . Therefore in order to distinguish between receptor defects and post -receptor defects the $K_{0.5}$ and Vmax values for the glucose transport process must be considered. In general, distinction between receptor and post- receptor defects have been made on the assumption that if the defect occurs at a receptor level then the $K_{0.5}$ for the transport process would increase, but the maximal response (Vmax) would remain unchanged.If however, the defect was at the post-receptor level then both the $K_{0.5}$ and Vmax values would be expected to change

In order to try to study receptor and post- receptor defects which could explain the human diseased state/s of diabetes mellitus, animal models of the disease have been used.

These models are generally mice or rats which display characteristics of Type I or Type II diabetic states.

1.1.4 Animal Models Of Diabetes

Type I - (IDDM)

Human insulin dependent diabetes mellitus (IDDM) is clinically characterized by the abrupt onset of symptoms such as hyperinsulinaemia, ketosis, hyperglycaemia and glycosuria. In order to sustain life, daily injection of insulin is required (Natioanal Diabetes Data Group 1979). This subclass 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 age groups.

Factors associated with the onset of this form of diabetes include abnormal immune responses (islet cell antibodies are often present at diagnosis) genetic inheritance (genes on chromosome 6 have been suggested) and viral infection. Chemically induced destruction of the B cells of the pancreas has been demonstrated to mimick the symptoms of type I / insulin dependent diabetes mellitus. Two chemicals in particular; streptozotocin (2-deoxy-2- (3-methyl - 3 - nitrosoureido) - D - glucopyranose) and alloxan (2,4,5,6-tetra oxo hexa hydro pyrimidine) have been used in rats and mice to chemically induce

an animal model for this form of diabetes. Both streptozotocin and alloxan can induce severe B cell necrosis (Wilson et al 1984) however, the mechanisms by which these drugs cause B cell damage is not entirely clear.

Streptozotocin

Streptozotocin has an LD_{50} value of 137.7 mg/Kg when administered to rats by intravenous injection (Merck Index nineth edition 1976) It has a molecular weight of 265.2 (the structure of this compound is illustrated in Appendix II, figure A2). This drug has been suggested (Uchigata et al 1982) to cause DNA strand breakage by the generation of carbonium ions (CH_3.) during decomposition of methylnitrourea from the drug itself. However, Wilson et al (1984) suggest that the generation of carbonium ions does not cause DNA strand breakage, but may alkylate DNA bases which leads to the activation of poly (ADP-ribose) synthetase to repair these modified sites. Cellular NAD is used as the enzyme's substrate and thereby subsequently results in NAD depletion and cell death occurs.

Robbins et al (1980) have suggested that the drug generates oxygen free radicals, but Wilson et al (1984) contest this proposal because they found no evidence that superoxide dismutase, a superoxide scavenger, gave any protection against streptozotocin's cytotoxic effects.

Rossini et al (1978) demonstrated that a combination of the non-metabolizable glucose analogue 3-O - methylglucose and antilymphatic serum can protect mice from low doses of streptozotocin. They proposed that the glucose analogue prevented hyperglycaemia and the antilymphocyte serum prevented the lymphocytic infiltration of pancreatic islets. The mechanism by which the glucose analogue prevented hyperglycaemia was not speculated upon. Perhaps the mechanism of protection was related to the similarity between the two structures with glucose.

Alloxan

Alloxan has an LD_{50} value of 200 mg/Kg when administered intravenously in mice (merck Index, Nineth Edition (1976)). The structure of the compound is illustrated in Appedix II, figure A1. Like streptozotocin, this drug has also been suggested (Uchigata et al (1982) to cause DNA strand breakage as a result of oxygen free radical generation . Others have questioned whether these free radicals generated by alloxan have the ability to break DNA strands (Wilson et al 1984; Sandler & Swene 1983).

Interestingly, Meglasson et al (1986) propose that alloxan inactivates glucokinase in pancreatic islet B cells and thereby prevents glucose metabolism in these cells. They suggest that glucokinase acts as the" glucose sensor"; that is, inactivation of this enzyme results in the abolition of glucose stimulated release of insulin from B cells. High levels of

glucose can " protect" glucokinase from inactivation by alloxan. This group did not comment on the mechanism by which alloxan causes B cell necrosis.

Finally, another group, Colca et al (1983) have proposed that alloxan inhibits a Ca^{2+} - calmodulin protein kinase in pancreatic islets and this inhibition leads to a loss of glucose stimulated insulin secretion. Again, this action can be prevented by the presence of glucose. Once again the mechanism by which alloxan causes this intracellular effect is unknown. Also do glucokinase and this enzyme share the same mechanism by which they control the secretion of insulin in response to glucose ? The answers appear to be unknown as is the precise mechanism by which alloxan conveys cytotoxicity specifically to islet B cells.

Type II - Non Insulin Dependent Diabetes Mellitus

This subclass of diabetes is fairly difficult to detect in its early stages because frequently minimal or no symptoms such as fasting hyperglycaemia may exist. (National Diabetes Data Group 1979). This type of diabetes has been termed "maturity onset diabetes", but this method of classification is not strictly correct because NIDDM may develope at any age, but it is true to say that most patients do develope this type of diabetes after the age of forty. It is this form of the disease which occurs most frequently in western society.

Also, 60 - 90 % of all NIDDM patients in western society are obese and treatment in some cases is restricted to diet alone. In other cases, controlled diet and sulphonylurea (see appendix II, figure A3) or biguanide (see appendix II , figure A5) therapy is administered. Clinical diagnosis of NIDDM, in the absence of observable symptoms , is generally made by the administration of an oral glucose tolerance test. Insulin resistance is characteristic and this has been associated with a defect at the post receptor level (Kolterman et al 1981). Hyperinsulinaemia and normoglycaemia is often also present. Secondary effects of NIDDM are more apparent.

The animal models used for this type of diabetes include obese Zucker rats and db/db mice. Only the Zucker rat will be discussed here because this is the model for NIDDM that I have used in my work. The Zucker rat adequately illustrates the common characteristics of a type II diabetic model.

The Zucker Rat

The "fatty" Zucker rat appeared as a spontaneous genetically obese condition in the Laboratory of Comparative Pathology, Stow, Massachusetts, U.S.A. in 1961 (Zucker & Zucker 1961). As the obese progeny were observed at a 25 % incidence per litter, the condition was believed to be due to a single recessive gene and the "fatty" condition is therefore a homozygous recessive expression. Obesity is observable within

three weeks of birth (see figure 5) and food intake is significantly increased in these animals. The blood plasma of "fatties" is of milky appearance. Indeed, the serum fatty acid level is ten times the level observed in lean Zucker rats and cholesterol is four times higher in serum from fat animals when compared to leans (Zucker & Zucker 1961). Low density lipoproteins (LDL) and high density lipoproteins (HDL) are increased two fold in serum from "fatty" rats (Bray 1977). Even when food intake is restricted, the "fatty" rats are still obese when compared to controls. However, there is no evidence of arterial thickening.

Hyperinsulinaemia is present in "fatties" before obesity is observed and plasma insulin concentration appears to be dependent on the age of the rat. However, when fat Zuckers are compared to lean controls with respect to their relative ages ; plasma insulin levels are calculated to be about four times higher in the obese rats than the leans (York et al 1972; Curry & Stern 1985) and B cell hypertrophy is also recorded (Stolz & Martin 1982). Glucagon levels in the pancreas appear to be normal, but circulating glucagon concentration is decreased in obese animals (Eaton et al 1976).

Interestingly, Clark et al (1983) have identified the presence of diabetes ; as assessed by a glucose tolerance test and the lowering of body fat and triglyceride, when compared to obese normoglycaemic rats. This condition appeared in obese

Figure shows a lean Zucker rat (447g) and its obese litter mate (1035g). Both are 10 months of age.
Figure is reproduced from Zucker & Zucker 1961

Zucker rats after six months of age and surprisingly occurred in more males than females.

Stolz & Martin (1982) tried chemically inducing diabetes in obese and lean Zucker rats in order to determine whether there was any relationship between plasma insulin levels, food intake and weight gain. They carefully controlled the plasma insulin levels in each set of animals by daily injection of exogenous insulin and they came up with some rather surprising results. The food intake and weight gain in the obese animals were reduced to that of the lean animals, but hepatic lipogenesis was still elevated and therefore independent of insulin concentration and food intake. This appears to be in agreement to some extent with the observation that obesity in these animals can be distinguished prior to the condition of hyperinsulinaemia being present (Curry & Stern 1985).

Finally, Guerre-Millo and colleagues (1985) have suggested that in adipocytes isolated from obese and lean Zucker rats, insulin mediated glucose transport is enhanced in the obese animals . Cytochalasin B binding studies suggest that this may be due to a significantly increased number of glucose carrier molecules in an intracellular microsomal "pool". This could obviously be considered as a hypersensitive response to insulin.

1.2 The Insulin Receptor

1.2.1 Structure

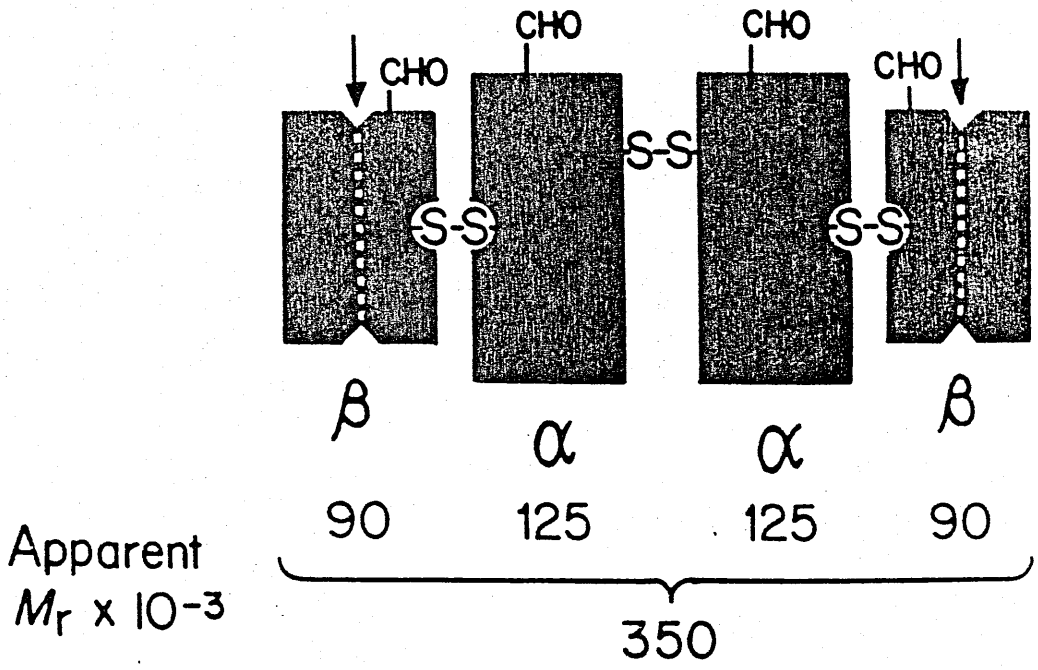
a) Protein Subunit Composition

The insulin receptor is an integral membrane glycoprotein generally accepted to be composed of two distinct polypepytide subunit species denoted α and β (Czech etal 1981). The proposed functional receptor, depicted in figure 6 , is comprised of an $(\alpha\beta)_2$ subunit complex which is stabilized by disulphide bridges. The α and β subunit molecular weights have been estimated to be about 120 K Da and 90 K Da respectively.(Pilch & Czech 1980).

Purification of the insulin receptor was originally achieved by solubilisation using non-ionic detergent, followed by chromatography using immobilized lectin columns and finally affinity chromatography utilizing immobilized insulin columns (Fujita & Yamaguchi 1984; Finn et al 1984). However, monoclonal antibodies raised against the insulin receptor now facilitates purification of the holomeric protein (Soos et al 1986 ; O'Brien et al 1986).

The use of photoaffinity labelled ^{125}I-insulin and the cross linking agent disuccinimidyl suberate (Pilch & Czech 1980) has greatly facilitated determination of its subunit composition. This has allowed identification of the binding site for insulin

Figure 6 Proposed Insulin Receptor Structure



Schematic diagram of the insulin receptor subunit structure illustrating the position of disuphide bridges and apparent molecular weights of each subunit. Intact complex molecular weight estimation was obtained under non-reducing conditions Figure is reproduced from Czech et al 1981.

to be the α subunit. The α subunits are transmembrane species, exposed at the external surface of the plasma membrane and the β subunits are exposed at both the extracellular and cytosolic surface of the membrane (Hedo & Simpson 1984).

Initially , determination of subunit stoichiometry was facilitated by the use of two experimental techniques; that is,the reduction of interchain disulphide bonds and specific *in vitro* proteolysis of the insulin receptor.

Interchain disulphide bond studies were performed by Jacobs *et al* (1979) & Massague *et al* (1980). Using various concentrations of the reducing agent dithiothreitol , they identified two catagories of disulphide bridges which were classified with respect to their differing sensitivity to reductants. The disulphide bridges most sensitive to reductant were found to link together the ($\alpha\beta$) receptor fragments. One receptor complex was found to yield two ($\alpha\beta$) fragments under mildly reducing conditions.

The other class of disulphide bridges were found to link together the α and B subunits. However, these disulphide bridges were only reduced in the presence of detergents which allowed solubilization of the insulin receptor complex. Hence, receptor complex formation must have prevented the exposure of these disulphide bridges and thereby protected them from reduction by dithiothreitol.

Studies using *in vitro* proteolysis of the receptor and subsequent receptor fragment resolution on S.D.S. gels (Massague *et al* 1981) has led to the identification of receptor fragments which have to some extent been isolated from partially purified membranes. This observation has led to suggestions that *in vivo* partial proteolysis of the receptor may occur. This may explain why other workers such as Baron & Sonksen (1983) have proposed three different molecular subunit types; the α , β and an additional δ subunit. This δ subunit was suggested to have a molecular mass of 65 K Da and is not a glycoprotein. Therefore, presumably this subunit would not face the outside of the cell. This polypeptide could conceivably be an artifact due to spontaneous proteolysis (for example of the β subunit which is sensitive to proteolysis) or if it does exist, it may be a protein which is associated with the insulin receptor in the plasma membrane.

Crosslinking (Baron & Sonksen 1983) and immunoprecipitation (Kasuga *et al* 1982a; Kasuga *et al* 1982b) studies suggest that there may be protein subunits non-covalently associated with the insulin receptor which would be removed during the solubilisation and purification procedure. In addition, Harmon *et al* (1980) have presented evidence for a protein species which may have the ability to reduce hormone binding upon association with the receptor.

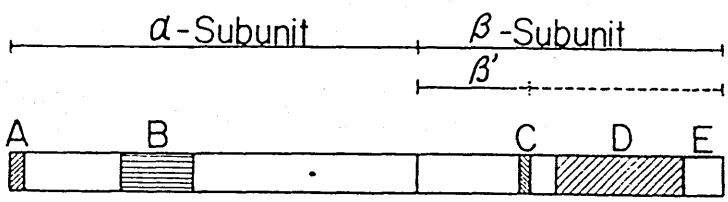
Finally, Graves *et al* (1985) have proposed a calmodulin binding domain either on the β subunit of the insulin receptor or

on a protein such as the ATPase which copurifies with the receptor. They suggest that upon Ca^{2+} - calmodulin interaction with the receptor, the affinity for insulin binding increases.

Probably one of the most exciting and most informative developements in structural elucidation has been the cloning of the insulin receptor from human placenta. This has been achieved by two independent groups (Ebina et al 1985; Ullrich et al 1985). It appears that the α and β subunits of the insulin receptor are derived from one polypeptide precursor molecule which is encoded for by a single mRNA and is therefore presumed to be the expression of a single gene which Ebina and colleagues have located on chromosome 19 (see figure 7).

Both groups indicate the presence of a 27 amino acid sequence at the N-terminal of the precursor which is characteristic of a hydrophobic "signal sequence" found in post-translationally modified proteins. The α subunit sequence follows this and consists of between 720 - 735 amino acid residues. Ebina predicts the molecular weight of the α subunit to be 84.2 K Da and identifies 15 possible glycosylation sites which could increase the molecular weight to the apparent 120-130 K Da level. The α subunit contains a cysteine rich domain with a very high proportion of hydrophilic residues. Ebina calls this a " cross-linking " region and this shows high homology to a cysteine rich region in the EGF receptor. The N - terminal of the α subunit also contains two short hydrophobic domains which

Figure 7 The Cloned Insulin Receptor Structure



A=putative signal peptide

B=crosslinking region

C= transmembrane region

D= cytoplasmic phosphokinase domain

E=C terminal region

Figure is reproduced from Ebina et al 1985 .

apparently are not large enough to span the membrane, but would conceivably form a hydrophobic interaction with the membrane or alternatively could form a hydrophobic " core " within itself.

The C - terminal of the α subunit shows no structural homology with the EGF receptor, but is speculated to contain the insulin binding site. There is also a potential glycosylation site in this region which may or may not play a role in insulin binding or recognition. Following the sequence coding for the α subunit is a domain which contains several proteolytic processing sites which would allow generation of the α and β subunits from the precursor.

The β subunit sequence contains a domain of 23 hydrophobic amino acids followed by 3 positively charged amino acids . This is suggested to be a " trans membrane domain " which would allow insertion of the polypeptide into the membrane . The polar amino acids would act as a " stop signal " to allow anchorage of the protein in the membrane. An ATP binding site has been identified on the β subunit sequence which shows slight homology with the EGF receptor and the V- ros gene product (an oncogene product known to exhibit tyrosyl kinase activity (Hunter 1986)).

The C-terminal region contains highly hydrophilic amino acids and is suggested to be the cytoplasmically exposed region which could be susceptible to intracellular proteolytic cleavage. Finally, Ebina and colleagues also indicate the presence of a so called β' segment which is present in the precursor β subunit

sequence. Upon analysis of solubilised and antibody purified insulin receptors, a 45 K Da polypeptide band was obtained. This has been identified as the β' segment found in the precursor β subunit sequence. The importance (if any) of this polypeptide is not known. However, it is possible that it could be related to the 65 K Da d peptide subunit proposed by Baron & Sonksen (1983) or indeed it could explain the high molecular weight form of the insulin receptor observed by Helmerhorst et al (1986).

Ebina's group (Ebina et al 1985 b) using the cloned insulin receptor cDNA , demonstrated that by construction of an expression plasmid they could express the human insulin receptor in both xenopus oocytes and chinese hamster ovary cells (CHO). They also demonstrated that in the CHO cells the human insulin receptor not only bound ^{125}I - insulin with high affinity and specificity, but could also stimulate autophosphorylation of the insulin receptor and 2-deoxyglucose uptake; actions characteristic of the functional insulin receptor.

b) Post translational Processing of The Insulin Receptor Subunits

In the early seventies Cuatracasas (1973a & 1973b) demonstrated that the insulin receptor binds to lectins, in particular wheat germ agglutinin and concanavalin A . This led to the suggestion that the insulin receptor is highly

glycosylated. This observation was later confirmed by Jarett and Smith (1974). More direct evidence for the presence of carbohydrate moieties on the insulin receptor came from Hedo *et al* (1981) when they demonstrated that both the α and the β subunits of the insulin receptor in IM-9-lymphocytes can incorporate ^{3}H carbohydrates such as ^{3}H-fucose and ^{3}H-mannose.

Further study of the insulin receptor biosynthetic pathway has been aided by the use of the inhibitor tunicamycin. This compound has the ability to inhibit the attachment of core oligosaccharide to asparaginyl residues of nascent glycoproteins. Ronnett & Lane (1981) and Reed *et al*(1981) demonstrated that glycosylation is essential for the post-translational formation of a functional insulin receptor in 3T3-L1 adipocytes . In the presence of this compound the number of cell surface insulin receptors were depleted and non - glycosylated receptor proteins accumulated intracellularly. Therefore, they proposed that glycosylation may be important in receptor movement to the cell surface.

More recently however, elaborate pulse - chase experiments using ^{3}H- mannose (Hedo & Simpson 1985) and ^{35}(S) -methionine (Ronnett *et al* 1984) have been performed. Both groups agree that very early (within minutes) in the receptor biosynthetic pathway, a 190 K Da "pro-receptor" protein appears.

However, Ronnett *et al* believe that prior to the appearance of this 190 K Da protein, there is a lower molecular

weight protein of about 180 K Da . This species, unlike the 190 K Da species, is not glycosylated. It is of course conceivable that this lower molecular weight species would be undetectable to Hedo & Simpson because they are pulse - chasing carbohydrate units, not protein units of the precursor molecule.

Both groups demonstrated glycosylation in an intracellular fraction (believed to be the endoplasmic reticulum) was followed by terminal glycosylation. This process was referred to by Ronnett's group as " sialic acid capping ".

Proteolytic cleavage of the α and β subunits occurs in the Golgi prior to receptor insertion into the plasma membrane.

However, the major discrepancy between the two groups is the question of whether or not a precursor molecule with a molecular weight of 205 - 210 K Da can bind ^{125}I - insulin . Ronnett's group suggest that only the processed insulin receptor inserted into the plasma membrane has the ability to bind ^{125}I - insulin.This point of contention has still to be resolved, but if the cloning studies of Ebina et al (1985a) are accurate then the insulin binding site is at the C - terminal end of the α polypeptide sequence. This therefore would not be accessible for interaction with insulin until cleavage of the α and β segments had occured . This process (both groups claim) occurs immediately prior to insertion of the receptor into the plasma membrane.

Now that the structure of the insulin receptor and its biosynthesis are known in more detail, it is hoped that these processes can be studied in human diseased states such as diabetes mellitus. To date, most receptor experimentation carried out on insulin resistant diabetic patients has been aimed at looking for changes in insulin binding affinity and receptor number, rather than changes in receptor structure. However, Khan et al (1976) using samples from type II diabetics and Chang et al (1975) using animal models of type II diabetes have suggested receptor structural changes.

Interestingly, Chandramouli et al (1977) have looked at lectin binding to liver plasma membranes in streptozotocin - induced diabetic rats . They concluded that this insulin deficient state led to significant generalized changes in cell surface glycoproteins. Perhaps such changes could occur on the insulin receptor . Certainly , lectins such as concanavalin A and wheat germ agglutinin have been suggested to compete with insulin to bind to the insulin receptor (Cuatracasas & Tell 1973) . These lectins were found to mimick insulin's ability to enhance glucose transport.

1.2.2 The Binding of Insulin To Its Receptor

Most insulin binding studies have been performed using the radiolabelled ligand ^{125}I - insulin . In the case of cell surface receptor number estimations, ^{125}I - insulin has been used

in conjunction with disuccinimidyl suberate to covalently cross - link and specifically label the receptors. Criticism concerning the specificity of insulin binding studies has been made (Cuatracasas 1974) . However , the radio-ligand technique has been very informative about kinetic parameters of binding, dissociation and movement of receptors . Two models of insulin receptor binding have been proposed :-

1) There are at least two distinct kinds of insulin receptors on the extracellular surface of the plasma membrane, each with a different affinity for insulin binding. These two receptor populations do not interact with each other in order to affect each other's binding activity.

2) There is only one form of insulin receptor on the extracellular surface of the plasma membrane, but receptors act in a negatively cooperative manner towards one another with respect to binding insulin such that, upon insulin binding to one receptor, the affinity of other receptors to bind insulin is reduced.

These models were proposed as a result of Scatchard binding analysis indicating curvilinear or biphasic characteristics. The evidence to support the two site model includes the following :-

a) Kahn et al (1974) submitted data which they proposed was indicative of the presence of two "sites" whose Km values were

dependent upon the incubation temperature.

b) Czech et al(1981) proteolytically cleaved the B subunit of the insulin receptor and were able to generate three insulin receptor forms . They suggest this could occur in vivo as a result of lysosomal protease processing.

c) Corin & Donner (1982) proposed two affinity states of the hormonal - receptor complex ie. the formation of a "high affinity" complex occurs upon insulin binding to its receptor.

The evidence to support the negative cooperativity model is as multitudinous as that for the two site model. One particularly strong piece of evidence in favour of this model is that presented by De Meyts et al (1976). This group demonstrated that the plant lectin concanavalin A was able to inhibit the site-site interactions between insulin receptors without binding to the insulin receptor binding site. This resulted in the linearization of the Scatchard plot.

When these binding studies are considered in context with the insulin receptor structural determinations, it is conceivable that either model may well be correct . For example, the proteolytic processing of the B subunit is evidently possible when the receptor cloning studies are considered. Indeed, Ebina et al identified a B' peptide which they suggest is cleaved from the B subunit sequence during pro-receptor processing. If however, the glycosylation studies are considered; the presence of a relatively small amount of glycosylation of the B

subunit (the subunit which is not generally accepted to be the insulin binding site) may serve the function of facilitating inter-receptor interactions. After all, insulin receptors do appear to aggregate prior to internalisation ; a process known as receptor mediated endocytosis (RME) . Also, the suggestion that plant lectins such as concanavalin A may compete with insulin for binding to the insulin receptor could be a result of their binding to carbohydrate moities. This could therefore reduce access to the insulin binding site. The observations by De Meyts et al are to some extent in agreement with observations by Cuatracasacs & Tell (1973). The ability of lectins to mimick insulin's stimulation of glucose transport in target cells could also be a result of receptor interactions and the endocytotic process.

In conclusion then, the precise model of insulin receptor binding is still a controversial issue. More recently , studies to investigate insulin binding in diabetic states, to determine whether insulin resistance could be attributed to insulin receptor abnormalities have been undertaken. Much literature is available in this area ,but only a few examples will be outlined here :

Livingston et al (1984) studied the binding of insulin in human adipocytes isolated from obese, insulin resistant and lean control subjects . They found no significant difference in binding affinity or capacity between these two classes of

subjects. It was therefore assumed that any defects were probably at the post-receptor level and not associated with insulin binding. Conversely, Salhanick et al (1985) and others such as Czech et al (1980) and Sampson et al (1982) have reported enhanced insulin binding in hepatocytes isolated from streptozocin - induced diabetic rats . This has been attributed to an "up regulation" or recruitment of receptors from intracellular locations. This would tend to suggest that the receptor levels themselves are controlled by the circulating plasma insulin concentration . Obviously in the case of this model of type I diabetes, the mechanism of insulin resistance does not appear to be reduced insulin binding capability.

Yki-Jarvinen et al (1984) looked at insulin binding in human adipocytes from control and type I diabetic subjects. They found a slight decrease in the total receptor number, but no change in receptor affinity. In addition, various hypoglycaemic agents therapeutically used in type II diabetes have been examined for possible effects on insulin binding. The literature published in this area is vast, but in general, the use of biguanides such as metformin (Vigneri et al 1982 ; Lord et al 1983 and Holle et al 1981) and phenformin (Iwamoto et al 1981) appear to increase the number of insulin receptors in a wide range of human cell lines. Increases in receptor number does not appear to involve de novo synthesis of receptors. Instead, receptors are recruited from an intracellular "pool".

Sulphonylureas have been reported in vitro (Maloff & Lockwood 1983; Vigneri et al 1982) to have no influence on insulin binding to its receptor. However, in vivo (Olefsky et al 1976; Feinglos & Leibovitz 1978 and Beck-Nielsen et al 1979) sulphonylureas appear to enhance insulin binding. This phenomenon has been attributed sulphonylureas in vivo increasing plasma insulin levels and therefore indirectly affecting insulin receptor number.

One very important point to mention is the comment on insulin binding studies which was made by Rizza et al (1981). That was, that many of the cell types used in such studies are not identified as insulin target cells. Therefore the value of such studies should be questioned.

1.2.3 Movement of Insulin Receptors From The Plasma Membrane

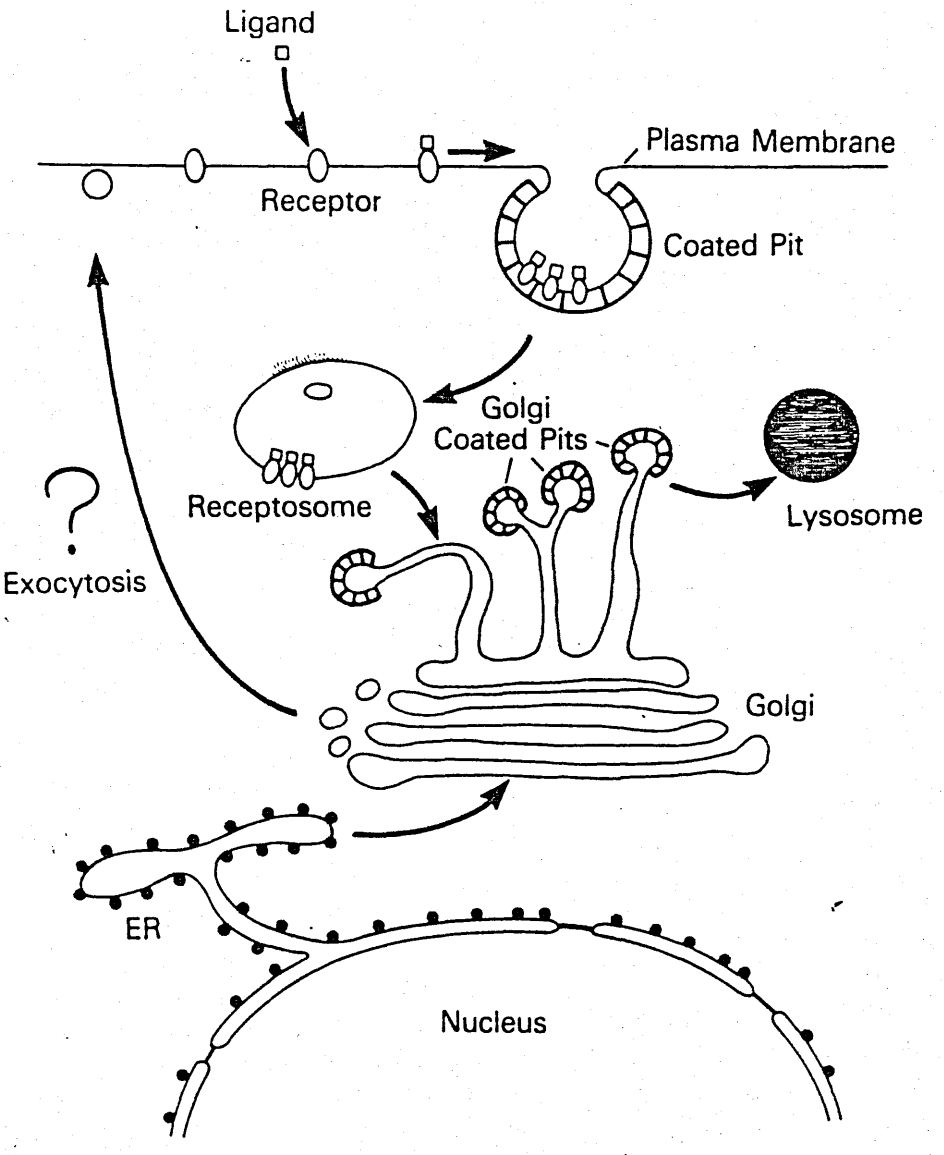
The process by which receptors are removed from the extracellular surface of the plasma membrane to intercellular localisations is known as "receptor mediated endocytosis " (RME). This process was first investigated by Goldfine et al (19 78) and Schlessinger et al (1978).Using radiolabelled or fluorescently labelled insulin, they demonstrated that occupied insulin receptors were internalized into hepatocytes. Similarly, utilization of photo-affinity labelled insulin has allowed the study of receptor lateral movement in the plasma membrane. It is proposed that occupied receptors must aggregate

or "cluster" prior to internalization in "pits" or plasma membrane indentations. These "pits" are coated on their cytoplasmic surface with a protein called clathrin (Pastan & Willingham 1983). Receptor aggregation has been reported to be important in insulin action (Kahn et al 1978) . Exposure of rat adipocytes to anti-insulin receptor antibodies resulted in the inhibition of ^{125}I- insulin binding. It was demonstrated that although both monovalent and bivalent Fab fragments affected radiolabelled insulin binding ,only bivalent Fab fragments triggered insulin - like effects in these cells. This therefore led to the proposal of receptor - hormone complex aggregation being an obligatory requirement for some of insulin's actions.

Compounds such as methylamine and ammonia have been shown to inhibit the clustering of a number of receptors (Maxfield et al 1979) . This inhibition of clustering has been demonstrated to block the internalization process. Indeed, activation of glucose transport has also been suggested to involve receptor movement in the plasma membrane (Simpson & Hedo 1984) . This process of receptor movement and internalization is proposed to occur in a series of distinctive stages (see figure 8).

The ligand - receptor complexes cluster into clathrin coated pits and become invaginated into vesicular structures inside the cell known as "endosomes". These endosomes may or may not be coated with clathrin (Pastan & Willingham 1983)

Figure 8 Schematic Diagram of The Process of Endocytosis



E.R. = endoplasmic reticulum. "Receptosomes" are endosomes containing the receptor - ligand complex.

Figure is reproduced from Pastan & Willingham 1983.

However, they do appear to have an internal acid pH which is proposed to trigger the dissociation of the ligand - receptor complex (Helenius et al 1983). There is strong evidence that the internalized receptors are recycled back to the plasma membrane , a process which appears to be independent of protein synthesis (Marshall et al 1981). This recycling process can be blocked by chloroquine and subsequently results in a net loss of cell surface receptors (a process known as "downregulation" of receptors). More recently, Hueckstedt et al (1986) used an iodinated , photoreactive analogue of insulin to covalently label the insulin receptor exposed on the extracellular surface of rat adipocytes.They demonstrated that insulin receptors may be recycled without the obligatory dissociation of the ligand-receptor complex. Thus, the process which "sorts" the ligand and the receptor moieties (Baenziger & Fiete 1986) may only distinguish with respect to the receptor and not the ligand.

It has also been demonstrated (Marshall et al1981) that in order for the internalisation process to occur, there is an obligatory requirement for occupancy of the receptor. Assuming that the insulin - receptor complex is internalised, dissociated and the receptor subsequently recycled, what then happens to the hormone ?

Insulin has been demonstrated to be proteolytically cleaved early after its internalization (Assoian & Tager 1981). However, this process was blocked using lysosomotropic agents

(Ward 1984). Interestingly, Blazar et al (1984) used chloroquine in vivo to inhibit insulin degradation in a diabetic patient who showed severe insulin resistance. This treatment was said to be very effective .

Now, "following" the internalization of insulin into hepatocytes, Ward (1984) found that the internalized hormone rapidly accumulated in the microsomal and / or Golgi fractions of the cells. However, the hormone did not accumulate in the lysosomes. The importance of this is not known at the moment, but much work is being continued in this area . It is of course possible that the internalization of insulin could be required for insulins' long-term effects such as specific protein synthesis induction. Perhaps an unidentified intracellular receptor for the hormone may exist . Or perhaps the internalization of the hormone acts only as a means to remove the hormone from the receptor and allow recycling of an unoccupied receptor back to the plasma membrane and thereby conserving the energetic expense of synthesizing a new receptor.

Of course as a result of this receptor movement it is possible that other membrane components are simultaneously translocated . There is evidence that insulin triggers the movement of IGF II receptors (Oppenheimer et al 1983) and transferrin receptors (Davis et al 1986) to the plasma membrane from intracellular (possibly microsomal) locations. The mechanism by which this occurs is unknown, but it does seem likely that this process of insulin stimulated RME would

influence such membrane movements.

Another process which seems to occur spontaneously upon insulin binding to its receptor is the autophosphorylation of the insulin receptor's β subnit. Over the past few years this area of insulin "action" has received a large amount of attention. A relatively brief outline of this process and its possible role in the normal and diabetic states will be presented.

1.2.4 Autophosphorylation Of The Insulin Receptor And Its possible Role In insulin Action

Kasuga et al (1982) were the first to demonstrate that insulin had the ability to stimulate phosphorylation of the β subunit of its own receptor. This was later confirmed by others and work performed by Shia & Pilch (1983) established the site of this insulin phosphorylation to be tyrosine specific. This autophosphorylation occured rapidly in the presence of insulin with a $K_{0.5}$ of 30 sec at 22 °C (White et al 1984) and a $K_{0.5}$ of 5 min at 4 °C (Zick et al 1983). The maximum and steady state levels of phosphorylation is achieved at between 10 and 20 min in the presence of insulin and is dependent upon the incubation temperature used. In the absence of insulin,

phosphorylation does occur, but only slowly (Gammeltoft & Van Obberghen 1986). Mn^{2+} and Mg^{2+} have been shown (Pike et al 1984) to augment the insulin stimulated phosphorylation of the insulin receptor, but the divalent cations Ca^{2+}, Zn^{2+} and Cr^{2+} were found to be ineffective, although Co^{2+} does have a slight effect (Avruch et al 1982; Zick et al 1983). The phosphate donor for the phosphorylation reaction is ATP (Kasuga et al 1982) and the Km value for ATP in the presence of insulin is in the order of 30 - 50 uM (Gammeltoft & Van Obberghen 1986). ADP inhibits ^{32}P ATP incorporation into the β subunit.

Insulin stimulation of this autophosphorylation process results in enhanced tyrosine kinase activity (Vmax increases , but Km remains unchanged). This autophosphorylation also results in phosphorylation of exogenous substrates in vitro such as caesin, actin, (Kasuga et al 1983) angiotensin II, histone 2B (Klein et al 1985)and synthetic peptides (Casnellie et al 1982; Zick et al 1985) on tyrosine residues.

Comparisons have been made between tyrosine kinase activity associated with the insulin receptor and the EGF receptor, particularly with respect to their specificities for exogenous substrates (Pike et al 1984; Klein et al 1985 ; Stadtmauer & Rosen 1983). It is believed that both receptor kinases phosphorylate similar exogenous substrates in vitro. However, it must be remembered that the conditions employed for in vitro studies are hardly physiological and when

the cellular localisation of some of these " exogenous substrates" are considered (such as histone 2B) there is no evidence as yet, to suggest that the insulin receptor and therefore the kinase has any direct contact with such substrates.

Rather interestingly however, is the observation that tyrosine kinase activity has been associated with a range of oncogene products (Hunter 1980) , the EGF receptor,the PDGF receptor (Ek et al 1982) and the IGF I receptor (Jacobs et al 1983). As a result of these observations, it may be feasible to suggest that the function of this tyrosyl kinase activity may well be associated with growth and cellular proliferation.

There are however differences in the sites of phosphorylation of the insulin receptor when in vitro and in vivo studies are compared. White and colleagues (1985) demonstrated that isolated receptor in vitro studies showed only phosphotyrosine containing β subunit , but in vivo receptor studies indicated that phosphoserine and phosphothreonine residues were also present in receptor β subunits stimulated with insulin in vivo before isolation. Using partially purified insulin receptor preparations, others (Zick et al 1983; Yu & Czech 1984) have suggested the presence of a serine kinase which is non-covalently associated with the receptor . This is proposed to be removed during the receptor purification procedure (see fig 9).

Since the insulin receptor has been demonstrated to

Figure 9 Schematic Representation of The Insulin Receptor Tyrosine Kinase And Associated Serine Kinase

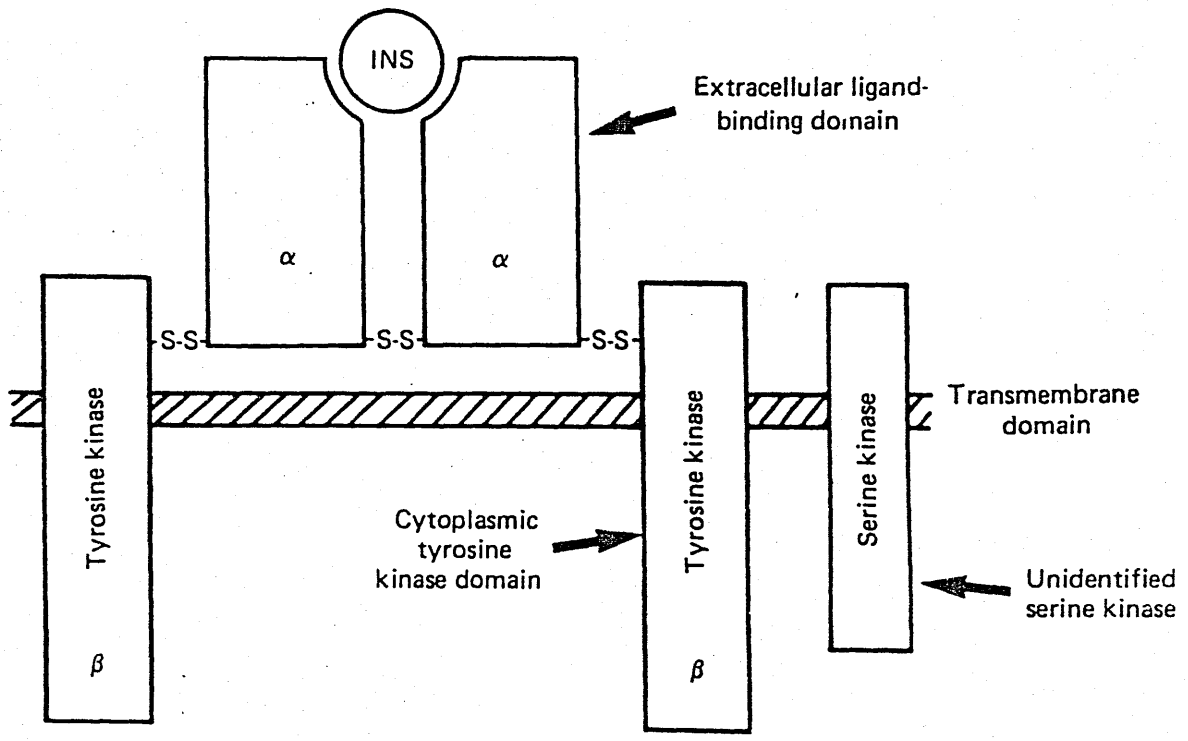


Figure reproduced from Gammeltoft & Van Obberghen 1986.

exhibit kinase activity , it has been the aim of many workers to ascribe a function to this action . Probably the most dramatic experiments in this field of research have been done by Ellis *et al* (1986). Site directed mutagenesis has been used to demonstrate a correlation between insulin's ability to autophosphorylate its receptor and insulin's ability to stimulate glucose transport. This group altered or removed tyrosine residues in the suggested autophosphorylation region of the β subunit and then expressed these mutant receptors in chinese hamster ovary cells (CHO) . They demonstrated that tyrosine kinase activity in these cells was subsequently reduced or abolished . Simultaneously, insulin stimulated 2-deoxyglucose transport was also demonstrated to be reduced or abolished whilst the insulin binding affinity remained unchanged. They therefore suggested that the autophosphorylation of the insulin receptor could regulate glucose transport *in vivo*. Similarly, Morgan & Roth (1987) used a monoclonal antibody which inhibits the insulin receptor kinase activity . This antibody was demonstrated to inhibit simultaneously the uptake of 2-deoxyglucose , phosphorylation of the S6 ribosomal protein and decrease glycogen synthesis in CHO cells, a human hepatoma cell line and rat adipocytes.

However, Zick *et al* (1984) demonstrated that polyclonal antisera directed against the insulin receptor inhibited insulin binding, stimulated lipogenesis in isolated rat adipocytes, but

did not stimulate the phosphorylation of the insulin receptor. Similarly, Simpson & Hedo (1984) reported that polyclonal antisera directed against the insulin receptor did not stimulate receptor phosphorylation ,but did stimulate glucose transport in intact isolated rat adipocytes. These polyclonal antisera experiments seem to suggest that activation of these rapid "short term" effects of insulin are a result of binding to the insulin receptor binding site rather than directly interacting with the tyrosine kinase activity (as in the case of the monoclonal antibody) . However, the important point to be made from these antisera experiments is that stimulation of receptor phosphorylation does not appear to be an obligatory prerequisite for these insulin actions.

It is of course possible that the receptor phosphorylation in the in vivo studies could stimulate this postulated serine, threonine kinase believed to be associated with the insulin receptor in the plasma membrane. This kinase could play a role in the regulation of these short term insulin actions. It is also conceivable that these site directed receptor mutants could affect non-covalent associations with peptides or other molecules at the cytoplasmic surface of the plasma membrane. Interference with insulin stimulated activities could thus occur.

The phorbol ester TPA (12-o-tetradecanoyl phorbol-13-acetate, see figure A7) has been demonstrated to activate protein kinase C in a variety of tissues (Niedel et al 1983; Castagna et al

1982). This phorbol ester and protein kinase C have been shown to serine and threonine phosphorylate the insulin receptor (Jacobs et al 1983b; Bollag et al 1986) .This resulted in the reduction of the insulin tyrosine kinase activity and decreased insulin stimulated glycogen synthase activity. This therefore suggested a link between tyrosine kinase activity and an early affect of insulin.

However, Van de Werve et al (1985) demonstrated that tumour promoting phorbol esters stimulated lipogenesis in rat adipocytes without altering insulin binding , but reduced insulin stimulated lipogenesis. Thus, phorbol esters themselves may have insulin like actions . In addition, insulin and TPA have been shown to phosphorylate an apparently common 40 K Da protein in adipocyte plasma membranes(Graves & McDonald 1985). The identity of this protein is unknown, but the phosphorylation of this protein does suggest another common feature between protein kinase C and insulin action. Of course the question of whether or not this membrane associated protein has kinase activity itself will be of great interest to answer . If this were found to be the case this would open up further potential research into the postulated serine kinase associated with the insulin receptor and its link with insulin action.

It is also of interest to note that activation of protein kinase C by diacylglycerol is believed to produce other insulin like effects such as activation of glucose and amino acid

transport (Farese et al 1986). Intriguingly, decreased autophosphorylation of the insulin receptor in the diabetic type I model has been suggested to occur (Kadowaki et al 1984). However, Amatruda et al (1985) reported normal receptor kinase activity in this model of diabetes. Therefore, this may or may not be a mechanism by which insulin resistance could occur.

Also, catecholamine treatment has been suggested to decrease tyrosine kinase activity of the insulin receptor. This treatment induces an " insulin resistant " state (Haring et al 1986). Pessin et al (1983) have also demonstrated that β- adrenergic agonists (catecholamines) inhibit both insulin and EGF binding to their respective receptors. This action appears to involve a cAMP - dependent process . As both receptor populations exhibit tyrosine autophosphorylation , it is a possibility that cAMP could be stimulating a protein kinase to phosphorylate the receptors themselves.This could inhibit ligand binding and subsequent activation of signal transduction in these systems. Such an action has not been proven , but it is a feasible speculation to make and does suggest "cross-talk" between hormones / receptor agonists with opposing metabolic actions.

1.3 Insulin Action

Insulin exerts many diverse biochemical effects within a wide variety of target tissues. The hormone displays both long

term and short term effects. Long term effects include changes in protein synthesis and breakdown . To date, this area of insulin action and mechanisms involved are poorly understood. Short term effects however, have been well characterisd and generally are a result of changes in the activity of existing enzymes and membrane transporters.

Principa lly, insulin controls carbohydrate and fat metabolism in liver, skeletal muscle and adipose tissue (Czech 1977; Denton et al 1981). The hormone's control of anabolic processes such as glycogen & lipid synthesis , in general are attributed to enhanced glucose transport and changes in covalent modification of key regulatory enzymes. Table I illustrates some of these short term effects and the mechanisms suggested via which these effects are mediated. As can be seen from the table, insulin has the ability to alter the activity of a range of enzymes in a variety of intracellular localisations . This observation has been intriguing to workers who have searched (and are still searching) for a classical" second messenger". Such a second messenger must have access to enzymes both in membrane - bound organelles and the cytosol.

Many second messenger proposals have been made and include ; elevated cGMP levels (Illiano et al 1973; Vydelingum et al 1975), cellular calcium flux (Clausen et al 1974; Kissebah et al 1975) and a peptide mediator released from the plasma membrane (Larner 1984). However, none of these proposals have been able to account for all of insulin's observed effects.

Table I Short-Term Effects of Insulin

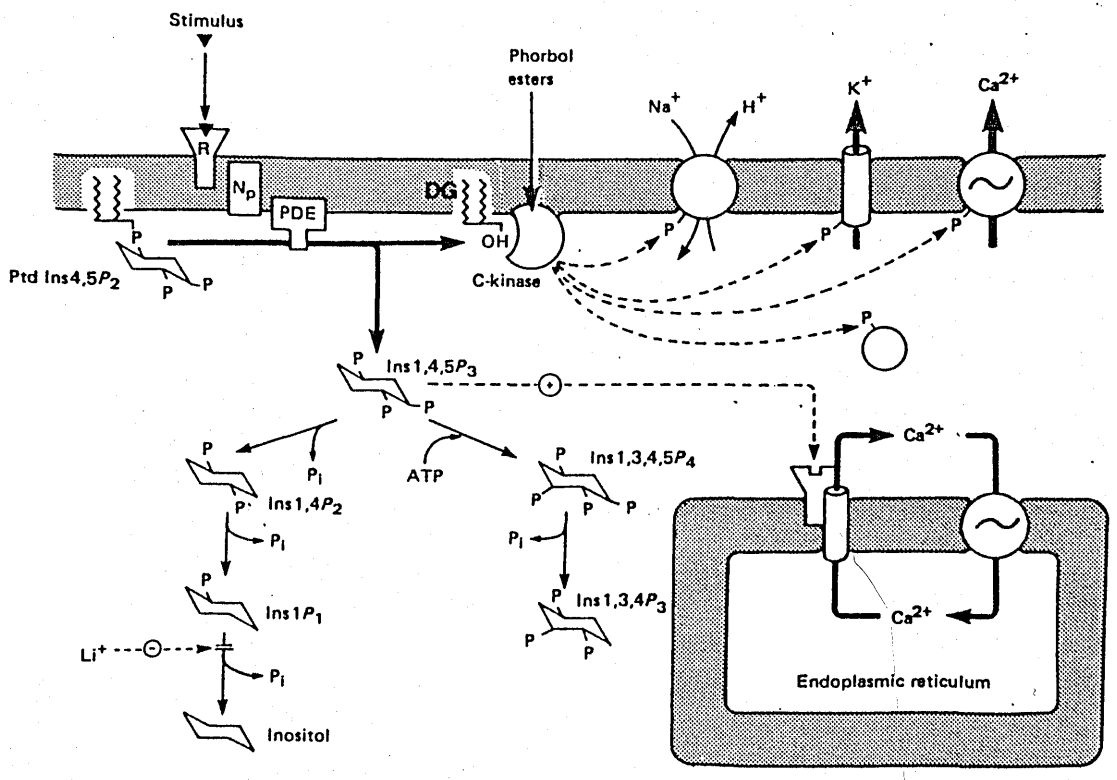
Tissue / Cell Type	Cell Location	Enzyme / Protein	Effect
Fat, Liver	cytoplasm	Acetyl CoA Carboxylase	increased phosphorylation activates enzyme
Fat, Liver, Heart	plasma membrane	Insulin receptor tyrosine kinase	Autophosphorylation of the receptor enhances tyrosine kinase activity
Fat,Liver,Heart	microsomes	S6 ribosomal protein	Increased serine phosphorylation correlates with increased protein synthesis
Fat,Liver,Muscle	glycogen complexes	Phosphorylase kinase	Decreased phosphorylation reduces enzyme activity
Fat,Liver,Muscle	glycogen complexes	phosphorylase	Decreased phosphorylation reduces enzyme activity
Liver,Fat	mitochondria	pyruvate dehydrogenase	Decreased phosphorylation ofenzyme enhances activity

Recently, it has been suggested that insulin might exert actions by stimulating other " second messenger " production or mediation systems. Some of these suggestions will now be briefly considered.

1.3.1 Phosphoinositol (P.I) Turnover

A wide variety of hormones (Berridge 1984) and neurotransmitters (Nahorski et al 1986) have been implicated in intracellular Ca^{2+} mobilization by the second mesenger transmembrane signalling system known as P.I. turnover. In brief, receptor activation leads to an increase in intracellular diacylglycerol and inositol 1,4,5 trisphosphate. Diacylglycerol is known to activate protein kinase C and inositol 1,4,5 trisphosphate is believed to release Ca^{2+} from the endoplasmic reticulum (Nishizuka 1984; Berridge & Irvine 1984; Downes & Michell 1985). As a result of protein kinase C activation and intracellular Ca^{2+} elevation, a number of responses such as smooth muscle contraction and secretory process are mediated. This P.I. "cycle" is very simplistically illustrated in fig 10 . This area of transmembrane signalling has become particularly active over the last few years and rapid advancement in the elucidation of phosphorylated inositol species and protein kinase C activation and localisation has been made.

Figure 10 The Phosphoinositide Pathway



Ligands binding to surface receptors (R) are coupled through a GTP-binding protein (N_p) to the phosphodiesterase (PDE) which cleaves phosphatidylinositol 4,5 diphosphate (PtdIns 4,5 P_2) to yield diacylglycerol (DG) and inositol triphosphate (Ins 1,4,5 P_3). DG activates protein Kinase C (C.Kinase) and Ins 1,4,5 P_3 releases calcium from the endoplasmic reticulum. The figure is reproduced from Berridge 1986

However, insulin's possible involvement in the P.I. cycle has been investigated since the nineteen sixties and seventies and its role (if any) remains controversial. Work has been published which supports the view that insulin can enhance the phosphorylation of phospholipids such as phosphatydlcholine, phosphatidylinositol , phosphatidylethanolamine and phosphatidic acid (Stein & Hales 1974; DeTorrontegue & Berthet 1966; Creba et al 1983). The problem with this work however, is that ^{32}Pi labelled ATP was used as the phosphate donor . This is a poor method for examining such effects because the hormone increases the specific activity of (^{32}P) ATP (Hepp et al 1968; Stein & Hales 1974). When this insulin induced increase in the specific activity of the ATP is considered, significant phosphate incorporation into specific phospholipids cannot necessarily be assumed.

More recently, Pennington & Martin (1985) have demonstrated , using ^{3}H- labelled inositol, that insulin may stimulate the de novo synthesis of phosphatidyl inositol and phosphatidyl 4,5 diphosphate. This of course must be a better way of quantifying insulin's specific effects upon P.I. metabolism, but one criticism which should perhaps be made is that the accumulation of phosphatidyl inositol was measured for 30 min . This incubation period is rather long when one considers the rapid actions of insulin , such as glucose transport, which occurs within a few minutes. Phorbol esters are

known to stimulate protein kinase C and have been suggested to also have the ability to stimulate glucose transport (Farese et al 1986). This could therefore conceivably be a possible effect of P.I. breakdown when diacylglycerol is released and stimulates protein kinase C activity. However, Creba et al (1983), Thomas & Williamson (1983) and Pennington & Martin (1985) have all reported that insulin does not have the ability to stimulate P.I. breakdown and thereby insulin would be assumed not to have the ability to stimulate the release of diacylglycerol for the activation of protein kinase C . It would therefore appear that the P.I cycle does not operate as a second messenger system for insulin in the same way as it does for hormones such as vasopressin or for neurotransmitters in the central nervous system. Although, it is possible that long term protein synthesis effects associated with insulin action could in some way involve phophorylation of phospholipids.

Indeed, some workers have suggested that there may be a relationship between the insulin receptor kinase activity and phosophatidylinositol phosphorylation (Machicao & Wieland 1984; Sale et al 1986). This phosphatidylinositol kinase actvity has been associated with both the partially purified insulin receptor and the homogeneously purified insulin receptor . Under these experimental conditions the serine kinase associated with the insulin receptor would be removed . This would therefore strongly suggest that the insulin receptor kinase or another

kinase activity strongly associated with the insulin receptor is responsible for this phosphorylastion event.

There is evidence for oncogene products of the rous sarcoma and avian sarcoma viruses being tyrosine kinases . These proteins may also phosphorylate phosphotidylinositides (Sugimoto et al 1984; Macara et al 1984) . In addition the suggestion of a relationship between the ras 21 oncogene product and P.I. breakdown occuring has been made (Wakelam et al 1986). Therefore should a more positive link between insulin and P.I. metabolism be established a breakthrough in understanding insulin's long term effects may be achieved.

Interestingly, Urumow & Wieland (1986) have demonstrated guanine nucleotide control of phosphotidylinositol 4,phosphate phosphorylation in human placental membranes.This event lead to activation of phospholipase C and subsequent P.I. turnover . This is similar to the suggestion that insulin also has the ability to interact with proteins whose activity is controlled by guanine nucleotides .

1.3.2 A Non-peptide Mediator Release

An American group (Saltiel & Cuatrecasas 1986 ; Saltiel et al 1986) have recently partially purified two novel mediators (see also Houslay & Wakelam 1987 for a review). They propose that unlike Larner's "peptide mediator" , these mediators are complex compounds containing glucosamine and inositol. Upon insulin stimulating an enzyme analagous to phospholipase C, this compound

is hydrolysed to a glucosamine containing inositol phosphate and diacylglycerol. They demonstrated that the enzyme catalyzing this action was distinct from phospholipase C which controls P.I. turnover.

The diacylglycerol produced has been suggested to contain myristoyl chains and would therefore be distinct from diacylglycerol produced from phosphatidyl inositol phosphates. This group also suggested that a specific species of protein kinase C could be activated by this distinctive diacylglycerol. Therefore it has been proposed that this diacylglycerol species could mediate insulin's so called "long term effects" such as the specific induction of protein synthesis and cellular growth.

1.3.3 The involvement of Guanine Nucleotide Regulatory Proteins in Insulin Action

General Introduction To Guanine Nucleotide Regulatory Proteins

In the late 1970's and early 1980's, guanine nucleotide regulatory proteins (G Proteins) began to be recognised as important components in hormone transmembrane signalling processes. Rapidly the G proteins Gi and Gs were postulated and a role in adenylate cyclase regulation ascribed to them (Stadel *et al* 1980). They were isolated, purified and their subunit structures determined (Northup *et al* 1983a ; Northup *et al* 1983b; Codina *et al* 1984). Another G protein termed transducin

was proposed to be involved in the regulation of cGMP phosphodiesterase activity in rod outer segments . This too, was isolated and purified and its structure determined (Fung 1983).

Hence, these G proteins (termed as such because their activity is regulated by guanine nucleotides) were identified as a "family" of proteins.Each has its own distinctive, but similar structural composition and each exhibits a crucial role in signal transduction between surface receptor and transmembrane effector systems (Houslay 1984).

Structurally, these proteins were initially believed to be of heterodimeric subunit composition. The subunits were denoted α and β and the holomeric complex had an ($\alpha\beta$) 1:1 stoichiometry. The α subunits of these G proteins was distinctive from and non-identical to each other. The guanine nucleotide binding site was identified as being located on the α subunits. The β subunit was identified as a 35 KDa molecular weight species which appeared to be identical in each member of the G protein family (Codina et al 1984; Manning & Gilman 1983).
The β subunit has recently been cloned (Sugimoto et al 1985) and both polyclonal and monoclonal antibodies have been raised against this polypeptide (Rosenthall et al 1986; Lingham et al 1986).

Codina et al (1984) isolated a third subunit species with a low molecular weight of between 10-15 K Da. This subunit, which was termed the γ subunit , was found to be

present in Gs, Gi and transducin protein complexes. The γ subunit is believed to associate so tightly with the β subunit that initial purification procedures used had not been able to resolve the two subunit species. Recently , the γ subunit in transducin has been shown to be structurally distinct from the γ subunits of Gs and Gi. However, the γ subunits of Gs and Gi appear to be identical (Manning & Gilman 1983; Hildebrandt et al 1985).

Also, in the early nineteen eighties ,a G protein was proposed to be involved in the mediation of two rapid insulin stimulated processes (Heyworth et al 1983 ;Heyworth & Houslay 1983 These processes were the activation of specific membrane associated cAMP phosphodiesterase activity and the inhibition of glucagon stimulated adenylate cyclase activity in isolated hepatocytes and liver plasma membranes. This G protein was termed " G ins "

Since these early G protein postulations and purifications , further G proteins have been identified and purified from brain and placenta (Sternweis & Robishaw 1984, Evans et al 1986). In particular, a G protein termed Go whose α subunit appears to be structurally similar to Gi, has been isolated and purified . The precise function of this G protein , which has been found in relative abundance in brain tissue, has yet to be determined. A G protein termed Gp has been purified from brain and placenta . This G protein has been suggested to be

involved in phospholipase C activation and therefore may be a modulator between receptors and an effector in the P.I. cycle signal pathway (Berridge 1986).

Other intracellular G proteins not associated with the plasma membrane have also been identified. These are believed to be invovled in processses such as cytoskeletal function and protein synthesis. Bacterial toxins have greatly aided the identification of G proteins both in terms of purification and functional studies. These studies will be discussed in more detail with respect to structural characteristics of Gs, Gi and Gins. My work has been primarily involved with one effector, that is , adenylate cyclase and its regulation by the G proteins Gs , Gi and the putative Gins. As a result of this, only these G proteins will be dealt with in greater detail. Also, the putative Gins will only be reviewed up to the time when my work began.

Structural & Functional Characteristics of Gs

Gs, is the guanine nucleotide regulatory protein which has the ability to stimulate the activity of the enzyme adenylate cyclase . It has been purified from a wide variety of tissues including brain and skeletal muscle. Its α subunit has a molecular weight of 45 K Da . An additional higher molecular weight form of 52 K Da was initially also found in rabbit skeletal muscle preparations. Recently,cDNA studies have

demonstrated the presence of two distinct forms of Gs α in a wide variety of tissues (Robishaw et al 1986 ; Harris et al 1985). The α subunit of this protein is ADP- ribosylated by cholera toxin in the presence of a membrane bound protein factor termed ARF (Gilman 1984). Using ^{32}P - labelled NADP as the ADP-ribose donor, this bacterial toxin prepared from Vibrae cholerae catalyzes this covalent modification process. This technique has been extensively used to identify the α subunit of this Gs protein. Upon cholera toxin ribosylation, the subunit binds GTP and remains in a persistantly active state . In this state , the α subunit remains undissociated from the (α β γ) holomeric complex.

Under hormonally activated conditions, G proteins bind GTP and subsequent activation of the α subunit occurs. The holomeric complex dissociates to liberate the free α and $\beta\gamma$ subunits (Northup et al 1983; Katada et al 1984) and the free α subunit is then able to interact with its effector system. It has been shown that Gs requires Mg^{2+} for activation (Iyengar 1981; Iyengar & Birnbaumer 1981). The Km for Mg^{2+} is 5-10 mM (Iyengar 1981), but the physiological concentration of intracellular Mg^{2+} is estimated to be about 0.5mM (Birnbaumer et al 1985). A role for hormonal / catecholamine stimulation of receptors has been postulated whereby receptor stimulation results in an increased affinity of the G protein for Mg^{2+} . Subsequent activation is then proposed to occur (see sectiom 1.4.2 " Dual Control of Adenylate Cyclase Activity By Hormonal Activation of Gs and Gi".)

The α subunit of Gs has been demomstrated to bind GDP, GTP and their analogues. The rate of Gs activation by non-hydrolysable analogues (such as GPP(NH)P and GTPγS) is slower than the rate of activtion by GTP itself (Schramm & Rodbell 1975) . Indeed, a distinctive "lag" period is observable prior to activation . This is believed to reflect a distinct activation process which occurs after nucleotide binding (Birnbaumer et al 1980 ; Iyengar et al 1980). Once activation of the α subunit by non-hydrolysable guanine nucleotides occurs , the activation state is persistent (Londos et al 1974).

The Km of Gs α for GTP and its analogues is about 1μM. Intracellular concentrations of GTP are about 0.1mM. Hence, under physiological conditions there is always saturating GTP levels present (Birnbaumer et al 1985). Under "basal" , that is , non-hormonally stimulating conditions , or when detergent solubilised Gs is used, GTP is unable to stimulate the activation and dissociation of the Gs complex. In order for GTP to activate Gs, reconstitution of the protein in the presence of stimulatory receptors is required (Brandt et al 1983; Cerione et al 1984).

Also, Gs α can be activated by NaF in the presence of Mg^{2+} and Al^{3+} . Indeed, these conditions were used in the earlier procedures for Gs isolation and purification (Sternweis & Gilman 1982). The precise mechanism by which NaF activates Gs is unknown, but the compound appears to promote persistent subunit dissociation.

Without doubt, one of the most useful tools which has been used to study G protein modulation of adenylate cyclase activity is the S 49 mouse lymphoma cell mutant which has been named "CYC^-". This cell mutant is deficient in Gs activity (Bourne et al 1975; Sternweis et al 1981). Originally, this cell mutant was believed to be devoid of adenylate cyclase activity because stimulatory ligands such as β- adrenergic agonists and guanine nucleotides were unable to stimulate this enzymes activity (Abramson et al 1985).

However, Ross & Gilman (1977) reconstituted Gs activity into CYC^- cells . Under these conditions they demonstrated the presence of a functional adenylate cyclase catalytic unit which could be stimulated by NaF , guanine nucleotides and β adrenergic agents.

Structural and Functional Characteristics of Gi

Gi, is the guanine nucleotide regulatory protein which has the ability to inhibit adenylate cyclase activity . It has been purified extensively from bovine brain (Neer et al 1984) and rat liver (Bokoch et al 1984). This protein is a heterotrimeric protein $\alpha\beta\gamma$ subunit composition . The α subunit has a molecular weight of 41 K Da and has been shown to have 75%

sequence homology with another related G protein Go. Gi α has been cloned by Michel et al (1986) and this group in addition have identified another G protein which they have called α h. This α h species has an apparent molecular weight of 39 K Da and also exhibits 75% sequence homology with Gi α .The function of both Gh and Go have yet to be determined.

Gi, Go and Gh are ribosylated by a component isolated from the bacterium Bordetella pertussis. This component, known as pertussis toxin or islet activating protein (IAP) catalyzes the ADP- ribosylation of a C-terminal region of the α subunits of these G proteins using NAD as the ADP -ribose donor. This pertussis toxin ribosylation technique has been extensively utilized to identify the 41 K Da Gi α subunit (Katada & Ui 1982; Bokoch et al 1984; Codina et al 1984). It is however now apparent that the use of this technique to identify this 41 K Da protein is not as specific a tool for recognition as had previously been assumed.

Pertussis toxin ribosylates Gi α when it is in its holomeric $\alpha\beta\gamma$ complex and resultantly prevents further activation of the α subunit by guanine nucleotides. Aktories et al (1983) demonstrated that when Gi was preactivated with GTPγS, deactivation by pertussis toxin treatment does not occur .

Gi α , like Gs α, binds GTP and its analogues upon hormonal stimulation in the presence of Mg^{2+} . The α subunit becomes activated and dissociates from the holomeric complex

form. Gi has a higher affinity for binding non-hydrolyzable GTP analogues than does Gs α, but both show the same potency order for these analogues of GTPγS > GPP(NH)P > GPPCH$_2$P. Half maximal activation of Gi α is achieved at a GTPγS concentration of 3nM, but half maximal activation of Gs α occurs at a GTP-γS concentration of 30nM (Jakobs et al 1985).

In the presence of GTP analogues, Gi α requires a lower Mg^{2+} concentration for activation than does Gs α (Ross & Gilman 1980; Birnbaumer & Iyengar 1982; Hildebrandt & Birnbaumer 1983; Jakobs et al 1983; Jakobs & Aktories 1983). The Km for Mg^{2+} is in the µM range (Hildebrandt et al 1983) and so under normal physiological conditions, Mg^{2+} is not a limiting factor for activation.

Gi can be selectively inactivated by treatment of intact cells or broken membranes with the sulphydryl reagent N- ethylmaleimide (NEM). It is believed that NEM affects receptor - G protein interactions specifically at low concentrations (Limbird & Speck 1983). Pretreatment of membranes with NEM also blocks the ADP-ribosylation of Gi by pertussis toxin , but the cholera toxin catalyzed ribosylation of Gs is minimally affected (Jakobs et al 1982; Brandt et al 1983; Aktories et al 1984).

Finally, Ca^{2+} and phorbol ester activated protein kinase C has been demonstrated to have the ability to phosphorylate Gi and thereby attenuate its activity in human

platelet membranes and CYC cells (Katada et al 1985; Watanbe et al 1985). Recently, purified Go and Gi were demonstrated to be phosphorylated in vitro by the insulin receptor tyrosyl kinase activity (O'Brien et al 1987). It has yet to be established whether these G proteins (and perhaps others) are phosphorylated by this kinase activity in vivo.

The Putative G Protein "Gins"

In the early nineteen eighties , Heyworth et al (1983) demonstrated that insulin had the ability to activate two distinct hepatic phosphodiesterase activities . These activities were resolved using a gentle homogenization and percoll gradient fractionation procedure. One of these phosphodiesterase activities termed the "plasma membrane enzyme" was shown to be activated 2 fold by insulin and 1.5 fold by cholera toxin treatment. The intriguing observation by this group was that although this enzyme was activated by cholera toxin treatment, it was not activated by glucagon or dibutyryl cAMP . Obviously, the cholera toxin activation was not a result of Gs activation by the toxin and subsequent cAMP elevation. Also of interest was the observation that glucagon pretreatment of hepatocytes blocked the activation of this enzyme by insulin.

Closely following this report was another publication suggesting that insulin could inhibit glucagon and GTP stimulated

adenylate cyclase activity in broken hepatocyte membranes. This action was guanine nucleotide dependent (Heyworth & Houslay 1983). This action of insulin was also dependent upon the concentrations of insulin and glucagon used. As the glucagon concentration was raised above 1nM , the ability of insulin to inhibit the glucagon stimulated adenylate cyclase activity was reduced. Intact isolated hepatocytes pretreated with glucagon in the presence of cAMP - phosphodiesterase inhibitors also demonstrated insulin's ability to reduce glucagon stimulated intracellular cAMP accumulation.

As discussed previously, the bacterial toxins; cholera toxin and pertussis toxin have been widely used to identify Gs α and Giα respectively. Heyworth et al (1985) demonstrated that cholera toxin also catalyzed the ribosylation of a plasma membrane protein with a molecular weight of about 25 K Da in isolated hepatocytes. This protein was identified as a GTP binding protein by the use of the photo-affinity GTP analogue azido-GTP. When insulin was present in the incubation medium, cholera toxin ribosylatin of this protein was reduced. This was therefore identified as a G protein which insulin (via its plasma membrane receptor) had the ability to interact with.

Together with the evidence that insulin and cholera toxin could activate a specific plasma membrane phosphodiesterase species , it was suggested that this process could involve this GTP binding protein. In addition, the activation of this enzyme

by insulin was unaffected by pertussis toxin pretreatment (Heyworth et al 1986). Similarly, this 25 K Da GTP binding protein was also apparently unaltered by pertussis toxin pretreatment. Also, the ability of insulin to inhibit adenylate cyclase activity in the presence of GTP suggested the requirement of a guanine nucleotide regulatory component in this action of insulin. As insulin only appeared to inhibit the cholera toxin catalyzed ribosylation of one G protein, it is probable that this protein is specifically coupled to the insulin receptor. Therefore, it is conceivable that this G protein could be involved in other short-term effects of insulin action.

There is evidence of the involvement of a G protein in insulin's ability to stimulate a cAMP-independent protein kinase in sarcolemma membranes from skeletal muscle(Walaas et al 1977; Walaas et al 1979). This protein kinase activity also appears to be sensitive to cholera toxin pretreatment (Walaas et al 1981). It has also been tentatively suggested that this G protein (termed "Gins") may be involved in glucose transport activation (Houslay & Wakelam 1987).

Finally, Horn et al (1986) suggested that insulin and GTP have the ability to regulate the dephosphorylation of the β subunit of the insulin receptor in sarcolemma membranes. It has been suggested that tyrosine kinase activity could be involved in activation of glucose transport in insulin's target tissues (see section 1.2.4) . Perhaps, Gins could be important as a link

between these two activities. Certainly, it has recently been shown that the insulin receptor kinase can phosphorylate G_i/G_o *in vitro* (O'Brien *et al* 1987). Perhaps other G proteins and in particular, perhaps Gins, could be covalently modified by this kinase activity in a similar manner.

1.4 The Adenylate Cyclase Effector System And Its Regulation

1.4.1 The Enzyme Adenylate Cyclase

Using forskolin - sepharose affinity and wheat germ lectin sepharose chromatography , the enzyme adenylate cyclase (E.C. 4.6.1.1) has been purified from rabbit myocardium and bovine brain cortex (Pfeuffer *et al* 1985a; Pfeuffer *et al* 1985b; Smigel 1986). The molecular weight of the catalytic unit ("C") is estimated to be between 115 - 190 K Da. Higher molecular weights of 270 K Da have been identified in brain cortex in the presence of the cross-linking agent disuccinimidyl suberate (Pfeuffer *et al* 1985b). These higher molecular weight species are believed to be aggregates of two or more adenylate cyclase catalytic units. However, these aggregates have not been observed in rabbit myocardial tissue (Pfeuffer *et al* 1985a).

The catalytic unit of adenylate cyclase was demonstrated to be a glycoprotein (Pfeuffer *et al* 1985b; Smigel 1986). It has therefore been suggested that the enzyme is an

integral membrane protein with exposed regions at both the extracellular and cytoplasmic surfaces of the plasma membrane. This suggestion is supported by the observation that isolation and purification of the enzyme requires significant amounts of non - ionic detergent to extract the protein from the membrane.

Purification of the non - activated ("basal") form of the enzyme has been achieved by the removal of forskolin from the forskolin - affinity purified enzyme preparation . This non - activated form of the enzyme is believed to be uncoupled from Gs (Pfeuffer 1985) and appears to be unresponsive to guanine nucleotide stimulation in the absence of additional Gs . Also, this "basal" form of the enzyme is not stimulated, but is inhibited by the addition of NaF.

Characteristically, bovine brain adenylate cyclase is proposed to exist in two forms; a Ca^{2+} / calmodulin sensitive and a Ca^{2+} / calmodulin insensitive form (Coussen et al 1985; Yeager et al 1985). However, forskolin and lectin purified adenylate cyclase activity appears to be insensitive to Ca^{2+} / calmodulin regulation . It is not known whether only one form of the enzyme is resolved during this purification procedure or whether a Ca^{2+} / calmodulin binding component important for activity regulation is lost during the procedure.

There is also speculation that the Ca^{2+} / calmodulin form of the enzyme occurs in other tissues such as pancreatic islets (Valverde et al 1979) smooth muscle (Piascik et al 1983),

cardiac muscle (Panchenko & Tkachuk 1984) and human platelets (Grigorian et al 1986). The mechanism by which Ca^{2+} and calmodulin can regulate adenylate cyclase activity remains controversial, but recently Asano et al (1986) suggested that calmodulin could interact with the G proteins Gi and Go. The significance of this observation is not clear at the moment.

Adenylate cyclase catalyzes the formation of cAMP from Mg^{2+}-ATP (see figure 11). The adenylate cyclase system is comprised of at least three distinct components ; namely , a receptor (or "discriminator molecule") of the type Rs or Ri (see section 1.4.2 for explanation) a stimulatory or inhibitory guanine nucleotide binding component termed a "G protein" and a catalytic unit ("C"). The catalytic unit is the effector system which upon stimulation by Rs activation of Gs or by direct activation of Gs results in the elevation of cAMP levels intracellularly . The mechanisms by which adenylate cyclase catalytic activity is regulated by these components will be discussed individually.

1.4.2 Dual Control of Adenylate Cyclase Activity By Hormonal Activation of Gs And Gi

Polypeptide hormones and catecholamines such as glucagon , adrenaline and thyroid stimulating hormone (TSH) bind to receptors on the extracellular surface of the plasma membrane.

Figure 11 G Protein Regulation of Adenylate Cyclase Activity

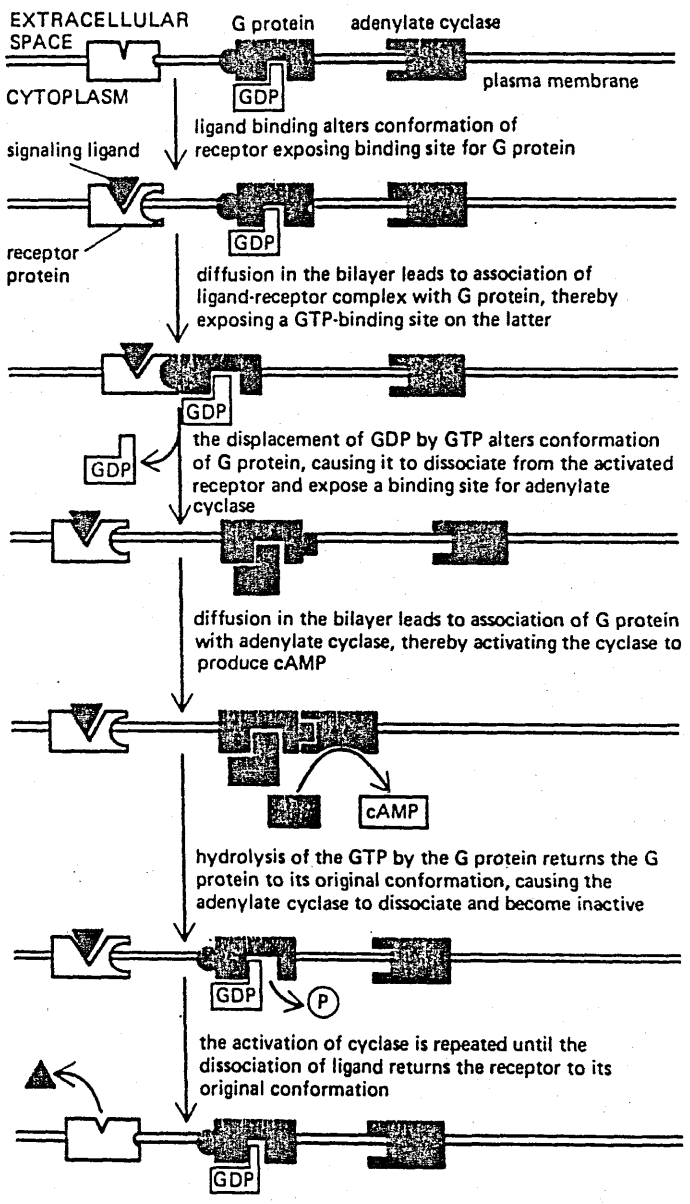


Figure is reproduced from "Molecular Biology of The Cell" Alberts et al 1983 (Garland Publishing inc. U.S.A.)

These receptors are believed to regulate G protein interactions with the adenylate cyclase catalytic unit ("C"). Receptors which interact with Gs to stimulate adenylate cyclase activity are denoted "Rs" and receptors which interact with Gi to inhibit adenylate cyclase activity are denoted "Ri" (Birnbaumer et al 1985).

Ligands which bind to specific receptors and cause activation of their respective G proteins are called "agonists". Agonists upon binding to Rs or Ri receptors cause changes in adenylate cyclase activity via this G protein activation process. However, some ligands have the ability to bind specifically to receptors , but do not have the ability to activate the respective G protein. These ligands are therefore unable to alter adenylate cyclase activity and are therefore known as "blockers" or "antagonists". There is also a further class of ligands called " partial agonists" which generally tend to be chemically modified analogues of agonists . These ligands are able to activate G proteins and cause changes in adenylate cyclase activity, but not to the same extent as their full agonist counterparts. These observations have led to suggestions that receptors exist in at least two or more states of activation (Birnbaumer et al 1985).

Addition of guanine nucleotides to membrane preparations has been demonstrated to reduce the affinity of receptors for their agonists (Rodbell et al 1971; Rubalcava &

Rodbell 1973). However, this effect is not observed for antagonists to the same receptors (Rubalcava & Rodbell (1973); Maguire et al 1976). This altered receptor affinity phenomenon has been experimentally characterised fairly extensively for glucagon and β-adrenergic receptors (Bird & Maguire 1978; Labarbera etal 1980).It was using the β-adrenergic system that the ability of guanine nucleotides to regulate receptor affinity was demonstrated to be Mg^{2+} dependent. Addition of guanine nucleotides to broken membranes in the absence of Mg^{2+} does not reduce receptor affinity for agonists . However, Mg^{2+} in the absence of nucleotides leads to an enhancement of receptor affinity for agonists. It appears that it is this Mg^{2+} enhanced receptor affinity that is reduced upon addition of guanine nucleotides.

It is believed that Mg^{2+} is the necessary component for activation of Gs and subsequent subunit dissociation . It may be therefore that activation and dissociation of Gs causes this receptor affinity attenuation. However, it was necessary to eliminate the possibility that Mg^{2+} was directly acting upon the receptor and thereby regulating agonist binding without altering Gs function. Therefore, Cyc^- cells which are deficient in functional Gs (Kent et al 1980) and reconstitution systems (Shorr et al 1981 ; Kalleher et al 1983) have been used to demonstrate that it is indeed the G protein which binds Mg^{2+} and thereby regulates receptor affinity.

Interestingly, in rat liver the glucagon receptor does not increase its affinity for agonists in the presence of Mg^{2+}. However, guanine nucleotides do reduce the receptor's affinity for agonists (Rojas et al 1983). The order of potency for guanine nucleotide transformation of glucagon and β-adrenergic receptors from a high to a low affinity for agonists has been studied. It appears that GDP is more potent than GTP (Rodbell et al 1971; Iyengar et al 1980) at initiating this process. These observations have led to the proposal that receptors in the low affinity state are "uncoupled" from the G protein Gs . This uncoupling process prevents further Rs and Gs interactions and may be concomitant with the G protein interacting with the adenylate cyclase catalytic unit .

Models to explain molecular interaction of the receptor, G protein and catalytic unit of the adenylate cyclase system have been proposed (Arad et al 1984) . Although the "Collision Coupling" model is probably one of the most favoured, other models cannot be entirely ruled out.

1.4.3 Models Proposed To Illustrate The Mechanism By Which Adenylate Cyclase ("C") , Gs And Rs Interact

Three major models have been proposed to explain the sequence of events occuring between the components C, Gs and Rs

upon hormonal stimulation of the adenylate cyclase system. These models are :

1. The Precoupled Model

$$Rs.Gs(GDP).C \longrightarrow HRs.Gs.'(GTP).C' \longrightarrow HRs.Gs.(GDP).C$$

This model proposes that all three components are associated with each each other at all times. Binding of agonist to the receptor in the presence of GTP results in the activation of the precoupled receptor-G protein-catalytic unit complex and subsequent stimulation of cAMP production by the catalytic unit. The activation "cycle" is terminated by hydrolysis of the GTP.

2. The Collision Coupling Model

$$Rs + Gs(GDP).C \longrightarrow \underset{\text{(Active)}}{H.Rs.Gs'.(GTP).C'} \longrightarrow \underset{\text{(Inactive)}}{HR + Gs(GDP).C}$$

This model proposes that the G protein and the catalytic unit are always associated with each other , but the receptor only briefly associates with the Gs.C complex upon agonist binding.

3. The Shuttle Model

$$Rs + Gs(GDP) \longrightarrow H.Rs.Gs'(GTP) \longrightarrow HRs + Gs'(GTP)$$

$$Gs'(GTP) + C \longrightarrow \underset{\text{(Active)}}{Gs'(GTP).C'} \longrightarrow \underset{\text{(Inactive)}}{Gs(GDP) + C}$$

This model proposes that all three components are separate entities which only interact in an ordered sequence. Initially, the agonist in the presence of GTP is proposed to associate with the G protein which in turn dissociates from the receptor complex and associates with the catalytic unit to activate the adenylate cyclase system. The "cycle" is once again terminated by the hydrolysis of GTP to yield the free (unassociated) G protein.

Detailed kinetic studies have been performed primarily using the β-adrenergic receptor system because major purification and classification advances have been made in this system. The precise mechanism by which hormonal stimulation of the adenylate cyclase activity is achieved remains controversial. In addition, the kinetic arguements are complex. However, evidence to support and / or discredit each model will be very briefly discussed below in order to try to demonstrate the complexity of the adenylate cyclase system and its control.

In general, the precoupled model has been superseded in popularity by the other two models. This is mainly due to the observation that β-adrenergic receptors in the presence of Mg^{2+} and GTP exhibit an altered affinity for agonist binding. This altered affinity has been suggested to be a result of Rs.Gs dissociation. Under the constraints of this model, the three components must remain associated at all times. Similarly, after challenging cells with catecholamines , a desensitized (ie. less

responsive) state with respect to further hormone challenge has been observed. This state is concomitant with the down regulation of β-adrenergic receptors from the cell surface. There is no evidence for simultaneous movement of Gs into intracellular fractions under these conditions (Levitzki 1986). Thus, there is evidence for the Rs and Gs unassociated state which is in major disagreement with the precoupled model proposed.

However, the collision coupling model assumes only a transient receptor-G protein -catalytic unit complex formation. So Rs and Gs dissociation is within the confines of this model. Also, probably the most convincing evidence for the persistently associated Rs-C complex state is the fact that Gs α has been copurified with the catalytic unit .

Finally, the shuttle model is in agreement with the observation that non-hydrolyzable GTP analogues are able to activate Gs in the absence of the catalytic unit. Under the constraints of the other two models, this would not be expected to occur because Gs and C would always be associated. Therefore activation of Gs would be dependent upon the presence of C. However, one piece of evidence which could discredit this model is the observation that Rs and Gs in the absence of GTP can form a complex in the presence of agonists (levitzki 1986). This should not occur under the constraints of the three separate component state of the shuttle model.

Thus , the model by which the components of the adenylate cyclase system interact remains unclear, although

complex kinetic analysis has been reported to favour the collision coupling model (Levitzki 1986).

1.4.4 Use of Forskolin To Stimulate Adenylate Cyclase Activity

Forskolin is a diterpene isolated from the plant Coleus forskolii. Its structure is illustrated in figure 6 (appendix II). This compound has been shown to have the ability to elevate adenylate cyclase activity in a wide variety of tissues and organisms (Seamon & Daly 1986). In rat liver, forskolin increases the Vmax of the enzyme without affecting the enzyme's Km for Mg.ATP (Birnbaumer et al 1983). Forskolin can activate solubilized adenylate cyclase in the presence of both ionic and non-ionic detergents. This observation has led to the suggestion that this compound does not activate membrane associated adenylate cyclase by causing perturbation of either membrane structure or interactions between the catalytic unit and phospholipids (Seamon & Daly 1986).

However, the precise mechanism by which this compound can activate adenylate cyclase activity is still unknown. Indeed, its site or sites of binding are still a controversial issue. Forskolin can bind directly to the catalytic unit of adenylate cyclase. This property has been used to purify this protein (Pfeuffer et al 1985; Smigel 1986). However, another site for forskolin binding has been suggested to involve the G

protein Gs. Forskolin binding studies (Seamon & Daly 1986) have indicated that there is an increase in the number of forskolin binding sites under conditions which activate Gs . There appears to be synergistic activation of adenylate cyclase in the presence of both forskolin and cholera toxin. Also, in the presence of Gs and stimulatory hormones , forskolin has been reported to have a higher affinity for activating adenylate cyclase (Clark et al 1982; Green & Clark 1982). It is therefore possible that cooperativity exists between Gs and forskolin activation of the adenylate cyclase catalytic unit.

Barber & Goka (1985) have suggested that Gs and the catalytic unit are at all times associated. They suggest that Upon forskolin binding to the catalytic unit , Gs activation is triggered by the dissociation of the β subunit from the Gs holomeric complex. However, one important observation to be considered is that forskolin does have the ability to activate adenylate cyclase in the absence of a functional Gs (Clark et al 1982). Similarly, Florio & Rosso (1983) have demonstrated that forskolin can activate adenylate cyclase in the absence of Gi.

In summary then, whether forskolin activates adenylate cyclase by direct interaction with the catalytic unit alone or whether forskolin stimulates both Gs and the catalytic unit is still a controversial issue. However, this compound is of great use as a tool by which one can stimulate the activity of adenylate cyclase without affecting receptor-catalytic unit

interactions. Hence, I have used this compound to elevate adenylate cyclase activity to allow me to study the function of both inhibitory and stimulatory G proteins without the requirement for receptor dependent processes . Receptor function could conceivably be altered in a diseased state such as diabetes and therefore cannot be assumed to be "normal" (see chapter 3).

1.5 The Aims of This Project

Initially, the aim of my project was to gain a more in-depth understanding of the role which G proteins play in insulin action. In particular, it was hoped that further evidence would be found to support the contention that insulin activates a specific G protein which has been termed "Gins". This G protein activation was proposed to be involved in the insulin mediated inhibition of adenylate cyclase activity (see section 1.3.3).

Work carried out previously within the group in which I worked demonstrated that pertussis toxin abolished this insulin mediated inhibition of adenylate cyclase activity (published Heyworth et al 1986). Therefore, there were new implications of a role for Gi in this inhibitory process. Thus, the main aim of my project became that of distinguishing between the role of Gi and Gins in this process.

Also, this action of insulin is a rapid "short-term" effect of insulin associated with the plasma membrane. It is therefore possible that a relationship between alterations in G protein function and insulin resistance observed in diabetic states could occur.

Therfore, in summary, the aims of my project were to :

1. Investigate the role of Gi and Gins in the ability of insulin to inhibit adenylate cyclase activity.

2. Investigate the possibility of a relationship between insulin resistance in the diabetic state and G protein function.

3. Investigate the possibility of the involvement of Gins in other short-term effects of insulin action.

2.0 Materials and Methods

2.1 Chemicals

Appendix 1 lists suppliers of chemicals and enzymes used.

2.2 Animals

Male Sprague - Dawley rats were obtained from the University of Glasgow breeding colony and unless otherwise stated were aged 8-10 weeks old and weighed 220 - 250 g.

Male Zucker rats aged 8 - 10 weeks old were obtained from Olac Ltd., Shaw's Farm, Blackthorn, Bicester, Oxon. U.K. Obese homozygotes (fa / fa) and both homozygote (+/+) and heterozygote (fa / +) lean Zucker rats were used.

2.3 Chemical Induction of Type I Diabetes

Diabetes Mellitus was induced in Sprague - Dawley rats by one intra - peritoneal injection of either streptozotocin (50 mg/Kg) in citrate buffer pH 4 as described by Sauerheber et al (1984) or alloxan (130 mg/Kg) in 0.9 % saline. Controls received one intra- peritoneal injection of citrate or saline respectively.Urine glucose was monitored using " Diabur - Test 5000 " sticks and animals were, sacrificed 7 or 3 days later in the case of streptozotocin and alloxan treated rats

respectively. At the time of sacrifice , blood samples were removed by intra - cardiac puncture and blood glucose concentration was determined using " Dextrostix " in conjunction with a glucometer (Ames, Slough, U.K.) Only when blood glucose concentrations were > 10 mM and glucosuria was detectable at the time of sacrifice were animals assumed to be diabetic and used.

2.4 Reversal of The Diabetic State

10 I.U. of insulin zinc suspension was injected subcutaneously 72 h after streptozotocin induction of the diabetic state. Subsequent daily injections for a period of 7 days were repeatedly administered and the presence of glucosuria carefully monitored. Glucosuria was generally absent within 24 - 48 h after initial administration of insulin.

2.5 Treatment of Sprague - Dawley Rats with Metformin.

The hypoglycaemic drug Metformin (Hermann 1979) was continuously administered orally in water at a carefully controlled daily dosage of 350 mg / Kg immediately after injection of streptozotocin or citrate buffer until the time of sacrifice.

2.6 Source of Human Blood For Platelet Preparations

Blood was obtained from the antecubital vein of normal (healthy) or type II diabetic volunteers. Normal (control) volunteers were not undergoing and had not been undergoing medication for at least one month before donating. Each diabetic patient donated only 30 - 40 ml of blood per session and gave their consent to its use.

2.7 Solutions

All solutions were prepared using double -distilled deionized water. pH values were measured using a Beckman 31 pH meter. Tris - HCl buffers were cooled to the required temperature prior to adjustment of the pH to the required value.

2.8 Preparation of Isolated Hepatocytes.

Preparation of isolated hepatocytes was essentially using the technique described by Elliot et al (1976) which is a modification of the method used by Berry and Friend (1969).

Rats were anaesthetised by one intra - peritoneal injection of 0.4ml of a 60mg / ml solution of Sagatal (Sodium Pentobarbitone solution containing 4mg/ml heparin). Upon loss of

the flexor and corneal responses, the abdominal cavity was opened up with a mid line incision to just below the diaphragm. The inferior vena cava and hepatic portal vein were then ligated. The inferior vena cava was cannulated with a 16 gauge needle containing a solution of heparin (10mg/ml) and the hepatic portal vein was then cannulated with a 19 gauge needle and the ligatures tightened to secure the positioning of the cannulae.

The liver was perfused in the physiological direction with 80 ml of Ca^{2+} - free Krebs Henseleit bicarbonate buffer pH 7.4 containing 1mg/ml EDTA at a constant temperature of 37 ^{o}C and a steady flow rate of 30ml/min using a Watson - Marlow peristaltic pump. The Krebs - Henseleit bicarbonate buffer had the following composition : -

25mM $NaHCO_3$, 1.2 mM $MgSO_4$, 1.2mM KH_2PO_4 , 5mM KCland 120mM NaCl and was freshly prepared for each perfusion. The pH was maintained at 7.4 units by thorough gassing with O_2 :CO_2 (95% :5% v/v). Air bubbles were prevented from entering the liver by the positioning of a "bubble trap" between the pump and the hepatic portal vein cannula.

The EDTA containing buffer was washed out with 50 ml of Ca^{2+} - free Krebs Henseleit bicarbonate buffer containing 20 mM glucose and discarded. 60 mg of collagenase was added to 100ml of this buffer and circulated for 30 min.

The thoracic cavity was opened and the blood vessel directly

above the diaphragm was occluded by the use of a haemostat. After 30 min the liver had a very "blotchy" appearance and the liver capsule was disrupted. When this stage was reached , the perfusion was terminated and the cannulae removed. The liver was transfered to a plastic beaker containing 35ml of Ca^{2+} - free Krebs Henseleit bicarbonate buffer and dispersed using plastic teaspoons. The subsequent cell suspension was gently filtered through nylon mesh with a pore size of 150 μm and washed with a further 20ml of buffer. The suspension was then centrifuged at 50g$_{av}$ for 2.5 min at room temperature in an MSE Centaur centrifuge. The supernatant was removed and discarded and the pellet resuspended in 40ml of Krebs Henseleit bicarbonate buffer containing 2.5mM $CaCl_2$ and centrifuged as before to remove erythrocytes. The final cell pellet was resuspended in Krebs Henseleit bicarbonate buffer containing 2.5mM $CaCl_2$ to give a cell dry weight of 20 - 30mg/ml.

2.9 Dry Weight Determinations

1ml portions of cell suspensions were pipetted into preweighed glass vials and dried to constant weight in an oven at 120 ^{o}C for 0.5 h. The mean dry weight of three samples from each cell suspension was determined.

2.10 Assessment of Cell Viability

2.10.1 Assay of Intracellular ATP Content

Intracellular ATP content was determined using the procedure of Stanley & Williams (1969). A 0.5ml aliquot of hepatocyte suspension (cell dry weight 5 - 6 mg/ml) was added to 50 µl of 20 % (w/v) perchloric acid, vortex mixed and put on ice for 10 min to allow protein precipitation. Subsequent centrifugation in a Hawksley - Gelman microfuge at 14,000 g_{av} for 2 min allowed sedimentation of the precipitate and removal of the supernatant. Neutralisation of the supernatant was achieved using a solution of 0.5 M triethanolamine containing 2M KOH.

10 µl of appropriately diluted supernatant containing 20 - 80 pmoles of ATP was added to 3ml of assay reagent. The assay reagent consisted of a freshly prepared mixture (1:1:1) of 80mM $MgSO_4$, 10mM K_2HPO_4/KH_2PO_4 (pH 7.4) and 100mM Na_2ASO_4. A range of standard ATP samples containing 0 - 150 pmoles of ATP were also prepared and all samples were left in the dark for 30 min. Preparation of the Firefly lantern extract (Sigma FLE - 50) involved homogenization of the extract with 10 ml of deionised water in the dark and 1 h later, centrifugation for 2 min in a Hawskley - Gelman microfuge at 14,000 g_{av}.

Initiation of the assay was by addition of 50 ul of this supernatant to each sample vial and chemiluminescencewas immediately measured using an LKB liquid scintilation counter in the absence of fluorescent light . ATP determinations were

routinely performed using 20 ml glass scintilation vials which had been previosly treated with 0.1M NaOH and 0.1M HCl to decrease non - specific photoemission. ATP content for viable cells was in the range of 9 - 11 nmoles / mg dry weight.

2.10.2 Trypan Blue Exclusion

100 µl of cell suspension (20 - 30 mg/ml dry weight) was added to a solution consisting of 800 µl of Krebs Henseleit bicarbonate buffer and 100 µl of trypan blue dye (0.1% w/v). The suspensiom was then inverted gently to allow mixing and viewed on a microscope under high power using a haemocytometer counting chamber. Cells were counted and those not excluding dye were assumed to be non - viable. Only cell preparations exhibiting > 90 % dye exclusion were used.

2.11 Incubation and Hormone/Drug Treatment of Isolated Hepatocytes

Incubation of hepatocytes was performed essentially by using the procedure described by Smith et al (1978).

1ml of isolated hepatocyte suspension (cell dry weight 20 - 30 mg / ml) was pipetted into 25 ml glass conical flasks (pretreated with dimethyldichlorosilane to prevent cell adhesion) containing 4ml of pregassed Krebs Henseleit bicarbonate buffer supplemented with 2.5mM $CaCl_2$, 10mM glucose and 2.5 % (w/v) bovine serum albumin. Vials were closed using "suba-seals"

(Gallenkamp & Co. Ltd., Widnes, Cheshire, U.K.) and gassed for 15 sec with O_2 : CO_2 (95 % : 5%) prior to incubation at 37 °C in a shaking water bath preset to 120 cycles min^{-1}. Cells were routinely preincubated for 20 min prior to drug or hormone treatment. Addition of hormones or drugs was by injection of solutions through the "suba-seals" using a 50µl glass syringe. Cells were gassed for 20 S every 10 min to maintain the buffer pH at 7.4 and prevent cell anoxia. After the required period of incubation, cell suspensions were put on ice to quench reactions and membranes were prepared as appropriate.

2.12 Preparation of A Crude Membrane Pellet From Isolated Hepatocytes

Crude membrane pellets were prepared as outlined by Houslay & Elliot (1979). In brief, hepatocytes were isolated as previously described and incubated with appropriate drugs or hormones for the required period of time and placed on ice. 1ml samples from each flask were pipetted into 1.5ml plastic vials and then centrifuged at 14,000 g_{av} in a "micro Rapide " temperature controlled bench centrifuge (A.R, Horwell, Hampstead, London, U.K.) at 4 °C for 6 min. The supernatant was discarded and the pellet resuspended and disrupted in 150µl of 1mM $KHCO_3$ pH 7.2 with 12 up and down actions using a 1ml syringe and a 25 G needle. A further 150µl of 1mM $KHCO_3$ solution was added and the

sample centrifuged at 14,000 g$_{av}$ for 6 min at 4^{o}C. The resultant washed and hypotonically lysed cell pellet was collected and resuspended to an appropriate volume (10 - 12 mg/ml protein content).

2.13 Hepatocyte Plasma Membrane Preparation Using Percoll Fractionation

Rat hepatocyte plasma membranes were prepared using the procedure developed by Heyworth et al (1985). The method involved washing hepatocytes, followed by homogenization and finally purification of plasma membranes from these cells on a percoll gradient. This is described below :

2.13.1 Preparation of Hepatocytes

Isolated hepatocytes (0.9 mg dry weight / rat liver) were resuspended to 30ml with Krebs Henseleit bicarbonate pH 7.4, washed with 80ml of ice - cold 0.25M sucrose containing 3mM imidazole pH 7.4 and centrifuged at 500 g$_{max}$ for 2.5 min in an MSE Centaur 2 bench centrifuge.

2.13.2 Homogenization of Hepatocytes

The cells were resuspended in 40 ml of 0.25M sucrose /3mM imidazole buffer pH 7.4 and placed in a 50ml Amicon Ultrafiltration unit and exposed to an oxygen - free , nitrogen

atmosphere at a pressure of 15lb / in^2 for 10 min at 4 ^{o}C. After which, the pressure was released rapidly .

The suspension was then homogenized by hand in a 50ml glass Potter - Elvejm homogenizer with two up and down strokes of a teflon pestle. The homogenate was centrifuged for 2.5 min at 500g$_{max}$, the supernatant collected and the pellet resuspended in 20 ml of the sucrose / imidazole buffer and subjected to the pressurisation and homogenization procedure as before.

The two supernatant fractions were then combined and centrifuged at 27,500 g$_{max}$ for 15 min. The resulting pellet was resuspended in 20 ml of the sucrose / imidazole buffer and placed on ice.

2.13.3 Percoll Gradient Purification

A working percoll solution was prepared consisting of 20 ml of a 90 % (v/v) percoll solution containing 0.25M sucrose, 20 ml of 6mM imidazole containing 0.25M sucrose pH 7.4 and 80ml of 3mM imidazole containing 0.25M sucrose pH 7.4 . The pH of the solution was adjusted to 7.4 using a solution of dilute HCl and cooled to 4 ^{o}C.

30 ml of this working solution was placed in a 50ml centrifuge tube and 2ml of 3mM imidazole containing 0.25M sucrose pH 7.4 was layered on top. 5ml of the hepatocyte homogenate was layered onto each gradient and centrifuged at 27,500 g$_{max}$ in an MSE 21 centrifuge using an 8 x 50 fixed angle rotor at 4 C for 15 min.

The first 2ml from each tube was discarded and the plasma membranes were collected in the next 5ml fraction. The plasma membrane fractions were combined , diluted 1:2 with 3mM imidazole containing 0.25M sucrose pH 7.4 and 30 ml fractions were layered onto a 10 ml "cushion" of 3mM imidazole containing 55 % sucrose pH 7.4 and centrifuged at 48,000 g for 15 min at 4^{o} C.
max

The membranes were collected from the bottom interface and washed by dilution (1:1) with 3mM imidazole containing 0.25M sucrose pH 7.4 and were again centrifuged at 48,000 g for 15 min at 4^{o} C. The plasma membranes recovered were resuspended in 2ml of 3mM imidazole containing 0.25M sucrose pH 7.4 and aliquoted for storage at - 80^{o} C.
max

2.14 Purification of Plasma Membranes From Whole Rat Liver

Liver plasma membranes were prepared using a modification (Marchmont et al ; 1981) of the method of Pilkis et al (1974).

3 rats were stunned and decapitated , their livers were removed, washed, and chopped in ice - cold 1mM $KHCO_3$ pH 7.2. Using 3 volumes of liver : 1 volume of $KHCO_3$ solution, the livers were homogenized with 6 strokes of a motor driven homogenizer (setting 3) and a teflon pestle. The homogenate was strained through 2 layers of muslin, diluted to 300ml with $KHCO_3$

buffer and centrifuged at 2,000 g$_{max}$ for 10 min at 4 ^{o}C using an MSE 21 cenrifuge and an 8 x 50 ml fixed angle rotor. The resultant supernatant was discarded and the pellets poured onto 72g of sucrose in a plastic beaker and the volume adjusted to 120 ml with the $KHCO_3$ buffer. This suspension was stirred at 4 ^{o}C for 30 min and then 20 ml portions were pipetted into clear centrifuge tubes. On top of this homogenate was carefully layered 12 ml of 48.2 % sucrose in 3mM imidazole pH 7.4 , followed by 6.5 ml of 42.5 % sucrose in 3mM imidazole pH 7.4 .

The gradients were then centrifuged at 100,000 g$_{max}$ for 3 h in a Beckman ultra centrifuge using an SW 28 rotor at 4 ^{o}C. Care was taken to ensure slow acceleration and deceleration speeds were selected to minimise disruption of the gradient. The plasma membranes collected at the 42.5 - 48.2 % sucrose interface were " hoovered off " using a pasteur pipette, diluted 1:1 with ice - cold $KHCO_3$ buffer and centrifuged at 25,000 g$_{max}$ for 15 min at 4 ^{o}C, resuspended in 5ml of $KHCO_3$ buffer and stored at - 80 ^{o}C in 0.25 ml aliquots.

2.15 Preparation of Intact Human Platelets

Intact human platelets were prepared as described by MacIntyre and Pollock (1983).

10 ml aliquots of freshly collected human blood were immediately added to plastic centrifuge tubes containing 1ml of trisodium citrate solution (3.8 % w/v) and inverted to allow mixing.

The suspension was centrifuged at 800 g$_{av}$ for 5 min at room temperature in a Centaur 2 bench centrifuge, the supernatants were removed and combined and to each 1ml of supernatant was added 40 ul of 100mM EGTA and 10 ul of 1M citric acid. The mixture was subsequently centrifuged at 1,000 g$_{av}$ for 10 min at room temperature and the pellet resuspended in Hepes' buffered Tyrodes' solution consisting of 129 mM NaCl, 10.9 mM trisodium citrate, 8.9 mM $NaHCO_3$, 0.56 mM D - glucose, 5mM Hepes, 2.8 mMKCl, 0.8 mM KH_2PO_4, 0.84 mM $MgCl_2$, 2.4 mM $CaCl_2$ and 0.35 % (w/v) bovine serum albumin pH 7.4 .

1 ml of this buffer was added per 10 ml of fresh blood used, and the suspension was incubated at 37 ^{o}C in siliconised glass conical flasks placed in a shaking water bath preset to 120 cycles min^{-1} for 15 min prior to any drug additions. The flasks were sealed using " suba-seal" stoppers and drug additions were made by injection through the stoppers using glass micro syringes. After the appropriate time of incubation with drugs the reaction was terminated by the addition of an equal volume of ice - cold Hepes' buffered Tyrodes' solution and the suspension centrifuged at 30,000 g$_{av}$ for 15 min using an MSE Hi - spin 21 centrifuge and an 8 x 50 fixed angle rotor cooled to 4 ^{o}C.

The resultant pellet was homogenized and washed using the procedure outlined below for preparation of a crude membrane pellet from human platelets.

2.16 Preparation of A Crude Membrane Pellet From Human Platelets

Membrane Pellets were prepared from platelets using the method outlined by Jakobs et al (1982).

10 ml portions of freshly collected blood were immediately mixed with 1ml of 3.8 % (w/v) trisodium citrate and centrifuged at 1,000g$_{av}$ for 5 min in a Centaur 2 bench centrifuge at room temperature. The supernatant was collected and centrifuged at 30,000g$_{av}$ for 15 min using an MSE Hi-Spin 21 centrifuge and an 8 x 50 rotor cooled to 4^{o}C .

The resulting pellet was homogenized in 15 ml of ice - cold 10 mM Tris containing 1 mM EDTA pH 7.4 using 10 up and down strokes with a glass Potter - Elvejm homogenizer and teflon pestle and recentrifuged at 30,000g$_{av}$ as before. The final pellet ; the "crude membrane pellet" , was then resuspended in 10 mM Tris / HCl pH 7.4 to 2 ml for every 100ml of fresh blood used and stored in 50 µl aliquots at - 80^{o}C.

2.17 Protein Estimations

Protein determinations were routinely made using the procedure of Peterson (1977) which is a modification of the method of Lowry et al (1951) . This procedure allows the initial removal of solutions which could interfere with the normal Folin-

Lowry protein estimation by solubilisation and subsequent precipitation of protein prior to the colourimetric estimation of protein content.

Standard solutions of bovine serum albumin were prepared containing 0 - 100 μg of protein ml^{-1} and unknown protein solutions were also made up to a 1ml volume in plastic centrifuge tubes. 0.1 ml of 0.15% (w/v) deoxycholate was added to each tube, mixed and incubated at room temperature for 10 min. 0.1ml of 72% T.C.A. (w/v) was then added , mixed and left at room temperature for 5 min. The samples were then centrifuged in a bench Centaur 2 at 3,000g $_{av}$ for 15 min at room temperature and the supernatants decanted and discarded.

1ml of distilled water was added to each sample and a " Lowry C" solution was prepared consisting of 50ml of 4% (w/v) Na_2CO_3 containing 0.4% (w/v) S.D.S. and 1ml of 1% (w/v) $CuSO_4.5H_2O$ in 2% (w/v) sodium citrate.

1ml of this Lowry C solution was added to each sample for exactly 10 min and then 0.5 ml of Folin & Ciocalteu's reagent (diluted 1:5 with water) was added and colour development was allowed to occur for 30 - 60 min before measuring absorbance at 650 nm with an LKB spectrophotometer linked up to an Apple 2e computer. Estimations were always performed in triplicate using an LKB "Wavescan" linear regression curve - fit program. (LKB , Surrey, U.K.).

2.18 Preparation of cAMP Binding Protein From Bovine Cardiac Muscle

The procedure used was that employed by Rubin et al (1974) and involves 3 distinct stages of purification:

2.18.1 Homogenization

The pericardium and fat tissue were removed from a fresh bovine heart and the heart was chopped into 2.5 cm cubes and minced as finely as possible using a Moulinex electric mincer. The minced heart was mixed with 4L of 40 mM KPO_4 buffer pH 6.1 containing 2 mM B mercaptoethanol and homogenized in a Waring blender for 1 min in small batches. The homogenate was centrifuged at 10,000g for 10 min at 4 °C and the supernatant was filtered through Whatman No. 54 filter paper using a Buchner funnel and flask under vacuum. The pellet was extracted twice more with 1 L of the same buffer and the filtered supernatants were combined.

2.18.2 $(NH_4)_2SO_4$ Precipitation

The combined supernatants were brought to 55% saturation by the addition of solid $(NH_4)_2SO_4$ at a concentration of 320 g / L . The pH was maintained between 7 and 8 by the addition of concentrated $(NH_4)OH$ solution. Protein precipitation was allowed to occur for 2.5 - 3 h, after which , the precipitate

was collected by centrifugation at 10,000g$_{av}$ for 10 min at 4 ^{o}C. The supernatant was discarded and the precipitate dissolved in 500ml of 50 mM Tris / HCl pH 7.6 containing 10mM NaCl and 4mM B mercaptoethanol . This solution was dialysed overnight against 5 L of the Tris buffer at 4 ^{o}C to remove the $(NH_4)_2SO_4$.

2.18.3 DE - 50 Cellulose Purification

In the original method used by Rubin et al (1974) DEAE - Sephadex was used. However, this was not available when this binding protein was first purified by us and so DE - 50 Cellulose was used as a substitute. This was found to be adequate for the preparation and so it was routinely used for each subsequent preparation.

2.18.4 Pretreatment of DE - 50 Cellulose Before Use

50g of DE - 50 Cellulose was stirred in 2 L of 0.5 M HCl and left for 30 min . The resin was then washed with distilled water until the pH rose to 4 units. The DE - 50 Cellulose was then stirred into 2 L of 0.5 M NaOH and then left for 30 min. The resin was then washed until the eluate was neutral and the whole acid / alkali cycle repeated once more. The resin was then equilibrated with 50 mM Tris / HCl pH 7.6 containing 10 mM NaCl and 4 mM B mercaptoethanol and a final

slurry was prepared to give a wet settled volume : final volume ratio of 2 : 1

2.18.5 Absorption of The Binding Protein By The Resin

The dialysed preparation was stirred for 1 h with 800 ml of equilibrated DE - 50 Cellulose and under these conditions cAMP - dependent protein kinase activity should be absorbed. The resin was collected by filtration using Whatman No. 54 filter paper on a Buchner funnel and flask under vacuum. The resin was washed with 3 L of Tris / HCl pH 7.6 containing 10 mM NaCl and 4mM B mercaptoethanol until the filtrate became colourless. The gel was then mixed with 200ml of 50 mM Tris /HCl pH 7.6 containing 0.3 mM NaCl and 4mM B mercaptoethanol for 45 min. Collection of the DE - 50 Cellulose was achieved by filtration and washing of the resin with 50 mM Tris/HCl buffer using a Buchner flask and funnel.

2.18.6 Isolation of The cAMP Binding Protein

The combined filtrates were brought to 35 % saturation with 119g / L of solid $(NH_4)_2SO_4$ and the pH maintained between 7 and 8 units with NH_4OH solution and after 1 h the precipitate was collected by centrifugation at 10,000g$_{av}$ for 10 min at 4 ^{o}C and discarded. The supernatant was brought to 75 % saturation by the addition of 258g / L of solid $(NH_4)_2SO_4$ and the precipitate

formed after 1 h was collected by centrifugation at 10,000g$_{av}$ as before and the pellet suspended in a minimum volume of 50 mM KPO_4 buffer containing 4mM B mercaptoethanol pH 7. This suspension was dialysed overnight at 4 °C against 2 L of the same buffer. The purified protein kinase was stored at - 80 °C in 0.25 ml aliquots. Under these conditions each preparation was found to be stable for 6 - 9 months. Aliquots were never refrozen, but could be used for up to 48 h after thawing if stored at 0 - 4 °C .

2.19 Assay of Adenylate Cyclase Activity

Adenylate cyclase (E.C. 4.6.1.1) was assayed as described by Houslay et al (1976). An assay cocktail was prepared containing 1.5 mM ATP, 5mM $MgSO_4$, 10mM theophylline, 1mM EDTA , 7.4 mg / ml phosphocreatine , 0.2 mg /ml creatine kinase, 25 mM triethanolamine hydrochloride , 1mM dithiothreitol and 0.8 mg / ml bovine serum albumin. The cocktail was then adjusted to pH 7.4 with KOH solution. Drugs, hormones and membranes were added as appropriate to the cocktail to give a final assay volume of 100µl.

These samples were then incubated at 30 °C for 10-15 min. The reaction was terminated by placing the samples in a water bath for 3 min at a temperature > 90 °C. Precipitated protein was then pelleted by centrifugation at 14,000g$_{av}$ for 5 min and the supernatants removed for cAMP determination.

Adenylate cyclase activity was expressed as pmoles cAMP produced min^{-1} mg^{-1} protein. The linearity of enzyme activity with respect to incubation time and protein concentrations used were always ensured.

2.19.1 cAMP Determination

Determination of cAMP content in 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).

Essentially this assay depends upon the specific binding of cAMP to a cAMP binding protein isolated and purified from bovine heart as described in section 2.18. Samples are incubated with the binding protein for a suitable period of time to allow a state of equilibrium to occur. The bound cAMP is then separated from unbound cAMP by the use of a charcoal / B.S.A. suspension . The charcoal absorbs free nucleotides and therefore estimation of ^{3}H-cAMP bound to the protein can be made.

Total cAMP binding to the protein is estimated by incubation of tritiated cAMP in the absence of unlabelled cAMP . Non-specific cAMP binding is estimated by the incubation of tritiated cAMP in the absence of both unlabelled cAMP and binding protein . Using a range of solutions of known unlabelled cAMP concentration it is possible to sequentially reduce the

proportion of labelled cAMP bound to the protein as the two cAMP species compete for a finite number of binding sites. Thus, a standard displacement curve can be constructed and estimation of cAMP content can be made.

A range of (unlabelled) cAMP standard solutions (0-320 pmoles / ml) were prepared in assay buffer containing 50mM Tris and 5mM EDTA pH 7.4. Tritiated cAMP (5,8-^{3}H-Adenosine 3',5' cyclic phosphate) in 50% ethanol was diluted in the Tris / EDTA assay buffer to give about 500,000 cpm /ml . 100µl of this solution was added to plastic vials containing 50µl of assay buffer. Then 50µl of standard cAMP solution or 50µl of supernatant from samples with unknown cAMP content were added to these vials and mixed gently. Finally 100µl of binding protein (prepared as described in section 2.18 and diluted 1/100 with assay buffer) was added to the samples and gently mixed. The samples were incubated at 4 ^{o}C for 1.5 - 3 h . Just prior to termination of the incubation period a charcoal solution was prepared consisting of 2% GSX-100 charcoal and 1% bovine serum albumin suspended in ice - cold assay buffer. The suspension was gently mixed at 4 ^{o}C for about 10 min prior to use.

0.25 ml of charcoal suspension was added to samples to terminate the incubation period and the charcoal was pelleted by centrifugation for 5 min at 14,000g $_{av}$ at a temperature of 4 ^{o}C. 0.4 ml of the supernatant was then removed , dispersed into

"Ecoscint" scintillation fluid and counted in an LKB scintillation counter.

In order to estimate cAMP content in the samples from adenylate cyclase assays ,it was necessary to construct a standard curve with known cAMP standard solutions each time an experiment was undertaken. The LKB scintillation counter used in our laboratory had an RIA curve fitting facility . Therefore, cAMP content in pmoles /sample was automatically calculated from each standard curve and printed out . Routinely the sensitivity of the binding assay was between 0.25 and 8, pmoles / sample.

2.20 Preparation of [γ-^{32}P] GTP

[^{32}P] GTP was prepared using the technique outlined by Maxam & Gilbert (1980) procedure 2.

$$\text{Glycerate-3-P} + \text{GTP} \overset{\text{PGK}}{\rightleftharpoons} \text{1,3 Diphosphoglycerate} + \text{GDP}$$

$$\text{1,3 Diphosphoglycerate} \overset{\text{GAP-DH}}{\rightleftharpoons} \text{Glyceraldehyde-3-P} + \text{Pi}$$

3 phosphoglycerate kinase (EC 2.7.2.3) catalyzes the reversible phosphorylation of glycerate 3-phosphate to 1,3 diphosphoglycerate using the nucleotides ATP or GTP as phosphate donors. In the presence of glyceraldehyde 3-phosphate

dehydrogenase (EC 1.2.1.12), the 1,3 diphosphoglycerate is converted to glyceraldehyde 3-phosphate.

However, under the conditions of the reaction mixture, [^{32}P] - labelled inorganic phosphate is also present and the position of equilibrium of the reaction is shifted so far to the left that the ^{32}Pi is incorporated into the 1,3 diphosphoglycerate and subsequently into GTP at the γ position. A theoretical conversion of 78% of the ^{32}Pi to [^{32}P] GTP is reported to be possible. However, 60-70 % conversion was routinely obtained.

The procedure used is a four stage process involving the removal of $(NH_4)_2SO_4$ from the commercially bought enzymes, incubation of the reaction mixture and PEI- cellulose thin layer chromatography identification and quantification of reactants and products. Finally, purification of the γ [^{32}P] GTP synthesized was achieved by the use of ion-exchange chromatography.

2.20.1 Removal of $(NH_4)_2SO_4$ From Commercial Enzyme Preparations

The purpose of this procedure is to remove residual $(NH_4)_2SO_4$ which could inhibit the reaction and also to remove any traces of inorganic phosphate which may be present in the enzyme preparations which would reduce the percentage conversion of the [^{32}P] labelled Pi into γ [^{32}P] GTP.

12.5 µl of yeast 3-Phosphoglycerate kinase (specific activity ca. 4.5U/µl) and 32.5 µl of rabbit muscle glyceraldehyde -3 phosphate-dehydrogenase (specific activity ca. 0.8 U / µl) were combined in a 1.5ml plastic vial and sedimented by centrifugation for 5 min at 14,000g$_{av}$ at 4°C. The supernatant was carefully removed and discarded.

The sediment was very gently resuspended in 50µl of a "wash buffer" consisting of 100mM Tris-HCl (pH8) containing 10mM B- mercaptoethanol , 1mM EDTA (pH8), 0.1mM NAD^+ and 3.2M$(NH_4)_2SO_4$ using a heat sealed glass capillary tube and again sedimented by centrifugation as before. The pellet was washed once more as before and recentrifuged. The walls of the vial and the top of the the pellet were then carefully washed with 12.5 µl of distilled water trying not to disturb the pellet. Finally, the enzymes were sedimented once more by centrifugation and the resultant pellet was very gently resuspended in 37.5µl of distilled water and placed on ice.

2.20.2 Incubation of The Reaction Mixture

500µl of [^{32}P] labelled inorganic phosphate (specific activity 1mCi / 100µl) was carefully neutralised with 10µl of 500mM NaOH using a heat - sealed glass capillary tube for mixing. To this was added 50µl of a 10 x concentrated solution containing (final concentrations) 500mM Tris-HCl (pH8), 50mM $MgCl_2$, 20mM

reduced glutathione , 10mM glycerate 3 phosphate , 1mM EDTA (pH8) and 0.1mM NAD^+. 5µl of 4mM GTP and 2µl of the washed enzyme suspension were added to initiate the reaction and the mixture was gently mixed and incubated at room temperature for 20 min. After which, the reaction was terminated by placing the reaction vial on ice.

2.20.3 PEI-Cellulose Thin Layer Chromatography

A 20 x 40 cm sheet of PEI-cellulose was cut in half and pre-run in water to take most of the yellow soluble material to the top. The plates were dried and marked in pencil into 10 x 5cm rectangles with an origin line 1.5 cm from the bottom. These rectangles were cut out and stored at 4 °C prior to use.

1µl reaction mixture samples were spotted onto these plates immediately prior to initiation of the reaction and immediately before termination of the reaction. The plates were left to dry at room temperature for 5 min and then placed in a chromatography tank containing 10ml of 0.75M KPO_4 buffer(pH3.5) until the buffer had travelled 3/4 of the way up the plate (within approximately 30 min). Under these conditions excellent separation of Pi and GTP is achieved; Pi runs near the front and GTP has an Rf of ca. 0.15-0.20.

The relative amount of [^{32}P] -labelled Pi and GTP were quantified by developing an autoradiograph of the dried

plate and cutting out the relevant areas for Cerenkov counting using a wide-open channel setting on an LKB scintillation counter.

As the specific activity of the ^{32}P was high; exposure of the film (Kodak XAR-5) to the PEI-cellulose plate was only required for 1 min . From these counts the percentage conversion of [^{32}P] -labelled Pi to γ [^{32}P] GTP could be calculated. It is also possible to ensure the [^{32}P] -labelled inorganic phosphate has been converted to γ [^{32}P] labelled GTP by the use of Norit A charcoal slurry.

1µl samples taken at 0 and 20 min incubation times were added to 200 µl of 50mM $NaPO_4$ pH 7. 100µl of this mixture was transfered to a plastic vial and to this was added 0.5ml of a 5% slurry of Norit A charcoal in 50mM $NaPO_4$ pH 7. The suspension was immediately vortex-mixed and centrifuged for 5 min at 14,000g$_{av}$ at 4 ^{o}C. 0.4ml of the supernatant was removed and 3ml of "Ecoscint" scintillation fluid was added. Under these conditions the charcoal is saturated with unlabelled inorganic phosphate and the radio -labelled GTP can thus be separated from the radiolabelled inorganic phosphate by specific absorption to the charcoal.

A 0 min reaction mixture sample allows the determination of non-specific binding to the charcoal by Pi. This "blank" is subtracted from all samples. Use of this relatively quick method enabled continuation of the preparation

to the purification stage without waiting for the PEI-cellulose chromatography quantification to have reached completion.

2.20.4 Purification of The [^{32}P] GTP

Purification of the radio-labelled GTP was by the use of the anion exchange resin Dowex 1-X2 (100-200 mesh Cl^- form). 50g of Dowex resin was suspended in 1.5L of 1M NaOH for 30 min and then filtered and washed with 5L of water on a large Buchner funnel. The Dowex was then suspended in 1.5L of 1M HCl for 30 min and the resin filtered and washed as before.

A 30 cm column of this washed Dowex resin was poured in a glass Pasteur pipette which had been "plugged" with a small amount of siliconised glass wool . The column was washed with 5 ml of 1M NaCl, followed by 10ml of distilled water and then the sample applied. This was then washed with 5 ml of water, followed by 20ml of 0.02M NH_4Cl / 0.02 M HCl to elute inorganic phosphate , GMP and GDP. In order to remove NH_4 ions the column was subsequently washed with a further 10ml of water and the GTP was eluted with 15 aliquots of 0.75 ml of 0.25M HCl. The 15 eluted fractions were collected in 1.5ml plastic vials containing 0.25 ml of 1M Tris (pH9).

These fractions were Cerenkov counted and the peak fractions (usually tubes 2-14) were pooled , aliquoted and stored at -80 °C. Using the Norit A charcoal method outlined above,

samples of this final GTP preparation stage were found to be 95 % absorbable.

Specific activity of the radio-labelled GTP was calculated by the following equation :

% Conversion X mCi [^{32}P] Used = Radioactivity / 20nmoles GTP.

2.21 Assay of GTPase Activity

GTPase activity was measured as described by Houslay et al (1986) which is based on the original GTPase assay developed by Cassel & Sellinger (1976).

The assay, in a final volume of 100µl contained 0.1µM [32P] GTP, 5mM $MgCl_2$, 100mM NaCl, 1mM dithiothreitol, 0.5mM EGTA, 1mM APP(NH)P, 0.6µM GTP, 1mM Oubain, 50mM Tris-HCl pH 7 and drugs as appropriate. The assay cocktail was prepared on ice and initiation of the reaction was by the addition of human platelet membranes. The cocktail was then incubated for exactly 5 min at 30 ^{o}C and the reaction was terminated by the addition of 0.5ml of a 2% ice-cold Norit A charcoal suspension in 50mM KPO_4 buffer pH 7.0 . This was immediately followed by vortex - mixing and centrifugation at 14,000g$_{av}$ for 5 min.

0.4ml of the supernatant was removed and Cerenkov counted in an LKB scintillation counter using a wide-open channel setting.

2.22 Pertussis Toxin Catalyzed Ribosylation of Human Platelet Broken Plasma Membranes

2.22.1 Pertussis Toxin Preactivation

100µl of pertussis toxin (500µg/ml) was added to 100µl of 40mM dithiothreitol and gently mixed. The solution was then incubated at 37 °C for 45 min. The incubation was terminated by placing the sample on ice.

2.22.2 Ribosylation of Membranes

A ribosylation cocktail containing 2mM NAD^+, 2mM ATP, 1mM GTP, 30mM thymidine and 100mM KPO_4 pH 7.4 was prepared. 0.5ml of this cocktail was added to the preactivated pertussis toxin sample. 300µl of platelet membranes (approximately 1.5mg of protein) were then added, gently mixed and incubated at 37 °C for 30 min. The sample was then centrifuged for 15 min at 14,000g$_{av}$ at a temperature of 4 °C to pellet the membranes. The pellet was then washed twice with 500µl of water and finally resuspended to the required volume in 10mM Tris / HCl pH 7.4.

2.23 Cholera Toxin Catalyzed Ribosylation of Human Platelet Broken Plasma Membranes

2.23.1 Cholera Toxin Preactivation

100µl of cholera toxin (1mg /ml) was added to 100µl of 40mM dithiothreitol and gently mixed. This solution was incubated at 30 °C for 20 min. The incubation was terminated by placing the sample on ice.

2.23.2 Cholera Toxin Ribosylation of Membranes

A ribosylation cocktail containing 2mM NAD^+, 1mM GTP, 30mM thymidine and 100mM KPO_4 pH 7.4 was prepared. 0.5ml of this cocktail was added to the preactivated cholera toxin sample. 300µl of platelet membranes (approximately 1.5mg of protein) were then added, gently mixed and incubated at 30 °C for 10 min. The sample was then centrifuged for 15 min at 14,000g$_{av}$ at 4 °C to pellet the membranes. The pellet was washed twice with 500µl of water and finally resuspended to the required volume in 10mM Tris -HCl pH 7.4 .

3. Characterization of G Protein Control of Hepatic Adenylate Cyclase Activity In Control And Diabetic Rats

Introduction

In this chapter, I have attempted to characterize the guanine nucleotide regulatory components Gi and Gs of the rat hepatic adenylate cyclase system in the "normal" (control), type I and type II diabetic states. GTP (Itoh et al 1984) and non-hydrolyzable GTP analogues (Hildebrandt et al 1982; Katada et al 1984) have been demonstrated to specifically activate Gi and Gs. Using the non-hydrolyzable GTP analogue , 5'-guanyly-imidodiphosphate (GPPNHP), I have attempted to demonstrate that the two G proteins can be sequentially activated due to their differing affinities to bind the nucleotide.

Hence, at low concentrations of the nucleotide (10^{-10} - 10^{-8} M) Gi is activated prior to Gs . At higher concentrations of the nucleotide (10^{-7} - 10^{-4} M) Gs also becomes activated and an overall stimulation of the adenylate cyclase catalytic unit is observable as Gs regulatory "input" counterbalances and overtakes Gi regulatory "input".

Under "basal" (resting) conditions, Gi inhibition of adenylate cyclase is not observable. In order to observe this inhibition, the adenylate cyclase activity must be elevated. This can be achieved either by the use of the diterpene , forskolin, or by the use of a stimulatory ligand such as a B-adrenergic agonist. As discussed earlier in the general introduction, forskolin has the ability primarily to increase the maximal velocity of adenylate cyclase without affecting the Km

for the enzyme's substrate ; Mg.ATP. The mechanism by which this compound has the ability to stimulate the catalytic unit remains relatively little understood. However, it is a useful tool to elevate the activity of the catalytic unit without the requirement for ligand-receptor interactions. Such interactions themselves of course could be altered in a diseased state such as diabetes.

Also, the use of this diterpene facilitates the characterization of the Gs component because although forskolin elevates the catalytic unit's activity (as does Gs) , they do so synergistically with each other (Seamon & Daly 1986). Thus, Gs stimulation of adenylate cyclase activity can be observed in the presence of forskolin.

Also in this chapter I have investigated the effect of metformin therapy on the activation of Gi and Gs by GPP(NH)P. Metformin, as briefly discussed in the general introduction, is a member of the biguanide family of hypoglycaemic drugs. It has been used in type II diabetic therapy and has been reported to have "post-receptor effects" which may attenuate insulin resistance (Lord et al 1983). As a G protein is a "post-receptor site" it was therefore conceivable that this drug could alter the function of one or more G proteins . Also, if this were found to be true , a relationship between G protein function and insulin resistance in the diabetic state may well prevail.

An additional method by which to assess Gi and Gs activity is to study glucagon's ability to stimulate adenylate

cyclase activity in the absence and presence of pertussis toxin treatment. Glucagon activates Gs and pertussis toxin attenuates Gi activity by promoting the ribosylation of the Gi α subunit. Resultingly, the holomeric Gi (α B Y) complex formation is favoured and this inactive state of Gi prevails.

Finally, a general characterization of the adenylate cyclase system in each of the control and diabetic states is undertaken using compounds such as sodium fluoride,forskolin , GTP and glucagon.

Additional Methods

Hepatocytes were isolated using the collagenase perfusion technique outlined in section 2.8.

Crude membranes were prepared from hepatocytes as described in section 2.12.

Adenylate cyclase activity was assayed as outlined in section 2.19 in the presence of forskolin (10^{-4} M) . Activity was assayed for 10 min at 30 ^{o}C with a final membrane protein concentration of 20-40 µg/ml. Forskolin was diluted to the required concentration using absolute ethanol and therefore basal activities were assayed in the presence of absolute alcohol also. Pertussis toxin and TPA pretreatment of hepatocytes was undertaken as described in section 2.11. Pertussis toxin (100ng/ml) and TPA (10ng/ml) pretreatments involved incubation

of each compound with hepatocyte suspensions as described in section 2.11 for 1 h and 20 min for each respective drug. Animal treatments are as described in sections 2. 3 - 2. 5 All assays were performed in triplicate and values given are mean +/- SEM values .

Monocomponent porcine insulin and porcine glucagon were used throughout.

"n" refers to the number of separate animals used .

3.1 Use of The Guanine Nucleotide GPP(NH)P To Regulate Forskolin Stimulated Adenylate Cyclase Activity

Figure 3.1.1 Guanine Nucleotide Regulation of Forskolin Stimulated Adenylate Cyclase Activity In Hepatocytes Isolated From Control Rats

Crude membrane pellets were prepared from hepatocytes isolated from control Sprague Dawley rats.

Adenylate cyclase activity was assayed in the presence of forskolin (10^{-4} M) and increasing concentrations of 5'- guanylyl-imidodiphosphate (GPP(NH)P) . Activity is expressed as a percentage of forskolin stimulated activity in the absence of GPP(NH)P.

All activities are expressed as mean +/_ S.E.M. values (n=5).

Basal and forskolin stimulated adenylate cyclase activities were 1.30 +/_ 0.10 and 18.20 +/_ 2.00 pmoles cAMP produced min^{-1} mg^{-1} respectively.

Figure 3.1.1 Guanine Nucleotide Regulation of Forskolin Stimulated Adenylate Cyclase Activity In Hepatocytes Isolated From Control Rats

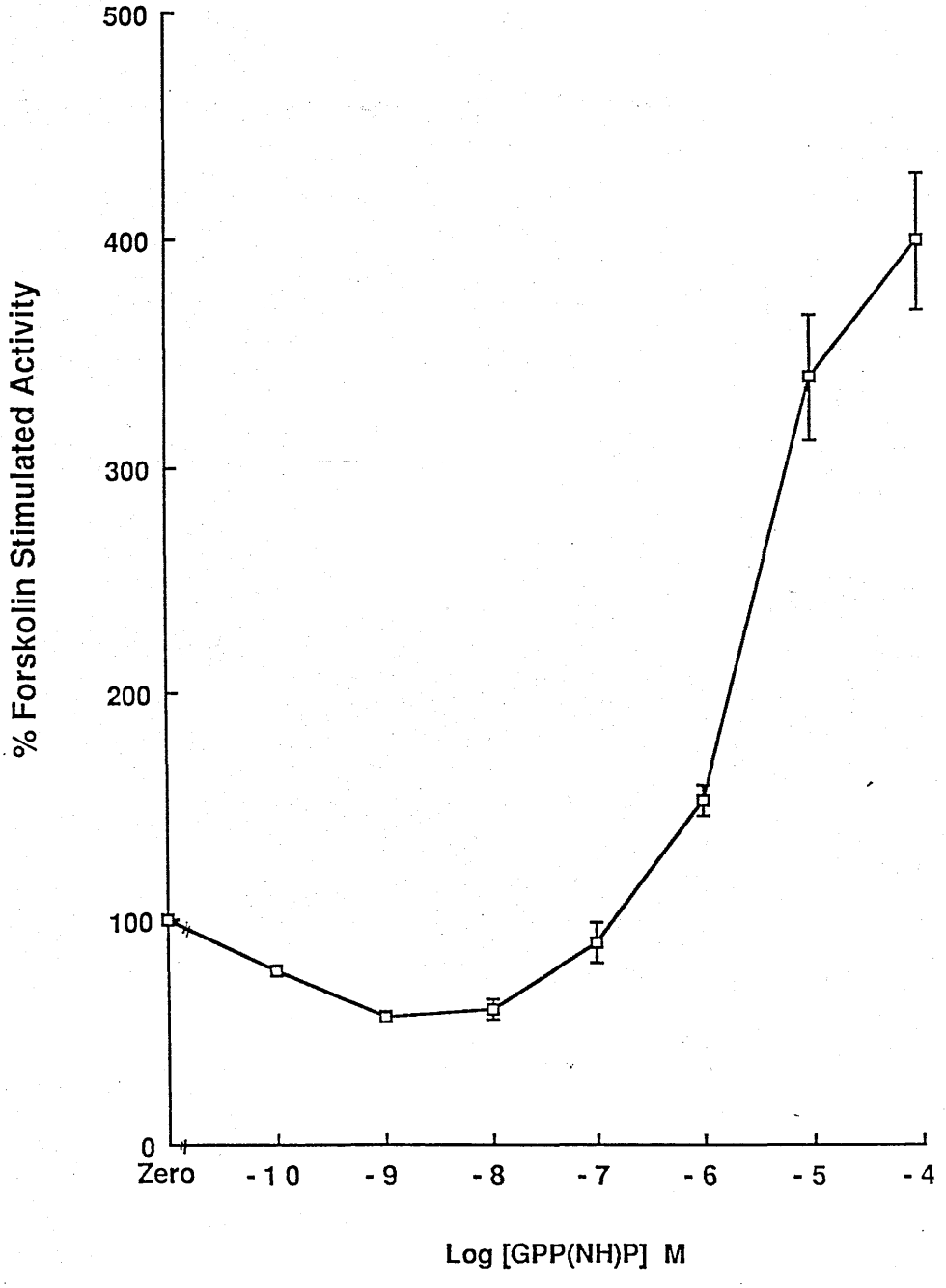


Figure 3.1.2 Guanine Nucleotide Regulation Of Forskolin Stimulated Adenylate Cyclase Activity In Hepatocytes Isolated From Streptozotocin-Induced Diabetic Rats

Type I diabetes (I.D.D.M.) was chemically induced in Sprague Dawley rats as outlined in Materials and Methods. Crude membrane pellets prepared from hepatocytes isolated from these animals were assayed for adenylate cyclase activity in the presence of forskolin (10^{-4} M) and increasing concentrations of 5 ' -guanylyl -imidodiphosphate (GPP(NH)P) . Activity is expressed as a percentage of forskolin stimulated activity in the absence of GPP(NH)P. All activities are expressed as mean +/_ S.E.M. values (n=5).

Basal and forskolin stimulated adenylate cyclase activities were 0.64 +/_ 0.02 and 11.20 +/- 1.00 pmoles cAMP produced min^{-1} mg-1 respectively.

Figure 3.1.2 Guanine Nucleotide Regulation of Forskolin Stimulated Adenylate Cyclase Activity In Hepatocytes Isolated From Streptozotocin - Induced Diabetic Rats

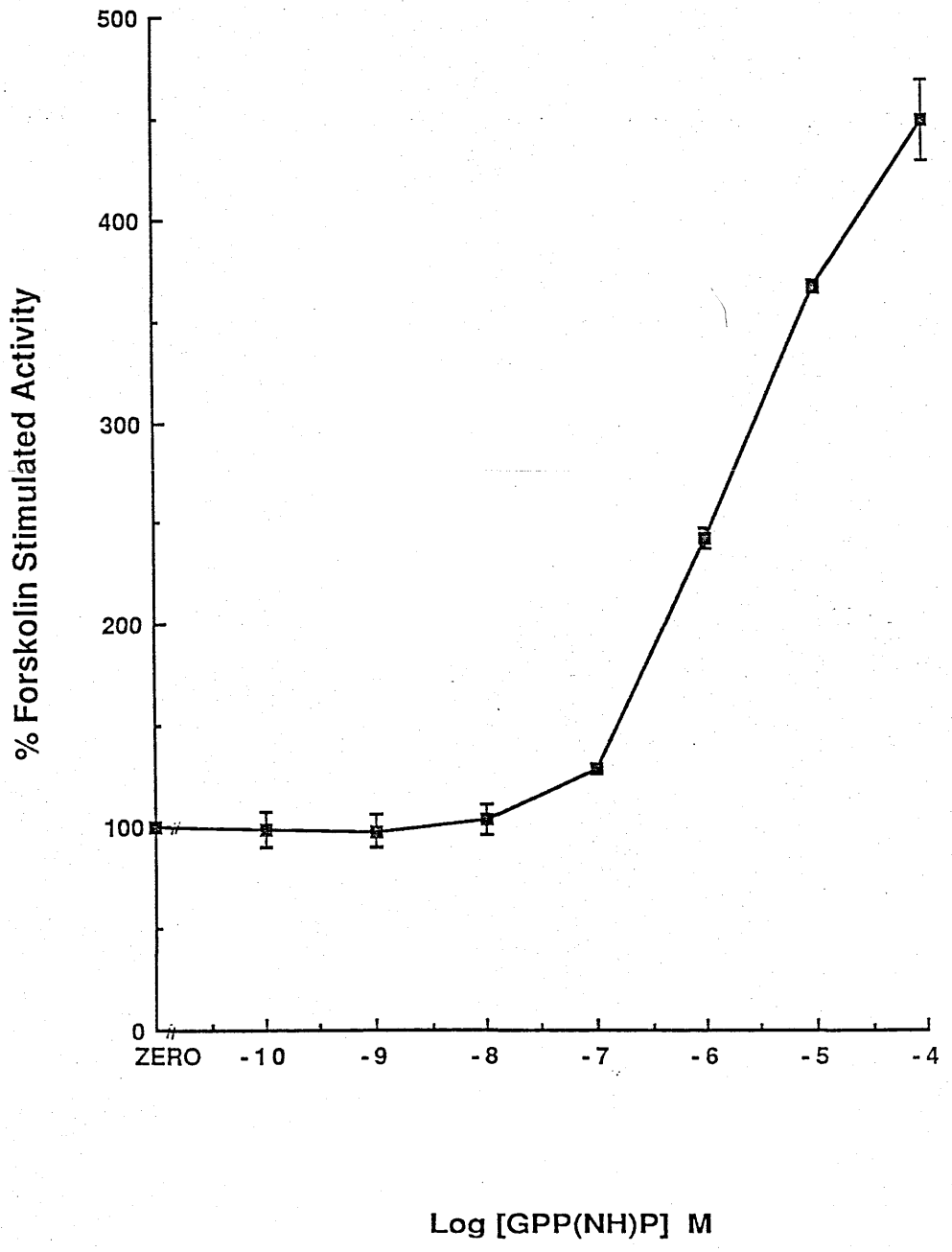


Figure 3.1.3 Guanine Nucleotide Regulation of Forskolin Stimulated Adenylate Cyclase Activity In Hepatocytes Isolated From Diabetic Rats Undergoing Insulin Therapy.

Type I diabetes (I.D.D.M.) was chemically induced in Sprague Dawley rats and Insulin administered daily as outlined in Materials and Methods.

Crude membrane pellets were prepared using hepatocytes isolated from these animals and assayed for adenylate cyclase activity in the presence of forskolin (10^{-4} M) and increasing concentrations of 5'- guanylyl-imidodipphosphate (GPP(NH)P). Activity is expressed as a percentage of forskolin stimulated activity in the absence of GPP(NH)P. Activities are mean +/- S.E.M. values (n=4).

Basal and forskolin stimulated activities were 0.68 +/_ 0.04 and 10.90 +/_ 0.90 pmoles cAMP produced min^{-1} mg^{-1} respectively.

Figure 3.1.3 Guanine Nucleotide Regulation of Forskolin Stimulated Adenylate Cyclase Activity In Hepatocytes Isolated From Diabetic Rats Undergoing Insulin Therapy

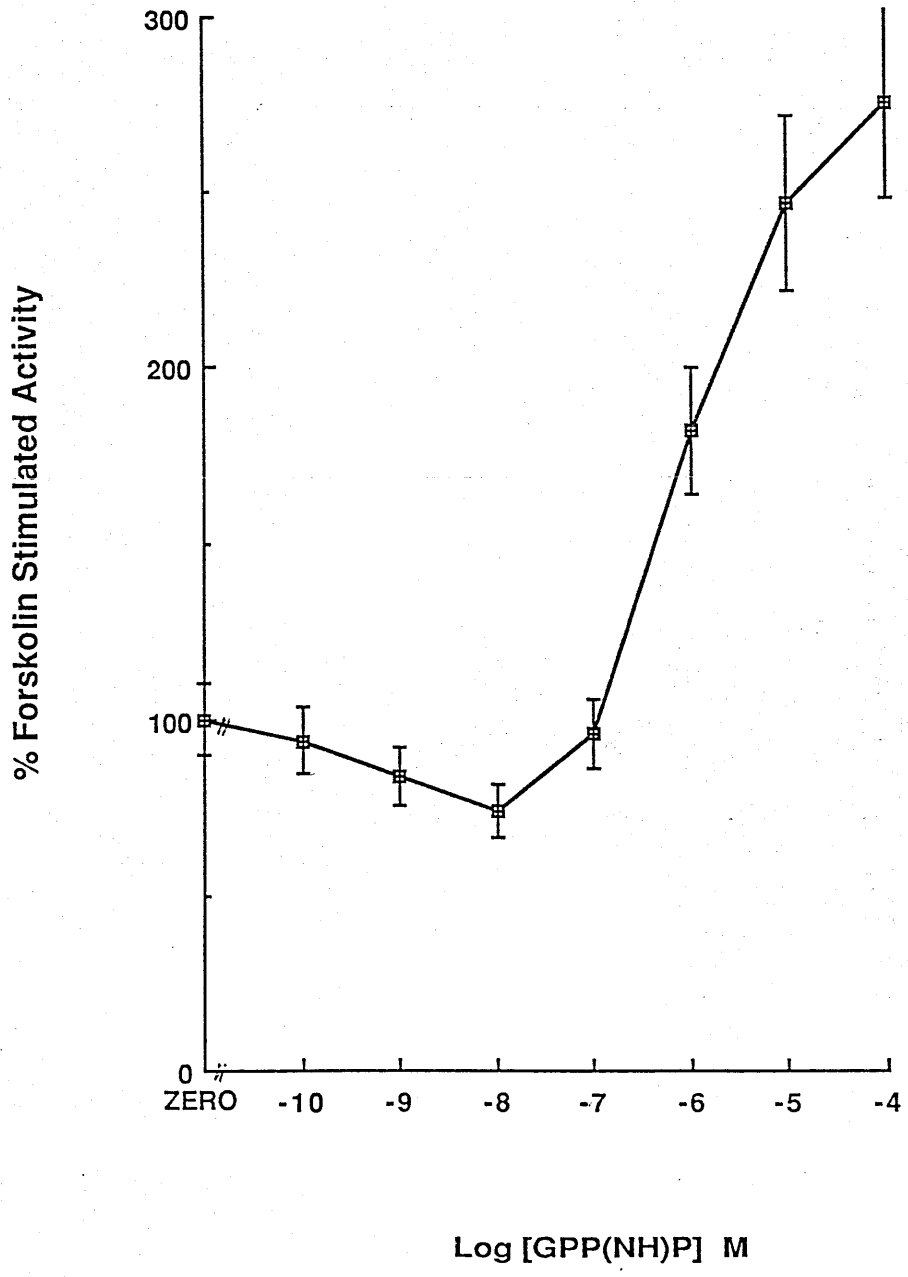


Figure 3.1.4 Guanine Nucleotide Regulation of Forskolin Stimulated Adenylate Cyclase Activity In Hepatocytes Isolated From Lean Zucker Rats

Crude membrane pellets were prepared from hepatocytes isolated from lean Zucker rats.

Adenylate cyclase activity was assayed in the presence of forskolin (10^{-4} M) and increasing concentrations of 5'-guanylyl-imidodiphosphate (GPP(NH)P). Activity is expressed as a percentage of forskolin stimulated activity in the absence of GPP(NH)P . All activities are expressed as mean +/_ S.E.M. values (n= 3).

Basal and forskolin stimulated adenylate cyclase activities were 1.14 +/_ 0.02 and 17.10 +/_ 2.00 pmoles cAMP produced min^{-1} mg^{-1} respectively.

Figure 3.1.4 Guanine Nucleotide Regulation of Forskolin Stimulated Adenylate Cyclase Activity In Hepatocytes Isolated From Lean Zucker Rats

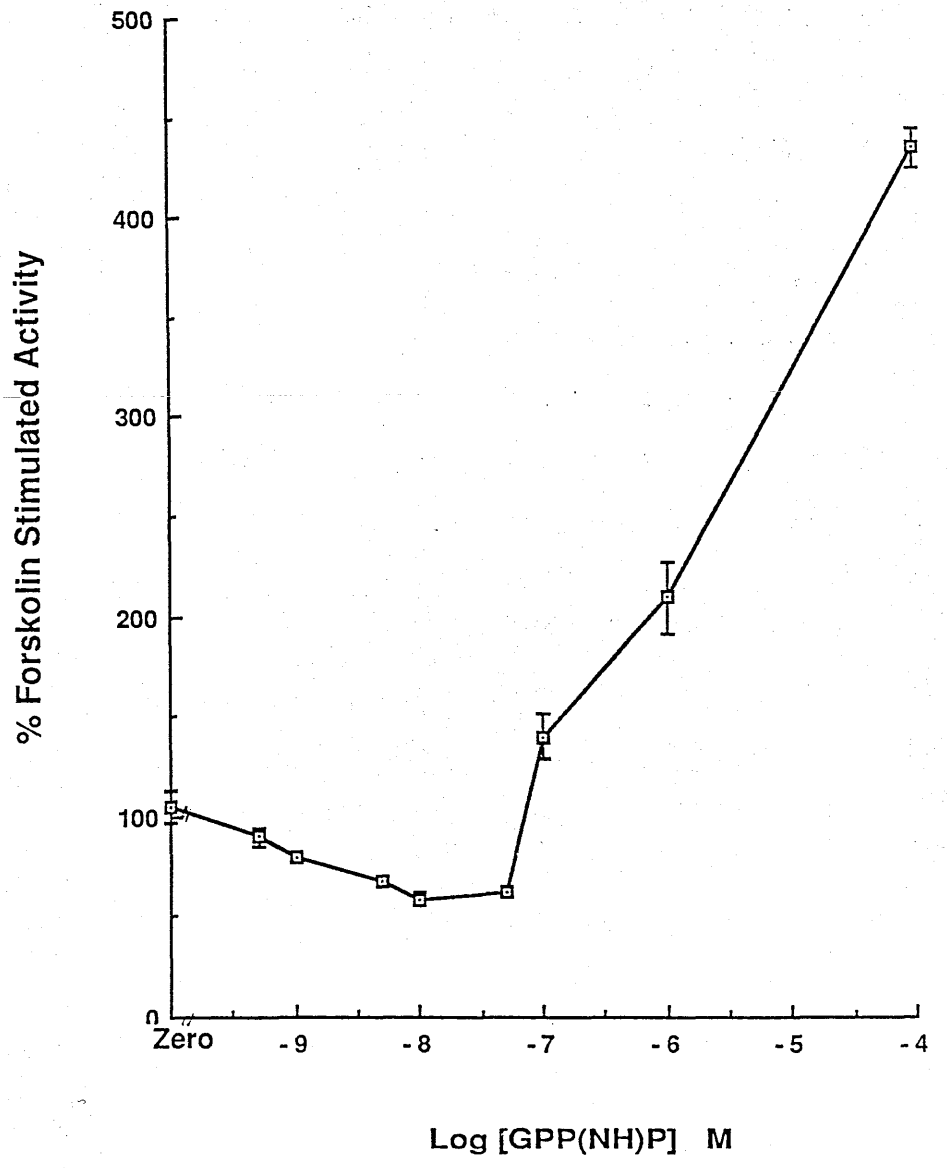


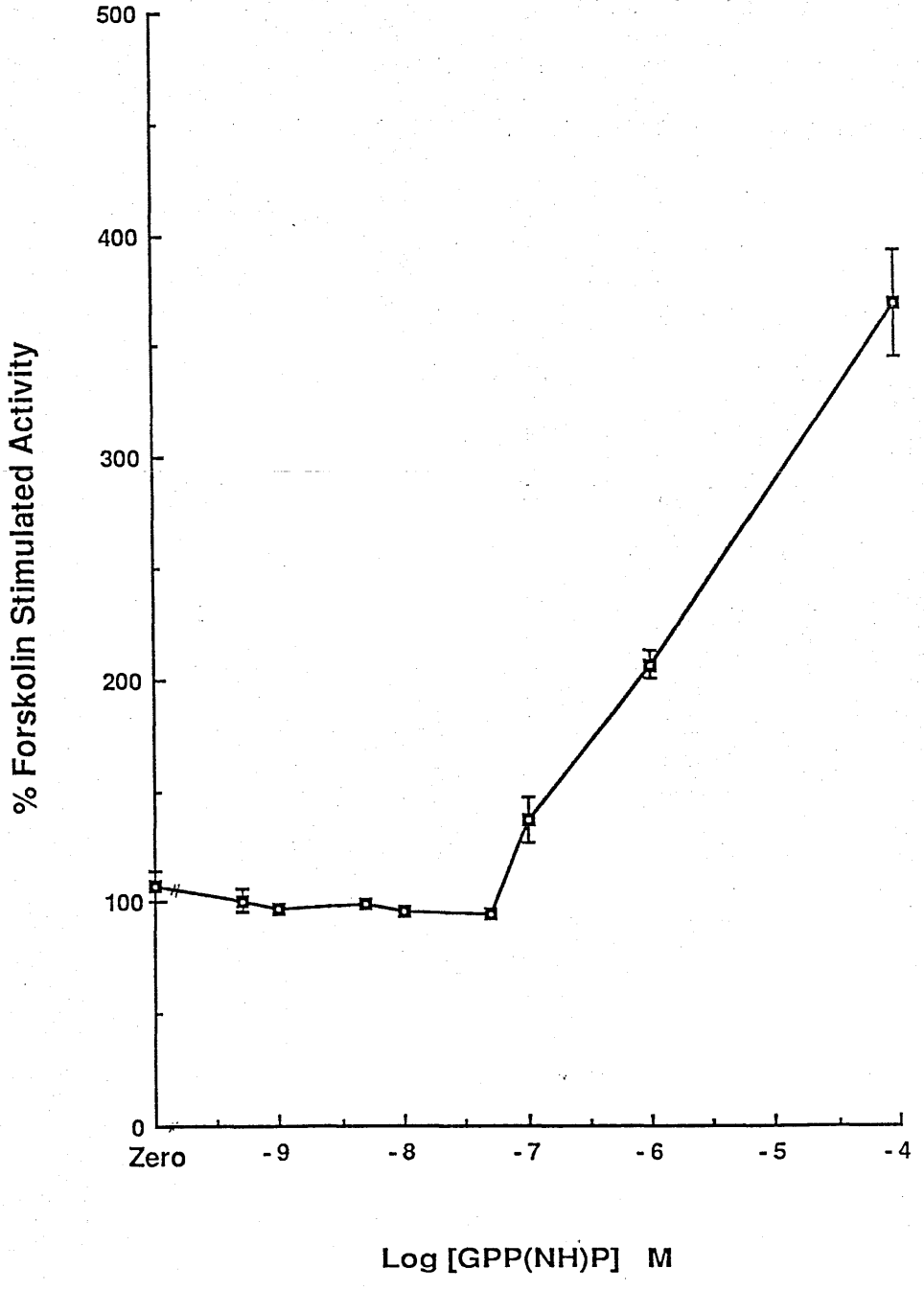
Figure 3.1.5 Guanine Nucleotide Regulation of Forskolin Stimulated Adenylate Cyclase Activity In Hepatocytes Isolated From Obese Zucker Rats

Crude membrane pellets were prepared from hepatocytes isolated from obese Zucker Rats.

Adenylate cyclase activity was assayed in the presence of forskolin (10^{-4} M) and increasing concentrations of 5'-guanylyl-imidodiphosphate (GPP(NH)P). Activity is expressed as a percentage of forskolin stimulated activity in the absence of GPP(NH)P. All activities are expressed as mean +/_ S.E.M. values (n=3).

Basal and forskolin stimulated adenylate cyclase activities were 1.24 +/_ 0.10 and 18.38 +/_ 1.60 pmoles cAMP produced min^{-1} mg^{-1} respectively.

Figure 3.1.5 Guanine Nucleotide Regulation of Forskolin Stimulated Adenylate Cyclase Activity In Hepatocytes Isolated From Obese Zucker Rats



3.2 GPP(NH)P Dependent Inhibition of Forskolin Stimulated Adenylate Cyclase Activity

Figure 3.2.1 GPP(NH)P - Dependent Inhibition of Forskolin Stimulated Adenylate Cyclase Activity In Hepatocytes Isolated From Control Rats

Crude membrane pellets were prepared from hepatocytes isolated from Sprague Dawley rats.

Adenylate cyclase activity was assayed in the presence of forskolin (10^{-4} M) and increasing concentrations of 5'- guanylyl-imidodiphospate (GPP(NH)P). Activity is expressed as a percentage of forskolin stimulated adenylate cyclase activity. All activities are expressed as mean +/_ S.E.M. values (n= 5).

Basal and forskolin stimulated adenylate cyclase activities were 1.30 +/_ 0.10 and 18.20 +/_ 2.00 pmoles cAMP produced min^{-1} mg^{-1} respectively.

Figure 3.2.1 GPP(NH)P - Dependent Inhibition of Forskolin Stimulated Adenylate Cyclase Activity In Hepatocytes Isolated From Control Rats

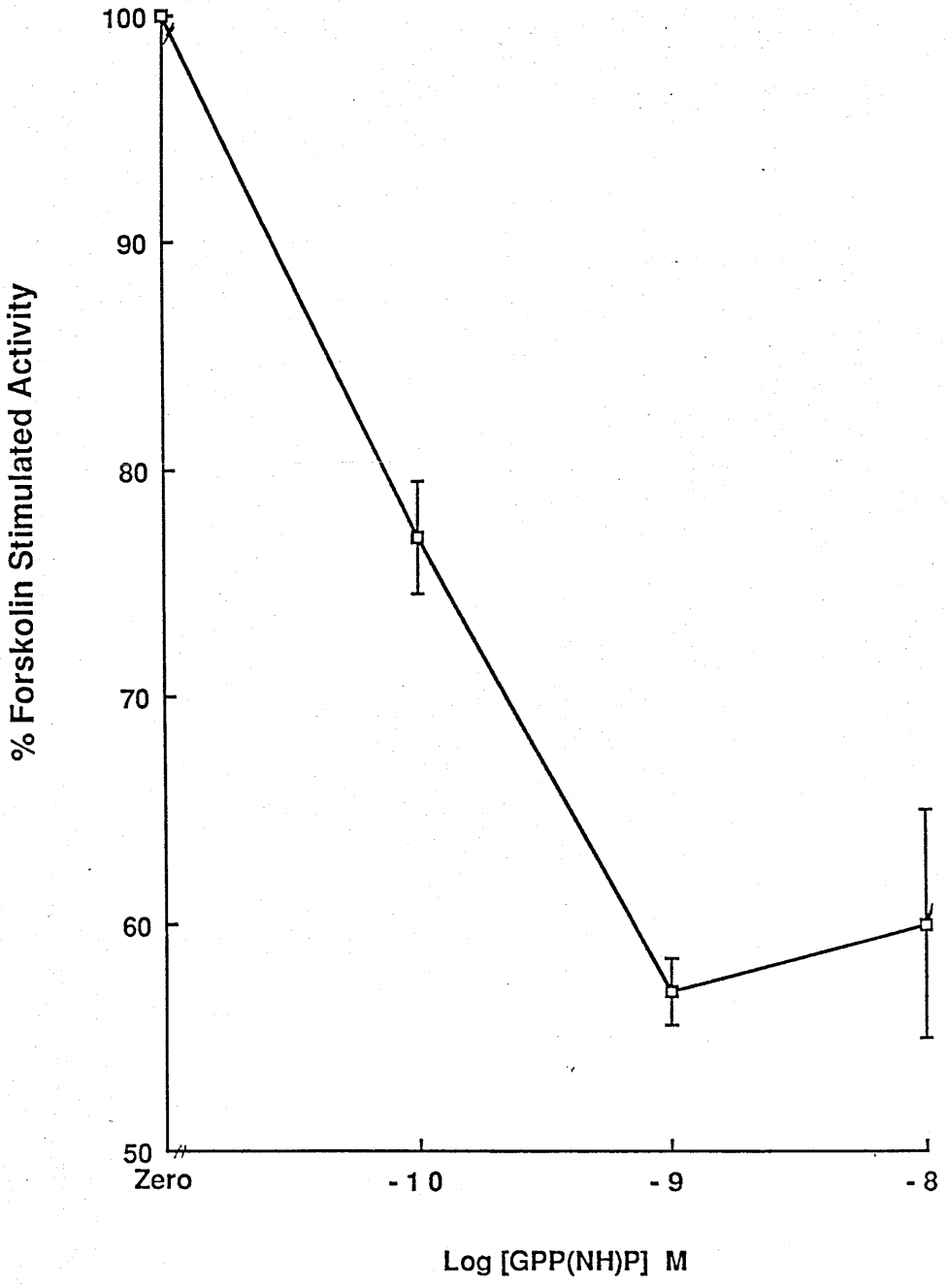


Figure 3..2.2 Effect of Streptozotocin Induced Diabetes On GPP(NH)P Mediated Inhibition of Adenylate Cyclase Activity In Isolated Hepatocytes

Type I diabetes (I.D.D.M.) was induced in Sprague Dawley rats as described in Materials and methods .

Crude membrane pellets were prepared from hepatocytes isolated from diabetic rats.Adenylate cyclase activity was assayed in the presence of forskolin (10^{-4} M) and increasing concentrations of 5'-guanylyl-imidodiphoshate (GPP(NH)P). Activity is expressed as a percentage of forskolin stimulated adenylate cyclase activity. All activities are expressed as mean +/_ S.E.M. values (n=5).

Basal and forskolin stimulated adenylate cyclase activities were 0.64 +/_ 0.02 and 11.20 +/- 1.00 cAMP produced min^{-1} mg^{-1} respectively.

Figure 3.2.2 Effect of Low Concentrations of GPP(NH)P On Forskolin Stimulated Adenylate Cyclase Activity In Hepatocytes Isolated From Streptozotocin- Induced Diabetic Rats

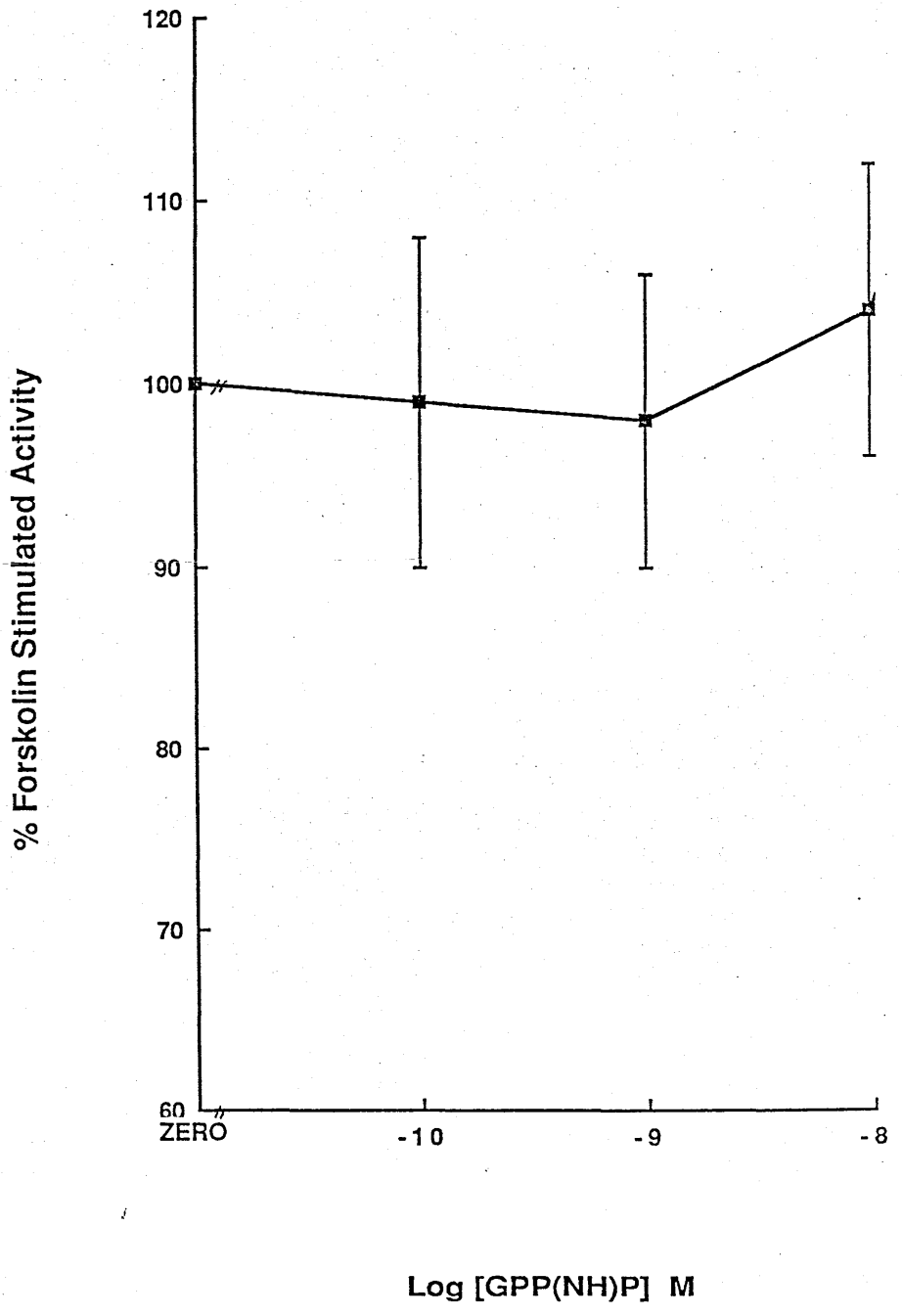


Figure 3..2.3 Effect of Alloxan Induced Diabetes On GPP(NH)P Mediated Inhibition of Adenylate Cyclase Activity In Isolated Hepatocytes

Type I diabetes (I.D.D.M.) was induced in Sprague Dawley rats as described in Materials and methods .

Crude membrane pellets were prepared from hepatocytes isolated from these diabetic rats.

Adenylate cyclase activity was assayed in the presence of forskolin (10^{-4} M) and increasing concentrations of 5'-guanylyl-imidodiphoshate (GPP(NH)P). Activity is expressed as a percentage of forskolin stimulated adenylate cyclase activity. All activities are expressed as mean +/_ S.E.M. values (n=5).

Basal and forskolin stimulated adenylate cyclase activities were 0.87 +/_ 0.07 and 12.30 +/- 1.50 pmoles cAMP produced min^{-1} mg^{-1} respectively.

Figure 3.2.3 Effect of Alloxan Induced Diabetes On GPP(NH)P Mediated Inhibition of Adenylate Cyclase Activity In Isolated Hepatocytes

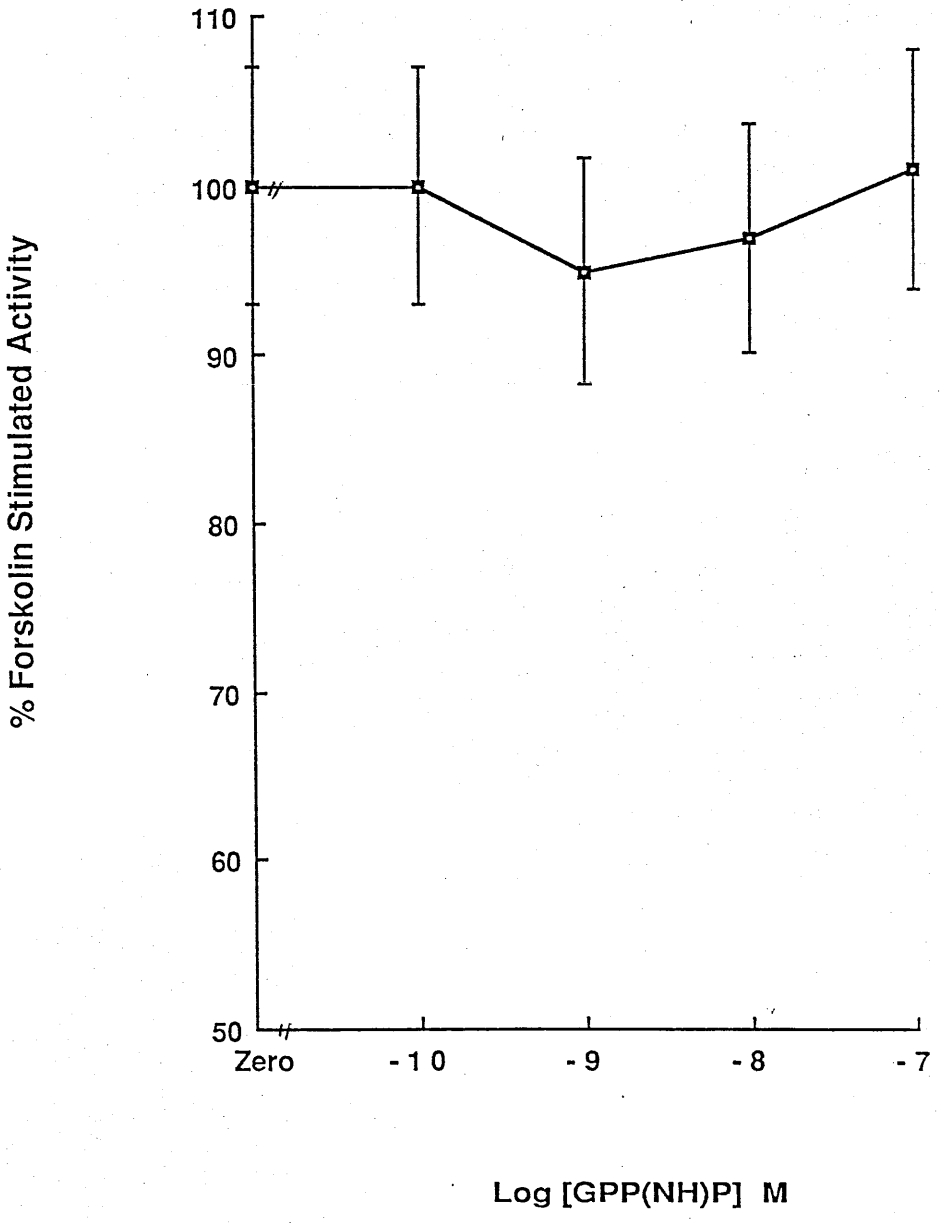


Figure 3.2.4 Effect of TPA And Pertussis Toxin Pretreatment On GPP(NH)P Mediated Inhibition of Adenylate Cyclase Activity In Hepatocytes Isolated From Control Rats

Crude membrane pellets were prepared from (□) control, (◇) TPA (10ng/ml) and (◆)Pertussis toxin (100ng/ml) pretreated hepatocytes isolated from Sprague Dawley rats.

Adenylate cyclase activity was assayed in the presence of forskolin (10^{-4} M) and increasing concentrations of 5'-guanylyl-imidodiphoshate (GPP(NH)P). Activity is expressed as a percentage of forskolin stimulated adenylate cyclase activity. All activities are expressed as mean +/_ S.E.M. values (n=5).

Basal adenylate cyclase activities were 1.30 +/_ 0.10 , 1.24 +/_ 0.10 and 1.35 +/_ 0.15 pmoles cAMP produced min^{-1} mg^{-1} for control, TPA and Pertussis toxin treated hepatocytes respectively. Similarly, the forskolin stimulated activities for these preparations were 18.20 +/_ 2.00 , 19.00 +/_ 2.04 and 19.60 +/_ 2.21 pmoles cAMP produced min^{-1} mg^{-1} respectively.

Figure 3.2.4 Effect of TPA And Pertussis Toxin Pretreatment On GPP(NH)P Mediated Inhibition of Adenylate Cyclase Activity In Hepatocytes Isolated From Control Rats

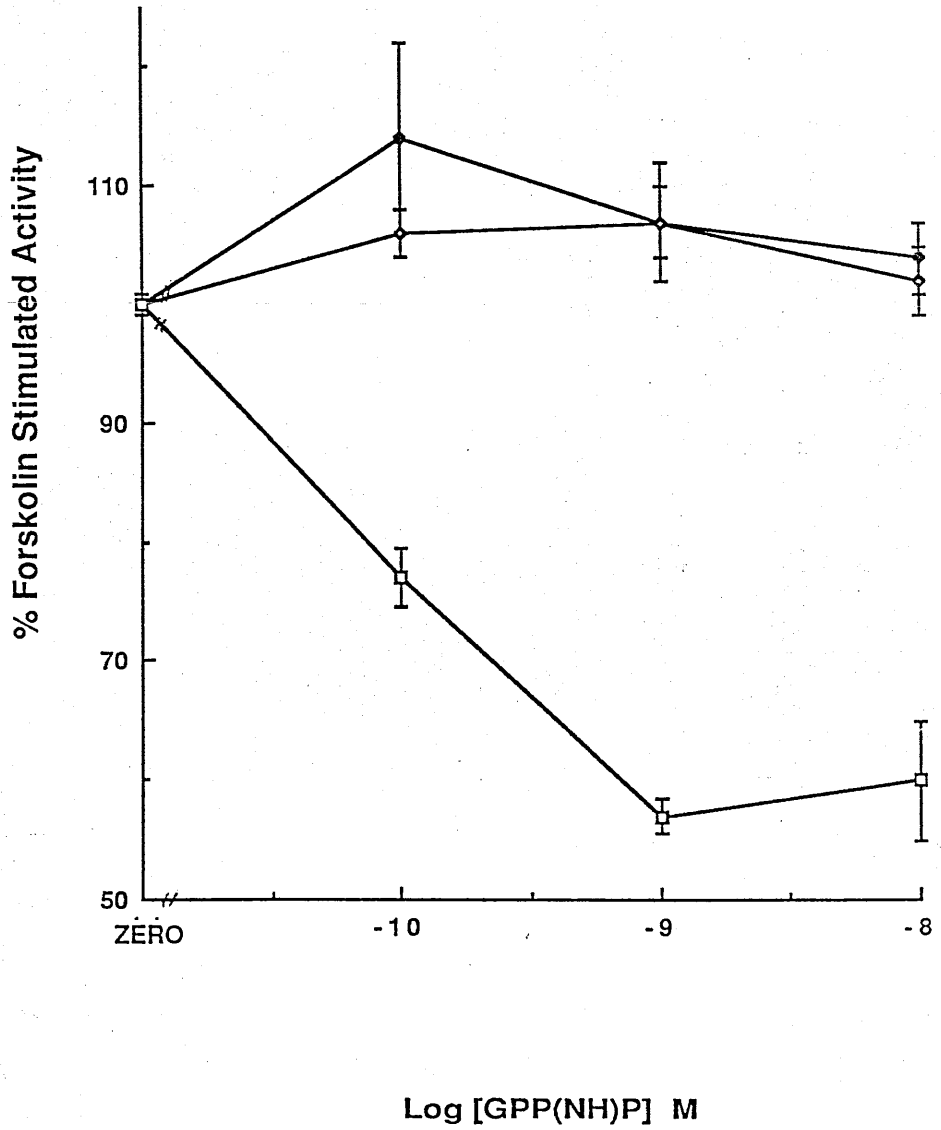


Figure 3..2.5 Insulin Reversal of Streptozotocin Induced Diabetes And Its Effect On GPP(NH)P Mediated Inhibition of Adenylate Cyclase Activity In Isolated Hepatocytes

Type I diabetes (I.D.D.M.) was induced in Sprague Dawley rats as described in Materials and methods .

Crude membrane pellets were prepared from hepatocytes isolated from (■) diabetic, (⊞) insulin treated diabetic and (□) control non treated rats.

Adenylate cyclase activity was assayed in the presence of forskolin (10^{-4} M) and increasing concentrations of 5'-guanylyl-imidodiphoshate (GPP(NH)P). Activity is expressed as a percentage of forskolin stimulated adenylate cyclase activity. All activities are expressed as mean +/_ S.E.M. values (n=5).

Basal adenylate cyclase activities were 0.64 +/_ 0 .02 0.68 +/_ 0.04 and 1.30 +/_ 0.10 pmoles cAMP produced min^{-1} mg^{-1} for diabetic animals treated with saline , insulin and control non treated animals respectively.

Forskolin stimulated activities were 11.20 +/_ 1.00 10.90 +/_0.90 and 18.20 +/_ 2.00 pmoles cAMP produced min^{-1} mg^{-1} respectively.

Figure 3.2.5 Insulin Reversal of Streptozotocin- Induced Diabetes And Its Effect On GPP(NH)P Mediated Inhibition Of Adenylate Cyclase Activity In Isolated Hepatocytes

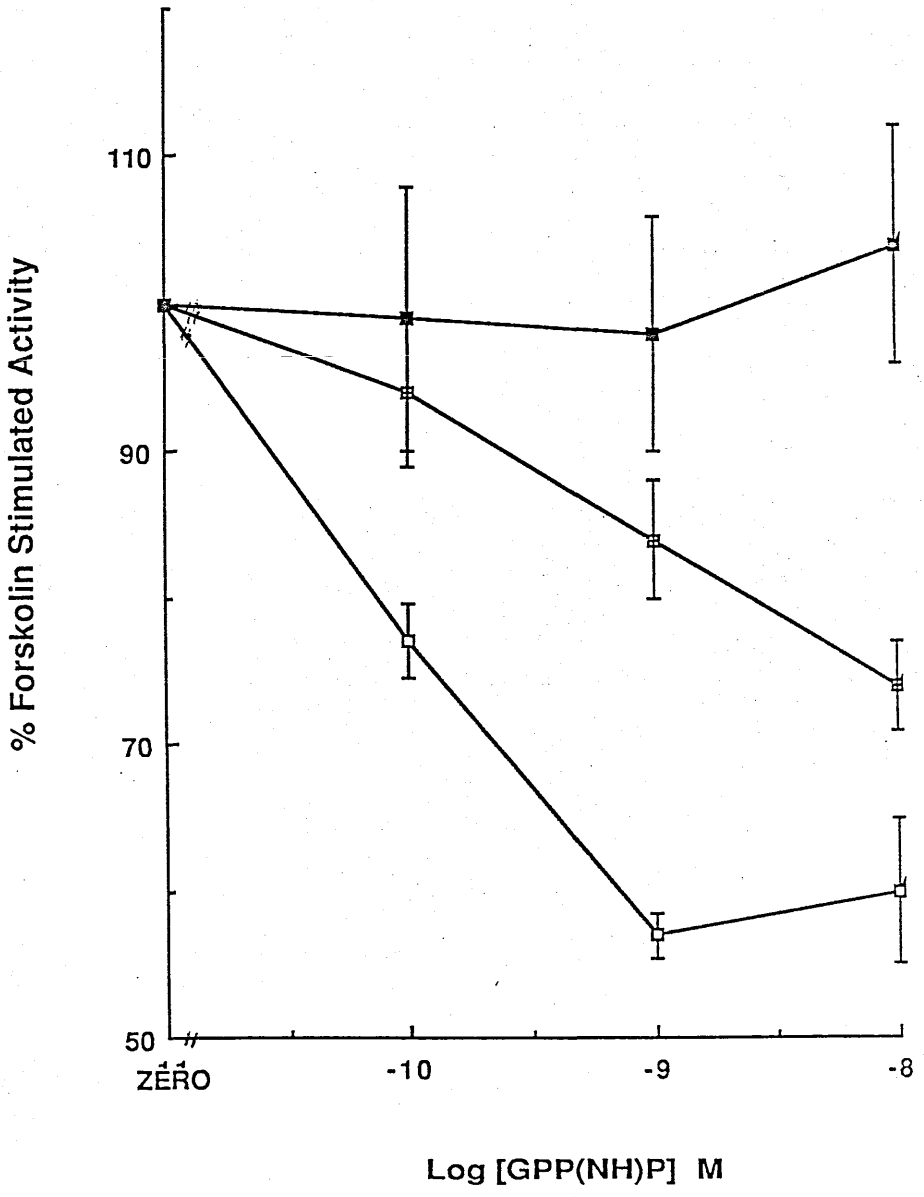


Figure 3.2.6 GPP(NH)P - Dependent Inhibition of Forskolin Stimulated Adenylate Cyclase Activity In Hepatocytes Isolated From Lean Zucker Rats

Crude membrane pellets were prepared from hepatocytes isolated from Lean Zucker rats.

Adenylate cyclase activity was assayed in the presence of forskolin (10^{-4} M) and increasing concentrations of 5'- guanylyl-imidodiphospate (GPP(NH)P). Activity is expressed as a percentage of forskolin stimulated adenylate cyclase activity. All activities are expressed as mean +/_ S.E.M. values (n= 5).

Basal and forskolin stimulated adenylate cyclase activities were 1.14 +/_ 0.02 and 17.10 +/_ 2.00 pmoles cAMP produced min^{-1} mg^{-1} respectively.

Figure 3.2.6 GPP(NH)P - Mediated Inhibition of Forskolin Stimulated Adenylate Cyclase Activity In Hepatocytes Isolated From Lean Zucker Rats

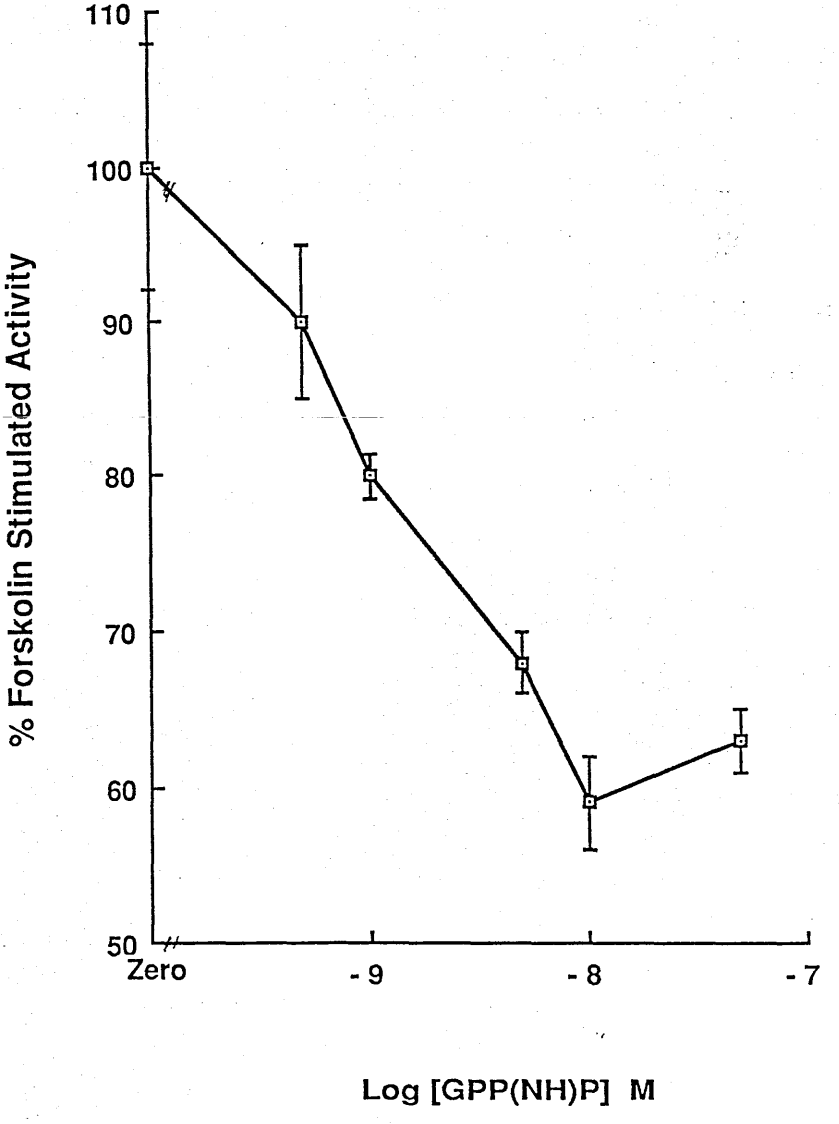


Figure 3.2.7 Effect of TPA And Pertussis Toxin Pretreatment On GPP(NH)P Mediated Inhibition of Adenylate Cyclase Activity In Hepatocytes Isolated From Lean Zucker Rats

Crude membrane pellets were prepared from (□) control, (◇) TPA (10ng/ml) and (◆)Pertussis toxin (100ng/ml) pretreated hepatocytes isolated from lean Zucker rats. Adenylate cyclase activity was assayed in the presence of forskolin (10^{-4} M) and increasing concentrations of 5'-guanylyl-imidodiphoshate (GPP(NH)P). Activity is expressed as a percentage of forskolin stimulated adenylate cyclase activity. All activities are expressed as mean +/_ S.E.M. values (n=4).

Basal adenylate cyclase activities were 1.14 +/_ 0.02, 1.20 +/- 0.10 and 1.24 +/_ 0.15 pmoles cAMP produced min^{-1} mg^{-1} for control, TPA and Pertussis toxin treated membranes respectively.

Similarly, forskolin stimulated activities were 17.10 +/_ 2.00 , 18.20 +/_ 1.95 and 19.00 +/_ 2.10 pmoles cAMP produced min^{-1} mg^{-1} respectively.

Figure 3.2.7 Effect of TPA And Pertussis Toxin Pretreatment On GPP(NH)P Mediated Inhibition of Adenylate Cyclase Activity In Hepatocytes Isolated From Lean Zucker Rats

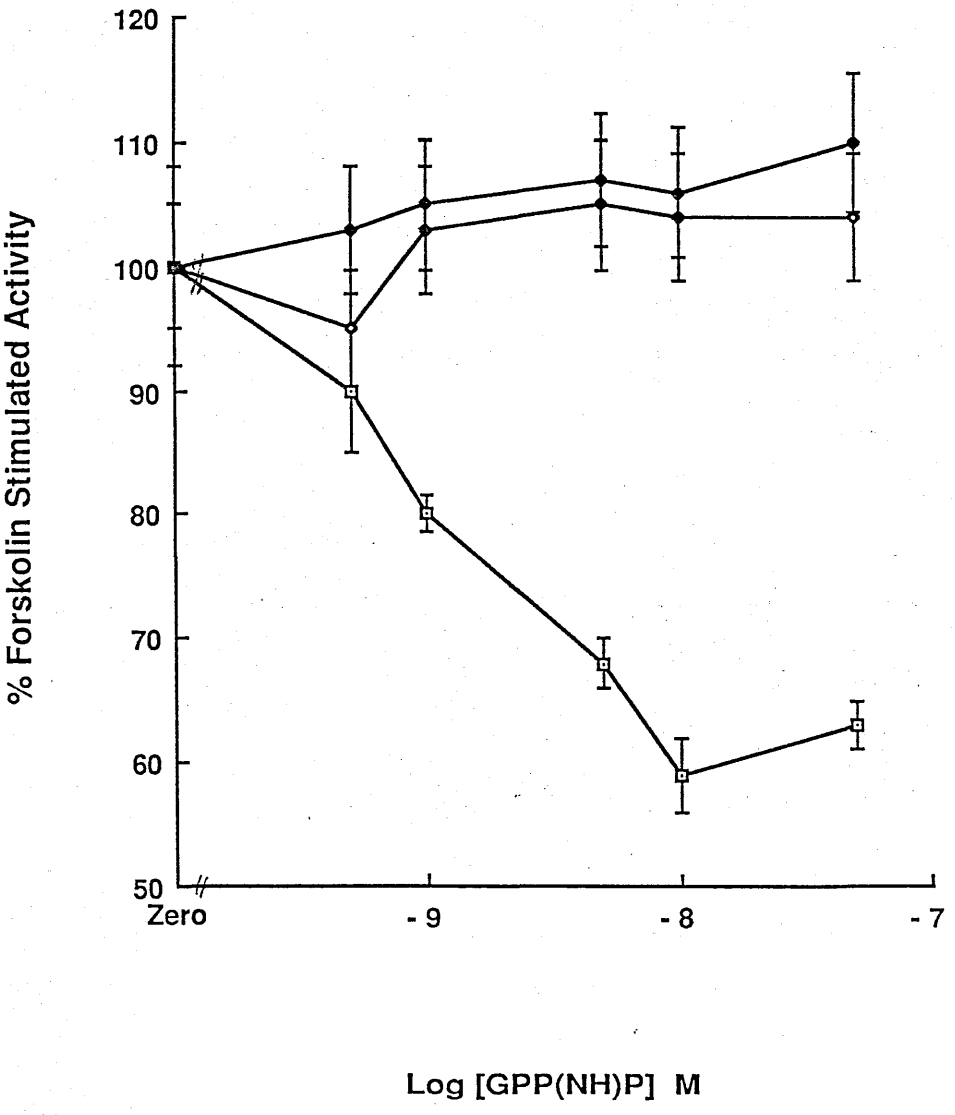


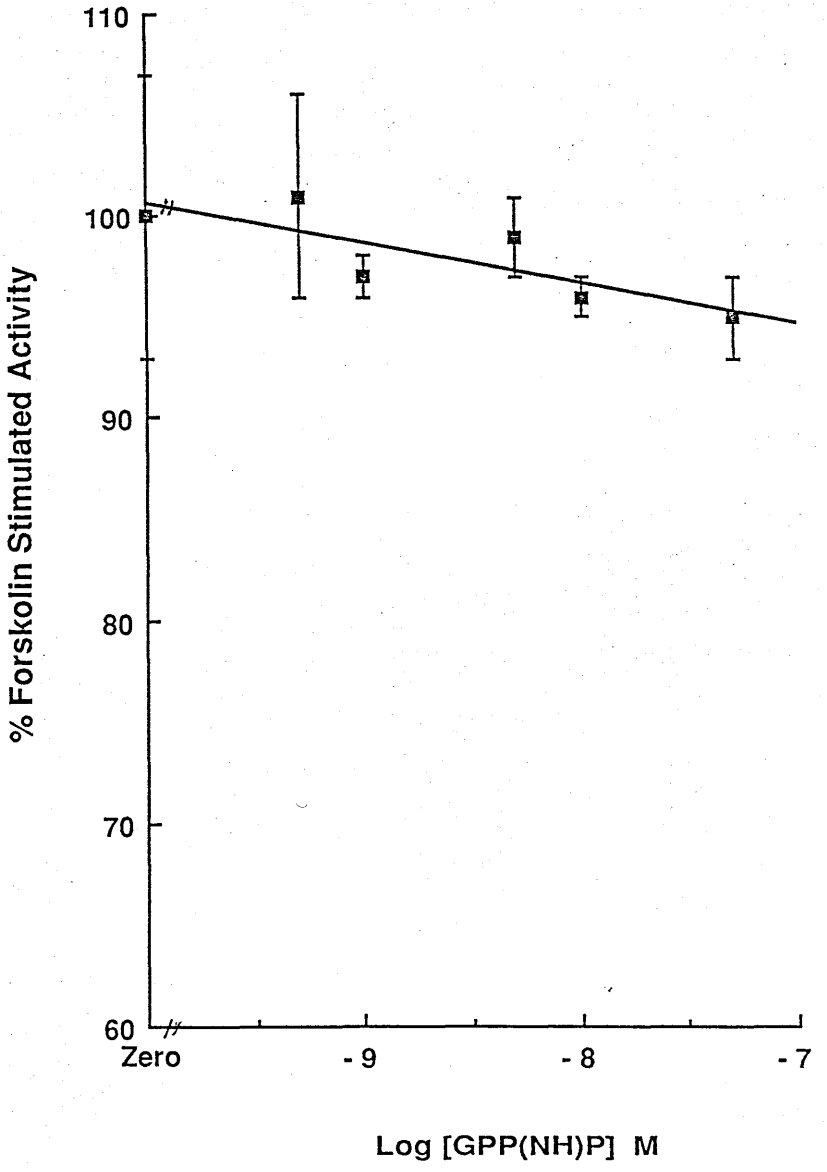
Figure 3.2.8 GPP(NH)P - Dependent Inhibition of Forskolin Stimulated Adenylate Cyclase Activity In Hepatocytes Isolated From Obese Zucker Rats

Crude membrane pellets were prepared from hepatocytes isolated from obese Zucker rats.

Adenylate cyclase activity was assayed in the presence of forskolin (10^{-4} M) and increasing concentrations of 5'- guanylyl-imidodiphospate (GPP(NH)P). Activity is expressed as a percentage of forskolin stimulated adenylate cyclase activity. All activities are expressed as mean +/_ S.E.M. values (n= 3)

Basal and forskolin stimulated adenylate cyclase activities were 1.24 +/_ 0.10 and 18.38 +/_ 1.60 pmoles cAMP produced min^{-1} mg^{-1} respectively.

Figure 3.2.8 GPP(NH)P-Dependent Inhibition of Forskolin Stimulated Adenylate Cyclase Activity In Hepatocytes Isolated From Obese Zucker Rats



3.3 GPP(NH)P Dependent Stimulation of Adenylate Cyclase Activity

Figure 3.3.1 GPP(NH)P-Dependent Stimulation of Adenylate Cyclase Activity In Hepatocytes Isolated From Control And Streptozotocin Induced Diabetic Rats

Crude membrane pellets were prepared from hepatocytes isolated from (▨) control and (■) streptozotocin treated Sprague Dawley rats. Adenylate cyclase activity was assayed in the presence of forskolin and increasing concentrations of 5'-guanylyl-imidodiphosphate (GPP(NH)P). Activity is expressed as a percentage of forskolin stimulated activity in the absence of GPP(NH)P. All activities are expressed as mean +/_ S.E.M. values(n=5).

Basal adenylate cyclase activities were 1.30 +/_ 0.10 and 0.64 +/_ 0.02 pmoles cAMP produced min^{-1} mg^{-1} for control and diabetic preparations respectively. Similarly, forskolin stimulated activities were 18.20 +/_ 2.00 and 11.20 +/_ 1.00 pmoles cAMP produced $min^{-1}mg^{-1}$ respectively.

Figure 3.3.1 GPP(NH)P-Dependent Stimulation of Adenylate Cyclase Activity In Hepatocytes Isolated From Control And Streptozotocin Induced Diabetic Rats

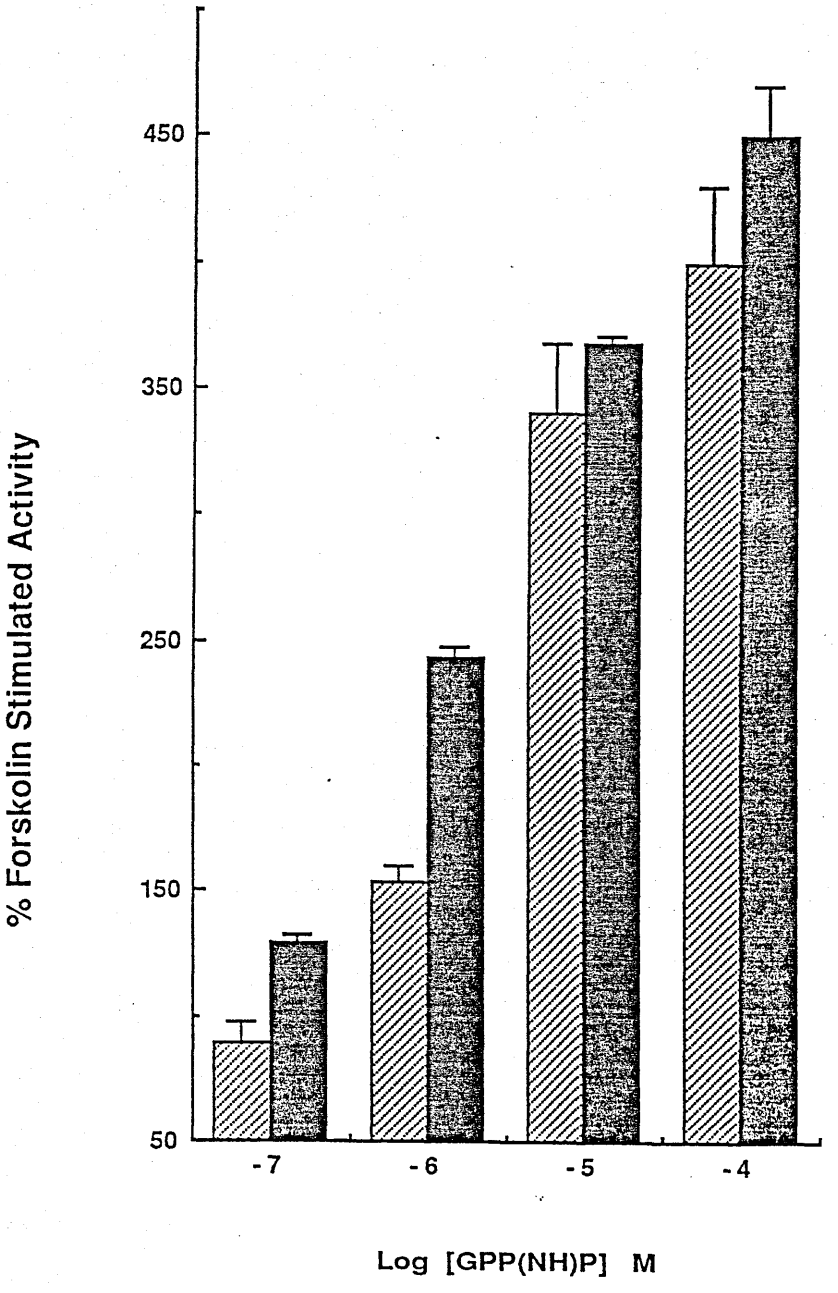
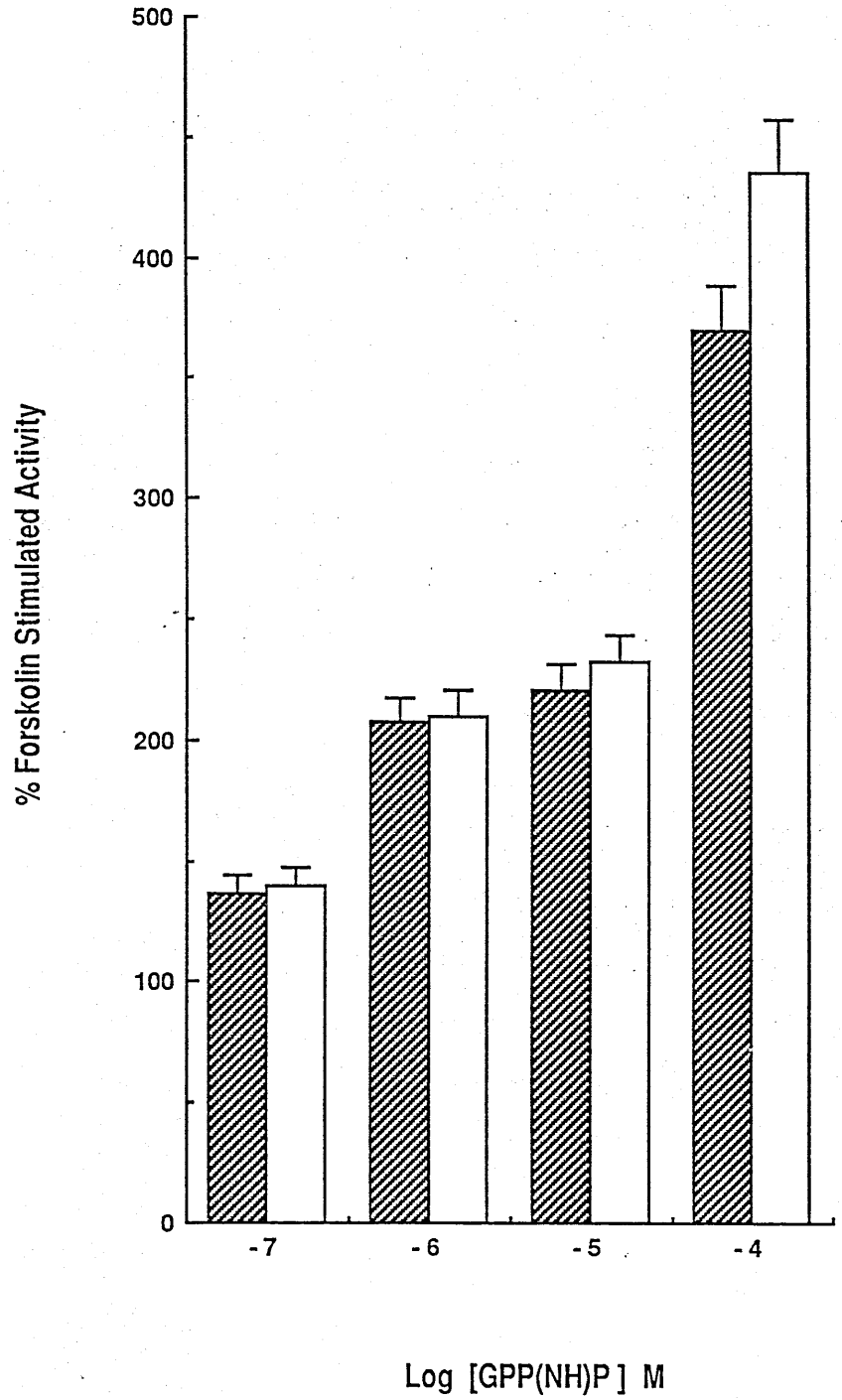


Figure 3.3.2 GPP(NH)P-Dependent Stimulation of Adenylate Cyclase Activity In Hepatocytes Isolated From Lean And Obese Zucker Rats

Crude membrane pellets were prepared from hepatocytes isolated from (□) lean and (▨) obese Zucker rats. Adenylate cyclase activity was assayed in the presence of forskolin and increasing concentrations of 5'-guanylyl-imidodiphosphate (GPP(NH)P). Activity is expressed as a percentage of forskolin stimulated activity in the absence of GPP(NH)P. All activities are expressed as mean +/_ S.E.M. values(n=3).

Basal adenylate cyclase activities were 1.14 +/_ 0.02 and 1.24+/_ 0.09 pmoles cAMP produced min^{-1} mg^{-1} for lean and obese rat preparations respectively. Similarly, forskolin stimulated activities were 17.10 +/_ 1.90 and 18.30 +/_ 2.01 pmoles cAMP produced $min^{-1}mg^{-1}$ respectively.

Figure 3.3.2 GPP(NH)P-Dependent Stimulation of Adenylate Cyclase Activity In Hepatocytes Isolated From Lean And Obese Zucker Rats



3.4 Effect of Biguanide Treatment On GPP(NH)P Regulation of Forskolin Stimulated Adenylate Cyclase Activity

Figure 3.4.1 Regulation of Adenylate Cyclase Activity Using The Guanine Nucleotide GPP(NH)P In Hepatocytes Isolated From Control Rats Undergoing Biguanide Therapy

Crude membrane pellets were prepared from hepatocytes isolated from control Sprague Dawley rats undergoing metformin therapy.

Adenylate cyclase activity was assayed in the presence of forskolin (10^{-4} M) and increasing concentrations of 5' - guanylyl-imidodiphosphate (GPP(NH)P) . Activity is expressed as a percentage of forskolin stimulated activity in the absence of GPP(NH)P.

All activities are expressed as mean +/_ S.E.M. values (n=6).

Basal and forskolin stimulated adenylate cyclase activities were 1.02 +/_ 0.15 and 17.34 +/_ 2.00 pmoles cAMP produced min^{-1} mg^{-1} respectively.

Figure 3.4.1 Regulation of Adenylate Cyclase Activity Using The Guanine Nucleotide GPP(NH)P In Hepatocyte Membranes Isolated From Control Rats Undergoing Biguanide Therapy

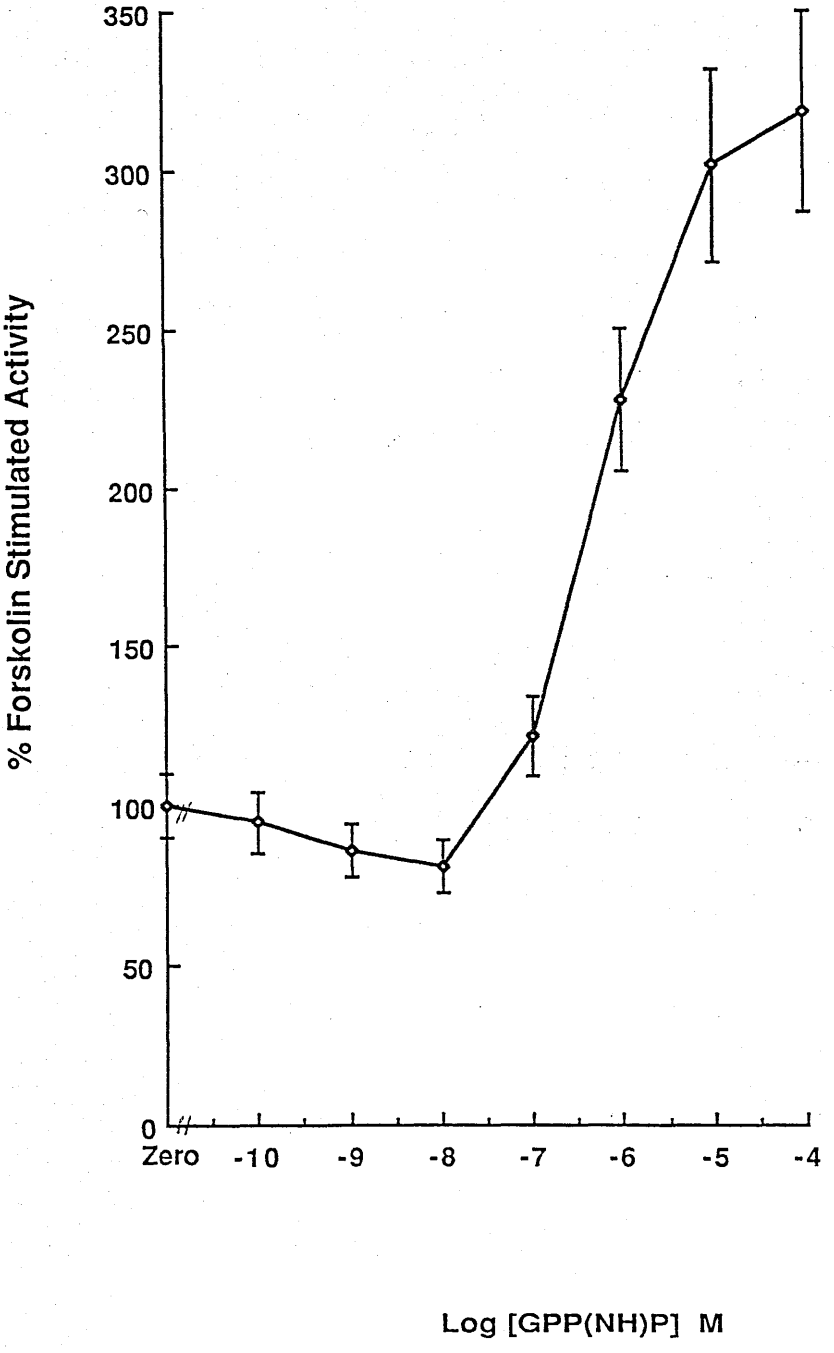


Figure 3.4.2 Regulation of Adenylate Cyclase Activity Using The Guanine Nucleotide GPP(NH)P In Hepatocytes Isolated From Streptozotocin induced diabetic Rats Undergoing Biguanide Therapy

Crude membrane pellets were prepared from hepatocytes isolated from streptozotocin induced diabetic Sprague Dawley rats undergoing metformin therapy.

Adenylate cyclase activity was assayed in the presence of forskolin (10^{-4} M) and increasing concentrations of 5' - guanylyl-imidodiphosphate (GPP(NH)P) . Activity is expressed as a percentage of forskolin stimulated activity in the absence of GPP(NH)P.

All activities are expressed as mean +/_ S.E.M. values (n=5).

Basal and forskolin stimulated adenylate cyclase activities were 0.60 +/_ 0.07 and 10.50 +/_ 0.30 pmoles cAMP produced min^{-1} mg^{-1} respectively

Figure 3.4.2 Regulation of Adenylate Cyclase Activity Using The Guanine Nucleotide GPP(NH)P In Hepatocytes Isolated From Streptozotocin Induced Diabetic Rats Undergoing Biguanide Therapy

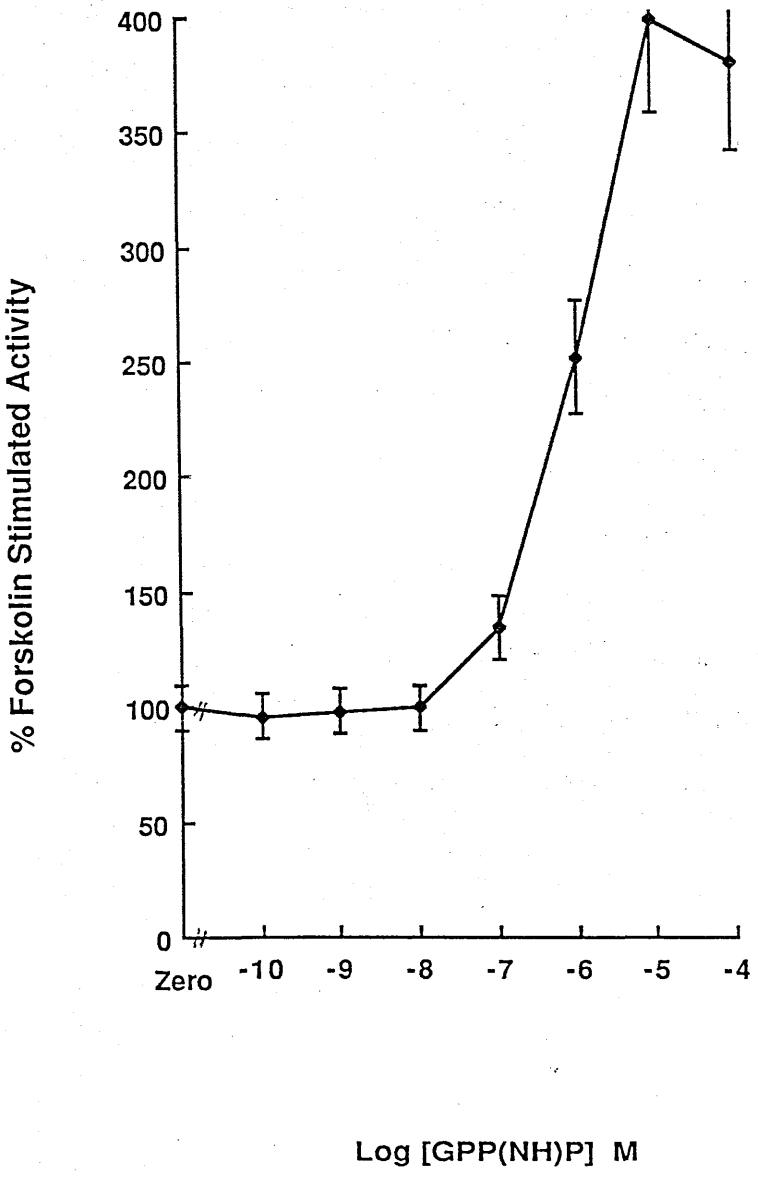


Figure 3.4.3 Regulation of Adenylate Cyclase Activity Using The Guanine Nucleotide GPP(NH)P In Hepatocytes Isolated From Lean Zucker Rats Undergoing Biguanide Therapy

Crude membrane pellets were prepared from hepatocytes isolated from lean Zucker rats undergoing metformin therapy.

Adenylate cyclase activity was assayed in the presence of forskolin (10^{-4} M) and increasing concentrations of 5' - guanylyl-imidodiphosphate (GPP(NH)P) . Activity is expressed as a percentage of forskolin stimulated activity in the absence of GPP(NH)P.

All activities are expressed as mean +/_ S.E.M. values (n=4).

Basal and forskolin stimulated adenylate cyclase activities were 1.23 +/_ 0.10 and 19.68 +/_ 2.10 pmoles cAMP produced min^{-1} mg^{-1} respectively.

Figure 3.4.3 Regulation of Adenylate Cyclase Activity Using The Guanine Nucleotide GPP(NH)P In Hepatocytes Isolated From Lean Zucker Rats Undergoing Biguanide Therapy

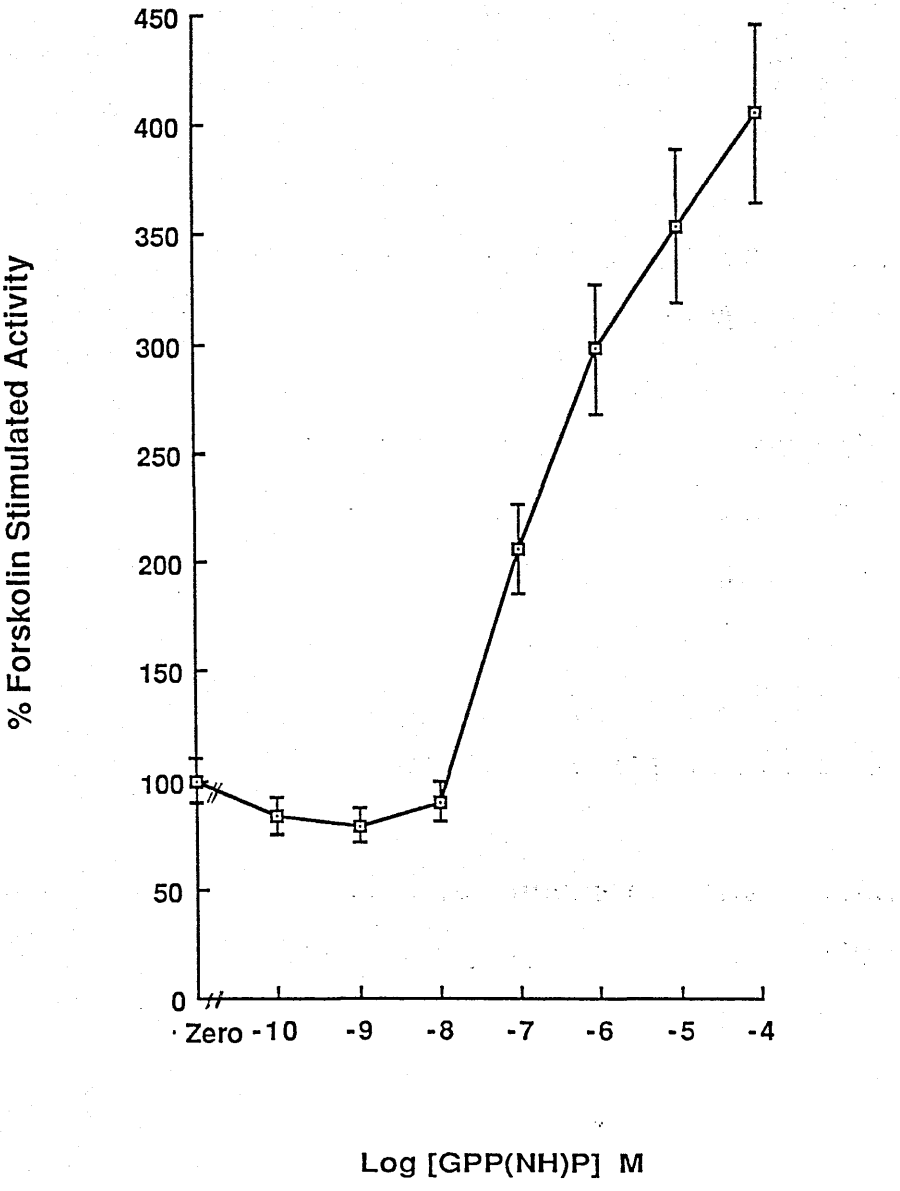


Figure 3.4.4 Regulation of Adenylate Cyclase Activity Using The Guanine Nucleotide GPP(NH)P In Hepatocytes Isolated From Obese Zucker Rats Undergoing Biguanide Therapy

Crude membrane pellets were prepared from hepatocytes isolated from obese Zucker rats undergoing metformin therapy.

Adenylate cyclase activity was assayed in the presence of forskolin (10^{-4} M) and increasing concentrations of 5' - guanylyl-imidodiphosphate (GPP(NH)P) . Activity is expressed as a percentage of forskolin stimulated activity in the absence of GPP(NH)P.

All activities are expressed as mean +/_ S.E.M. values (n=5).

Basal and forskolin stimulated adenylate cyclase activities were 1.00 +/_ 0.10 and 15.00 +/_ 2.01 pmoles cAMP produced min^{-1} mg^{-1} respectively.

Figure 3.4.4 Regulation of Adenylate Cyclase Activity Using The Guanine Nucleotide GPP(NH)P In Hepatocytes Isolated From Obese Zucker Rats Undergoing Biguanide Therapy

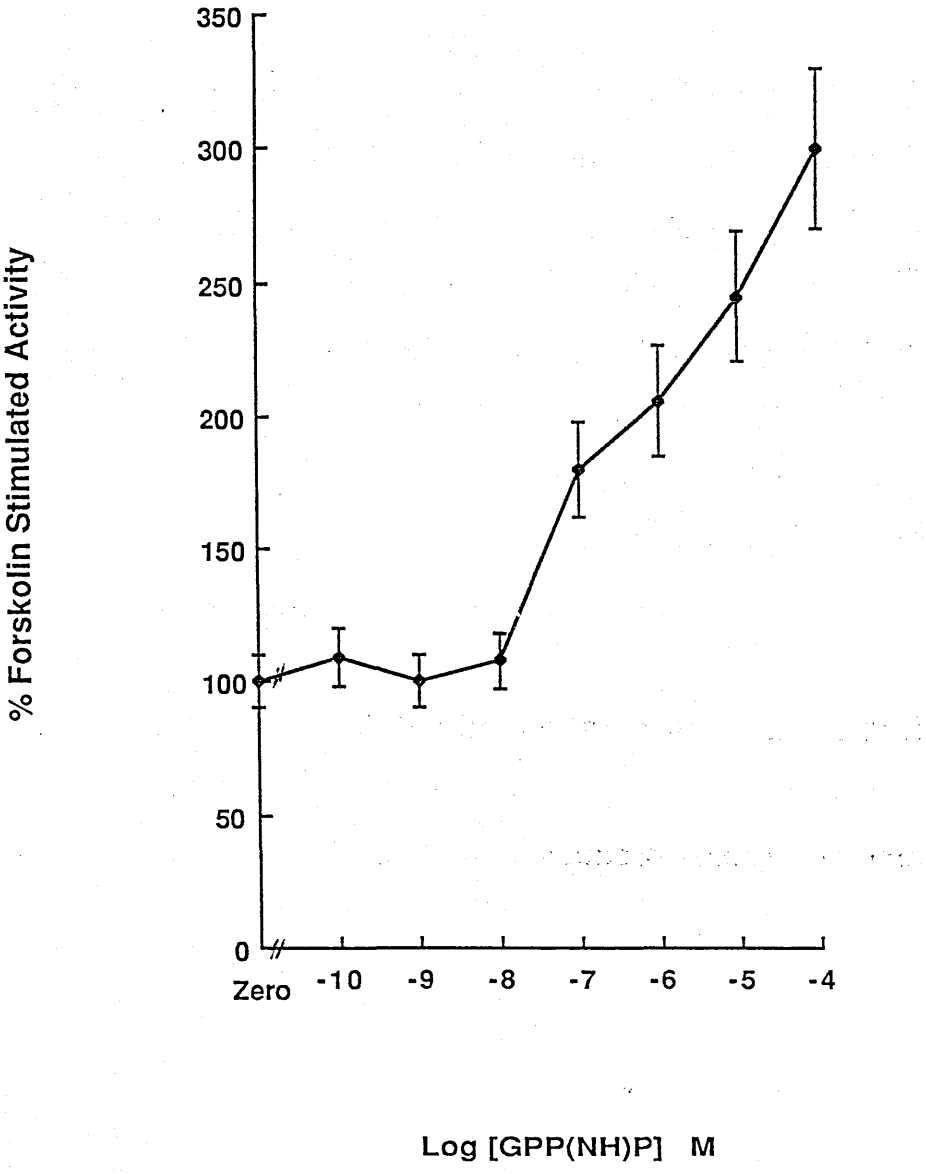


Figure 3.4.5 Effect of Biguanide Treatment On GPP(NH)P - Dependent Inhibition of Forskolin Stimulated Adenylate Cyclase Activity In Hepatocytes Isolated From Control Rats

Crude membrane pellets were prepared from hepatocytes isolated from Sprague Dawley rats which (◘)had or(⊡) had not undergone metformin therapy.

Adenylate cyclase activity was assayed in the presence of forskolin (10^{-4} M) and increasing concentrations of 5'- guanylyl-imidodiphospate (GPP(NH)P). Activity is expressed as a percentage of forskolin stimulated adenylate cyclase activity. All activities are expressed as mean +/_ S.E.M. values (n= 6).

Basal adenylate cyclase activities were 1.02 +/_ 0.15 and 1.30 +/- 0.10 pmoles cAMP produced min^{-1} mg^{-1} for treated and untreated animals respectively. Similarly, forskolin stimulated activities were 17.34 +/_ 2.00 and 18.20 +/_ 2.00 pmoles cAMP produced min^{-1} mg^{-1} respectively.

Figure 3.4.5 Effect of Biguanide Treatment on GPP(NH)P-Dependent Inhibition of Forskolin Stimulated Adenylate Cyclase Activity In Hepatocytes Isolated From Control Rats

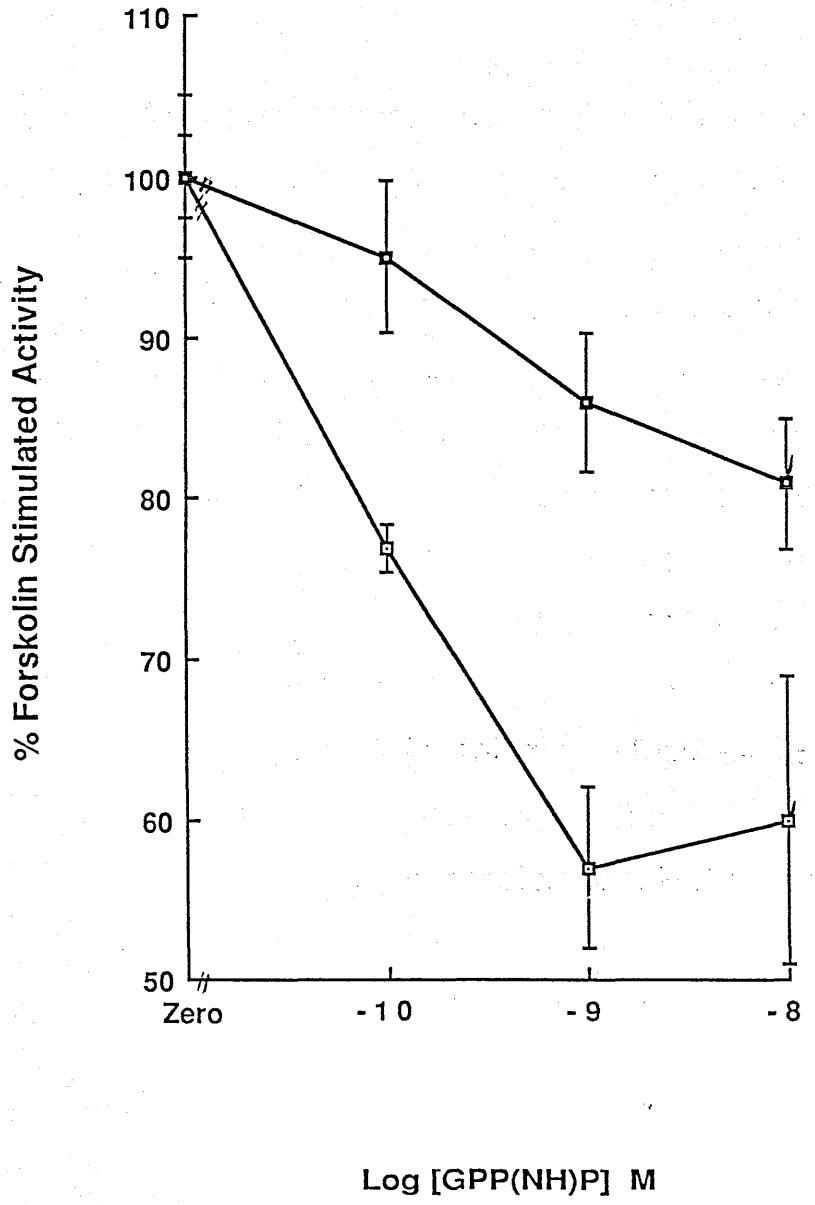


Figure 3.4.6 Effect of Biguanide Treatment On GPP(NH)P - Dependent Inhibition of Forskolin Stimulated Adenylate Cyclase Activity In Hepatocytes Isolated From Streptozotocin Induced Diabetic Rats

Crude membrane pellets were prepared from hepatocytes isolated from Streptozotocin induced diabetic rats which (■)had or(□) had not undergone metformin therapy.
Adenylate cyclase activity was assayed in the presence of forskolin (10^{-4} M) and increasing concentrations of 5'- guanylyl-imidodiphospate (GPP(NH)P). Activity is expressed as a percentage of forskolin stimulated adenylate cyclase activity. All activities are expressed as mean +/_ S.E.M. values (n= 5).
Basal adenylate cyclase activities were 0.60 +/_ 0.07 and 0.64 +/_ 0.02 pmoles cAMP produced min^{-1} mg^{-1} for treated and untreated animals respectively. Similarly, forskolin stimulated activities were 10.50 +/_ 0.30 and 11.20 +/_1.00 pmoles cAMP produced min^{-1} mg^{-1} respectively.

Figure 3.4.6 Effect of Biguanide Treatment On GPP(NH)P-Dependent Inhibition of Forskolin Stimulated Adenylate Cyclase Activity In Hepatocytes Isolated From Streptozotocin Induced Diabetic Rats

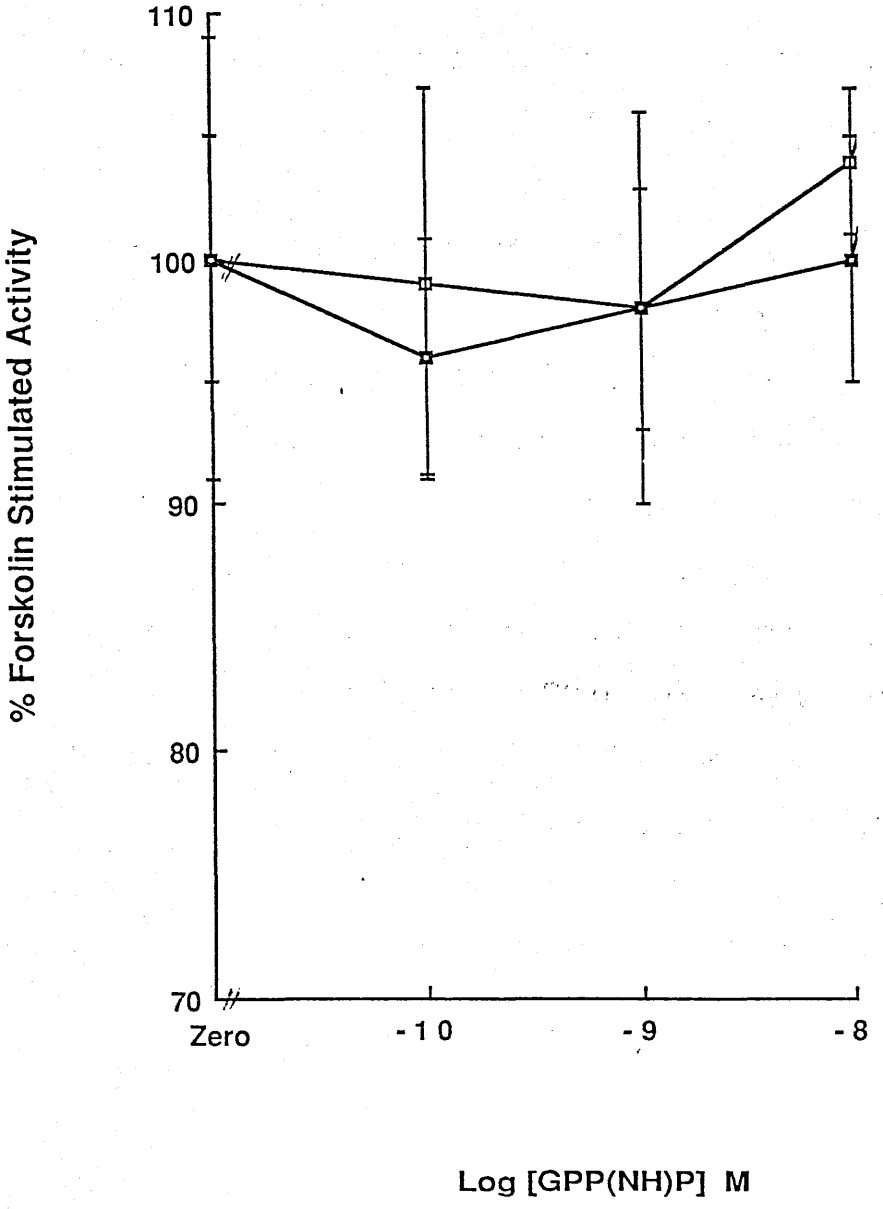


Figure 3.4.7 Effect of Biguanide Treatment On GPP(NH)P - Dependent Inhibition of Forskolin Stimulated Adenylate Cyclase Activity In Hepatocytes Isolated From Lean Zucker Rats

Crude membrane pellets were prepared from hepatocytes isolated from lean Zucker rats which (◘)had or(◻) had not undergone metformin therapy.

Adenylate cyclase activity was assayed in the presence of forskolin (10^{-4} M) and increasing concentrations of 5'- guanylyl-imidodiphospate (GPP(NH)P). Activity is expressed as a percentage of forskolin stimulated adenylate cyclase activity. All activities are expressed as mean +/_ S.E.M. values (n= 4).

Basal adenylate cyclase activities were 1.23 +/_ 0.10 and 1.14 +/_ 0.02 pmoles cAMP produced min^{-1} mg^{-1} for treated and untreated animals respectively. Similarly, forskolin stimulated activities were 19.68 +/_ 2.10 and 17.10 +/_ 1.90 pmoles cAMP produced min^{-1} mg^{-1} respectively.

Figure 3.4.7 Effect of Biguanide Treatment On GPP(NH)P-Dependent Inhibition of Forskolin Stimulated Adenylate Cyclase Activity In Hepatocytes Isolated From Lean Zucker Rats

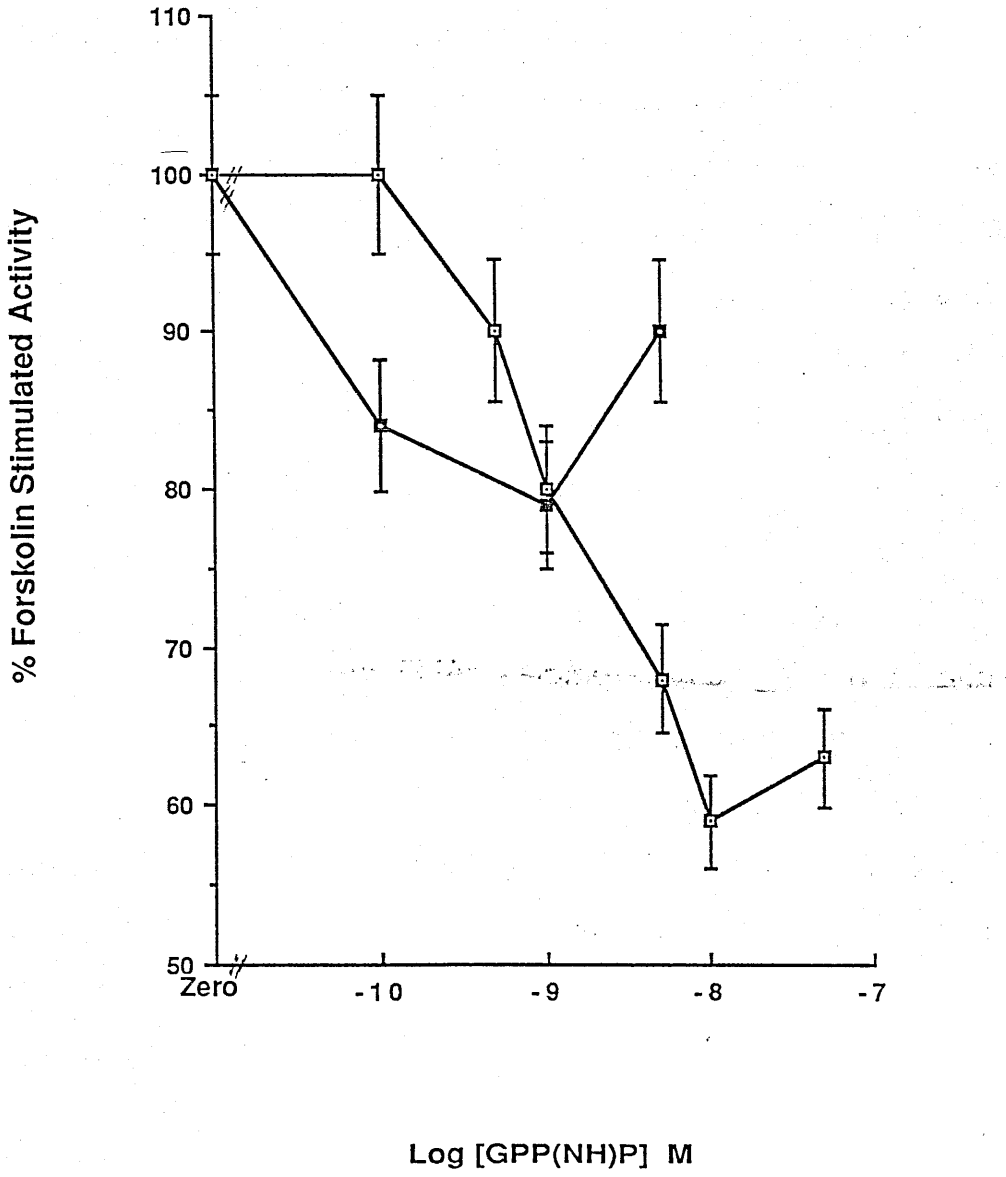


Figure 3.4.8 Effect of Biguanide Treatment On GPP(NH)P - Dependent Inhibition of Forskolin Stimulated Adenylate Cyclase Activity In Hepatocytes Isolated From Obese Zucker Rats

Crude membrane pellets were prepared from hepatocytes isolated from obese Zucker rats which (■)had or(□) had not undergone metformin therapy.

Adenylate cyclase activity was assayed in the presence of forskolin (10^{-4} M) and increasing concentrations of 5'- guanylyl-imidodiphospate (GPP(NH)P). Activity is expressed as a percentage of forskolin stimulated adenylate cyclase activity. All activities are expressed as mean +/_ S.E.M. values (n= 5).

Basal adenylate cyclase activities were 1.00 +/_ 0.10 and 1.24 +/_ 0.09 pmoles cAMP produced min^{-1} mg^{-1} for treated and untreated animals respectively. Similarly, forskolin stimulated activities were 15.00 +/_ 2.01 and 18.30 +/_ 2.01 pmoles cAMP produced min^{-1} mg^{-1} respectively.

Figure 3.4.8 Effect of Biguanide Treatment On GPP(NH)P Mediated Inhibition of Adenylate Cyclase Activity In Hepatocytes Isolated From Obese Zucker Rats

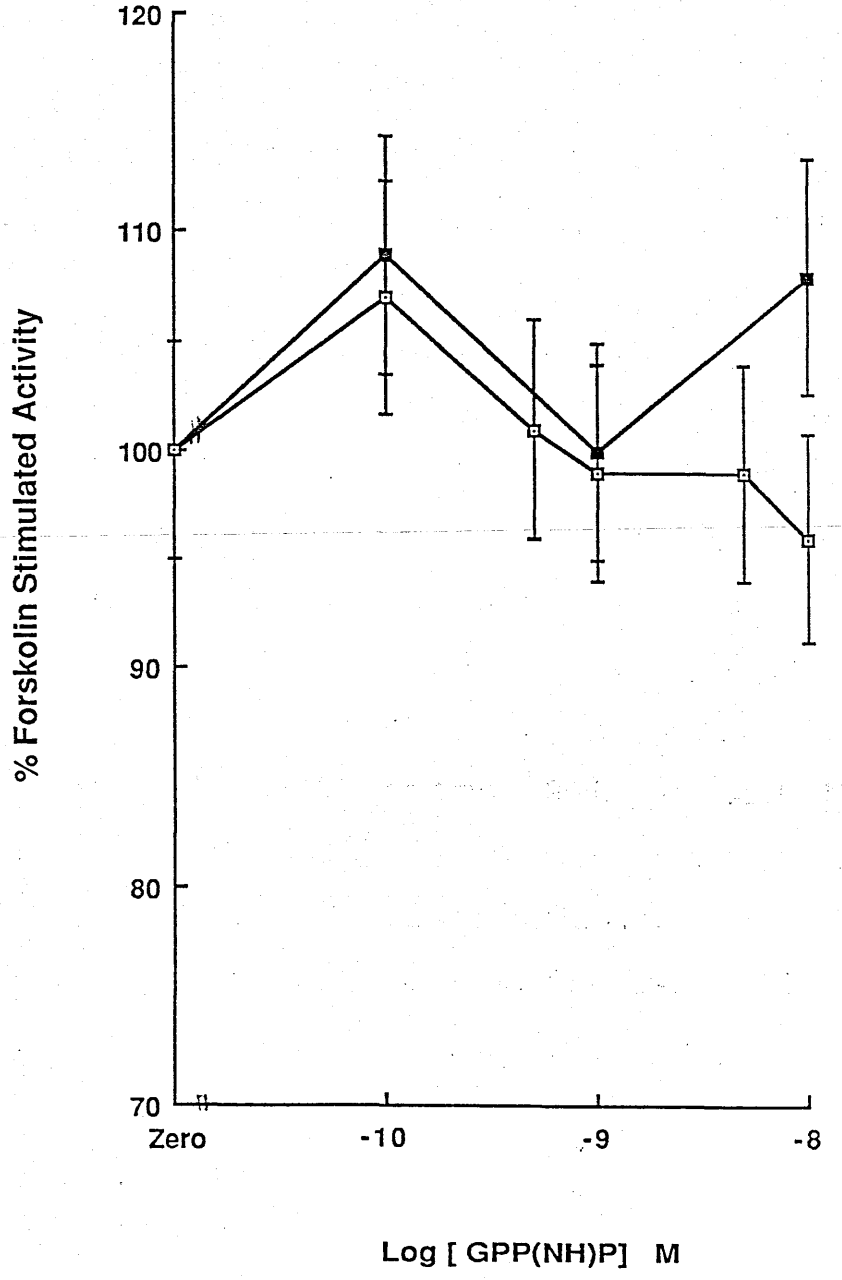


Figure 3.4.9 Effect of Biguanide Therapy On GPP(NH)P Mediated Stimulation of Adenylate Cyclase Activity In Hepatocytes Isolated From Control Rats

Hepatocytes were isolated from (■) Metformin treated and (▨) non treated Sprague Dawley rats.
Crude membrane pellets were prepared and adenylate cyclase activity assayed in the presence of forskolin (10^{-4} M) and increasing concentrations of 5'-guanylyl-imdodiphosphate (GPP(NH)P).
Activity is expressed as a percentage of forskolin stimulated activity in the absence of GPP(NH)P. All activities are given as mean +/_ S.E.M. values (n= 6).
Basal adenylate cyclase activities for metformin treated and untreated animal preparations were 1.02 +/_ 0.15 and 1.30 +/_ 0.10 pmoles cAMP produced min^{-1} mg^{-1} respectively. Similarly, forskolin stimulated activities were 17.34 +/_ 2.00 and 18.20 +/_ 2.00 pmoles cAMP produced min^{-1} mg^{-1} respectively.

Figure 3.4.9 Effect of Biguanide Therapy On GPP(NH)P Mediated Stimulation of Adenylate Cyclase Activity In Hepatocytes Isolated From Control Rats

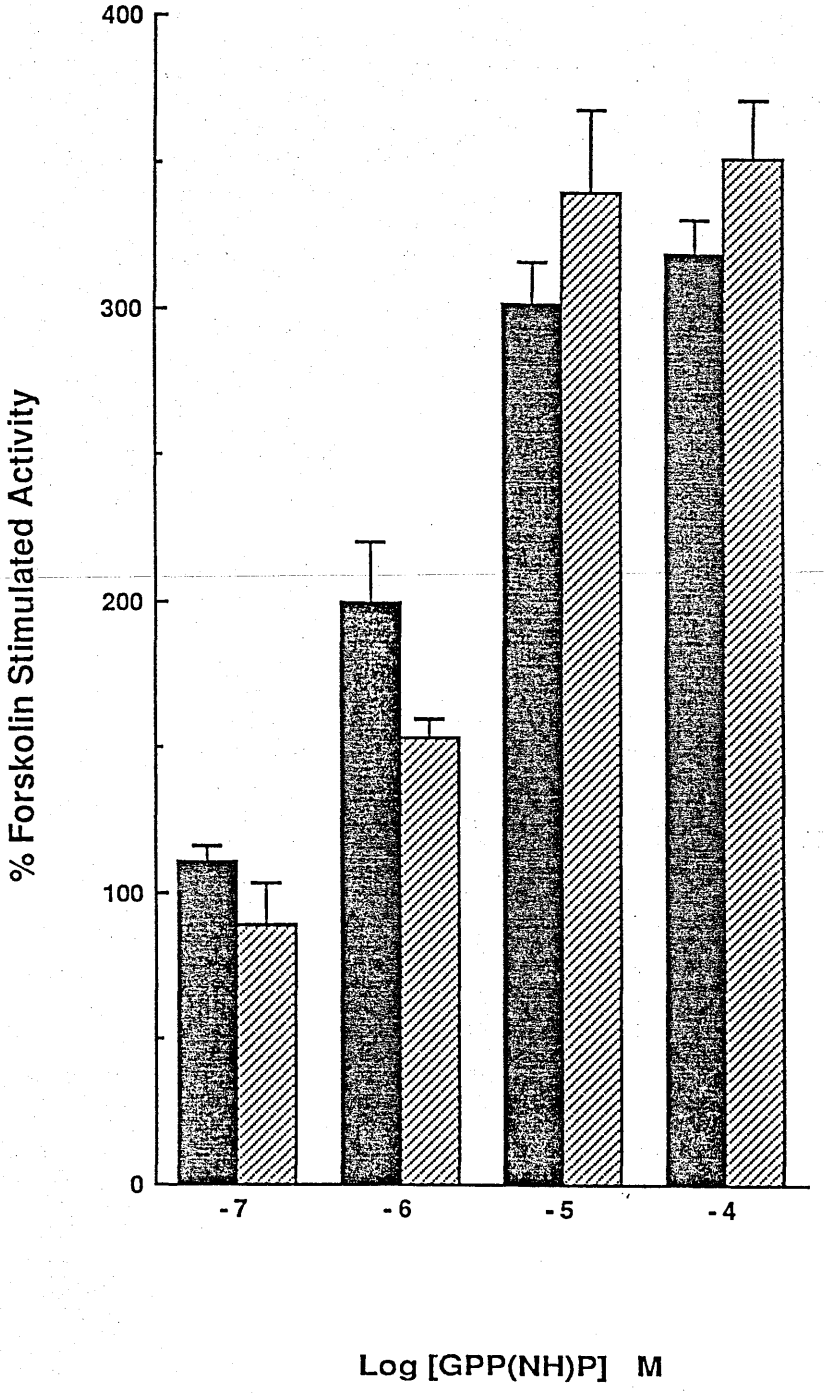


Figure 3.4.10 Effect of Biguanide Therapy On GPP(NH)P Mediated Stimulation of Adenylate Cyclase Activity In Hepatocytes Isolated From Streptozotocin Induced Diabetic Rats

Hepatocytes were isolated from (⊞) Metformin treated and (⊟) non treated diabetic rats.

Crude membrane pellets were prepared and adenylate cyclase activity assayed in the presence of forskolin (10^{-4} M) and increasing concentrations of 5'-guanylyl-imdodiphosphate (GPP(NH)P).

Activity is expressed as a percentage of forskolin stimulated activity in the absence of GPP(NH)P. All activities are given as mean +/_ S.E.M. values (n= 5).

Basal adenylate cyclase activities for metformin treated and untreated animal preparations were 0.60 +/_ 0.07 and 0.64 +/_ 0.02 pmoles cAMP produced min^{-1} mg^{-1} respectively. Similarly, forskolin stimulated activities were 10.50 +/_ 0.30 and 11.20 +/_ 1.00 pmoles cAMP produced min^{-1} mg^{-1} respectively.

Figure 3.4.10 Effect of Biguanide Therapy On GPP(NH)P Mediated Stimulation of Adenylate Cyclase Activity In Hepatocytes Isolated From Streptozotocin Induced Diabetic Rats

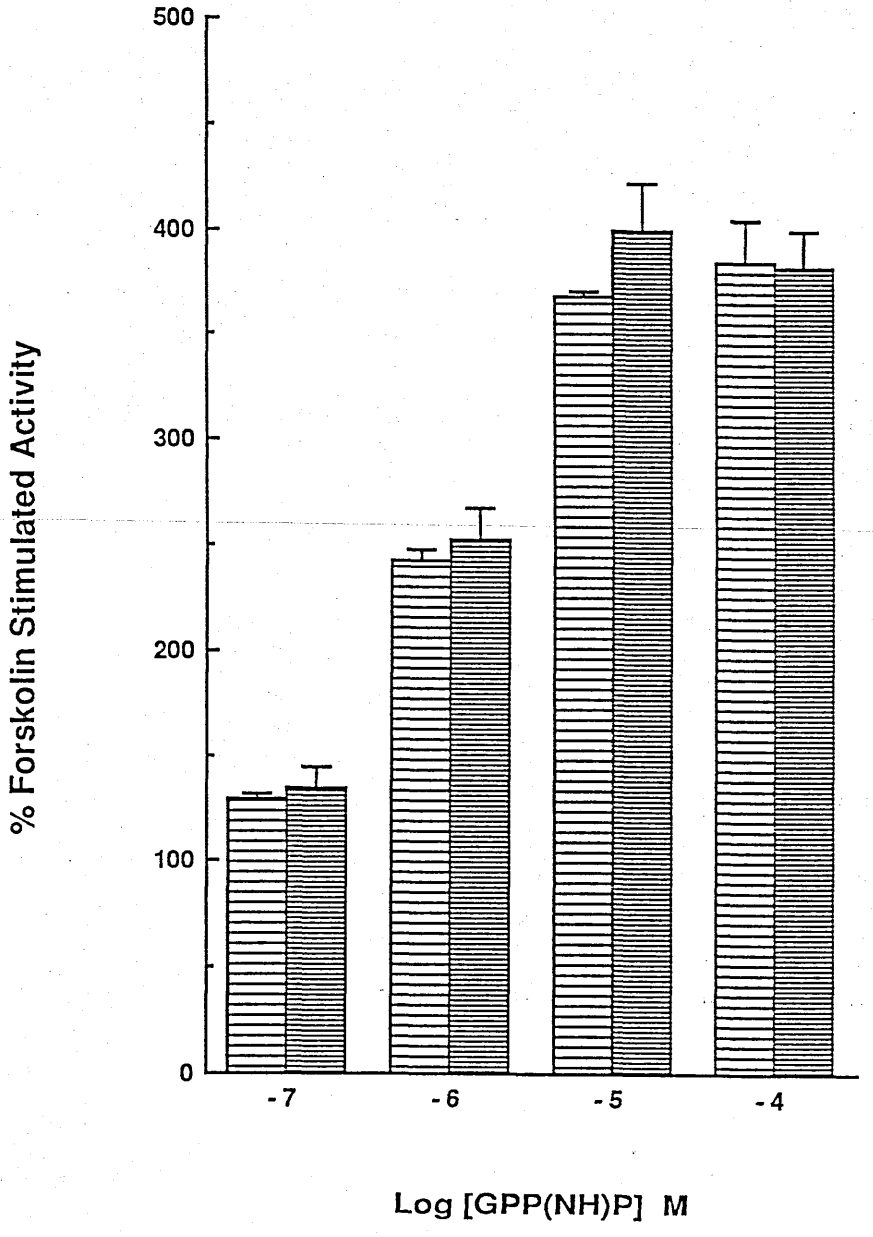


Figure 3.4.11 Effect of Biguanide Therapy On GPP(NH)P Mediated Stimulation of Adenylate Cyclase Activity In Hepatocytes Isolated From Lean Zucker Rats

Hepatocytes were isolated from (■) Metformin treated and (□) non treated lean Zucker rats.

Crude membrane pellets were prepared and adenylate cyclase activity assayed in the presence of forskolin (10^{-4} M) and increasing concentrations of 5'-guanylyl-imdodiphosphate (GPP(NH)P).

Activity is expressed as a percentage of forskolin stimulated activity in the absence of GPP(NH)P. All activities are given as mean +/_ S.E.M. values (n= 4).

Basal adenylate cyclase activities for metformin treated and untreated animal preparations were 1.23 +/_ 0.10 and 1.14 +/_ 0.02 pmoles cAMP produced min^{-1} mg^{-1} respectively. Similarly, forskolin stimulated activities were 19.68 +/_ 2.10 and 17.10 +/_ 1.90 pmoles cAMP produced min^{-1} mg^{-1} respectively.

Figure 3.4.11 Effect of Biguanide Therapy On GPP(NH)P Mediated Stimulation of Adenylate Cyclase Activity In Hepatocytes Isolated From Lean Zucker Rats

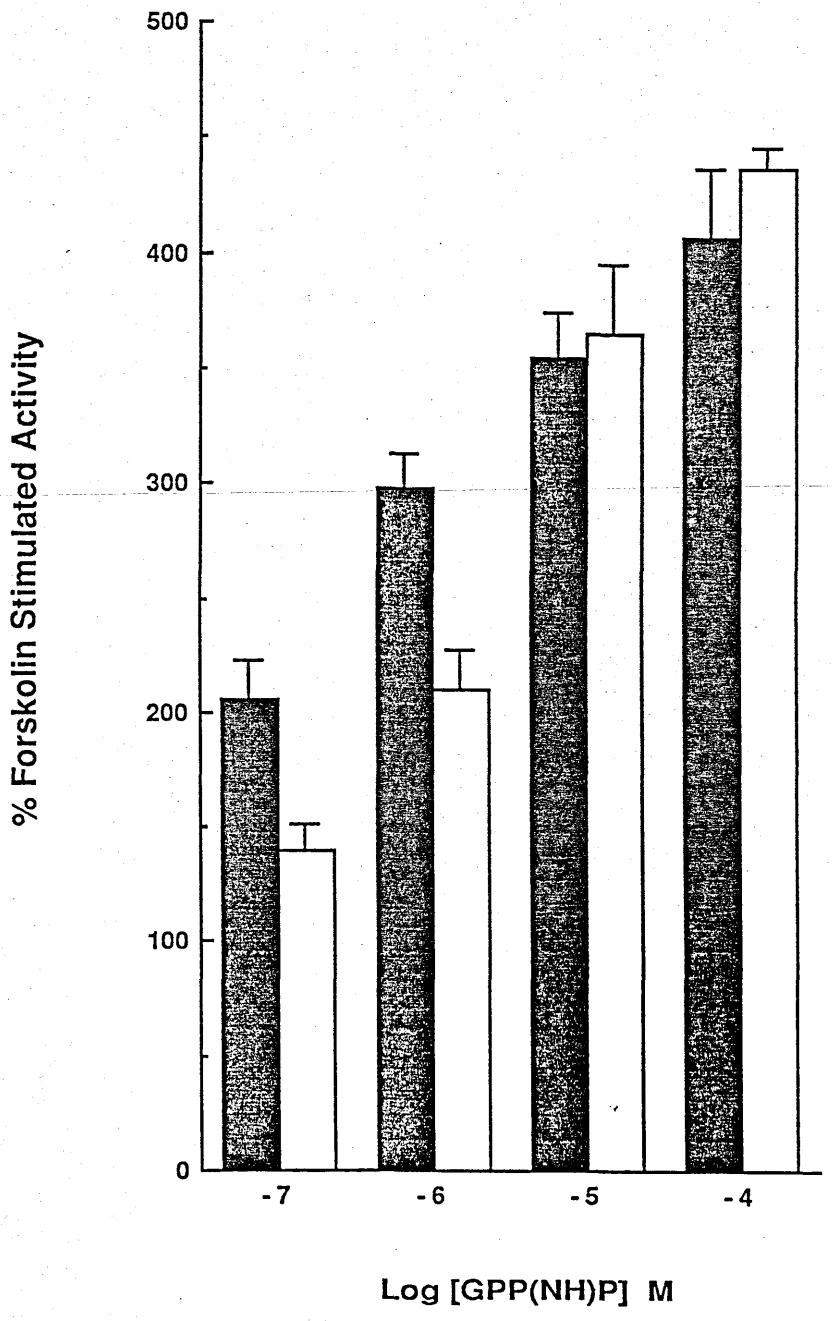


Figure 3.4.12 Effect of Biguanide Therapy On GPP(NH)P Mediated Stimulation of Adenylate Cyclase Activity In Hepatocytes Isolated From Obese Zucker Rats

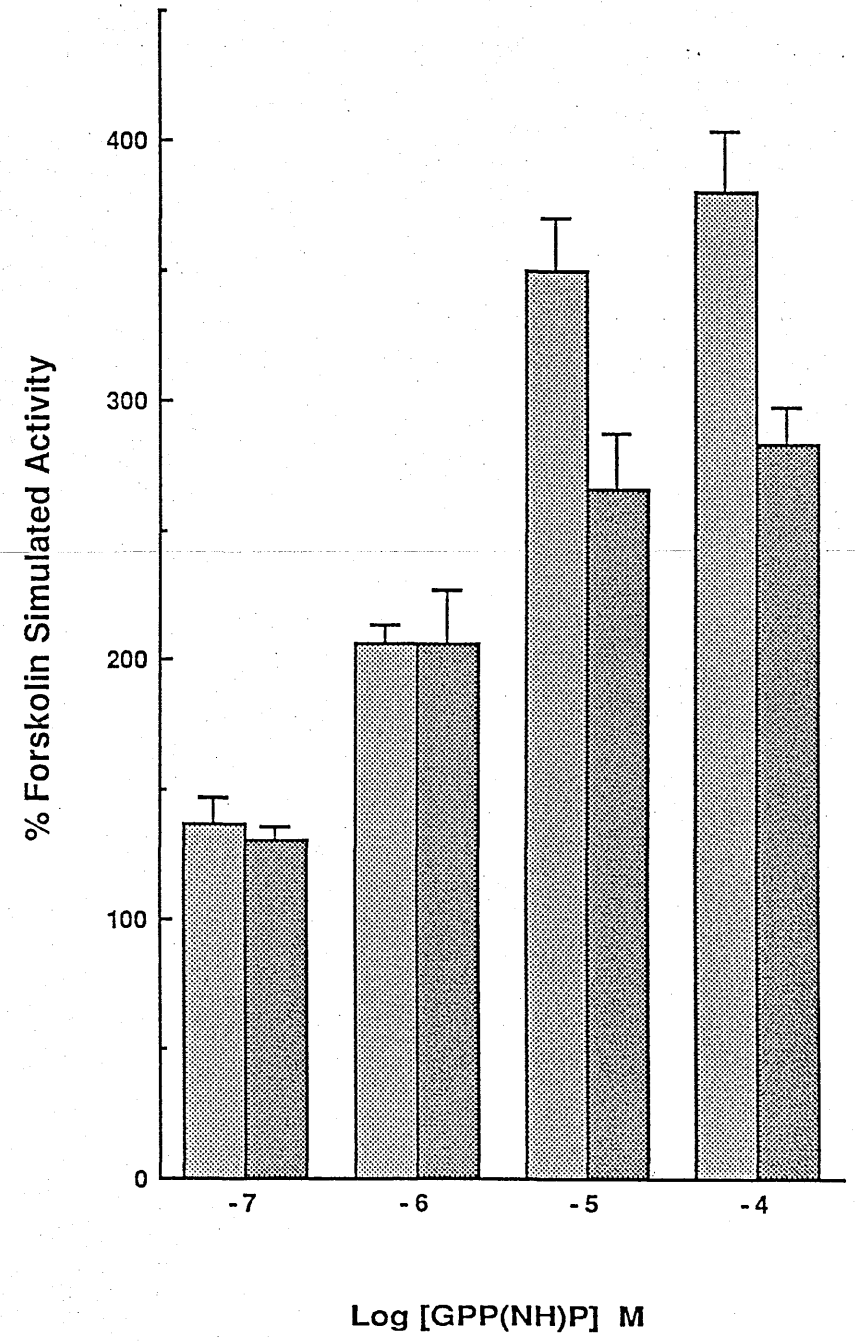
Hepatocytes were isolated from (■) Metformin treated and (□) non treated obese Zucker rats.

Crude membrane pellets were prepared and adenylate cyclase activity assayed in the presence of forskolin (10^{-4} M) and increasing concentrations of 5'-guanylyl-imdodiphosphate (GPP(NH)P).

Activity is expressed as a percentage of forskolin stimulated activity in the absence of GPP(NH)P. All activities are given as mean +/_ S.E.M. values (n= 5).

Basal adenylate cyclase activities for metformin treated and untreated animal preparations were 1.00 +/_ 0.10 and 1.24 +/_ 0.09 pmoles cAMP produced min^{-1} mg^{-1} respectively. Similarly, forskolin stimulated activities were 15.00 +/_ 2.01 and 18.30 +/_ 2.01 pmoles cAMP produced min^{-1} mg^{-1} respectively.

Figure 3.4.12 Effect of Biguanide Therapy On GPP(NH)P Mediated Stimulation of Adenylate Cyclase Activity In Hepatocytes Isolated From Obese Zucker Rats



3.5 Glucagon Stimulated Adenylate Cyclase Activity In The Presence And Absence of Pertussis Toxin Treatment

Figure 3.5.1 Effect of Streptozotocin Induced Diabetes On Glucagon Stimulation of Adenylate Cyclase Activity In Isolated Hepatocytes

Crude membrane pellets were prepared from hepatocytes isolated from (□) control and (■) streptozotocin induced diabetic Sprague Dawley rats.

Adenylate cyclase activity was assayed in the presence of GTP (10^{-4}M) and increasing concentrations of glucagon. Activity is expressed as fold stimulation over basal (GTP alone) activity and values presented are mean +/_ S.E.M. values (n=5).

Basal adenylate cyclase activities for membranes prepared from control and diabetic animals were 1.82 +/_ 0.10 and 0.90 +/_ 0.06 pmoles cAMP produced min^{-1} mg^{-1} respectively.

Figure 3.5.1 Effect of Streptozotocin Diabetes

On Glucagon Stimulation of Adenylate Cyclase Activity In

Isolated Hepatocytes

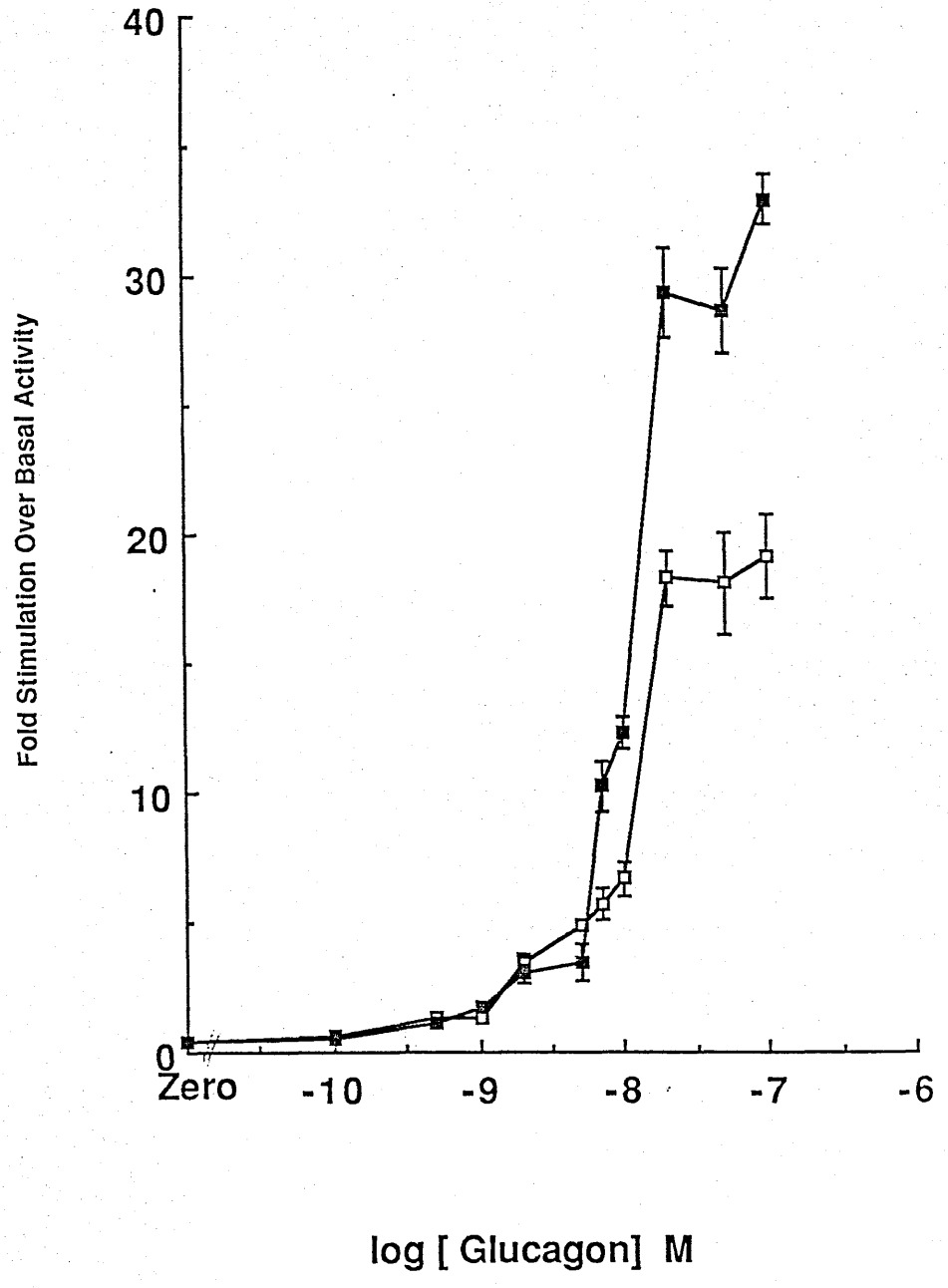


Figure 3.5.2 Effect of Diabetic State On Glucagon Stimulation of Adenylate Cyclase Activity In Isolated Hepatocytes

Crude membrane pellets were prepared from hepatocytes isolated from (□) control and (■) streptozotocin induced diabetic and (▦) insulin treated diabetic Sprague Dawley rats.

Adenylate cyclase activity was assayed in the presence of GTP (10^{-4}M) and increasing concentrations of glucagon. Activity is expressed as fold stimulation over basal (GTP alone) activity and values presented are mean +/_ S.E.M. values (n=5).

Basal adenylate cyclase activities for membranes prepared from control , diabetic and insulin treated animals were 1.82 +/_ 0.10 , 0.90 +/_ 0.06 and 0.95 +/_ 0.07 pmoles cAMP produced min^{-1} mg^{-1} respectively.

Figure 3.5.2 Effect of Diabetic State On Glucagon Stimulation of Adenylate Cyclase Activity In Isolated Hepatocytes

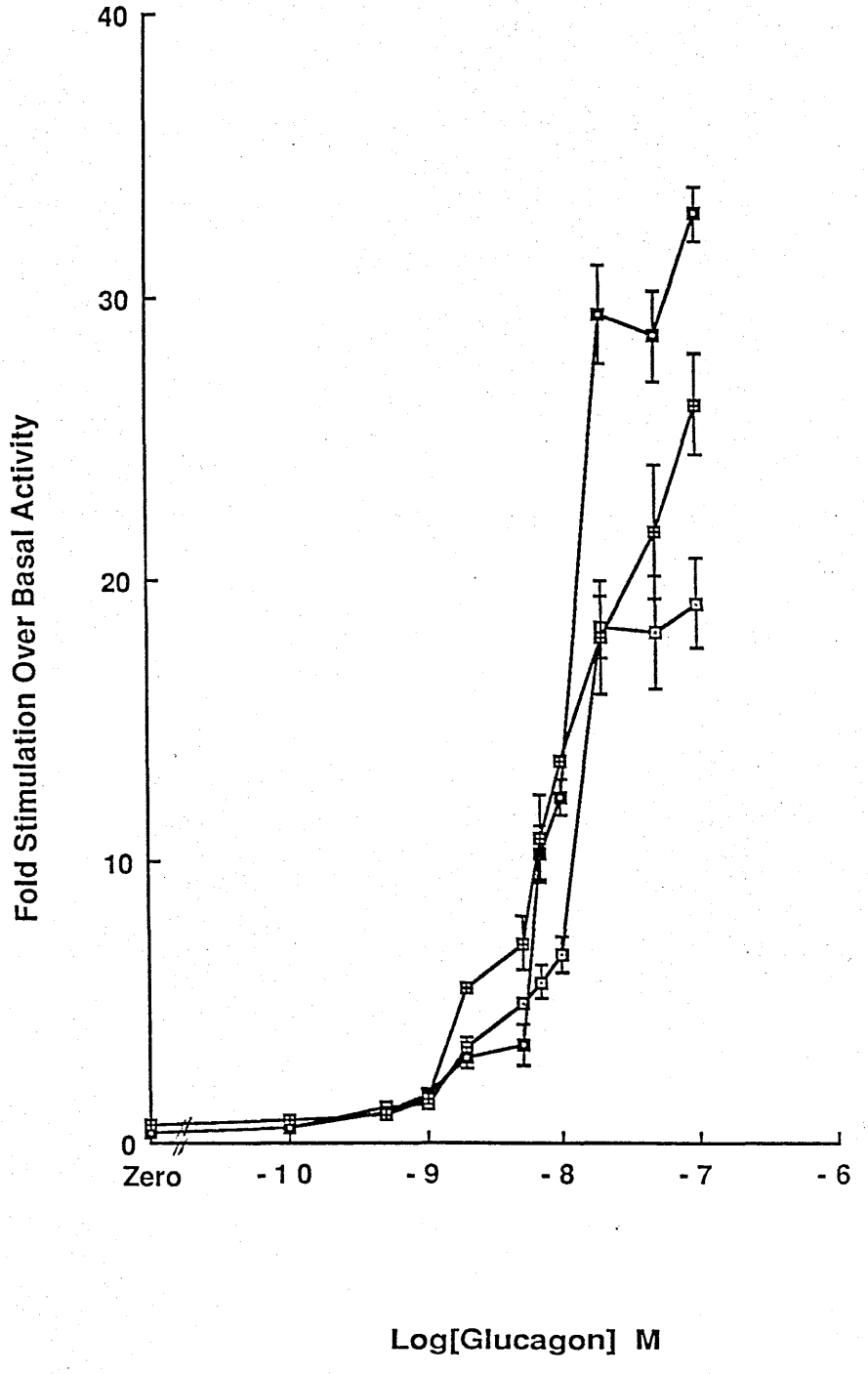


Figure 3.5.3 Effect of Pertussis Toxin On Glucagon Stimulation of Adenylate Cyclase Activity In HepatocytesIsolated From Control Rats

Crude membrane pellets were prepared from hepatocytes isolated from control Sprague Dawley rats (●) with, or (□) without pertussis toxin pretreatment.

Adenylate cyclase activity was assayed in the presence of GTP (10^{-4}M) and increasing concentrations of glucagon. Activity is expressed as fold stimulation over basal (GTP alone) activity and values presented are mean +/_ S.E.M. values (n=5).

Basal adenylate cyclase activity for membranes prepared from non treated and treated hepatocytes were 1.82 +/_ 0.10 and 1.90 +/_ 0.20 pmoles cAMP produced min^{-1} mg^{-1} respectively.

Figure 3.5.3 Effect of Pertussis Toxin On Glucagon Stimulation of Adenylate Cyclase Activity In Hepatocytes Isolated From Control Rats

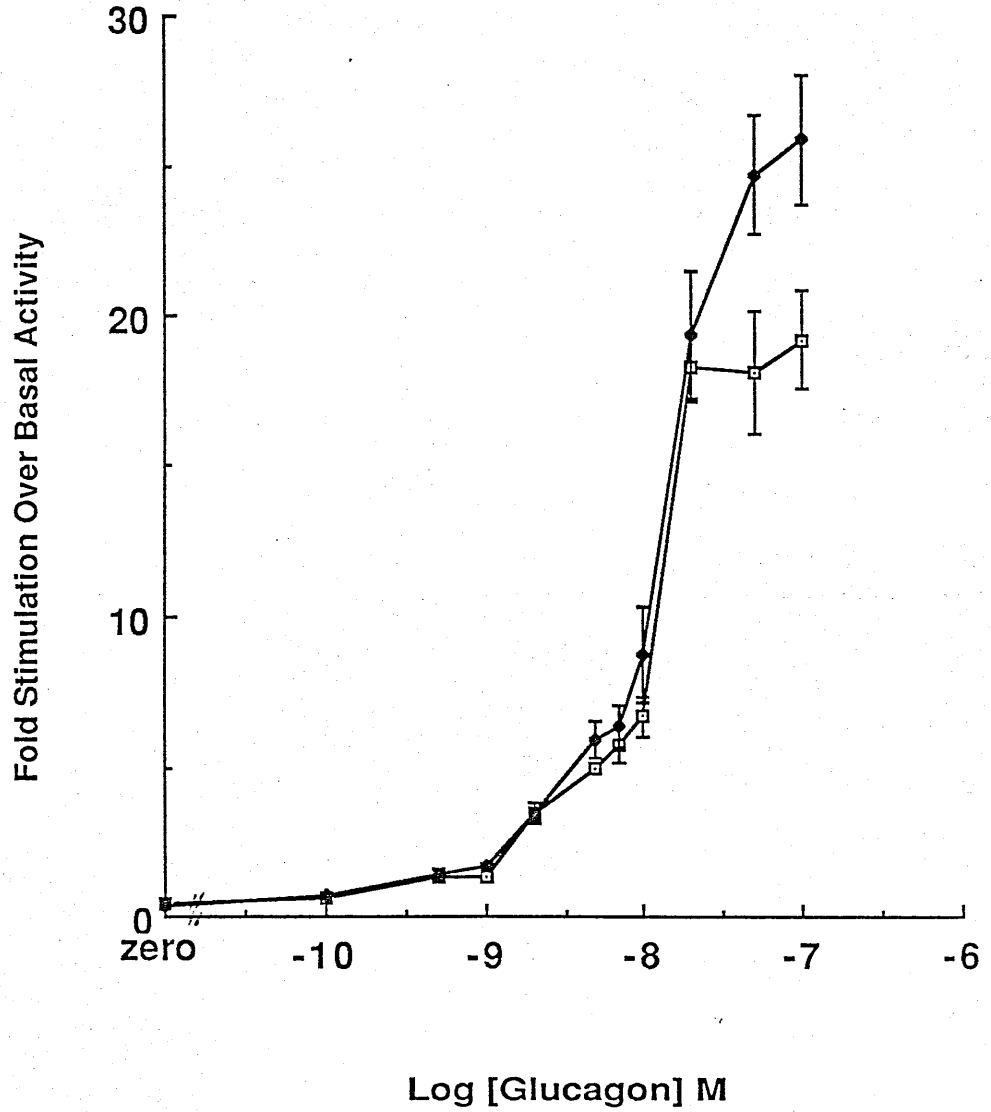


Figure 3.5.4 Effect of Pertussis Toxin On Glucagon Stimulation of Adenylate Cyclase Activity In Hepatocytes Isolated From Streptozotocin Induced Diabetic Rats

Crude membrane pellets were prepared from hepatocytes isolated from diabetic Sprague Dawley rats (●) with, or (▯) without pertussis toxin pretreatment.

Adenylate cyclase activity was assayed in the presence of GTP (10^{-4}M) and increasing concentrations of glucagon. Activity is expressed as fold stimulation over basal (GTP alone) activity and values presented are mean +/_ S.E.M. values (n=5).

Basal adenylate cyclase activity for membranes prepared from non treated and treated hepatocytes were 0.90 +/_ 0.06 and 0.95 +/_ 0.08 pmoles cAMP produced min^{-1} mg^{-1} respectively.

Figure 3.5.4 Effect of Pertussis Toxin On Glucagon

Stimulation of Adenylate Cyclase Activity In Hepatocytes

Isolated From Streptozotocin Induced Diabetic Rats

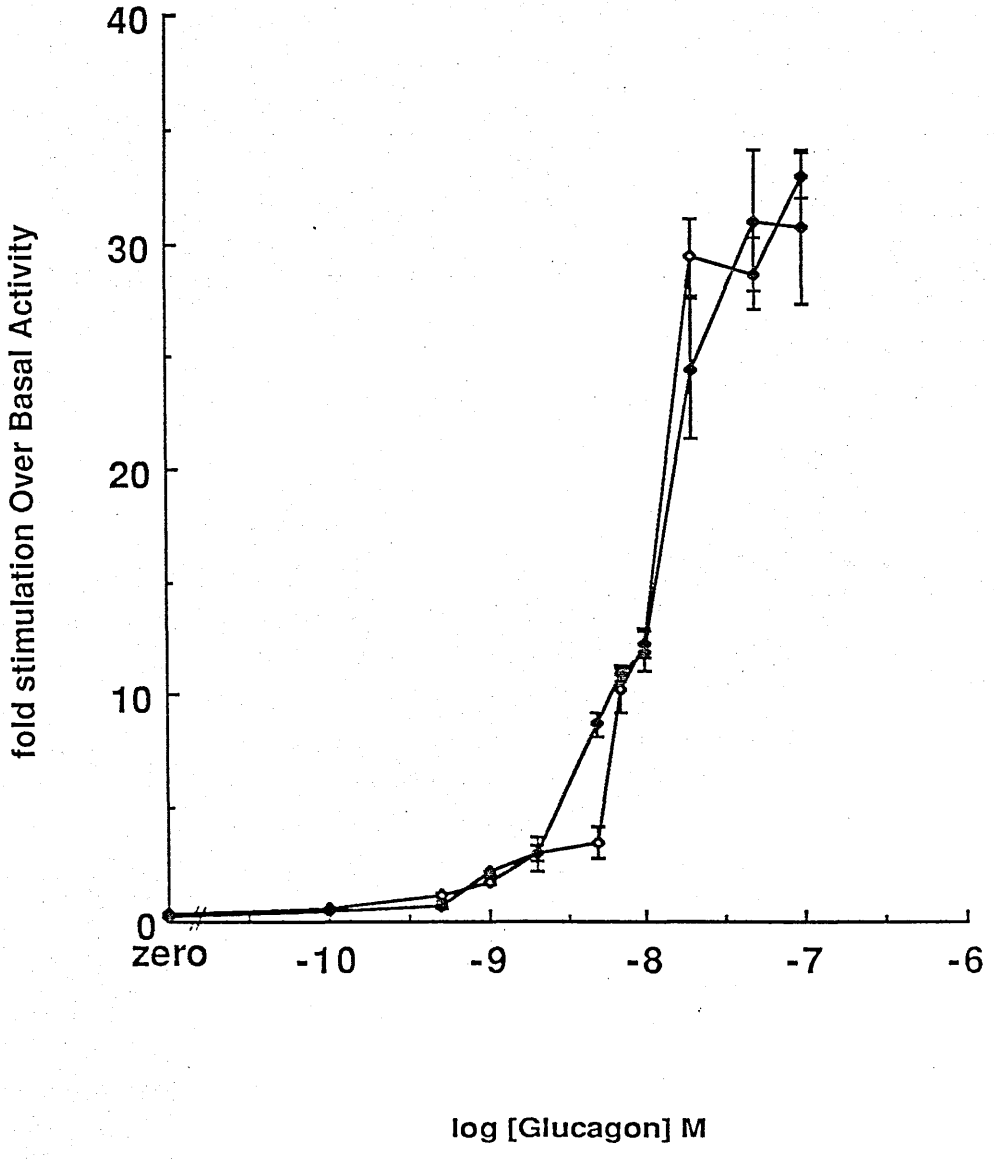


Figure 3.5.5 Glucagon Dose Dependent Stimulation of Adenylate Cyclase Activity In Hepatocytes Pretreated with Pertussis Toxin And Isolated From Streptozotocin Induced Diabetic And Control Rats

Hepatocytes were isolated from (●)diabetic and (□) controlSprague Dawley rats and pretreated with pertussis toxin. Crude membrane pellets were prepared and adenylate cyclase activity was assayed in the presence of GTP (10^{-4}M) and increasing concentrations of glucagon. Activity is expressed as fold stimulation over basal (GTP alone) activity and values presented are mean +/_ S.E.M. values (n=5).

Basal adenylate cyclase activities for pertussis toxin treated membranes prepared from control and diabetic animals were 1.90 +/_ 0.20 and 0.95 +/_ 0.08 pmoles cAMP produced min^{-1} mg^{-1} respectively.

Figure 3.5.5 Glucagon Dose Dependent Stimulation of Adenylate Cyclase Activity In Hepatocytes Pretreated With Pertussis Toxin And Isolated From Streptozotocin Induced Diabetic And Control Rats

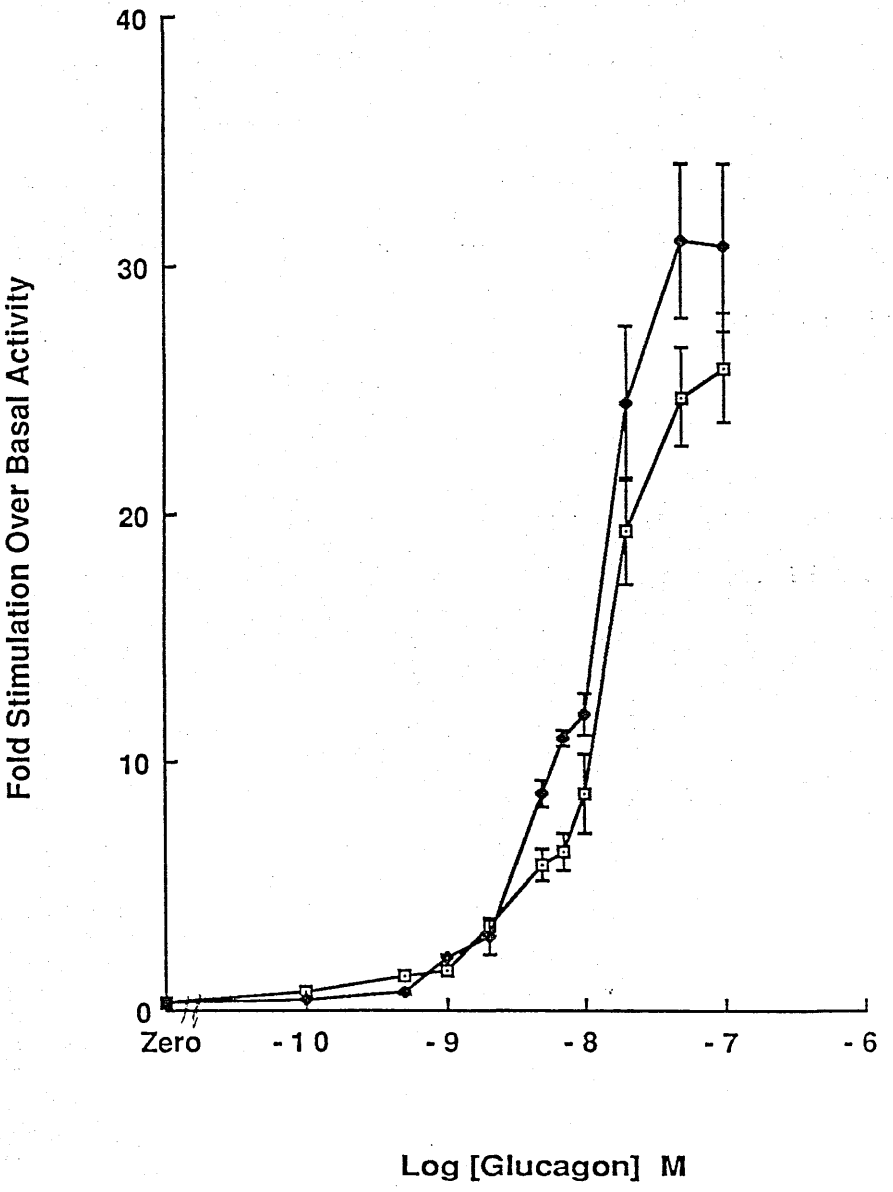


Figure 3.5.6 Effect of Pertussis Toxin Treatment On Glucagon Stimulation of Adenylate Cyclase Activity In Hepatocytes Isolated From Diabetic, Insulin Treated Diabetic And Control Rats

Hepatocytes were isolated from (■)diabetic , (o) insulin treated diabetic and (□) controlSprague Dawley rats These hepatocytes were pretreated with pertussis toxin and crude membrane pellets were prepared . Adenylate cyclase activity was assayed in the presence of GTP (10^{-4}M) and increasing concentrations of glucagon. Activity is expressed as fold stimulation over basal (GTP alone) activity and values presented are mean +/_ S.E.M. values (n=5).

Basal adenylate cyclase activities for pertussis toxin treated membranes prepared from control diabetic and insulin treated diabetic animals were 1.90 +/_ 0.20 , 0.95 +/_ 0.08 and 1.00 +/_ 0.08 pmoles cAMP produced min^{-1} mg^{-1} respectively.

Figure 3.5.6 Effect of Pertussis Toxin Treatment On Glucagon Stimulation of Adenylate Cyclase Activity In Hepatocytes Isolated From Diabetic, Insulin Treated Diabetic And Control Rats

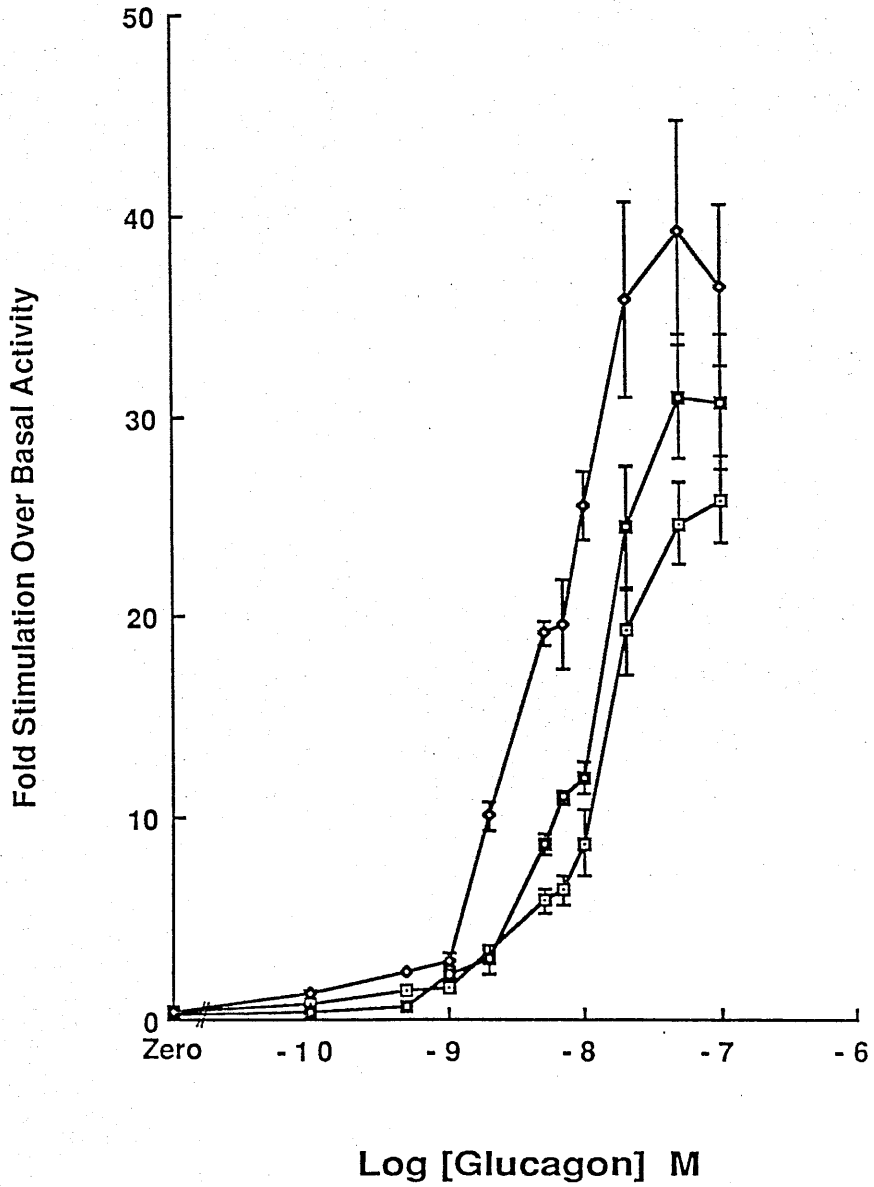


Figure 3.5.7 Glucagon Stimulation of Adenylate Cyclase Activity In Hepatocytes Isolated From Lean And obese Zucker Rats

Hepatocytes were isolated from (○)lean and (●) obese Zucker rats Crude membrane pellets were prepared and adenylate cyclase activity was assayed in the presence of GTP (10^{-4}M) and increasing concentrations of glucagon. Activity is expressed as fold stimulation over basal (GTP alone) activity and values presented are mean +/_ S.E.M. values (n=5).

Basal adenylate cyclase activities for membranes prepared from lean and obese animals were 1.60 +/_ 0.12 and 1.74 +/_ 0.20 pmoles cAMP produced min^{-1} mg^{-1} respectively.

Figure 3.5.7 Glucagon Stimulation of Adenylate Cyclase Activity In Hepatocytes Isolated From Lean And Obese Zucker Rats

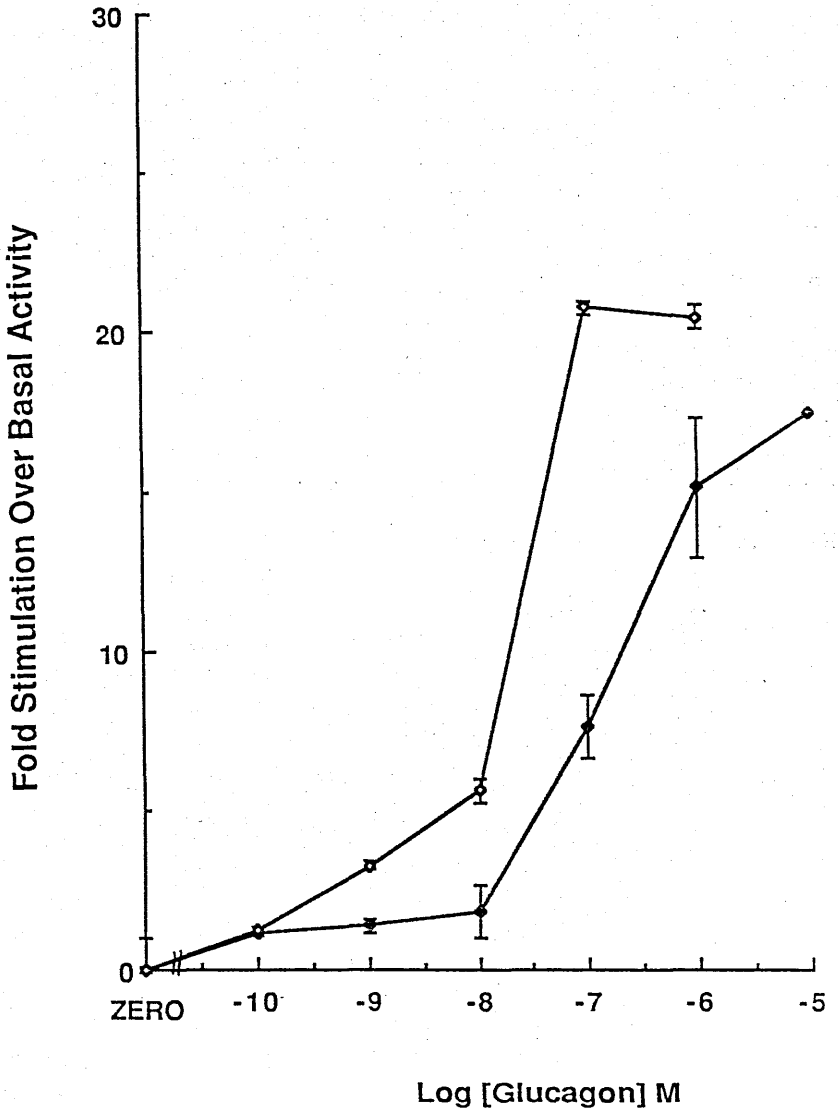


Figure 3.5.8 Effect of Pertussis Toxin On Glucagon Stimulation of Adenylate Cyclase Activity In Hepatocytes Isolated From Lean Zucker Rats

Crude membrane pellets were prepared from hepatocytes and pretreated (●)with or (o) without pertussis toxin. Adenylate cyclase activity was assayed in the presence of GTP (10^{-4}M) and increasing concentrations of glucagon. Activity is expressed as fold stimulation over basal (GTP alone) activity and values presented are mean +/_ S.E.M. values (n=5).

Basal adenylate cyclase activities for membranes prepared from pertussis toxin treated and untreated hepatocytes were 1.64 +/_ 0.04 and 1.60 +/_ 0.12 pmoles cAMP produced min^{-1} mg^{-1} respectively.

Figure 3.5.8 Effect of Pertussis Toxin on Glucagon

Stimulation of Adenylate Cyclase Activity In Hepatocytes Isolated

From Lean Zucker Rats

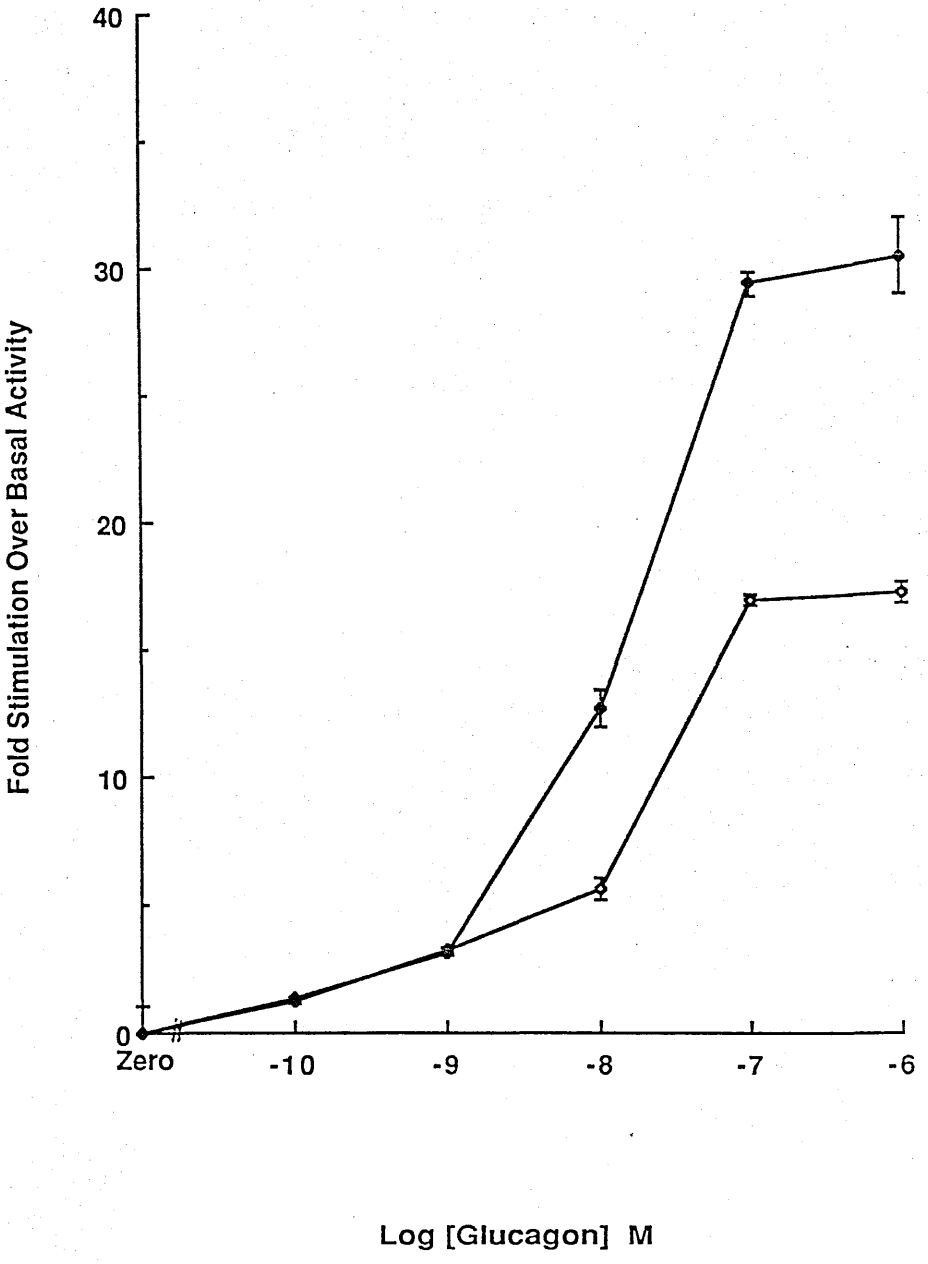


Figure 3.5.9 Effect of Pertussis Toxin On Glucagon Stimulation of Adenylate Cyclase Activity In Hepatocytes Isolated From Obese Zucker Rats

Crude membrane pellets were prepared from hepatocytes and pretreated (▲)with or (△) without pertussis toxin. Adenylate cyclase activity was assayed in the presence of GTP (10^{-4}M) and increasing concentrations of glucagon. Activity is expressed as fold stimulation over basal (GTP alone) activity and values presented are mean +/_ S.E.M. values (n=5).

Basal adenylate cyclase activities for membranes prepared from pertussis toxin treated and untreated hepatocytes were 1.80 +/_ 0.12 and 1.74 +/_ 0.20 pmoles cAMP produced min^{-1} mg^{-1} respectively.

Figure 3.5.9 Effect of Pertussis Toxin On Glucagon Stimulation of Adenylate Cyclase Activity In Hepatocytes Isolated From Obese Zucker Rats

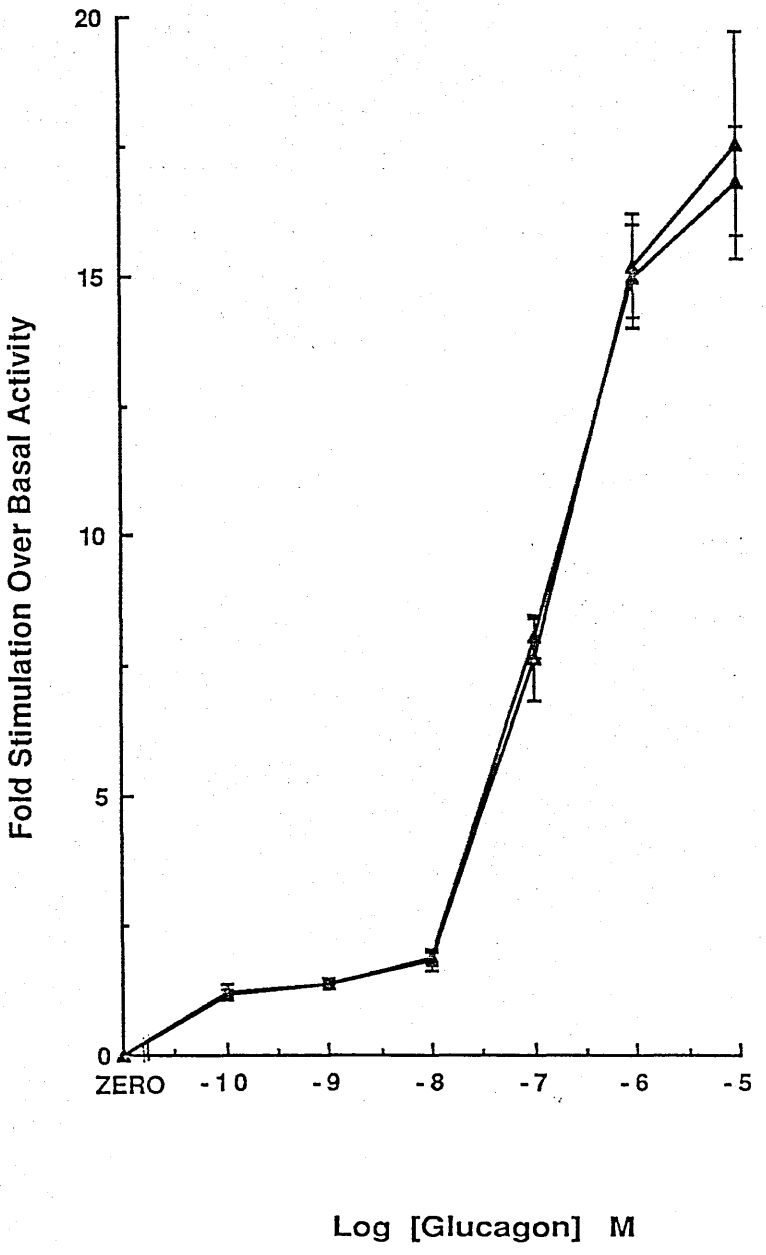
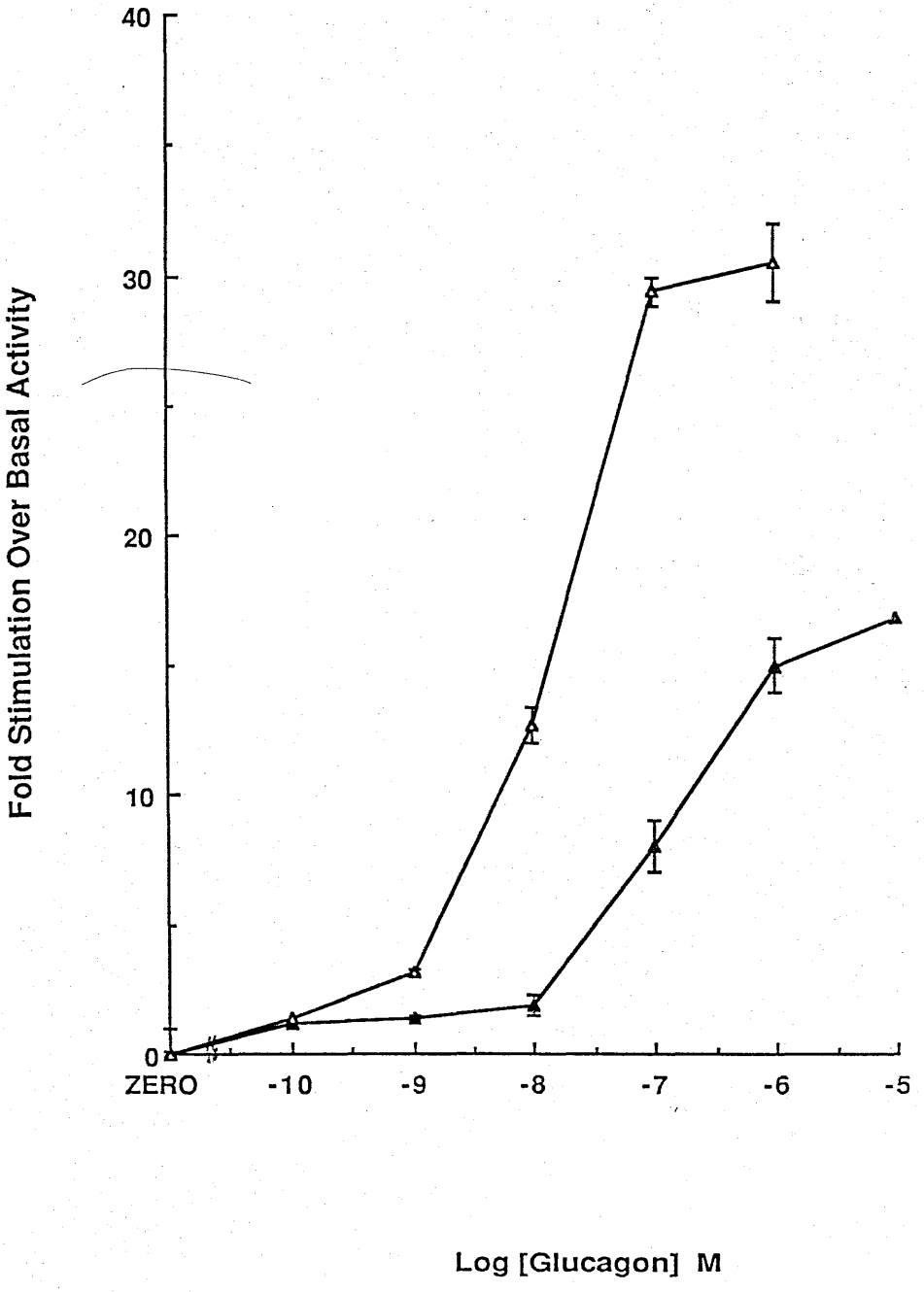


Figure 3.5.10 Glucagon Stimulation of Adenylate Cyclase Activity In Hepatocytes Isolated From Zucker Rats And Pretreated With Pertussis Toxin

Hepatocytes were isolated from (△)lean and (▲) obese Zucker rats . Hepatocytes were pretreated with pertussis toxin and crude membrane pellets were prepared . Adenylate cyclase activity was assayed in the presence of GTP (10^{-4}M) and increasing concentrations of glucagon. Activity is expressed as fold stimulation over basal (GTP alone) activity and values presented are mean +/_ S.E.M. values (n=5).

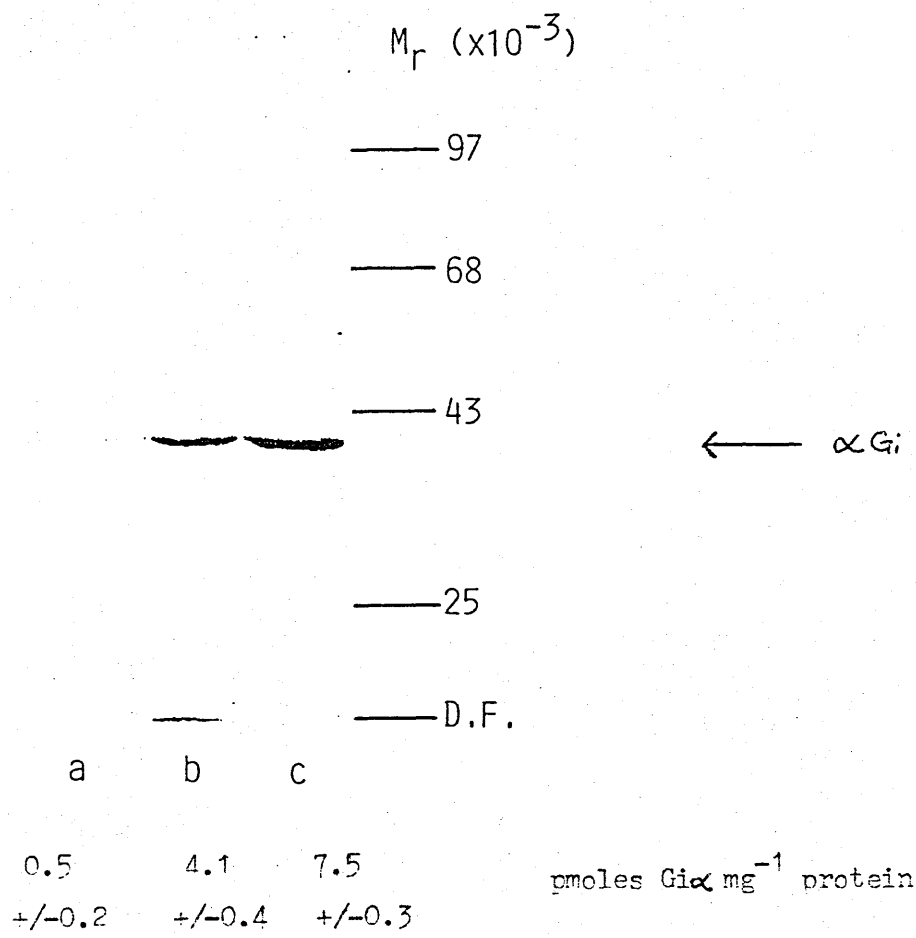
Basal adenylate cyclase activities for membranes prepared from lean and obese animals after pertussis toxin treatment were 1.64 +/_ 0.14 and 1.80 +/_ 0.12 pmoles cAMP produced min^{-1} mg^{-1} respectively.

Figure 3.5.10 Glucagon Stimulation of Adenylate Cyclase Activity In Hepatocytes Isolated From Zucker Rats And Pretreated With Pertussis Toxin



3.6 Gi Quantification In Control And Diabetic States

Figure 3.6.1 Quantification of Giα In Type I Diabetic, Insulin Treated And Control Hepatocyte Membrane Preparations

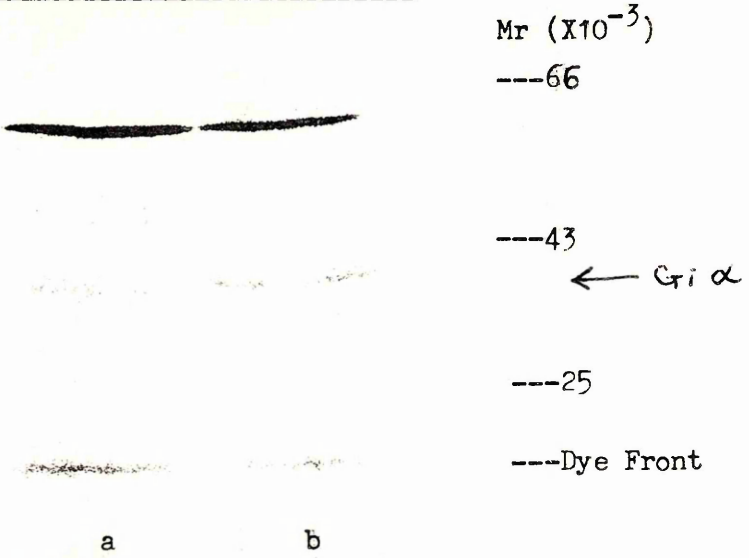


Track a = Streptozotocin diabetic plasma membranes

Track b = Insulin treated streptozotocin diabetic plasma membranes

Track c = control plasma membranes

Figure 3.6.2 Quantification of Gi In Hepatocyte Membranes Prepared From Lean & Obese Zucker Rats



Track a = Lean Zucker rat preparations

Track b = Obese Zucker rat preparations

The identity of the higher molecular weight band (approx.60KDa) is unknown , but as the antibody used to recognize Giα was affinity purified prior to use, it is believed to be a form of Gi α present in these rat preparations, but this form of Giα was not apparant in the Sprague Dawley rat species.

3.7 Characterization of Gs Function

Table 2 Comparison of Hepatic Gs Function In Control And Diabetic States

Membrane Source	NaF(15mM) (Fold Stimulation)	Forskolin(0.1mM) (Fold Stimulation)	GTP (0.1mM) (Fold Stimulation)	Glucagon (0.1uM) (Fold Stimulation)	Basal Specific Activity (pmoles/min/mg)
Control					
Sprague	14.47+/-1.65	14.00+/-1.00	1.45+/-0.20	18.00 +/- 1.20	1.30 +/- 0.09
Streptozotocin					
Diabetic	14.10+/-1.00	14.62+/-1.50	1.40+/-0.13	30.00+/-2.00	0.64 +/- 0.02
Alloxan					
Diabetic	14.70+/-2.00	15.00+/-1.30	1.50+/-0.20	28.70 +/- 3.00	0.60 +/- 0.08
Streptozotocin					
& Insulin					
Treated	15.00+/-2.00	16.03+/-1.43	1.43+/-0.18	26.00 +/- 2.00	0.68 +/- 0.04
Lean					
Zucker	14.50+/-1.20	15.00+/-1.22	1.40+/-0.15	17.00 +/- 1.00	1.14 +/- 0.02
Obese					
Zucker	14.23+/-1.50	14.82+/-1.65	1.36+/-0.12	17.30 +/- 1.05	1.24 +/- 0.09

Table 3 Comparison of Hepatic Gs Function In Metformin Treated Control And Diabetic Rats

Membrane Source	NaF(15mM) (Fold Stimulation)	Forskolin (0.1mM) (Fold Stimulation)	GTP (0.1mM) (Fold Stimulation)	Basal (Specific Activity) pmoles/min/mg
Control Sprague	12.08 +/-1.06	17.00 +/- 2.00	1.23 +/- 0.10	1.02 +/- 0.15
Streptozotocin Diabetic	14.17 +/-1.50	16.54 +/-1.90	1.30 +/- 0.20	0.60 +/- 0.07
Alloxan Diabetic	14.36 +/-1.80	15.50 +/-2.00	1.35 +/- 0.10	0.62 +/- 0.09
Lean Zucker	15.33 +/-1.10	16.00 +/-1.20	1.38 +/- 0.09	1.23 +/- 0.10
Obese Zucker	15.00 +/- 1.60	15.00 +/-1.35	1.40 +/- 0.08	1.00 +/- 0.10

Discussion of Results

The non-hydrolyzable analogue, GPP(NH)P, has been used to demonstrate specific activation of two distinct G proteins which characteristically regulate adenylate cyclase activity. These two G proteins; Gi and Gs, which inhibit and stimulate adenylate cyclase activity respectively, are selectively activated by the nucleotide as a result of their differing affinities to bind the nucleotide.

Characteristically by sequentially activating each G protein , biphasic GPP(NH)P dose response curves are obtained (see figures 3.1.1 - 3.1.5). When each G protein activation "phase" is separated out , it can be clearly observed that Gi activation is abolished in the chemically-induced type I diabetic model. This abolition of Gi function occurred when two different drugs; streptozotocin (figure 3.2.2) and alloxan (figure 3.2.3) were used independently to destroy β cell function. This suggests that the diabetic condition rather than the drugs themselves are causing Gi function to be lost. Indeed, when insulin therapy was administered to streptozotocin induced diabetic rats, Gi function was restored in hepatic membrane preparations. However, the maximum inhibition of adenylate cyclase activity achieved under these conditions was reduced from 42% (controls) to 27% (insulin treated diabetic preparations).

This may be due to the rather crude experimental procedure adopted to reverse the diabetic condition by insulin administration. Insulin was administered by daily intramuscular injection of 10I.U. of an insulin-zinc suspension to each 250g rat. This dose of insulin appeared to normalize blood glucose levels to those found in control animals (4.6 +/- 0.6 mM) and glycosuria was not observed within 24-48 h after initial insulin administration. However, it is possible that this dose of insulin could have been rather high when compared to other workers (Chatzipanteli & Saggerson 1983). Although the same maximal Gi activity was not observed, the concentration of GPP(NH)P which elicited 1/2 maximal inhibition (ie. 1/2 maximal Gi activation) was comparable in the reversed diabetic state and controls. This 1/2 maximal Gi activation is estimated to occur with a GPP(NH)P concentration of 0.5nM ,compared with 0.1nM for controls. It would appear therefore that the affinity for GPP(NH)P binding to Gi was not significantly altered.Therefore, the suggestion that the reduced maximal Gi function may be reflective of reduced levels of Gi protein itself rather than changes in the nucleotide binding affinity may be proposed .

When the Gi function profiles for lean and obese Zucker rats are examined (see figures 3.2.6 - 3.2.8) it is clear that the same trends are observed. In lean (control) Zucker rat membranes GPP(NH)P activates Gi to the same extent (40 %) with a comparable 1/2 maximal concentration of 1nM. However, in the type II diabetic model (the obese Zucker rat) , Gi function

is once again abolished. These obese Zucker rats are characteristically hyperinsulinaemic (see section 1. 1.4) . Therefore this Gi function abolition cannot be due to insulin deficiency as proposed in the type I diabetic model above.

In summary therefore, in both models of type I and type II diabetes the activation of hepatic Gi mediated by low concentrations of the guanine nucleotide GPP(NH)P is abolished. Insulin administration to chemically induced (type I) diabetic rats partially restores hepatic Gi function. Preliminary evidence suggested that insulin deficiency in type I diabetic animals reduced the levels of Gi protein in hepatic plasma membranes.

Hepatic Gi function in the type II diabetic model (the obese Zucker rat) was also absent. The control for this diabetic model (the lean Zucker rat) exhibited a slightly higher KO.5 value fo Gi function , but a maximal activity value which is comparable to those obtained in control Sprague rats. It is therefore suggested that the mechanism by which hepatic Gi activity is abolished in these two diabetic models may be different. In the type I diabetic model, insulin deficiency prevails. Reversal of this condition significantly reverses the Gi function loss. In the type II diabetic model hyperinsulinaemia is characteristic.

Using high concentrations of GPP(NH)P it was possible to activate the G protein Gs. As Gs activation of adenylate cyclase is synergistic with forskolin stimulation of adenylate cyclase (Seamon & Daly 1986) it is possible to observe activation of this G protein in the presence of this diterpene.

Figure 3.3.1 demonstrates that inducement of the type I diabetic state using the compound streptozotocin had no significant effect upon Gs activation by high concentrations of the nucleotide. Similarly using this technique there appeared to be no significant difference between hepatic Gs function in lean and obese (type II diabetic model) Zucker rats. It is therefore proposed that hepatic Gs function in both models of diabetes remains unaltered. This observation is very encouraging because it suggests that alterations in G protein function in these diabetic models are specific and not necessarily reflective of a wholely dysfunctional adenylate cyclase system.

Throughout my studies with type I diabetic animal preparations, it was very noticeable that the basal (resting) adenylate cyclase activities were routinely about half the value of those for control animals. Similarly the forskolin stimulated activities were also about half the level in diabetic (type I) animal preparations than those in controls. The reason for this is unknown, but I would suggest that this could be a reflection of a reduction in the absolute amount of the catalytic unit in the insulin deficient state. For this reason all data in this chapter is expressed as a percentage of the basal or forskolin stimulated states. The fold stimulation of adenylate cyclase activity over the basal activity for control and type I diabetic states show no significant differences in the presence of forskolin (see table 2). Therefore as Gs activation and the

forskolin fold stimulation of adenylate cyclase activities are comparable in each system, it is suggested that activities of Gs and C (the catalytic unit) remain unaltered.

The type II diabetic preparations did not show reduced adenylate cyclase activities when compared with controls (the lean Zucker rat).However, once again only a Gi dysfunction is proposed.

As an "internal" control, the effect of TPA and pertussis toxin upon Gi function was investigated. Both these compounds have been shown to attenuate Gi function (Katada et al 1985; Watanbe et al 1985; Aktories et al 1983). Thus in both the Sprague and Zucker rat preparations (figures 3.2.4 and 3.2.7) these agents were observed to abolish this GPP(NH)P mediated inhibition of adenylate cyclase activity . Hence, it is believed that this apparent GPP(NH)P mediated inhibition is indeed due to the activation of Gi alone.

The effect of the hypoglycaemic drug metformin was investigated with respect to its effect upon GPP(NH)P mediated inhibition and stimulation of adenylate cyclase activity. Metformin therapy appeared to reduce the maximal inhibition of adenylate cyclase activity from 42% in control animal preparations to 20% in metformin treated animal preparations. The estimated concentration at which 1/2 maximal inhibition was attained was not significantly altered (0.1 & 0.3 nM for control and metformin treated respectively). The streptozotocin induced type I diabetic preparations (figure 3.4.6) still showed no Gi

function irrespective of metformin treatment. The type II diabetic preparations (figures 3.4.7 and 3.4.8) showed similar trends to the Sprague rat preparations. That is, the maximal inhibition observed in the metformin treated animal preparations was reduced from 45 % (in non-treated treated lean Zucker rats) to 20 % (in metformin treated lean Zucker rat preparations). However, this time the concentration of GPP(NH)P eliciting 1/2 maximal inhibition was reduced from 1nM (control preparations) to 0.1nM (metformin treated preparations). Metformin treatment did not restore Gi function in the obese Zucker (type II diabetic model) rat preparations.

Metformin therapy was also found to have no significant effect upon stimulation of adenylate cyclase activity using high concentrations of GPP(NH)P in control or diabetic type I preparations . However, this drug did reduce the maximal activation of Gs in the obese Zucker (type II diabetic model) rat preparations. The reason for this is unknown.

From this section of work it was therefore concluded that metformin did not restore hepatic Gi function in the diabetic state preparations. Indeed, in controls it reduce the maximal inhibition of adenylate cyclase activity elicited by Gi. Also, this drug did not alter Gs function in controls or type I diabetic preparations. However, the type II diabetic model preparations exhibited a reduced maximal Gs stimulation of adenylate cyclase activity.

In order to confirm the proposal of altered hepatic Gi function in the type I and II diabetic models used ,the effect of pertussis toxin pretreatment of hepatocytes was investigated. The rationale being that if hepatic Gi function is not present in these diabetic model states, then pertu ssis toxin (which attenuates Gi function) should have no appreciable effect upon the adenylate cyclase system in these preparations. As adenylate cyclase is under the dual control of Gi and Gs in "normal" systems, if Gi "input" is removed by pertussis toxin treatment, then Gs stimulation of the system should be enhanced.

Figure 3.5.1 demonstrates that maximal glucagon stimulation of adenylate cyclase activity in control Sprague Dawley rat preparations was 20 fold over basal with an estimated $K_{0.5}$ of 10nM. However, the chemically induced diabetic animal preparations exhibited a maximal glucagon stimulation of 30 fold over basal with a $K_{0.5}$ of 9nM (figure 3.5.2).

Pertussis toxin pretreatment of control hepatocytes resulted in an enhanced maximal glucagon stimulation of adenylate cyclase activity of 25 fold over basal with an estimated $K_{0.5}$ of 10nM (figure 3.5.3). However, pertussis toxin had no effect upon glucagon stimulation in the chemically induced diabetic rat hepatocyte preparations (figure 3.5.4).

Also, when pertussis toxin pretreated hepatocyte preparations from both control and type I diabetic rats are compared the glucagon dose response curves are almost superimposable (figure 3.5.5). The insulin treated diabetic rat hepatocyte preparations

displayed a slightly elevated maximal glucagon stimulation of adenylate cyclase activity when compared with controls, but less so than that observed in the type I untreated diabetic state (figure 3.5.2).

Thus, it would appear that the proposal that hepatic Gi function is abolished in the type I model of diabetes is in agreement with the pertussis toxin studies undertaken.

Hepatic glucagon stimulation of adenylate cyclase activity in both lean and obese zucker rat preparations exhibited similar characteristics to those observed in the Sprague Dawley rat control preparations. That is, maximal glucagon stimulation was 17 and 20 fold over basal for lean and obese zucker rat preparations respectively (figure 3.5.7). Pertussis toxin pretreatment of hepatocytes from lean Zucker rats exhibited an enhanced maximal glucagon stimulation of adenylate cyclase activity. The elevated stimulation was 24 fold over basal and the estimated $K_{0.5}$ was unaltered at 10nM (figure 3.5.8). However, pertussis toxin pretreatment of hepatocytes isolated from obese Zucker rats (the typeII diabetic model) did not result in an elevated maximal glucagon stimulation of adenylate cyclase activity (figure 3.5.9). In fact, the two curves displaying glucagon dose dependent stimulation of adenylate cyclase activity for non-treated and pertussis toxin treated hepatocytes from these animals were superimposable.

In summary therefore, it appears that pertussis toxin pretreatment of hepatocytes isolated from the type II diabetic model , the obese Zucker rat, has no ability to enhance hepatic glucagon stimulation of adenylate cyclase activity because Gi function is already abolished. However, as no enhanced glucagon responsive state was apparent in the non-pertussis toxin treated hepatocytes from these animals, it would appear that the mechanism by which Gi function is abolished may be different from that observed in the type I diabetic model state.

In order to distinguish whether Gi function was lost in these diabetic animals as a result of reduced synthesis of the protein or else due to a covalent modification of this protein , collaboration with Dr. G. Milligan (also in the Biochemistry department at Glasgow university) was undertaken. Dr. Milligan had an antibody raised against a ten amino acid peptide sequence found in the α subunits of Gi and transducin. As transducin has only been isolated from rod outer segments, this antibody only recognised Gi in liver . Plasma membranes were purified from isolated hepatocytes as described in section 2.13 and Dr. Milligan quantified Giα in membranes prepared from all control and diabetic animal types using a Western blotting technique. This technique involved the separation of membrane proteins using SDS polyacrylamide gel electrophoresis , followed by transference of proteins to nitrocellulose and specific recognition of the Gi α subunit protein using the antibody and appropriate stain.

The results obtained are illustrated in figures 3.6.1 and 3.6.2 . Figure 3.6.1 shows the relative amounts of Giα found in streptozotocin diabetic, insulin treated streptozotocin diabetic and control preparations. It can be clearly seen that there is no appreciable Giα protein in the plasma membranes isolated from diabetic animals. Insulin reversal of the diabetic state brought back Giα protein in hepatocyte membranes by about 50 % when compared to control levels of the protein. This is in good agreement with the Gi functional studies which demonstrated a return of Gi function after insulin treatment by approximately 60 %.

Figure 3.6.2 demonstrates that the levels of Gi protein in plasma membranes prepared from lean and obese Zucker rats were not significantly different. Therefore it appears that although Gi function is lost in obese Zucker rat preparations , Gi α protein is not reduced in plasma membranes from these preparations.

I would therefore suggest that hepatic Gi function in the type I diabetic animal model is abolished because Gi synthesis is prevented. However, hepatic Gi function in obese Zucker rat preparations is abolished , but Gi levels in these hepatocyte plasma membranes are "normal". Therefore I suggest that Gi function is absent in these membranes because there may be a modification of the protein which results in attenuated activity . The antibody used to recognize Gi also recognizes the protein when it is ribosylated or phosphorylated.

It would be tempting to speculate that in the hyperinsulinaemic obese Zucker rat , the constantly high levels of insulin in the blood stream could have triggered the phosphorylation of the G protein Gi. Certainly, in vitro studies have demonstrated that the insulin receptor kinase has the ability to phosphorylate Gi . However, more studies would be required to determine more convincingly the kind of covalent modification occurring in these membranes.

Finally, as these obese zucker rat preparations do not exhibit an enhanced glucagon stimulated adenylate cyclase activity in the presence or absence of pertussis toxin pretreatment, I would suggest that this proposed Gi α subunit modification prevents the reassociation of B γ subunits to form the holomeric Gi protein complex and thereby does not allow Gi to act as a "sink" for these B γ subunits. Therefore, by mass action, once the Gs α subunits are released they may reassociate with the free B γ which would "normally" be complexed with Gi α subunits as a result of the pertussis toxin pretreatment. Therefore no enhancement of glucagon stimulated adenylate cyclase activity would be observed. To test this hypothesis of course, an antibody directed against B γ subunits would be required to quantify free B γ levels in each control and diabetic state. As there is a constantly elevated glucagon stimulated state in the chemically induced diabetic model, I would suggest that B γ levels in these preparations are reduced. I am therefore proposing that insulin has the ability (mechanism unknown) to

regulate both the expression of Gi α and Bγ subunits in rat hepatocyte plasma membranes.

Finally, Tables 2 and 3 demonstrate that although the basal adenylate cyclase activity in hepatocyte plasma membranes derived from type I diabetic animals was half that found in control animals, the fold stimulation elicited by forskolin (0.1mM), GTP (0.1mM), and NaF (15mM) in the presence or absence of metformin treatment was not significantly altered. Therefore, it is suggested that the only component in the hepatic adenylate cyclase system which is altered in the diabetic state is Gi . Gs and C appear to be functionally "normal". The reason for the reduced adenylate cyclase activity in the chemically induced diabetic animals is unknown. However, this may reflect reduced amounts of the catalytic unit or a general change in enzyme activities within the plasma membrane as a result of altered physical properties of the membrane structure itself .

4. Insulin Mediated Inhibition of Hepatic Adenylate Cyclase Activity In Control And Diabetic States

Introduction

Insulin was first shown to have the ability to inhibit glucagon and GTP stimulated adenylate cyclase activity by Heyworth *et al* (1983). This action of insulin was demonstrated to be dependent upon the insulin and glucagon concentrations used. When a glucagon concentration greater than 1nM is used, insulin's ability to inhibit adenylate cyclase activity is significantly reduced.

As this action of insulin was dependent upon guanine nucleotide concentration, this process is proposed to involve a G protein. I have attempted to investigate this action of insulin in control and diabetic states. This process could be considered as providing an index by which to assess insulin sensitivity in hepatocytes with respect to one insulin action. Therefore the effect of the hypoglycaemic drug , metformin, was investigated.

As pertussis toxin and the phorbol ester TPA have been shown to attenuate Gi activity, these compounds were used to try to establish whether this insulin mediated process involes this G protein.

Additional Methods

All studies, with the exception of experiments using TPA and pertussis toxin , were carried out using sucrose gradient purified liver plasma membranes (see section 2.14). TPA and pertussis toxin experiments were carried out using isolated hepatocyte crude membrane pellets (see section 2.8 - 2.12).

Animal treatments are described in section 2.3 - 2.6. Monocomponent porcine insulin and porcine glucagon were used throughout. Adenylate cyclase activity was assayed at 30 °C for 12 min using a final membrane protein concentration of 50 - 60 µg/ml.

Pertussis toxin was incubated with hepatocyte suspensions for 1 h using the procedure outlined in section 2.12 at a final concentration of 100ng / ml. TPA was incubated with hepatocyte suspensions for 0 - 15 min at a final concentration of o.1ng / ml. At each time point, a 1ml sample of the suspension was removed and centrifuged at 14,000g for 6 min at 4 °C. The hepatocyte pellet was then washed twice with ice - cold $KHCO_3$ pH 7 and the final lysed cellular pellet was resuspended to an appropriate volume and assayed for Gi and insulin mediated inhibition of adenylate cyclase activity. Only the concentrations of insulin (10^{-8} M) and glucagon (0.5nM) eliciting maximal response in these two processes were used in these studies.

Finally , the effect of metformin incubation with hepatocytes isolated from Sprague Dawley control rats was investigated. A series of metformin concentrations were incubated with a suspension of hepatocytes for 5 min. Samples were then extracted ,washed and the cells lysed.A crude membrane pellet was prepared and basal adenylate cyclase activity was assessed.

"n" refers to the number of independent membrane preparations used unless otherwise stated.

Figure 4.1 Insulin Mediated Inhibition of Adenylate Cyclase Activity In Liver Membranes Prepared From Control Rats

Liver plasma membranes were prepared from control Sprague Dawley rats as outlined in Materials & Methods. Adenylate Cyclase Activity was assayed for 12 min at 30°C in the presence of GTP (10^{-4}M), glucagon (5×10^{-10} M) and increasing concentrations of insulin. Inhibition is shown as a percentage of adenylate cyclase activity in the presence of GTP and glucagon , but in the absence of insulin .

Basal (GTP alone) specific activity was10.47 +/- 0.36 and glucagon stimulated activity in the presence of GTP was 15.71 +/- 1.21 pmoles cAMP produced /min/mg.

(n=4)

Figure 4.1 Insulin Mediated Inhibition of Adenylate Cyclase Activity In Liver Membranes Prepared From Control Rats

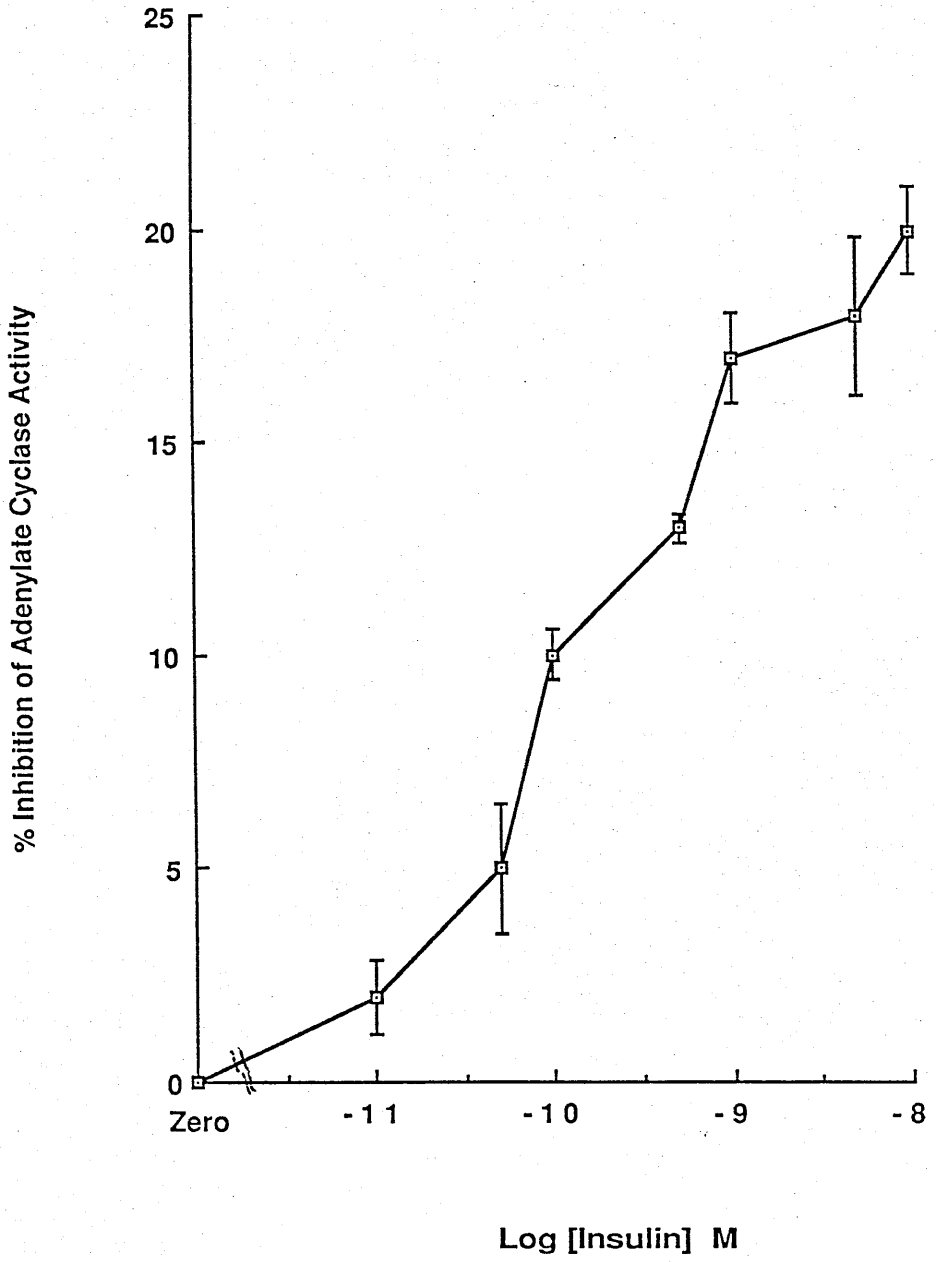


Figure 4.2 Effect of Streptozotocin Induced Diabetes On Insulin's Ability To Inhibit Adenylate Cyclase Activity In Liver Membranes

Liver plasma membranes were prepared from (□)control and (■)Streptozotocin - induced diabetic Sprague Dawley rats as outlined in Materials & Methods.

Adenylate Cyclase Activity was assayed for 12 min at 30°C in the presence of GTP (10^{-4}M), glucagon (5×10^{-10} M) and increasing concentrations of insulin.

Inhibition is shown as a percentage of adenylate cyclase activity in the presence of GTP and glucagon , but in the absence of insulin .

Basal (GTP alone) specific activities were 10.47 +/- 0.36 and 5.24 +/- 0.19 pmoles cAMP produced /min/mg for control and diabetic preparations respectively.

Similarly, glucagon stimulated activities in the presence of GTP were 15.71 +/- 1.21 and 7.65 +/- 0.51 pmoles cAMP produced /min/mg respectively.

(n = 4 for control and n = 6 for diabetic preparations)

Figure 4.2 Effect of Streptozotocin Induced Diabetes On Insulin's Ability To Inhibit Adenylate Cyclase Activity In Liver Membranes

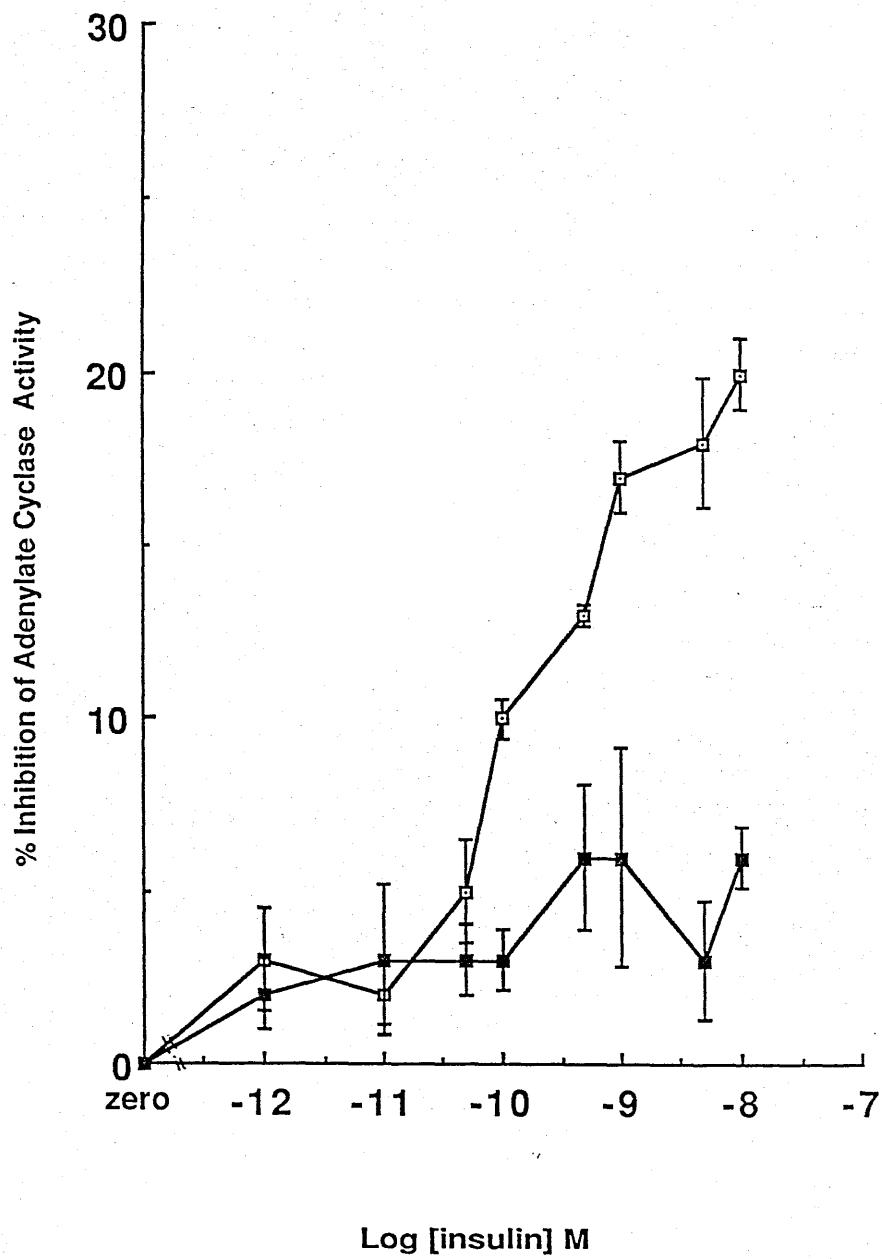


Figure 4.3 Effect of Alloxan Induced Diabetes On Insulin's Ability To Inhibit Adenylate Cyclase Activity In Liver Membranes

Liver plasma membranes were prepared from (□)control and (■)Alloxan - induced diabetic Sprague Dawley rats as outlined in Materials & Methods.

Adenylate Cyclase Activity was assayed for 12 min at 30°C in the presence of GTP (10^{-4}M), glucagon (5×10^{-10} M) and increasing concentrations of insulin.

Inhibition is shown as a percentage of adenylate cyclase activity in the presence of GTP and glucagon , but in the absence of insulin .

Basal (GTP alone) specific activities were 10.47 +/- 0.36 and 4.82 +/- 0.21 pmoles cAMP produced /min/mg for control and diabetic preparations respectively.

Similarly, glucagon stimulated activities in the presence of GTP were 15.71 +/- 1.21 and 6.90 +/- 0.70 pmoles cAMP produced /min/mg.

(n=4 for control and n=3 for diabetic preparations)

Figure 4.3 Effect of Alloxan Induced Diabetes

On Insulin's Ability To Inhibit Adenylate Cyclase

Activity In Liver Membranes

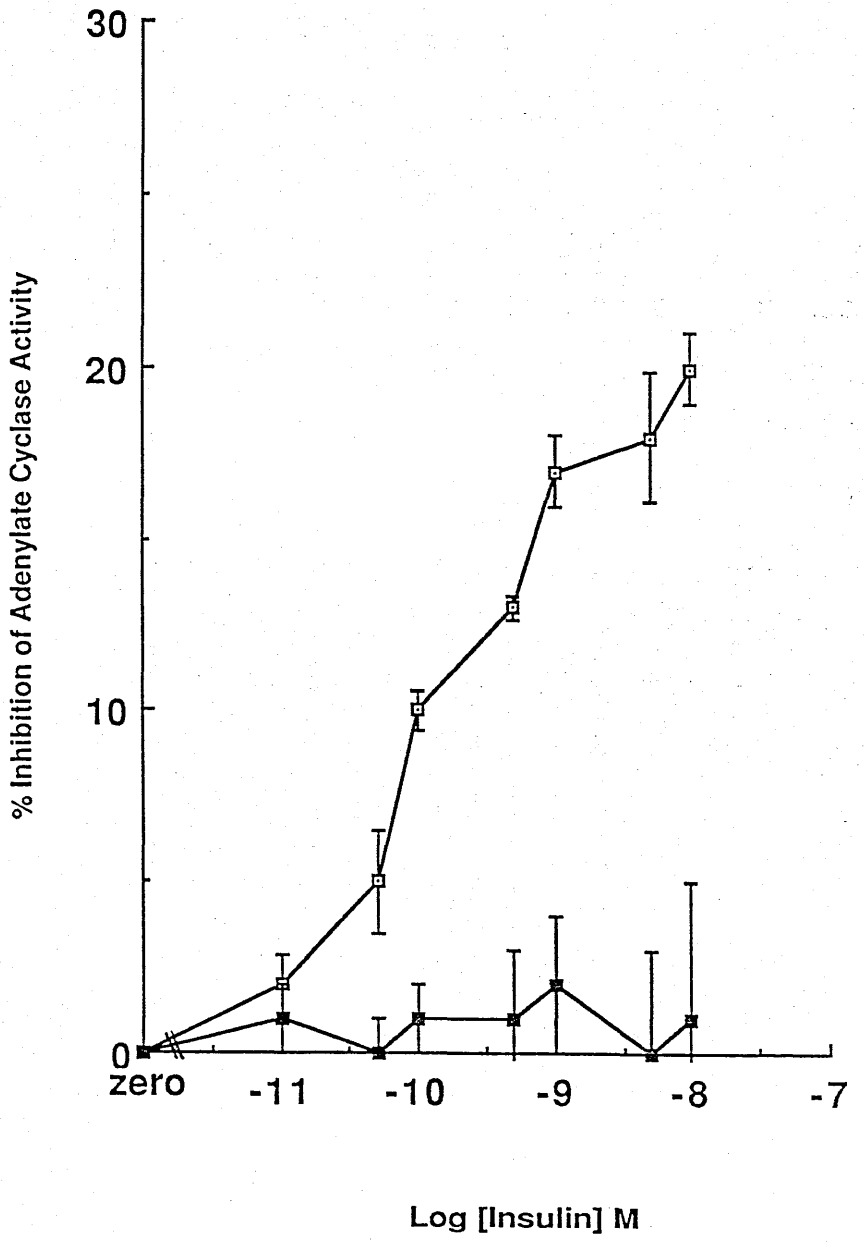


Figure 4.4 Effect of Diabetic State On Insulin's Ability To Inhibit Adenylate Cyclase Activity In Liver Membranes

Liver plasma membranes were prepared from (□)control (▩) Streptozotocin - induced diabetic and (⊞) Streptozotocin and insulin treated Sprague Dawley rats as outlined in Materials & Methods.

Adenylate Cyclase Activity was assayed for 12 min at 30°C in the presence of GTP (10^{-4}M) , glucagon (5 x 10^{-10} M) and increasing concentrations of insulin. Inhibition is shown as a percentage of adenylate cyclase activity in the presence of GTP and glucagon , but in the absence of insulin .

Basal (GTP alone) specific activities were 10.47 +/- 0.36 , 5.24 +/-0.19 and 6.60 +/- 0.48 pmoles cAMP produced /min/mg for control , diabetic and insulin treated preparations respectively. Similarly, glucagon stimulated activities in the presence of GTP were 15.71 +/- 1.21 ,7.65 +/- 0.51 and 9.77 +/- 0.41 pmoles cAMP produced /min/mg.

(n = 4 for control, n = 4 for insulin treated and n = 6 for diabetic preparations respectively).

Figure 4.4 Effect of Diabetic State On Insulin's Ability To Inhibit Adenylate Cyclase Activity In Liver Membranes

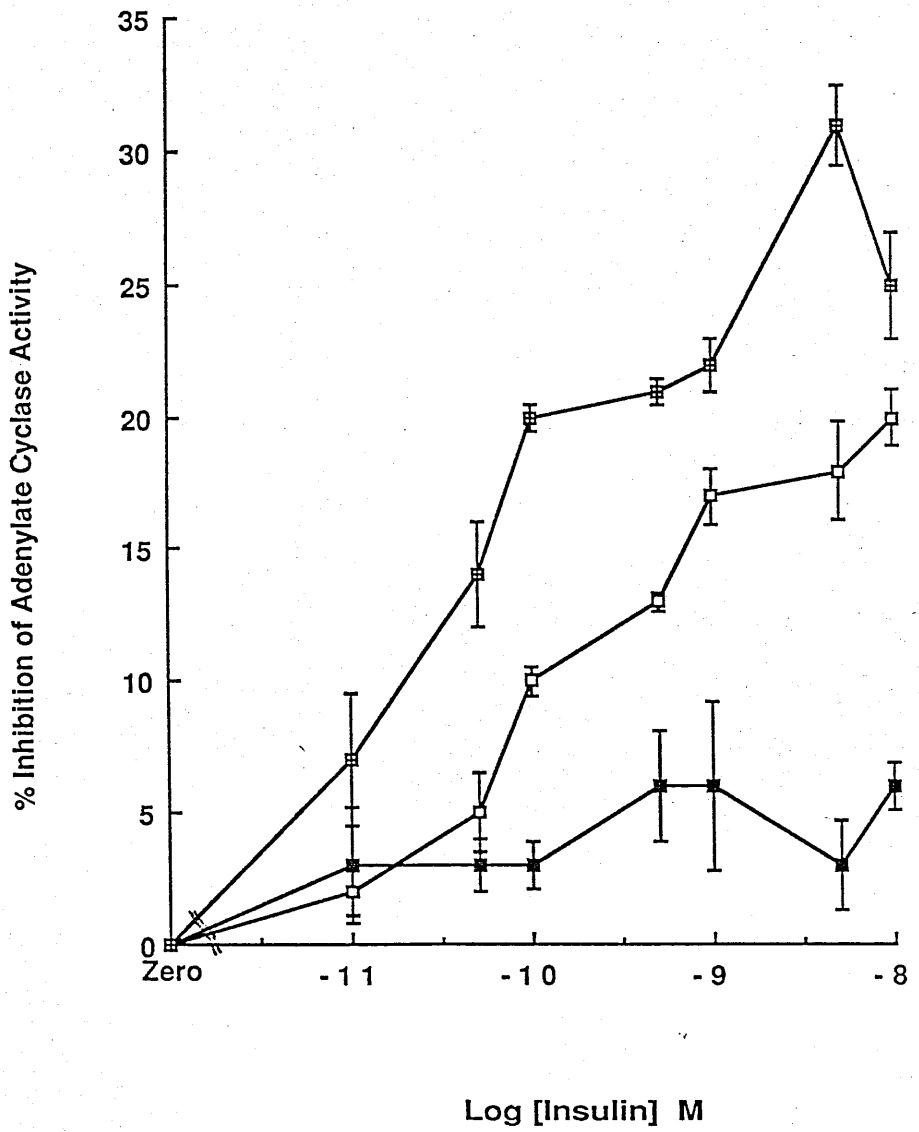


Figure 4.5 Insulin Mediated Inhibition of Adenylate Cyclase Activity In Liver Membranes Isolated From Lean Zucker Rats

Liver plasma membranes were prepared from lean Zucker rats as outlined in Materials & Methods.

Adenylate Cyclase Activity was assayed for 12 min at 30°C in the presence of GTP (10^{-4}M), glucagon (5×10^{-10} M) and increasing concentrations of insulin.

Inhibition is shown as a percentage of adenylate cyclase activity in the presence of GTP and glucagon , but in the absence of insulin .

Basal (GTP alone) and glucagon stimulated specific activities in the presence of GTP were 10.11 +/- 1.12 and 15.37 +/-1.20 pmoles cAMP produced /min/mg respectively.

(n=5).

Figure 4.5 Insulin Mediated Inhibition of Adenylate Cyclase Activity In Liver Membranes Isolated From Lean Zucker Rats

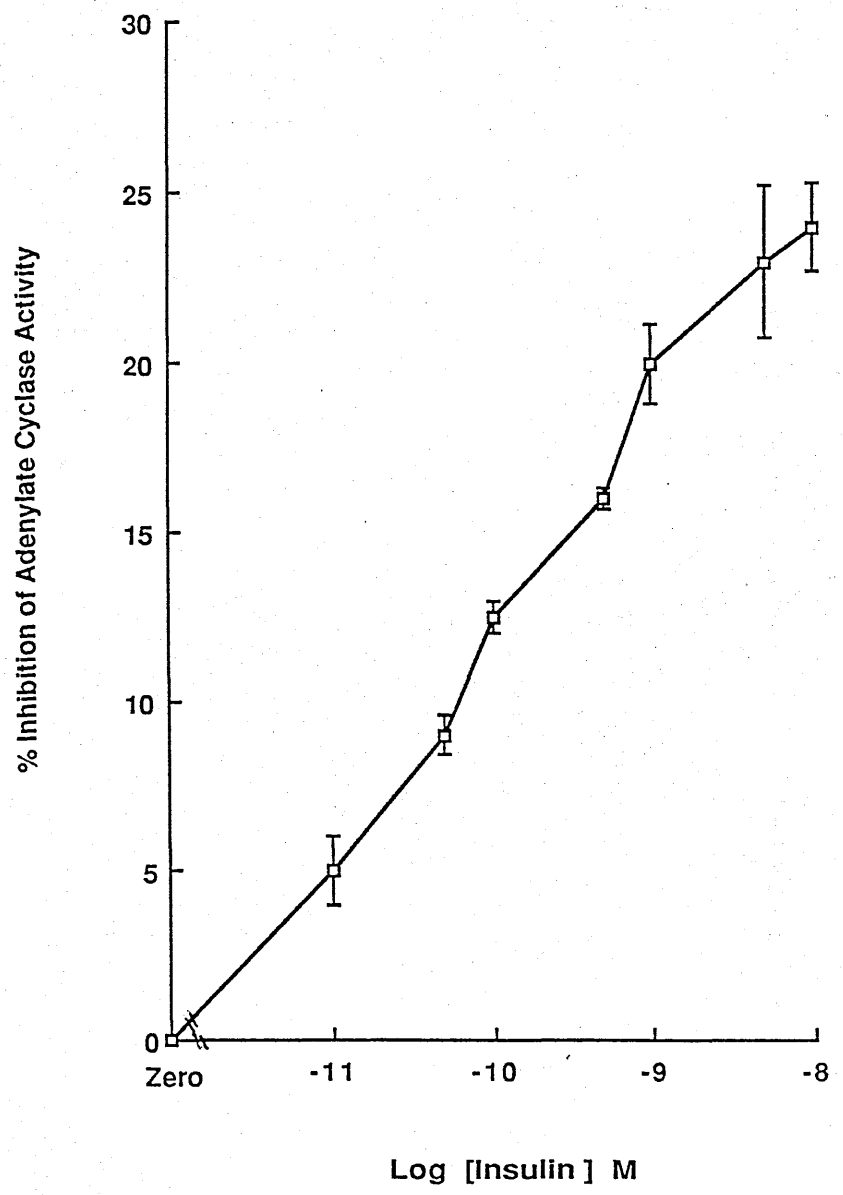


Figure 4.6 Insulin Mediated Inhibition of Adenylate Cyclase Activity In Liver Membranes Prepared From Lean And Obese Zucker Rats

Liver plasma membranes were prepared from (⊡) lean and (■) obese Zucker rats as outlined in Materials & Methods.

Adenylate Cyclase Activity was assayed for 12 min at 30°C in the presence of GTP (10^{-4}M), glucagon (5×10^{-10} M) and increasing concentrations of insulin.

Inhibition is shown as a percentage of adenylate cyclase activity in the presence of GTP and glucagon , but in the absence of insulin .

Basal (GTP alone) specific activities were 10.11 +/- 1.12 and 10.32 +/- 0.81 pmoles cAMP produced /min/mg for lean and obese animal preparations respectively.

Similarly, glucagon stimulated activities in the presence of GTP were 15.37 +/- 1.20 and 16.10 +/- 0.99 pmoles cAMP produced /min/mg.

(n=5 for lean and n=3 for obese preparations respectively)

Figure 4.6 Insulin Mediated Inhibition of Adenylate Cyclase Activity In Liver Membranes Prepared From Lean And Obese Zucker Rats

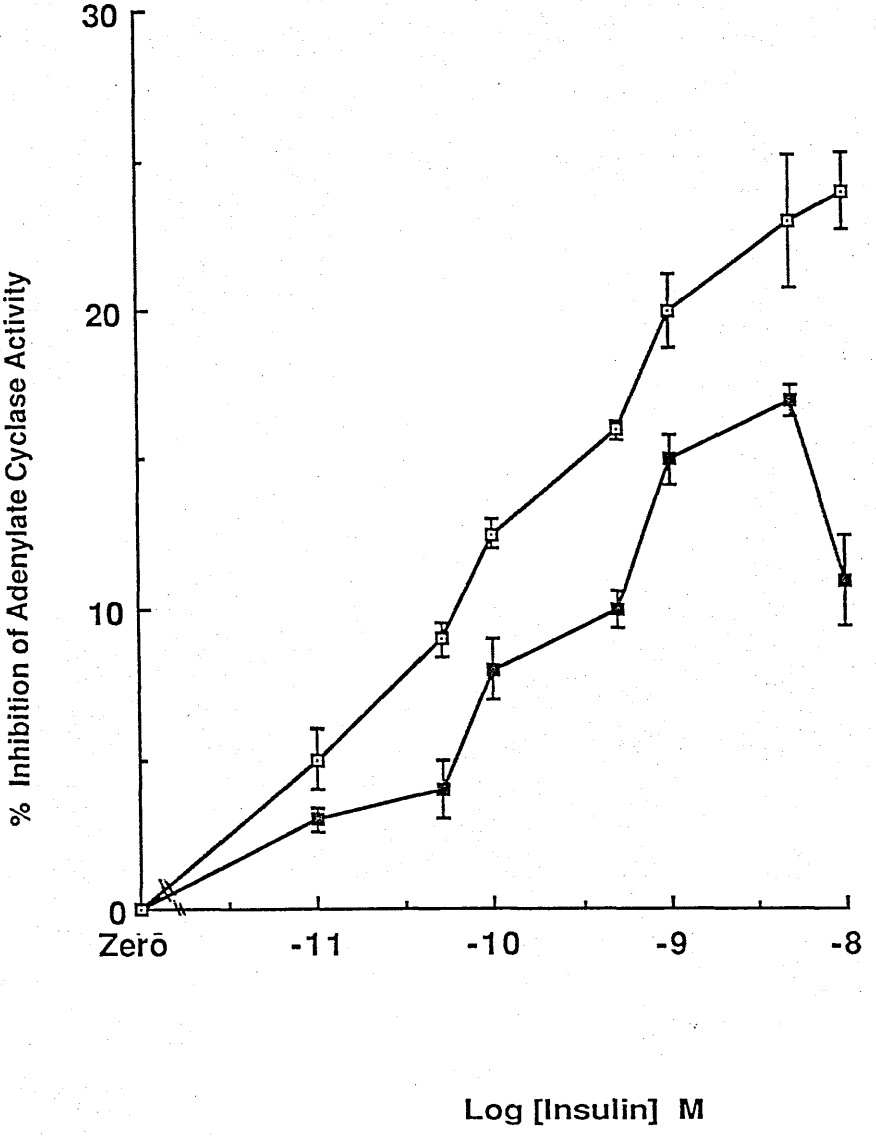


Figure 4.7 Effect of Biguanide Treatment On Insulin's Ability To Inhibit Adenylate Cyclase Activity In Control Liver Membranes

Liver plasma membranes were prepared from control Sprague Dawley rats which (▲) had or (□) had not undergone oral metformin therapy prior to sacrifice. Adenylate Cyclase Activity was assayed for 12 min at 30°C in the presence of GTP (10^{-4}M), glucagon (5×10^{-10} M) and increasing concentrations of insulin. Inhibition is shown as a percentage of adenylate cyclase activity in the presence of GTP and glucagon , but in the absence of insulin .

Basal (GTP alone) specific activities were 9.46 +/- 1.01 and 10.47 +/- 0.36 pmoles cAMP produced /min/mg for for metformin treated and non - treated animal preparations respectively. Similarly, glucagon stimulated activities in the presence of GTP were 13.99 +/- 1.70 and 15.71 +/- 1.21 pmoles cAMP produced /min/mg.

(n= 4 for control and n = 5 for biguanide treated preparations respectively).

Figure 4.7 Effect of Biguanide Treatment On

Insulin's Ability To Inhibit Adenylate Cyclase

Activity In Control Liver Membranes

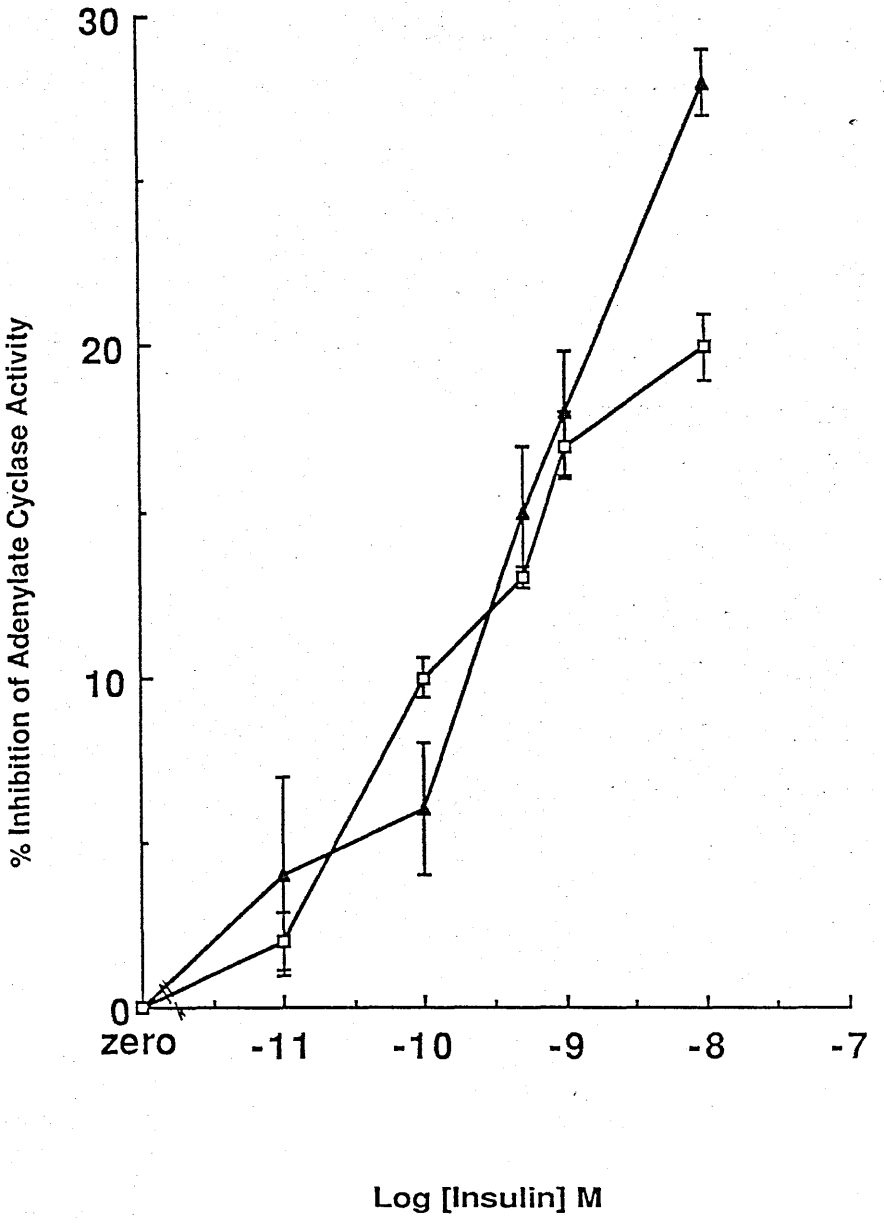


Figure 4.8 Effect of Biguanide Treatment On Insulin's Ability To Inhibit Adenylate Cyclase Activity In Liver Membranes Prepared From Streptozotocin Induced Diabetic Rats

Liver plasma membranes were prepared from Streptozotocin induced diabetic Sprague Dawley rats which (●) had or (o) had not undergone oral metformin therapy prior to sacrifice.

Adenylate Cyclase Activity was assayed for 12 min at 30°C in the presence of GTP (10^{-4}M), glucagon (5×10^{-10} M) and increasing concentrations of insulin. Inhibition is shown as a percentage of adenylate cyclase activity in the presence of GTP and glucagon , but in the absence of insulin .

Basal (GTP alone) specific activities were 3.43 +/- 0.29 and 5.24 +/= 0.19 pmoles cAMP produced /min/mg for for metformin treated and non - treated animal preparations respectively. Similarly, glucagon stimulated activities in the presence of GTP were 5.09 +/- 0.32 and 7.65 +/- 0.51 pmoles cAMP produced /min/mg.

(n = 6 for diabetic and n = 5 for metformin treated diabetic preparations respectively).

Figure 4.8 Effect of Biguanide Treatment On Insulin's Ability To Inhibit Adenylate Cyclase Activity In Liver Membranes Prepared From Streptozotocin Induced Diabetic Rats

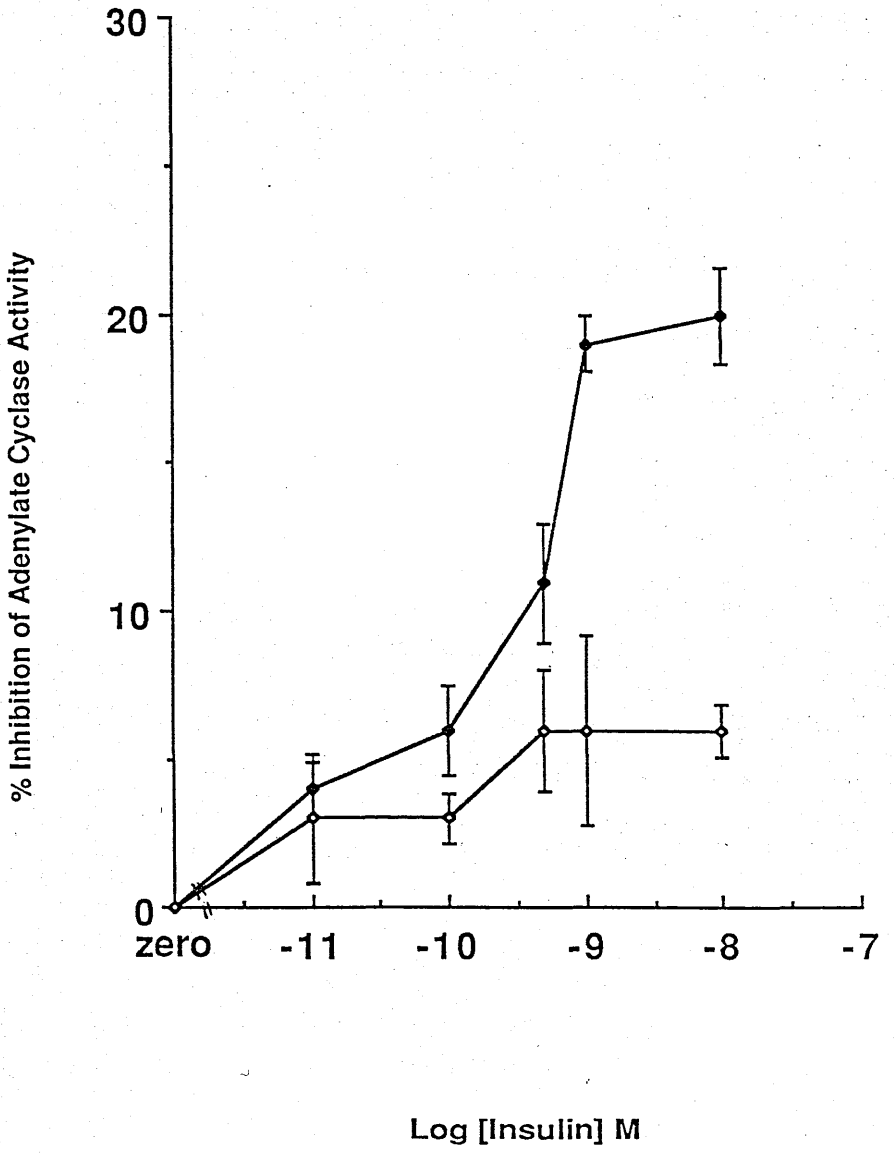


Figure 4.9 Effect of Biguanide Treatment On Insulin's Ability To Inhibit Adenylate Cyclase Activity In Liver Membranes Prepared From Control And Diabetic Rats

Liver plasma membranes were prepared from (△)control and (▲) Streptozotocin induced diabetic Sprague Dawley rats which had undergone oral metformin therapy prior to sacrifice.

Adenylate Cyclase Activity was assayed for 12 min at 30°C in the presence of GTP (10^{-4}M), glucagon (5×10^{-10} M) and increasing concentrations of insulin.

Inhibition is shown as a percentage of adenylate cyclase activity in the presence of GTP and glucagon , but in the absence of insulin .

Basal (GTP alone) specific activities were 9.46 +/- 1.01 and 3.43 +/- 0.29 pmoles cAMP produced /min/mg for for control and diabetic animal preparations respectively. Similarly, glucagon stimulated activities in the presence of GTP were 13.99 +/- 1.70 and 5.09 +/- 0.32 pmoles cAMP produced /min/mg.

(n=5 for control and n=5 for diabetic preparations respectively).

Figure 4.9 Effect of BiguanideTreatment On Insulin's Ability To Inhibit Adenylate Cyclase Activity In Liver Membranes Prepared From Control And Diabetic Rats

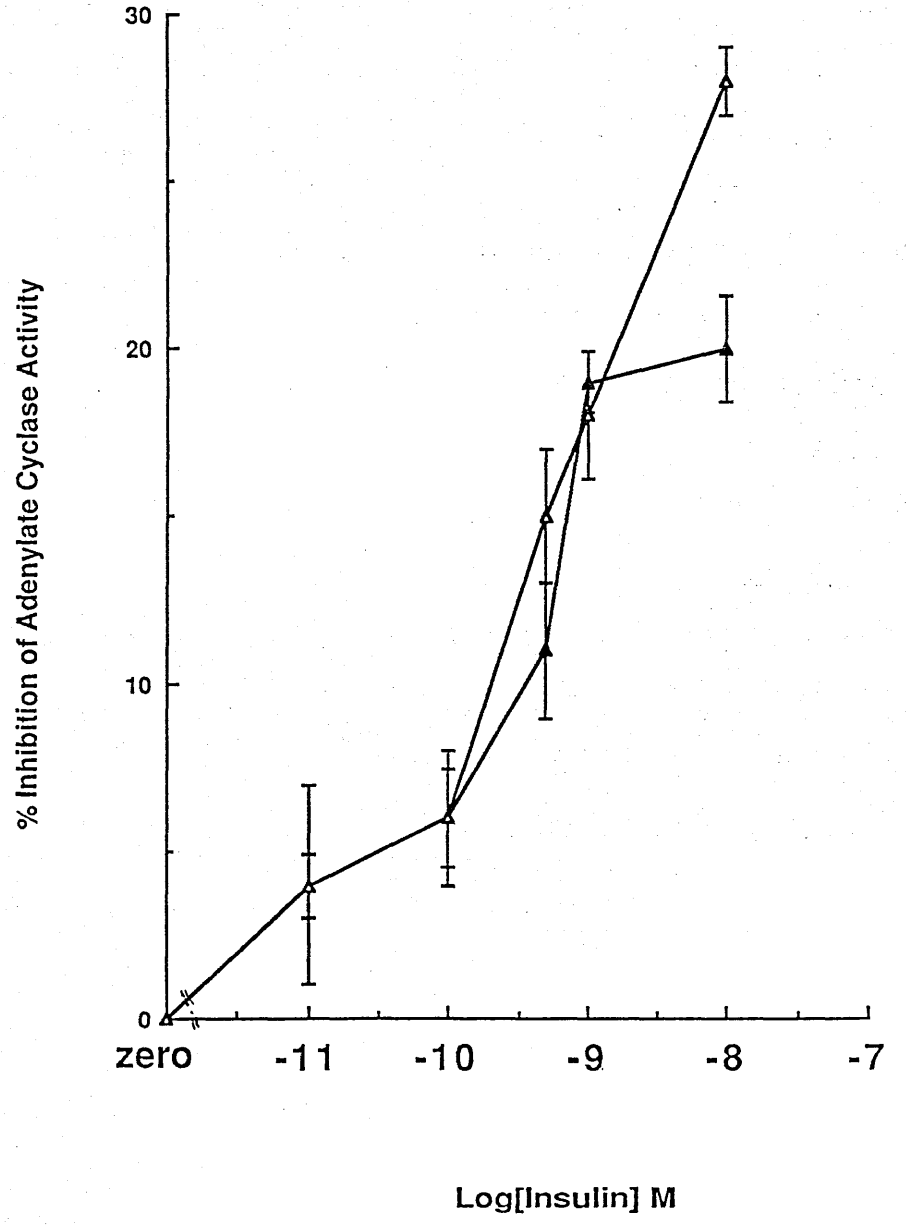


Figure 4.10 Effect of Biguanide Treatment On Insulin's Ability To Inhibit Adenylate Cyclase Activity In Liver Membranes Prepared From Lean Zucker Rats

Liver plasma membranes were prepared from lean Zucker rats which (■) had or (□) had not undergone oral metformin therapy prior to sacrifice.

Adenylate Cyclase Activity was assayed for 12 min at 30°C in the presence of GTP (10^{-4}M), glucagon (5×10^{-10} M) and increasing concentrations of insulin.

Inhibition is shown as a percentage of adenylate cyclase activity in the presence of GTP and glucagon , but in the absence of insulin .

Basal (GTP alone) specific activities were 9.00 +/- 1.05 and 10.11 +/- 1.12 pmoles cAMP produced /min/mg for for treated and non-treated animal preparations respectively. Similarly, glucagon stimulated activities in the presence of GTP were 13.62 +/- 0.86 and 15.37 +/- 1.20 pmoles cAMP produced /min/mg.

(n=5 for lean and n=4 for metformin treated lean preparations respectively)

Figure 4.10 Effect of Biguanide Treatment On Insulin's Ability To Inhibit Adenylate Cyclase Activity In Liver Membranes Prepared From Lean Zucker Rats

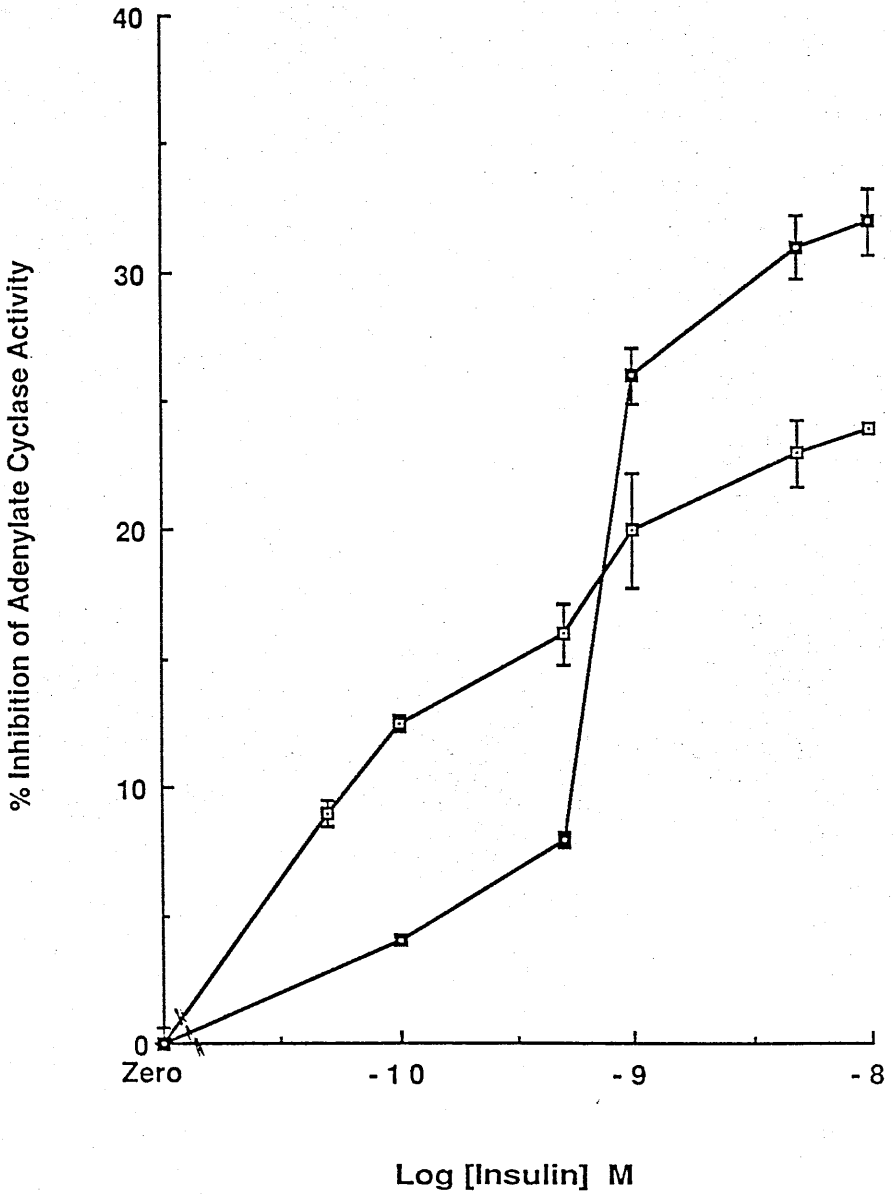


Figure 4.11 Effect of Biguanide Treatment On Insulin's Ability To Inhibit Adenylate Cyclase Activity In Liver Membranes Prepared From Obese Zucker Rats

Liver plasma membranes were prepared from obese Zucker rats which (▲) had or (△) had not undergone oral metformin therapy prior to sacrifice.

Adenylate Cyclase Activity was assayed for 12 min at 30°C in the presence of GTP (10^{-4}M), glucagon (5×10^{-10} M) and increasing concentrations of insulin.

Inhibition is shown as a percentage of adenylate cyclase activity in the presence of GTP and glucagon , but in the absence of insulin .

Basal (GTP alone) specific activities were 9.55 +/- 1.20 and 10.32 +/- 0.81 pmoles cAMP produced /min/mg for treated and non-treated animal preparations respectively. Similarly, glucagon stimulated activities in the presence of GTP were 14.72 +/- 1.66 and 16.10 +/- 0.99 pmoles cAMP produced /min/mg.

(n=3 for both obese and metformin treated obese rat preparations)

Figure 4.11 Effect of Biguanide Treatment On Insulin's Ability To Inhibit Adenylate Cyclase Activity In Liver Membranes Prepared From Obese Zucker Rats

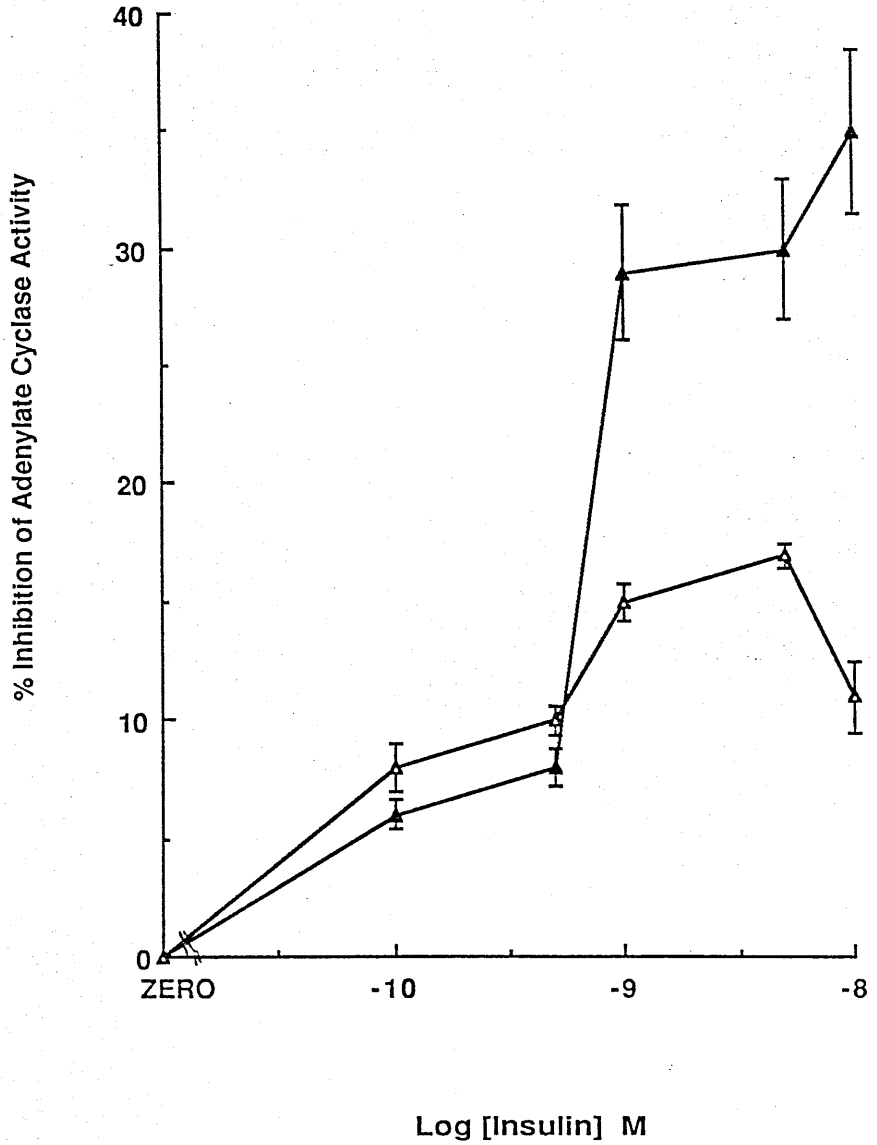


Figure 4.12 Insulin Mediated Inhibition of Adenylate Cyclase Activity In Liver Membranes Prepared From Zucker Rats Undergoing Biguanide Therapy

Liver plasma membranes were prepared from (△)lean and (▲) obese Zucker rats which had undergone metformin therapy prior to sacrifice.

Adenylate Cyclase Activity was assayed for 12 min at 30°C in the presence of GTP (10^{-4}M) , glucagon (5×10^{-10} M) and increasing concentrations of insulin.

Inhibition is shown as a percentage of adenylate cyclase activity in the presence of GTP and glucagon , but in the absence of insulin .

Basal (GTP alone) specific activities were 9.00 +/- 1.05 and 9.55 +/- 1.20 pmoles cAMP produced /min/mg for lean and obese animal preparations respectively. Similarly, glucagon stimulated activities in the presence of GTP were 13.62 +/- 0.86 and 14.72 +/- 1.66 pmoles cAMP produced /min/mg.

(n= 4 for lean and n= 3 for obese rat preparations respectively)

Figure 4.12 Insulin Mediated Inhibition of Adenylate Cyclase Activity In Liver membranes Prepared From Zucker Rats Undergoing Biguanide Therapy

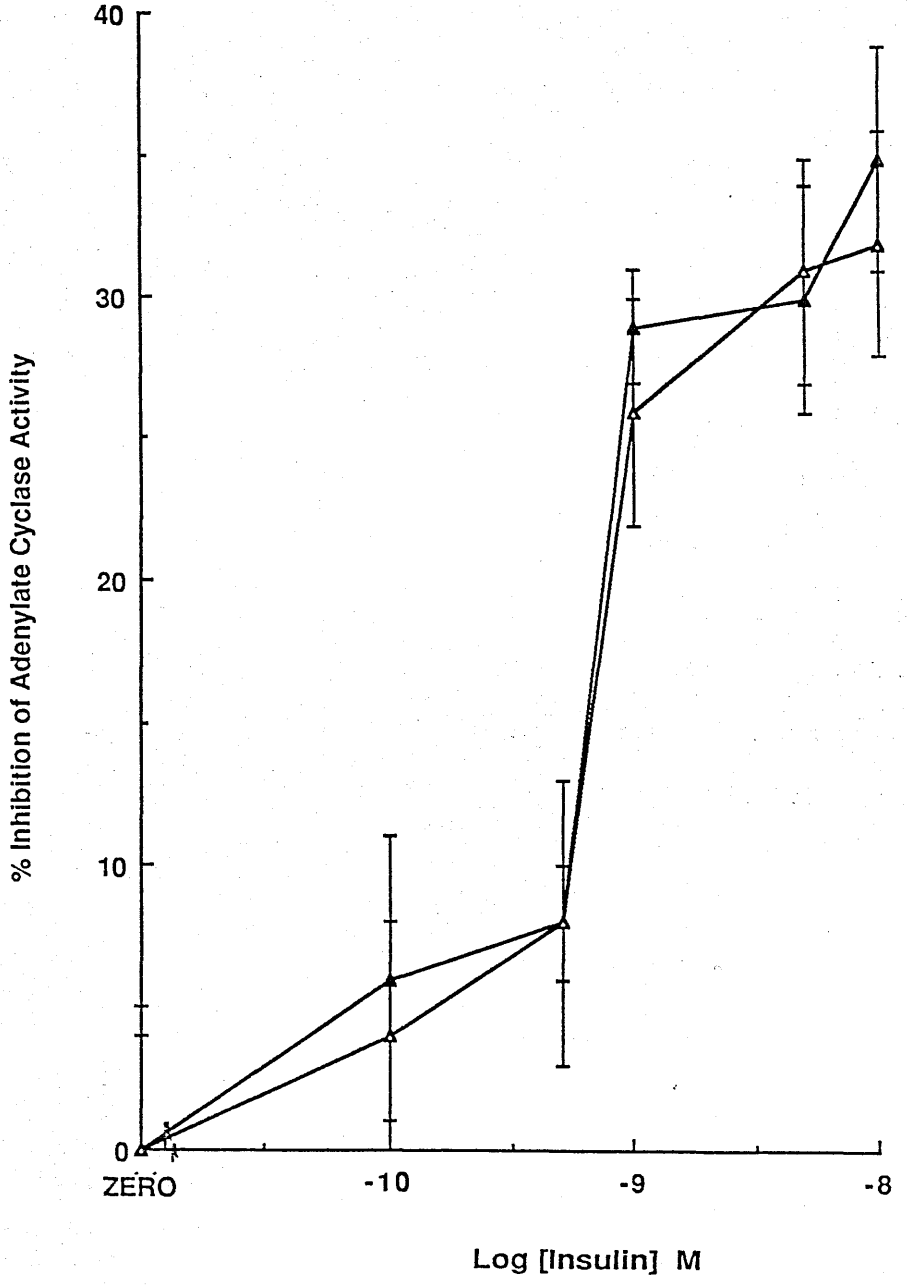


Table 4 Incubation of Hepatocytes With Metformin: Effect Upon Adenylate Cyclase Activity

Metformin Concentration (mM)	Adenylate Cyclase Activity pmoles min^{-1} mg^{-1}
0	1.20 +/- 0.10
2	0.20 +/- 0.05
4	0.00 -
6	0.00 -
8	0.00

n=3 experiments. Values given are mean +/- SEM values.

Figure 4.13 Effect of Pertussis Toxin Treatment

Crude membranes were prepared from (□) control and (●) pertussis pretreated hepatocytes isolated from Sprague Dawley rats as outlined in Materials & Methods.
Adenylate Cyclase Activity was assayed for 12 min at 30°C in the presence of GTP (10^{-4}M), glucagon (5×10^{-10} M) and increasing concentrations of insulin.
Inhibition is shown as a percentage of adenylate cyclase activity in the presence of GTP and glucagon, but in the absence of insulin.
Basal (GTP alone) specific activities were 1.32 +/- 0.08 and 1.26 +/- 0.60 pmoles cAMP produced /min/mg for control and pertussis toxin treated preparations respectively. Similarly, glucagon stimulated activities in the presence of GTP were 2.02 +/- 0.15 and 1.85 +/- 0.12 pmoles cAMP produced /min/mg respectively.
(n = 3 animals for both control and toxin treated hepatocyte preparations)

Figure 4.13 Effect of Pertussis Toxin Pretreatment

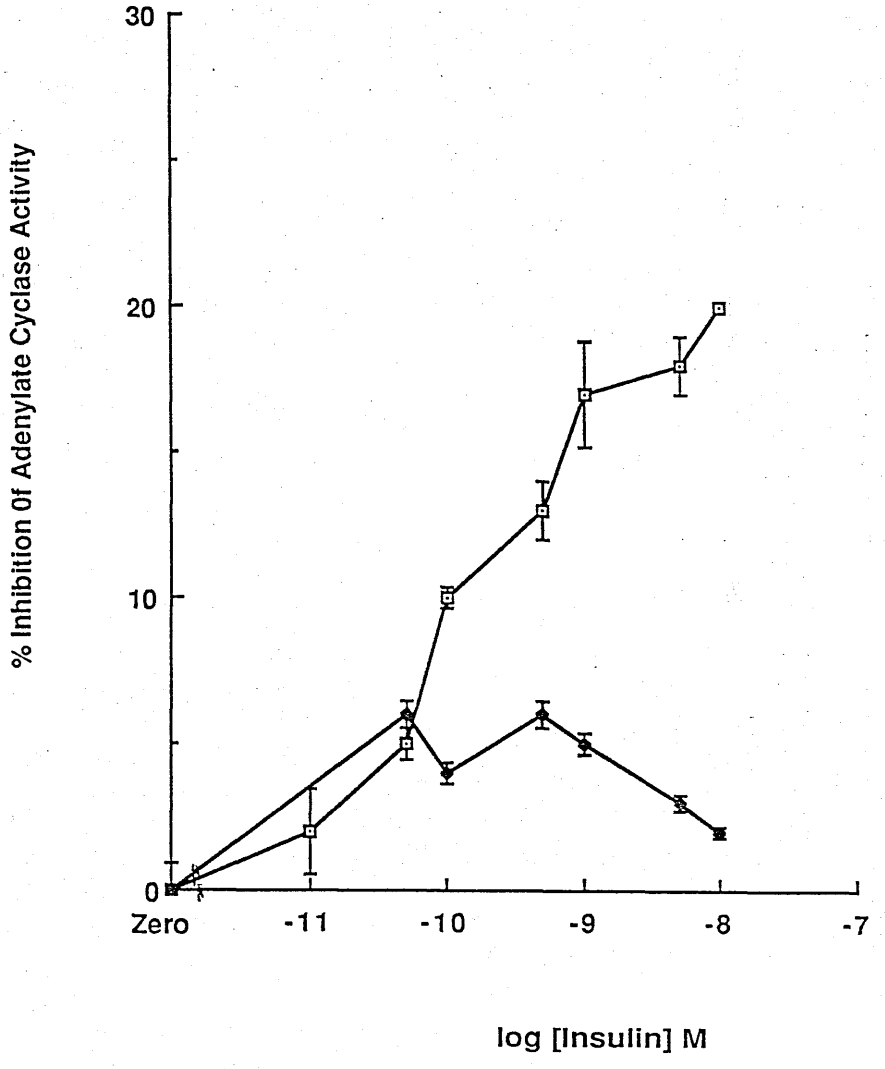
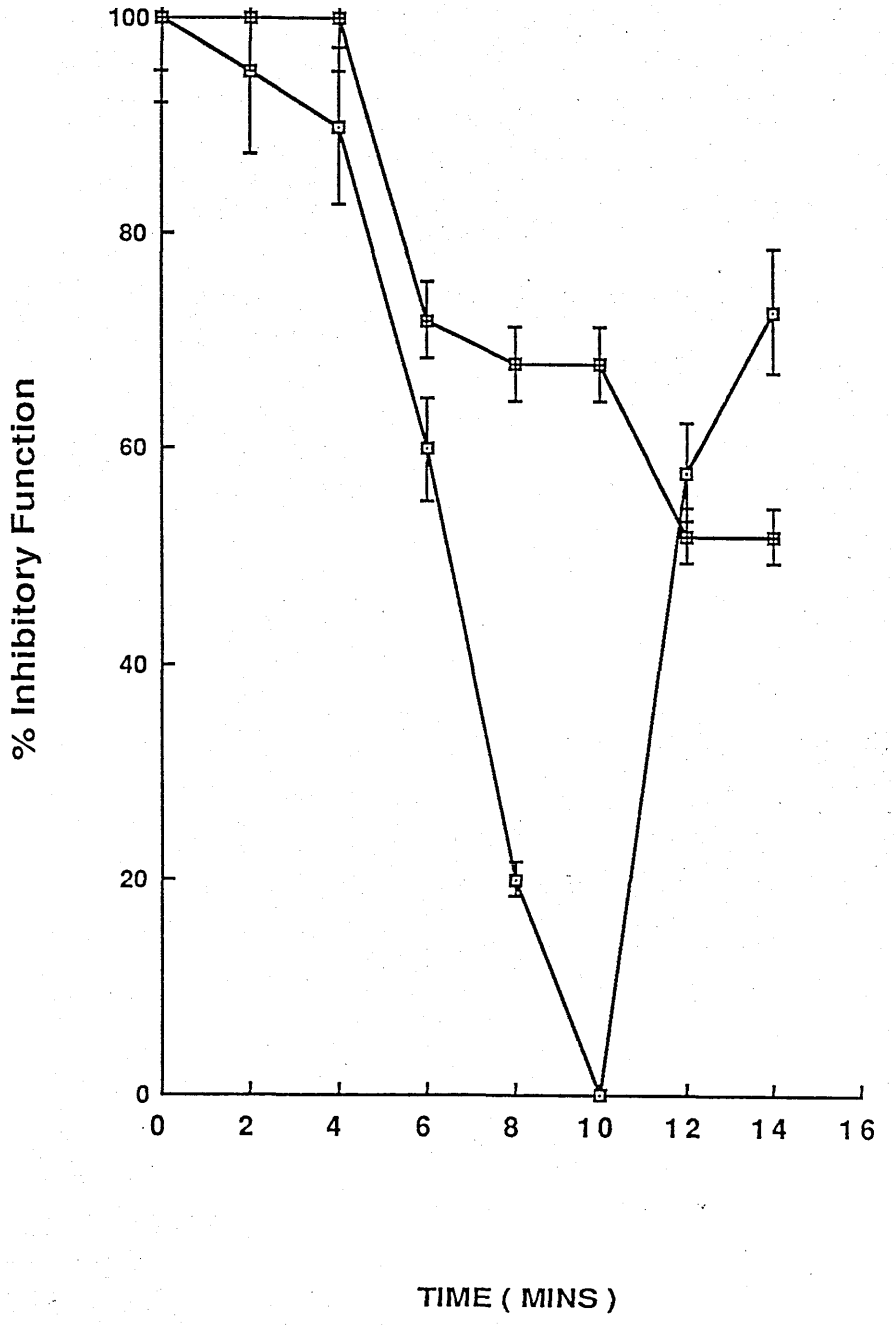


Figure 4.14 Effect of TPA Treatment Upon The Inhibition of Adenylate Cyclase Activity Mediated By Insulin And GPP(NH)P

Hepatocytes were incubated with TPA (0.1ng/ml) for 0-16 minutes at 37^{0}C in a shaking water bath. Hepatocyte suspension samples were removed at the appropriate time point and crude membrane pellets were rapidly prepared. Gi function was assessed (□) for each sample using forskolin (10^{-4} M) and GPP(NH)P (10^{-8} M) as described in chapter3. Insulin mediated inhibition was assessed (⊞) for each sample using insulin (10^{-8} M), GTP (10^{-4}M) and glucagon ($5x10^{-10}$ M)as described in chapter 4. The inhibition of adenylate cyclase mediated by each process is expressed as a percentage of the inhibitory function of each process.
Values shown are mean +/- SEM values for n=4 experiments (4 animals).

Figure 4.14 Effect of TPA Treatment Upon The Inhibition of Adenylate Cyclase Activity Mediated By Insulin And GPP(NH)P



Discussion of Results

Insulin has the ability to inhibit glucagon (0.1nM) stimulated adenylate cyclase activity in the presence of GTP (0.1mM) (see figure 4.1). The estimated concentration of insulin which elicits 1/2 maximal inhibition of glucagon stimulated adenylate cyclase activity is 0.1nM. Maximal inhibition (20 +/- 3 %) occurs at 10nM insulin. This insulin mediated inhibition of adenylate cyclase activity is abolished in the chemically induced diabetic state (figures 4.2 & 4.3). This diabetic state was induced using two different chemicals; streptozotocin and alloxan. It was therefore assumed that the effects observed were not due to toxic effects upon the adenylate cyclase system ,or upon the liver itself. Instead ,these effects were assumed to be due to the chemical induction of an insulin deficient condition in these animals.

Evidence to confirm this assumption is that insulin treatment of these diabetic animals reverse the physical characteristics associated with type I diabetes displayed in these animals. This included the reversal of such charateristics as elevated plasma glucose levels and the presence of glycosuria. In addition, the ability of insulin to inhibit glucagon stimulated adenylate cyclase activity in the presence of GTP returns (figure 4.4). Not only do these insulin reversed diabetic preparations display an enhanced maximal inhibition (30

+/- 2 %), but also the $K_{0.5}$ (the concentration of insulin which elicits 1/2 maximal inhibition) was reduced from 0.1nM (in controls) to 0.04nM (in insulin treated preparations).

When this action of insulin is examined in liver membranes prepared from Zucker rats, membranes from the lean (control) animals displayed an insulin mediated inhibtion of adenylate cyclase activity with a maximal inhibitory response of 24 +/- 3%), and a $K_{0.5}$ estimated value for the process of 0.1nM (see figure 4.5). However, the type II diabetic model, the obese Zucker rat, displayed a reduced maximal inhibition of 17 +/- 1% and an estimated $K_{0.5}$ of 0.1nM. It was also noticable that the maximal response for the process occurred at an insulin concentration of 5nM which is slightly lower than that observed in the control animals.

Insulin resistance is well documented in this type II diabetic state , but it is clearly evident (figure 4.6) that insulin action on the adenylate cyclase system in this state does not display an increased $K_{0.5}$ for the process. However, a reduced maximal response in this state is observed.

Administration of the hypoglycaemic drug metformin was once again examined. Metformin was found to enhance the maximal inhibition mediated by insulin by 35 % when compared to controls. Also, the $K_{0.5}$ for the process in the presence of metformin treatment was increased from 0.1nM to 0.5nM.

When the effect of metformin therapy in streptozotocin induced diabetic animal preparations was investigated , the

results obtained were very surprising (figure 4.8). Drug therapy appears to return this previously abolished inhibitory action of insulin. The maximal response is 20 +/- 2 % which is very similar to that observed in controls and the K0.5 estimated value for the process in these preparations is 0.5nM.

When metformin treated control and diabetic states are compared (figure 4.9) there appears to be an elevated maximal response ,but apart from this , the two curves are superimposable. Metformin therapy administered to lean Zucker rats resulted in similar effects to those observed in Sprague Dawley rat preparations. Metformin treatment enhanced the maximal inhibition by approximately 29 % to 31 +/- 5 % and the K0.5 estimated for the process was increased from 0.1nM to 0.9nM.

Similarly, membrane preparations obtained from metformin treated obese Zucker rats were more responsive to insulin . The maximal inhibition mediated by the hormone in these preparations was increased from 17 +/- 1 to 30 +/- 2 % when compared to controls. Also, when the insulin response in metformin treated lean and obese Zucker rat preparations were compared , superimposable curves were obtained.

In summary therefore, it appears that insulin inhibition of glucagon stimulated adenylate cyclase activity is abolished completely in the type I diabetic model, but this action of insulin returns upon metformin therapy with responses comparable to control non-diabetic animal preparations. Insulin

therapy also restored this inhibitory action of insulin, but resulted in a more pronounced response and a slightly lower $K_{0.5}$ value.

Similarly, Metformin treatment of lean and obese Zucker rats resulted in a heightened response to insulin with a slightly increased $K_{0.5}$ value . Again, the non-treated diabetic model (type II) was less responsive than the control ,but this action was not totally absent as in the case of the type I diabetic model. In every case of metformin administration, the maximal response to insulin was enhanced and the $K_{0.5}$ values were also increased. However, considering the limits of accuracy in estimating these values, it is not possible to comment on the significance of these $K_{0.5}$ changes.

Pertussis toxim pretreatment of control Sprague Dawley rat hepatocytes abolished insulin's ability to inhibit adenylate cyclase activity (figure 4.13). This confirms the findings of Heyworth et al (1986).

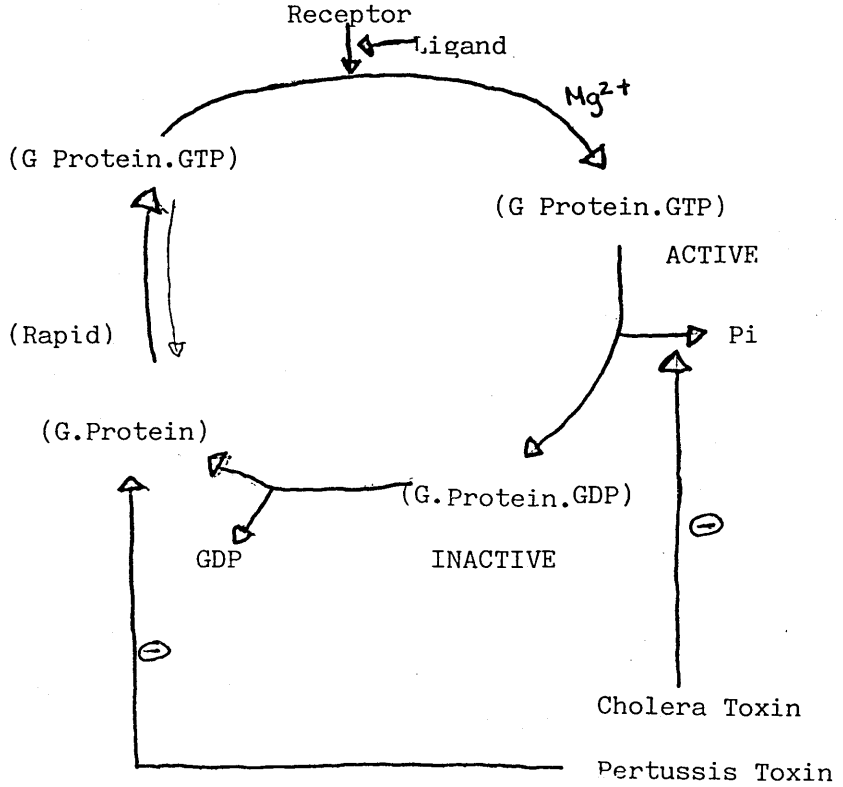
Using the phorbol ester , TPA, at a final concentration of 0.1ng /ml, Gi function and insulin's ability to inhibit adenylate cyclase activity was investigated. Following an incubation time-course with this compound (figure 4.14), it is clearly observable that Gi function is abolished within 10 min. However, at this time point only 30 % of insulin's ability to inhibit adenylate cyclase activity is removed. It is therefore suggested that insulin inhibits adenylate cyclase activity by a process which is independent of the inhibitory G protein Gi.

Finally, the effect of metformin incubation with hepatocytes isolated from control Sprague Dawley rats was investigated (table 4). It was found that metformin at a concentration greater than 2mM obliterated adenylate cyclase activity all together. When the equivalent oral metformin dose is 8mM. However, in vitro studies with this drug are generally in the 1μM-10μM range. It therefore seems unlikely that the effects observed in preparations from animals which have undergone metformin therapy are direct membrane effects. It is also probable that metformin is metabolized in the gut of these animals prior to reaching the blood stream.

5. High Affinity GTPase Activity Within Human Platelet Broken Plasma Membranes

Introduction

Guanine nucleotide regulatory proteins undergo a cycle of activation and deactivation under agonist stimulated conditions. The activation process is described in detail in the general introduction and involves Mg^{2+}, receptors (Rs or Ri) and the binding of GTP. The deactivation process is known as the GTPase activity of the protein. This activation-deactivation cycle is summarized schematically as follows :



GTPase activity is an intrinsic activity within the G protein and in the presence of specific agonists this GTPase activity can be stimulated.

This phenomenon was first demonstrated by Cassel & Selinger (1976) and has since been demonstrated by others in a variety of cell types using a variety of receptor ligands (Grandt et al 1986 Fain et al 1985; Houslay et al 1986).

Essentially, the cell type chosen must have a low endogenous GTPase activity as most cellular GTPase activity is a result of low affinity GTP hydrolysis. This low affinity GTPase activity is regarded as "non-specific" or "background" activity and must be accounted for and subtracted from the total cellular GTPase activity in order to "observe " GTPase activity associated with G proteins.

Also, utilization of GTP as a phosphate donor must be reduced in order to "observe" this high affinity GTPase activity. Thus, the inclusion of the non-hydrolyzable ATP analogue, APP(NH)P adenylyl -imidodiphosphate, and the Na^+/K^+ ATPase inhibitor, ouabain is often made to GTPase assay systems.

In this chapter, I have attempted to demonstrate that in human platelet membranes , insulin has the ability to stimulate the GTPase activity of a G protein distinct from that associated with Gs, Gi and the G protein involved in phospholipase C activation, Gp (Blackmore et al 1985; Bradford & Rubin 1986.).

Additional Methods

[^{32}P] GTP was prepared as outlined in section 2.20 . GTPase activity was assayed as described in section 2.21. Toxin pretreatment of membranes were undertaken as described in sections 2.22 and 2.23. Unless otherwise stated, the ligand concentrations were as follows :

Isoproterenol 1μM, PGE1 20μM, Adrenaline 100μM, Propranolol 1uM, Insulin 10nM and vasopressin 10nM.

Unless otherwise indicated , the final GTP concentration was 0.6μM. Values given are mean +/- SEM values and n = number of experiments performed. All assays were performed in triplicate and linearity of GTPase activity was ensured for incubation times and membrane protein concentrations used.

GTPase activity shown is the high affinity GTPase activity associated with G protein function. Non - specific GTPase activity has been eliminated by the inclusion of a high concentration (0.1mM) of non-radiolabelled GTP during each experiment. Under these conditions (Cassel & Selinger 1976; Houslay et al 1986) only high affinity GTPase activity is observable.

The additivity ratio shown in table 6 was calculated as follows :

$$\text{Additivity Ratio} = \frac{\text{Stimulation Observed For Drug Combination}}{\sum(\text{Stimulation By Drug1} + \text{Stimulation By Drug 2})}$$

Therefore for ligands which stimulate one GTPase activity, the additivity ratio equals 0.5 . For ligands which stimulate distinct GTPase activities, the additivity ratio equals 1.0.

5.1 Dose-Dependent Ligand Stimulation Of Specific GTPase Activity

Figure 5.1.1 Dose Response Curve For Stimulation of GTPase Activity By Adrenaline

Specific GTPase activity was assayed in the presence of 0.6 µM GTP , 1µM propranolol and increasing concentrations of adrenaline. Activity is expressed as a percentage stimulation of the basal specific GTPase activity. Basal specific activity was 27.59 +/- 1.89 pmoles GTP hydrolysed min^{-1} mg^{-1}.

Values shown are mean +/- SEM values for n=5 experiments (3 independent blood donors).

Figure 5.1.1 Dose Response Curve For Stimulation of GTPase Activity By Adrenaline

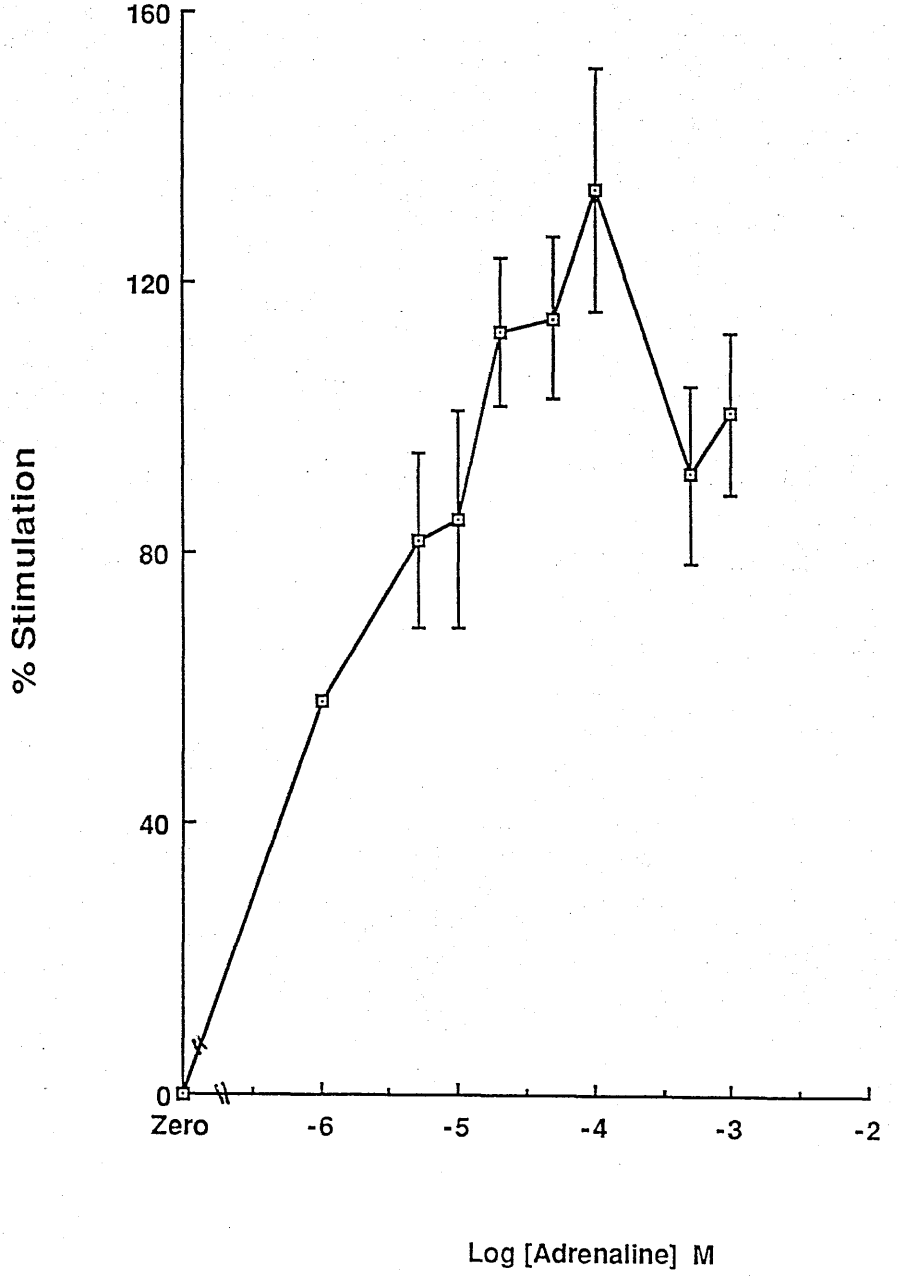


Figure 5.1.2 Dose response curve for GTPase stimulation By PGE1

Specific GTPase activity was assayed in the presence of 0.6 μM GTP and increasing concentrations of PGE1. Activity is expressed as a percentage stimulation of the basal specific GTPase activity. Basal specific activity was 27.59 +/- 1.89 pmoles GTP hydrolysed min^{-1} mg^{-1}. Values shown are mean +/- SEM values for n=5 experiments (3 independent blood donors).

Figure 5.1.2 Dose Respomse Curve For Stimulation of GTPase Activity By PGE1

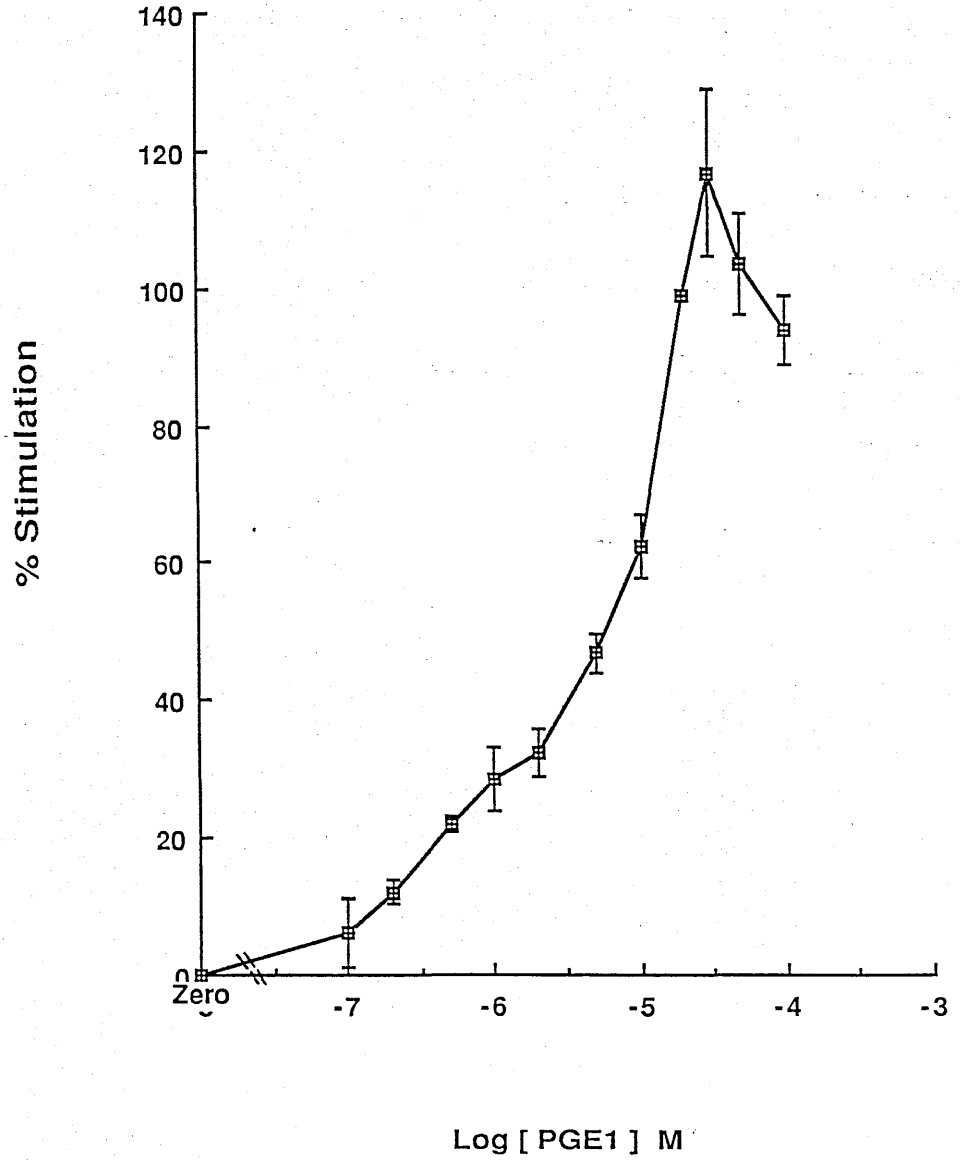
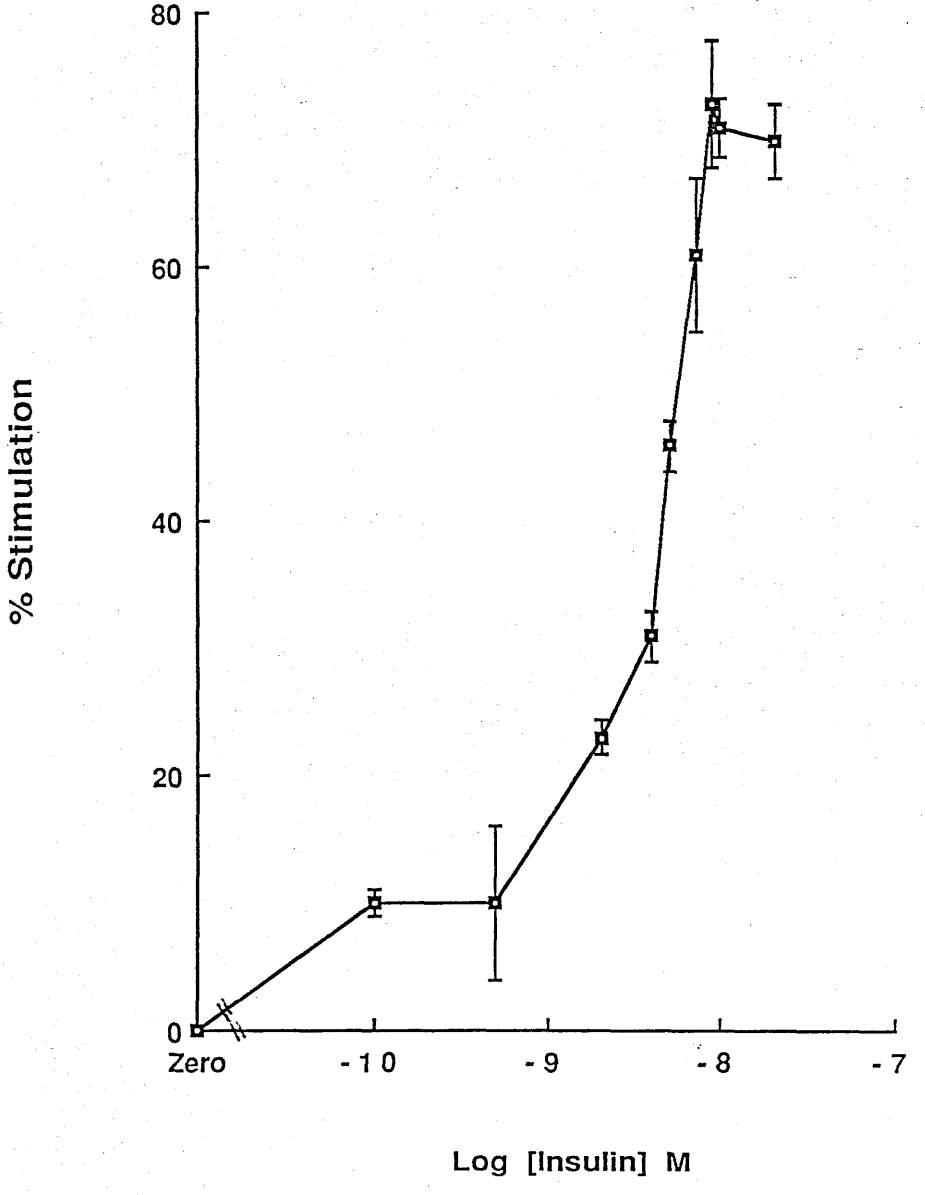


Figure 5.1.3 Dose Response Curve For Stimulation of GTPase Activity By Insulin

Specific GTPase activity was assayed in the presence of 0.6 μM GTP and increasing concentrations of insulin. Activity is expressed as a percentage stimulation of the basal specific GTPase activity. Basal specific GTPase activity was 27.59 +/- 1.89 pmoles GTP hydrolysed min^{-1} mg^{-1}.

Values shown are mean +/- SEM values for n=5 experiments (3 independent blood donors).

Figure 5.1.3 Dose Response Curve For Stimulation of GTPase Activity By Insulin



5.2 Effect of GTP Concentration On Ligand Stimulation of Specific GTPase Activity

Figure 5.2.1 Effect of GTP concentration On Adrenaline Stimulation of GTPase Activity

Specific GTPase activity was assayed in the presence of 0.1mM adrenaline, 1μM propranolol and increasing concentrations of GTP. Activity is expressed as a percentage stimulation of the basal specific GTPase activity at each corresponding GTP concentration.
Values shown are mean +/- SEM values for n=5 experiments (3 independent blood donors).

Figure 5.2.1 Effect of GTP Concentration On Adrenaline Stimulation of GTPase Activity

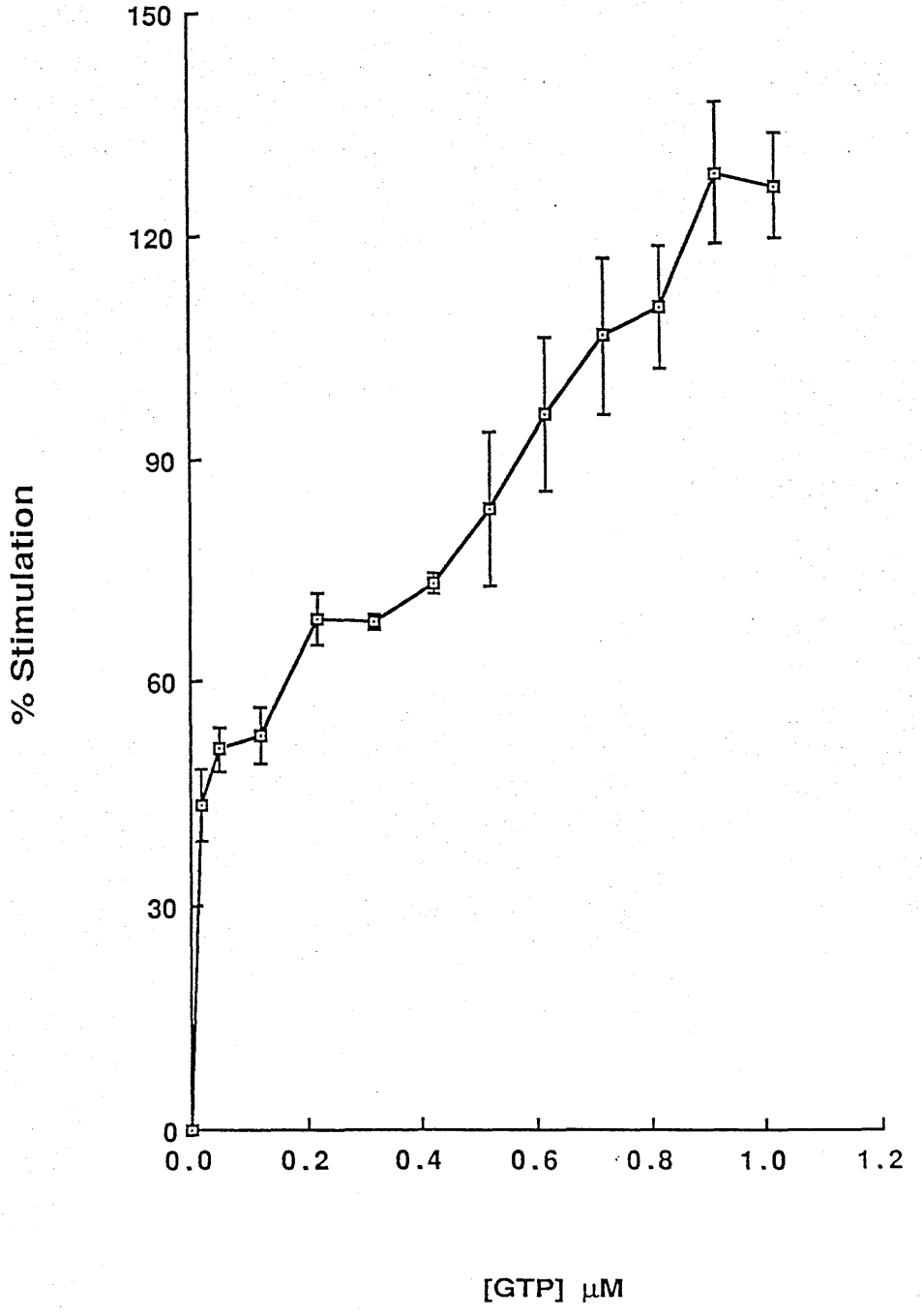


Figure 5.2.2 Effect of GTP concentration On PGE1 Stimulation Of GTPase Activity

Specific GTPase activity was assayed in the presence of 20 μM PGE1 and increasing concentrations of GTP. Activity is expressed as a percentage stimulation of the basal specific GTPase activity at each corresponding GTP concentration.

Values shown are mean +/- SEM values for n=5 experiments (3 independent blood donors).

Figure 5.2.2 Effect of GTP Concentration On PGE1 Stimulation of GTPase Activity

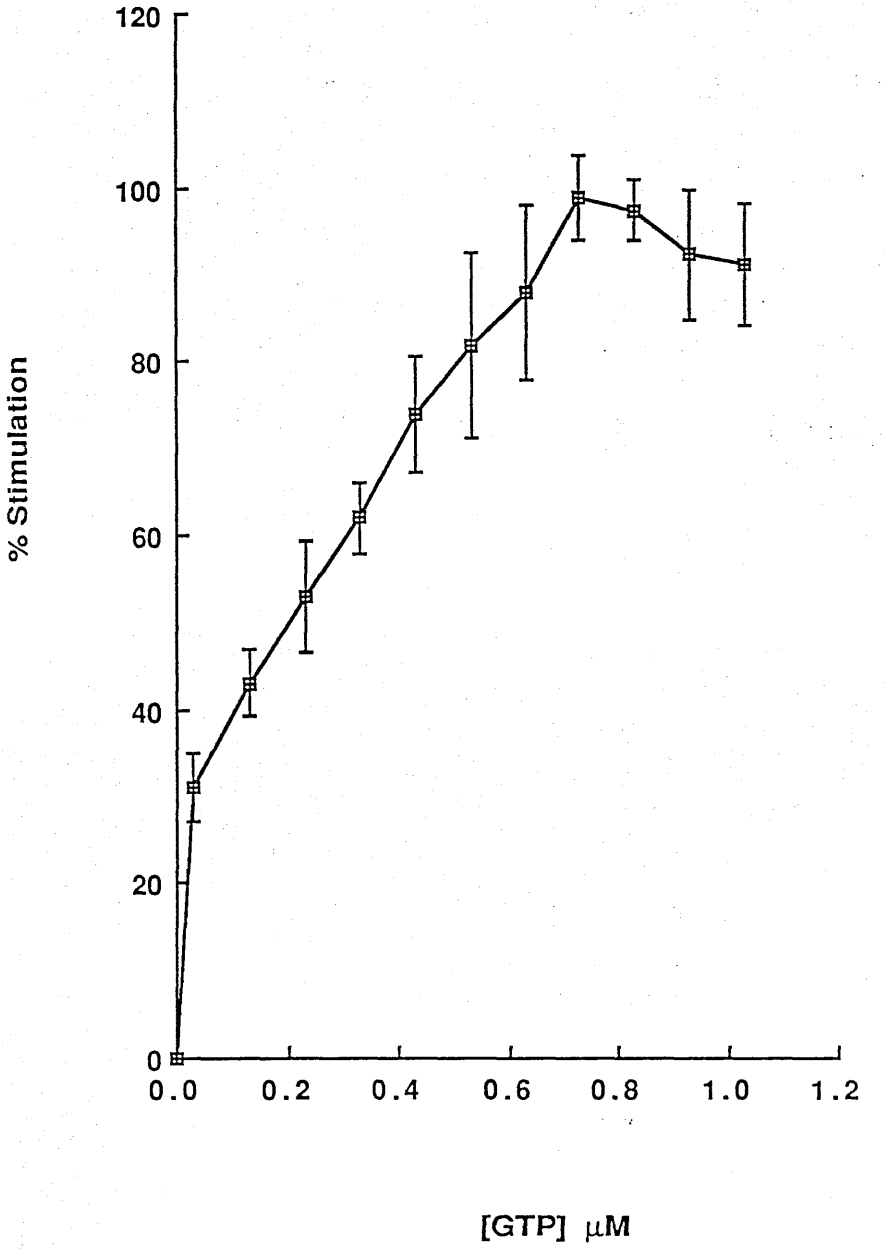
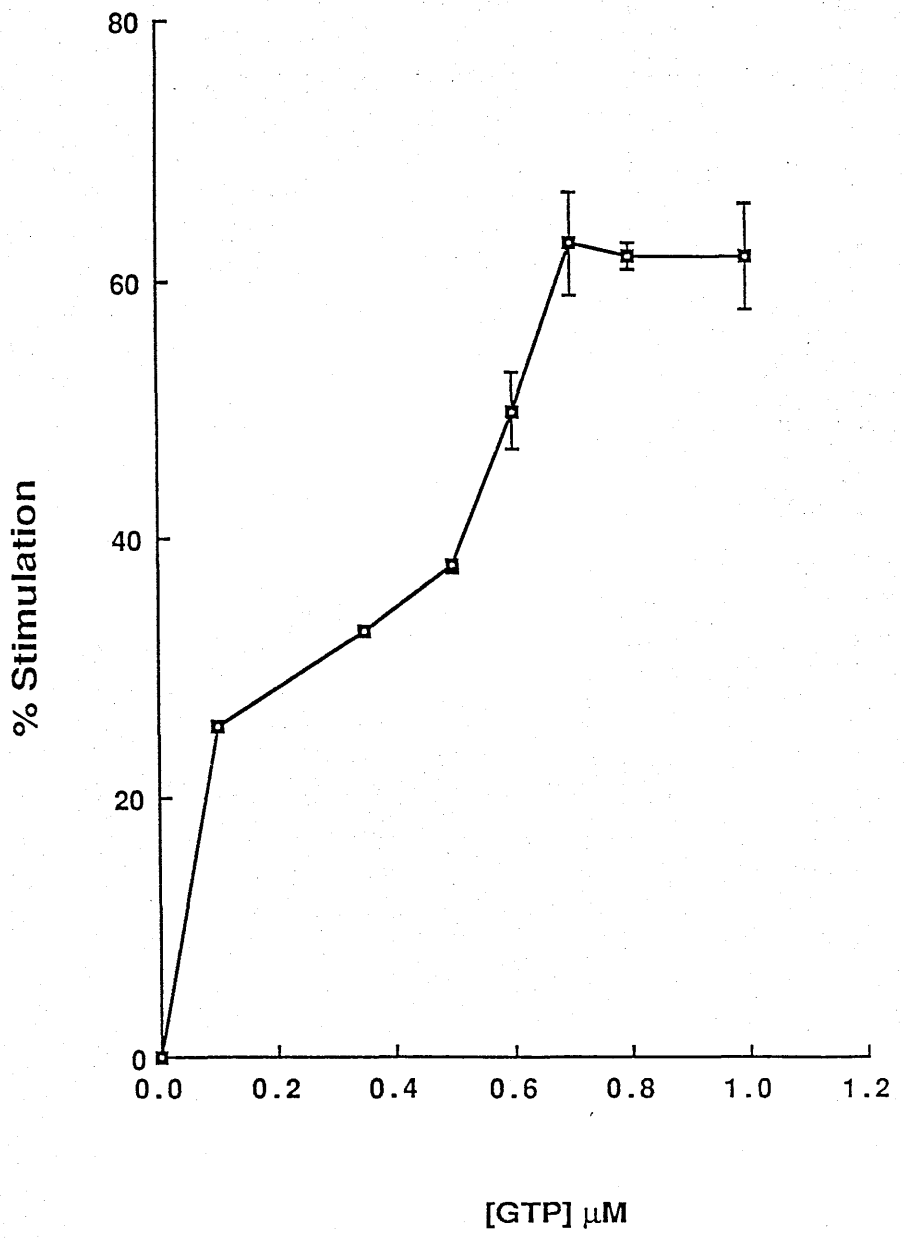


Figure 5.2.3 Effect of GTP concentration On Insulin Stimulation of GTPase Activity

Specific GTPase activity was assayed in the presence of 10nM Insulin and increasing concentrations of GTP. Activity is expressed as a percentage stimulation of the basal specific GTPase activity at each corresponding GTP concentration.

Values shown are mean +/- SEM values for n=5 experiments (3 independent blood donors).

Figure 5.2.3 Effect of GTP Concentration On Insulin Stimulation of GTPase Activity



5.3 Kinetic Analysis of Ligand Stimulated Specific GTPase Activity

Figure 5.3.1 Lineweaver - Burke Plot For Control Samples

Specific GTPase activity was assayed as described in "Additional Methods " section in the presence of increasing concentrations of GTP, but in the absence of receptor ligands.

Estimated Km and Vmax values are given in table 5. Values shown are mean +/- SEM values for n=6 experiments (3 individual blood donors).

Figure 5.3.1 Lineweaver - Burke Plot For Control Samples

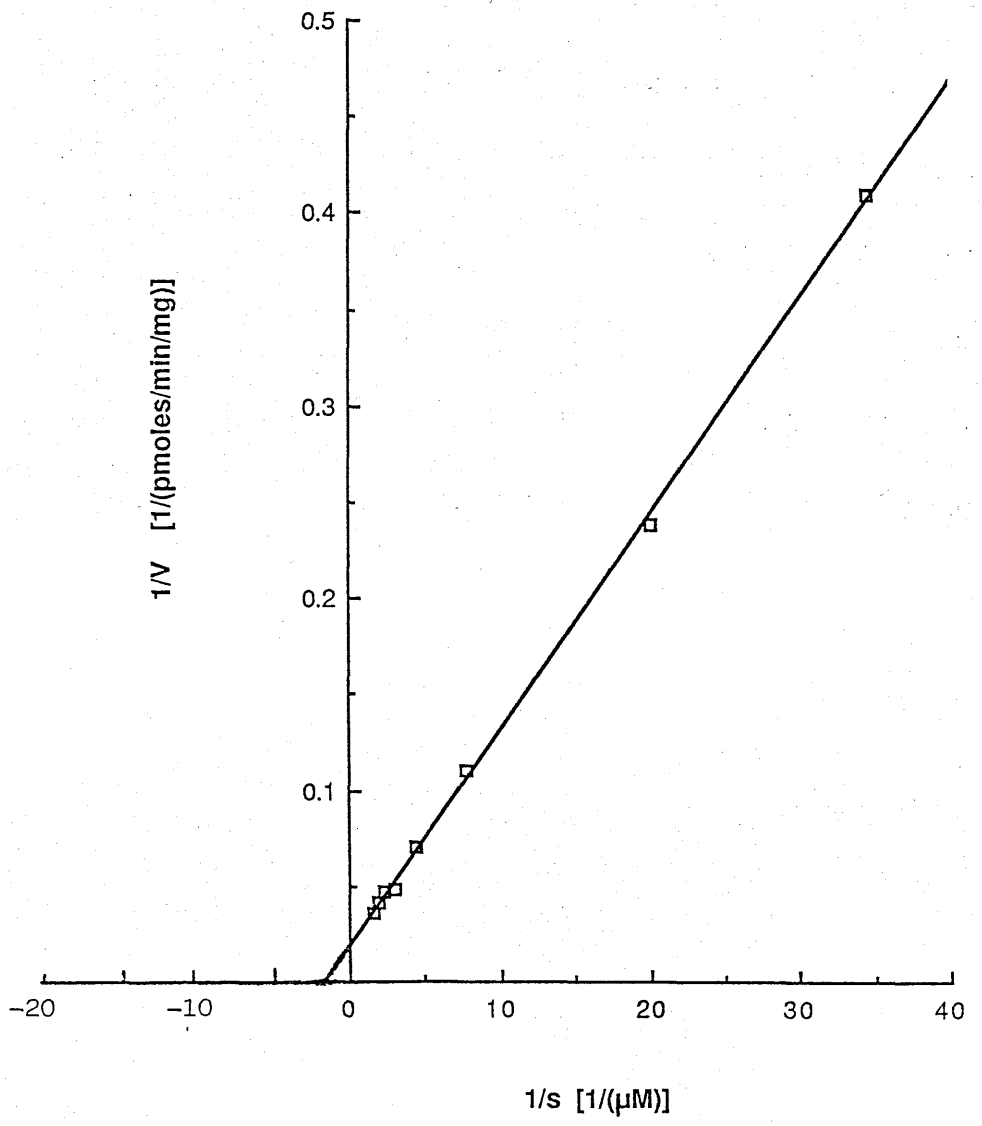


Figure 5.3.2 Lineweaver - Burke Plot For Adrenaline Treated Samples

Specific GTPase activity was assayed as described in "Additional Methods " section in the presence of 100 uM adrenaline , 1 μM propranolol and increasing concentrations of GTP .

Estimated Km and Vmax values are given in table 5. Values shown are mean +/- SEM values for n=6 experiments (3 individual blood donors).

Figure 5.3.2 Lineweaver- Burke Plot For Adrenaline Treated Samples

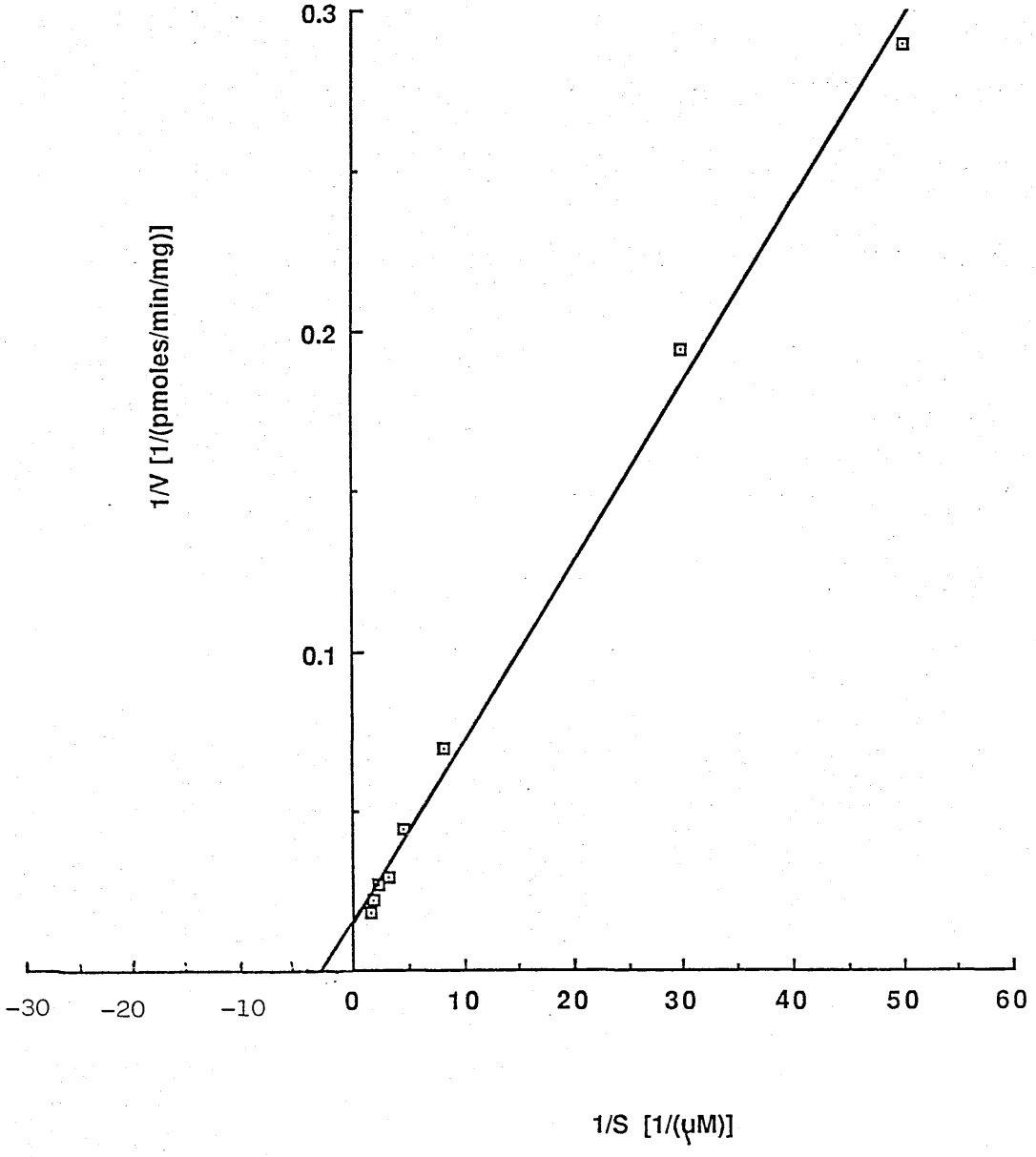


Figure 5.3.3 Lineweaver - Burke Plot For PGE1 Treated Samples

Specific GTPase activity was assayed as described in "Additional Methods " section in the presence of 20 μM PGE1 and increasing concentrations of GTP.

Estimated Km and Vmax values are given in table 5. Values shown are mean +/- SEM values for n=6 experiments (3 individual blood donors).

Figure 5.3.3 LIneweaver -Burke Plot For PGE1 Treated Samples

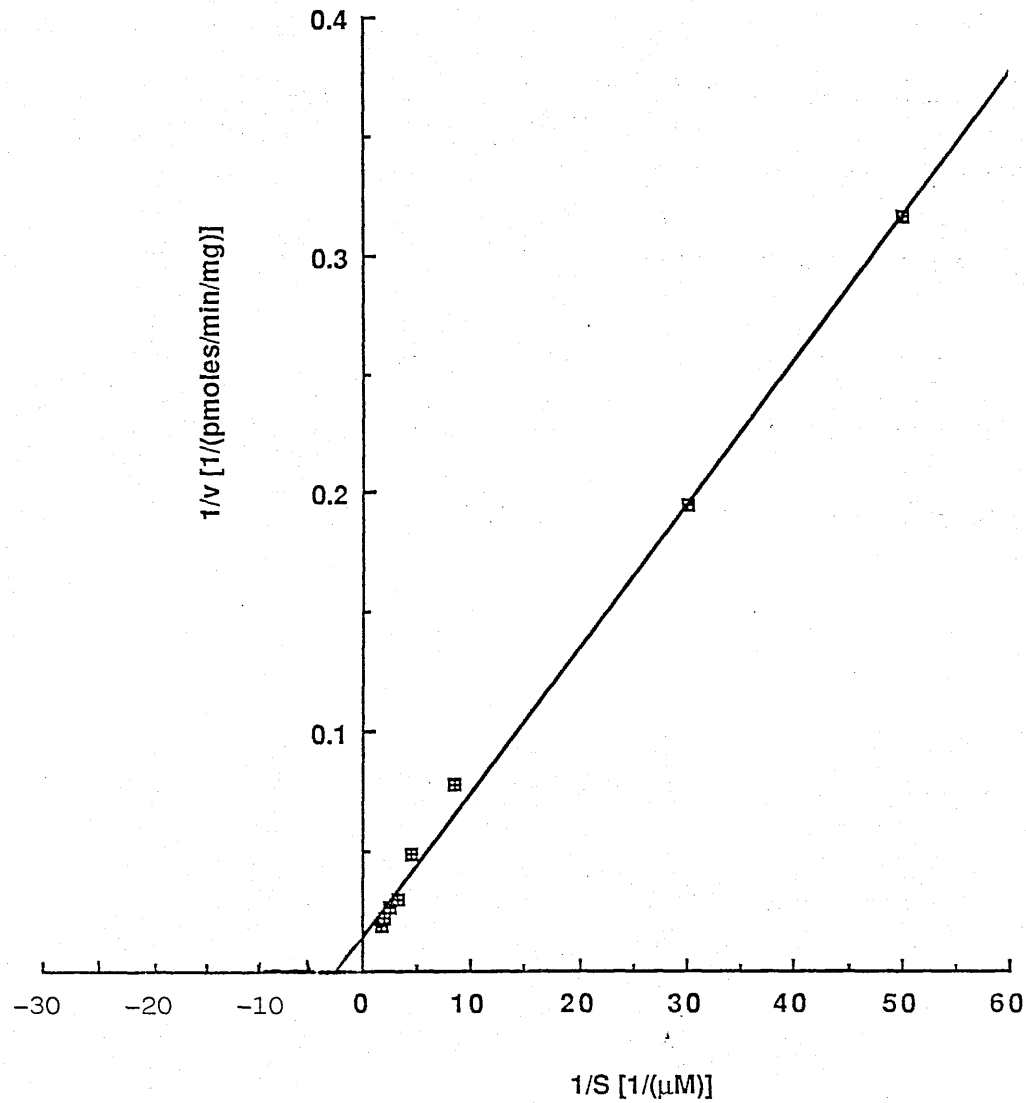


Figure 5.3.4 Lineweaver - Burke Plot for Insulin Treated Samples

Specific GTPase activity was assayed as described in "Additional Methods " section in the presence of 10 nM insulin and increasing concentrations of GTP.

Estimated Km and Vmax values are given in table 5. Values shown are mean +/- SEM values for n=6 experiments (3 individual blood donors).

Figure 5.3.4 Lineweaver-Burke Plot For Insulin Treated Samples

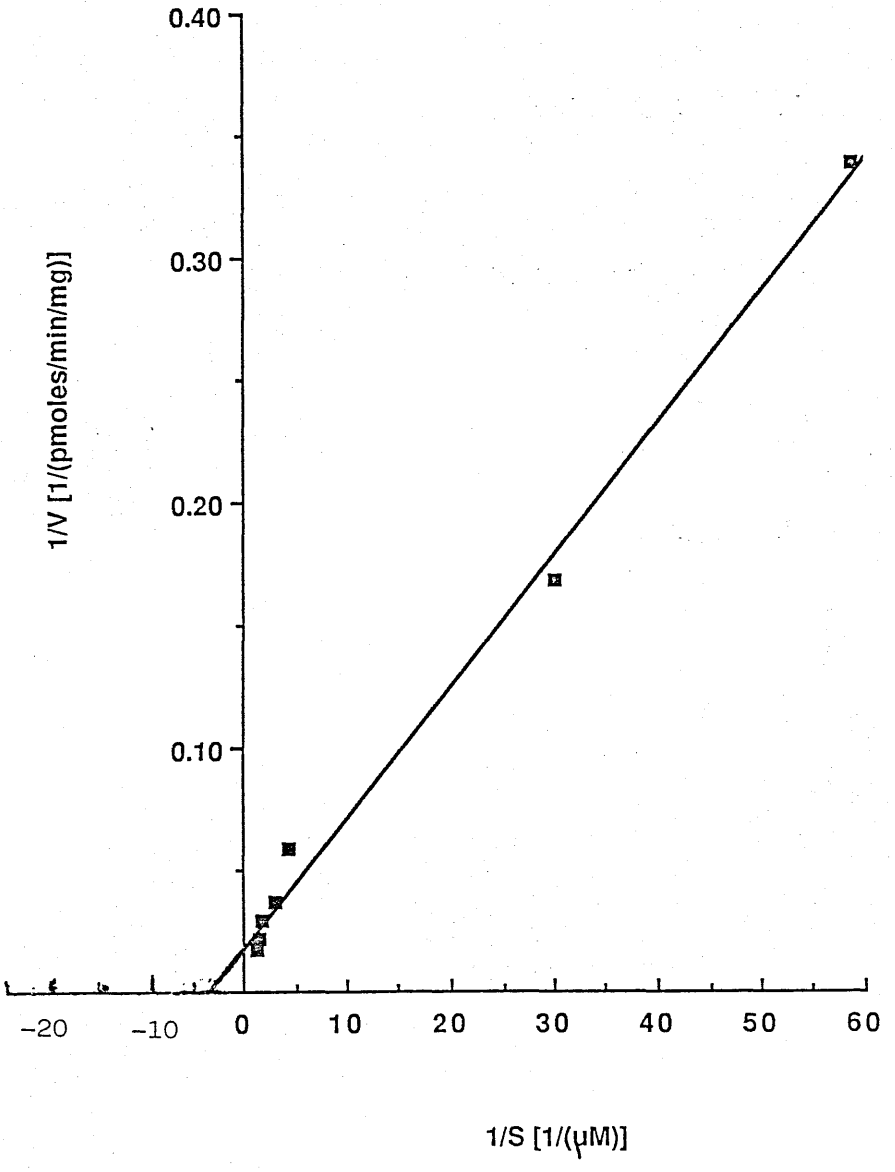


Table 5 Kinetic Analysis of High Affinity GTPase Activity In Human Platelet Membranes

Treatment	Lineweaver-Burke		Eadie-Hofstee	
	Km	Vmax	Km	Vmax
Control	0.6	53	0.6	52
Adrenaline/ Propranolol	0.4	66	0.5	82
PGE_1	0.4	66	0.6	85
Insulin	0.3	59	0.4	66

Km units are μM and Vmax units are pmoles min^{-1} mg^{-1} .
Drug concentrations are as outlined in "additional methods".

5.4 Evidence For Insulin Activation of A Novel G Protein

Table 6 Stimulation of GTPase Activity Using Drug Combinations

Drug combination	Observed Stimulation	Expected Stimulation	Additivity Ratio
Isoproterenol	18 +/- 3%	-	-
Adrenaline/ Propranalol	135 +/- 10%	-	-
Insulin	58 +/- 7%	-	-
PGE_1	94 +/- 6 %	-	-
Iso.+Adren/prop.	137 +/- 10%	153 %	0.9
Iso. + Insulin	70 +/- 5 %	76 %	0.9
Iso. + PGE_1	52 +/- 3 %	112 %	0.5
Adren./Prop. + Insulin	200 +/- 15 %	193%	1.0
Adren./Prop.+ PGE_1	250 +/- 10%	229%	1.1
Insulin+PGE_1	135 +/- 16%	152%	0.9
Insulin + PGE_1 + Adren./Prop.	245 +/- 18%	287%	0.8

Abbreviations used above:
Iso. (isoproterenol) ; Adren. (adrenaline)

Drug concentrations are as detailed in "Additional Methods".
(n=6 experiments, 3 blood donors).

Table 7 Cholera Toxin And Pertussis Toxin Pretreatment of Human Platelet Membranes

Hormone / Ligand	No Treatment (%) Stimulation	+ Cholera Toxin (%) Stimulation	+ Pertussis Toxin (%) Stimulation
PGE_1	98 +/- 5	26 +/- 3	101 +/- 13
Adrenaline/propranolol	124 +/- 15	111 +/- 13	55 +/- 3
Insulin	62 +/- 4	6 +/- 2	54 +/- 4
Vasopressin	34 +/- 2	26 +/- 3	31 +/- 2

Values shown are mean +/- SEM values for n=6 experiments (3 blood donors).
Experimental details are given in "Additional Methods" section.

Discussion of Results

High affinity GTPase activity was observed in human platelet membranes. This activity was stimulated by the drugs isoproterenol, adrenaline, PGE1, vasopressin and insulin . Isolproterenol and PGE1 are agonists of Rs type receptors which activate Gs (Lester et al 1982; Aktories et al 1982). Adrenaline has the ability to bind to both α_2 and β- adrenergic receptors. α_2 receptors are able to activate Gi (Jakobs et al 1985) and β- adrenergic receptors activate Gs Brandt (et al 1983) . Hence, in order to stimulate only Gi activity with this ligand, it is necessary to use the β "blocker" or antagonist propranolol.

Dose - response curves were constructed for adrenaline, PGE1 and insulin stimulated GTPase activity (figures 5.1.1 - 5.1.3). This allowed estimation of the saturating concentration (that is the concentration of ligand required to fully activate their specific G protein) for each ligand. These ligand concentrations were then used to estimate the affinity of each stimulated GTPase activity for the substrate GTP. Two reciprocal plots ; the Lineweaver-Burke and Eadie - Hofstee plots were derived and Km and Vmax values were estimated in the presence and absence of receptor agonists (figures 5.3.1 - 5.3.4 , also table 5).

It can be concluded that adrenaline, PGE1 and insulin all increase the Vmax value when compared to the control (no agonist) state by about 40%, 40% and 20% respectively. Although,

the estimated Km values were all of comparable magnitude (0.4-0.6μM). These estimated values are also of comparable magnitude to those estimated for Gi and Gs by other workers using platelet membranes (Lester et al 1982; Avdonin et al 1985).

Also, when the effect of combinations of ligands upon GTPase activity was examined (table 6) , it is clearly observable that when insulin was present in combination with adrenaline/propranolol, or PGE1 or vasopressin, this hormone stimulated a GTPase activity distinct from those stimulated by each of the other drugs. Only the combination of isoproterenol and PGE1 exhibited an additivity ratio equal to 0.5 and this is of course because both ligands stimulate the same G protein Gs.

Finally, when the effect of pertussis toxin and cholera toxin pretreatment was investigated, it was found (table 7) that pertussis toxin reduced only adrenaline / propranolol stimulated GTPase activity to any significant extent (56 %). However, cholera toxin reduced both PGE1 and insulin stimulated GTPase activity by 73 % and 90 % respectively.

In summary therefore, it is proposed that insulin has the ability to stimulate a high affinity GTPase activity in human platelet membranes which has an estimated Km for GTP of 0.4 μM. The insulin stimulated GTPase activity is both insulin and GTP concentration dependent ; maximal stimulation of GTPase activity occurs at an insulin concentration of 10nM. The insulin concentration which elicits 1/2 maximal stimulation of this GTPase activity (at a GTP concentration of 0.6μM) is estimated to

be 5nM. The GTPase activity stimulated by insulin appears to be distinct from that stimulated by ligands which activate Gi, Gs and the putative Gp . It is proposed therefore that insulin stimulates a distinct G protein whose activity is unaltered by pertussis toxin pretreatment, but is significantly reduced by cholera toxin pretreatment.It is therefore suggested that this G protein has the characteristics which were observed for the putative Gins G protein (Heyworth et al 1985). There is no evidence for insulin stimulated GTPase activity associated with the G protein Gi.

6. Conclusions Drawn

Animal models of diabetes have been used to try to gain an insight into possible G protein alterations occurring in the diabetic state. In order to assess G protein function, the adenylate cyclase effector system has been studied. This effector system not only allowed the characterization of Gs and Gi, but also presented an opportunity to study a rapid, short term effect of insulin.

Insulin's ability to inhibit adenylate cyclase activity is a guanine nucleotide dependent process and therefore may well involve one or more G proteins. An important aim of the project was to attempt to elucidate the mechanism by which insulin has the ability to mediate this action . G protein function in normal and diabetic states was characterized on the basis of G protein interactions with the adenylate cyclase effector system.

As detailed discussion sections have been included at the end of each results chapter, only a brief summary of conclusions drawn from the data will now be presented :

The results obtained suggest that Gi function in both type I and type II diabetic animal models is abolished. Quantification of Gi in hepatocyte membranes prepared from each animal type suggested that hepatic Giα subunits were not present to any appreciable extent in type I diabetic animals.However, Gi α was present at levels comparable to control animals in the type II diabetic preparations. It is therefore proposed that loss of hepatic Gi function in type I diabetic animals reflects

the lack of Gi α expression in these cells. However, loss of hepatic Gi function in type II diabetic preparations reflects the occurrance of a covalent modification of Gi which leads to attenuation of this G proteins acivity. It is suggested that hepatic Gi α expression in type I diabetic animals is controlled either directly or indirectly by circulating plasma insulin concentration. It is also possible that loss of Gi function in the type II diabetic animals may be a result of elevated plasma insulin levels.

Use of the hypoglycaemic drug , Metformin, indicated that Gi function was not involved in insulin mediated inhibition of adenylate cyclase activity. Indeed, oral administration of this drug to control animals resulted in the attenuation of Gi function, but enhanced insulin's ability to inhibit adenylate cyclase activity. This action of insulin was abolished in type I diabetic preparations and reduced in preparations from type II diabetic animals, although Gi function in both these animal types was abolished. Metformin restored this action of insulin in the type I diabetic state and enhanced this action in the type II diabetic condition. The mechanism by which metformin restores or enhances this action is unknown , but it is unlikely that this drug mediates these effects by directly interacting with the membrane or the adenylate cyclase system as the effects observed are selective. Tables 2 and 3 demonstrate that this drug has no significant effect upon the function of Gs or C. Also, incubation of hepatocytes in the presence of this drug at concentrations

comparable to those orally administered to animals resulted in the abolition of basal adenylate cyclase activity. When the structure of this compound is considered, this non-specific effect upon the adenylate cyclase system may well reflect the intercalation of the drug into the plasma membrane. It is therefore suggested that this drug may be metabolized in the gut of these animals and it may be a modified form of this drug which elicits these effects. However,lower concentrations of the drug have been used by other workers in cell culture studies and post-receptor effects have been suggested. It is therefore possible that the drug may have the ability to enter the cells and induce its effects intracellularly. However, it does appear that this drug displays some aspects of selectivity with respect to the adenylate cyclase system and perhaps these are important in diabetic therapy. In addition, this drug provided evidence that Gi function and insulin's ability to inhibit adenylate cyclase activity are unrelated.

Further evidence to support this theory was obtained using the phorbol ester TPA. This compound abolished Gi function prior to the abolition of this insulin action. However, using the bacterial toxin ,pertussis toxin, both Gi function and insulin mediated inhibition of adenylate cyclase activity was removed. It is proposed that pertussis toxin ribosylates Giα subunits and thereby promotes the Gi ($\alpha\beta\gamma$) holomeric state. Hence, if insulin binding to its receptor activated a G protein it could stimulate the liberation of $\beta\gamma$ subunits from an ($\alpha\beta\gamma$)

holomeric complex. If these Bγ subunits associated with Gs α subunits , then the glucagon (Gs) stimulated adenylate cyclase activity would be "turned off" as the inactive (αB γ) complex prevails. Thus, if insulin inhibited glucagon stimulated adenylate cyclase activity by a Bγ reassociation process, then pertussis toxin pretreatment would promote Bγ subunit association with Giα, prior to Gs subunit reassociation.

Evidence to support this theory comes from studying glucagon's ability to stimulate adenylate cyclase activity in each of the control and diabetic states. It appears that enhanced maximal glucagon stimulation occurs in type I, but not type II diabetic animals. This enhanced glucagon stimulated condition is induced if control hepatocytes are preincubated with pertussis toxin. Therefore it is proposed that under these conditions, pertussis toxin promotes Bγ reassociation with free Gi α subunits. Resultantly this would remove inhibitory input into the adnylate cyclase system and Gs activation would be enhanced. This enhanced stimulatory response may only be apparent at high stimulatory ligand concentrations, after all, Gi inhibitory input is only observable when the catalytic unit's activity is elevated (for example, by the use of forskolin).

It is also suggested that a modified form of Giα could be unable to associate with Bγ subunits. This would explain the inability of pertussis toxin to induce an enhanced glucagon stimulated state observed in controls. Therefore, it is suggested that insulin mediates the inhibition of glucagon stimulated

adenylate cyclase activity by the release of B γ subunits from a G protein distinct from Gi.

Finally, in chapter 5 , I have presented evidence for insulin having the ability to promote stimulation of a high affinity GTPase activity distinct from Gs, Gi and the putative Gp in human platelet plasma membranes. This stimulation of GTPae is abolished by cholera toxin preteatment, but remains unaltered by pertussis toxin pretreatment. It is therefore suggested that this GTPase activity could be associated with the putative G protein Gins because this GTP binding protein is cholera toxin, but not pertussis toxin ribosylated.

It was unfortunate that this GTPase work could not be undertaken using the rat platelet system. When membranes prepared from rat platelets were prepared and incubated with ligands which are known to stimulate GTPase activity associated with Gs or Gi, no high affinity GTPase stimulation in this system was observable. The reason for this is not known, but perhaps different receptor populations (or numbers) exist in the rat platelet system.

Therefore, in summary, it is proposed that insulin inhibits adenylate cyclase activity by activating a G protein distinct from Gi, Gs and Gp. Upon activation of this G protein, B γ subunits are released and attenuation of Gs stimulation of the catalytic unit is achieved. In the diabetic state, Gi function is absent. In the type I diabetic model, insulin is unable to inhibit adenylate cyclase activity. In the type II

diabetic model this action of insulin is present , but reduced. Gs function remains unaltered in both diabetic states.

It is proposed that alterations in G protein function may reflect post-receptor defects in the diabetic state. In particular, a G protein, distinct from Gs, Gi and Gp, may be important in insulin action. This G protein's activity also appears to be reduced in the diabetic state. The attenuation of this G protein's activity may be reversed upon biguanide therapy.

References

Aktories, K.,Hungerer, K.D., Robbel, L. & Jakobs, K.H. (1984), Proc. Intern. Congr. Pharmacol., London 1984, Abstr.1655,Macmillan, London.

Aktories, K., Schultz, G., & Jakobs, K.H. (1982), Biochim. Biophys. Acta. 719, 58-64

Aktories, K., Schultz, G., & Jakobs, K.H. (1983), Mol. Pharmacol. 24, 183-188

Amatruda, J.M., & Roncone, M. (1985), Biochem. Biophys. Res. Comm. 129, 163-170

Arad, H., Rosenbusch, J.P. & Levitzki., A.(1984), Proc. Natl. Acad. Sci. (USA) 81, 6579-6583

Asano, T. , Ogasawara, M., Kitajima, S. & Sano, M. (1986), FEBS Letters 203, 135-138

Assoian, R.K. & Tager, H.S. (1981), J. Biol. Chem. 256, 4042-4049

Avdonin, P.V., Svitua-Ullitiua, I.V. & Kulikov, V.I. (1985), Biochem. Biophys. Res. Comm. 131, 307-313

Avruch, J.,Nemenoff, R.A., Blackshear, P.J., Pierce, M.N. & Osathanondh, R. (1982), J. Biol.Chem. 257, 15162-15166

Baldwin, S.A. & Lienhard, G.E. (1981), Trends. Biochem. Sci. 6, 208-211

Barber, R. & Goka, T.J. (1985), J.Cyc. Nucl. Pro. Phos. Res. 10, 23-29

Baron, M.D. & Sonksen, P.H. (1983), Biochem. J. 212, 79-84

Beck-Nielsen, H., Pedersen, O. & Linskov, H.O. (1979) , Acta. Endocrinol. 90, 451-458

Berridge, M.J. (1984), Biochem.J. 220, 345-352

Berridge, M.J. (1986), Biol. Chem. Hoppe-Seyler 367, 447-456

Berridge,M.J. & Irvine, R.F. (1984), Nature (Lond). 312, 315-319

Berry, M.N. & Friend, D.S. (1969), J. Cell. Biol. 43, 506-512

Bird, S.J. & Maguire, M.G. (1978), J.Biol. Chem. 253, 8826-8834

Birnbaumer, L., Codina, J., Mattera, R., Cerione, R.A., Hildebrandt, J.D., Sunyer, T., Rojas, F., Caron, M.G., Lefkovitz, R.J. & Iyenger, R. (1985) , in "Molecular Mechanisms of Transmembrane Signalling" (Cohen,P. & Houslay,M.D. eds.) pp131-182, Elsevier Publ. Amsterdam.

Birnbaumer, L. & Iyenger, R. (1982), in Cyclic Nucleotides, Handbook of Experimental Pharmacology, Vol. 58, ed. Nathanson , J.A. & Kebabian, J.W., pp 153-183, Springer-Verlag, Berlin, Heidelberg, New York)

Birnbaumer, L., Stengel, D., Desmier,M. & Hanoune, J. (1983) , Eur. J. Biochem. 136, 107-112

Birnbaumer,L., Swartz, T.L., Abramowitz, J.Mintz, P.W. & Iyenger, R. (1980), J.Biol. Chem. 255, 3542-3551

Blackmore, P.F., Bocckino, S.B., Waynick, L.E. & Exton, E. (1985), J. Biol. Chem. 260, 14477-14483

Blazar, B.R., Whitley, C.B., Kitabchi, A.E., Tsai, M.Y., Santiago, J., White, N., Stentz, F.B. & Brown, D.M. (1984), Diabetes 33, 1133-1137

Bokoch, G.M., Katada, T., Northup, J.K., Ui, M. & Gilman, A.G. (1984), J.Biol. Chem. 259, 3560-3567

Bollag, G.E., Roth, R.A., Beaudoin, J., Mochly-Rosen, D. & Koshland, D.E. (1986), Proc. Natl. Acad. Sci. (USA) 83, 5822-5824

Bourne, H.R., Coffino, P. & Tomkins, G.M. (1975), Sci. 187, 750-752

Bradford,P.G. & Rubin, R.P. (1986), Biochem. J. 239, 97-102

Brandt,D.R., Asano,T., Pedersen, S.E. & Ross, E.M. (1983), Biochemistry, 22, 4357-4362

Bray,G.A.(1977) Fed.Proc. 36, 148-153

Brown,B.l., Albano,J.D.M., Ekins,R.P., Sqherzi, M.A. & Tampion, W. (1972), Biochem. J. 121, 561-563

Ca snellie, J.E., Harrison, M.L., Pike,L.J., Hellstrom, K.E. & Krebs, E.G. (1982), Proc. Natl. Acad. Sci. (USA) 79, 282-286

Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kiddawa,U. & Nishizuka, Y., (1982), J. Biol. Chem. 257, 7847-7851

Cassel, D. & Selinger, Z. (1976), Biochim. Biophys. Acta. 452, 538-551

Cerione, R.A., Codina, J., Benovic, J.L., Lefkovitz, R.J., Birnbaumer,L. & Caron , M.G. (1984), Biochemistry 23, 4519-4525

Chamdramouli, V., Williams, S., Marshall, J.S. & Carter, J.R. (1977), Biochim.Biophys.Acta. 465, 19-33

Ciaraldi, T.P., Kolterman, O.G. & Olefsky, J.M. (1981), J.Clin. Invest. 68, 875-880

Clark, R.B., Goka, T.J., Green, D.A., Barber, R. & Butcher, R.W. (1982), Mol. Pharmacol. 22, 609-613

Clark,R.B., Palmer, C.J. & Shaw,W.N., (1983), Proc. Soc. Expt.Biol. Med. 173, 68-75

Clausen, T., Elbrink, J. & Martin, B.R. (1974), Acta. Endocrinol. 77, Supl.191, 137-143

Codina, J., Hildebrandt, J.D., Sekura, R.D., Birnbaumer,M., Bryan, J., Manclark,C.R., Iyenger, R. & Birnbaumer, L. (1984), J. Biol. Chem. 259, 58712-5886

Colca, J.R., Kotagal,N., Brooks, C.L., Lacy, P.E., Landt, M. & Mcdaniel, M.l. (1983), J. Biol. Chem. 258, 7260-7263

Corin, R.E. & Donner, D.B. (1982), J. Biol. Chem. 257, 104-110

Coussen,F., Guermah, M., D'Alayer, J., Monneron, A., Haiech, J. & Cavadore, J-C (1986), FEBS letters 206, 213-217

Creba, J.A., Downes, C.P., Howkins,P.T., Brewster, G., Michell,R.H. & Kirk, C.J. (1983), Biochem. J. 212, 733-747

Cuatrecasas, P. (1973) Biochemistry 12, 1312-1322

Cuatrecasas, P. (1974), Ann. Rev. Biochem. 43, 169-214

Cuatrecasas, P. & Tell, G.P.E. (1973) Proc. Natl. Acad. Sci. (USA) 70, 485-589

Curry,D.l. & Stern, J.S. (1985), Metabolism 34, 791-796

Cushman, S.W. & Wardala, L.J. (1980), J. Biol. Chem. 255, 4758-4762

Czech, M.P. (1977) , Ann. Rev. Biochem. 46, 359-384

Czech, M.P. (1980), Diabetes 29, 399-409

Czech,M.P., Freeman, R.B., Caro, J.F. & Armatruda, M. (1980), Biochem. J. 188, 839-845

Czech, M.P., Massague, J. & Pilch, P.F. (1981), Trends. Biochem. Sci. 6, 222-225

Davis, R.J., Corvera, S. & Czech, M.P. (1986), J. Biol. Chem. 261, 8708-8711

De Meyts P., Bianco, A.R. & Roth, J. (1976), J. Biol. Chem. 251, 1877-1888

Denton,R.M., Brownsey,R.W. & Belsham,G.L. (1981), Diabetologia 21, 347-362

DeTorrontegue, G. & Berthat, J. (1966), Biochim. Biophys. Acta. 116, 477-481

Downes, C.P. & Michell, R.H. (1985), in "Molecular Mechanisms of Transmembrane Signalling " (Cohen,P. & Houslay, M.D. eds.) pp 3-56 , Elsevier Sci. Publ. Amsterdam

Dustin, M.L., Jacobson, G.R. & Peterson, S.W. (1984), J. Biol. Chem. 259, 13660-13663

Eaton, R.P., Oase, R. & Schade, D.S. (1976), Metabolism 25, 245-249

Ebina, Y., Edery, M., Ellis, L., Standring, D., Beaudoin, J., Roth, R.A. & Rutter, W.J. (1985a), Proc. Natl. Acad. Sci. (USA) 82, 8014-8018

Ebina, Y., Ellis, L., Jarnagin, K., Edery, M., Graf, L., Clauser,E., Ou,J.H., Masairz,F., Kan, Y.W., Goldfine,I.D., Roth,R.A. & Rutter, W.J. (1985b) , Cell 40, 747-758

Ek,B., Westernmark, B., Wasterson,A. & Heldin,C.K. (1982), Nature (Lond) 295, 419-421

Elliot, K.R.F., Ash, R., Pogson,C.I., Smith, S.A. & Crisp, D.M. (1976) in "Use of Isolated Liver Cells & Kidney Tubules In Metabolic Studies" (Tager, J.M., Sohing, H-D. & Williamson, J.R. eds.) pp139-143 North-Holland, Amsterdam

Ellis,L., Clauser,E., Morgan, D.O., Edery, M., Roth, R.A. & Rutter, W.J. (1986), Cell 45, 721-732

Evans, T., Brown, M.L., Fraser, E.D. & Northup, J.K. (1986), J. Biol. Chem. 261, 7052-7059

Fain, J.N., Brindley,D.N., Pittner, R.A. & Hawthorne, J.N. (1985), FEBS. Letters 192, 251-254

Farese, R.V., Kuo, J.Y., Babischkin, J.S. & Davis, J.S.(1986), J. Biol. Chem. 261, 8589-8592

Feinglos, M.N. & Lebovitz, H.E. (1978), Nature (Lond) 276, 184-185

Finn, F.M., Titus, G., Horstman, D. & Hofman, K. (1984), Proc. Natl. Acad. Sci. (USA) 81, 7328-7332

Florio, V.A. & Ross, E.M. (1983), Mol. Phamacol. 24, 195-202

Fung,B.K.K. (1983), J. Biol. Chem. 258, 10495-10502

Fujita-Yamaguchi, Y. (1984), J. Biol. Chem. 259, 1206-1211

Gammeltoft,S. (1984), Physiol. Rev. 64, 1321-1378

Gammeltoft, S. & Van-Obberghen,E., (1986), Biochem.J. 235,1-11

Gilman, A.G. (1984), Cell 36, 577-579

Goldfine, I.D., Jones, A.L., Hradet,G.T., Wong,.K.Y. & Mooney, J.S. (1978), Sci. 202, 760-763

Grandt, R., Greiner,C., Zubin, P. & Jakobs, K. (1986), FEBS. Letters 196, 279-283

Graves, C.B., Goewert, R.R. & McDonald, J.M. (1985), Sci. 230, 827-830

Graves, C.B. & McDonald, J.M. (1985), J. Biol. Chem. 260, 11286-11292

Green,D.A. & Clark, R.B. (1982), J. Cycl. Nucl.Res. 8, 337-346

Gregorian,G.Y.,Resink,T.J., Stucki, S. & Buhler, F.R. (1986), Cell Calcium 7, 261-273

Guerre-Millo, M., Lavau, M., Horne, J.S & Wardzala, L.J. (1985), J. Biol. Chem. 260, 2197-2201

Haring,H., Kirsch,D., Obermaier,B., Ermal, B. & Machicao, F. (1986) , J. Biol. Chem. 261, 3869-3875

Harmon, J.T., Kahn. C.R. , Kempner, E.S. & Schlergal, W. (1980), J. Biol. Chem. 255, 3412-3419

Harris, B.A., Robishaw, J.D., Mumby,S.M. & Gilman, A.G. (1985), Sci. 229, 1274-1277

Hedo, J.A., Kasuga, M., Van-Obberghen, E., Roth, J. & Khan, C.R. (1981), Proc. Natl. Acad. Sci. (USA) 78, 4791-4795

Hedo, J.A. & Simpson, I.A. (1985), Biochem. J. 232, 71-78

Hellenius, A., Mellman, I., Wall, D. & Hubbard, A. (1983), Trends Biochem. Sci. 8, 245-249

Helmerhorst,E., Ng,D.S., Moule, M.l. & Yip, C.C. (1986), Biochemistry, 25, 2060-2065

Hepp, D., Challoner,D.R. & Williams, R.M. (1968), J. Biol. Chem. 243, 4020-4026

*
Heyworth,C.M., Grey,A-M.,Wilson, S.R., Hanski, E. & Houslay, M.D. (1986) Biochem. J. 235, 149-154

Heyworth, C.M. & Houslay, M.D. (1983), Biochem. J. 214, 547-552

Heyworth, C.M., Wallace, A.V. & Houslay, M.D. (1983), Biochem. J. 214, 99-110

Heyworth, C.M., Whetton, A.D., Wong, S., Martin, B.R. & Houslay, M.D. (1985), Biochem. J. 228, 593-603

Hildebrandt, J. & Birnbaumr, L. (1983), J. Biol. Chem. 258, 13141-13147

Hildebrandt, J.D., Codina, J., Rosenthal,W. & Birnbaumer, L. (1985) J. Biol. Chem. 260, 14867-14872

Hidebrandt, J.D., Hanoune,J. & Birnbaumer, L. (1982), J. Biol. Chem. 257, 14723-14725

Holle, A., Dreyer,M., Kuhnau,J., Mangels, W., Maack, P., Siemers, U. & Rudiger, H.W. (1981), N.Eng. J. Med. 305, 563-566

Horn, R.S., Lysted,E., Adler, A. & Walaas, O. (1986), Biochem. J. 234, 527-533

Houslay,M.D. (1984), Trends Biochem. Sci. 9, 39-40

Houslay, M.D., Bojanic, D. & Wilson, A., (1986), Biochem. J. 234, 737-740

Houslay, M.D. & Elliot, K.R.F. (1979), FEBS. Letters 104, 359-363

Houslay, M.D., Metcalfe, J.C., Warren, G.B., Hesketh, T.R. & Smith, G.A. (1976), Biochim. Biophys.Acta. 436,489-494

Houslay, M.D. & Wakelam,M.J.O. (1986), in press

Hueckstead, T., Olefsky, J.M., Brandenberg,D. & Heidenreich, K.A. (1986), J. Biol. Chem. 261, 8655-8659

Hunter, T. (1980), Cell 22, 647-648

Hunter, T. (1985), Nature (Lond). 313, 740-741

Illiano, G., Tell, G.P. E., Siegel, M.I. & Cuatrecasas, P. (1973), Proc. Natl. Acad. Sci. (USA), 70, 2443-2447

Itoh, H., Okajima,F. & Ui, M. (1984), J. Biol. Chem. 259, 15464-15473

IYengar,R., Abramowitz, J., Bordelon-Riser, L.G., Blume, A.J. & Birnbaumer, L. (1980a), J. Biol. Chem. 255, 10312-10321

Iyengar, R., Abramovitz, J. Riser,M. & Birnbaumer, L. (1980b), J. Biol. Chem. 255, 3558-3564

Iyengar, R. (1981), J. Biol. Chem. 256, 11042-11050

Iyengar, R. & Birnbaumer, L. (1981), J. Biol. Chem. 256, 11036-11041

Jacobs,S., Hazum,E., Schnechter,T. & Cuatrecasas, P. (1979), Proc. Natl.Acad. Sci. (USA) 76, 4918-4920

Jacobs, S., Kull, F.C., Suoboda,M.E., Van Wyk, J.J. & Cuatrecasas, P. (1983), J. Biol. Chem. 258, 9581-9584

Jakobs,K.H., & Aktories, K. (1983), Biochim. Biophys. Acta. 732, 352-358

Jakobs, K.H., Aktories, K., Minuth,M. & Schultz,G. (1985), Adv. Cyc. Nucl. Pro. Phos. Res. 19, 137-150

Jakobs,K.H., Bauer,S. & Watanbe,Y. (1985), Eur. J. Biochem. 151, 425-430

Jakobs, K.H., Gehring, U., Gaugler, B., Pfeuffer,T. & Schultz, G. (1983), Eur. J. Biochem. 130, 605-611

Jakobs, K.H., Lasch,P., Minuth, M., Aktories, K. & Schultz, G. (1982), J. Biol. Chem. 257, 2829-2833

Jamieson, J.D. & Palade, G.E., (1968), J. Cell Biol. 39, 589-596

Jarett,L. & Smith, R.M., (1974), J. Biol. Chem. 249, 5195-5199

Jarvinen, Y.H., Taskinen,M.R., Kivilvoto, T.,Hilden, H., Helve, E., Koivosto, V.A. & Nikkila, E.A.(1984), J. Clin. Endocrinol. Met. 59, 1183-1191

Kadawaki,T., Kasuga, M., Akanuma, Y., Ezaki, O. & Takaku, F. (1984), J. Biol. Chem. 259, 14208-14216

Kahn,C.R., Baird, K.L., Jarrett, D.B. & Flier, J.S. (1978), Diabetes 27, suppl. 2 449-458

Kahn,C.R., Flier, J.S., Bar, R.S., Archer, J.I., Gorden, P., Martin, M.M. & Roth, J. (1976), N. Engl. J. Med . 294, 739-745

Kahn, C.R., Freychet,P., Roth, J. & Neville, D.M., (1974), J. Biol. Chem, 249, 2249-2257

Kalleher, D.J., Rashidbaig, A., Ruoho, A.G. & Johnson, G.L. (1983), J. Biol. Chem. 258, 12881-12885

Karnieli, E.,Zarnowski, M.J., Hissin, P.J., Simpson, I., Salans, L.B. & Cushman, S.W. (1981), J. Biol. Chem. 256, 4772-4777

Kasuga, M., Fujita-Yamaguchi, Y., Blithe, D.L., White, N.F. & Kahn, C.R., (1983), J. Biol. Chem. 258, 10973-10980

Kasuga, M., Karlsson, F.A. & Kahn, C.R. (1982), Sci. 215, 185-186

Kasuga, M., Zick,Y., Blithe,D.L.,Karlsson, F.A., Haring, H.V. & Kahn,C.R. (1982), J. Biol. Chem. 257, 9891-9894

Katada,T., Bokoch, G,.M., Northup, J.k., Ui., M. & Gilman, A.G. (1984), J. Biol. Chem. 259, 3568-3577

Katada,T., Gilman, A.G., Watanbe, Y., Bauer, S. & Jakobs, K.H., (1985), Eur. J. Biochem. 151, 431-437

Katada, T. & Ui. M., (1982), J. Biol. Chem. 257, 7210-7216

Kent,R.S., Delean, A. & Lefkovitz, R.J. (1980), Mol. Pharmacol. 17, 14-23

Kissebah,A.H., Hope-Gill, H., Vydelingum, N., Tulloch,B.R., Clarke, P.V. & Fraser, T.R. (1975), Lancet 1, 144-147

Klein,H.H, Freidenberg, G.R., Codera, R. & Olefsky, J.M. (1985), Biochem. Biophys. Res. Comms. 127,254-263

Kolterman, O.G., Gray, R.S.& Griffin, J. (1981), J. Clin. Invest. 68, 957-969

Labarbera,A.R., Richert,N.D. & Ryan, R.J., (1980), Arch. Biochem. Biophys. 200, 177-185

Larner, J.(1984), Trends Pharmacol. Sci. 5, 67-70

Lehninger, A.L. (1979), in "Biochemistry" (2nd edition) Worth publ. inc.

Lester, H.A., Steer, M.L. & Levitzki, A. (1982), Proc. Natl. Acad. Sci. (USA) 79, 719-723

Levitzki, A., (1986), Physiol. Rev. 65, 819-854

Limbird, L.E., & Speck, J.L. (1983), J. Cyc. Nucl. Pro. Phos. Res. 9, 191-201

Lingham, R.B., Brown, P.J., Holcombe, V. & Schreiber, C.L. (1986), Biochem. J. 236, 267-271

Livingston, J.N., Lerea, K.M., Bolinder, J. ,Kager, L. , Backman,L. & Arner, P. (1984), Diabetologia 27, 447-453

Londos, C., Salomon, Y., Lin, M.C., Harwood, J.P., Schramm, M., Wolff, J. & Rodbell, M. (1974), Proc. Natl. Acad. Sci. (USA) 71, 3087-3090

Lord, J.M., White, S.I., Bailey, C.J., Watkins, T., Fletcher, R.F. & Taylor, K.G. (1983), Brit. Med. J. 1-5

Lowry, O.H., Rosenburgh, N.J., Farr, A.L. & Randall, R.J. (1951), J. Biol. Chem. 193, 265-275

Macara,I.G., Marinetti, G.V. & Balduzzi, P.C. (1984), Proc. Natl. Acad. Sci. (USA) 81, 2728-2732

Machicao, E. & Wieland, O.H. (1984), FEBS. Letters 175, 113-116

MacIntyre, D.E. & Pollock, W.K. (1983), Biochem. J. 121, 433-437

Maguire, M.E., Van Arsdale, P.M. & Gilman, A.G. (1976), Mol. Pharmacol. 12, 335-339

Maloff, B.L. & Lockwood, D.H. (1981), J. Clin. Invest. 68, 85-90

Manning, D.R. & Gilman, A.G. (1983), J. Biol. Chem. 258, 7059-7063

Marchmont, R.J., Ayad, S.R. & Houslay, M.D. (1981), Biochem. J. 195, 645-652

Marshall,S., Green,A. & Olefsky, J.M. (1981), J. Biol. Chem. 256, 11464-11470

Massague,J., Pilch,P.F. & Czech,M.P.(1980), Proc. Natl. Acad. Sci. (USA) 77, 7137-7143

Massague,J., Pilch, P.F. & Czech, M.P. (1981), J.Biol. Chem. 256, 3182-3190

Maxam,A.M. & Gilbert, W. (1980), Meth. Enzymol. 65, 499-560

Maxfield, F.R., Willingham, M.C., Davies, P.J. & Pastan, I. (1979), Nature (Lond) 277, 661-663

Meglasson,M.D., Burch,P.T., Berner, D.K., Najafi, H. & Matschinsky,F.M. (1986), Diabetes 35, 1163-1173

Michel,T., WWinslow,J.W., Smith,J.A., Seidman,J.G. & Neer, E.J. (1986), Proc. Natl. Acad. Sci. (USA), 83, 7663-7667

Morgan,D.O. & Roth,R.A. (1987), Proc. Natl. Acad. Sci. (USA) 84, 41-45

Nahorski,S.R., Kendall,D.A. & Batty, I. (1986), Biochem. Pharmacol. 35, 2447-2453

National Diabetes Data Group, (1979), 28, 1039-1057

Neer, E.J., Lok, J.M. & Wolf, L.G. (1984), J. Biol. Chem. 259, 14222-14229

Niedel, J.E., Kuhn, L.J. & Vandenbark, G.R. (1983), Proc. Natl. Acad. Sci. (USA) 80, 36-40

Nishizuka,Y. (1984), Nature (Lond) 308, 693-698

Northup,J.K., Smigel,M.D., Sternweis, P.C. & Gilman, A.G. (1983a), J.Biol. Chem. 258, 11369-11376

Northup, J.K., Sternweis, P.C. & Gilman, A.G. (1983b), J. Biol. Chem. 258, 11361-11368

O'Brien,R.M., Houslay,M.D., Milligan,G. & Siddle, K. (1987), FEBS. Letters 212, 281-288

O'Brien,R.M., Soos, M.A. & Siddle, K. (1986), Biochem. Soc. Trans. 14, 316-317

Olefsky,J.M. & Raven, G.M. (1976), Am. J. Med. 60, 89-95

Oppenheimer,C.L., Pessin, J.E., Massague, J., Gitomer, W. & Czech, M.P. (1983), J. Biol. Chem. 258, 4824-4830

Pagano, G., Cassander, M., Cavallo-Perin, P., Bruno, A., Masciola,P., Ozzello, A., Dall'ono, A.M. & Foco, A. (1984), Metab. 33, 976-981

Panchenko,M.P. & Tkachuk, V.A. (1984), FEBS. Letters 174, 50-53

Pastan, I . & Willingham, M.C. (1983), Trends Biochem. Sci. 8, 250-253

Pedersen,O. & Gliemann, J. (1981), Diabetologia 20, 630-635

Pennington,S.R. & Martin, B.R. (1985), J.Biol. Chem. 260, 11039-11045

Pessin, J.E., Gitomer, W., Oka, Y, Oppenheimer, C.L. & Czech, M.P. (1983), J. Biol. Chem. 258, 7386-7394

Peterson, G.L. (1977), Anal. Biochem. 83, 346-356

Pfeuffer, .E., Dreher, R-M., Metzger,H. & Pfeuffer, T. (1985a), Proc. Natl. Acad. Sci. 82, 3086-3090

Pfeuffer, E., Mollner, S. & Pfeuffer, T. (1985b), EMBO. J. 4, 3675-3679

Piascik, M.T., Babich, M. & Rush, M.E. (1983), J. Biol. Chem. 258, 10913-10918

Pike, L.J., Kuenzel, E.A., Casnellie, J.E. & Krebs, E.G. (1984), J. Biol. Chem. 259, 9913-9921

Pilch,P.F. & Czech, M.P. (1980), J. Biol. Chem. 254, 3375-3381

Pilkis,S.J., Exton, J.H., Johnson,R.A. & Park, C.R. (1974), Biochim. Biophys. Acta. 343, 250-267

Pillion, D.J. & Czech, M.P. (1978), J. Biol. Chem. 253, 3761-3764

Pillion, D.J., Grantham, J.R. & Czech, M.P. (1979), J. Biol. Chem. 254, 3211-3220

Reed,B.C., Ronnett, G.V. & Lane, M.D. (1981), Proc. Natl. Acad. Sci. (USA) 78, 2908-2912

Robbins, M.J., Sharp, R.A., Slonim,A.E. & Burr, I.M. (1980), Diabetologia 18, 55-58

Robishaw, J.D., Smigel, M.D. & Gilman, A.G. (1986), J. Biol. Chem. 261, 9587-9590

Rodbell, M., Krans, H.M.J., Pohl,S.L. & Birnbaumer, L. (1971), J. Biol. Chem. 246, 1872-1876

Rojas,F.J., Garber, A.J. & Birnbaumer,L. (1983), Diabetes 32, (suppl.1) Abst.175

Ronnett,G.V., Knutson, V.P., Kohanski, R.A., Simpson, T.L. & Lane, M.D. (1984), J. Biol. Chem. 259, 4566-4575

Ronnett,G.V. & Lane, M.D. (1981), J. Biol. Chem. 256, 4704-4707

Rosenthal,W., Koesling, D., Rudolph,V., Kleuss, C. & Pallast, M. (1986), Eur. J. Biochem. 158, 255-263

Ross, E.M. & Gilman, A.G. (1977), J. Biol. Chem. 252, 6966-6969

Ross, E.M. & Gilman, A.G. (1980), Ann. Rev. Biochem. 49, 533-564

Rossini, A.A., Williams, R.M., Appel, M.C. & Like, A.A., (1971), Nature (Lond.) 276, 182-184

Rubalcava, B. & Rodbell, M. (1973), J. Biol. Chem. 248, 3831-3837

Rubin, C.S., Ehlichman, J. & Rosen, O. M. (1974), Meth. Enzymol. 38, 308-315

Sale, G.J., Fujita-Yamaguchi, Y. & Kahn, C.R., (1986), Eur. J. Biochem, 155, 345-351

Salhanick, A.I., West, H. & Amatruda, J.M. (1985), J. Biol. Chem. 260, 16232-16236

Saltiel, A.R. & Cuatrecasas, P. (1986), Proc. Natl. Acad. Sci. (USA) 83, 5793-5797

Saltiel, A.R., Fox, J.A. ,Sherline, P. & Cuatrecasas , P. (1986), Sci. 233, 967-972

Samson, M., Fehlman,M. Morin, O., Dolais-Kitabgi, J. & Freychet, P. (1982), Metab. 31, 766-772

Sandler,S. & Swenne,I. (1983), Diabetologia 25, 444-447

Sanger,F.& Thompson, E.O.P.(1963), Biochem. J, 53, 353-374

Sanger, F. & Tuppy, H. (1961), Biochem. J. 49, 463-490

Sauerheber, R.D., Kuhn, C.E., & Hyslop, P.A. (1984), Diabetes 33, 258-265

Schlessinger,J.Y., Schechter,Y., Willingham,M.C. & Pastan, I. (1978), Proc. Natl. Acad. Sci. (USA) 75, 2659-2663

Schramm,M. & Rodbell,M. (1975), J. Biol. Chem. 250, 2232-2237

Schroer,D.W., Frost, S.C., Kohanski,R.A., Lane, M.D. & Lienhard, G.E. (1986), Biochim. Biophys. Acta. 885, 317-326

Seamon, K.B. & Daly, J.W. (1986), Advs. Cyc. Nucl. Pro. Phos. Res. 20, (Greengard, P. & Robison, G.A. eds.) Raven Press New York pp 1-150

Shia,M.A. & Pilch, P.F. (1983), Biochemistry 22, 717-720

Shorr, R.G.L., Lefkowitz,R.J. & Caron, M.g. (1981), J. Biol. Chem. 256, 5820-5826

Smigel,M.D. (1986), J. Biol. Chem. 261, 1976-1982

Smith, S.A., Elliot, K.R.F. & Pogson, C.I. (1978), Biochem. J. 176, 817-825

Soos,M.A., Siddle, K., Baron, M.D., Heward,J.M., Luzio,J.P., Bellatin, J. & Lennox, E.S. (1986), Biochem. J. 235, 199-208

Stadel, J.M., Delean, A. & Lefkowitz, R.J. (1980), Ann. Rev. Biochem. 1-37

Stadtmauer, L.A. & Rosen, O.M. (1983), J. Biol. Chem. 258, 6682-6685

Stanley, P.E. & Williams, S.G. (1969), Anal. Biochem. 29, 381-392

Stein, J.M. & Hales, C.N. (1974), Biochim. Biophys. Acta. 337, 41-49

Steiner,D.F. (1977), Diabetes 26, 322-340

Steiner, D.F. (1973) , Nature (Lond.) 243, 528-530

Steiner, D.F., Kemmler, W., Tager, H.S. & Petertson, J.D. (1974), Fed. Proc. 33, 2105-2115

Sternweis,P.C. & Gilman, A.G. (1982), Proc. Natl. Acad. Sci. (USA) 79, 4888-4891

Sternweis, P.C., Northup, J.K., Smigel, M.D. & Gilman, A.G. (1981), J. Biol. Chem. 256, 11517-11526

Sternweis,P.C. & Robishaw, J.D. (1984), J. Biol. Chem. 259, 13806-13813

Stolz, D.J. & Martin, R.J. (1982), J. Nutrition 112, 997-1002

Sugimoto, K., Nukada,T., Tanabe, T., Takahashi, H., Noda, M.,

Minamino, N., Kangawa,K., Matsuo,H., Hirose, T., Inayama, S. & Noma, S. (1985), FEBS. Letters 191, 235-240

Sugimoto,Y., Whitman,M., Cantley,L.C. & Erikson, R.L. (1984), Proc. Natl. Acad. Sci. 81, 2117-2121

Tager,H.,Given,B., Baldwin,D., Mako, M., Markese,J., Rubenstein,A., Olefsky, J., Kobayashi,M., Kolterman, O & Poucher, R. (1979), Nature (Lond.) 281, 122-125

Thomas, A.P. & Williamson, J.R. (1983), J.Biol. Chem. 258, 1411-1414

Uchigata,Y., Yamamoto,H., Kawamura,A. & Okamoto,H. (1982), J. Biol. Chem. 257, 6084-6088

Ullrich, A., Bell, J.R., Chen,E.Y., Herrera,R., Petruzzelli, L.M., Dull, T.J., Gray,A.,Coussens, L., Liao,Y.C., Tsubokawa,M., Mason,A., Seeburg, P.H., Grunfield, C., Rossen, O.M. & Ramachandran, J. (1985), Nature (Lond.) 313, 756-761

Urumow,T. & Wieland, O.H. (1986), FEBS. Letters 207, 253-257

Valverde,I., Vandermeers,A., Anguanejulu, R. & Malaisse, W.J. (1979), Sci. 206, 225-227

Van DE Werve,G., Proietto,J. & Jeanrenaud, B. (1985), Biochem. J. 225, 523-527

Vigneri,R., Pezzino, V., Wong, K.Y. & Goldfine, I.D. (1982), J. Clin. End.Metab. 54, 95-100

Vydelingum, N., Kissebah, A.H., Wynn, V. & Sampson,A.(1975), Diabetologia 11, 382-387

Walaas, O., Horn,R.S., Lystad, E. & Adler, A. (1981), FEBS. Letters 128, 133-136

Walaas, O., Walaas, E., Lystad, E., Alertsen, A.R. & Horn, R.S. (1979), Mol. Cell. Endocrionol. 16, 45-55

Walaas,O., Walaas, E., Lystad,E., Alertsen, A.R., Horn, R.S. & Fossum, S. (1977), FEBS. Letters 80, 417-422

Wakelam,M.J.O., Davies,S.A., Houslay, M.D., McKay,I., Marshall, C.J. & Hall, A. (1986), Nature (Lond.) 323, 173-176

Ward, W.F. (1984), Horm. Metabol. Res. 16, 509-512

Watanbe,Y., Horn, F., Bauer,S. & Jakobs, K.H. (1985), FEBS. Letters 192, 23-27

Whetton, A.D., Needham,L., Dodd, N.J.F., Heyworth, C.M. & Houslay, M.D. (1983)¦ Biochem. Pharmacol. 32, 1601-1608

White, M.F., Haring, H.U., Kasuga,M. & Kahn, C.R. (1984), J. Biol. Chem. 259, 255-264

White,M.F., Takayama,S. & Kahn.C.R. (1985), J. Biol. Chem. 260, 9470-9478

Whitesell,R.R. & Gliemann,J. (1979), J. Biol. Chem. 254, 5276-5283

Wilson, G.L., Patton, N.J., McCord,J.M., Mullins,D.W. & Nossman, B.T. (1984), Diabetologia 27, 587-591

Yeager,R.E., Heideman,W., Rosenberg, G.B. & Storm, D.R. (1985), Biochemistry 24, 3776-3783

York, D.A., Steinke,J. & Bray, G.A. (1972), Metabolism 21, 277-284

Yu,K.T. & Czech, M.P. (1984), J. Biol. Chem. 259, 5277-5286

Zick, Y., Grunberger, G.,Rees-Jones, R.W. & Comi,R.J. (1985), Eur. J. Biochem. 148, 177-182

Zick,Y., Rees-Jones, R.W., Taylor,S.I., Gorden, P. & Roth, J. (1984), J. Biol. Chem. 259, 4396-4400

Zick,Y., Whittaker,J. & Roth,J. (1983), J.Biol. Chem. 258, 3431-3434

Zucker,L.M. & Zucker, T.F.(1961), J. Hered. 52, 275-278

Additional References (*)

Baenziger, J., & Fiete, D., (1986) J. Biol. Chem. 261, 7445 -7454

Chatzipanteli,K., & Saggerson, D., (1983) FEBS. Lett. 155, 135 - 138

Hermann,L.S., (1979) Diabete & Metabolisme 3, 233 - 245

Jacobs,S., Sahyoun,N.E., Saltiel, A.R., & Cuatrecasas,P., (1983b) Proc. Natl. Acad. Sci. (USA) 80, 6211 - 6213

Rizza,R.A., Mandarino,L.J., & Gerich,J.E., (1981) Am. J. Med. 70, 169 - 176

Appendix I

Chemical & Enzyme Suppliers

All reagents used were of analytical grade.

Supplier	Chemical / Enzyme
Sigma Chemical Co.,	Alloxan
Poole,	BSA
Dorset.	Cholera Toxin
	DE-50 Cellulose
	Dowex 1-X2 resin
	Firefly Lantern Extract (FLE-50)
	Glutathione
	Norit A Charcoal
	Oubain
	Streptozotocin
	Theophylline
	TPA
	Trypan Blue
Boehringer (U.K.) Ltd.,	
Lewes,	
East Sussex.	
	ATP
	Collagenase
	Creatine Kinase
	Diabur Test 5,000
	Dithiothreitol
	Glyceraldehyde 3 phosphate dehydrogenase

Supplier	Chemical / Enzyme
	Glycerate 3-phosphate
	NAD
	Phosphocreatine
	Phosphoglycerate Kinase
	Triethanolamine-HCL
	Tris
May & Baker Ltd., Dagenham, Essex.	Hydrochloric Acid
	Sagatal
	Trisoium Citrate
Ames, Slough.	Dextrstix
Calbiochem, Cambridge.	Forskolin
National Diagnostics Aylesbury, Buckinghamshire.	"Ecoscint" Scintillation fluid
Pharmacia, Milton Keynes.	Percoll

Prof. Freer,

Dept. Microbiology,

University of Glasgow. Pertussis Toxin

The following reagents were generously donated as gifts :

Insulin and glucagon were donated by Dr. W.W. Brommer, Eli Lilly & Co., I.N. (USA).

Metformin was donated by Dr. Michel Noel, Aron-S.A., Suresnes, France.

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

Appendix II

Structural Formulae

Figure A1 Structure of Alloxan

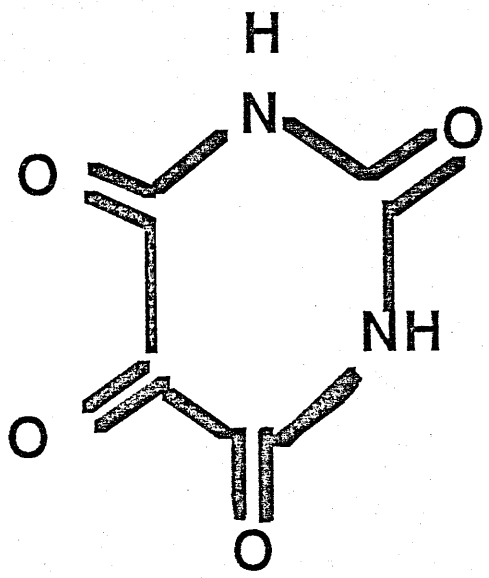


Figure A2 Structural Formula of Streptozotocin

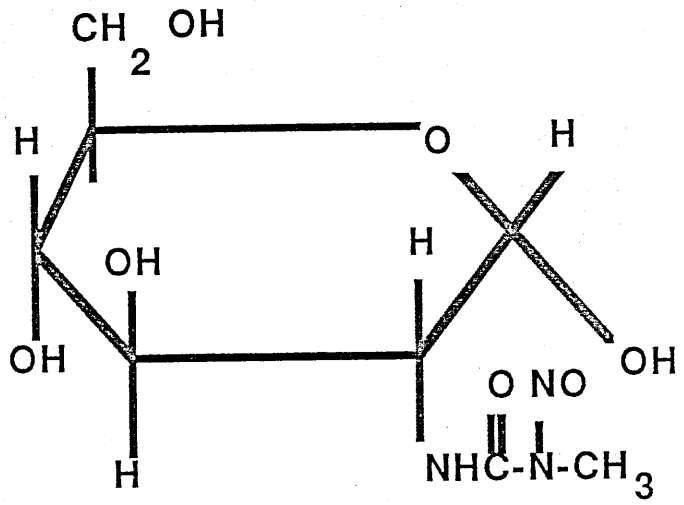
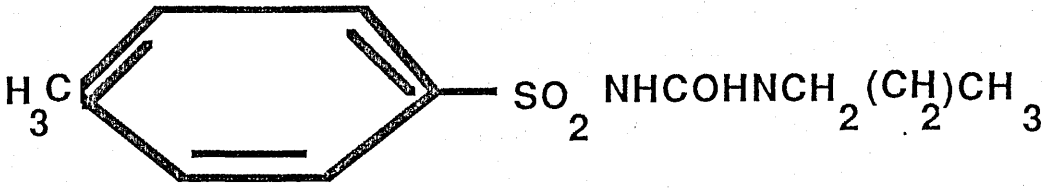


Figure A3 Examples of Sulphonylureas

a) Tolbutamide



b) Glipizide

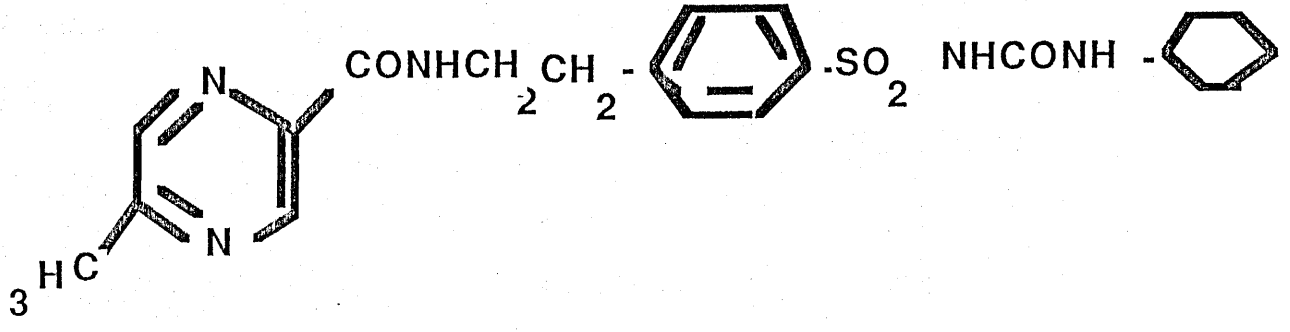


Figure A4 Structural Formula of cAMP

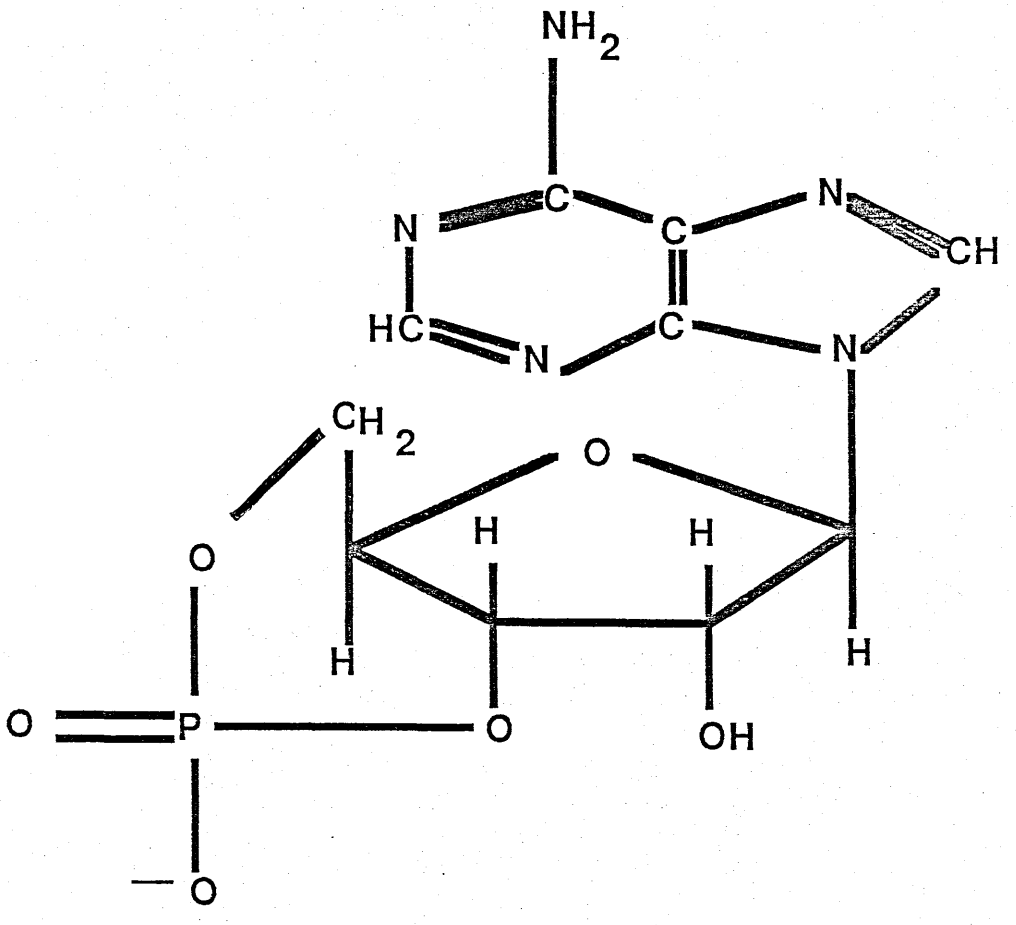
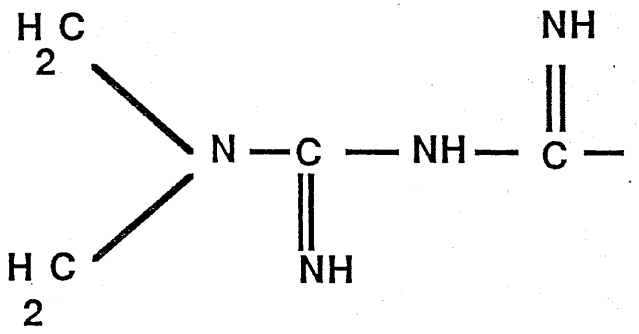


Figure A5 Structural Formula of Metformin



(N',N'-dimethylbiguanide)

Figure A6 Structural Formula of Forskolin

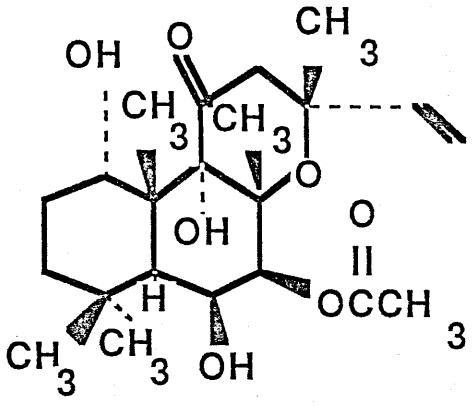
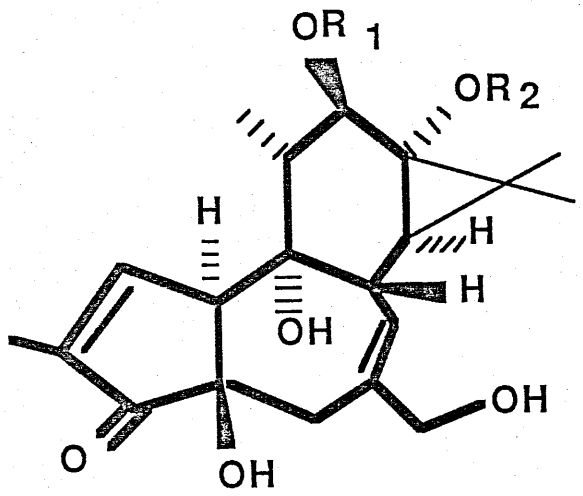
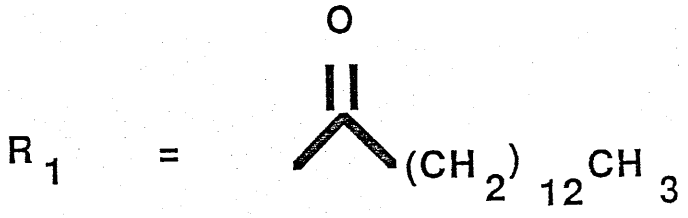
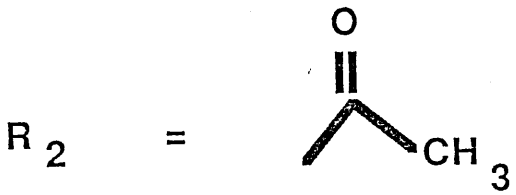


Figure A7 Structural Formula of Phorbol Esters



TPA





Publications Obtained During The Undertaking of This Research Studentship

Heyworth C.M., Wilson S.P., Gawler D.G., & Houslay M.D., (1985) FEBS Lett. 187, 196 - 200

Houslay M.D., Bojanic D., Gawler D., O'Hagan S., & Wilson A., (1986) Biochem. J. 238, 109 - 113

Gawler D.J., & Houslay M.D., (1987) FEBS Lett. 216, 94 - 98

Gawler D.J., Milligan G., Spiegal A.M., Unson C.G., & Houslay M.D., (1987) Nature (Lond). 327, 229 - 237

Houslay M.D., Wakelam M.J.O., Murphy G.J., Gawler D.J., & Pyne N.J., (1987) Biochem. Soc. Trans. 15, 21 - 24

Gawler D.J., Milligan G., & Houslay M.D., (1987) Submitted to Biochem. J.