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# **Interactions between insulin and adenylyl cyclase signalling**

A thesis submitted to the  
FACULTY OF MEDICINE  
for  
the degree of  
DOCTOR OF PHILOSOPHY

by

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## Summary

Hepatocytes immortalised by transfection with SV40 viral DNA have been well studied with respect to marker protein expression and the effects of immortalisation on cell phenotype. Here, a study was undertaken of adenylyl cyclase signalling in one such cell line P9, in order to consider its suitability as a model system for studying this system and insulin action.

Cells were found to contain glucose-6-phosphatase activity. Plasma membranes possessed adenylyl cyclase activity, comparable to rat hepatocytes with dose- and receptor-dependent stimulation by PGE<sub>1</sub> and receptor-independent stimulation by forskolin, sodium fluoride, GTP and GppNHp. There was no stimulation by glucagon challenge alone. However, glucagon did enhance the ability of forskolin to stimulate adenylyl cyclase activity and elevate P9 cell intracellular cAMP. G<sub>s</sub>- $\alpha$  forms and G<sub>i</sub>- $\alpha$  subtypes expressed in the cells were the same as those in hepatocytes but all G-proteins, with the exception of the 42kDa form of G<sub>s</sub>- $\alpha$ , were present at higher levels in P9 cell than in rat hepatocyte plasma membranes. There was little evidence of G<sub>i</sub> function in the cells. Unlike the situation in rat hepatocytes, there was no uptake of labelled phosphate into P9 cell G<sub>i</sub>-2 $\alpha$  in response to agonist challenge, suggesting either that G<sub>i</sub>-2 $\alpha$  subunit was already phosphorylated in P9 cells or that there are differences in kinase and phosphatase activities in P9 cells as compared to rat hepatocytes.

Exposure of the cells to a high concentration of insulin (0.5 $\mu$ M for 16 hours) failed to produce any morphological change or to restore glucagon responsiveness, but did have a small mitogenic effect and enhanced both receptor-dependent and -independent adenylyl cyclase stimulation in plasma membranes derived from insulin-treated cells.

At a concentration of 1nM, insulin impaired the ability of PGE<sub>1</sub> to elevate intracellular cAMP in P9 cells, over the same time period. There was no impairment of the response to forskolin and the inhibition was not blocked by IBMX, cycloheximide or pertussis toxin pre-treatment, suggesting it to be a functional effect, at the level of the membrane, independent of protein synthesis or G<sub>i</sub> activation and exerted through the high affinity insulin receptor at a site proximal to the catalytic subunit of adenylyl cyclase.

An examination of the time course for the effect revealed that insulin acutely enhanced the ability of PGE<sub>1</sub> to elevate intracellular cAMP, which later proceeded to inhibition of the response. This potentiation of the response was dose-dependent for insulin and not observed in plasma membranes from insulin-exposed cells or when plasma membranes were directly exposed to insulin. It is concluded that adenylyl cyclase signalling in the P9 cell line, unlike marker protein expression is considerably divergent from that of native rat hepatocytes. However, the cell line may still be useful at low passage for studying aspects of cyclase signalling, insulin action and the activities of other second messenger systems.

G-protein levels and adenylyl cyclase activity were examined in mononuclear leucocyte plasma membranes from type 2 diabetic subjects and corresponding age- and sex-matched control subjects. No significant differences in G-protein levels were noted between plasma membranes from the two groups. Likewise there was no significant difference in the GTP- or forskolin-stimulated adenylyl cyclase activities. There was a reduction in PGE<sub>1</sub>-stimulated adenylyl cyclase in the male diabetic group compared to their controls, but without any difference between the groups in fold stimulation over basal cyclase activity. Given the sex-specific nature of this difference it seems unlikely that it is linked to the reduction in immune functioning characteristic of the diabetic state.

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## Abbreviations

AC	adenylyl cyclase
ADP	adenosine 5'-diphosphate
ANSA	1-amino-2-naphthol-4-sulphonic acid
ATP	adenosine 5'-triphosphate
BSA	bovine serum albumin
cAMP	adenosine 3',-5'-cyclic monophosphate
CTX	cholera toxin
CV	coefficient of variation
DMEM	Dulbecco's modification of Eagle's medium
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol bis ( $\beta$ -aminoethyl ether)-N', N', N', N',- tetraacetic acid
FCS	foetal calf serum
GDP	guanosine 5'-diphosphate
GDP $\beta$ S	guanosine 5'-( $\beta$ -thio) diphosphate
GppNHp	guanosine 5'-( $\beta$ - $\gamma$ -imido) triphosphate
G-protein	guanine nucleotide regulatory protein
G <sub>i</sub>	inhibitory guanine nucleotide regulatory protein
G <sub>s</sub>	stimulatory guanine nucleotide regulatory protein
GTP	guanosine 5'-triphosphate
GTP $\gamma$ S	guanosine 5'-( $\gamma$ -thio) triphosphate
IBMX	3-isobutyl-1-methylxanthine



Ig	immunoglobulin
IGF	insulin-like growth factor
K <sub>d</sub>	dissociation constant
K <sub>i</sub>	concentration giving half maximal inhibition
K <sub>0.5</sub>	concentration giving half maximal activation
mRNA	messenger ribonucleic acid
NAD	nicotinamide adenine dinucleotide
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCA	perchloric acid
PGE <sub>1</sub>	prostaglandin E <sub>1</sub>
P <sub>i</sub>	inorganic phosphate
PI	phosphatidylinositol
PK-A	cAMP-dependent protein kinase
PK-C	protein kinase-C
PMSF	phenylmethylsulphonyl fluoride
PP <sub>i</sub>	pyrophosphate
PTX	pertussis toxin
r	correlation coefficient
R <sub>i</sub>	inhibitory receptor
R <sub>s</sub>	stimulatory receptor
SD	standard deviation
SDS	sodium dodecyl sulphate
SEM	standard error of mean
TBS	tris-buffered saline
TCA	trichloroacetic acid
TEMED	N, N, N', N'-tetramethylethylenediamine
TPA	12-O-tetradecanoylphorbol 13-acetate
Tris	2-amino-2-hydroxymethyl-propane-1,3-diol

## Contents

	<u>PAGE</u>
Title	i
Summary	ii
Acknowledgements	iv
Abbreviations	v
Contents	vii
List of figures	xii
List of tables	xvii

### **Chapter 1 Introduction**

1.1	The molecular basis of insulin signalling	1
1.1.1	Signal generation by insulin	1
1.1.2	The insulin receptor tyrosine kinase	2
1.1.3	Serine and threonine phosphorylation in response to insulin	5
1.1.4	Control of cell responsiveness to insulin	6
1.1.5	Regulation of growth and gene expression by insulin	8
1.1.6	Other mechanisms of insulin action	10
1.2	Insulin-like growth factors	12
1.3	Adenylyl cyclase signalling	14
1.3.1	The role of cAMP in metabolism	16
1.3.2	Regulation of adenylyl cyclase activity	17
1.3.3	G-protein subtypes	22
1.3.4	Adenylyl cyclase subtypes	24
1.3.5	Forskolin	27
1.3.6	Regulation of G-protein levels	28

1.3.6.1	Hormonal regulation of G-protein levels	29
1.3.6.2	G-proteins in differentiation	31
1.3.6.3	G-proteins in disease	31
1.3.7	G-proteins in growth factor signal transduction	33
1.3.8	G-proteins in the immune system	35
1.3.9	G-protein phosphorylation	36
1.4	Prostaglandins	37
1.4.1	The E series prostaglandins	38
1.4.1.1	PGE action in hepatocytes	40
1.4.1.2	PGE action in the immune system	41
1.4.1.3	Interactions between PGE and insulin signalling	42
1.5	Interactions between insulin and adenylyl cyclase signalling	43
1.5.1	Insulin's modulation of adenylyl cyclase signalling	43
1.5.2	Adenylyl cyclase signalling in diabetes	46
1.5.2.1	Defects in hepatocyte adenylyl cyclase signalling in diabetes	47
1.5.2.2	Defects in adipocyte adenylyl cyclase signalling in diabetes	50
1.5.2.3	Defective adenylyl cyclase signalling in other tissues in diabetes	51
1.6	Cell culture systems in the study of hepatocyte signal transduction	52
1.6.1	Native hepatocytes	52
1.6.2	Hepatoma cell lines	54
1.6.3	SV40-immortalised hepatocytes	55
1.7	Aims of project	59

## **Chapter 2 Materials and methods**

2.1	Materials	60
2.1 (a)	General reagents	60
2.1 (b)	Tissue culture plasticware	62

2.2	Tissue culture media	63
2.3	Radiochemicals	63
2.4	Standard buffers	64
2.5	Hepatocyte preparation	66
2.5 (a)	Hepatocyte isolation	66
2.5 (b)	Transfection	66
2.5 (c)	Plasma membrane preparation	66
2.5 (d)	Cell counting	67
2.5 (e)	Collagen coating of plasticware	67
2.6	Tissue culture	67
2.6 (a)	Cell growth	67
2.6 (b)	Cell subculture	68
2.6 (c)	Cell freezing	68
2.6 (d)	Cell harvesting	68
2.6 (e)	Plasma membrane preparation	69
2.6 (f)	Toxin treatment of cells	69
2.6 (g)	Mycoplasma screening	69
2.7	Rat cerebral cortex plasma membrane preparation	70
2.8	Antibody production	70
2.9	Protein determination	71
2.10	Gel electrophoresis	71
2.10 (a)	Resolving gel preparation	72
2.10 (b)	Stacking gel preparation	73
2.10 (c)	Sample preparation	74
2.10 (d)	Gel protein staining	74
2.10 (e)	Autoradiography	74
2.10 (f)	Densitometry	75
2.11	Immunoblotting	75

2.12	Toxin-ribosylation of membranes	76
2.12 (a)	Toxin preactivation	76
2.12 (b)	ADP-ribosylation	76
2.13	Adenylyl cyclase assay	77
2.13 (a)	Assay conditions	77
2.13 (b)	Preparation of chromatography columns	78
2.13 (c)	Calibration of chromatography columns	78
2.13 (d)	Isolation of cAMP on chromatography columns	79
2.14	Assay of cAMP	79
2.14 (a)	Cell stimulation and cAMP extraction	79
2.14 (b)	Preparation of bovine adrenal cAMP binding protein	80
2.14 (c)	Cyclic AMP determination	80
2.15	Assay of glucose-6-phosphatase activity	81
2.16	Measurement of [ <sup>3</sup> H]-Thymidine uptake	82
2.17	Measurement of Gi-2 $\alpha$ subunit phosphorylation	82
2.18	Measurement of labelled glucagon binding	84
2.19	Methods used in study of leucocytes from diabetic subjects	84
2.19 (a)	Subjects	84
2.19 (b)	Leucocyte isolation	84
2.19 (c)	Leucocyte plasma membrane preparation	85
2.20	Data analysis	86

**Chapter 3 The P9 cell line as a model system for studying adenylyl cyclase signalling**

3.1	Introduction	90
3.2	Results	92
3.3	Discussion	99

<b><u>Chapter 4</u></b>	<b><u>Low affinity effects of insulin on adenylyl cyclase signalling in P9 cells</u></b>	
4.1	Introduction	130
4.2	Results	131
4.3	Discussion	133
<b><u>Chapter 5</u></b>	<b><u>High affinity effects of insulin on adenylyl cyclase signalling in P9 cells</u></b>	
5.1	Introduction	153
5.2	Results	154
5.3	Discussion	157
<b><u>Chapter 6</u></b>	<b><u>G-protein expression and adenylyl cyclase activity in mononuclear leucocytes from type 2 diabetic subjects</u></b>	
6.1	Introduction	181
6.2	Results	184
6.3	Discussion	186
<b><u>Chapter 7</u></b>	<b><u>General discussion</u></b>	198
References		202
Appendix of chemical structures		245

## List of Figures

	<u>PAGE</u>	
Fig. 1.1	Insulin and insulin-like growth factor receptors.	13
Fig. 1.2	The reaction catalysed by adenylyl cyclase.	15
Fig. 1.3	The G-protein GTPase cycle.	19
Fig. 1.4	Hormonal regulation of adenylyl cyclase.	20
Fig. 1.5	Structure of eukaryotic adenylyl cyclase.	25
Fig. 1.6	Biosynthesis of PGE <sub>1</sub> .	39
Fig. 2.1	Elution profiles of [ $\alpha$ - <sup>32</sup> P]-ATP and [8- <sup>3</sup> H]-cAMP from dowex and alumina columns.	87
Fig. 2.2	Standard curve for cAMP determination.	88
Fig. 2.3	Standard curve for phosphate determination.	89
Fig. 3.1	P9 cells and rat hepatocytes in culture.	111
Fig. 3.2	Effect of increasing concentrations of PGE <sub>1</sub> on P9 cell intracellular cAMP.	112
Fig. 3.3	Elevation of rat hepatocyte intracellular cAMP in response to forskolin and glucagon.	113
Fig. 3.4	Effect of increasing concentrations of glucagon on forskolin- stimulated adenylyl cyclase activity in P9 cell plasma membranes.	114
Fig. 3.5	Elevation of P9 cell intracellular cAMP in response to forskolin combined with increasing concentrations of glucagon.	115
Fig. 3.6	Effect of increasing concentrations of forskolin on P9 cell intracellular cAMP.	116
Fig. 3.7	Time course for elevation of P9 cell intracellular cAMP in response to forskolin, alone and combined with glucagon.	117
Fig. 3.8	Time course for elevation of P9 cell intracellular cAMP in response to PGE <sub>1</sub> .	118

Fig. 3.9	Immunological identification of Gi-2 $\alpha$ in P9 cell plasma membranes.	119
Fig. 3.10	Immunological identification of Gi-3 $\alpha$ in P9 cell plasma membranes.	120
Fig. 3.11	Immunological identification of G <sub>s</sub> - $\alpha$ in P9 cell plasma membranes.	121
Fig. 3.12	Immunological identification of G-protein $\beta$ subunit in P9 cell plasma membranes.	122
Fig. 3.13	Expression of Gi- $\alpha$ subtypes in P9 cells and rat tissues.	123
Fig. 3.14	Expression of G <sub>s</sub> - $\alpha$ forms and G-protein $\beta$ subunit in P9 cells and rat tissues.	124
Fig. 3.15	Toxin-catalysed ADP-ribosylation of P9 cell plasma membranes.	125
Fig. 3.16	Effect of increasing concentrations of GppNHp on forskolin-stimulated adenylyl cyclase activity in P9 cell plasma membranes.	126
Fig. 3.17	Effect of increasing concentrations of GTP on adenylyl cyclase activity in P9 cell plasma membranes.	127
Fig. 3.18	Effect of pertussis toxin pre-treatment on glucagon response and toxin labelling in P9 cells.	128
Fig. 3.19	Phosphorylation of Gi-2 $\alpha$ in intact P9 cells.	129
Fig. 4.1	Effect of increasing concentrations of PGE <sub>1</sub> on adenylyl cyclase activity in plasma membranes from control P9 cells and cells exposed to insulin (0.5 $\mu$ M, 16 hours).	142
Fig. 4.2	Effect of increasing concentrations of GppNHp on adenylyl cyclase activity in plasma membranes from control P9 cells and cells exposed to insulin (0.5 $\mu$ M, 16 hours).	143



Fig. 4.3	Effect of increasing concentrations of forskolin on adenylyl cyclase activity in plasma membranes from control P9 cells and cells exposed to insulin (0.5 $\mu$ M, 16 hours).	144
Fig. 4.4	Time course for change in forskolin-stimulated adenylyl cyclase activity after addition of insulin (0.5 $\mu$ M) to P9 cells.	145
Fig. 4.5	Uptake of [ <sup>3</sup> H]-Thymidine by P9 cells in response to insulin and IGF-1.	146
Fig. 4.6	Effect of increasing concentrations of PGE <sub>1</sub> on P9 cell intracellular cAMP after exposure to insulin (0.5 $\mu$ M, 16 hours).	147
Fig. 4.7	Effect of increasing concentrations of insulin (16 hours exposure) on PGE <sub>1</sub> -elevated intracellular cAMP in P9 cells.	148
Fig. 4.8	Effect of increasing concentrations of insulin (16 hours exposure) on forskolin-elevated intracellular cAMP in P9 cells.	149
Fig. 4.9	Effect of cycloheximide pre-treatment on insulin's modulation of PGE <sub>1</sub> - and forskolin-elevated intracellular cAMP in P9 cells.	150
Fig. 4.10	Toxin-catalysed ADP-ribosylation of G-proteins in plasma membranes from P9 cells exposed to insulin (0.5 $\mu$ M, 16 hours).	151
Fig. 4.11	G-protein levels in plasma membranes from P9 cells exposed to insulin (0.5 $\mu$ M, 16 hours).	152
Fig. 5.1	Effect of IBMX and cycloheximide pre-treatment on insulin's modulation of the PGE <sub>1</sub> -elevated intracellular cAMP in P9 cells.	167

Fig. 5.2	Effect of IBMX and cycloheximide pre-treatment on insulin's modulation of the forskolin-elevated intracellular cAMP in P9 cells.	168
Fig. 5.3	Elevation of P9 cell intracellular cAMP by increasing concentrations of PGE <sub>1</sub> after exposure to insulin (1nM, 16 hours).	169
Fig. 5.4	Elevation of P9 cell intracellular cAMP by increasing concentrations of forskolin after exposure to insulin (1nM, 16 hours).	170
Fig. 5.5	Effect of pertussis toxin pre-treatment on impairment of the PGE <sub>1</sub> -elevated P9 cell intracellular cAMP by insulin (1nM, 16 hours).	171
Fig. 5.6	Time course for insulin's (1nM) modulation of the PGE <sub>1</sub> elevated intracellular cAMP in P9 cells.	172
Fig. 5.7	Effect of increasing concentrations of GTP on PGE <sub>1</sub> -stimulated adenylyl cyclase activity after exposure of P9 cells to insulin (1nM, 16 hours).	173
Fig. 5.8	Effect of increasing concentrations of insulin (10 minutes exposure) on PGE <sub>1</sub> -elevated intracellular cAMP in P9 cells.	174
Fig. 5.9	Effect of increasing concentrations of GTP on PGE <sub>1</sub> -stimulated adenylyl cyclase activity after exposure of P9 cells to insulin (1nM, 10 minutes).	175
Fig. 5.10	Effect of increasing concentrations of GTP on PGE <sub>1</sub> -stimulated adenylyl cyclase activity in the presence of insulin.	176
Fig. 5.11	Effect of increasing concentrations of insulin (10 minutes exposure) on forskolin-elevated intracellular cAMP in P9 cells.	177

Fig. 5.12	Effect of insulin exposure on glucagon- and forskolin-elevated intracellular cAMP in P9 cells.	178
Fig. 5.13	Effect of insulin exposure (1nM, 16 hours) on toxin labelling of G-proteins in P9 cell plasma membranes.	179
Fig. 5.14	Effect of insulin exposure (1nM, 16 hours) on G-protein levels in P9 cell plasma membranes.	180
Fig. 6.1	Immunological identification of Gi-2 $\alpha$ in mononuclear leucocyte plasma membranes.	192
Fig. 6.2	Immunological identification of Gs- $\alpha$ in mononuclear leucocyte plasma membranes.	193
Fig. 6.3	Immunological identification of G-protein $\beta$ subunit in mononuclear leucocyte plasma membranes.	194
Fig. 6.4	G-protein levels in mononuclear leucocyte plasma membranes from control and type 2 diabetic subjects.	195
Fig. 6.5	Comparison of G-protein levels in mononuclear leucocytes from control and type 2 diabetic subjects.	196
Fig. 6.6	Comparison of adenylyl cyclase activities in mononuclear leucocyte plasma membranes from control and type 2 diabetic subjects.	197
Fig. A1	Forskolin	245
Fig. A2	GppNHp	246
Fig. A3	IBMX	247
Fig. A4	The E-series prostaglandins	248

## List of Tables

	<u>PAGE</u>
Table 2.1 Decapeptides used in raising G-protein antisera.	70
Table 3.1 Glucose-6-phosphatase activity in P9 cells and rat hepatocytes.	107
Table 3.2 Agonist-stimulated adenylyl cyclase activity in P9 cell and rat hepatocyte plasma membranes.	108
Table 3.3 Elevation of P9 cell intracellular cAMP in response to forskolin and PGE <sub>1</sub> , alone and combined.	109
Table 3.4 Quantitative comparison of G-protein levels in P9 cells and rat hepatocyte plasma membranes.	110
Table 4.1 Agonist-stimulated adenylyl cyclase activity in plasma membranes from P9 cells exposed to insulin (0.5 $\mu$ M, 16 hours).	140
Table 4.2 Quantitative comparison of toxin labelling and G-protein levels in plasma membranes from P9 cells exposed to insulin (0.5 $\mu$ M, 16 hours).	141
Table 5.1 Parameters for GTP/PGE <sub>1</sub> stimulated adenylyl cyclase activity in P9 cell plasma membranes.	165
Table 5.2 Quantitative comparison of toxin labelling and G-protein levels in plasma membranes from P9 cells exposed to insulin (1nM, 16 hours).	166
Table 6.1 Plasma glucose measurements from control and type 2 diabetic subjects.	191

**Chapter 1**  
**Introduction**

## **1.1 The molecular basis of insulin signalling**

The anabolic hormone insulin is a 5.7kDa protein of 52 amino acids, secreted from pancreatic beta cells in response to the fed state. Insulin acts upon its target cells to stimulate glucose, protein and lipid metabolism as well as nucleic acid synthesis, by altering the activity of various enzymes and transport processes. Considerable effort has been made in trying to understand the molecular basis of insulin action, as this is necessary for understanding the pathogenesis of diabetes, where there is either an absolute deficiency of insulin (type 1) or relative deficiency (type 2). Knowledge of the molecular basis of insulin action is also important for understanding a number of insulin resistant states such as obesity, uraemia, glucocorticoid and growth hormone excess and severe insulin resistance resulting from insulin receptor defects.

### **1.1.1 Signal generation by insulin**

Insulin is thought to exert its effects on target cells by binding to a trans-plasma membrane receptor and activation of a receptor-associated tyrosine kinase (Kasuga *et al.*, 1982a) (Fig. 1.1). The cDNA for the insulin receptor precursor has been cloned from humans (Ullrich *et al.*, 1985) and *Drosophila* (Petruzzelli *et al.*, 1986) allowing the amino acid sequences to be identified. The sequences are very similar, showing that the receptor structure has been well conserved in evolution. The receptor is present in almost all mammalian tissues but varies considerably in its level of expression between tissues. In liver, a major target tissue for insulin, there are some 50-100 000 copies per cell (Kahn, 1976). The hormone binds in a specific and saturable manner, with a  $K_d$  in the pM to low nM range. This corresponds to the concentration of insulin required for half maximal tyrosine kinase activation (Kasuga *et al.*, 1982a). There is a strong correlation between binding affinity of insulin for its receptor and its biological action

(Anderson *et al.*, 1977). As no competitive antagonists of insulin have yet been discovered, probably all the structural requirements for binding include all the features required for biological action.

The insulin receptor has been purified from various tissue sources and is an approximately 450kDa heterotetrameric glycoprotein consisting of two  $\alpha$  subunits ( $M_r = 135\text{kDa}$ ) and two  $\beta$  subunits ( $M_r = 95\text{kDa}$ ) linked by five disulphide bonds in a  $\beta$ - $\alpha$ - $\alpha$ - $\beta$  structure (Massague *et al.*, 1981). The  $\alpha$  subunit is entirely extracellular and contains the insulin binding site as determined both by cross linking of labelled insulin to the receptor (Massague *et al.*, 1981) and photoaffinity insulin analogues (Yip *et al.*, 1978). The  $\beta$  subunits traverse the plasma membrane and have an ATP binding domain in their cytosolic portion responsible for the tyrosine kinase activity (Kasuga *et al.*, 1982a). Complementary DNA cloning of the receptor has shown that the intracellular domain of the  $\beta$  subunit is homologous to other tyrosine kinases including receptors for epidermal growth factor, platelet-derived growth factor and IGF-1, as well as various oncogene products (Ebina *et al.*, 1985, White and Kahn, 1986).

### **1.1.2 The insulin receptor tyrosine kinase**

Insulin binding activates both multisite tyrosine phosphorylation of the receptor and phosphorylation of intracellular protein substrates. Autophosphorylation of the receptor occurs by an intramolecular cascade, resulting in phosphorylation of at least seven tyrosine residues in the  $\beta$  subunit (Sale and Smith, 1989) which then phosphorylates endogenous substrates. This activity was found to be maximal within one minute of insulin binding (Pang *et al.*, 1985) and to remain for as long as the receptor remained phosphorylated, even after insulin dissociation, suggesting that it was important for signal transduction (Yu and Czech, 1984). Dephosphorylation of the receptor by phosphatases caused a reduction in tyrosine kinase activity, showing that

loss of phosphate from tyrosine residues probably terminates the response to insulin (King and Sale, 1990).

A number of endogenous substrates for the tyrosine kinase have been found which may mediate intracellular responses. The first substrate was detected by White *et al.* (1985) who immunoprecipitated a 185kDa protein from insulin-stimulated Fao hepatoma cells. This substrate, termed IRS-1 (formerly pp185) and similar high molecular weight proteins have been observed in all cells studied (White *et al.*, 1987, Gibbs *et al.*, 1986). Their phosphorylation is stimulated by insulin and IGF-1 but not by other receptor tyrosine kinases.

As a result of studies on a range of receptor tyrosine kinases, it appears that they select their targets by recognising a conserved protein sequence of about 100 amino acids called a SH2 (src homology) domain, found in a range of different signalling proteins (Koch *et al.*, 1991). This specific binding may be a means by which receptor tyrosine kinases can recruit SH2-containing proteins to the plasma membrane, allowing them to act as substrates for phosphorylation (Pawson and Gish, 1992). The substrate IRS-1 contains multiple potential tyrosine phosphorylation sites that are located within SH2 binding elements (Sun *et al.*, 1991). Tyrosine phosphorylated IRS-1 has been shown to bind to the enzyme phosphatidyl inositol-3-kinase (PI3-kinase), the enzyme which converts PI-4,5-bisphosphate to PI-3,4,5-trisphosphate (Lavan *et al.*, 1992). In addition progress has been made by the demonstration that PI3-kinase itself is a substrate for the receptor tyrosine kinase, consistent with a role for phosphatidyl inositol-3-phosphate in insulin action (Yonezawa *et al.*, 1990), though it remains unclear what this role is.

It is possible that alteration in the functioning of kinase substrates may play a role in the pathogenesis of insulin resistance and diabetes. Scott Thies *et al.* (1990)



showed in a study that the phosphorylation of the IRS-1 was reduced in adipocytes from type 2 diabetic subjects, though it is not possible to say whether the finding was due to a defect in the substrate itself. Alteration in the level<sup>of</sup> a tyrosine kinase substrate has been noted in association with insulin resistance in at least one model system. In 3T3 L1 cells, a fibroblast cell line which will differentiate into adipocytes in response to a variety of agents, glucose transport can be down-regulated by prolonged insulin exposure (Kozka *et al.*, 1991). This down-regulation is associated with a decrease in the level of IRS-1 (Rice *et al.*, 1992). It seems likely then that tyrosine phosphorylation of IRS-1 by the insulin receptor may be important for the stimulation of glucose transport. However, there is as yet no conclusive evidence that any of the endogenous tyrosine kinase substrates are mediators of insulin action.

The most convincing evidence that the activity of the receptor tyrosine kinase is important for insulin's action on cells has come from mutagenesis experiments. When lysine 1030, a residue thought to be involved in the ATP binding site was changed to various other amino acids, the mutant receptors bound insulin normally but were inactive both as kinases and in stimulating cellular metabolism (Ebina *et al.*, 1987). Antibodies to the intracellular domain of the receptor (Morgan and Roth, 1987) and to phosphotyrosine (Takayama *et al.*, 1988a) inhibited the tyrosine kinase and insulin's effects. The tyrosine residues in positions 1162-1163 have proven to be important sites of phosphorylation and are well conserved amongst tyrosine kinases (Stadtmauer and Rosen, 1986). Ellis *et al.* (1986) demonstrated by mutagenesis that these residues play a role in deoxy-glucose transport. Interestingly however, mutating these residues did not impair the mitogenic effect of low concentrations of insulin, suggesting that the metabolic and mitogenic pathways of insulin signalling may diverge early, at the level of the receptor (Debant *et al.*, 1990). Receptors mutated at tyrosine 960 did not phosphorylate IRS-1, nor were they able to transmit insulin's action (White *et al.*, 1988) suggesting that this residue is critical for insulin signalling.

### 1.1.3 Serine and threonine phosphorylation in response to insulin

The insulin receptor is also subject to serine and threonine phosphorylation (Kasuga *et al.*, 1982b). This is much slower than tyrosine phosphorylation, requiring about ten minutes to reach maximal levels (Pang *et al.*, 1985). It can be stimulated by phorbol esters (Takayama *et al.*, 1984) and cAMP analogues (Roth and Beaudoin, 1987), showing that the two protein kinases, protein kinase C (PK-C) and cAMP-dependent protein kinase (PK-A) may elicit this phosphorylation. These authors demonstrated that, in contrast to tyrosine phosphorylation, serine phosphorylation inactivates the kinase. This has led to the suggestion that serine phosphorylation of the receptor may be the end point of a negative feedback loop, allowing insulin to regulate its own action (Häring *et al.*, 1986) and may also be involved in the action of some hormones which antagonise insulin by activation of PK-C or PK-A (Van Obberghen *et al.*, 1990). Serine phosphorylation of the receptor may therefore in part represent crosstalk from other signalling systems.

A pathological increase in serine phosphorylation might explain the reversible decrease in receptor tyrosine kinase activity which has been observed in diabetes (Becker and Roth, 1990). Indeed elevation of PK-C activity has been suggested to mediate desensitisation of the insulin receptor observed in starved rats and the insulin resistance in obese subjects (Pfeiffer and Schatz, 1992). Elevation of diacylglycerol levels (the endogenous PK-C activator) by generation via cellular glucose metabolism during hyperglycemia may explain this increase in kinase activity (Greene *et al.*, 1987).

Although it is unclear how phosphorylation of the insulin receptor leads to action of the hormone on metabolism, most evidence favours some form of phosphorylation cascade, as has been described for the PK-A system (Taylor, 1989).

The phosphorylation cascade hypothesis is complicated by insulin's ability to influence both positively and negatively the phosphorylation state of several key metabolic enzymes. Those dephosphorylated include glycogen synthase (Sheorain *et al.*, 1982), hormone-sensitive lipase (Stralfros *et al.*, 1984) and pyruvate dehydrogenase (Hughes *et al.*, 1980). Those phosphorylated include ATP citrate lyase (Alexander *et al.*, 1979), acetyl CoA carboxylase (Brownsey and Denton, 1982), phosphofructokinase (Sale and Denton, 1985) and S6 kinase (Cobb and Rosen, 1983). In order to substantiate the protein kinase cascade hypothesis it has been considered important to identify the serine kinases involved in these phosphorylations and the mechanisms by which they are activated in response to insulin. At least seven serine/threonine kinases are known to be involved in insulin signalling (Sale and Smith, 1989). Their activation is stable to cell extraction in the presence of phosphatase inhibitors suggesting that they themselves are activated by phosphorylation. None of the serine kinases have however been shown to be directly activated by the receptor tyrosine kinase. Presumably more intermediary kinases await discovery.

The model in its present state also has difficulty in accounting for the large number of different actions of insulin with different time scales, apparently initiated by a single phosphorylation event. It is possible that some of insulin's actions may be independent of phosphorylation. Other mechanisms of insulin action are discussed below in section 1.1.6.

#### **1.1.4 Control of cell responsiveness to insulin**

Regulation of a particular cell's response to insulin can occur at the level of insulin receptor number, tyrosine kinase activity, dephosphorylation of the receptor and post receptor events. There is evidence for all occurring *in vitro* and influencing insulin's action in disease states.

Regulation of the number of cell surface receptors is a means by which the organism can determine specificity in the response of target cells to hormone and adapt to changes in its circumstances. Internalisation of the receptor follows ligand binding, serving to terminate the signal at the cell surface and allowing control of receptor number by degradation and recycling. In cells so far studied, the insulin receptor has a half life of 8-12 hours (Hedo *et al.*, 1983, Deutsch *et al.*, 1983) and the phosphorylation state of the receptor has been implicated in its internalisation and recycling (Knutson, 1991). Insulin receptors recycled back to the plasma membrane are devoid of phosphotyrosine, suggesting that they must be dephosphorylated before recycling and implicating a phosphatase enzyme as a regulatory component (Backer *et al.*, 1989). Insulin exposure causes a down-regulation of cell surface insulin receptors and whether this occurs by means of increased receptor inactivation or decreased synthesis depends on the cell type (Knutson, 1991). Exposure of rat hepatoma cells (Crettaz and Kahn, 1976) or cultured rat hepatocytes (Melin *et al.*, 1990) to high insulin concentrations was found to result in a loss of cell surface insulin receptors leading to a state of insulin resistance with respect to insulin's metabolic action.

Level of insulin receptor expression also depend on cell type, state of differentiation, other hormones eg. glucocorticoids and the cell's metabolic status. Receptor levels are lower in growing 3T3 L1 pre-adipocytes but approach a maximum as cells reach a stationary phase (Karlsson *et al.*, 1979). In contrast, receptor levels are high in HL-60 cells (a human promyelocytic cell line) and decrease during chemically-induced differentiation. Presumably there are tissue specific and developmental factors which regulate expression of the gene.

Altered insulin sensitivity can occur in pathological states. The lowered sensitivity of target tissues in the hyperinsulinaemia of obesity and type 2 diabetes mellitus can be accompanied by a decrease in receptor number (Czech, 1985). I<sub>1</sub>

addition, the insulin receptor tyrosine kinase activity has been reported to be impaired in starvation (Karasik *et al.*, 1990), insulin resistance (Takayama *et al.*, 1988b), obesity (Le Marchand-Brustel *et al.*, 1985) and in cases of severe insulin resistance resulting from receptor mutations (Becker and Roth, 1990). The diabetic alteration in tyrosine kinase activity correlates well with altered sensitivity to glucose transport and antilipolysis by insulin (Takayama *et al.*, 1988b) though it is not known whether the defect is intrinsic to or secondary to the diabetic condition. Presumably it does not result from a mutation as it is reversible with insulin treatment. In rodent models of insulin deficient (type 1) diabetes, a decrease in tyrosine kinase activity has been observed, despite an increase in receptor number (Okamoto *et al.*, 1986). Conversely, tyrosine kinase activity is increased in insulin hyperresponsive adipocytes from young obese Zucker rats (Debant *et al.*, 1987). An increase in phosphotyrosine phosphatase activity has been observed in adipocytes and liver from diabetic animals, where it has been proposed to contribute to insulin resistance (Begum *et al.*, 1991). Consequently, the receptor tyrosine kinase remains a possible site for future therapeutic intervention in diabetes, since enhancing signal generation by insulin might enhance sensitivity of target tissues to the hormone.

### **1.1.5 Regulation of growth and gene expression by insulin**

As well as acutely regulating metabolic flux, insulin over longer time periods and at higher concentrations than those present *in vivo*, is known to influence protein synthesis and cell growth (O'Brien and Granner, 1991). Some of these effects may be explained by insulin's low affinity action at the IGF-1 receptor (Florini *et al.*, 1991) (and see below, section 1.2) whilst in a few cell types only insulin can increase DNA synthesis at physiological concentrations by acting through its own receptor (Koontz and Iwahashi, 1981, Taub *et al.*, 1987).

There are at least 50 genes known to be regulated in response to insulin, the best studied being those encoding enzymes involved in regulation of hepatic glucose metabolism *viz.* phosphoenolpyruvate carboxykinase (PEPCK), whose synthesis is repressed by insulin and glucokinase, whose expression is enhanced. The mechanisms and physiological significance of insulin's regulation of genes are not fully understood, but it is clear that insulin can influence gene expression positively and negatively at the level of transcription, RNA processing and degradation and translation (Granner, 1987). In many cases the action of insulin on gene expression is permissive rather than overriding, in that its effect is only observed in the presence of other hormones or substrates. This tonic role of insulin means that significant changes in gene expression are only observed in abnormal circumstances, for example in diabetes or starvation. The rapidity of insulin's action on gene expression varies considerably between genes, probably a reflection of its different modes of action. Extensive work is underway to identify insulin response sequences in genes.

Insulin's action on gene expression, like its actions on energy metabolism are cell specific. For example, insulin stimulates DNA synthesis (McGowan *et al.*, 1981) and increases the amount of mRNA for albumin in primary cultured rat hepatocytes (Lloyd *et al.*, 1987) but does not do so in the cultured rat hepatoma (H4-II-E) cell line (Straus and Takemoto, 1987). Differential effects of insulin on transcription in the two cell types are responsible here. For some genes, the effect of insulin varies within clones of the same cell line (Stanley, 1988). Thus, cell type and state of differentiation are both important in determining insulin responsiveness. In addition, insulin itself can influence the state of differentiation of some cell types. It has been shown to promote differentiation in 3T3 L1 fibroblasts (Watkins *et al.*, 1987), chick embryo muscle cells (Schmid *et al.*, 1983) and rat muscle satellite cells (Allen and Boxhorn, 1989).

### 1.1.6 Other mechanisms of insulin action

Various mechanisms have been proposed by which insulin may exert its effects on cells in addition to tyrosine kinase activation. There is considerable evidence that some of insulin's actions may involve established second messenger systems. The involvement of adenylyl cyclase in insulin signalling is discussed in section 1.5.

Mediators have been isolated from insulin-exposed cells which, after extraction, can mimic some of insulin's actions *in vitro* (Mato, 1989). Mediators have been generated from plasma membranes in response to insulin, indicating that all the components for their generation are located in the plasma membrane and evidence has been presented for their regulation of kinases, phosphatases and direct action on pyruvate dehydrogenase. Romero *et al.* (1988) proposed that a phospho-oligosaccharide second messenger could be generated extracellularly by an insulin-specific PLC, the substrate for the phospholipase being glycosyl phosphatidyl inositol (GPI), released from a GPI-anchored membrane protein by concomitant protease activation. Antibodies which recognise these GPI-derived mediators can specifically inhibit intracellular actions of insulin (Larner *et al.*, 1990), suggesting that they may play a role in insulin's action. The insulin mediator hypothesis has also been extended to explain insulin's effect on intracellular cAMP levels. Saltiel and Cuatrecasas (1986) isolated a mediator from liver following insulin treatment which could inhibit plasma membrane adenylyl cyclase activity. A cAMP-lowering mediator isolated from adipocyte plasma membranes mimicked insulin's action in lowering cAMP and in stimulating lipogenesis and antilipolysis (Zhang *et al.*, 1983). Problems with this hypothesis are that the mediators are generated extracellularly, requiring an explanation for their uptake into the cell. Moreover, it is unclear how insulin activates the protease required for mediator generation and how mediators bring about their

intracellular effects on metabolism. At best, the mediator hypothesis can account for only some of insulin's actions.

A reduction in plasma membrane fluidity, shown using extrinsic probes follows the binding of insulin to its receptor, and occurs at physiological concentrations of the hormone (Farias, 1987). This action of insulin may have a role in regulating the activities of membrane bound enzymes or components of second messenger systems. In several cell lines and in human fibroblasts it has been shown that increasing membrane fluidity by supplementation with polyunsaturated fatty acids is associated with an increase in insulin receptor number, decreased insulin binding affinity and increased insulin sensitivity (Ginsberg *et al.*, 1990). In studies on insulin resistance in cultured hepatoma cells, Bruneau *et al.* (1987) noted that alteration of the membrane lipid composition of hepatoma cells by 25-hydroxycholesterol treatment made the cells insulin resistant with respect to insulin's action on glycogen synthesis. Ginsberg *et al.* (1990) proposed that a less fluid membrane may favour receptor aggregation into clusters, whereas at high fluidity the clusters dissociate exposing more hormone binding sites. Interestingly, these fluidity effects are not observed with the IGF-1 receptor (Ginsberg *et al.*, 1990). This may be a result of the relatively low homology of the transmembrane regions of the insulin and IGF-1 receptors (Ullrich *et al.*, 1986), the region presumably most subject to the influence of membrane lipids. However, as many other agents are capable of altering membrane fluidity, this effect of insulin is of questionable importance in specific actions of the hormone.

As membrane lipid fluidity and hence insulin sensitivity is open to modification by dietary means, considerable interest has developed in this facet of insulin's action with regard to management of diabetes, particularly as type 2 diabetes is commonly associated with hyperlipidaemia. Pathological alteration in lipid fluidity in diabetic states has been shown to respond to dietary and insulin treatment. Neufeld *et al.*, (1986),



working with mononuclear leucocytes from obese type 2 diabetic subjects, showed that a reduced receptor number was dependent on an increased cholesterol /phospholipid ratio which affected lipid fluidity. An increase in receptor number followed a period of caloric restriction in these subjects. Similar findings have been reported in platelets from type 1 and type 2 diabetic subjects (Winocour *et al.*, 1990). In addition, erythrocyte membrane microviscosity is known to be higher in type 1 diabetes (Bryszewska *et al.*, 1986). These findings have been reported to be reduced on insulin treatment.

Another relatively non-specific means by which insulin has been proposed to influence hepatic enzyme activity is its ability to regulate potassium balance and cell volume (Vom Dahl *et al.*, 1991). In addition, most of the established intracellular second messengers have been suggested to mediate certain of insulin's actions in specific cell types viz. calcium ions, diacylglycerol, cGMP and cAMP (Martin, 1987), but no one second messenger has emerged as being responsible for all insulin's effects. The role of cAMP in insulin signalling is further discussed in section 1.5.

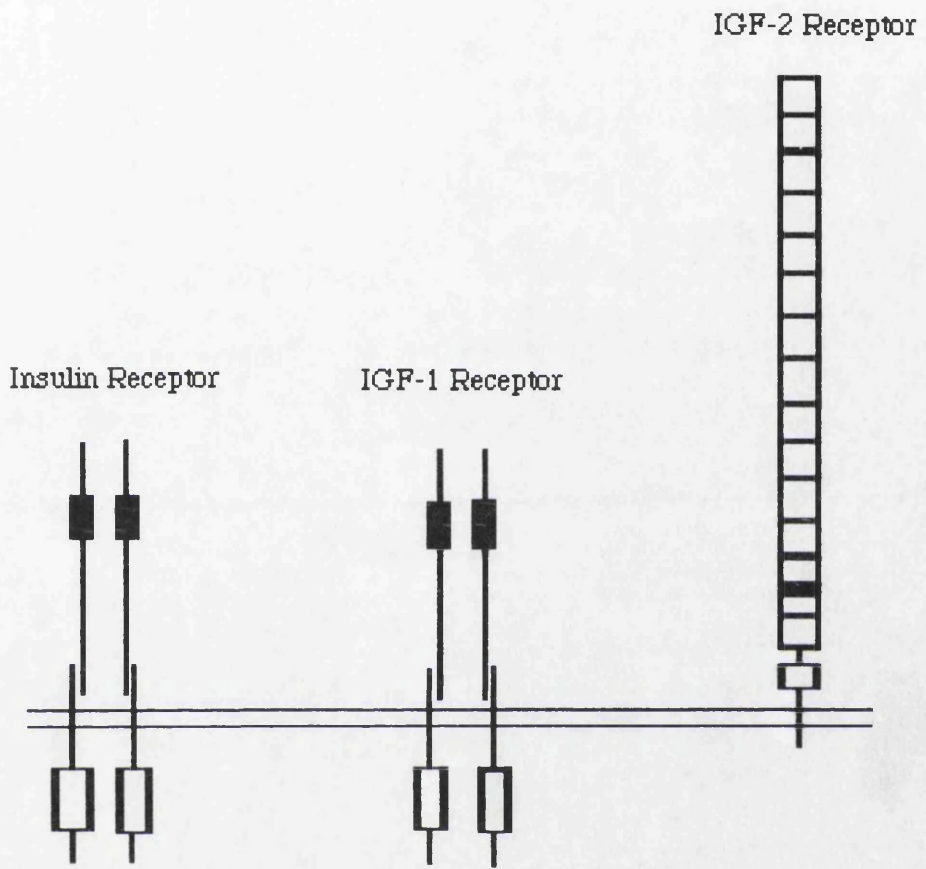
## **1.2 Insulin-like growth factors**

Insulin-like growth factors (IGFs) (or somatomedins) are pro-insulin-like hormones found in the circulation whose primary source is thought to be liver (Froesch and Zapf, 1985). They have a variety of anabolic actions including glucose uptake, amino acid uptake and incorporation into protein, DNA synthesis and cell proliferation (Baxter, 1988). IGF-1 is the main mediator for the growth promoting effects of growth hormone. Elevated levels of plasma IGF-1 are thought to play a role in the pathogenesis of proliferative diabetic retinopathy (Dills *et al.*, 1991).

**Fig. 1.1**      **Insulin and insulin-like growth factor receptors**

The insulin and IGF-1 receptors possess similar structural features with two extracellular, ligand-binding  $\alpha$  subunits and two intracellular  $\beta$  subunits with tyrosine kinase activity. The white boxes represent the tyrosine kinase region and the black boxes cysteine rich, homologous regions. The IGF-2 receptor is a monomer, largely extracellular and with 15 repeats of 150 amino acid residues in the extracellular portion. It bears no structural homology to the other two receptors. (Adapted from Roth *et al.*, 1990).

**Figure 1.1**



The activities of IGFs are well documented in cell lines as well as native cells. IGF-1 is a 7.6kDa protein of 70 amino acid residues and IGF-2, a 7.5kDa protein of 67 amino acids (Florini *et al.*, 1991). The IGF-1 receptor resembles that for insulin in general structure, molecular weight (350kDa) and in possessing tyrosine kinase activity (Rechler and Nissley, 1985) (Fig. 1.1). It has specificity in the order IGF-1 > IGF-2 > insulin. At high ( $\mu\text{M}$ ) concentrations then, insulin is thought to stimulate cell growth and differentiation of cells by binding with low affinity to the IGF-1 receptor (Florini *et al.*, 1991). In this way insulin can stimulate DNA synthesis in hepatocytes, which contain both insulin and IGF-1 receptors.

The IGF-2 receptor is a 250kDa monomer with no known intrinsic activity (Roth, 1988). It does not bind insulin, is not linked by disulphide bonds to other subunits and its physiological role is unclear. One possibility is that it plays a role in IGF-2 metabolism.

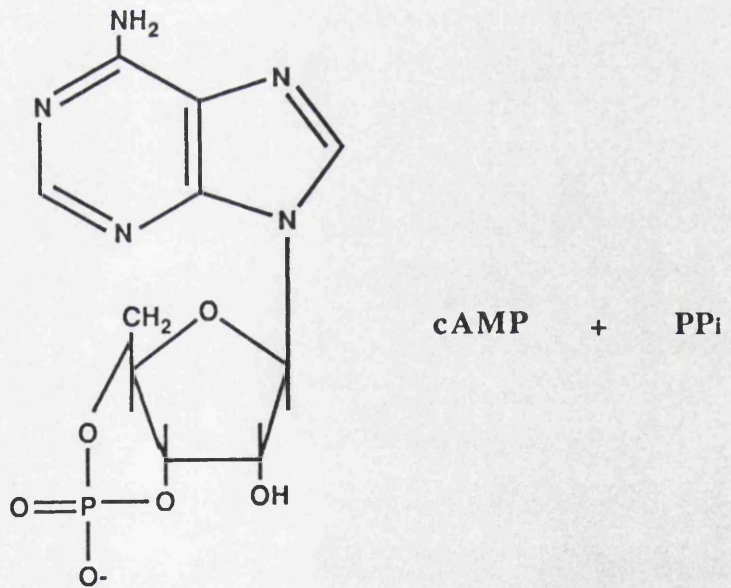
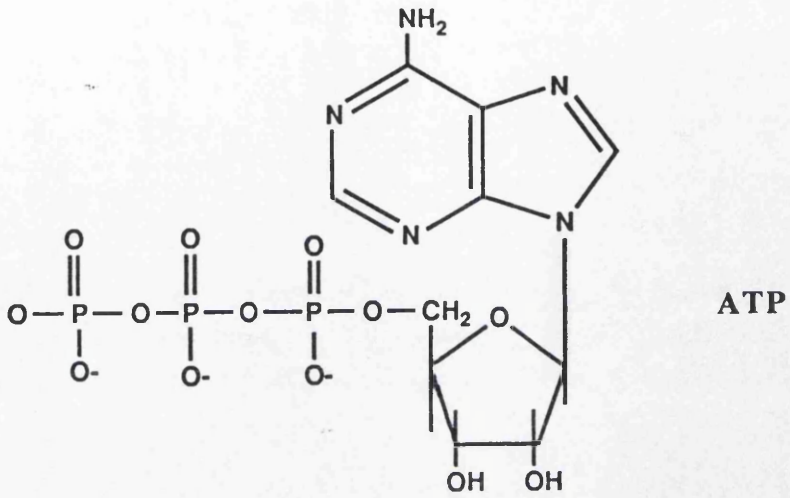
### **1.3 Adenylyl cyclase signalling**

The second messenger cAMP was discovered during studies of hormone effects on liver tissue (Rall *et al.*, 1967). The workers reported production of a heat stable adenine ribonucleotide which was able to activate glycogen phosphorylase in the supernatant fraction. This was the first example of a liver enzyme altered in activity by cAMP. The enzyme producing cAMP, adenylyl cyclase, is a plasma membrane-bound enzyme which catalyses the conversion of ATP to cAMP and  $\text{PPi}$  (Fig. 1.2). Adenylyl cyclase activity was found in all tissues studied except for dog erythrocytes and the highest activity was found in brain (Sutherland *et al.*, 1962).

**Fig. 1.2      The reaction catalysed by adenylyl cyclase**

Adenylyl cyclase catalyses the conversion of ATP to cAMP and P<sub>Pi</sub>.

Figure 1.2



### 1.3.1 The role of cAMP in metabolism

Cyclic AMP exerts its action on cells by activating cAMP-dependent protein kinase (PK-A) and thence a protein kinase cascade in which there is amplification of the response before enzyme activity is altered. The action of cAMP is terminated by its hydrolysis to 5'AMP by cAMP phosphodiesterase activity (Beavo, 1990). Since the discovery that it could stimulate liver glycogen phosphorylase, cAMP has been found to be a major regulator of liver and adipose tissue metabolism. It is a ubiquitous intracellular messenger and has a variety of different functions in different cells.

In liver, cAMP exerts its influence at two levels. Firstly, it acutely regulates the activity of the key enzymes involved in glycolysis, gluconeogenesis and fatty acid metabolism. Secondly, it regulates gene transcription. Glucagon, the main gluconeogenic hormone elevates cAMP in liver thereby activating PK-A and leading to the phosphorylation and activation of phosphorylase kinase and inhibition of the glycolytic enzyme pyruvate kinase (Johnson and Veneziale, 1980). This coordinate regulation is also evident at the level of gene expression where cAMP abolishes the induction of pyruvate kinase (Munnich *et al.*, 1984) and impairs glucokinase expression (Pilkis, 1970). Cyclic AMP is the major stimulus for the increased synthesis and impaired degradation of mRNA for the gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK) (Hod *et al.*, 1986). The other important enzyme regulated in response to cAMP levels is 6-phosphofructo-2-kinase / fructose-2,6-bisphosphatase. PK-A phosphorylates and inhibits the kinase and activates the bisphosphatase (El-Maghrabi *et al.*, 1982), thereby impairing glycolytic flux and enhancing gluconeogenic flux.

Insulin opposes the action of glucagon and catecholamines on these processes. By restraining the level of cAMP it opposes PK-A-mediated phosphorylation and gene transcription and has cAMP-independent effects on gene expression which favour the anabolic pathways. It enhances pyruvate kinase levels and restores glucokinase levels which have been lowered by diabetes mellitus (Spence, 1983). In addition to its effects on carbohydrate metabolism, insulin has long been known to oppose the lipolytic action of cAMP both in liver and adipose tissue (Illiano and Cuatrecasas, 1972). This mechanism of this restraint is further discussed in section 1.5.1.

### **1.3.2 Regulation of adenylyl cyclase activity**

A possible role for GTP in the regulation of adenylyl cyclase was suggested 14 years after the discovery of cAMP (Rodbell *et al.*, 1971). The group found that GTP regulated binding of glucagon to hepatocyte membranes and that GTP was necessary for effective stimulation of adenylyl cyclase by the hormone. They went on to discover that GTP could be used both in stimulation and inhibition of the enzyme, which were suggested to be separate processes (Yamamura *et al.*, 1977). Their introduction of the non-hydrolysable GTP analogue, GppNHp (see appendix) and the discovery of catecholamine-induced GTP hydrolysis by Cassel and Selinger (1976) led to the proposal of the GTPase cycle (Fig. 1.3). The molecular basis of the GTP binding components did not emerge until the introduction of bacterial toxins. The stimulatory component  $G_s$  was shown to be the site of action of cholera toxin which ADP-ribosylated and persistently activated the protein (Johnson *et al.*, 1978). The inhibitory component  $G_i$  was shown to be the site of action of pertussis toxin, another ADP ribosyltransferase (Katada and Ui, 1982). These two toxin substrates were subsequently purified. In 1986, Feder *et al.* demonstrated that adenylyl cyclase could be activated by hormone after reconstitution of the purified receptor, G-protein and catalytic moiety into phospholipid vesicles, showing that these three components



were the minimum necessary to form a signal transduction unit (Fig. 1.4).

G-proteins consist of  $\alpha$  (39-52kDa),  $\beta$  (35-36kDa) and  $\gamma$  (7-10kDa) subunits. The  $\alpha$  subunit appears to subserve the most important functions associated with the protein. Activation of adenylyl cyclase by stimulatory agonists is thought to proceed by the following mechanism (Gilman, 1987). Binding of agonist to the plasma membrane receptor results in a conformational change and activation of the receptor which increases its affinity for GDP-bound  $G_s$ . On colliding with  $G_s$ , the receptor catalyses exchange of GTP for GDP, thus promoting activation of  $G_s$ - $\alpha$  and its dissociation into constituent  $\alpha$ -GTP and  $\beta\gamma$  components. The  $\alpha$  subunit is then able to interact with and activate the effector adenylyl cyclase. Amplification of the response occurs at both the receptor- $G_s$  interaction and the  $G_s$ -AC interaction. Termination of the response occurs due to the intrinsic GTPase activity of the  $\alpha$  subunit which hydrolyses GTP to GDP, restoring the  $\alpha$  subunit to its GDP bound, inactive form and permitting reassociation of the trimer. Fluoride, when complexed with aluminium is thought to activate adenylyl cyclase by binding to the GDP-bound form of  $G_s$ - $\alpha$  where it mimics the  $\gamma$  phosphate of GTP, preventing restoration to the inactive form. Distinct domains on G-protein  $\alpha$  subunits subserve the functions of GTP binding, receptor and effector interaction. The C terminus is considered the site of receptor interaction and N terminus the site of  $\beta\gamma$  interaction (Kaziro *et al.*, 1991).

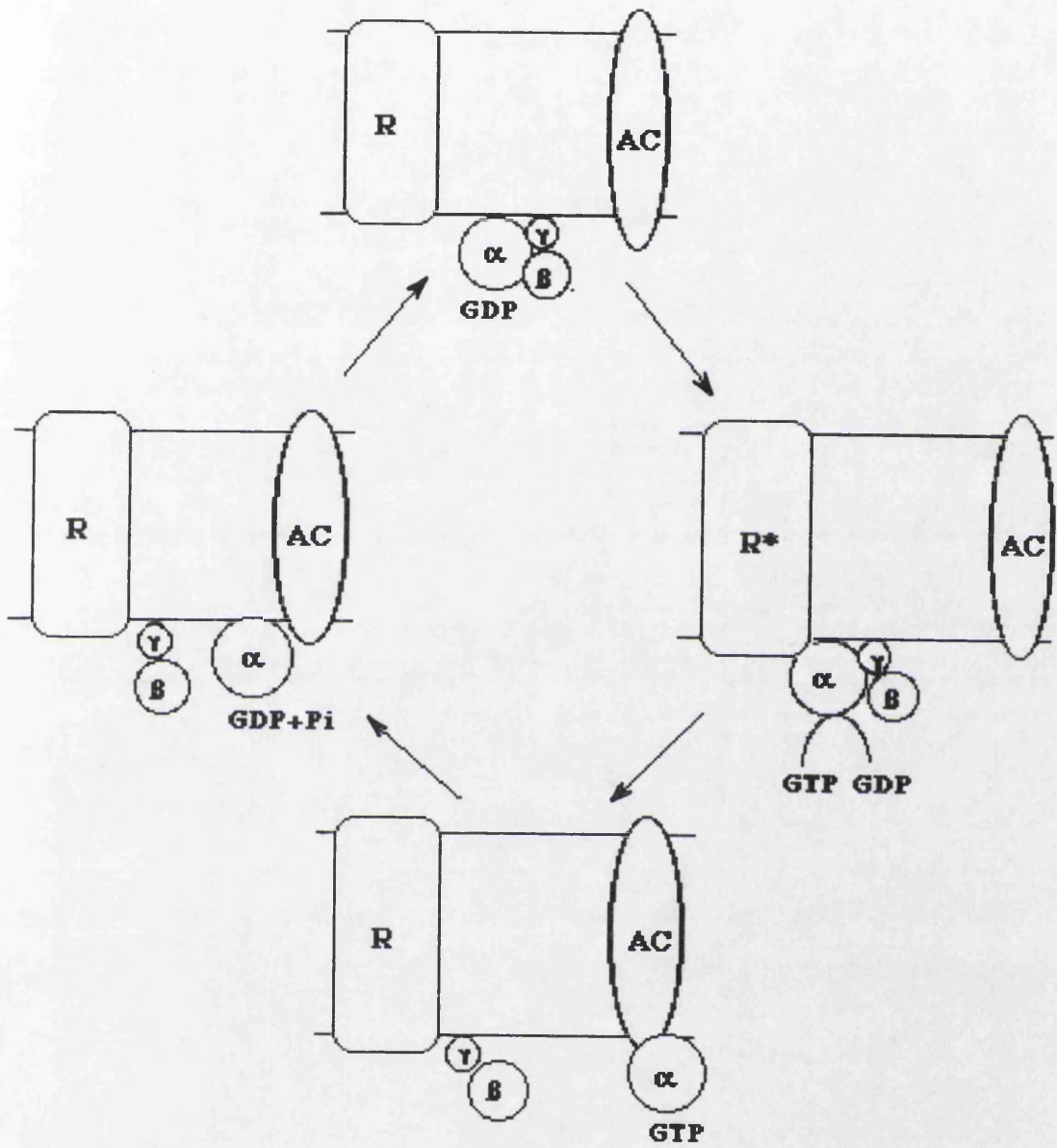
Agonist-induced inhibition of adenylyl cyclase is thought to occur in two different ways (Gilman, 1987). Firstly, activated  $G_i$ - $\alpha$  may interact directly with AC in a similar manner to  $G_s$ - $\alpha$  but possibly binding at a different site, so as to inhibit its activity. Alternatively, a dissociation mechanism has been proposed (Katada *et al.*, 1984) whereby  $\beta\gamma$  subunits released from activated  $G_i$  shift the equilibrium for  $G_s$  dissociation in favour of the inactive trimer. Probably both these mechanisms are functional in cells but their contributions to receptor-mediated inhibition will depend

**Fig. 1.3      The G-protein GTPase cycle**

- (a) G-protein in the basal state is holomeric and has GTP bound to the  $\alpha$  subunit.
- (b) Activated receptor ( $R^*$ ) catalyses exchange of GDP for GTP;  $\alpha$  subunit dissociates from the  $\beta\gamma$  component.
- (c) GTP-bound  $\alpha$  subunit interacts with the effector.
- (d) Intrinsic GTPase activity of the  $\alpha$  subunit hydrolyses GTP and the trimer reassociates.

(Adapted from Spiegel, 1992)

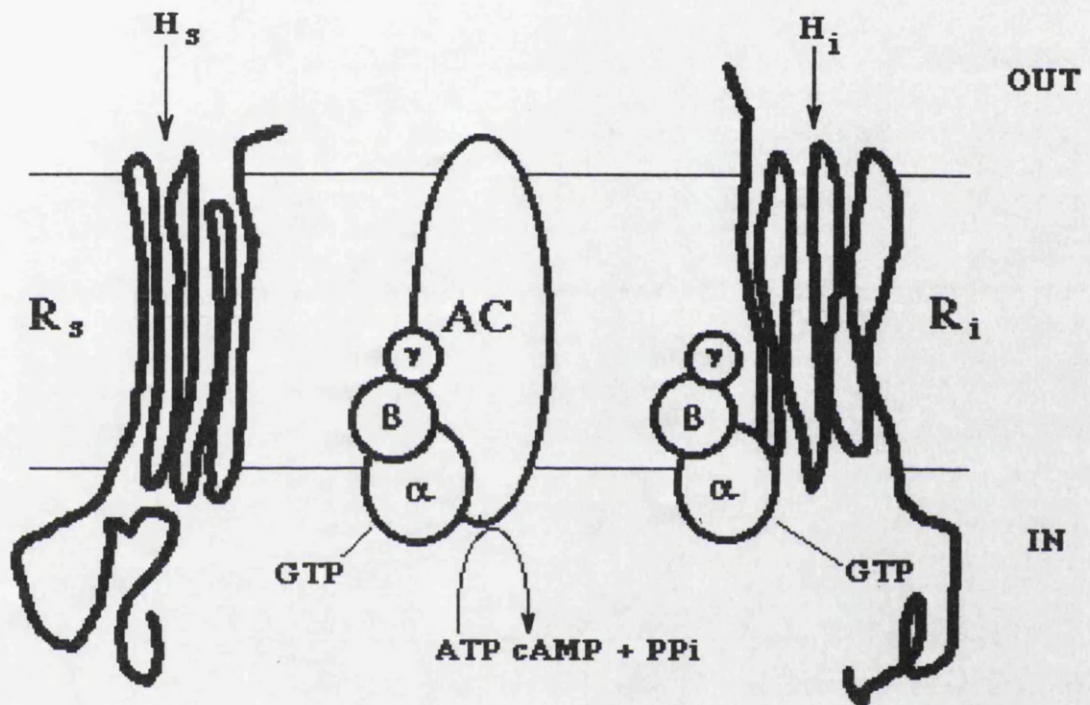
**Figure 1.3**



**Fig. 1.4            Hormonal regulation of adenylyl cyclase**

Stimulatory hormones (H<sub>s</sub>) bind to receptors which have a positive effect on adenylyl cyclase (AC) activity through G<sub>s</sub>. Inhibitory hormones (H<sub>i</sub>), on activating their receptors, exert a negative influence on adenylyl cyclase through G<sub>i</sub>. Both stimulatory and inhibitory G-protein-linked receptors are thought to consist of a hydrophobic, transmembrane domain of seven  $\alpha$  helices, as well as intracellular and extracellular domains. (Adapted from Levitski, 1988).

**Figure 1.4**



on the relative concentrations of  $G_i$  and  $G_s$  (Birnbaumer *et al.*, 1985). Certain types of adenylyl cyclase are also thought to be directly regulated by  $\beta\gamma$  dimers (Tang and Gilman, 1992). Clearly, the extent of inhibition by this mechanism will depend on the  $\beta\gamma$  and adenylyl cyclase subtypes present in the vicinity. Possibilities for control of G-protein function in addition to those mentioned above are becoming apparent. The retinal protein phosducin inhibited the activity of several G-proteins, including  $G_s$ - $\alpha$  mediated adenylyl cyclase activity. In turn, phosducin was inhibited by PK-A, suggesting that it may be part of a complicated regulatory network (Bauer *et al.*, 1992).

The signalling functions of G-proteins appear to require that they remain in close proximity to the plasma membrane. Although in some systems there is evidence for G-proteins leaving the membrane on activation (McArdle *et al.*, 1988) and their location at subcellular sites (Bokoch *et al.*, 1988) there is no evidence for G-proteins performing signalling functions at sites other than at the plasma membrane.

Cholera toxin from *Vibrio cholerae*, in the presence of  $NAD^+$ , ADP-ribosylates GTP-bound  $G_s$ - $\alpha$ , reducing its intrinsic GTPase activity and rendering the protein permanently active (Northup *et al.*, 1980). Its site of action is an arginine residue present in all variants of  $G_s$ - $\alpha$  (residue 201/202 in the long forms and 187/188 in the short forms). This persistent activation results in elevated intracellular cAMP levels, responsible for the symptoms of cholera. Pertussis toxin from *Bordetella pertussis*, the causative organism of whooping cough, ADP-ribosylates and inactivates the GDP-bound (trimeric) form of  $G_i$ - $\alpha$ , preventing its dissociation and abolishing both receptor-mediated and tonic inhibition of adenylyl cyclase. The site of action of the toxin is a cysteine residue located four residues from the C terminus. The  $\alpha$  subunits of at least six G-proteins can be modified by pertussis toxin-catalysed ADP-ribosylation (Freissmuth *et al.*, 1989). Pertussis toxin has proven a particularly useful tool in identifying  $G_i$ -mediated events and substrates involved in signal transduction.

Pertussis toxin treatment of cells, by removing inhibitory  $G_i$  input into the activity of the catalytic subunit, can enhance responses to stimulatory hormones (Heyworth *et al.*, 1984). Hormonal activity not mediated by G-proteins is not influenced by pertussis toxin treatment.

### **1.3.3 G-protein subtypes**

It has become apparent that G-protein  $\alpha$  subunits are members of an extensive GTP binding protein family which have structural features in common (Bourne *et al.*, 1991) and some 16  $\alpha$  subunits have now been cloned (Birnbaumer, 1992). In mammalian cells there are at least nine genes coding for these  $\alpha$  subunits, some of which give rise to more than one polypeptide by alternative splicing mechanisms (Kaziro *et al.*, 1991). Many of the polypeptides have been well characterised but for others very little information is available at present on their tissue distribution and receptor-effector coupling.

$G_s$  is expressed ubiquitously in mammalian cells. Its presence is essential for stimulatory regulation of adenylyl cyclase by hormones. GTP at  $\mu\text{M}$  levels is required for efficient coupling.  $G_s$ - $\alpha$  is encoded by a single gene, but differential splicing can produce up to four different polypeptides (Bray *et al.*, 1986) which have a tissue specific distribution (Mumby *et al.*, 1986). On finding that two forms of  $G_s$ - $\alpha$  could reconstitute fluoride and guanine nucleotide regulatory activity in the  $G_s$ -lacking mutant S49 cyc- cells with equal efficacy, Graziano *et al.* (1987) proposed that there is no functional difference between the different forms, though evidence has also been presented that the shorter form may have a greater ability to activate adenylyl cyclase (Walseth *et al.*, 1989). It seems likely that differences will be uncovered in their receptor coupling abilities, as in the case of  $G_i$ - $\alpha$  forms.

Complementary DNA cloning of Gi- $\alpha$  has revealed three different Gi- $\alpha$  cDNAs viz. Gi-1 $\alpha$ , Gi-2 $\alpha$  and Gi-3 $\alpha$  (Kaziro *et al.*, 1991) all of which are encoded by distinct genes. All three polypeptides have been purified from tissue sources and their distribution characterised. They show 85% amino acid sequence identity. Gi-2 $\alpha$  (40kDa) and Gi-3 $\alpha$  (41kDa) are ubiquitous but their relative amounts vary between tissues (Milligan, 1990). Gi-2 $\alpha$  is generally thought to be the form responsible for inhibition of adenylyl cyclase (Bushfield *et al.* 1990a), though there is evidence both for (Pobiner *et al.*, 1991) and against (McClue *et al.*, 1992) Gi-3 $\alpha$  also having such a role. Gi-3 $\alpha$  has also been proposed to regulate sodium channels (Cantiello *et al.*, 1990), potassium channels (Mattera *et al.*, 1989) and Golgi trafficking (Stow *et al.*, 1991). Gi-1 $\alpha$  (41kDa) has a more limited tissue distribution, notably present in cerebral cortex (where it is the most abundant Gi form), adipocytes and neural tissue-derived cell lines. Its function is at present undefined, but Attali and Vogel (1989) proposed that it may serve to couple opiate receptors to inhibition of adenylyl cyclase. Go- $\alpha$  another pertussis toxin substrate, is expressed predominantly in neural tissues where it is thought to regulate calcium channels (McFadzean *et al.*, 1989). The pertussis toxin-insensitive G<sub>q</sub> is a group of  $\alpha$  subunits thought to couple receptor activation to phospholipid hydrolysis by activating phospholipase C (Simon *et al.*, 1991). Interestingly, only the  $\beta$  isoforms of PLC appear G-protein sensitive. Thus G<sub>q</sub> shows a high degree of specificity in its effector coupling.

There is very strong conservation of  $\alpha$  subunit structure between species. For example only one residue out of 394 differs between rat and human G<sub>s</sub>- $\alpha$ . The amino acid sequences of Gi-2 $\alpha$ , Gi-3 $\alpha$  and G<sub>o</sub>- $\alpha$  are 98% identical between mammalian species. This demonstrates that there is strong evolutionary pressure to maintain G-protein function.

At least four different  $\beta$  and five  $\gamma$  subtypes exist on the basis of cDNA



cloning (Birnbaumer, 1992). The significance of this heterogeneity is unclear but presumably different polypeptides may have different functions increasing the number of possibilities for specific interactions.  $\beta\gamma$  components have been considered to have roles in anchoring of  $\alpha$  subunits, promotion of GDP exchange and attenuation of  $\alpha$  subunit functions. However, it is becoming clear that  $\beta\gamma$  components may activate effectors in their own right. They have been suggested to regulate potassium channels (Logothetis *et al.*, 1987), phospholipase A2 (Bourne, 1989), phospholipase C (Katz *et al.*, 1992) and calmodulin (Katada *et al.*, 1987). A recent study by Kleuss *et al.* (1992) showed that in rat pituitary GH3 cells which express four  $\beta$  isoforms, only two of the isoforms,  $\beta_1$  and  $\beta_3$  couple specific receptors to calcium channels. This demonstrates that  $\beta\gamma$  signalling can be hormone-specific, increasing the likelihood of its having a physiological role.

It has been proposed (Birnbaumer, 1992), that in certain tissues, signalling may occur both through  $\alpha$  subunit and  $\beta\gamma$  subunits at respectively low and high receptor occupancy. This dual signalling may account for the ability of certain receptors to stimulate both PLC and adenylyl cyclase at respectively low and high concentrations. Alternatively, receptors stimulating both effectors may be capable of coupling to both  $G_q$  and  $G_s$ .

#### 1.3.4 Adenylyl cyclase subtypes

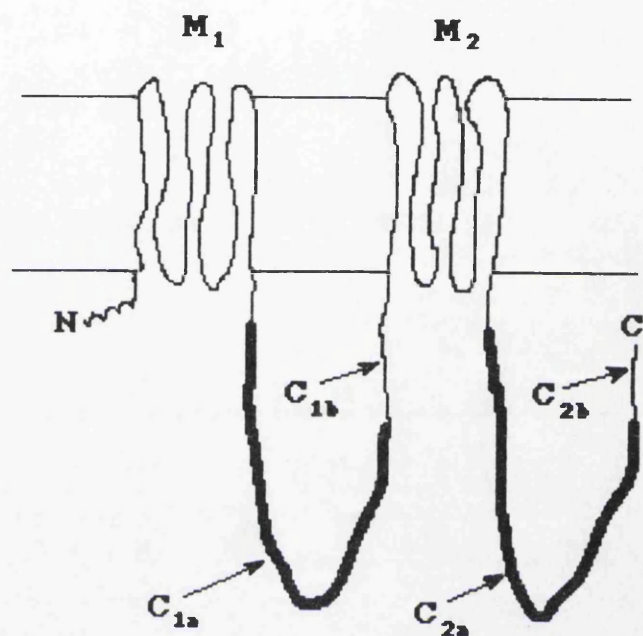
Although adenylyl cyclase signalling has been well studied in a range of tissues, characterisation of the enzyme itself has been relatively slow due to its presence in small amounts in biological membranes and difficulties in purification because of its hydrophobic nature. Major progress was made with the development of its affinity purification on forskolin agarose (Pfeuffer, 1991) and the cloning of calmodulin-sensitive adenylyl cyclase from brain (Krupinski *et al.*, 1989). At least

**Fig. 1.5**

**The structure of eukaryotic adenylyl cyclase**

The adenylyl cyclase catalytic subunit consists of two hydrophobic membrane spanning regions (M1 and M2), an N terminal region representing a motif in eukaryotes and two 40kDa cytoplasmic domains (C1 and C2). Regions of homology between different subtypes are shown in bold. C1<sub>a</sub> and C2<sub>a</sub> are well conserved, forming the likely nucleotide binding site. Point mutations in these regions considerably reduce activity. The hydrophobic domains are the presumed site of forskolin binding. (Adapted from Tang and Gilman, 1992)

**Figure 1.5**



six adenylyl cyclase isoforms have now been cloned from mammalian tissues and can be divided into four subfamilies on the basis of sequence homology (70-75% between members) and regulatory properties. All have similar overall structure (Fig. 1.5) with 12 hydrophobic membrane spanning sequences, two hydrophilic intracellular domains essential for activity (Kelley Bentley and Beavo, 1992) and both N and C termini intracellularly located. All are regulated in a stimulatory fashion by G<sub>s</sub>. Brain adenylyl cyclase was originally estimated to have a molecular weight of 200-250kDa (Neer *et al.*, 1980) but the actual molecular weights of the cloned constituent species are now known to be approximately 120kDa. *In vivo* therefore, the molecule may function as a dimer, or in close association with other proteins.

AC-I (120kDa) is calmodulin sensitive, inhibited by G-protein  $\beta\gamma$  component (Gao and Gilman, 1991) and may have a role in long term potentiation of synaptic transmission. AC-III (129kDa) is present high levels in olfactory epithelia and has high homology with type I within the hydrophilic domains but is unaffected by  $\beta\gamma$  (Bakalyar and Reed, 1990). AC-II (123kDa) and AC-IV (110kDa) have high sequence homology and are stimulated by  $\beta\gamma$  in the presence of G<sub>s</sub> (Gao and Gilman, 1991). In the case of AC-II, this stimulation has been shown to occur in the intact cell (Federmann *et al.*, 1992). This stimulatory effect of  $\beta\gamma$  on these isoforms may explain the ability of agonists which are not themselves activators of adenylyl cyclase to potentiate those that are, via release of  $\beta\gamma$  components from other G-proteins. AC-V and AC-VI have been recently cloned from rat liver (Premont *et al.*, 1992). They appear widely distributed and are not stimulated by  $\beta\gamma$ .

Sensitisation of adenylyl cyclase to stimulation has been noted in response to phorbol ester treatment and attributed to PK-C phosphorylation of adenylyl cyclase (Simmoreit *et al.*, 1991). A recent study by Jacobowitz *et al.* (1993) showed that cyclase subtypes differ in their sensitivity to phorbol ester treatment. The activities

of AC-I, -II and -III increased considerably in response to PK-C activation, while AC-IV, -V and -VI showed only very small increases in activity. Types I-III then, may be signal effectors for extracellular signals not mediated by activation of the stimulatory G-protein  $G_s$ .

The presence of multiple isoforms of adenylyl cyclase provides extra possibilities for control, both by selective expression of the isoforms in target tissues and by interaction with specific G-proteins and other regulatory influences. Thus, the number of mechanisms for regulating cAMP synthesis is probably considerably greater than previously imagined.

### **1.3.5 Forskolin**

The lipid soluble diterpene forskolin (see appendix) was discovered when plant extracts were screened for cardiovascular activities (Bhat *et al.*, 1977). Its ability to stimulate adenylyl cyclase activity in plasma membranes was discovered by Seamon *et al.* (1981) and noted to occur directly on the catalytic subunit. Since then, forskolin has been widely used in investigating the role of cAMP in cell functioning and found to cause large increases in cAMP in almost all mammalian cells. The stimulation of adenylyl cyclase is rapid and potent, with  $10\mu\text{M}$  forskolin generally giving half maximal activation. Maximal forskolin stimulation seen at  $100\mu\text{M}$  is generally greater than that achieved with fluoride or non-hydrolysable GTP analogues and there is little variation in the kinetics of its action between cell types. Forskolin-elevated intracellular cAMP usually reaches a maximum level after 5-10 minutes. Thereafter, any fall in cAMP can be attributed to cAMP phosphodiesterase activity or cAMP extrusion, as homologous desensitisation to forskolin has not been observed in any cell type.

Forskolin can act synergistically with both weak and strong agonists in elevating cAMP (Seamon and Daly, 1983) and can reveal responses to hormones which are too low to observe in the absence of forskolin (Darfler *et al.*, 1982). This synergistic action is more easily observed in intact cells than in plasma membranes and is half maximal at less than 0.1 $\mu$ M. These findings led to the suggestion that forskolin has two sites of action (Barovsky *et al.*, 1984) *viz.* a low affinity site for its action on the catalytic subunit and a high affinity site responsible for its potentiation of hormone action. The physical nature of these sites is not known. However, studies with S49 lymphoma mutants have shown that the high affinity response requires functional G<sub>s</sub> (Darfler *et al.*, 1982), suggesting that forskolin potentiates other hormones by an action on G<sub>s</sub>. The finding that cycloheximide pre-treatment disrupted forskolin responsiveness (Brooker *et al.*, 1983) prompted the suggestion that forskolin, in exerting its low affinity effect on the catalytic subunit, may act on a distinct protein susceptible to inhibition of protein synthesis. The majority of forskolin's pharmacological actions can be attributed to cAMP elevation as it alters cell physiology in a manner consistent with cAMP elevation and PK-A activation (Seamon and Daly, 1986). However, a number of cAMP-independent actions of forskolin have also been reported, for example its inhibitory effect on glucose transport (Klip *et al.*, 1988).

### **1.3.6 Regulation of G-protein levels**

Alterations in tissue G-protein expression can occur on agonist exposure, during differentiation and in a number of diseases. Some of these changes are discussed below.

### 1.3.6.1 Hormonal regulation of G-protein levels

Whilst down regulation of plasma membrane receptors on prolonged exposure to agonist is responsible for reducing responses to specific hormones ie. homologous desensitisation, altered expression of G-proteins may play a role in heterologous desensitisation ie. desensitisation to multiple hormonal stimuli (Milligan and Green, 1991).

Adaptive alterations in both  $G_s$  and  $G_i$  expression have been observed in a direction consistent with current knowledge of their effects on adenylyl cyclase.  $G_s$ - $\alpha$  can be down regulated by stimulatory signals. Prolonged exposure of NG108-15 cells (a neuroblastoma x glioma hybrid) to PGE<sub>1</sub> caused a down regulation of  $G_s$ - $\alpha$  whose time course paralleled that of the prostanoid receptor (McKenzie and Milligan, 1990). The levels of other G-proteins were unaltered. Rich *et al.* (1984) showed that exposure of an MDCK (canine kidney) cell line to glucagon caused a decrease in glucagon-, PGE<sub>1</sub>- and fluoride-stimulated adenylyl cyclase activity. A twofold increase in  $G_i$  was detected. Prolonged exposure of cardiac muscle cells to the  $\beta$ -adrenergic agonist noradrenaline resulted in down regulation of  $\beta_1$ -adrenoceptors and up regulation of  $G_i$  (Reithmann *et al.*, 1989). These changes were accompanied by a reduction in both receptor-dependent and -independent (forskolin) stimulation of adenylyl cyclase.

Similarly, exposure to inhibitory agonists can cause down regulation of  $G_i$ . Treatment of adipocytes with the adenosine analogue N<sup>6</sup>-phenylisopropyladenosine down regulated all three  $G_i$  forms along with the  $\beta$  subunit, but there was no effect on  $G_s$  levels (Green and Johnson, 1989). Both the mechanisms by which receptor sequestration and altered G-protein expression are triggered remain unknown, though it has been suggested that up-regulation of  $G_i$  may be a relatively non-specific response to elevated cAMP levels (Reithmann *et al.*, 1991). The transcription of a number of genes

has been shown to be regulated by cAMP acting through cAMP regulatory elements (Roesler *et al.*, 1988). It is conceivable therefore, that cAMP may have a direct influence on transcription of the  $G_i-2\alpha$  gene. In support of this is the finding of Weinstein *et al.* (1988) that the promoter region of the  $G_i-2\alpha$  gene contained binding sites for a factor (AP-2) which may mediate the transcriptional effects of cAMP. In contrast, no such cAMP response elements were observed on the gene for  $G_s-\alpha$  (Kozasa *et al.*, 1988). In S49 lymphoma cells, the increase in  $G_i-2\alpha$  expression on prolonged adenylyl cyclase stimulation has been shown to be mediated by transcription of the  $G_i-2\alpha$  gene (Hadcock *et al.*, 1990). Interestingly, in a variant of these cells lacking PK-A, there was no up-regulation of  $G_i$  by stimulatory agonists, suggesting that this kinase is essential for control of the level of  $G_i$  expression. However, the cAMP analogue dibutyryl cAMP has been noted to cause a decrease in  $G_i$  levels in NG108-15 cells (Mullaney *et al.*, 1988). Therefore, the up-regulatory effect of cAMP on  $G_i$  is not universal and cAMP is probably not the sole influence on expression of the  $G_i-2\alpha$  gene.

Hormones not acting through G-protein activation can also affect the levels of G-protein expression in cells and consequently adenylyl cyclase signalling. Glucocorticoids can also increase  $G_s-\alpha$  levels in pituitary GH3 cells (Chang and Bourne, 1987). Corticosterone has been noted to increase  $G_s$  forms and decrease  $G_i-1\alpha$  and  $G_i-2\alpha$  in cerebral cortex, leaving  $\beta$  subunit levels unaltered (Saito *et al.*, 1989). All these changes are consistent with the ability of glucocorticoids to enhance hormonal stimulation in tissues (Davies and Lefkowitz, 1984) and the cAMP-elevating effect of these changes probably contributes to the diabetogenic effect of glucocorticoids. The molecular basis of cAMP elevation in liver from diabetic animals is further discussed in section 1.5.2.1 and the influence of insulin on G-protein expression in section 1.5.1.



### **1.3.6.2 G-proteins in differentiation**

There is evidence that G-protein levels can alter in ageing (Green and Johnson, 1989), during development of organs (Luetje *et al.*, 1987) and cell types (Musk *et al.*, 1992) and in response to a number of differentiation agents. Insulin and dexamethasone (Watkins *et al.*, 1987, 1989) can promote differentiation of 3T3 L1 fibroblasts into adipocytes associated with an increased expression of G<sub>s</sub>- $\alpha$ , Gi- $\alpha$  (except Gi-2 $\alpha$ ) and  $\beta$ . These changes were accompanied by an increased adenylyl cyclase response to the  $\beta$ -adrenergic agonist isoproterenol. Retinoids inhibit proliferation and promote differentiation in various non-differentiated cell types. For example, in ROS 17/2.8 cells, an osteosarcoma cell line, retinoic acid was found to inhibit the increased adenylyl cyclase responses associated with cell growth and achieved this both by lowering stimulatory receptor number and levels of the G-proteins Gi and G<sub>s</sub>. Dexamethasone, a potent glucocorticoid analogue, influenced stimulatory hormone receptor expression and G<sub>s</sub> and Gi expression in the opposite direction (Imai *et al.*, 1988). Differentiation of HL-60 cells by exposure to DMSO can produce an increase in Gi-2 $\alpha$  levels (Uhing *et al.*, 1987). Whilst it is clear that G-protein expression and cyclase functioning can be altered in differentiation, various factors are involved in this process, and it is not clear to what extent they are individually involved in the changes. Given these findings, it seems likely that G-proteins play an important role in differentiation *in vivo* and in altering the signalling capability of differentiated cells. However, the significance and mechanisms of these changes remain to be established.

### **1.3.6.3 G-proteins in disease**

Tissue-specific alterations in G-protein expression and function have been noted in a number of endocrine and other diseases, which have extended our

knowledge of the molecular basis of these diseases.

Hypothyroidism is associated with impaired stimulatory signalling by adrenaline resulting in reduced lipolysis and the obesity observed in the clinical state (Goodman and Knobil, 1959). Studies of animal models of hypothyroidism have consistently shown increases in adipocyte expression of all three Gi- $\alpha$  forms, G $\alpha$ - $\alpha$  and G $\beta$  at the level of protein and mRNA (Saggerson, 1992). These changes are associated with increased Gi-mediated responses by N<sup>6</sup>-phenylispropyladenosine, PGE<sub>1</sub> and nicotinate and impaired stimulatory regulation by adrenaline through the  $\beta$ -adrenergic pathway (Saggerson, 1986). Significant increases in Gi-1 $\alpha$ , Gi-2 $\alpha$  and G $\alpha$ - $\alpha$  have also been observed in areas in the brain from hypothyroid animal models (Orford *et al.*, 1991). The mechanism of altered G-protein expression in hypothyroidism is unknown.

Both glucocorticoid excess and deficiency can have marked effects on adenylyl cyclase signalling (Hadcock and Malbon, 1992). Removal of the site of glucocorticoid production by adrenalectomy, impairs catecholamine-stimulated cAMP accumulation and lipolysis in adipose tissue (Ros *et al.*, 1989a), a defect which is partly reversible by glucocorticoid replacement. These changes are accompanied by a fall in G $s$ - $\alpha$ , G $\beta$ 1 and G $\beta$ 2 levels in tissues along with their associated mRNAs (Ros *et al.*, 1989b).

In congestive cardiac failure in humans there is reduced  $\beta$ -adrenergic responsiveness in cardiac muscle along with reduced G $s$  function, increased Gi function and decreased GppNHp and fluoride-stimulated adenylyl cyclase activity (Urasawa and Insel, 1992). As only G $s$  defects have been observed in ischaemic heart disease alone, a role for the loss of Gi function is implicated in the progression to cardiac failure. G-protein-mediated signalling has also been extensively studied in primary hypertension. However, there is no evidence for any changes found in

adenylyl cyclase signalling being associated with the pathogenesis of the condition.

Ethanol exposure of cultured cells has been shown to cause changes in G-protein expression and evidence is growing that impaired adenylyl cyclase signalling may play a role in alcoholism (Gordon *et al.*, 1992). Prolonged exposure of cultured cells to ethanol impairs adenosine uptake. The excess extracellular adenosine stimulates intracellular cAMP accumulation through adenosine (A<sub>2</sub>) receptors, which ultimately leads to desensitisation of stimulatory receptors coupled to G<sub>s</sub> by down-regulation of G<sub>s</sub> protein and its mRNA (Mochly-Rosen *et al.*, 1988). This heterologous desensitisation due to G<sub>s</sub>- $\alpha$  down-regulation has also been observed in lymphocytes from alcoholic patients and may be considered a marker for the disease. Similarly, studies in platelets from these patients showed a significant reduction in PGE<sub>1</sub>- and fluoride-stimulated adenylyl cyclase (Tabakoff *et al.*, 1988). A phenotypic abnormality may therefore predispose certain patients to alcoholism by resulting in a disturbance of G-protein mediated signalling.

### **1.3.7 G-proteins in growth factor signal transduction**

G-proteins were first suspected to be involved in growth regulation in 1987 when Vallar *et al.*, on studying growth hormone-producing pituitary tumours, found that the high basal cAMP levels in these cells were due to a constitutively active G<sub>s</sub>. The sequence of G<sub>s</sub>- $\alpha$  from cells of these tumours was in each case found to contain a point mutation in the codon for Glutamine 227 or Arginine 201, the site of action of cholera toxin, which considerably reduced the GTPase activity of the protein, leading to a permanently active polypeptide (Landis *et al.*, 1989). Evidence that constitutively active adenylyl cyclase can result in increased cell division came from the work of Zachary *et al.* (1990) who transfected Swiss 3T3 cells with G<sub>s</sub>- $\alpha$  mutated at Glutamine 227. The transfected cells were exquisitely responsive to insulin as a mitogen, suggesting that

constitutive cAMP production may potentiate growth factors.

There is extensive evidence that Gi-2 $\alpha$  is involved in growth regulation. Serotonin, which acts through receptors negatively coupled to adenylyl cyclase is mitogenic in some cells (Seuwen *et al.*, 1988) and this action is blocked by pertussis toxin. On transfection of the inhibitory  $\alpha_2$ -adrenergic receptor into fibroblasts, Seuwen *et al.* (1990) found that it conferred mitogenicity upon the cells. Hence a Gi-mediated reduction in intracellular cAMP may be the growth signal in certain systems. It is likely that Gi can also couple to effectors other than adenylyl cyclase in enhancing cell growth; the mitogenic effect of growth factors sensitive to pertussis toxin in NIH 3T3 cells was not affected by cAMP (Taylor *et al.*, 1988) or by transfection with mutant Gi-2 $\alpha$  (Pace *et al.*, 1991). Likewise, in smooth muscle cells, the stimulatory action of serotonin on DNA synthesis was blocked by pertussis toxin but did not involve any alteration in intracellular cAMP levels (Kavanaugh *et al.*, 1988). Two likely effectors to which Gi-like proteins may couple in transducing mitogenic signals are the enzymes PLC and PI3-kinase, as there are numerous examples of PI turnover being stimulated by mitogens and its blockade by pertussis toxin (Ives, 1991).

Mutant Gi-2 $\alpha$  can also lead to abnormal growth regulation. Cyclic AMP impairs growth in Rat-1 cells. Transfection of these cells with a constitutively active mutant Gi-2 $\alpha$  increased the growth rate of the cells and increased their tumorigenic potential (Pace *et al.*, 1991). Lyons *et al.* (1990) carried out an extensive study of different tumours and found that 5-10% of tumours had mutations in Gs- $\alpha$  or Gi-2 $\alpha$  at the same sites as described above or in corresponding codons. It seems then, that G-protein mutations are not universal in tumour cells, but probably restricted to certain endocrine tumours where they may have a role in oncogenesis.

### 1.3.8 G-proteins in the immune system

There is considerable evidence that G-protein-mediated signalling is involved in lymphocyte activation, though the precise roles of the individual G-proteins are elusive. In T cells, agents known to activate G-proteins such as aluminium fluoride (O'Shea *et al.*, 1987) and GTP $\gamma$ S (Sasaki and Hasegawa-Sasaki, 1987) induce generation of inositol phosphates via PLC activity, a biochemical event characteristic of antigen receptor activation. Similarly, increases in GTP hydrolysis and GTP $\gamma$ S binding of T cell membranes result from treatment with mitogenic antibodies (Kvanta *et al.*, 1989). Changes in G-protein expression are known to accompany T lymphocyte activation. On mitogenic activation, mRNAs for G<sub>s</sub>- $\alpha$  and Gi-3 $\alpha$  (but not for Gi-2 $\alpha$ ) were shown to increase, along with the levels of these  $\alpha$  subunits in plasma membranes (Holter *et al.*, 1991). The increased level of G<sub>s</sub>- $\alpha$  and numbers of PGE<sub>2</sub> receptors in these cells resulted in increased cAMP generation, which the authors proposed may be a physiological mechanism for limiting the immune response.

As agonists elevating cAMP can enhance immunoglobulin production by lymphocytes (Roper *et al.*, 1990) (and see below in sections 1.4.1.2 and 6.1), it is likely that G-proteins play a role in B lymphocyte activation, though again their precise roles are unclear. The surface immunoglobulin signalling pathway in B cells exhibits features of a G-protein-coupled inositol phospholipid signalling pathway (Harnett and Rigley, 1992), in that signalling can be blocked in permeabilised cells by the GDP analogue, GDP $\beta$ S whereas the non-hydrolysable GTP analogue GTP $\gamma$ S stimulates inositol phospholipid hydrolysis. The G-protein involved is thought to be pertussis toxin-insensitive.

There is extensive evidence that neutrophil polymorphonuclear leucocyte and macrophage chemoattractant receptors interact with and have their signals transduced by

G-proteins. Increases in cellular cAMP levels induced by hormones such as PGE<sub>1</sub> or  $\beta$ -adrenergic agonists or by forskolin, cholera toxin and dibutyryl cAMP antagonise neutrophil functions *viz.* granule enzyme release and superoxide generation (Bokoch, 1990). Chemotaxis, shape change, aggregation, granule enzyme secretion and superoxide production by neutrophils have all been shown to be sensitive to pertussis toxin treatment. These actions can be directly correlated with the ability of pertussis toxin to ADP-ribosylate a 40kDa toxin substrate in neutrophils (Bokoch and Gilman, 1984). Furthermore, inactivation of neutrophil G<sub>i</sub> by pertussis toxin in a reconstituted system prevents binding of N-formyl peptide (a chemoattractant) (Kikuchi *et al.*, 1986). Non-hydrolysable GTP analogues and sodium fluoride enhance superoxide production (Ligeti *et al.*, 1988). In addition, the N-formyl peptide chemoattractant receptor in neutrophils has been shown to copurify with a 40kDa pertussis toxin substrate, suggesting a direct interaction between the receptor and G-protein (Polakis *et al.*, 1988). The pertussis toxin substrate present in neutrophil membranes consists of G<sub>i</sub>-2 $\alpha$  and G<sub>i</sub>-3 $\alpha$ , 80-90% of which is G<sub>i</sub>-2 $\alpha$  (Bokoch, 1990). One of these must be responsible for transduction of the response, as neutrophils do not contain any G<sub>o</sub> or G<sub>i</sub>-1 (Bokoch *et al.*, 1987, Goldsmith *et al.*, 1987).

### **1.3.9 G-protein phosphorylation**

Phosphorylation, a major control mechanism in metabolic regulation has been implicated in regulation of G-protein function (Houslay, 1991a). Phosphorylation of GDP-bound G<sub>i</sub> and G<sub>o</sub>- $\alpha$  subunits by the purified insulin receptor tyrosine kinase (O'Brien *et al.*, 1987) and phosphorylation of G<sub>i</sub>-2 $\alpha$  (Krupinski *et al.*, 1988) have been recorded, but not the phosphorylation of G<sub>s</sub>- $\alpha$ . Insulin however, does not appear to elicit G<sub>i</sub> phosphorylation in intact cells (Pyne *et al.*, 1989a), raising the possibility that the action of the insulin receptor is only an *in vitro* phenomenon. Phosphorylation of G<sub>i</sub>-2 $\alpha$  has also been detected in response to the phorbol ester, TPA and

and calcium-mobilising hormones, which cause the production of diacylglycerol, suggesting that PK-C may elicit the phosphorylation .

It is unclear what role these phosphorylations play *in vivo*, but considering the amount of evidence emerging for interactions between G-proteins and the insulin receptor (discussed below in section 1.5.1) it seems likely that phosphorylation represents a real form of control. Whilst receptor interaction and GTP hydrolysis account for the major part of G-protein regulation, phosphorylation may be a more subtle form of regulation in response to certain hormones and disease states, indicative of crosstalk between the two signalling systems (Houslay, 1991b). The role of  $G_i-2\alpha$  phosphorylation in diabetes is discussed in section 1.4.2.

#### **1.4 Prostaglandins**

Prostaglandins are a family of potent, locally and short acting (autocrine) hormones, synthesised by almost every tissue in the body, in response to various hormonal and neural stimuli and in inflammation and disease (Norman and Litwack, 1987). All are derived from fatty acids stored in cellular membranes as phospholipids. Following an appropriate signal, phospholipases are activated yielding arachidonic acid and fatty acids, which are acted upon by prostaglandin synthetase to yield cyclic endoperoxides. A series of synthetic reactions then give rise to prostaglandins. The prostaglandins synthesised may either bind to membrane receptors or be released from the cell, producing effects on neighbouring cells. The main site of their metabolism is lung, but different prostaglandins are also rapidly metabolised in tissues such as spleen, liver, kidney and small intestine.

All have the same general structure of a 5-membered ring with two chains

extending from the ring. Members of each subclass have the same ring substituents but different numbers of double bonds in the chains, denoted by the subscript number. Prostaglandins are bound in serum by non-covalent interactions, the tightness of binding being in reverse order to their polarity.

There are thought to be five main types of PG receptors, *viz.* DP, EP, FP, IP and TP ie. one for each naturally occurring prostanoid (Coleman, 1987). Their most potent agonists are prostaglandin D<sub>2</sub>, E<sub>2</sub>, F<sub>2α</sub>, I<sub>2</sub> and A<sub>2</sub> respectively. In addition there are subclasses for the different types of receptor.

#### **1.4.1 The E series prostaglandins**

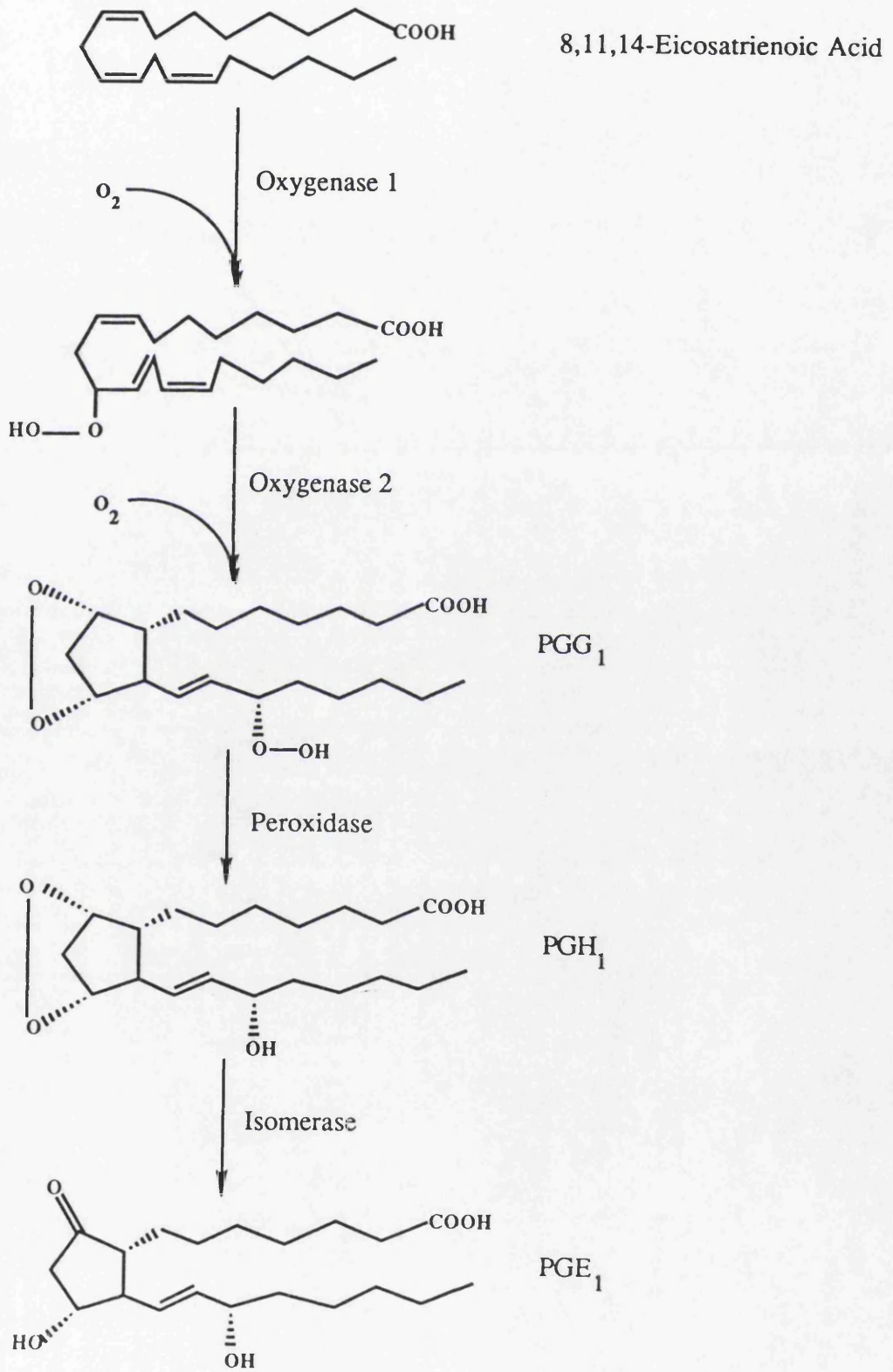
PGE (see appendix) is intermediate in solubility between PGA (lipid soluble) and PGF (water soluble) and shares ring substituents and activities with both these subclasses. The biosynthetic pathway for PGE<sub>1</sub> is shown in Fig. 1.6. Receptors for PGE<sub>1</sub> are present in liver, adipocytes, endocrine tissue and smooth muscle. PGE<sub>1</sub> lowers arterial blood pressure, inhibits gastric acid secretion, inhibits platelet aggregation and has a bronchodilatory action. By producing vasodilatation in vascular beds and inducing vascular leakage it has a pro-inflammatory effect. Of particular medical interest are its stimulation of bone resorption, its cytoprotective effect on gastric and duodenal mucosa and production of fever. PGE<sub>1</sub> has been used therapeutically in Raynaud's disease, vascular insufficiency resulting from connective tissue disorders and in cardiac malformations of the newborn. It has an important 'algesic' effect in sensitising pain receptors to stimuli. The stimulus producing pain also causes release of fatty acids from membranes leading to the formation of PGEs. However, it is unclear how cAMP elevation thereafter leads to the production of pain.



**Fig. 1.6    Biosynthesis of PGE<sub>1</sub>**

The pathway shows the biosynthesis of PGE<sub>1</sub> from 8,11,14-Eicosatrienoic acid (dihomo- $\gamma$ -linoleic acid). All enzymes catalysing the reactions form part of the complex of enzymes constituting PG synthetase. The synthesis of both PGE<sub>2</sub> and PGE<sub>3</sub> proceed through similar pathways involving oxygenation and cyclisation reactions which yield cyclic endoperoxides. These are acted upon by isomerases to produce prostaglandins. The synthesis of PGE<sub>2</sub> starts with 5,8,11,14-Eicosatetraenoic acid (arachidonic acid) and PGE<sub>3</sub> synthesis starts with 5,8,11,14,17-Eicosapentaenoic acid). (Adapted from Norman and Litwack, 1987)

Figure 1.6



Both inhibition (Engelhard *et al.*, 1978) and stimulation (Kassis and Fishman, 1982) of cAMP formation by PGE have been observed in tissues. In most cell types including platelets and fibroblasts, PGE<sub>1</sub> potently elevates intracellular cAMP which mediates its intracellular effects. By this mechanism it impairs reabsorption of water and electrolytes by the small intestine and like cholera toxin has a diarrhoea-inducing effect. In adipocytes, where the receptor is negatively coupled to adenylyl cyclase (Green and Johnson, 1991) its action is antilipolytic. PGE<sub>1</sub> is also known to impair cAMP extrusion from pigeon erythrocytes (Brunton and Mayer, 1979) and to be involved in the action of releasing hormones on pituitary cells (Norman and Litwack, 1987).

Stimulation of PGE<sub>1</sub> release is itself under hormonal and neural control. Glucagon stimulates its release from liver and stimulation of the sympathetic nervous system is the signal for its release from various other tissues. As a result of the widespread expression of prostanoid receptors coupled to adenylyl cyclase in tissues and cell lines, PGE<sub>1</sub> has proven a useful tool in studying hormonal regulation of adenylyl cyclase activity.

#### **1.4.1.1 PGE action in hepatocytes**

In hepatocytes the physiological role of the E series prostaglandins remains rather unclear, but evidence supports PGE<sub>1</sub> having a regulatory effect on hepatic glucose metabolism and output. The E series prostaglandins have been reported to impair glucagon-stimulated glycogenolysis in isolated hepatocytes (Okamura *et al.*, 1988) and glucagon-stimulated cAMP accumulation and fatty acid oxidation (Brass *et al.*, 1988). The transition from the fed to the fasted state has been found to cause a significant decrease in PGE binding site density in rat hepatocytes (Garrity *et al.*,

1988). One possible explanation for this finding is that hepatic PGE levels may rise during starvation. In addition, PGE is rapidly degraded in liver, implying that it does have an important regulatory role (Garrity *et al.*, 1984).

Receptors for E series prostaglandins have been described in isolated hepatocytes (Robertson *et al.*, 1980). Both high affinity ( $K_d = 9.9 \times 10^{-10}M$ ) and low affinity ( $K_d = 8 \times 10^{-9}M$ ) binding sites have been demonstrated. Garrity *et al.* (1988) showed that guanine nucleotide analogues inhibited the binding of PGE to its hepatocyte receptors. This inhibition was absent in membranes from animals pre-treated with pertussis toxin, suggesting that receptor-binding of PGE<sub>1</sub> in hepatocytes is regulated by Gi.

#### **1.4.1.2 PGE action in the immune system**

E series prostaglandins are generally considered to suppress immune responsiveness by inhibiting lymphocyte proliferation (Simkin *et al.*, 1987, Galizzi *et al.*, 1988, Phipps *et al.*, 1989). If exposed to T lymphocytes after mitogenic stimulation, they inhibit interleukin production and cell proliferation. This is probably achieved via elevation of intracellular cAMP (Rodbell, 1980, Rappaport and Dodge, 1982) as a number of agents elevating cAMP have been shown to inhibit lymphocyte proliferation. However, E series prostaglandins have been shown to have stimulatory effects on antibody production. Roper *et al.* (1990) demonstrated that both PGE<sub>1</sub> and PGE<sub>2</sub> increased IgE and IgG production by murine B lymphocytes. At least three cell types in the spleen are known to secrete PGE *viz.* monocytes (Kurland and Bockman, 1978), follicular dendritic cells (Heinen *et al.*, 1986) and fibroblasts (Frey *et al.*, 1986). This may provide a mechanism by which these cells are able to favourably affect immune responsiveness.

PGE may also play a role in atopy *in vivo*, where PGE-secreting cells can promote increased IgE synthesis by B lymphocytes. Monocytes from patients with atopy due to hyper-IgE syndrome secrete constitutively high levels of PGE (Leung *et al.*, 1988). Interestingly, T lymphocytes from atopic subjects possess fewer PGE receptors and respond less to PGE in functional assays than T lymphocytes from normal controls (Rocklin and Thistle, 1986). This may represent a form of desensitisation following prolonged elevation of PGE levels.

#### **1.4.1.3 Interactions between PGE and insulin signalling**

Prostaglandins have been shown to produce a regulatory effect on pancreatic beta cell function through the inhibition of glucose-stimulated insulin secretion (Robertson and Chen, 1977). This may account for the ability of certain inhibitors of prostaglandin synthesis to augment the secretion of insulin (Widstrom, 1977).

Human erythrocyte membranes contain highly specific PGE<sub>1</sub> binding sites (Dutta-Roy and Sinha, 1985). On exposure of these membranes to PGE<sub>1</sub> there is an increase in insulin receptor number, without any apparent change in receptor affinity (Ray *et al.*, 1986). Interestingly, this effect was specific for PGE<sub>1</sub>. In addition, the presence of nM concentrations of PGE<sub>1</sub> decreases the concentration of insulin required for reduction in membrane microviscosity in erythrocytes. As erythrocyte deformability, which is inversely related to membrane microviscosity is important for oxygenation of tissues in the microcirculation (Brownlee and Cerami, 1981), this effect of PGE<sub>1</sub> may allow it to modify favourably tissue oxygenation by erythrocytes, by lowering the concentration of insulin required for decrease in membrane viscosity. Ray *et al.* (1986) also reported that PGE<sub>1</sub> increased the binding of insulin in human lymphocytes.

## **1.5 Interactions between insulin and adenylyl cyclase signalling**

As discussed above, insulin is thought to signal primarily by activation of its receptor tyrosine kinase. However, insulin has been shown in various tissues and cell culture systems, to modulate adenylyl cyclase signalling, suggesting that it may exert some its actions on cells by modifying the activity of this signalling system. There is evidence for its modulation at the level of the receptor, G-protein and adenylyl cyclase catalytic subunit itself. Examples of each are discussed below.

### **1.5.1 Insulin's modulation of adenylyl cyclase signalling**

Insulin cannot modify unstimulated cAMP levels in intact hepatocytes (Pilkis *et al.*, 1975) nor was it noted to affect basal cAMP levels (Illiano and Cuatrecasas, 1972). However, it is well known that insulin can lower cAMP levels which have been previously raised by another hormone and that it can accomplish this both by activation of cAMP phosphodiesterases (Beavo, 1990) and by an inhibitory effect on adenylyl cyclase (Heyworth and Houslay, 1983). At least three high affinity hepatic phosphodiesterases are known to be activated in response to insulin and its activation of a 52kDa plasma membrane phosphodiesterase is thought to be achieved by tyrosyl phosphorylation (Pyne *et al.*, 1989b). The mechanism of activation of other phosphodiesterases is less clear. The direct, high affinity, inhibitory effect of insulin on the adenylyl cyclase catalytic subunit was found to be GTP dependent, overcome by high concentrations of glucagon and dose dependent for insulin with concentrations higher than 10nM abolishing the inhibition. Inhibition was demonstrable on isolated plasma membranes as well as intact cells. These findings, along with the knowledge that pertussis toxin pre-treatment blocks this effect of insulin (Heyworth *et al.*, 1986) led to the proposal that insulin might exert its action on adenylyl cyclase through an

insulin-specific G-protein.

Insulin has been shown to modulate adenylyl cyclase signalling in various blood cell types. Challenge of whole platelets with insulin enhanced stimulation of adenylyl cyclase by PGE<sub>1</sub> in plasma membranes made from these platelets but insulin had no effect on the response when membranes alone were exposed to the hormone. Increased PGE<sub>1</sub> binding in these membranes was shown to be a result of increased receptor number, rather than increased affinity of the binding site (Kahn and Sinha, 1990). More importantly, insulin treatment decreased by 50% the amount of PGE<sub>1</sub> required to inhibit platelet aggregation suggesting that this action of insulin is indeed relevant to platelet physiology. Diabetes is known to be associated with increased responsiveness of platelets to agents promoting aggregation (Halushka *et al.*, 1985) and diabetic subjects have impaired responsiveness to the anti-aggregatory effect of prostaglandins (Davi *et al.*, 1982). It may be that in the absence of insulin, platelets are less sensitive to the anti-aggregatory effect of PGE<sub>1</sub> because of an impairment of the ability of PGE<sub>1</sub> to elevate intracellular cAMP. However, no studies have yet examined the correlation between platelet function and PGE<sub>1</sub> binding in diabetic subjects.

In mononuclear leucocytes, insulin exposure did not alter the basal cAMP level (Sager *et al.*, 1990) but acutely increased the number of cell surface  $\beta$ -adrenoceptors and the response to isoproterenol, which thereafter showed a time dependent decline. The authors proposed that insulin altered both  $\beta$ -adrenoceptor density and coupling to adenylyl cyclase. However, the relevance of this particular effect to leucocyte function is unclear.

A recent study on  $\beta$ -adrenoceptor function in DDTMF-2 cells, a smooth muscle cell line, showed insulin to phosphorylate and attenuate functioning of the  $\beta$ -adrenoceptor (Hadcock *et al.*, 1992). This may be part of the mechanism by which

insulin can antagonise catecholamine action in these cells, though it remains to be seen whether such a mechanism will apply to insulin's action in other cell systems.

There are various reports of insulin inhibiting pertussis toxin-catalysed ADP-ribosylation of G-proteins. Rothenberg and Kahn (1988) showed a 50% reduction in labelling after a short (10 minute) exposure of isolated hepatocytes to insulin. The effect was half maximal at 20nM insulin (a supraphysiological concentration) and independent of GTP. This suggested a functional link between the insulin receptor and G-protein system, possibly altering conformation or dissociation of the heterotrimer. Given the relatively high concentrations of insulin required for the effect however, it is doubtful whether this interaction represents a physiological phenomenon. Pyne *et al.* (1989a) showed a similar inhibition of labelling by insulin in hepatocyte plasma membranes, demonstrating that all the components required for this effect are located within the plasma membrane. A short exposure of whole cells to insulin prior to making membranes had no effect on labelling.

There are a number of reports of exposure of cultured cells to insulin and IGF-1 causing alterations in G-protein expression. Both hormones enhanced intracellular cAMP elevation by adrenocorticotrophic hormone in cultured bovine adrenal cells and this was shown to be as a result of increased expression of G<sub>s</sub>. At physiological concentrations, IGF-1 was more potent in its action than insulin and the increase affected both high and low molecular weight forms of G<sub>s</sub>- $\alpha$  (Begeot *et al.*, 1989). In the same cell type, both peptides have been shown to increase G<sub>i</sub> expression (Langlois *et al.*, 1990). Functionally this change was effected as an increase in phosphoinositide breakdown in response to the hormone angiotensin-II.

Evidence in various other forms has been produced, implicating G-protein involvement in insulin action. Firstly, a number of recent studies have shown that GTP



analogues can modulate insulin binding and action. In rat adipocytes, the non-hydrolysable GTP analogue GTP $\gamma$ S can modulate both insulin binding and receptor tyrosine kinase activity (Davis and McDonald, 1990). Mortensen *et al.* (1992) showed that GTP $\gamma$ S decreased insulin binding in turkey erythrocytes but only in membranes reduced beforehand by dithiothreitol exposure. Kellerer *et al.* (1991) found that in adipocyte and skeletal muscle plasma membranes, insulin at low concentrations rapidly increased GTP $\gamma$ S binding which inhibited subsequent insulin binding and receptor tyrosine kinase activity. On further examination using a photoreactive GTP $\gamma$ S analogue, the binding site was found to be a 40kDa GTP-binding protein. The authors proposed that the insulin receptor may interact with this 40kDa G-protein but did not identify the protein. Secondly, insulin has been reported to stimulate a high affinity GTPase in human platelets suggesting an interaction of the insulin receptor with the G-protein system (Gawler and Houslay, 1987). However, this finding has not been observed in other systems. All these examples provide evidence that aspects of insulin signalling may involve the adenylyl cyclase second messenger system and that crosstalk can occur between the two signalling systems. However, the form of interaction between insulin signalling and G-proteins is taking longer to unravel than the interaction between the classical G-protein-linked receptors and their G-proteins. This may be because the effect of insulin on the system is more subtle, providing fine tuning of the responses to other hormones, rather than producing a signal in its own right.

### **1.5.2 Adenylyl cyclase signalling in diabetes**

Diabetes is a major health problem, accounting for 2-5% of mortality in the western world. Around 80-90% of diabetics in developed countries have type 2 (non-insulin dependent) diabetes. These patients have persistent hyperglycaemia in the presence of normal or elevated levels of insulin and therefore by definition some degree of insulin resistance, though the relative contributions of defective insulin secretion and

peripheral insulin resistance to the pathogenesis of type 2 diabetes is still a matter of debate. Insulin resistance is present in the major insulin target tissues *viz.* adipose tissue, muscle and liver, that in liver being most responsible for the fasting hyperglycemia observed in diabetes (Gerich, 1990). At the molecular level, insulin resistance probably encompasses a number of defects, including impaired receptor tyrosine kinase activity, reduced glucose transport, reduction in activities of enzymes involved in glucose metabolism and abnormal responses to other hormones. The relative contributions of these various factors are not known with certainty.

Diabetics have long been known to have higher stress responses than non-diabetics *ie.* the catabolic pathways of glycogenolysis and lipolysis are more readily activated in response to catecholamines (Shamoon *et al.*, 1980). In recent years the molecular basis of this increased responsiveness has started to become apparent, particularly in liver. Firstly, as glucagon, catecholamines and insulin are all involved in regulation of hepatic cAMP levels, removal of insulin's acute restraint results in elevated levels of the second messenger and enhanced gluconeogenesis, glycogenolysis and ketogenesis. Secondly, defects at all levels of the signal transduction cascade are well documented, which result in elevated basal cAMP levels and increased responses to stimulatory agonists.

#### **1.5.2.1 Defects in hepatocyte adenylyl cyclase signalling in diabetes**

Liver, being an insulin sensitive tissue has been extensively used for studies of adenylyl cyclase signalling in diabetic animals. In diabetes, liver turns from a glucose-storing to a glucose-producing tissue. This follows a decrease in plasma insulin and increased plasma levels of the anti-insulin hormones, noradrenaline, adrenaline, glucagon and cortisol (Tamborlane *et al.*, 1979, Cryer, 1980). This

increased liver responsiveness to anti-insulin hormones has also been observed in insulin-treated diabetic animals (Shamoon *et al.*, 1980).

A common event in animal models of diabetes appears to be elevation of resting intracellular cAMP levels in liver. Elevated cAMP was observed in liver from animal models of both type 1 diabetes eg. streptozotocin diabetic rats (Pilkis *et al.*, 1974) and type 2 diabetes eg. the obese Zucker rat and db/db mice (Herberg and Coleman, 1977). The molecular mechanism by which this elevated liver cAMP is achieved is hotly debated (Lynch and Exton, 1992) and probably varies between systems.

Studies of diabetic rats have revealed either increased (Soman and Felig, 1978), decreased (Srikant *et al.*, 1977, Bhatena *et al.*, 1978) or unchanged (Chamras *et al.*, 1980) numbers of hepatic glucagon receptors, as well as elevated (Lynch *et al.*, 1989) or reduced (Pilkis *et al.*, 1974) basal and agonist-stimulated adenylyl cyclase activity in liver plasma membranes from chemically-induced diabetic rats. One possible explanation for these discrepancies is that animals differ in their toxic responses to diabetogenic agents. There is ample evidence for changes in G-protein expression and functioning in liver from diabetic animals as being in part responsible for the altered adenylyl cyclase signalling. Bushfield *et al.* (1990b) found a reduction in the levels of Gi-2 $\alpha$ , Gi-3 $\alpha$  and the 42kDa form of G<sub>s</sub>- $\alpha$  in hepatocyte plasma membranes from diabetic rats, as well as increased expression of the adenylyl cyclase catalytic subunit. The findings of Lynch *et al.* (1989) are in contrast to the above changes. These workers found no change in Gi- $\alpha$  expression and increased G<sub>s</sub>- $\alpha$  expression in diabetic animals. Whether these inconsistencies have arisen from differences in the methodology or from genuine differences between diabetic animals is a matter not yet resolved. Bushfield *et al.* (1990b) also reported an abnormality of Gi function in diabetic animals, resulting in a reduced ability of low concentrations of GppNHp to inhibit forskolin-stimulated adenylyl cyclase activity. As Gi-1 $\alpha$  is not expressed in liver

and Gi-3 $\alpha$  is not thought to mediate adenylyl cyclase inhibition this finding suggested a dysfunctional Gi-2 $\alpha$ . It was suggested that this dysfunction may be as a result of phosphorylation of the  $\alpha$  subunit under basal conditions. Since such phosphorylation can be mimicked by phorbol ester-activation of PK-C, an abnormally active kinase may be the causal factor. Activated PK-C has indeed been reported in diabetes as a result of increased diacylglycerol levels formed from excess glucose (Greene *et al.*, 1987) and may impair insulin action by increasing insulin receptor phosphorylation.

Changes in the ability of G-proteins to be labelled by pertussis toxin have been observed in animal models and human diabetes. Lynch *et al.* (1989) found reduced pertussis toxin-catalysed ADP-ribosylation of total Gi in hepatocyte membranes from diabetic animals. The ability of pertussis toxin to label Gi in human diabetic liver has also been noted to be impaired (Caro *et al.*, 1991). These authors also showed that the ability of insulin to attenuate pertussis toxin-catalysed ADP-ribosylation of Gi was impaired in liver tissue from diabetic humans. These findings are interesting in view of the work by Rothenberg and Kahn (1988) demonstrating insulin's ability to inhibit pertussis toxin-labelling of G-proteins which suggested a functional interaction between the two signalling systems, possibly by insulin influencing Gi conformation. This again raises the possibility of an abnormality of Gi function in diabetes.

As well as defects at the level of the membrane, decreased cAMP phosphodiesterase activity may account in part for the increased catecholamine and glucagon responsiveness of diabetic liver. Solomon *et al.* (1986) found a reduction in activity of the high affinity low  $K_m$  cAMP phosphodiesterase in liver from streptozotocin diabetic rats which would allow exaggerated intracellular cAMP responses to stimulatory hormones. This is an area which has been given less attention than the membrane protein defects occurring in cAMP generation, but is undoubtedly an important facet of insulin action, which is potentially defective in diabetes.

### 1.5.2.2 Defects in adipocyte adenylyl cyclase signalling in diabetes

In adipose tissue,  $\beta$ -adrenoceptors and glucagon receptors couple through  $G_s$  to adenylyl cyclase, mediating the stimulatory effects of these hormones on lipolysis (Zaagsma and Nahorski, 1990). As in liver, adenylyl cyclase in adipose tissue from chemically induced diabetic animals shows increased sensitivity to the anti-insulin hormones (Zapf *et al.*, 1978) and unstimulated cAMP levels are elevated both in the basal state and in response to stress (Schimmel, 1976). Increased sensitivity and expression of the adipocyte  $\beta$ -adrenoceptor has been noted in streptozotocin-induced and human diabetes (Wahrenberg *et al.*, 1989, Solomon *et al.*, 1990), but glucagon receptor numbers appear to be decreased (Sato *et al.*, 1989). No changes have been noted in  $G_s$ - $\alpha$ ,  $G_i$ -1 $\alpha$  or  $G_i$ -2 $\alpha$  levels in adipocytes from diabetic animals (Strassheim *et al.*, 1990). Interestingly, there is an increase in  $G_i$ -3 $\alpha$  expression and mRNA for both  $G_i$ -3 $\alpha$  and  $G_i$ -1 $\alpha$ , though it is difficult to assess the significance of this finding, as it is not known to which effector  $G_i$ -3 $\alpha$  couples in adipocytes. In one study on adipocytes from human type 1 diabetic subjects, no changes in G-protein levels were noted, though the study was too small to be conclusive and did not investigate adenylyl cyclase activity and G-protein function in the adipocyte membrane preparations (Ohisalo *et al.*, 1989).

In db/db mice, a genetic animal model of diabetes and obesity an elevation of  $G_i$ -1 $\alpha$  was noted in adipocyte plasma membranes as compared to lean controls but no change in the other  $G_i$ - $\alpha$  subtypes,  $G_s$ - $\alpha$  or  $\beta$  subunit. The change in  $G_i$ -1 $\alpha$  expression had little effect on regulation of adenylyl cyclase by inhibitory hormones, though the functioning of specific stimulatory receptors was attenuated in the animals (Strassheim *et al.*, 1991). Palmer *et al.* (1992), on studying adipocyte adenylyl cyclase from obese

diabetic CBA/Ca mice, an animal model of insulin resistance, noted impaired receptor-mediated stimulation of adenylyl cyclase by isopoteranol and glucagon. Levels of all three  $G_i$  forms were reduced in adipocyte plasma membranes from the diabetic animals, but  $G_s$  levels were unaltered. These changes correlated with measurements of impaired  $G_i$  function.

It seems likely that  $G_i$  dysfunction may contribute to the increased stimulatory agonist responsiveness observed in adipocytes from diabetic animals and humans. Strassheim *et al.* (1990) reported loss of GTP dependent- but not receptor-stimulated  $G_i$  function in adipocytes from diabetic animals. As in liver, an increase in  $G_i$ -2 $\alpha$  phosphorylation has been proposed to account for the reduction in  $G_i$  function (Bushfield *et al.*, 1990c). Green and Johnson (1991) found a functional uncoupling of  $G_i$  from the inhibitory PGE<sub>1</sub> and A<sub>1</sub>-adenosine receptors in adipocytes from diabetic rats. This change was accompanied by reduced pertussis toxin labelling of  $G_i$ - $\alpha$  labelling in plasma membranes from the adipocytes.

### **1.5.2.3 Defective adenylyl cyclase signalling in other tissues in diabetes**

Stimulation of  $\beta$ -adrenoceptors in cardiac muscle normally increases heart rate and contractility. In cardiac muscle from diabetic humans and rats which have some degree of diabetic neuropathy, there is reduced cardiac responsiveness to  $\beta$  agonists (Zola *et al.*, 1988, Berlin *et al.*, 1986, Almira and Misbin, 1989). There is a well documented reduction in cAMP responsiveness (Gotzsche, 1983) and  $\beta$ -adrenoceptor number (Ingebretsen *et al.*, 1983), which is probably largely responsible for the impaired  $\beta$ -adrenergic responsiveness in this tissue, as  $G_s$ - $\alpha$  and  $G_i$ - $\alpha$  expression have been noted to be unaltered in this tissue (Bushfield *et al.*, 1990c). In diabetic skeletal muscle there is reduced  $\beta$ -adrenergic stimulation of adenylyl cyclase (Garber, 1980).

Decreased G<sub>s</sub> and G<sub>i</sub> expression have also been noted, but with their ratio unaltered, therefore the significant change is probably at the level of the receptor.

Absent G<sub>i</sub>-mediated inhibition of adenylyl cyclase has also been noted in areas of the brain (Abbracchio *et al.*, 1989) and retina and labelling of the G-protein transducin was reduced, in rod outer segments from diabetic animals (Kowluru *et al.*, 1992). Therefore, this is not a phenomenon which is restricted to the main target tissues of insulin. No detailed study has been made of G-protein expression in specific areas of the brain in diabetes.

Observations of G-protein mediated signalling defects have also been made in platelets from diabetic animals and humans (Abraham *et al.*, 1986, Connell *et al.*, 1986, Livingstone *et al.*, 1991). Although many signalling defects have been reported which may be responsible for the altered physiology in animal models and diabetic patients, it is still unclear how these changes come about. Blood cell functioning in diabetes is further discussed in section 6.1.

## **1.6 Cell culture systems in the study of hepatocyte signal transduction**

Studies on hepatocytes can be carried out on primary cultured cells, tumour cell lines, or virally-immortalised cells. Each of these is discussed in detail below, with regard to the study of hepatocyte function.

### **1.6.1 Native hepatocytes**

Hepatocytes isolated by collagenase perfusion closely resemble *in vivo* adult liver cells both structurally (Sattler *et al.*, 1978) and biochemically (Tanaka *et al.*, 1978)

and preparations are relatively free from non-parenchymal cells (Berry and Friend, 1969), despite the number of other cell types present in whole liver. They can be seeded out on collagen coated plates in order to increase the lifespan of the cells in culture (Strom and Michalopoulos, 1982). Hepatocytes plated on collagen membranes do not flatten out like those seeded on a rigid substratum, and retain endoplasmic reticulum, Golgi apparatus and desmosomes for at least ten days (Sattler *et al.*, 1978). However, all these monolayer cultures have the major disadvantage that they do not proliferate (Richman *et al.*, 1976), so that studies on signal transduction are limited to this time period during which cells retain relatively hepatocyte-like features and shorter still (a few hours) if cells are isolated under non-sterile conditions. As a result of this short lifespan in primary culture, fresh cells must be prepared on each occasion of experiment.

Many features distinguish native hepatocytes considerably from undifferentiated cells. Notably, native hepatocytes are highly specialised and polarised cells with specific functions in particular areas of plasma membrane. For example, it is possible to isolate distinct plasma membrane fractions with different protein levels, Gi- $\alpha$  and G-protein  $\beta$  subunit being highest in the bile canalicular fraction than in the sinusoidal membrane fraction (Ali *et al.*, 1989). Secondly, liver tissue is thought to show zonal heterogeneity regarding metabolic processes (Jungermann, 1985), with gluconeogenesis, oxidative energy metabolism and urea synthesis concentrated in the periportal zone and glucose uptake, glycolysis, glycogen synthesis and lipogenesis predominating in the perivenous zone. Zonal differences are also apparent in enzyme distribution (Katz *et al.*, 1977) and hormone responsiveness with glucagon-stimulated adenylyl cyclase located predominantly in the perivenous area. The presence of these highly differentiated features in hepatocytes means that de-differentiated cells, if chosen for study, will only be to a limited extent comparable with native cells and also makes it possible that there will be considerable heterogeneity amongst cell lines derived from



hepatocytes.

### **1.6.2 Hepatoma cell lines**

Clearly there are numerous advantages of having a hepatocyte cell line as a model system for studying signal transduction. Cells are uniform and can be passaged continuously, as they proliferate spontaneously, providing a large amount of material and avoiding the repeated use of animals. Cultured cells can be grown under controlled conditions and unlike rat hepatocytes or whole animal studies, are free from the influences of counter-regulatory hormones if experiments are performed in serum-free media. However, hepatocyte cell lines have a number of disadvantages, in particular their lack of comparability to the native cell type. They may de-differentiate or diverge phenotypically, as indicated by the lack of cell specific markers (Nairn *et al.*, 1990) and the uniform monolayers lack the characteristic features of native liver cells discussed above *viz.* polarisation and zonation.

Though their responses are not entirely similar to those of hepatocytes, hepatoma cell lines have been extensively used in the study of insulin action and have proven useful as model systems. For example, the H4-II-E cell line (Straus and Takemoto, 1987) and Hep G2 (Hatada *et al.*, 1989) cells have been widely used in studying transmembrane signalling by insulin, and regulation of gene transcription. The H-35 hepatoma cell line has been used in studies on hormonal regulation of serum protein secretion (Tsukada *et al.*, 1985) and in identification of an insulin-sensitive glycopospholipid (Mato *et al.*, 1987). The hepatoma line Fao was the cell system first used in studies on the insulin receptor tyrosine kinase substrate IRS-1 (White *et al.*, 1985) and another hepatoma cell line ZHC, has been used in studies on the effects of membrane lipid composition on insulin signalling (Staedel *et al.*, 1990).

### 1.6.2 SV40-immortalised hepatocytes

Immortalisation means acquisition of an unlimited proliferative potential. Cell cultures can be defined as immortalised when they are capable of growth for at least 100 doublings as compared to a maximum lifespan of about 30 doublings in control cells (Spandidos and Wilkie, 1984). It can be measured by the ability of cells to grow at low cell density (Petit et al, 1983), a technique based on the finding that native rodent cells do not multiply at low density (Todaro and Green, 1963). Immortalisation is generally considered to be an early event in the process of malignant transformation, as treatment of embryonic cells with carcinogens established cell lines, which although untransformed at low passage number, progressed to anchorage independence and malignancy after *in vitro* culture (Newbold *et al.*, 1982).

Several viral and cellular oncogenes have been shown to immortalise cells, suggesting that various mechanisms may lead to escape from senescence (Linder and Marshall, 1990). In order to replicate in non-dividing host cells, small DNA viruses induce cellular DNA synthesis. They accomplish this by encoding proteins which relax growth control. Specific immortalising genes have been identified for adenovirus (Houweling *et al.*, 1980), polyoma virus (Jat and Sharp, 1986), SV40 (Petit *et al.*, 1983) and papillomavirus (Matlashewski *et al.*, 1987). The SV40 virus can be used to immortalise and transform various cell types by transfection, using genetic material prepared from virions. This leads to the establishment of a stable cell line (Chou, 1989). Alternatively, DNA contained within a plasmid vector can be used. Immortalised cells which have not acquired the non-differentiated phenotype of transformed cells can be isolated at low passage (Nagata *et al.*, 1983).

On integration into the host cell genome, two proteins can be expressed, the large and small T antigens, 19kDa and 17kDa in size, respectively. The large T antigen induces changes in the host cell, bringing about immortalisation and eventually transforming them to tumorigenicity. It is a 708 amino acid protein with several domains possessing different functions (Livingston and Bradley, 1987). It binds DNA (Fanning *et al.*, 1989) and in a separate domain possesses an ATPase thought to be required for its unwinding of double stranded DNA, prior to its induction of gene expression (Beard and Bruggmann, 1989). A high concentration of T antigen is thought to be required for immortalisation (Lanford *et al.*, 1985) and expression above a certain level is necessary for tumorigenicity (Efrat and Hanahan, 1989). T antigen is also able to associate with various cellular proteins such as DNA polymerase and transcription factors (Knippers, 1989). The SV40 large T antigen can cooperate with activated oncogenes to induce the conversion of normal embryonal fibroblasts to tumour cells. Glucocorticoid hormones can substitute for T antigen in the immortalisation of rodent fibroblasts (Martens *et al.*, 1988). The mechanism of this action is unknown but it may be related to their ability to regulate the transcription of the same genes as viral gene products (Offringa *et al.*, 1988).

Woodworth *et al.* (1986) generated cell lines by transfection of rat hepatocytes with SV40. These cells did not initially show a fully transformed morphology, but developed this after a time in culture. The cells produced albumin in amounts comparable to rat hepatocytes and, as albumin synthesis is a major feature of hepatocytes accounting for 11% of protein synthesis, this was an indication that the cell line retained at least one feature of differentiated cells. However, the cells were all fibroblast-like in morphology and in this respect more closely resembled hepatoma cell lines than primary cultured hepatocytes. Native hepatocytes produce many proteins associated with their differentiated state, a feature advantageous in monitoring the

effects of SV40 transfection on phenotype. Various markers have been identified in transfected hepatocytes, for example tyrosine aminotransferase (Isom *et al.*, 1980) and transferrin, hemopexin and glucose-6-phosphatase (Woodworth *et al.*, 1986) but are present in differing amounts between cell lines generated (Isom *et al.*, 1980).

Woodworth *et al.* (1988) showed that a variety of liver-specific gene products were expressed in SV40-immortalised cells, at levels similar to those in native hepatocytes, for up to 22 passages after transfection. Reduction in expression of these genes occurred with time in culture and correlated with increased tumorigenic potential. A large number of these cell lines have now been generated and proven particularly useful in the study of changes in gene expression on progression from a normal cell to hepatocellular carcinoma and mechanisms regulating gene expression and differentiation.

Chromosomal damage and cytogenetic abnormalities observed in SV40-immortalised cells as compared to native rat hepatocytes are lower than in other established cell lines (MacDonald *et al.*, 1991). This closer genotypic relation to native cells has caused workers to consider the value of SV40-immortalised cells as model systems. One such cell line, SV40 RH1, has been examined as a model system for studying drug metabolism, as non-differentiated tumour cell lines have reduced glutathione synthesis and are therefore a poor substitute for native hepatocytes in this case. Interestingly, the immortalised cell line was found to retain the pathway for glutathione synthesis and possessed glutathione S-transferases, comparable to rat hepatocytes (Nairn *et al.*, 1990). This cell line then, at least at low passage, is more useful than hepatoma cells as a model system for the study of drug metabolism.

As cell lines differ phenotypically from their native cell types, some have been subjected to agents and conditions which promote differentiation in order to produce cells with more native characteristics. This may allow their responses to be more readily

compared to those of adult, non-proliferating cells (Watkins *et al.*, 1987). Conditions favouring cell differentiation include high cell density (more than  $10^5$  cells /cm<sup>2</sup>) (Frame *et al.*, 1984), high intracellular calcium-ion concentration and the presence of various differentiation inducers. These include glucocorticoid hormones, insulin, nerve growth factor, the polar solvent DMSO and IBMX, a phosphodiesterase inhibitor (see appendix). Matrix interaction can also promote differentiation. For example, collagen and fibronectin substrata can be used to aid adherence of cells and allow the development of cell polarity (Strom and Michalopoulos, 1982), avoiding the use of the above agents. However, the ability to differentiate under the conditions mentioned is a feature only of certain cell types.

Rapid proliferation in a cell line and the maintenance of a fully differentiated state are mutually exclusive. However, in generating cell lines for study, the hope is to obtain cells which, having a capacity to divide, retain at least some of the features of differentiated cells.

## **1.7 Aims of project**

In contrast to hepatoma cell lines, SV40-immortalised hepatocytes have some phenotypic similarity to native hepatocytes. SV40-immortalised cells then, are potentially a useful model system for studying signal transduction mechanisms. The aim of this project at the outset was to characterise adenylyl cyclase signalling in one SV40-immortalised, hepatocyte-derived cell line, P9, comparing it with native hepatocytes and to consider the value of this line as a model system for studying this second messenger system. Secondly, the project sought to examine aspects of insulin's modulation of the signalling system in this cell line. A separate aim of the project, but still in the area of insulin signalling was to examine adenylyl cyclase signalling and G-protein expression in human diabetic subjects. Mononuclear leucocytes were chosen as the tissue for study.

**Chapter 2**  
**Materials and Methods**

## **2.1 Materials**

Reagents were obtained from the following suppliers:

### **2.1a General reagents**

Sigma chemical Co.

Poole, Dorset

Alumina (neutral)

ANSA

Arginine hydrochloride

ATP (disodium salt)

BSA

Bromophenol blue

Cyclic AMP (disodium salt) and 8-bromo-cAMP

Charcoal (Norit A)

Cholera toxin

Collagen (Rat tail, type 1, acid soluble)

Dexamethasone

Dextran T-500

Dowex (AG50W-X4, 200-400 mesh)

Glucagon

Imidazole

Insulin (porcine pancreas)

Nonidet-P40

O-dianisidine

Phosphoserine

Phosphothreonine

PGE<sub>1</sub>

Protein A-agarose

TEMED

60



Thimerosal

TPA

Triton X-100

Trypsin

Boehringer (UK) Ltd

Lewes, East Sussex

Creatine phosphate

Creatine phosphokinase

Dithiothreitol

GTP

GppNHp

Triethanolamine hydrochloride

Tris

Thymidine

Calbiochem, Cambridge

Forskolin

National Diagnostics,

Aylesbury,

Buckinghamshire

'Ecoscint' scintillation fluid

Porton products,

Porton Down,

Salisbury,

Wiltshire

Pertussis toxin

Koch Light Lab Ltd,

Haverhill,

Suffolk

DMSO

Sodium potassium tartrate

FSA Lab Supplies	N,N'-methylenebisacrylamide
M and B, Dagenham, UK.	Ammonium persulphate
Whatman International Ltd.	3mm chromatography paper
BRL, Paisley, UK	Pre-stained molecular weight markers
Pharmacia Ltd., Milton Keynes, Bucks. UK.	Ficoll-Paque
Antibody production Unit, Law Hospital, Lanarkshire, Scotland	HRP-linked sheep anti-rabbit IgG
BOC	Carbon dioxide
Analytichem International	Frits Chromatography columns

### **2.1b Tissue culture plasticware**

Costar Cryotubes	Filters (0.22 $\mu$ m)
Becton Dickenson	Falcon tissue culture plates

Corning	Multiwell plates
Elkay Products	50ml centrifuge tubes Tissue culture pipette tips

## 2.2 Tissue culture media

Gibco Life Technologies, Paisley, UK	Dulbecco's modification of Eagle's medium Foetal calf serum Glutamine (200mM) Sodium bicarbonate (7.5%) Penicillin (10 <sup>4</sup> IU/ml) Streptomycin (100mg/ml)
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## 2.3 Radiochemicals

Amersham plc Amersham, Buckinghamshire	[8- <sup>3</sup> H] Adenosine 3', 5'-cyclic monophosphate. [5',8- <sup>3</sup> H] Adenosine 3', 5'-cyclic monophosphate [α- <sup>32</sup> P]-Adenosine 5'-triphosphate [ <sup>125</sup> I]-Glucagon [ <sup>32</sup> P]Pi [ <sup>125</sup> I]-Sheep anti-rabbit IgG [5-Me- <sup>3</sup> H]-Thymidine
Du Pont	[Adenylate- <sup>32</sup> P]-NAD

All other reagents were from BDH, Poole, Dorset, England.

## 2.4 Standard buffers

Phosphate-buffered saline: (PBS)	NaCl 145mM KCl 5.4mM KH <sub>2</sub> PO <sub>4</sub> 1.5mM Na <sub>2</sub> HPO <sub>4</sub> 8.1mM, pH 7.4
Tris-buffered saline: (TBS)	NaCl 500mM Tris HCl 20mM, pH 7.5
Hepes-buffered salt solution: (HBSS)	NaCl 145mM KCl 6mM Glucose 10mM Hepes 25mM, pH 7.4
Trypsin solution:	Trypsin 0.1% (w/v) Glucose 10mM EDTA 0.025% (w/v)
Laemmli buffer:	Urea 5M SDS 0.17M Dithiothreitol 0.4M Bromophenol blue 0.01% (w/v) Tris HCl 50mM, pH 8.0
Rat hepatocyte plasma membrane preparation buffer:	EDTA 1mM EGTA 1mM Benzamidine 2mM β-glycerophosphate 10mM

Sucrose 0.25M  
Phosphoserine 1mM  
Phosphothreonine 1mM  
Tris HCl 10mM, pH 7.4

Krebs /Henseleit /BSA:

NaCl 120mM  
NaHCO<sub>3</sub> 25.3mM  
KCl 4.8mM  
MgSO<sub>4</sub> 1.2mM  
KH<sub>2</sub>PO<sub>4</sub> 1.2mM  
CaCl<sub>2</sub> 1.28mM  
Glucose 5mM  
Lactate 10mM  
Glutamine 2mM  
BSA 2% (w/v), pH 7.4.

Cell-solubilising buffer:

Triton X-100 1% (v/v)  
SDS 1% (w/v)  
EDTA 10mM  
NaH<sub>2</sub>PO<sub>4</sub> 10mM  
NaF 10mM  
Na<sub>3</sub>VO<sub>4</sub> 100μM  
β-glycerophosphate 10mM  
Phosphoserine 1mM  
Phosphothreonine 1mM  
Hepes 50mM, pH 7.2

Wash buffer:

Triton X-100 1% (v/v)  
SDS 0.1% (w/v)

NaCl 100mM

NaF 100mM

NaH<sub>2</sub>PO<sub>4</sub> 50mM

Hepes 50mM, pH 7.2

## **2.5 Hepatocyte preparation**

### **2.5a Hepatocyte isolation and culture**

Rat hepatocytes were routinely available in the laboratory. They were isolated by the method of Berry and Friend (1969) involving a two step perfusion of the liver *in situ*. Cells for agonist stimulation were cultured as described in section 2.14b.

### **2.5b Transfection**

The P9 cell line was generated by Dr C. MacDonald, University of Strathclyde, Glasgow, U.K., according to the method of Woodworth *et al.* (1986). Briefly, cells were incubated in a 1: 1 mix of William's medium E and Ham's F12 (Gibco BRL) supplemented with 5% (v/v) FCS and 2mM glutamine. Transfection was achieved by exposure of the rat hepatocytes to a precipitate of SV40 viral DNA for five hours. After this period the monolayer was washed with fresh medium. Immortalised cells were selected by their ability to grow at low cell density after subculturing (Petit *et al.*, 1983), as discussed in section 1.6.3. Thereafter, cells were maintained and cultured as described in section 2.6.

### **2.5c Plasma membrane preparation**

Cells were pelleted by centrifugation for two minutes at 800 x g in a Centaur-2 bench centrifuge. Three volumes of ice-cold membrane preparation buffer were added

and cells homogenized with 30 strokes of a Potter-Elvehjem teflon /glass homogeniser. The homogenate was centrifuged at 1000 x g for 10 minutes at 4°C. The supernatant was collected and centrifuged at 100 000 x g for one hour. The resulting plasma membrane pellet was resuspended in 5ml of buffer A, aliquoted and stored at -80°C until use.

### **2.5d Cell counting**

Both rat hepatocytes and P9 cells were counted using a Neubauer haemocytometer.

### **2.5e Collagen coating of plasticware**

The method used was that of Strom and Michalopoulos (1982). Type-1 (acid soluble) collagen from rat tail was dissolved in 3% (v/v) acetic acid at 10mg/ml and stored at 4°C. Before use it was diluted to 0.03% (w/v) collagen in 3% acetic acid and applied to plasticware using a needle and syringe. Plates were allowed to dry overnight under UV irradiation in the laminar flow hood. Before use, plates were rinsed with sterile PBS to remove any residual acetic acid.

## **2.6 Tissue culture**

### **2.6a Cell growth**

Cells were grown in 100 x 20mm cell culture plates in 0.0375% (w/v) sodium bicarbonate-buffered Dulbecco's modification of Eagle's medium (DMEM), containing 10% (v/v) foetal calf serum which was heat-inactivated at 56°C for 30 minutes before use (complete medium). The medium was supplemented with glutamine (2mM), penicillin (100IU/ml) and streptomycin (1mg/ml). Cells were grown in a humidified

atmosphere of 5% CO<sub>2</sub>/95% air. Medium was changed every two days.

### **2.6b Cell subculture**

Confluent cells ( $3.8 \times 10^5$  cells/cm<sup>2</sup>) were passaged by removing medium and adding 3ml of trypsin solution to the monolayer. When cells had detached from the plate, trypsinisation was stopped by the addition of 10ml complete medium. Cells were removed from the plate and centrifuged at 800 x g for two minutes in an MSE Centaur-2 bench centrifuge. The cell pellet was resuspended in complete medium and seeded at a split ratio of 1:10 into cell culture plates.

### **2.6c Cell freezing**

The procedure for subculturing the cells was followed up to centrifugation after which cells were suspended in 'freezing medium' (10% (v/v) DMSO and 25% (v/v) FCS in complete medium) at a final concentration of  $5 \times 10^6$  cells/ml or greater. Aliquots of the cell suspension (1ml) were placed in cryotubes and frozen to -80°C at a rate of 1°C per minute. The cryotubes were transferred to liquid nitrogen until required. Cells were thawed rapidly by placing the frozen vial at 37°C. The aliquot of cell suspension was transferred to a cell culture plate and made up to a volume of 10ml by slow addition of complete medium. Cells were incubated overnight to allow adherence to the plate and the following day medium changed to remove DMSO and the cells grown to confluence.

### **2.6d Cell harvesting**

Complete medium was aspirated off and the monolayer rinsed twice with HBSS. Cells were then scraped off the plates in HBSS and the cell suspension centrifuged as for subculturing. The resulting cell pellet was washed with buffer,



re-centrifuged and stored at -80°C until use.

### **2.6e Plasma membrane preparation**

Membranes were prepared according to Koski and Klee (1981). Frozen cell pellets were thawed and suspended in 2ml ice-cold 1mM EDTA, 10mM Tris HCl, pH 7.4 containing freshly added protease inhibitors, 1mM PMSF, 3mM benzamidine, 1µM leupeptin, 2µg/ml aprotinin and 0.5µg/ml pepstatin A and homogenised with 15 strokes of a Potter/Elvehjem teflon/glass homogeniser. The homogenate was centrifuged at 500 x g for 10 minutes in a Beckman L5-50B centrifuge in a Ti50 rotor, to sediment unbroken cells and nuclei. Plasma membranes were collected by centrifugation of the supernatant at 48 000 x g for 10 minutes, washed in 10 volumes of the same buffer and after a second centrifugation were resuspended in the same buffer to a final protein concentration of 2-4 µg/ml, aliquoted and stored at -80°C until required.

### **2.6f Toxin treatment of cells**

After removing complete medium the monolayer was washed with serum-free medium then the cells incubated with serum-free medium supplemented with pertussis toxin at the concentration required, before agonist challenge.

### **2.6g Mycoplasma screening**

Cells were screened for mycoplasma by the fluorescence staining method described by Hay (1989).

## **2.7 Rat cerebral cortex plasma membrane preparation**

This was done by the method of Milligan *et al.* (1987). Animals were killed by cervical dislocation after which the cerebral cortex was dissected out and transferred to 0.32M sucrose on ice. The tissue was hand homogenised in ice-cold 0.32M sucrose using 35 up and down strokes of a Potter/Elvehjem teflon/glass homogeniser. This was centrifuged at 500 x g for 10 minutes and the supernatant collected and recentrifuged at 48 000 x g for 10 minutes. The pellet from this spin was washed in 0.32M sucrose, re-centrifuged under the same conditions and the final pellet resuspended in 3ml of 0.32M sucrose buffer. The sample was aliquoted and stored at -80°C until use.

## **2.8 Antibody production**

Antisera used for immunoblotting were raised in New Zealand White rabbits against synthetic peptides conjugated to keyhole limpet haemocyanin as described by Goldsmith *et al.* (1987). Decapeptides used are listed in table 2.1 below.

**Table 2.1**

Antiserum	Peptide used	G-protein sequence	Antiserum identifies
CS1/CS2	RMHLRQYELL	G <sub>s</sub> - $\alpha$ 385-394	G <sub>s</sub> - $\alpha$
I3C	KNNLKECGLY	Gi-3 $\alpha$ 345-354	Gi-3 $\alpha$
1432	KNNLKDCGLF	Gi-2 $\alpha$ 345-354	Gi-1 $\alpha$ , Gi-2 $\alpha$
BN1/BN3	MSELDQLRQE	$\beta$ 1-10	$\beta$ 1, $\beta$ 2

Peptides RMHLRQYELL and MSELQDLRQE were from Dr C.G. Unson, Rockefeller University, New York, USA. The other peptides were synthesised by Biomac Ltd., Glasgow, UK.

## **2.9 Protein determination**

The method used was based on that of Lowry *et al.* (1951). Stock solutions:

1. Copper tartrate carbonate (10% (w/v)  $\text{Na}_2\text{CO}_3$ , 0.1%  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.2% sodium potassium tartrate)
2. 0.8M NaOH
3. 10% SDS

Immediately before use, one part of each of these solutions was mixed along with one part  $\text{H}_2\text{O}$  to give reagent 'A', which was gently warmed to dissolve any precipitate. Standard solutions of BSA were prepared in duplicate containing 0-100 $\mu\text{g}$  of protein per ml and unknown protein solutions in triplicate. All samples were made up to 1ml with  $\text{H}_2\text{O}$  and then 1ml reagent 'A' added to each sample. After 10 minutes 0.5ml reagent 'B' was added (Folin and Ciocalteu's reagent diluted 1: 6 with distilled  $\text{H}_2\text{O}$ ), mixed and colour development allowed to occur for 30 minutes before measuring absorbance at 750nm in an LKB Ultrospec-2 spectrophotometer.

## **2.10 Gel electrophoresis**

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) was carried out by the method of Laemmli (1970).

## 2.10a Resolving gel preparation

All stock solutions were stored at 4°C. Acrylamide solutions were filtered through Whatman No. 1 filter paper.

Buffer 1: 0.4% (w/v) SDS, 1.5M Tris HCl, pH 8.8

Buffer 2: 0.4% (w/v) SDS, 0.5M Tris HCl , pH 6.8

Acrylamide 'A': 30% (w/v) acrylamide, 0.8% (w/v) N,N'-methylene bisacrylamide

Acrylamide 'B': 30% (w/v) acrylamide, 0.15% (w/v) N,N'-methylene bisacrylamide

50% (v/v) glycerol

10% (w/v) APS (made immediately before use)

TEMED

Running buffer:                      0.025M Tris  
   0.192 Glycine  
   0.1% (w/v) SDS

10% (w/v) acrylamide/0.27% N,N'-methylenebisacrylamide (w/v) gels were prepared as follows :

	Volume (ml)
Distilled H <sub>2</sub> O	8.2
Buffer 1	6
Acrylamide 'A'	8
50% glycerol	1.6
APS	0.16
TEMED	0.028

The solution was mixed and poured between 180 x 160mm glass plates separated by 1.5mm spacers. The gel was layered with 25% (v/v) buffer 1 to exclude air and left to set

at room temperature.

12.5% (w/v) acrylamide/ 0.0625% (w/v) N,N'-methylenebisacrylamide gels were prepared as follows :

	Volume (ml)
Distilled H <sub>2</sub> O	11.6
Buffer 1	12
Acrylamide 'B'	20
50% glycerol	4
APS	0.16
TEMED	0.028

The solution was mixed and poured between 200 x 200mm glass plates separated by 1.5mm spacers. The gel was layered with 25% (v/v) buffer 1 to exclude air and left to set at room temperature.

### **2.10b Stacking gel preparation**

Stacking gels were prepared as follows :

	Volume (ml)
Distilled H <sub>2</sub> O	9.75
Buffer 2	3.75
Acrylamide 'A'	1.5
APS	0.16
TEMED	0.028

The solution was mixed, layered on top of the resolving gel and allowed to set around a 10-well teflon comb. Electrophoresis was performed at 60mA over three hours in the

case of the 10% acrylamide gel system and 40mA overnight in the case of the 12.5% acrylamide gel system.

### **2.10c Sample preparation**

Protein was precipitated from plasma membranes by addition of 6.25µl of 2% (w/v) sodium deoxycholate, followed by 250µl of 24% (w/v) TCA then samples were made up to 1.15ml with H<sub>2</sub>O and centrifuged at 13 000 x g for 20 minutes in an MSE Microcentaur centrifuge. Supernatants were discarded and 20µl of 1M Tris base added to the pellets which were then solubilised by addition of 20µl of Laemmli buffer. Laemmli buffer (50µl) was also added to the marker proteins then all samples vortexed and boiled for five minutes before loading onto the gel.

### **2.10d Gel protein staining**

The gel was covered in stain solution which consisted of 0.1% (w/v) Coomassie blue in 50% (v/v) H<sub>2</sub>O, 40% (v/v) methanol, 10% (v/v) glacial acetic acid and placed on a shaker for one hour. The stain solution was then discarded and the gel shaken overnight in destain solution (identical to stain solution but without Coomassie blue).

### **2.10e Autoradiography**

Gels to be autoradiographed were stained for protein and dried down onto Whatman 3mm filter paper at 80°C for two hours under vacuum. The dried gel was placed against Fuji NIF RX 100 X-Ray film in a cassette with intensification screens for 3-4 days at -80°C. Films were developed using a Kodak X-OMAT developer.

## **2.10f Densitometry**

Densitometric scanning of autoradiographs and blots was performed using a Shimadzu CS-9000 densitometer. Absorbance for autoradiographs and reflectance for blots were measured in arbitrary units.

## **2.11 Immunoblotting**

Proteins were electrophoresed from SDS gels to a nitrocellulose sheet in an LKB transblot apparatus (Towbin *et al.*, 1979) at 350mA for two hours. The tank buffer consisted of 0.192M glycine, 25mM Tris and 20% (v/v) methanol. The nitrocellulose sheet was 'blocked' for 90 minutes in 5% dried skimmed milk in TBS after which the blot was washed in distilled H<sub>2</sub>O and incubated overnight with the antiserum, appropriately diluted in 1% (w/v) dried skimmed milk in TBS. The antiserum was then removed and the sheet subjected to washes in distilled H<sub>2</sub>O, 0.2% (v/v) Nonidet-P40-containing TBS and TBS alone for 10 minutes each, after which the blot was incubated with a second antibody (peroxidase-conjugated sheep anti-rabbit IgG or <sup>125</sup>I-labelled sheep anti-rabbit IgG) for two hours at room temperature. The second antibody was then removed and the sheet subjected to the same series of washes. In the case of the peroxidase-conjugated second antibody, the blot was developed in 40ml of 10mM Tris pH 7.5 with 0.025% (v/v) H<sub>2</sub>O<sub>2</sub> and 0.025% (w/v) o-dianisidine as a substrate. Where <sup>125</sup>I-labelled second antibody was used, the blot was autoradiographed as described in section 2.10e. Both first and second antibodies could be used up to three times and were stored at 4°C using 0.004% (w/v) thimerosal as an antibacterial agent.

## 2.12 Toxin ribosylation of membranes

### 2.12a Toxin pre-activation

This was done by the method of Hildebrandt *et al.* (1983). Stock pertussis toxin was added to an equal volume 100mM dithiothreitol and vortexed. The solution was incubated for one hour at room temperature and the incubation terminated by placing the sample on ice. Cholera toxin was pre-activated in a similar manner. Final concentrations in the assay mixture were 10µg/ml for pertussis toxin and 20µg/ml for cholera toxin.

### 2.12b ADP-ribosylation

Membranes to be ADP-ribosylated were diluted in 1mM EDTA, 10mM Tris HCl, pH 7.4 to a protein concentration of 1-2.5mg/ml. 20µl aliquots of diluted membrane protein were assayed in a final volume of 50µl containing :

[<sup>32</sup>P]-NAD, 2µCi per assay

20mM Thymidine

1mM ATP, pH 7.5

100µM GTP, pH 7.5

20mM Arginine HCl

0.25M Potassium phosphate buffer, pH 7.0

The ribosylation assay was initiated by addition of membranes and continued for one hour at 37°C. Assays were terminated by transfer to ice followed by addition of sodium deoxycholate and TCA precipitation as detailed in section 2.10c. Proteins were then resolved using SDS PAGE and gels dried, stained and autoradiographed as described in sections 2.10d. and e.



## 2.13 Adenylyl cyclase assay

### 2.13a Assay conditions

Assays were carried out by the method of Johnson and Salomon (1991) using sequential chromatography on dowex and alumina columns to separate the labelled product, [ $^{32}\text{P}$ ]-cAMP from labelled substrate, [ $\alpha^{32}\text{P}$ ]-ATP. Assays were carried out in an incubation medium containing at final concentrations in a 100 $\mu\text{l}$  volume:

2mM  $\text{MgCl}_2$

0.1mM EGTA

1mM IBMX

1mM dithiothreitol

50mM triethanolamine HCl, pH7.4

The assay also contained creatine phosphate (5mM) and creatine phosphokinase (140U/ml) as an ATP regenerating system and BSA (2mg/ml), cAMP (0.1mM), ATP (50 $\mu\text{M}$ ) and [ $\alpha^{32}\text{P}$ ]-ATP (1 $\mu\text{Ci}$  per assay). Ligands and GTP were present at the concentrations required. Plasma membranes for assay were diluted in 1mM EDTA, 10mM Tris, pH7.4. Reaction tubes were set up on ice and the reaction started by transfer to a 30 $^\circ\text{C}$  water bath and addition of membrane protein at a final concentration of 5-10 $\mu\text{g}$  per assay. Reactions were terminated by addition of ice-cold stopping solution which consisted of 2% (w/v) SDS, 40mM ATP and 1.4mM cAMP, pH7.5. This was followed by 100 $\mu\text{l}$  [ $8\text{-}^3\text{H}$ ]-cAMP (about 5000cpm per sample) and 700 $\mu\text{l}$   $\text{H}_2\text{O}$ . Total counts of [ $\alpha^{32}\text{P}$ ]-ATP and [ $8\text{-}^3\text{H}$ ]-cAMP were retained as well as assay blanks, where no membrane protein was added, so that unseparated [ $\alpha^{32}\text{P}$ ]-ATP counts could be subtracted from sample counts.

### **2.13b Preparation of chromatography columns**

Dowex AG50W-X4 resin (200-400 mesh, H<sup>+</sup> form) was washed by mixing in 1M HCl. After allowing it to settle and decanting the supernatant the wash process was repeated several times with distilled H<sub>2</sub>O. The resin was resuspended in H<sub>2</sub>O at twice its packed volume and 2ml of the slurry added to glass wool-plugged plastic columns to give a packed bed volume of 1ml per column. The columns were then washed with 2ml of 1M HCl. Before use, the columns were washed with 2 x 1ml of HCl and then 3 x 10ml of distilled H<sub>2</sub>O. Alumina columns used for the second stage were plugged with glass wool and filled with 1g of neutral alumina. Before use the columns were washed with 8ml of 0.1M imidazole HCl buffer, pH 7.5.

### **2.13c Calibration of chromatography columns**

Elution profiles of dowex and alumina columns were determined before use in sample chromatography in order to allow for variation between batches of resin and obtain optimal recovery of labelled product. A mixture of [8-<sup>3</sup>H]-cAMP and [α-<sup>32</sup>P]-ATP was applied to a dowex column in 1ml of H<sub>2</sub>O. Water was added in 1ml fractions and 1ml volumes of the eluate collected as shown in Fig. 2.1a. 3ml 0.1M imidazole, pH 7.5 and 8ml 'Ecoscint' were added and the samples counted using a dual label program. For the alumina columns only [8-<sup>3</sup>H]-cAMP was added to the the dowex column and eluted onto alumina using 3ml H<sub>2</sub>O. Counts were eluted from the alumina column by successive additions of 1ml volumes of 0.1M imidazole, pH 7.5. The recovery and optimal elution volumes could then be determined, Fig. 2.1. Recovery of [8-<sup>3</sup>H]-cAMP was generally 60-80%. After the recovery fell to 40%, dowex was discarded and new columns prepared. Alumina columns were discarded when, after progressive accumulation of [α-<sup>32</sup>P]-ATP, the radioactivity level became unacceptably high.

### **2.13d Isolation of cAMP on chromatography columns**

Samples were carefully layered onto the surface of the dowex resin using Pasteur pipettes. Two 1ml volumes of H<sub>2</sub>O were added and the eluate from these additions containing most of the [ $\alpha$ -<sup>32</sup>P]-ATP was discarded. The remaining label was then eluted onto alumina columns with 3ml of water and the eluate discarded. Cyclic AMP was eluted from the alumina columns into scintillation vials by addition of 4ml 0.1M imidazole, pH7.5 to the columns. The eluate was collected in scintillation vials, mixed with 8ml 'Ecoscint' and counted using the dual label program. Columns were then regenerated before the next assay as described in section 2.13b.

### **2.14 Assay of cAMP**

#### **2.14a Cell stimulation and cAMP extraction**

Cells were grown to confluence in 24-well plates then the medium removed, the monolayer washed in serum-free medium then incubated with 0.5ml serum-free medium for 16 hours before agonist stimulation. The cells were then incubated on a heated plate at 37°C and agonist added to the medium, generally in a 5 $\mu$ l volume, but occasionally in a greater volume when high agonist concentrations were required. After the desired period, medium was aspirated and 2% (w/v) ice-cold perchloric acid (150 $\mu$ l per well) was added to the monolayer in order to extract the cAMP. Each well contained approximately  $9.66 \times 10^5$  cells.

In the case of rat hepatocytes, cells were loaded onto collagen coated 6-well plates in Krebs/BSA and left for 40 minutes in the tissue culture incubator. Cells adhered at a density of approximately  $9.7 \times 10^5$  cells per well. After this time period non-adherent cells were aspirated off and the monolayer washed with buffer. This was

then replaced with medium containing agonist, added in a 5 or 10 $\mu$ l volume. After the desired time period, the buffer was aspirated off and cAMP extracted in 2% (w/v) ice-cold perchloric acid (250 $\mu$ l per well). All plates were incubated for 15-30 minutes on ice then the cell debris scraped off and centrifuged at 13 000 x g for two minutes in an MSE Microcentaur centrifuge. The supernatant was neutralised with 0.5M triethanolamine in 2M potassium hydroxide. Neutrality was checked with universal indicator after which the precipitate was pelleted as above and an aliquot of supernatant taken for assay of cAMP by the binding protein method.

### **2.14b Preparation of bovine adrenal cAMP binding protein**

The method used was that of Brown *et al.* (1972). Bovine adrenal glands (about 20-30) were transported to the laboratory on ice. After removal of excessive fat, the glands were hemisected and the medulla removed. Cortex was scraped from the gland capsule, pooled and transferred into a pre-cooled Waring blender along with 1.5 volumes of ice-cold homogenisation buffer (0.25M sucrose, 25mM KCl, 5mM MgCl<sub>2</sub> and 50mM Tris HCl, pH7.4). The tissue was homogenised at maximum speed then transferred to centrifuge tubes on ice and centrifuged at 2000 x g for five minutes at 4°C. The supernatant was re-centrifuged at 6000 x g for 15 minutes at 4°C and the final supernatant pooled, aliquoted and stored at -20°C until use.

### **2.14c Cyclic AMP determination**

The method used was based on the binding assay described by Tovey *et al.* (1974). The assay depends on competition of labelled and unlabelled cAMP for a limited number of binding sites on a binding protein purified from bovine adrenal glands, as described in section 2.14b. Increasing amounts of unlabelled cAMP (0-320pmols/ml) were incubated with a fixed amount of [5',8-<sup>3</sup>H]-cAMP and binding

protein. Standard solutions of cAMP were prepared in assay buffer containing 4mM EDTA and 50mM Tris, pH7.4. [5',8-<sup>3</sup>H]-cAMP was diluted in assay buffer to give approximately 500 000cpm/ml. Labelled cAMP (0.1ml) was added to all standards and unknowns and the volume made up to 0.2ml with assay buffer. The incubation was started by addition of binding protein (0.1ml) which had been diluted 1: 30 in assay buffer before use. After incubation for 2-3 hours at 4°C, the binding reaction was terminated by addition of 0.25ml of a suspension of 2% (w/v) Norit-GSX charcoal and 1% (w/v) BSA in ice-cold assay buffer which had been allowed to mix for 45 minutes beforehand. Tubes were rapidly mixed and centrifuged at 13 000 x g in an MSE Microcentaur centrifuge to pellet the charcoal, after which 0.3ml of each supernatant was taken for counting by addition to 4ml of 'Ecoscint' in a scintillation vial. Vials were then counted using an curve-fitting program which constructed a standard curve allowing unknown cAMP values to be calculated. A typical standard curve is shown in Fig. 2.2.

### **2.15 Assay of glucose-6-phosphatase activity**

Glucose-6-phosphatase activity was assayed by the method of Houslay and Palmer (1978). The assay contained in a final volume of 75µl: 20mM glucose-6-phosphate in 40mM sodium cacodylate HCl, pH6.5 and 35µl of crude cell homogenate. For each sample a boiled blank was also assayed in order to allow for endogenous phosphate present in the sample. The reaction was started by addition of homogenate, allowed to continue for 30 minutes at 37°C then stopped by addition of 50µl of ice-cold 20% (w/v) TCA. Samples were centrifuged at 13 000 x g for two minutes in an MSE Microcentaur centrifuge and a sample of supernatant taken for assay of phosphate.

Phosphate was assayed by the method of Fiske and Subbarow (1925). Distilled H<sub>2</sub>O (0.1ml) along with 0.1ml 2.5% (w/v) ammonium molybdate in 5N sulphuric acid, 40µl of ANSA reducing agent then 0.685 ml of distilled H<sub>2</sub>O. A standard phosphate sample was also prepared containing 19.25nmols of phosphate in order to calculate unknown Pi. Samples were vortexed and heated at 37°C for 30 minutes and absorbance measured at 660nm in an LKB spectrophotometer. In this assay there was shown to be a linear increase in absorbance over two orders of magnitude of phosphate concentration (Fig. 2.3). All unknowns were within this range.

### **2.16 Measurement of [<sup>3</sup>H]-Thymidine uptake**

This was done according to the method of McNulty *et al.* (1990). After growing cells to confluence in 24 well plates, medium was removed and the monolayer rinsed with serum-free medium. The cells were incubated for 16 hours in serum-free medium containing 1µCi/ml of [<sup>3</sup>H]-Thymidine along with the agonist of interest. The medium was removed and the monolayer washed twice with HBSS. Cells were washed twice with ice-cold 5% TCA, twice with ethanol then solubilised in 0.5ml of 0.3M NaOH. This was added to a scintillation vial containing 0.1ml 1.5M HCl and 4ml 'Ecoscint' and the samples counted.

### **2.17 Measurement of Gi-2α subunit phosphorylation**

Cells were grown to confluence in 6 well plates and preincubated for 16 hours in serum-free medium. Cells (3.9 x 10<sup>6</sup> cells per well) were then labelled for three hours in a 1ml volume of medium, with 0.25mCi of [<sup>32</sup>P]Pi. During this period the cells were kept in the tissue culture incubator under the conditions described for growth (section 2.6a). Ligands were added in a 10µl volume. After the appropriate

time period, reactions were stopped by removal of medium and addition of 1ml of ice-cold cell solubilising buffer to each well. Cells were scraped and left at 4°C for one hour then centrifuged at 13 000 x g for five minutes at 4°C in an MSE Microcentaur centrifuge in order to pellet any insoluble material. The supernatant was taken and incubated for 16 hours at 4°C with 50µl of antiserum 1432. After this period, 50µl of protein A-agarose was added and incubation continued for three hours with frequent vortexing of samples. The agarose with attached immune complex was pelleted by centrifugation as above and the pellets washed three times in wash buffer then once in SDS-free wash buffer. Immune complex was detached from the pellets by boiling for five minutes in 50µl of Laemmli buffer. Agarose was sedimented by centrifugation as above, but at room temperature and proteins in the supernatant resolved by SDS PAGE as described in section 2.10.

### **2.18 Measurement of labelled glucagon binding**

Measurement of [<sup>125</sup>I]-glucagon binding to plasma membranes was by the method of Rojas and Birnbaumer (1985). Briefly, membranes were diluted to a protein concentration of 1mg/ml with 5mM EDTA/20mM Tris, pH7.5 then incubated in a total volume of 100µl containing 50µl of 2 x binding buffer, 5µl plasma membranes, 5µl unlabelled glucagon in wash buffer (1µM final concentration) and 10µl of [<sup>125</sup>I]-glucagon (3 x 10<sup>4</sup>cpm). Binding buffer was 0.2% BSA, 2mM EDTA and 40mM Tris, pH7.5. The reaction proceeded for 20 minutes at 32°C after which it was stopped with 5ml of wash buffer and samples filtered immediately. Cellulose acetate filters (0.45µm) which had been pre-soaked in 10% BSA were washed once with wash buffer before use. After filtering of samples, the filters were washed with 5ml wash buffer then removed and counted using an LKB-1275 Minigamma γ counter.

## **2.19 Methods used in study of leucocytes from diabetic subjects.**

### **2.19a Subjects**

Type 2 diabetic subjects were recruited at the diabetic out-patient clinic. All had a diagnosis established on the basis of fasting hyperglycemia (the hospital quote a reference range for normal plasma glucose of 2.8-6.0mmols/l). All were hyperglycemic at the time of sampling and were either newly diagnosed or had been managed by dietary measures alone since the time of diagnosis. Patients were chosen who were as far as possible free from other disorders and taking a minimum of medications. None had clinical signs of active infection at the time of sampling. The male diabetics ranged from 30-73 and female diabetics from 40-77 years of age. Control subjects were recruited from the general surgical out-patient clinic, attending with minor surgical conditions. They were sex-matched with the diabetic patients and, within the limitations of the patients available, also age-matched.

### **2.19b Leucocyte isolation**

Leucocytes were isolated by the method of Böyum (1968). Approximately 80ml of blood was taken from the antecubital vein, anticoagulated by the addition of 1ml of 0.2M EDTA per 10ml of blood and transported within one hour to the laboratory. A high molecular weight dextran solution (6% dextran T-500, 149mM NaCl, 5mM KCl) was added (5ml per 20ml whole blood) and samples incubated for 30 minutes at 37°C. The leucocyte-rich plasma was removed by Pasteur pipette and loaded onto 'Ficoll-Paque' solution (5.7% Ficoll-400, 9% diatrizoate sodium with calcium edetate) at approximately 15ml plasma per 10ml Ficoll. This was centrifuged at 1500 x g for 30 minutes at room temperature to obtain:



1. A neutrophil and erythrocyte pellet (also containing basophils and eosinophils)
2. Platelet-containing plasma as supernatant.
3. A lymphocyte/monocyte/platelet-containing band at the interface between plasma and Ficoll.

The lymphocyte/monocyte band was removed by Pasteur pipette, taking as little Ficoll as possible and three volumes of HBSS added. This was centrifuged for five minutes at 1000 x g to pellet the lymphocytes, leaving a platelet-containing supernatant. The cell pellet was washed in HBSS and the centrifugation repeated after which supernatants were pooled for platelet membrane preparation and the leucocyte pellet stored at -80°C until required for membrane preparation.

### **2.19c Leucocyte plasma membrane preparation**

Leucocytes isolated by density gradient centrifugation were resuspended in ice-cold lysing buffer (10mM Tris, 1mM EDTA, pH7.4, containing 0.1mM PMSF, 2mM benzamidine and 2µg/ml of aprotinin). This was homogenised by 10 up and down strokes of a Potter/Elvehjem teflon/glass homogeniser and the sample centrifuged at 500 x g for 10 minutes in a Beckman L5-50B centrifuge in a Ti50 rotor, in order to sediment unbroken cells and nuclei. Plasma membranes were collected by centrifugation of the supernatant at 48 000 x g for 10 minutes, washed in 10mM Tris, pH7.4 (containing protease inhibitors at the same concentrations as above) and recentrifuged under the same conditions to give a final membrane pellet. This was resuspended in 50µl of 10mM Tris buffer per 10ml original whole blood and aliquoted for storage at -80°C until used for immunoblotting or assay of plasma membrane adenylyl cyclase activity.

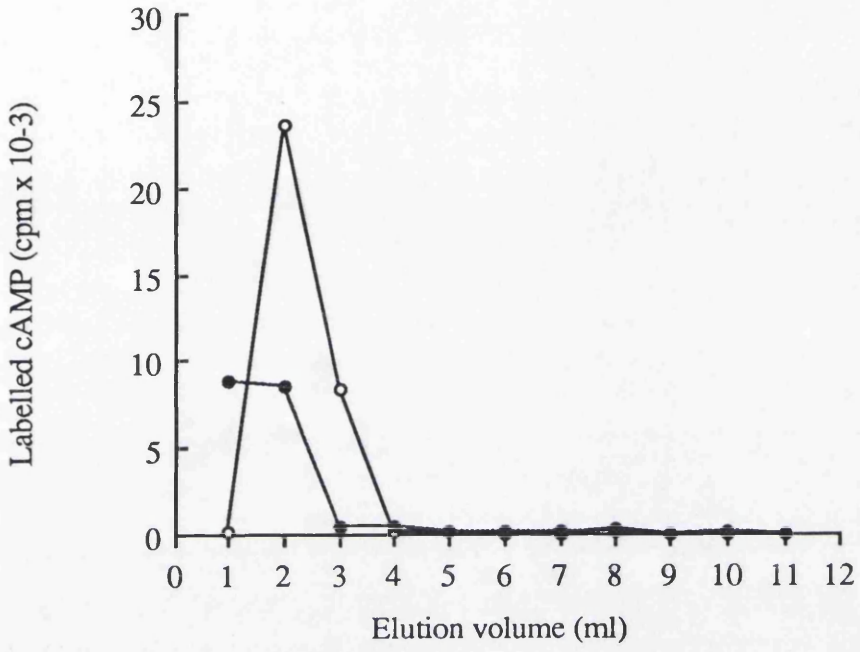
## **2.20 Data analysis**

Where appropriate, data was analysed for statistical significance using Student's t-test for unpaired data.

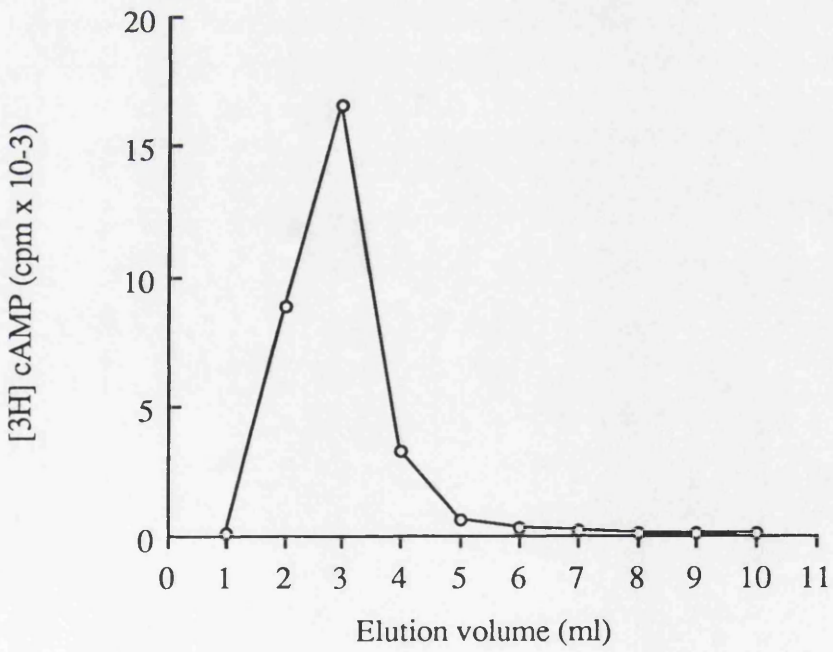
**Fig. 2.1**            **Elution profiles of [ $\alpha$ -<sup>32</sup>P]-ATP and**  
**[8-<sup>3</sup>H]-cAMP from dowex and alumina**  
**columns.**

[8-<sup>3</sup>H]-cAMP (34 000 cpm in 0.5ml) (open circles) and [ $\alpha$ -<sup>32</sup>P] (19 000cpm in 0.5ml) (closed circles) were added to a dowex column then eluted from the column by repeated additions of 1ml volumes of H<sub>2</sub>O (Fig. 2.1a) and counted as described in section 2.13b. [8-<sup>3</sup>H]-cAMP (34 000cpm in 1ml) was added to a dowex column and eluted onto alumina by the addition of 5ml H<sub>2</sub>O. Counts were then eluted from the alumina column by repeated addition of 1ml volumes of imidazole, pH7.5 (Fig. 1.1b). Fractions were collected and counted as above.

**Figure 2.1 (a)**



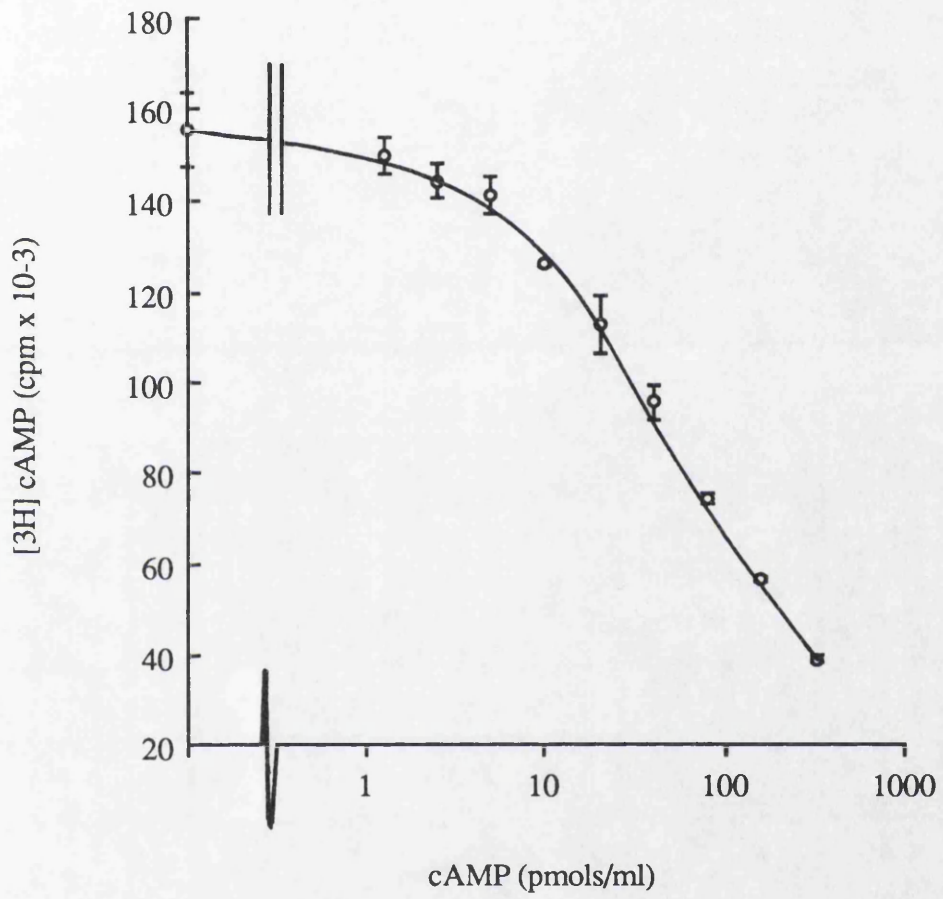
**Figure 2.1 (b)**



**Fig. 2.2**      **Standard curve for cAMP determination.**

Standard solutions of cAMP (from 0-320pmoles/ml) were set up and assayed as described in section 2.14c. Counts from non-specifically bound [<sup>3</sup>H]-cAMP have been subtracted from each value (350cpm). Data are mean+/-SD for one standard curve performed in duplicate. Significant displacement of labelled cAMP from specific binding sites was obtained from upwards of 10pmols/ml ( $p < 0.05$ ). Similar results were obtained in all successful cAMP assays.

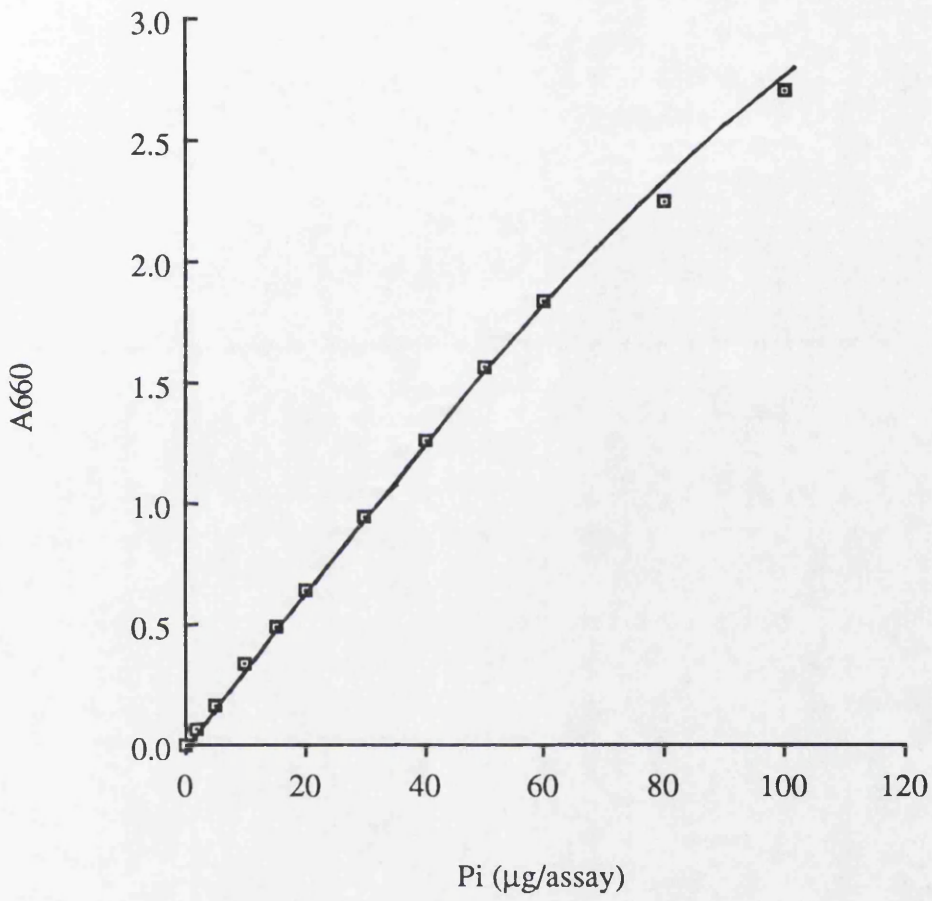
**Figure 2.2**



**Fig. 2.3**      **Standard curve for phosphate determination.**

A standard curve was prepared in which phosphate solutions containing phosphate (0-100 $\mu$ g) were assayed and absorbance measured spectrophotometrically as described in section 2.15.

**Figure 2.3**





## Chapter 3

### The P9 cell line as a model system for studying adenylyl cyclase signalling

### 3.1 Introduction

The P9 cell line was generated by transfection of native rat hepatocytes with DNA from the SV40 virus and expresses the large T antigen, evidence that DNA has entered the cell genome resulting in immortalisation of the cells. Cell lines, whether tumour-derived or virally-transformed show considerable phenotypic differences when compared to their native, differentiated counterparts. Before carrying out extensive work on the cell line, it was judged important to examine basic characteristics and agonist responses in the cells in order to compare them to rat hepatocytes. Rat hepatocytes transfected in the same manner by other workers have yielded stable cell lines which, despite their fibroblast-like morphology express albumin, tyrosine aminotransferase (Isom *et al.*, 1980) and transferrin, haemopexin and glucose-6-phosphatase (Woodworth *et al.*, 1986) all of which are specific to hepatocytes as opposed to the non-parenchymal cells present in liver. However, the quantity of markers expressed was variable, indicative of phenotypic differences between cell lines generated.

Previous work done on the P9 hepatocyte-derived cell line (MacDonald, C., unpublished work) has demonstrated that whilst the cells are de-differentiated, having a fibroblast-like morphology in culture, they possess bilirubin glucuronyl transferase activity, a hepatocyte-specific enzyme involved in conjugation of bilirubin, convincing evidence that the cells were indeed derived from hepatocytes and retain at least one differentiated characteristic. They can be passaged up to 20 times without any change in morphology. No previous study however, has been made of adenylyl cyclase signalling or insulin action in the P9 cell line. The purpose of this chapter then, was firstly to study basic adenylyl cyclase responses in P9 cells, *viz.* agonist-stimulated plasma membrane adenylyl cyclase activity and agonist-elevated intracellular cAMP, to examine how comparable they were to those of rat hepatocytes and to consider the

usefulness of the cell line as a model system for studying adenylyl cyclase signalling.

Secondly, G-protein subtypes expressed by the cells were identified immunologically and quantitatively compared to those of rat hepatocytes. Finally, attempts at promoting differentiation of the cells are briefly discussed.

## 3.2 Results

The P9 cell line in monolayer culture is fibroblast-like in morphology and rapidly growing as a uniform monolayer, reaching confluence 3-4 days after subculture and showing no morphological change at confluence (Fig. 3.1). Cells retained the same morphology with repeated subculture. Glucose-6-phosphatase activity was found to be present in a crude homogenate from the cells at about a fifth of the level found in native rat hepatocytes (Table 3.1).

As shown in Table 3.2, P9 cell plasma membranes possess adenylyl cyclase activity which can be potently stimulated by the diterpene forskolin in the same manner as that of rat hepatocyte adenylyl cyclase. Maximum stimulation in membranes and maximal cAMP accumulation in whole cells in response to forskolin was at  $10^{-4}$ M, resembling other mammalian adenylyl cyclase systems. The small stimulation by GTP and the receptor-mediated stimulatory response to PGE<sub>1</sub> in the presence of GTP demonstrates that the cells possess functional G<sub>s</sub> coupled to adenylyl cyclase stimulation (GTP must be present at saturating concentrations in order to obtain optimal receptor mediated responses). The response to the prostanoid was slightly smaller in P9 cells than in rat hepatocytes. PGE<sub>1</sub> potently elevated intracellular cAMP when intact P9 cells were challenged with the hormone (Table 3.3 and Fig. 3.2). Dose response curves for agonist-stimulated adenylyl cyclase activity in P9 cell plasma membranes are given in Chapter 4.

There was no stimulatory response to the hormone glucagon in P9 cells, which gave potent stimulation of adenylyl cyclase activity in rat hepatocyte plasma membranes (Table 3.2). Similarly, glucagon over a range of concentrations ( $10^{-9}$ - $10^{-6}$ M) did not elevate intracellular cAMP above basal levels in the cells when intact cells were

challenged with the hormone (not shown), whilst rat hepatocytes showed a considerable elevation over basal levels when challenged with glucagon ( $10^{-8}\text{M}$ ) (Fig. 3.3). These data were in line with glucagon binding data from the two cell types, where plasma membranes from P9 cells showed specific glucagon binding at 3.7% of the level observed in rat hepatocyte plasma membranes.

Forskolin in combination with glucagon showed considerable synergism in its action in intact rat hepatocytes. After two hours in culture however, the response had fallen in proportion to the response to glucagon alone. This synergism between the two agonists was also observed in P9 cells. Glucagon, when incubated with P9 cell plasma membranes, in the presence of GTP ( $10^{-4}\text{M}$ ), enhanced in a dose-dependent manner, the ability of forskolin to stimulate adenylyl cyclase activity (Fig. 3.4). Dose dependence on glucagon for enhancement of the response was of the same order as that in rat hepatocytes where  $1.4 \times 10^{-8}\text{M}$  yields a half maximal response and approximately  $10^{-7}\text{M}$  yields a maximal response (Gawler et al, 1987). Glucagon and forskolin also showed synergism in their ability to elevate intracellular cAMP in intact P9 cells (Fig. 3.5). The potency of the synergism was greater in intact cells, an effect generally observed with forskolin's synergistic responses. Similarly, PGE<sub>1</sub> showed marked synergism with forskolin in elevating intracellular cAMP, but more potent than in the case of glucagon given its ability alone to elevate intracellular cAMP above basal levels in the cells (Table 3.3).

The ability of forskolin to elevate intracellular cAMP was considerably greater in P9 cells than in rat hepatocytes (Fig. 3.6). As forskolin similarly stimulated plasma membrane adenylyl cyclase activity in both cell types in the presence of the phosphodiesterase inhibitor IBMX, this finding suggests that the P9 cells possess lower phosphodiesterase activity than rat hepatocytes. In further support of this, was the finding that in P9 cells IBMX pre-incubation did not generally cause much

enhancement of intact cell cAMP accumulation in response to agonist. In one experiment, challenge of intact P9 cells with  $5 \times 10^{-5}$ M PGE<sub>1</sub> elevated intracellular cAMP to  $24 \pm 3$  pmols/50 $\mu$ l extract (SEM for triplicate observations), whilst in cells pre-incubated with 1mM IBMX, the level was  $28 \pm 2$  pmols/50 $\mu$ l extract.

On examination of the time course for agonist elevation of intracellular cAMP, forskolin caused a rapid and potent elevation of intracellular cAMP reaching a plateau and not returning to basal levels during the time period of the experiment (Fig. 3.7). The response to glucagon and forskolin combined yielded a time course entirely parallel to that of forskolin. Like forskolin, PGE<sub>1</sub> caused a rapid elevation in intracellular cAMP again reaching a plateau (Fig. 3.8). When the time course here was repeated in the presence of IBMX, a similar shape of curve was obtained. As both agonists at  $10^{-5}$ M over a period of two minutes gave readily measurable and submaximal responses, this concentration and time period were used in later studies of intracellular cAMP elevation by these agonists.

P9 cell plasma membranes were immunoblotted using specific antisera in order to identify G-proteins expressed by the cells. P9 cells were shown to express Gi-2 $\alpha$  (40kDa) (Fig. 3.9), Gi-3 $\alpha$  (41kDa) (Fig. 3.10), G<sub>s</sub>- $\alpha$  (45kDa and 42kDa forms) (Fig. 3.11) and G-protein  $\beta$  subunit (35kDa) (Fig. 3.12). The immunological data showed that these antisera were selective and could be used as probes for examining expression of specific G-proteins. Signals obtained from all polypeptides on loading increasing amounts of P9 cell membrane protein were found to be linear up to about 100 $\mu$ g of protein. Thereafter, this amount of membrane protein was loaded in order to maximise the signals obtained and ease quantification.

G-protein levels in P9 cell plasma membranes were quantitatively compared to those in rat hepatocytes. The signals obtained from Gi-2 $\alpha$  and Gi-3 $\alpha$  co-migrated with

signals from rat cerebral cortex, a tissue in which both these G-proteins are expressed (Fig. 3.13). The gel system used in Fig. 3.13a. provides greater resolution in the 35-45kDa region and is adequate for resolving Gi-1 $\alpha$  and Gi-2 $\alpha$ , but no signal was obtained from P9 cells or rat hepatocytes corresponding to Gi-1 $\alpha$ , expressed by rat cerebral cortex. The higher molecular weight band observed using the I3C antiserum may represent a cross reaction with another G-protein  $\alpha$  subunit, possibly the 45kDa form of G<sub>s</sub>- $\alpha$ , as this antiserum is already known to recognise other  $\alpha$  subunits at high titres. The signal obtained from each Gi subtype was greater in P9 cells than in rat hepatocytes.

On probing with the CS1 antiserum, signals were obtained at 45kDa and 42kDa (Fig. 3.14) representing G<sub>s</sub>- $\alpha$  subunits. The 45kDa form reacted more strongly with the antiserum, presumably being the more prevalent of the two. The signal obtained from the 45kDa subunit was greater in the case of P9 cells than for rat hepatocytes, whilst the 42kDa  $\alpha$  subunit in P9 cells reacted more weakly with the antiserum than in rat hepatocytes.

The BN3 antiserum reacted strongly with a 35kDa polypeptide from P9 cell plasma membranes, which co-migrated with a signal from rat cerebral cortex, demonstrating that the cells express at least one form of the G-protein  $\beta$  subunit (Fig. 3.14). The electrophoresis system used here is inadequate for resolving the different G $\beta$  subtypes. Again the signal obtained from P9 cells was much larger than that from rat hepatocytes.

Toxin-catalysed ADP-ribosylation of P9 cell plasma membranes was carried out in order to confirm that the bands observed on immunoblotting co-migrated with toxin substrates and therefore represented G-protein  $\alpha$  subunits. Cholera toxin-catalysed ADP-ribosylation of P9 cell plasma membranes yielded labelled bands at approximately

45kDa and 42kDa representing the two  $G_s$ - $\alpha$  forms (Fig. 3.15). These bands co-migrated with signals obtained when membrane proteins were blotted and probed using an antiserum to  $G_s$ - $\alpha$  (Fig. 3.14). As in immunoblotting experiments, the 45kDa form appeared the more prevalent in the cells. Radioactivity was incorporated into other bands, but in none was the incorporation as great as in the  $G_s$ - $\alpha$  forms. Pertussis toxin catalysed ADP-ribosylation yielded a broad band at approximately 40kDa (Fig. 3.15). As the gel system used here is not adequate to resolve toxin-labelled  $G_i$ -2 $\alpha$  and  $G_i$ -3 $\alpha$ , this band must represent a combination of at least these two  $\alpha$  subunits, expressed by the cells. There was very little labelling of other polypeptides present in the membranes.

A range of GppNHp concentrations <sup>was used</sup> to influence forskolin-stimulated adenylyl cyclase activity in P9 cell plasma membranes but no inhibitory phase was obtained, although there was a marked low affinity, stimulatory phase (Fig. 3.16). Using a range of GTP concentrations to influence basal adenylyl cyclase activity in P9 cell plasma membranes, it was possible to demonstrate a consistent impairment of the optimal response by  $10^{-3}$ M GTP (Fig. 3.17). Pertussis toxin pre-treatment of P9 cells, at a concentration adequate to ADP-ribosylate 92% of the  $G_i$  present in P9 cell plasma membranes, had no influence on the response to glucagon (Fig. 3.18).

Experiments to measure phosphate incorporation into  $G_i$ -2 $\alpha$  upon agonist challenge of P9 cells showed no uptake of labelled phosphate over basal on challenge with TPA or 8-bromo-cAMP even in the presence of the phosphatase inhibitor okadaic acid (Fig. 3.19). Experiments done in parallel on rat hepatocytes showed considerable labelling of the immunoprecipitated  $G_i$ -2 $\alpha$  on agonist challenge (not shown).

Elevation of intracellular cAMP levels and plasma membrane adenylyl cyclase responses to agonist stimulation showed considerable variation in magnitude between experiments. Possible reasons for this are integrity of the agonist itself (concentration



and activity) and cell factors, for example the precise degree of cell confluence at stimulation and actual differences in receptor expression between batches of cells. It was noted that at passage 13, the cells lost the stimulatory response to PGE<sub>1</sub>, presumably due to loss of receptor expression and thereafter, cells were not used for experiment after passage 12. However, there was no obvious relationship between passage number and response to agonist to suggest a gradual reduction in receptor number. Variation between batches of serum used in the growth medium could be responsible for variation between cells. In addition, other authors have reported that rat hepatocytes transformed in a chemically-defined medium show a greater tendency to retain differentiated functions than those transformed in serum-supplemented medium (Woodworth *et al.*, 1986). These factors emphasise the desirability of using defined media where possible for cell culture work. Whatever the cause of this variation in responsiveness, it limits to some extent, the usefulness of the cells as a model system.

On calculating values for fold stimulation by agonist over basal intracellular cAMP levels it is important to remember that basal intracellular cAMP levels are within the range in which cAMP cannot measurably displace labelled cAMP from the binding protein, ie below 0.5pmols/50µl, as discussed in section 2.14c. The assay can only reliably detect cAMP at levels greater than this. This suggests that basal cAMP levels are likely to be inaccurately measured by the binding protein assay. Evidence that these values are indeed inaccurate is provided by their large percentage errors, in comparison to the errors on stimulated cAMP level measurements. For example, in the dose response curve for forskolin given in Fig. 3.5, basal cAMP was measured as 0.50±0.20 (Mean±SEM for triplicate observations), a 40% error and maximal forskolin stimulation gave 105±6pmols/50µl extract, a 6% error. Values for fold stimulation over basal are therefore also likely to be inaccurate. Likewise, basal values for plasma membrane adenylyl cyclase activity were also generally less accurate than those for stimulated activity, as indicated by the errors. Less weight then, should be

given to values for fold stimulation than to absolute values for agonist stimulation.

All attempts to promote differentiation of the cells failed. Growing the cells on collagen-coated plates had no effect on cell morphology or glucagon responsiveness. Nor did exposure of cells to dexamethasone (0.25 $\mu$ M, 16 hours) or insulin (0.5 $\mu$ M, 16 hours) affect morphology or restore glucagon responsiveness. However, insulin at this concentration did stimulate cell growth and had marked effects on adenylyl cyclase signalling which are discussed in Chapter 4.

### 3.3 Discussion

Previous studies have shown that rat hepatocytes, although immortalised or transformed by SV40, retain a variety of differentiated cell characteristics. The P9 cell line expresses glucose-6-phosphatase and bilirubin glucuronyl transferase. These findings are consistent with those of other authors who have examined SV40-immortalised, rat hepatocyte-derived cell lines (Isom *et al.*, 1980, Woodworth *et al.*, 1986). This, along with the high degree of cell purity in the preparations from which they were derived, makes it hard to imagine how the cell line could have arisen from any cell type other than hepatocytes, present during the transfection procedure. The situation in these fibroblast-like cells however, is clearly very different from that in 3T3 L1 fibroblasts. Whilst the latter can be encouraged to differentiate in response to various agents (Watkins *et al.*, 1987), P9 cells appear irreversibly fixed in their relatively de-differentiated, rapidly proliferating form. In this way P9 cells more closely resemble established hepatoma cell lines than other fibroblast cell lines.

Despite the presence of these two hepatocyte-specific marker proteins the adenylyl cyclase signalling system in the cells, on close examination, showed considerable divergence from that of rat hepatocytes. There are clear functional alterations in signalling at the level of the receptor and G-protein in the P9 line as compared to rat hepatocytes. Firstly, the cells have lost elevation of intracellular cAMP in response to glucagon, a highly specialised cellular response, implying that their cyclase functioning, in addition to their morphology, has moved in the direction of a non-differentiated state. This loss of responsiveness appears to be due to the low level of glucagon receptors in the plasma membrane, though we cannot say whether it reflects an actual reduction in receptor expression or simply receptor sequestration at a subcellular site. This is in line with the findings of Houslay *et al.* (1980) who showed that lost responsiveness would require at least 75% of the receptor to be absent from the plasma membrane. Changes in receptor expression are a major factor in control of

responses and cells undergoing transformation, are commonly known to lose receptor-mediated responses. However, down-regulation of the glucagon response observed on culture of rat hepatocytes suggests that the low responsiveness of P9 cells may also be a consequence of their removal from the original tissue and adherence to plates rather than as a result of transfection alone. In this respect, the absent glucagon response may reflect the artificial environment of the cultured cells, rather than their state of differentiation. In addition, receptor phosphorylation, a form of modification known to be involved in desensitisation of receptor-mediated responses may be contributing to the loss of glucagon responsiveness (Houslay, 1991b).

There are several factors that may contribute to the plateau observed on examining the time courses for intracellular cAMP elevation in response to agonist. This levelling-off of the response may be achieved at the level of cAMP generation, hydrolysis or by its extrusion from the cell. Phosphodiesterase activation can be excluded as a cause, as the phosphodiesterase inhibitor IBMX had no influence on the plateau. Both PGE<sub>1</sub> (Brunton and Mayer, 1979) and phenol red (King and Mayer, 1974) present in the serum-free medium surrounding the cells, have been reported to inhibit cAMP extrusion. Indeed, Brunton et al reported in their system a K<sub>0.5</sub> of 10 μM PGE<sub>1</sub> for the effect. This suggests that cAMP extrusion is unlikely to be responsible for the plateau. More likely it may reflect desensitisation to or metabolism of the agonist, or feedback by high levels of cAMP on the cAMP generating system. Similar causes may be proposed for the plateau observed on the time course for forskolin-elevated intracellular cAMP, except for desensitisation as forskolin is not known to elicit desensitisation in any cell type. That the time course with glucagon and forskolin in combination was entirely parallel, suggests that the glucagon response too showed no ability to desensitise, unlike that in rat hepatocytes (Murphy *et al.*, 1989).

The synergism observed between forskolin and glucagon action in P9 cells is further evidence that the cells retain at least small numbers of functional glucagon

receptors (with apparently normal kinetics for cAMP generation). As synergism is indicative of forskolin's high affinity action on the adenylyl cyclase system, this finding also suggests that, like rat hepatocytes, they possess both high and low affinity sites for forskolin action. In common with diverse cell types, P9 cells retain a stimulatory response to PGE<sub>1</sub>, though the ability of this agonist to stimulate plasma membrane adenylyl cyclase activity was smaller in P9 cells than in rat hepatocytes.

P9 cells like rat hepatocytes express at least two out of a possible four G<sub>s</sub>- $\alpha$  variants (Bray *et al.*, 1986) and two G<sub>i</sub> forms, but the polypeptides were expressed in different relative amounts between the two cell types. There are a number of possible reasons for the change in G-protein levels observed in the immortalised cells. Although a change in expression could potentially occur due to the differences in hormonal or physical environment between rat liver and cell culture plate, it is much more likely that the changes are indicative of a change in cell phenotype as observed in other cells on differentiation or de-differentiation (Uhing *et al.*, 1987), except that the changes in P9 cells, like their fibroblast-like morphology, are not subject to change on challenge with differentiation agents. Furthermore, if for example the level of glucocorticoid hormones in the cell culture serum as opposed to the animal were responsible for the differences, one might expect to see inconsistencies in the pattern of expression between different batches of cells, which was not the case. Similarly, the changes are unlikely to be in response to a chronic elevation in basal cAMP as basal cAMP levels in both P9 cells and rat hepatocytes were barely detectable. The greater expression of G<sub>i</sub>- $\alpha$  and the  $\beta$  subunit suggests that these polypeptides may have specific functions in the immortalised cell that require their presence in larger amounts, relative to other plasma membrane proteins. Mobilisation of G-proteins to the plasma membrane has been proposed as a mechanism by which cells can regulate the activity of receptors which signal through these G-proteins. Indeed these changes may confer specificity for responses which have not been examined in the P9 cells. The changes in G-protein expression may be driven by the cell in an attempt to compensate for the loss of

stimulatory receptor or  $G_i$  input into adenylyl cyclase. Alternatively, the increase in plasma membrane  $G_i$  levels in P9 cells may, like the level of glucagon responsiveness, be a consequence of primary culturing the cells rather than a result of immortalisation alone. Fujinaga *et al.* (1989), on studying of cultured rat hepatocytes, noted that a sustained increase in pertussis toxin substrate occurred early during their primary culture. In addition they found that pertussis toxin preincubation could abolish DNA synthesis in response to insulin only at an early stage after plating the cultures, but not after 24 hours. The authors concluded that a pertussis toxin sensitive G-protein may play a role in cell cycle control in the transition from the resting ( $G_0$ ) state to the state in which DNA synthesis occurs ( $G_1$ ). The mechanism of the increased level of  $G_i$ - $\alpha$  in P9 cells could be increased expression, decreased degradation or greater message stability and may be involved in the high growth rate of P9 cells as compared to rat hepatocytes.

It is particularly difficult to speculate about the reasons for co-ordinate regulation of the two  $G_s$ - $\alpha$  forms as nothing is known with certainty about the specific activities and functions of these splice variants. However, the increase in one form without the other shows that they are differentially regulated, presumably with consequences for adenylyl cyclase signalling in the cells. This finding raises the question of the site of the regulation. Granneman *et al.* (1990) in studies on  $G_s$  expression in various rat tissues showed that the levels of individual splice variants in tissues correlated well with the levels of their respective mRNAs. In liver the level of mRNA for the higher molecular weight form of  $G_s$  was marginally more abundant than the mRNA encoding the lower molecular weight splice variant. It would be interesting to measure the levels of mRNAs for the  $G_s$  forms in P9 cells in order to find whether there is a similar correlation with protein levels and therefore to provide evidence for the site of control of their levels being at transcription.

In rat hepatocyte plasma membranes there is a characteristic dual regulation of adenylyl cyclase by GppNHp. Low concentrations inhibit the response which has

been stimulated by forskolin, an effect half maximal at 0.1nM and high concentrations further stimulate the response (Gawler and Houslay, 1987). This is thought to be due to  $G_i$  and  $G_s$  having respectively high and low affinities for this non-hydrolysable GTP analogue. Using this method there was however, no evidence of  $G_i$  function in P9 cell plasma membranes, though there was ample  $G_s$  function as evidenced by the low affinity, stimulatory phase. In addition to the lack of GppNHp-mediated inhibition of adenylyl cyclase we have no evidence of receptor-mediated  $G_i$  function in the cells. However, an inhibitory phase was observed in the GTP dose response curve. This has been proposed to represent  $G_i$  function in other systems studied for example rat adipocytes (Strassheim *et al.*, 1990), mouse adipocytes (Palmer *et al.*, 1992) and rat hepatocytes (Heyworth *et al.*, 1984) as  $G_i$  has a low affinity for the nucleotide as opposed to  $G_s$ , so that the inhibitory phase follows the stimulatory phase. This inhibitory phase as a measure of  $G_i$  function in P9 cells however, took place at much higher concentrations and was of much smaller magnitude than in rat hepatocytes, where the authors noted half maximal inhibition at 1 $\mu$ M GTP.

It is particularly intriguing that some measure of  $G_i$  function could be demonstrated by one method but not the other. Presumably this reflects a difference in the mechanics of guanine nucleotide binding to  $G_i$  in the two cell types. That adenylyl cyclase inhibition by these two guanine nucleotides can be differentially influenced by circumstances is a phenomenon which has been previously observed. Palmer *et al.* (1992) noted on studying mouse adipocytes, that maximal inhibition of agonist-stimulated adenylyl cyclase was achieved at much higher concentrations of GppNHp ( $10^{-6}$ M) than in hepatocyte plasma membranes. The authors also found that inhibition of agonist-stimulated adenylyl cyclase by GppNHp was similar in control and diabetic animals, whilst the inhibitory phase on employing GTP was considerably reduced in diabetic animals.  $G_i$  function, as measured by the ability of low concentrations of GppNHp to inhibit forskolin-stimulated adenylyl cyclase activity, has been noted to be abolished in both adipocyte (Strassheim *et al.*, 1990) and hepatocyte

(Gawler *et al.*, 1987) plasma membranes from streptozotocin-induced diabetic rats. A similar influence to that present in diabetes which brings about these functional changes, may be at work in P9 cells. There appears then to be considerable scope for variation in  $G_i$  functional measurements between systems and their alteration in disease states.

In addition to disease states, changes in the physical environment of the signalling system are known to influence  $G_i$  function. High sodium chloride concentrations for example, are known to decrease agonist affinity of G-protein-coupled receptors and to affect the interaction of G-proteins with GTP, thereby modulating G-protein function (Gierschik *et al.*, 1988). However, it seems unlikely that such a major alteration in the internal environment could be responsible for the change observed in P9 cells and one would expect such an influence, if present in the cells, to be removed on preparing and assaying membranes.

Another possibility for the lack of functional cyclase inhibition is that  $G_i$ -2 has been largely inactivated by a covalent modification in the cells.  $G_i$ -2 $\alpha$  phosphorylation has been shown to occur in response to a number of stimuli (Pyne *et al.*, 1989a) and has been proposed as the mechanism of  $G_i$ -2 inactivation in diabetes (Bushfield *et al.*, 1990b). Again, one might expect such a modification to be reversible on making membranes, as the cells were harvested and membranes prepared for these experiments in the absence of phosphatase inhibitors. The inability to show agonist-induced phosphorylation of  $G_i$ -2 $\alpha$  in P9 cells distinguishes them from rat hepatocytes, where agonists resulting in PK-A or PK-C activation stimulate incorporation of labelled phosphate into the  $\alpha$  subunit of  $G_i$ -2 (Bushfield *et al.*, 1990a). The failure to incorporate phosphate into P9 cell  $G_i$ -2 $\alpha$  in response to agonists could be as a result of the G-protein being already phosphorylated in the basal state, possibly due to overactivity of one of these kinases. Another possible mechanism of  $G_i$ -2 phosphorylation that could be envisaged in P9 cells, is as a result of the activity of the



large T antigen which is known to activate a kinase capable of phosphorylating and potentiating the activity of the protein p53 involved in cell transformation (Scheidtmann, 1989). It is possible that this kinase may have other, as yet unidentified substrates, including signal transduction components. Alternatively, rather than reflecting phosphorylation *in vivo*, the failure to incorporate phosphate into P9 cell Gi-2 $\alpha$  may be due to the G-protein being a poorer kinase substrate as a result of its conformation or, may result from kinases responsible for G-protein phosphorylation being less abundant in P9 cells than in rat hepatocytes. In order to further investigate these possibilities, we would need to examine Gi function in P9 cell plasma membranes after phosphatase treatment and study expression of the above kinases by the cell line.

Whatever the cause of reduced cyclase inhibition it does suggest that the Gi- $\alpha$  subtypes may subserve other functions in the cells, as both Gi-2 $\alpha$  and Gi-3 $\alpha$  are expressed in abundance. Possibly the coupling of Gi to other effectors is of greater priority to the immortalised phenotype than cyclase inhibition. Indeed, we have not been able to demonstrate any receptor-mediated inhibitory responses in P9 cells presumably due to lack of expression of the appropriate receptors, which would mean that Gi was in any case redundant in this regard. Further evidence that there is little or no Gi input into adenylyl cyclase signalling in P9 cells came from the finding that pertussis toxin pre-treatment had no effect on the response to glucagon or PGE<sub>1</sub> (see Chapter 5). The low stimulatory response to glucagon in P9 cells is probably entirely due to reduced receptor expression rather than any change in Gi or G<sub>s</sub> input.

Indirect evidence has been provided for differences in cAMP phosphodiesterase activity between P9 cells and their native rat hepatocyte counterparts. The nature of the cAMP degradation pathway depends on the cell phenotype and evidence has previously been presented that the complexity of this process may be greater in differentiated cells (Conti *et al.*, 1991). As P9 cells are rapidly dividing and cAMP is inextricably involved in growth control (Roesler *et al.*, 1988), there is good reason to expect differences in

its regulation between the two cell types. The phosphodiesterases expressed in P9 cells would merit formal investigation.

The adenylyl cyclase catalytic subunit is ubiquitous in mammalian cells so it is not surprising to find that the cells have this enzyme activity. However, given the differences in receptor and G-protein expression in P9 cells as compared to native hepatocytes, it is also possible that the cells express a different array of catalyst subtypes and that this may account in part for the differences in stimulatory and inhibitory cyclase regulation between the two cell types. This would be interesting to investigate when antisera to the various subtypes are more readily available.

Various cell lines have been studied extensively and are currently in use as model systems. The P9 cell line does not possess the normal responses of the cell type from which it was derived and shows marked phenotypic differences from rat liver, both functional and morphological. As the most useful model systems express a wide variety of receptors and responses comparable to their native tissues, this mitigates against use of the P9 cell line as a hepatocyte model system. The main problems with the cell line which limit its usefulness are a poor variety of responses and loss of responsiveness after a finite number of passages. It is clearly not possible by using the cell line to gain further insight into some of the most interesting aspects of hepatocyte adenylyl cyclase signalling such as glucagon desensitisation and the influence  $G_i$  on responses. Its advantages are ease of growth thus obtaining substantial amounts of material for experiment. Studying the cell line may give us more information about signalling and change in expression of markers as a result of the viral transfection. Presumably, some of the features noted will apply to virally-immortalised cells in general and whilst we cannot hope to compare the cells more than superficially to hepatocytes, we can certainly use them to examine features of adenylyl cyclase signalling, some of which are common to all mammalian cells.

**Table 3.1**      **Glucose-6-phosphatase activity in P9 cells and rat hepatocytes.**

Confluent P9 cells were harvested and stored at -80°C as described in section 2.6d. Freshly isolated rat hepatocytes were stored at -80°C until use. Cells were thawed, homogenised in 1mM EDTA, 10mM Tris HCl, pH 7.4 and glucose-6-phosphatase activity assayed as described in section 2.15. Samples of homogenate were retained for protein assay. Data are mean+/-SEM for triplicate observations obtained in three experiments.

**Table 3.1**

Cell type	Glucose-6-phosphatase activity ( $\mu\text{g Pi/mg protein / minute}$ )
P9 cells	1.9 $\pm$ 0.2
Rat hepatocytes	9.2 $\pm$ 0.2

**Table 3.2      Agonist-stimulated adenylyl cyclase activity in  
P9 cell and rat hepatocyte plasma membranes.**

Plasma membrane preparations from P9 cells and rat hepatocytes were assayed for adenylyl cyclase activity in response to agonists as described in section 2.13. Agonists were used alone or in combination at the following concentrations GTP ( $10^{-4}\text{M}$ ), glucagon ( $10^{-5}\text{M}$ ), PGE<sub>1</sub> ( $10^{-4}\text{M}$ ), GppNHp ( $10^{-4}\text{M}$ ), forskolin ( $10^{-4}\text{M}$ ) and NaF ( $1.5 \times 10^{-2}\text{M}$ ). Data are mean $\pm$ SEM for triplicate observations obtained in one experiment. Figures in brackets are values for fold stimulation over the respective basal values. Similar results were obtained in another experiment.

**Table 3.2**

Agonist	Adenylyl cyclase activity (pmols/mg protein/min)	
	P9 cells	Rat hepatocytes
-	19 $\pm$ 1	38 $\pm$ 3
GTP	40 $\pm$ 6 (2)	105 $\pm$ 3 (3)
Glucagon + GTP	41 $\pm$ 4 (2)	367 $\pm$ 26 (10)
PGE <sub>1</sub> + GTP	95 $\pm$ 4 (5)	341 $\pm$ 14 (9)
GppNHp	130 $\pm$ 7 (7)	330 $\pm$ 3 (9)
Forskolin	611 $\pm$ 26 (32)	870 $\pm$ 8 (23)
NaF	653 $\pm$ 24 (27)	620 $\pm$ 8 (16)

**Table 3.3**      **Elevation of P9 cell intracellular cAMP in response to forskolin and PGE<sub>1</sub>, alone and combined.**

Confluent P9 cells were incubated for 16 hours in serum-free medium then challenged for 2 minutes with forskolin ( $10^{-5}\text{M}$ ), PGE<sub>1</sub> ( $10^{-5}\text{M}$ ) and forskolin and PGE<sub>1</sub> combined at the same concentrations. Cyclic AMP was extracted and assayed as described in section 2.14. Results are mean $\pm$ -SEM for triplicate observations in one experiment. Values in brackets are fold stimulation over basal cAMP. Similar results were obtained in another experiment.

**Table 3.3**

Agonist	Intracellular cAMP (pmols/50µl extract)
-	0.1+/-0.04
PGE <sub>1</sub>	25.1+/-0.7 (224)
Forskolin	15.4+/-1.4 (137)
PGE <sub>1</sub> + Forskolin	49.4+/-1.6 (442)



**Table 3.4**      **Quantitative comparison of G-protein levels in P9 cell and rat hepatocyte plasma membranes.**

G-proteins expressed in P9 cells and rat hepatocytes were identified by an immunoblotting procedure using specific antisera as described in the legend to Figure 3.10. Membrane protein, 100 $\mu$ g was blotted in every case. Blots were scanned densitometrically as described in section 2.10f. and the density of the band obtained from P9 cells given as a percentage band density of the signals obtained from rat hepatocytes for each polypeptide. Similar results were obtained in two other experiments. The blots from one experiment are shown in Figs. 3.14 and 3.15.

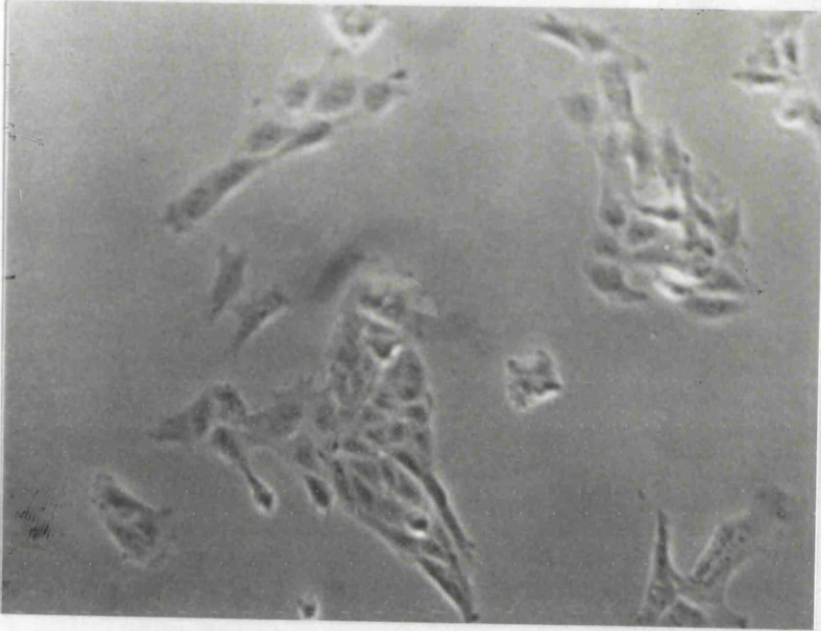
**Table 3.4**

Polypeptide	P9 cells/Rat hepatocytes (%)
G <sub>s</sub> - $\alpha$ (45kDa)	377
G <sub>s</sub> - $\alpha$ (42kDa)	50
G $\beta$	258
G <sub>i</sub> -2 $\alpha$	210
G <sub>i</sub> -3 $\alpha$	543

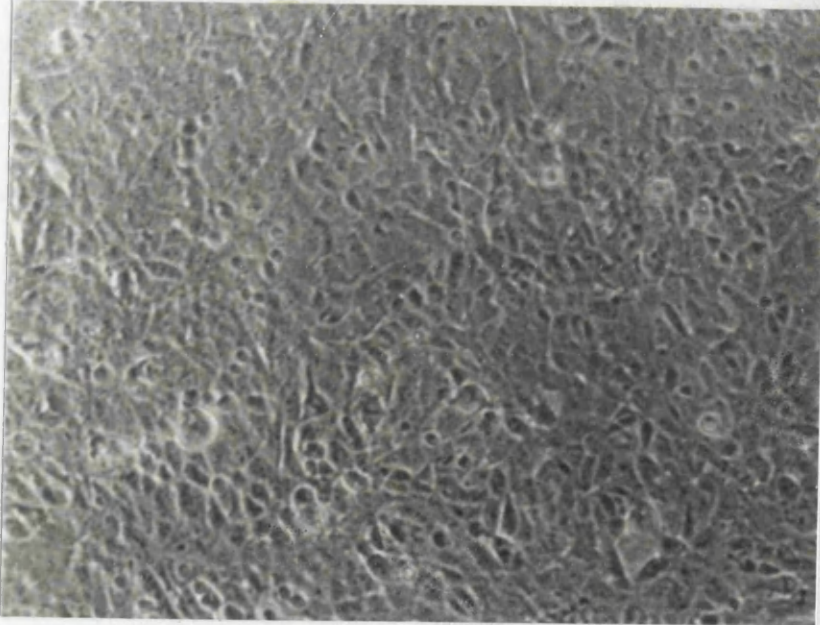
**Fig. 3.1      P9 cells and rat hepatocytes in culture.**

P9 cells in culture (Magnification x 90), a. one day after subculture and b. at confluence. c. rat hepatocytes in primary culture (Magnification x 90) two hours after isolation.

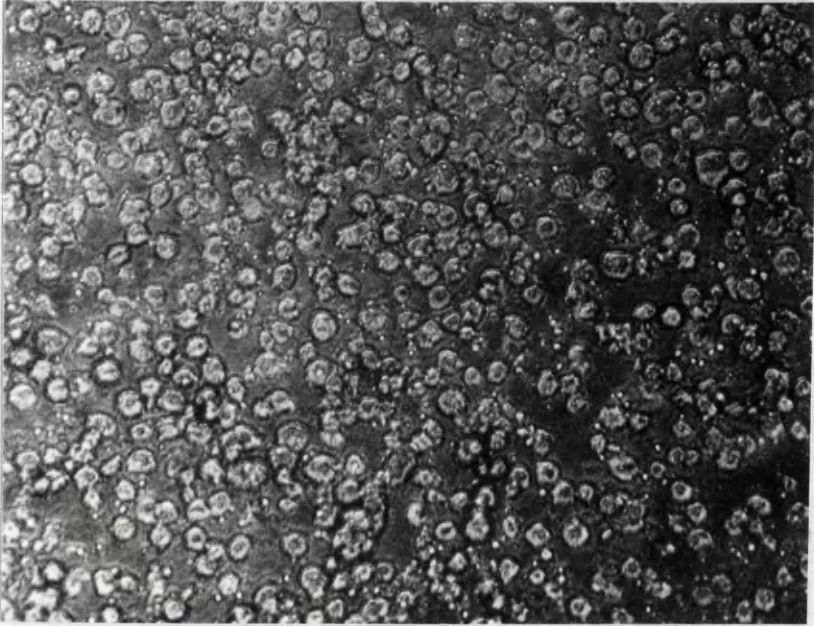
**Figure 3.1 (a)**



**Figure 3.1 (b)**



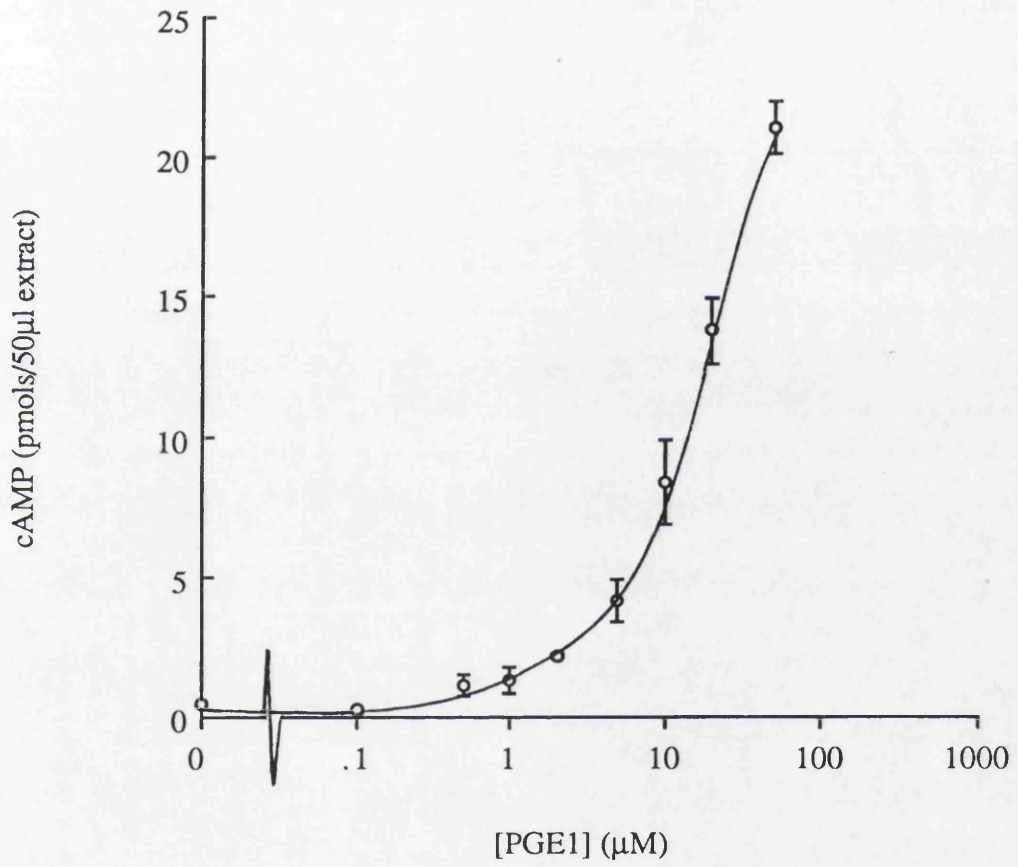
**Figure 3.1 (c)**



**Fig. 3.2**      **Effect of increasing concentrations of PGE<sub>1</sub> on P9 cell intracellular cAMP.**

P9 cells were challenged for 2 minutes with increasing concentrations of PGE<sub>1</sub> ( $10^{-7}\text{M}$ - $10^{-4}\text{M}$ ) then cAMP extracted and assayed as described in section 2.14. Fold stimulation over basal was  $48\pm 2$ .  $K_{0.5}$  for PGE<sub>1</sub> was  $1.35 \times 10^{-5}\text{M}$ . Basal cAMP was  $0.43\pm 0.06$  pmols cAMP /50 $\mu\text{l}$  extract. All data are mean $\pm$ SEM for triplicate observations obtained in one experiment. Similar results were obtained in another experiment.

**Figure 3.2**

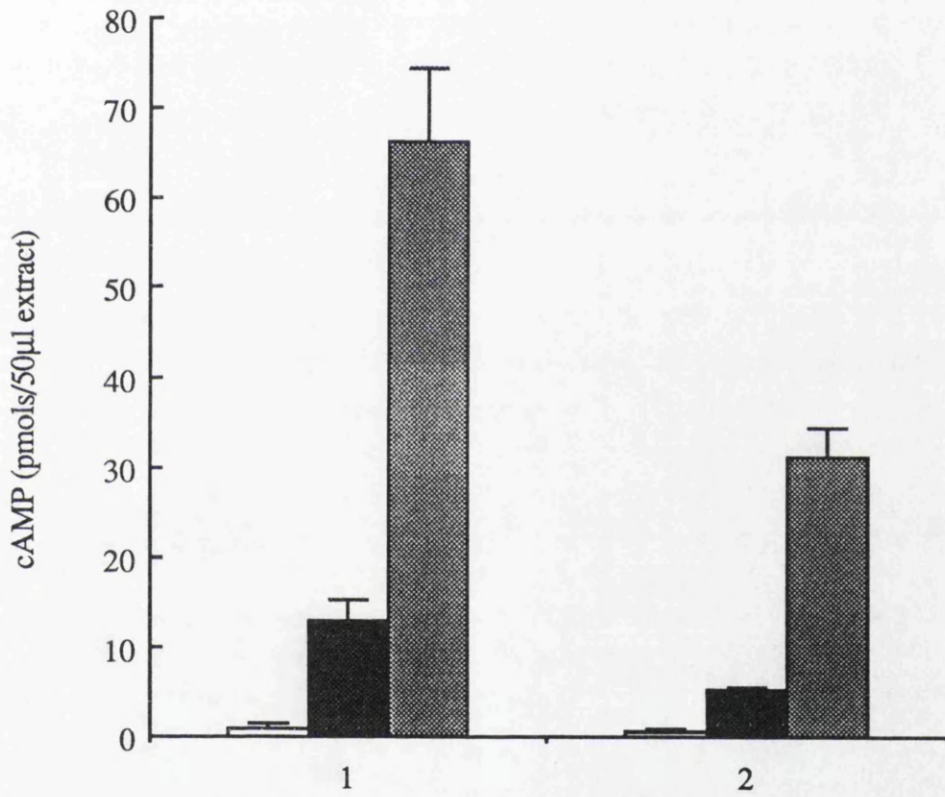


**Fig. 3.3**      **Elevation of rat hepatocyte intracellular cAMP  
in response to forskolin and glucagon, alone  
and combined.**

Freshly isolated rat hepatocytes were kept in primary culture as described in section 2.5a. and after 40 minutes (column 1) and 2 hours (column 2) were challenged with forskolin ( $10^{-5}\text{M}$ ) alone (white bars), glucagon ( $10^{-8}\text{M}$ ) alone (black bars), or forskolin and glucagon combined at the same concentrations (grey bars). After 2 minutes buffer was aspirated and cAMP extracted and assayed as described in section 2.14. Basal cAMP levels were  $0.17 \pm 0.06$  pmols/50 $\mu\text{l}$  extract (40 minutes) and  $0.07 \pm 0.02$  pmols/50 $\mu\text{l}$  extract (2 hours). Data are mean $\pm$ SEM for triplicate observations obtained in two experiments. Glucagon response was enhanced significantly by forskolin ( $p < 0.001$ ) at both time points and both glucagon and glucagon/forskolin responses were significantly reduced ( $p < 0.01$ ) after 2 hours.



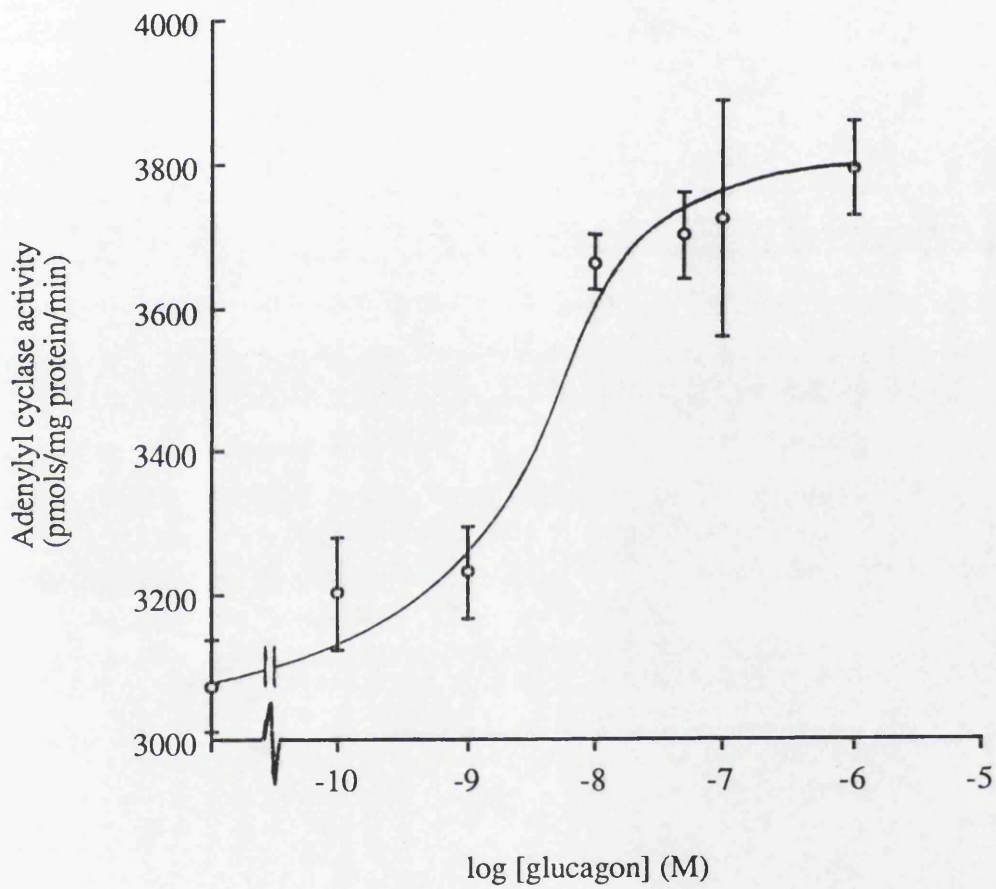
**Figure 3.3**



**Fig. 3.4**      **Effect of increasing concentrations of glucagon on forskolin-stimulated adenylyl cyclase activity in P9 cell plasma membranes.**

P9 cell plasma membranes incubated with forskolin ( $10^{-5}\text{M}$ ), GTP ( $10^{-4}\text{M}$ ) and glucagon ( $0-10^{-6}\text{M}$ ) were assayed for adenylyl cyclase activity, as described in section 2.13. The maximum response to glucagon (at  $10^{-6}\text{M}$ ) was  $123\pm 4\%$  of that to forskolin alone ( $p < 0.01$ ). The  $K_{0.5}$  for glucagon's effect was  $2.9 \times 10^{-9}\text{M}$ . Data are mean $\pm$ SEM for triplicate observations obtained in one experiment. Similar results were obtained in another experiment.

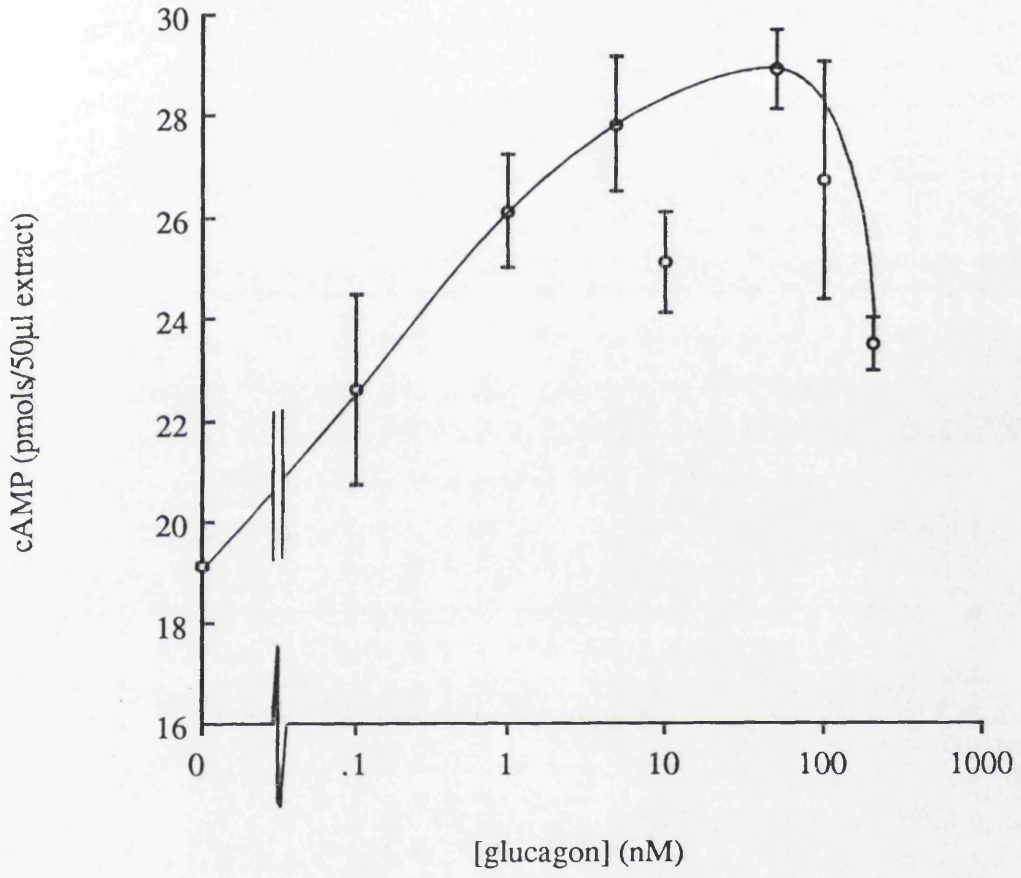
**Figure 3.4**



**Fig. 3.5    Elevation of P9 cell intracellular cAMP in response to forskolin combined with increasing concentrations of glucagon.**

P9 cells grown to confluence in 24 well plates and exposed to serum free medium for 16 hours were challenged for 2 minutes with forskolin ( $10^{-5}\text{M}$ ) and increasing concentrations of glucagon (0 to  $2 \times 10^{-7}\text{M}$ ). Cyclic AMP was extracted and assayed as described in section 2.14. The maximum response to glucagon (at  $5 \times 10^{-8}\text{M}$ ) was  $151 \pm 7\%$  of the response to forskolin alone ( $p < 0.001$ ) The  $K_{0.5}$  for glucagon's action in enhancing the response to forskolin was  $3 \times 10^{-10}\text{M}$ . Data are mean  $\pm$  SEM for triplicate observations obtained in one experiment. Similar results were obtained in two other experiments.

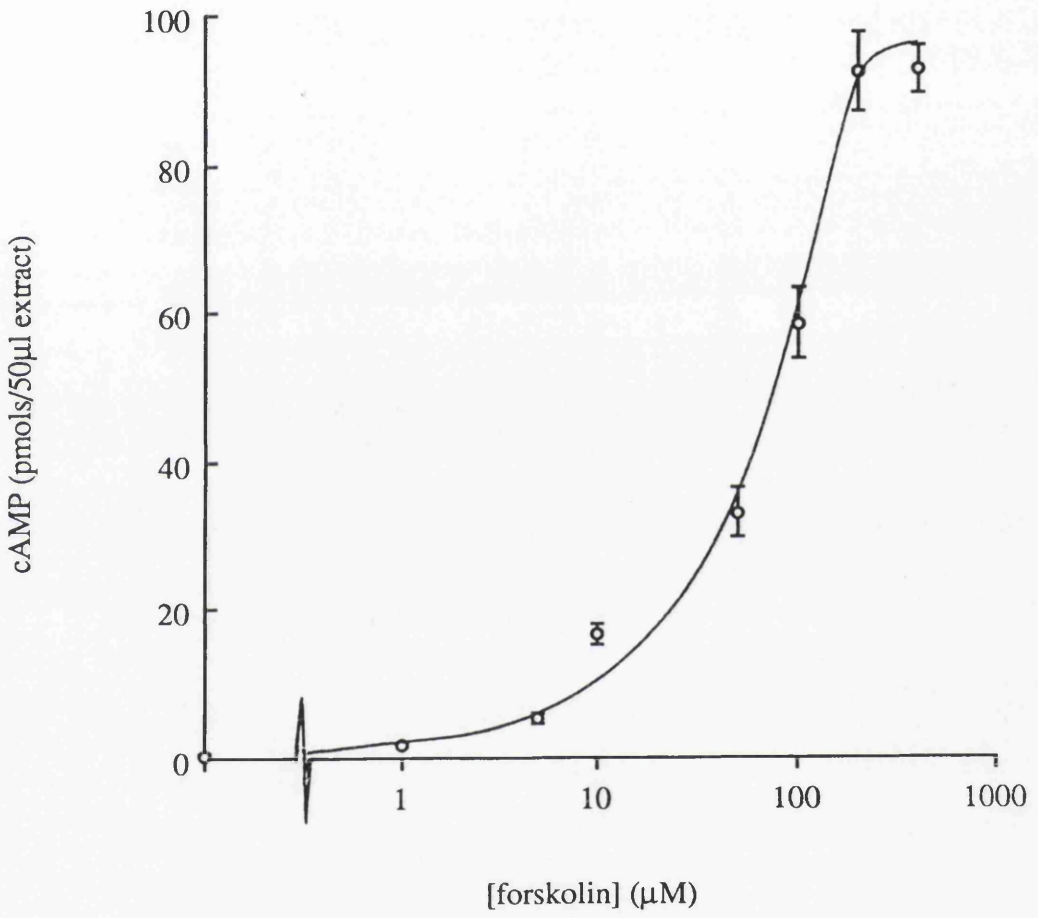
**Figure 3.5**



**Fig. 3.6**      **Effect of increasing concentrations of forskolin**  
**on P9 cell intracellular cAMP.**

P9 cells were challenged for 2 minutes with increasing concentrations of forskolin ( $10^{-6}\text{M}$  to  $4.0 \times 10^{-4}\text{M}$ ) then cAMP was extracted and assayed as described in section 2.14. Fold stimulation over basal was  $186 \pm 6$ .  $K_{0.5}$  for forskolin was  $7.2 \times 10^{-5}\text{M}$ . Basal cAMP was  $0.50 \pm 0.35$  pmols/50 $\mu\text{l}$  extract. Results are mean $\pm$ -SEM for triplicate observations obtained in one experiment. Similar results were obtained in another experiment.

**Figure 3.6**

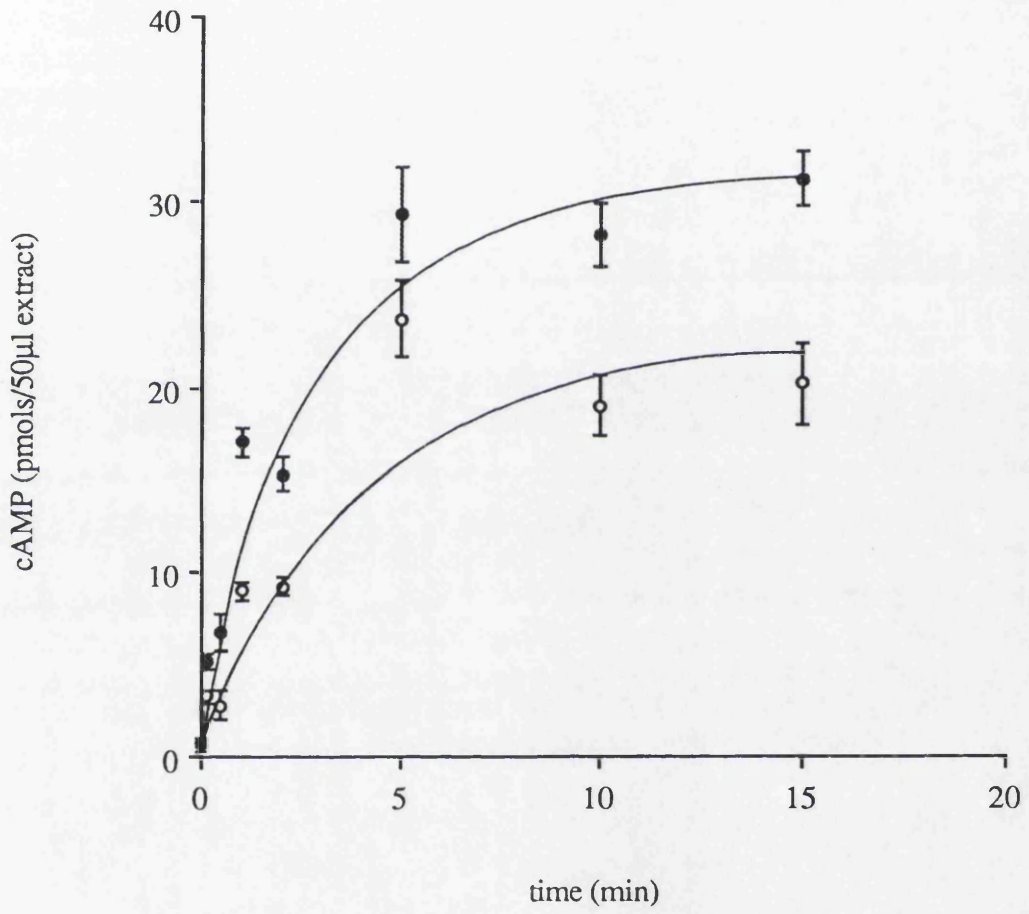


**Fig. 3.7**      **Time course for elevation of P9 cell**  
**intracellular cAMP in response to forskolin,**  
**alone and combined with glucagon.**

P9 cells were challenged with forskolin ( $10^{-5}\text{M}$ ) alone (open circles) or in combination with glucagon ( $10^{-8}\text{M}$ ) (closed circles) for 2 minutes and cAMP extracted at intervals. Samples were neutralised and assayed for cAMP content as described in section 2.14. Data are mean $\pm$ SEM for triplicate observations obtained in one experiment. Similar results were obtained in three other experiments.



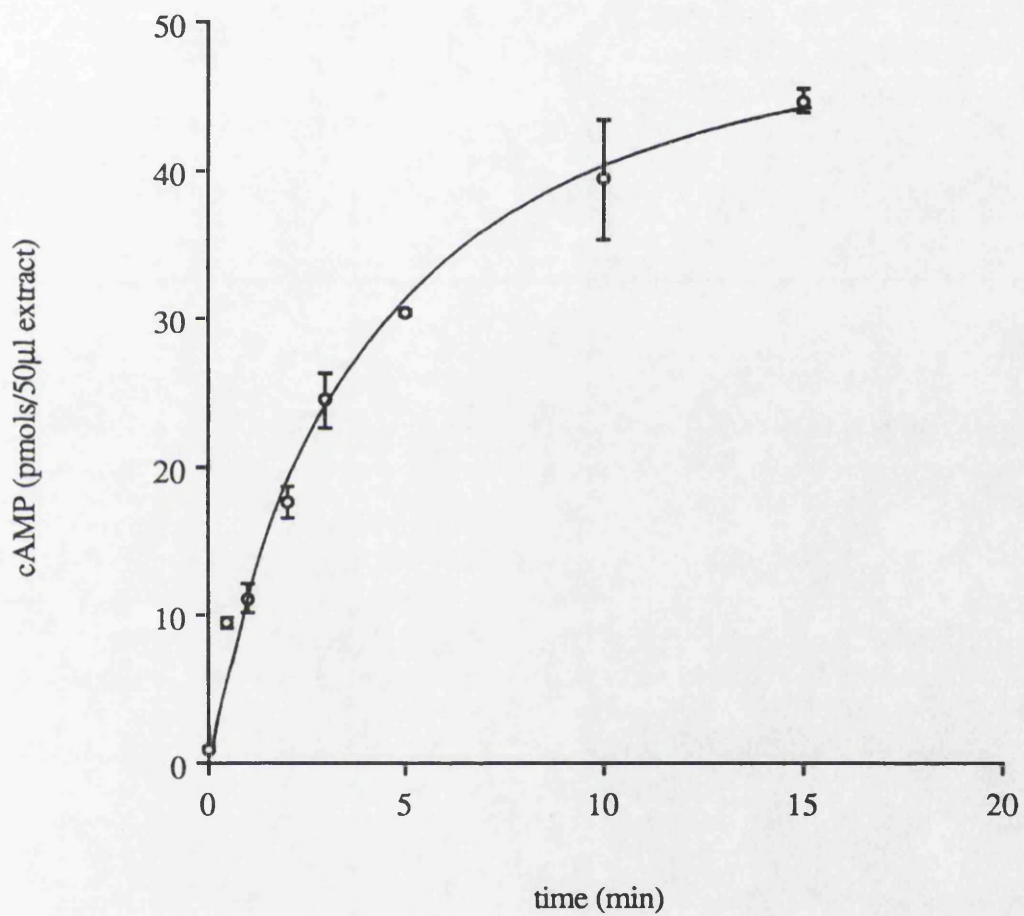
**Figure 3.7**



**Fig. 3.8**      **Time course for elevation of P9 cell**  
**intracellular cAMP in response to PGE<sub>1</sub>.**

P9 cells were challenged with PGE<sub>1</sub> (10<sup>-5</sup>M) and cAMP extracted at intervals then samples neutralised and assayed for cAMP as described in section 2.14. Data are mean±SEM for triplicate observations obtained in one experiment. Similar results were obtained in three other experiments.

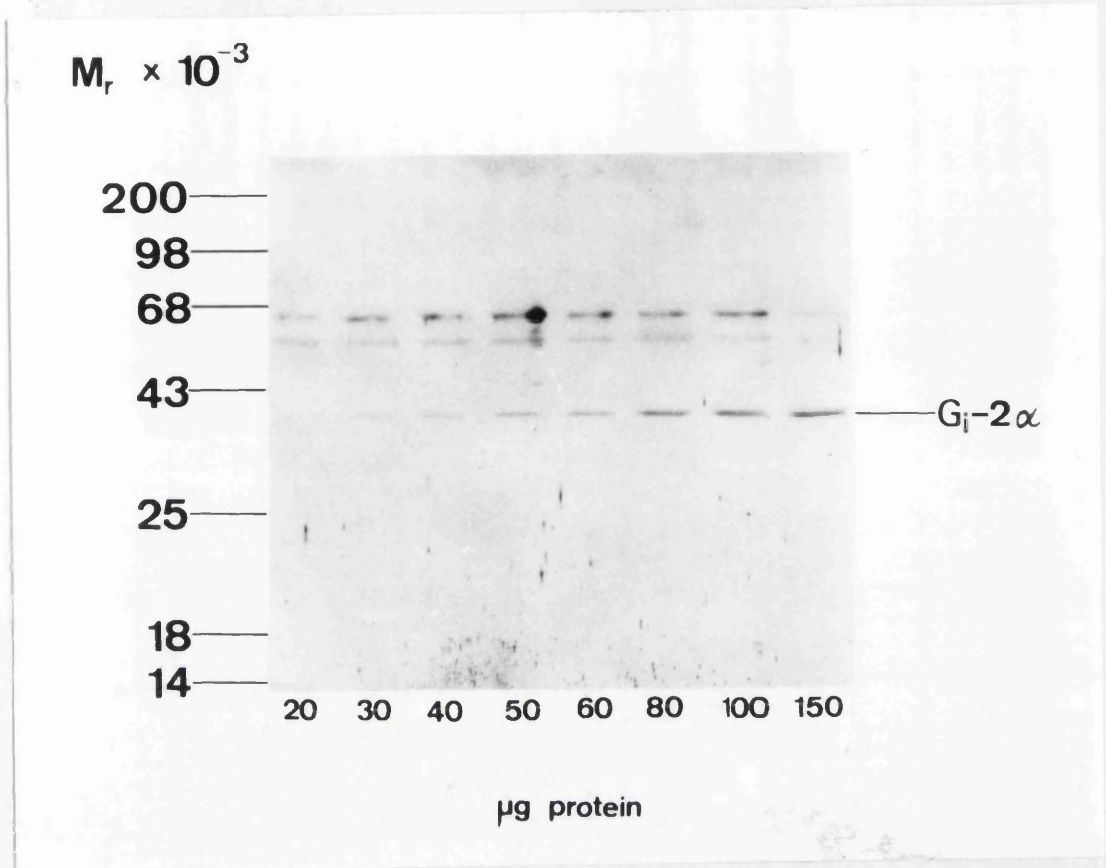
**Figure 3.8**



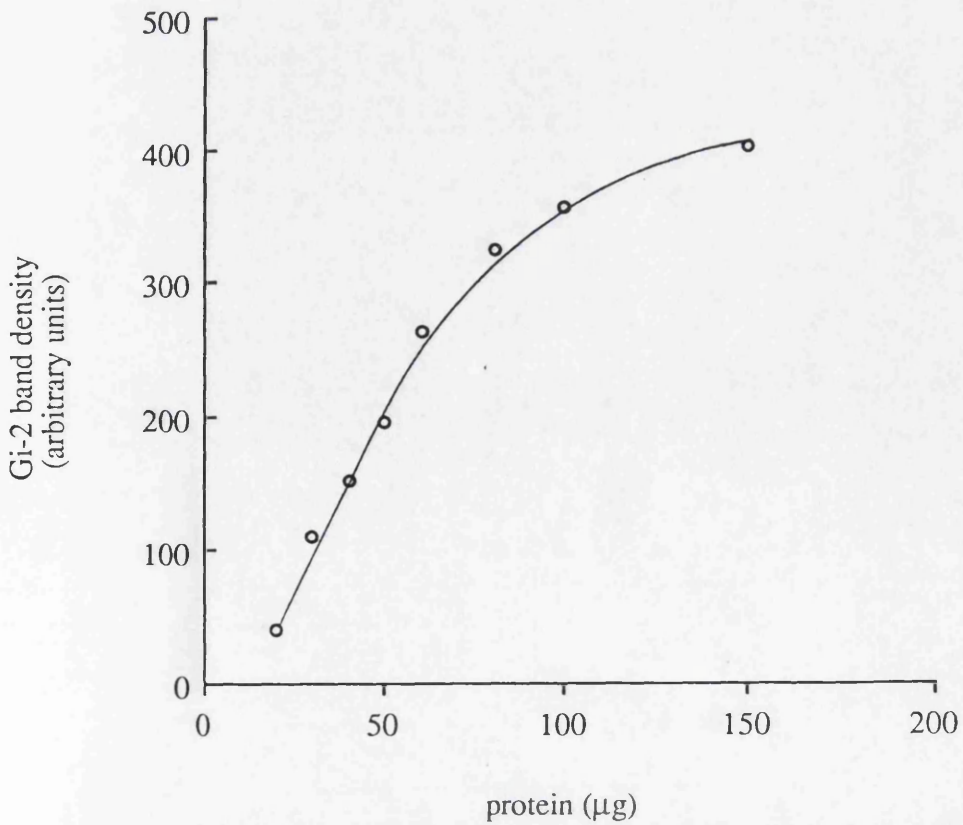
**Fig.3.9**                    **Immunological identification of Gt-2 $\alpha$  in P9 cell plasma membranes.**

Samples of P9 cell plasma membrane protein (20 $\mu$ g to 150 $\mu$ g) were TCA precipitated and resolved using SDS PAGE as described in section 2.10 then transferred onto nitrocellulose membrane as described in section 2.11. Immunoblotting was performed using a 1: 200 dilution of antiserum 1432. Detection of the primary antiserum was achieved using anti-rabbit IgG at 1: 500 dilution which was coupled to horseradish peroxidase (Fig. 3.9a). The substrate for the peroxidase was o-dianisidine. Bands were scanned densitometrically and results plotted graphically (Fig. 3.9b), using arbitrary units for band density. Data shown are from one experiment. Similar results were obtained in another experiment.

**Figure 3.9 (a)**



**Figure 3.9 (b)**



**Fig. 3.10      Immunological identification of Gi-3 $\alpha$  in P9  
cell plasma membranes.**

Samples of P9 cell plasma membrane protein (20 $\mu$ g to 150 $\mu$ g) were TCA precipitated, resolved and transblotted as described in the legend to Figure 3.9 then Gi-3 $\alpha$  detected using a 1: 1000 dilution of the antiserum I3C and horseradish peroxidase-linked IgG as second antibody (Fig. 3.10a). Bands were scanned densitometrically and results plotted graphically (Fig. 3.10b). Data shown are from one experiment. Similar results were obtained in another experiment.

Figure 3.10 (a)

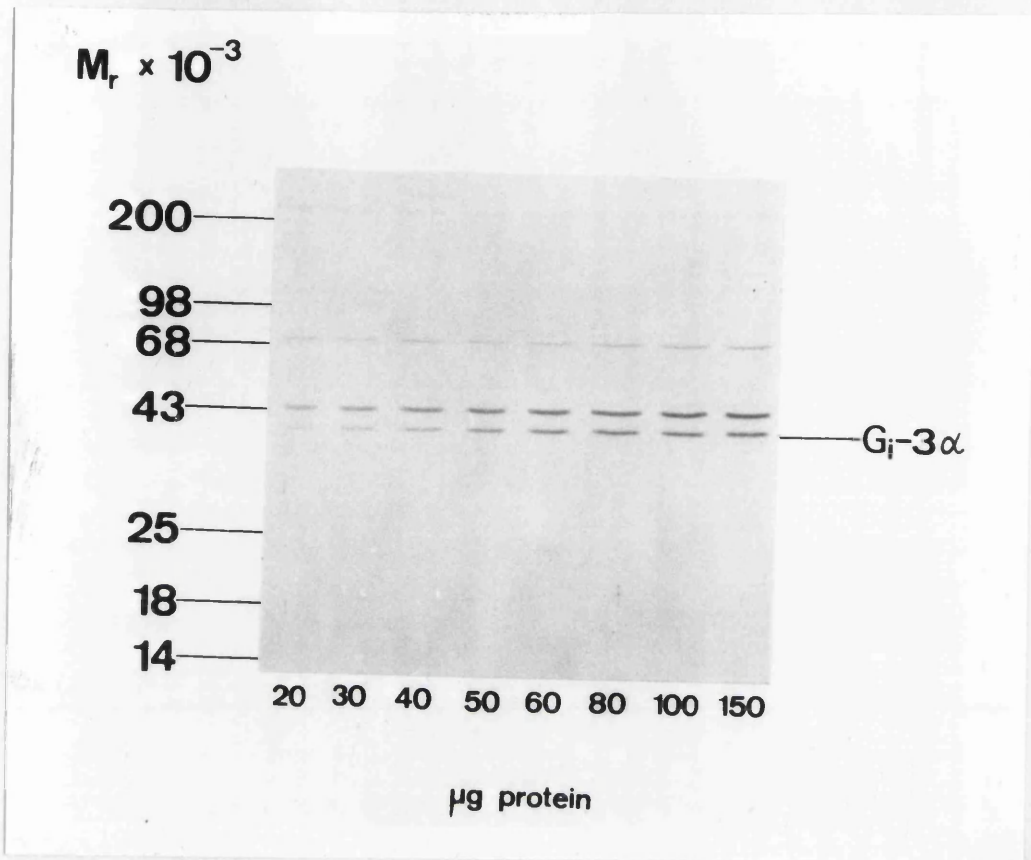
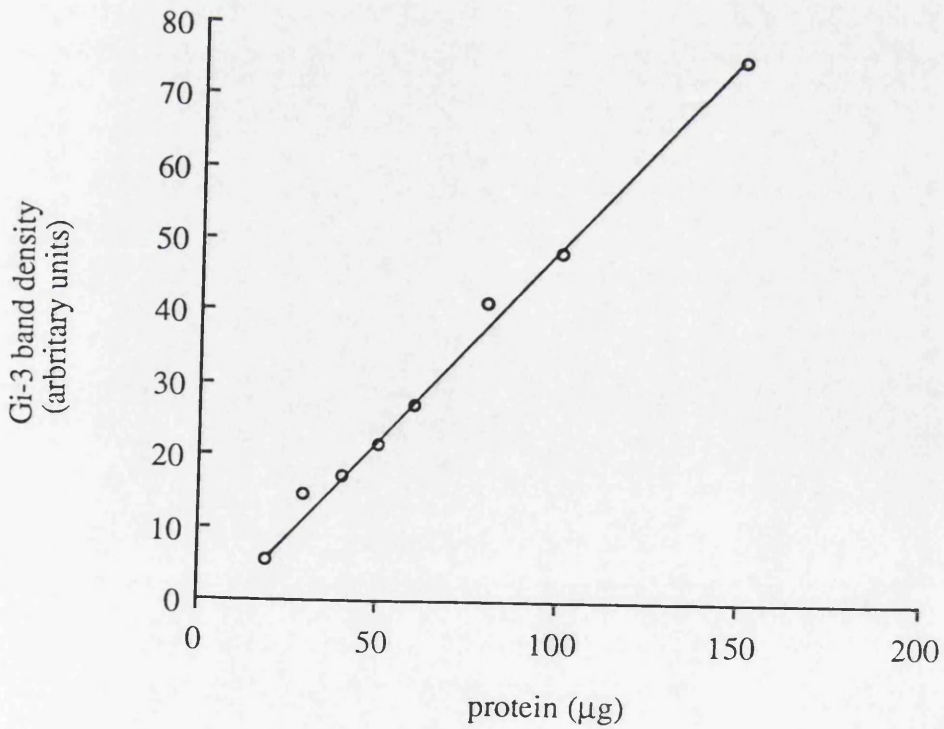


Figure 3.10 (b)



**Fig. 3.11      Immunological identification of G<sub>s</sub>- $\alpha$  in P9 cell plasma membranes.**

Samples of P9 cell plasma membrane protein (20 $\mu$ g to 200 $\mu$ g) were TCA precipitated, resolved and transblotted as described in the legend to Figure 3.9 then G<sub>s</sub>- $\alpha$  detected using a 1:200 dilution of the antiserum CS1 and horseradish peroxidase-linked IgG as second antibody (Fig. 3.11a). Bands were scanned densitometrically and results plotted graphically (Fig 3.11b). Data shown are from one experiment. Similar results were obtained in another experiment.



Figure 3.11 (a)

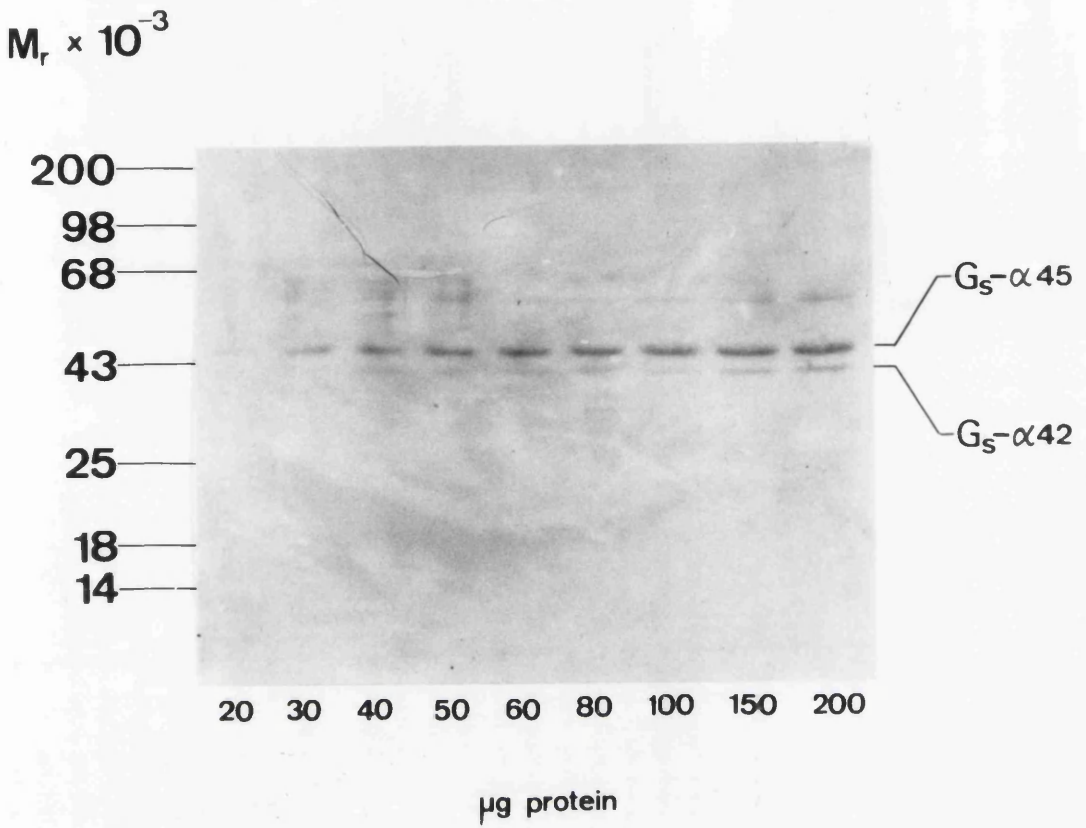
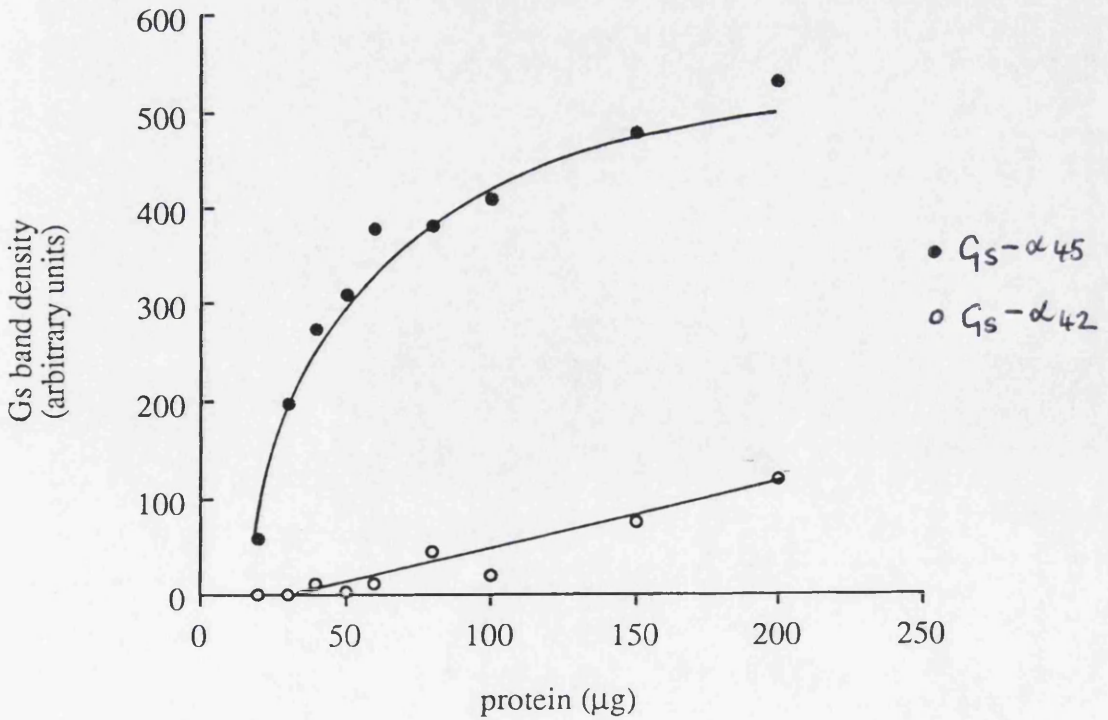


Figure 3.11 (b)



**Fig. 3.12      Immunological identification of G-protein  $\beta$   
subunit in P9 cell plasma membranes.**

Samples of P9 cell plasma membrane protein (20 $\mu$ g to 200 $\mu$ g) were TCA precipitated, resolved and transblotted as described in the legend to Fig. 3.9 then G-protein  $\beta$  subunit detected using a 1: 200 dilution of the antiserum BN3 and horseradish peroxidase-linked IgG as second antibody (Fig. 3.12a). Bands were scanned densitometrically and results plotted graphically (Fig. 3.12b). Data shown are from one experiment. Similar results were obtained in another experiment.

Figure 3.12 (a)

$M_r \times 10^{-3}$

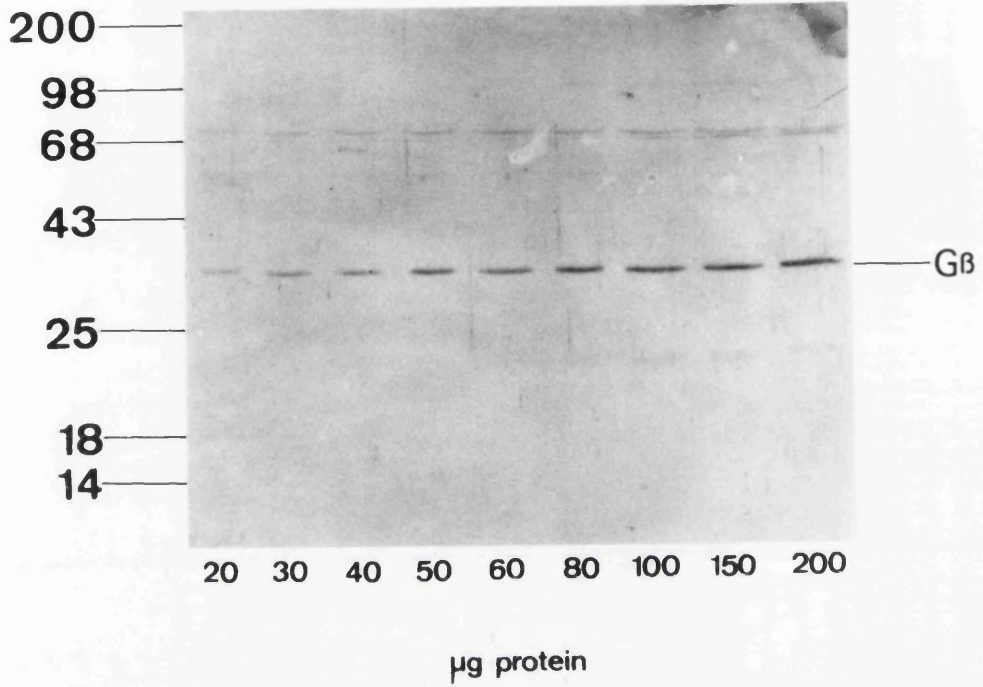
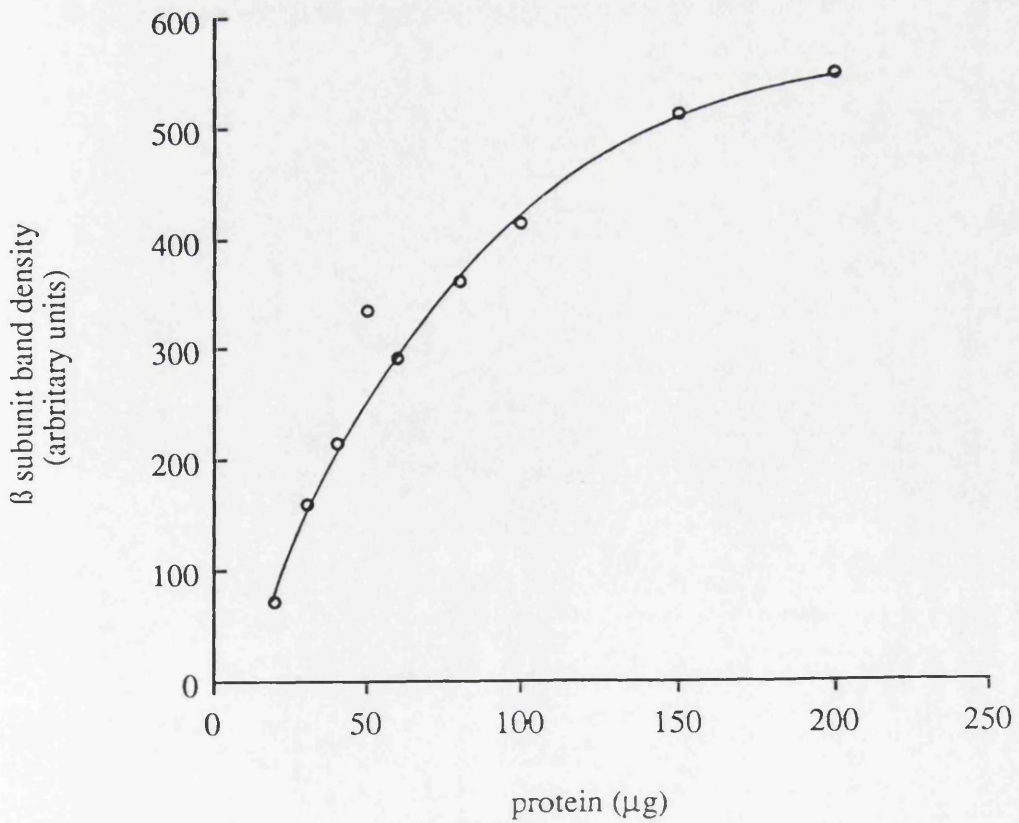


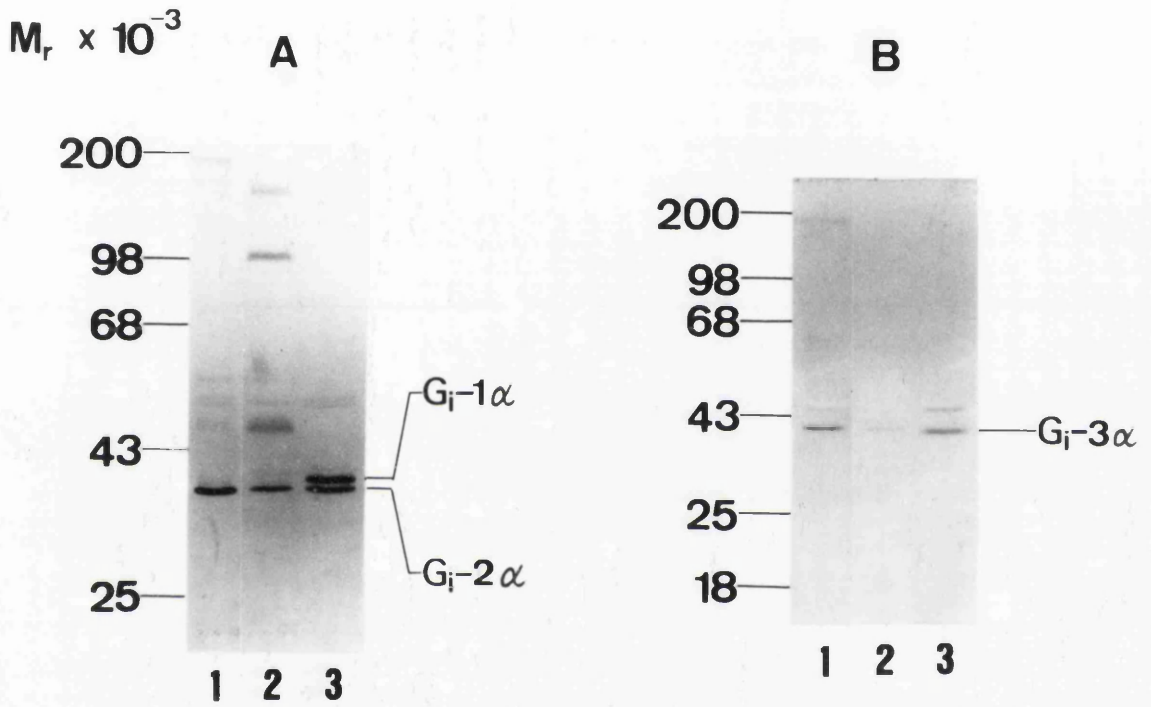
Figure 3.12 (b)



**Fig. 3.13**      **Expression of Gi- $\alpha$  subtypes in P9 cells and rat tissues.**

Samples of membrane protein (100 $\mu$ g) from P9 cells (lane 1) , rat hepatocytes (lane 2) and rat brain (lane 3) were resolved using SDS PAGE and immunoblotted to compare quantitatively G-protein levels in plasma membranes from the respective tissues. In panel A, samples were resolved using a 12.5% acrylamide/0.0625% bisacrylamide resolving gel as described in section 2.10a. In panel B samples were resolved using a 10% acrylamide, 0.27% bisacrylamide resolving gel. Thereafter, samples were immunoblotted and proteins detected using the antisera: 1432 (panel A) and I3C (panel B) at the same dilutions as given in Figs. 3.9 and 3.10. The primary antisera were detected and bands scanned densitometrically as in Fig. 3.10. Densitometric data are presented in Table 3.4. Data shown are from one experiment. Similar results were obtained in two other experiments.

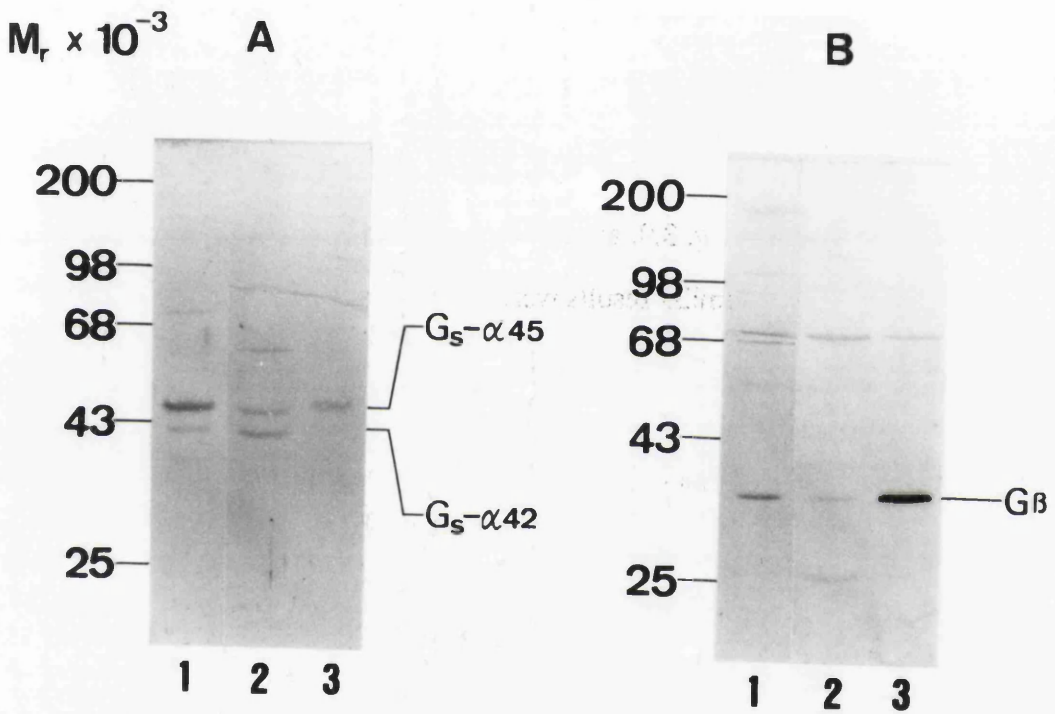
**Figure 3.13**



**Fig. 3.14      Expression of G<sub>s</sub>- $\alpha$  forms and G-protein  $\beta$  subunit in P9 cells and rat tissues.**

Samples of membrane protein (100 $\mu$ g) from P9 cells (lane 1), rat hepatocytes (lane 2) and rat brain (lane 3) were resolved using SDS PAGE and immunoblotted in order to quantitatively compare levels of G<sub>s</sub>- $\alpha$  and G $\beta$  in the respective tissues. In both panels samples were resolved using a 10% acrylamide/0.27% bisacrylamide resolving gel. Thereafter proteins were detected using the antisera CS1 (panel A) and BN3 (panel B) at the same dilutions as in Figures 3.11 and 3.12. The primary antisera were detected and bands scanned densitometrically as in Fig. 3.9. and data presented in Table 3.4. Data shown are from one experiment. Similar results were obtained in two other experiments.

**Figure 3.14**



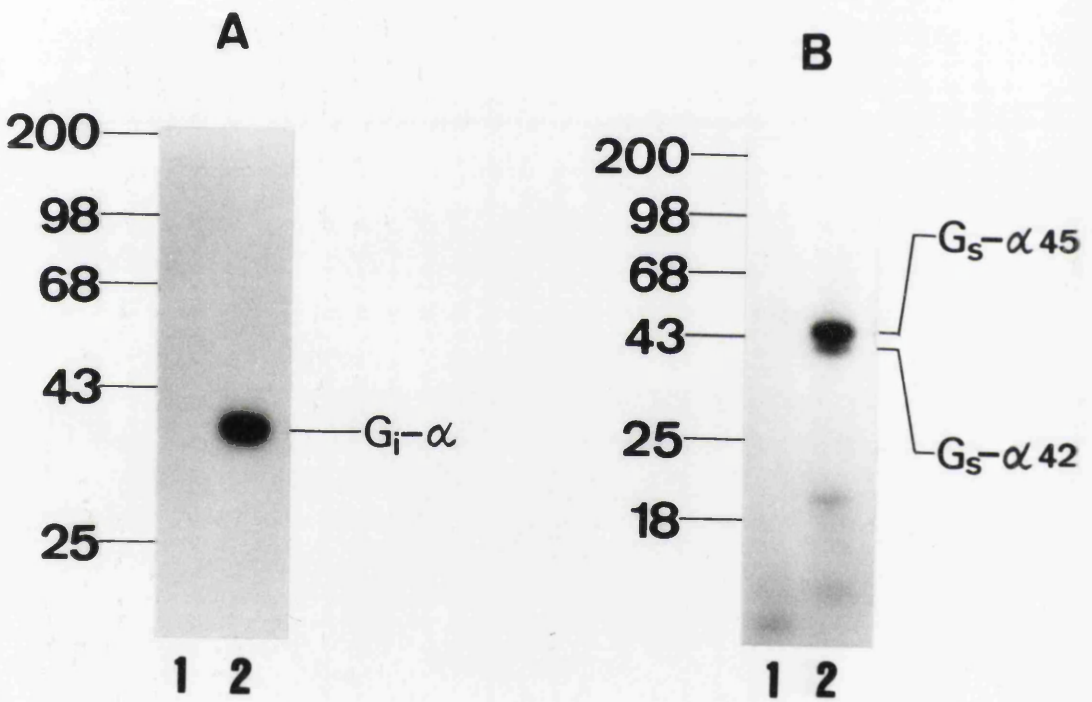
**Fig. 3.15      Toxin catalysed ADP-ribosylation of P9 cell plasma membranes.**

Toxin substrates expressed by P9 cells were examined. Plasma membranes from P9 cells (lane 2) were ADP-ribosylated using thiol pre-activated pertussis toxin (panel A) and cholera toxin (panel B) and [<sup>32</sup>P]-NAD<sup>+</sup> as described in section 2.12. P9 cell plasma membranes were also subjected to the same treatment in the absence of toxin (lane 1). Samples were resolved using SDS PAGE (10% acrylamide/0.27% bisacrylamide) as detailed in section 2.10. Data shown are from one experiment. Similar results were obtained in two other experiments.



**Figure 3.15**

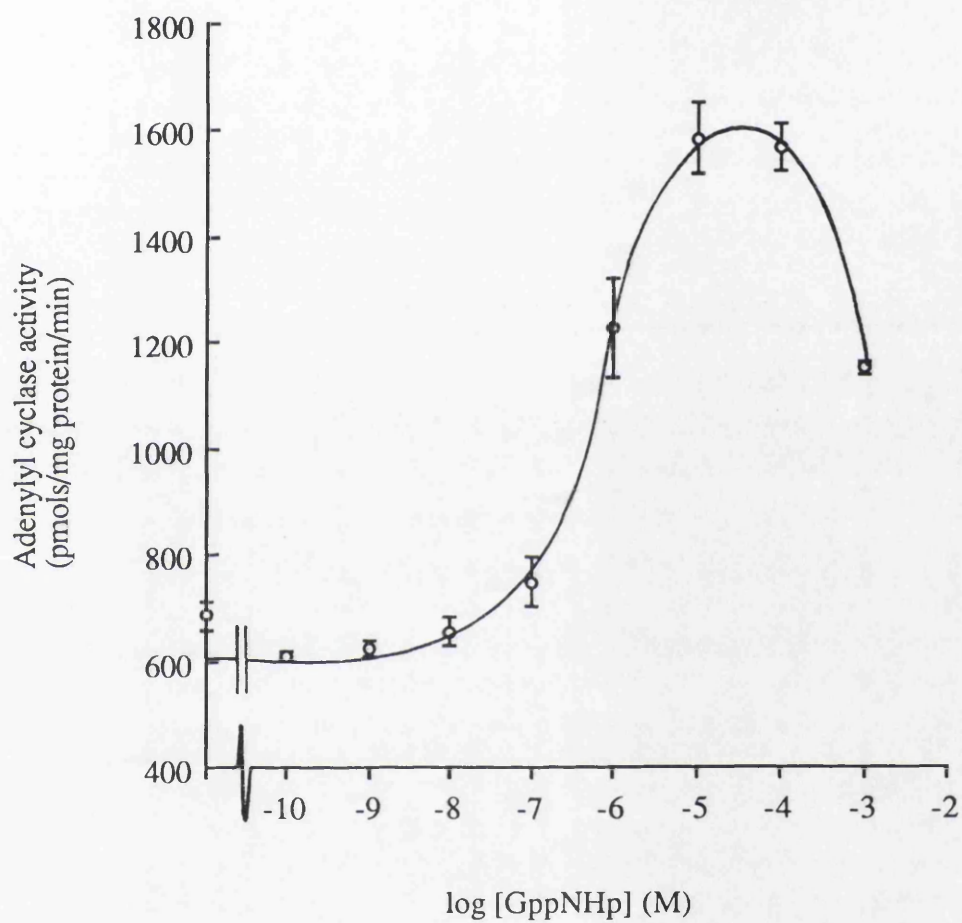
$M_r \times 10^{-3}$



**Fig. 3.16**      **Effect of increasing concentrations of GppNHp on forskolin-stimulated adenylyl cyclase activity in P9 cell plasma membranes.**

P9 cell plasma membranes incubated with forskolin ( $10^{-4}\text{M}$ ) and increasing concentrations of GppNHp ( $10^{-10}\text{M}$  to  $10^{-3}\text{M}$ ) were assayed for adenylyl cyclase activity as described in section 2.13. The maximum response to GppNHp ( at  $10^{-5}\text{M}$ ) was  $231\pm 10\%$  of that to forskolin alone. There was no significant inhibition of the response to forskolin by  $10^{-10}\text{M}$  GppNHp. The  $K_{0.5}$  of GppNHp for enhancement of the forskolin response by GppNHp was  $6.2 \times 10^{-7}\text{M}$ . Data are mean $\pm$ SEM for triplicate observations obtained in one experiment. Similar results were obtained in two other experiments.

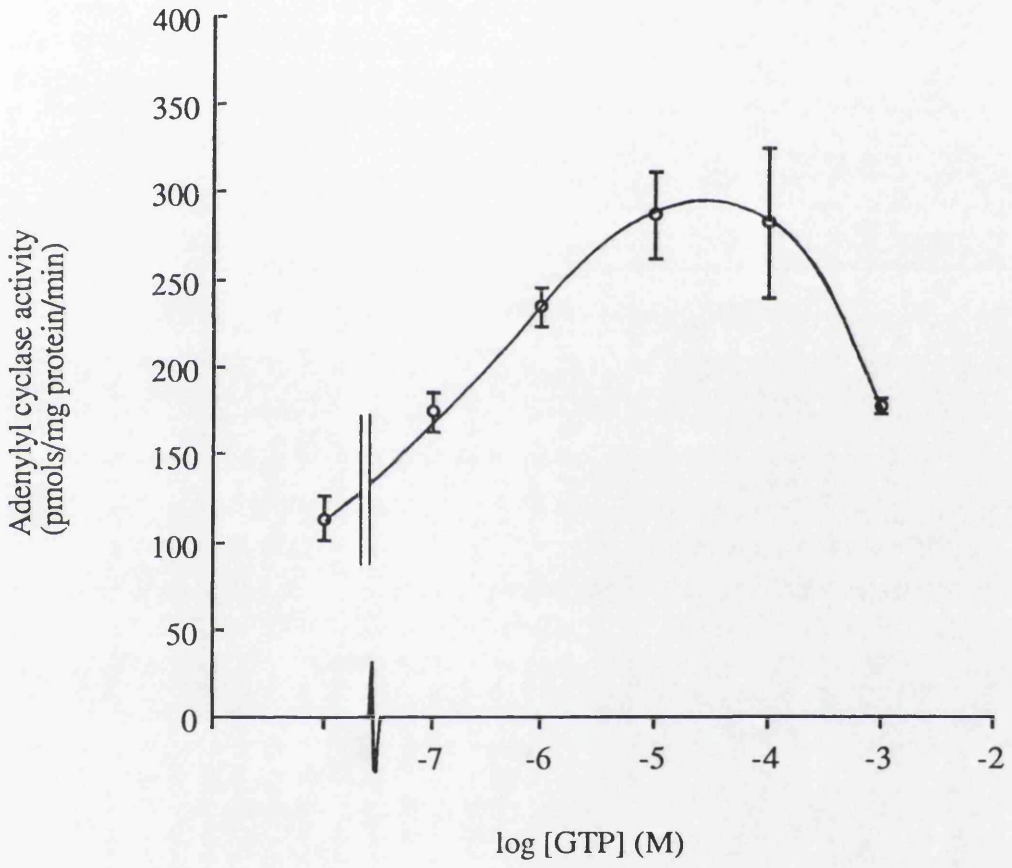
**Figure 3.16**



**Fig. 3.17      Effect of increasing concentrations of GTP on adenylyl cyclase activity in P9 cell plasma membranes.**

P9 cell plasma membranes incubated with increasing concentrations of GTP ( $10^{-7}\text{M}$ - $10^{-3}\text{M}$ ) were assayed for adenylyl cyclase activity as described in section 2.13. The maximum response to GTP (at  $10^{-5}\text{M}$ ) was  $253\pm 22\%$  of the basal adenylyl cyclase activity. The  $K_{0.5}$  for GTP for stimulation of adenylyl cyclase was  $2.7 \times 10^{-7}\text{M}$ . Inhibition of the optimal response by  $10^{-3}\text{M}$  GTP was  $38\pm 6\%$  (CV) Data are mean $\pm$ SEM for triplicate observations obtained in one experiment. Similar results were obtained in two other experiments.

**Figure 3.17**

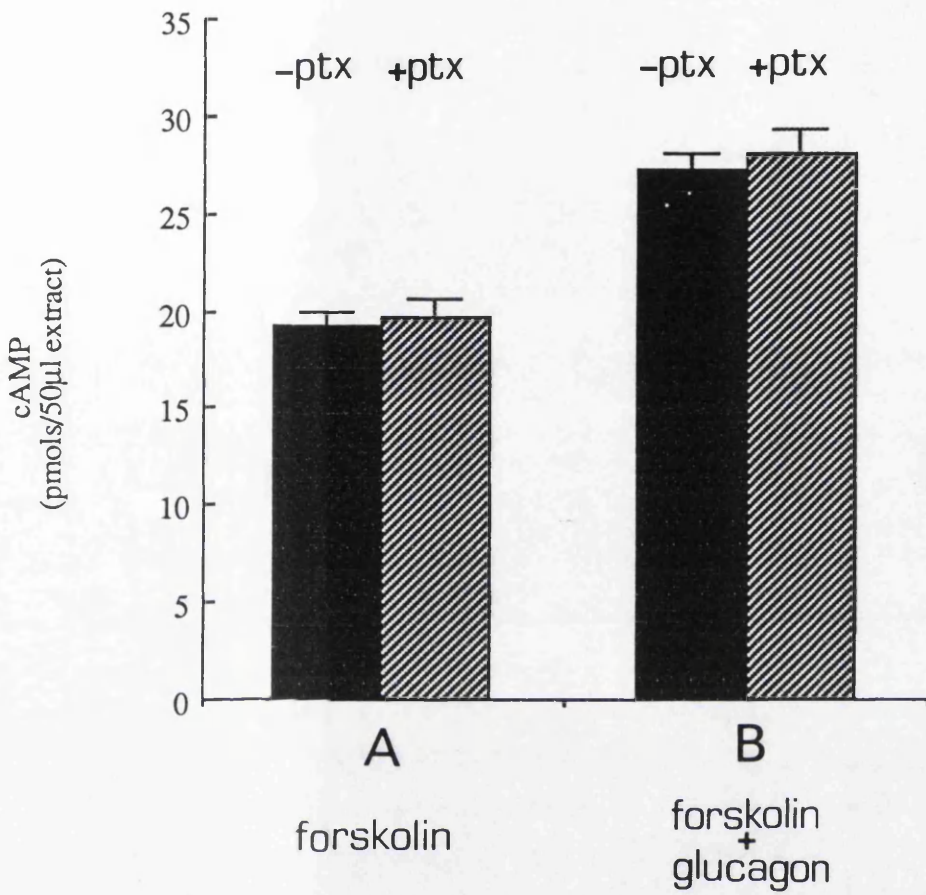


**Fig. 3.18**      **Effect of pertussis toxin pretreatment on glucagon response and toxin labelling in P9 cells.**

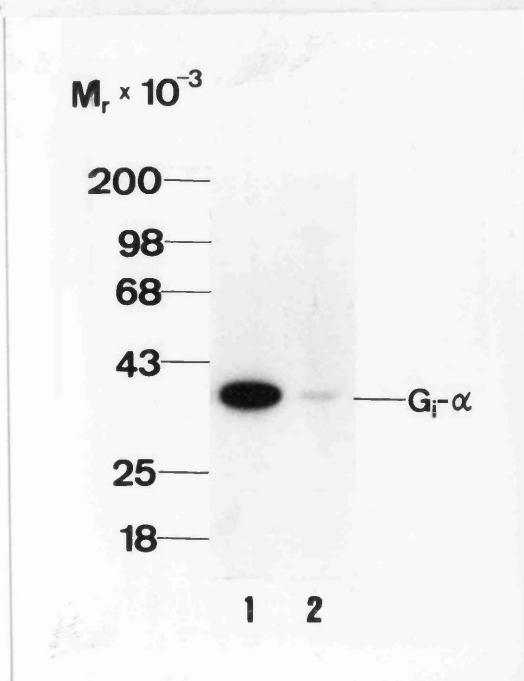
In Fig. 3.18a. confluent P9 cells were exposed to serum-free medium for 16 hours alone, (black bars) or with pertussis toxin (100ng/ml) present during the final 2 hours of the incubation (hatched bars). Cells were then challenged with forskolin ( $10^{-5}\text{M}$ ) (column 1) or forskolin ( $10^{-5}\text{M}$ ) combined with glucagon ( $10^{-8}\text{M}$ ) (column 2), for 2 minutes in each case. Cyclic AMP was then extracted and assayed in section 2.14. Glucagon responses were significant in each case, but there was no significant difference in the presence of pertussis toxin. Data are mean  $\pm$  SEM for triplicate observations obtained in four experiments.

In Fig. 3.18b. P9 cells exposed to serum-free medium alone, (lane 1) or containing pertussis toxin as in (A) during the final 2 hours of exposure, (lane 2) were harvested, membranes prepared and ADP-ribosylated using pertussis toxin and [ $^{32}\text{P}$ ]-NAD<sup>+</sup> as described in section 2.12. Pertussis toxin treatment in this manner ADP-ribosylated 92% of the substrate available. Data shown are from one experiment. Similar results were obtained in two other experiments.

**Figure 3.18 (a)**



**Figure 3.18 (b)**

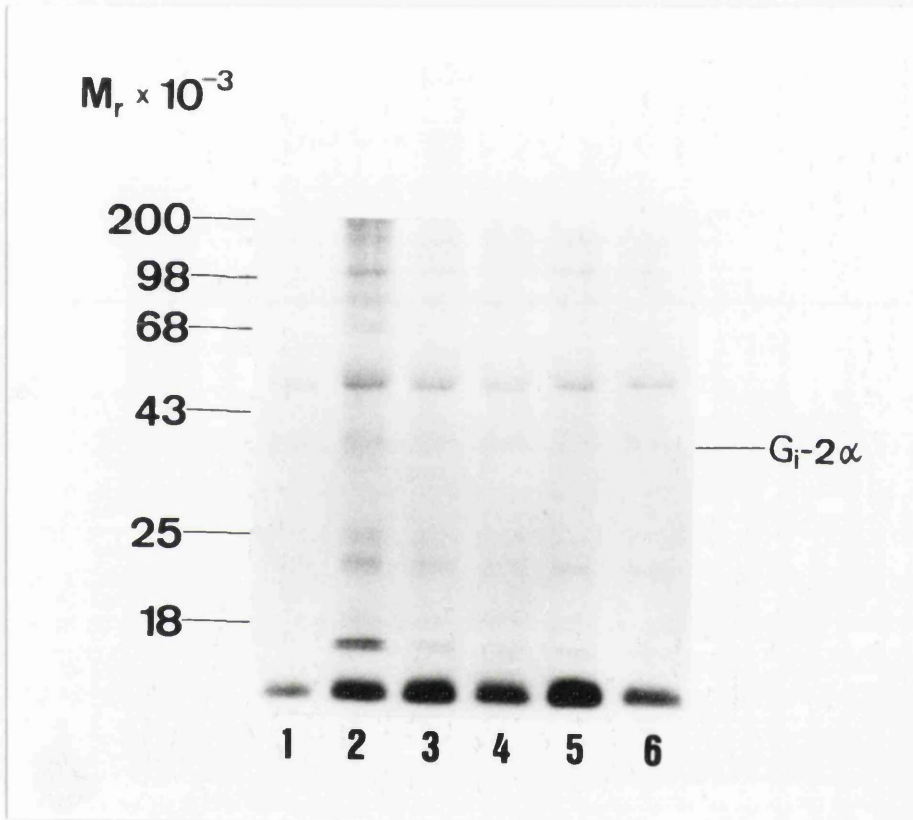


**Fig. 3.19      Phosphorylation of Gi-2 $\alpha$  in intact P9 cells.**

Confluent P9 cells were pre-incubated with [ $^{32}$ P]Pi for 3 hours then challenged for 15 minutes with no agonist (lane 1), TPA, 10ng/ml (lane 2), TPA, 10ng/ml and okadaic acid,  $10^{-6}$ M (lane 3), 8-bromo-cAMP, 300 $\mu$ M (lane 4), 8-bromo-cAMP, 300 $\mu$ M and okadaic acid,  $10^{-6}$ M (lane 5) and okadaic acid,  $10^{-6}$ M alone (lane 6). Gi-2 $\alpha$  was immunoprecipitated from the solubilised cell extract using the 1432 antiserum, as described in section 2.17. Samples were resolved by SDS PAGE and autoradiography performed as described in section 2.10. Results shown are from one experiment. Similar results were obtained in another experiment using 8-bromo-cAMP and TPA alone.



**Figure 3.19**



## **Chapter 4**

### **Low affinity effects of insulin on adenylyl cyclase signalling in P9 cells**

## **4.1 Introduction**

As discussed in Chapter 3, the initial aim of exposing confluent P9 cells to a high concentration of insulin ( $0.5\mu\text{M}$  for 16 hours) was to observe whether it could promote any morphological differentiation of the cells or restore glucagon responsiveness. Whilst it had neither of these effects, insulin was found to have low affinity effects on cyclase signalling. The aim of this chapter then was to characterise these effects and to observe whether changes in signalling in P9 cells on insulin exposure resemble those in other systems.

## 4.2 Results

Exposure of P9 cells to a high concentration of insulin (0.5 $\mu$ M) for 16 hours was found to have pronounced effects on the responses to stimulatory agonists in plasma membranes derived from these cells (Table 4.1 and Figs. 4.1-4.3). The responses to PGE<sub>1</sub> and GppNHp were markedly enhanced as was the response to forskolin, although there was no significant increase in basal adenylyl cyclase activity. For PGE<sub>1</sub> and GppNHp there was a slight reduction in the concentration of agonist required to give a half maximal response but with forskolin there was no left shift of the dose response curve. An examination of the time course for the effect on the forskolin response, showed that increased cyclase activity was visible two hours and maximal 16 hours after insulin exposure, typical for a growth related effect of insulin (Fig. 4.4).

Insulin and IGF-1 both caused a small stimulation of [<sup>3</sup>H]-Thymidine uptake into P9 cells at concentrations at which these agonists would be expected to act as growth factors (Fig. 4.5). In addition, 0.5 $\mu$ M insulin markedly enhanced the ability of stimulatory agonists to elevate intracellular cAMP as shown for PGE<sub>1</sub> in Fig. 4.6. The basal cAMP level was not significantly altered. Dose dependence for insulin of this increased intact cell responsiveness is shown for PGE<sub>1</sub> (Fig. 4.7) and forskolin (Fig. 4.8). The low affinity stimulatory effect of insulin on both responses paralleled dose dependence on insulin for stimulation of [<sup>3</sup>H]-Thymidine uptake and both were blocked by concurrent exposure to the protein synthesis inhibitor cycloheximide (1 $\mu$ M) (Fig. 4.9). The fold enhancement by 0.5 $\mu$ M insulin of stimulatory agonist responses was considerably larger than the stimulation of [<sup>3</sup>H]-Thymidine uptake by the same insulin concentration. This experiment showed that approximately 18% of the enhanced cAMP accumulation was due to increased cell number and the remainder to increased cAMP generation by plasma membrane adenylyl cyclase. On examining dose dependence for insulin it was noted that insulin at low concentrations impaired cAMP accumulation in

response to PGE<sub>1</sub> and enhanced cAMP accumulation in response to forskolin. These high affinity effects are discussed in Chapter 5.

Membranes from insulin-exposed P9 cells were subjected to toxin labelling with pertussis toxin and cholera toxin (Fig. 4.10 and Table 4.2) and G-proteins quantitated by immunoblotting using specific antisera (Fig. 4.11 and Table 4.2). After insulin exposure there was a considerable reduction in labelling of toxin substrates. Labelling with cholera toxin of both 45kDa and 42kDa G<sub>s</sub>- $\alpha$  splice variants was reduced and a reduction in labelling of the 'Gi' band with pertussis toxin. This change in labelling was not the result of a reduction in G-protein levels, as no changes in G-protein  $\alpha$  or  $\beta$  subunit levels were observed on immunoblotting.

The low affinity effect of insulin on adenylyl cyclase functioning was considerably variable in magnitude. Presumably this reflects differences in expression of IGF-1/insulin receptors or other proteins required for insulin responsiveness between batches of cells. There was no obvious relationship between insulin responsiveness in this manner and passage number. Again the possibility is raised of variability between cells as a result of the growth factor content and age of the serum which will not be the same on every occasion. Receptor number has been shown to vary widely in cells, depending on the batch of foetal calf serum used (Devedjian *et al.*, 1991). Alternatively, the variability may arise from the state of the cells at the time of insulin exposure. Nakamura *et al.* (1983) showed in studies on cultured hepatocytes that induction of DNA synthesis by insulin took place after a time lag, the duration of which was dependent on cell density. At a high cell density near confluence, induction of DNA synthesis required a longer exposure time. The situation may be similar in P9 cells where the precise cell density could influence insulin's effect on cell growth and plasma membrane adenylyl cyclase activity.

### 4.3 Discussion

In this section, the ability of insulin (at a high concentration over a long time period) to modulate P9 cell adenylyl cyclase signalling was investigated. Increased intracellular cAMP accumulation in response to PGE<sub>1</sub> was shown to be associated with increased stimulatory responses of plasma membrane adenylyl cyclase. The long time course for the effect and its abolition by cycloheximide in intact cells suggest that protein synthesis underlies the effect. An influence on cAMP phosphodiesterase activity is not implicated in this action of insulin, as the non-specific phosphodiesterase inhibitor IBMX was present routinely in the adenylyl cyclase assay buffer.

As forskolin at high concentrations is thought to act directly on the adenylyl cyclase catalytic subunit, (ie. more distally in the signalling system than PGE<sub>1</sub> and GppNHp), this suggests that insulin was having a positive effect at the catalytic subunit of adenylyl cyclase, possibly by increasing its quantity in the membranes, disproportionately over other membrane proteins. This could be further investigated by immunological quantitation of the catalytic subunit when antisera become available, or by measuring binding of radiolabelled forskolin to the membranes.

Apart from increasing the quantity of effector in plasma membranes, there are other possible ways in which insulin might bring about functional cyclase activation which should be considered. Firstly, sensitisation of adenylyl cyclase to stimulatory agonists has been noted under conditions where PK-C is active. Phorbol esters, which activate PK-C, sensitised intact human astrocytoma cells to cAMP-elevating agonists (Johnson and Toews, 1990) and Summers and Cronin (1986) found that phorbol ester treatment of a pituitary cell line enhanced forskolin- and PGE<sub>1</sub>-stimulated cAMP accumulation and adenylyl cyclase activity. Simmoteit *et al.* (1991) attributed the increase in adenylyl cyclase activity in the presence of phorbol esters to

phosphorylation of the adenylyl cyclase catalytic subunit. In a recent report Jacobowitz *et al.* (1993) showed that specific adenylyl cyclase subtypes *viz.* AC-I, -II and -III are targets for PK-C activation. Insulin itself has been suggested to activate PK-C in some cell types (Houslay, 1991b) and, if doing so in P9 cells, could potentially account for the cyclase sensitisation observed here. However, if a phosphorylation event were responsible for the sensitisation, we would have expected a shorter time course for the effect, unless expression of the kinase itself was induced by insulin.

Insulin sensitisation to adenylyl cyclase-activating hormones has also been observed in a rat sarcoma cell line (Hickman and McElduff, 1990). These authors proposed that a lessening of  $G_i$  input was responsible in their system. It seems unlikely that this could apply to P9 cells, as we have little evidence of  $G_i$  input into cyclase activity in P9 cells.

It has recently become clear that certain adenylyl cyclase subtypes are subject to activation following receptor-mediated  $\beta\gamma$  release from G-proteins. AC-II and -IV are known to be regulated in this manner (Gao and Gilman, 1991) and AC-IV is expressed in liver (Kelley Bentley and Beavo, 1992). Such a mechanism has been proposed to explain why hormones not signalling directly through adenylyl cyclase can potentiate those that do. We would expect such an effect to be most prominent at high concentrations of stimulatory agonist and not observable in absence of agonist, *ie.* on basal cyclase activity or intracellular cAMP concentrations which was the result observed. Insulin, if it were acting in the cells to promote G-protein dissociation, could potentiate cyclase activation in this manner. Evidence that insulin exposure of P9 cells may indeed promote dissociation of  $G_i$ , with release of  $\beta\gamma$  components, was provided by the reduction in pertussis-toxin labelling of  $G_i$  observed after insulin exposure, implying a reduction in proportion of the undissociated holomer. The released  $\beta\gamma$  components would presumably be available to interact with effectors such as adenylyl

cyclase. However, without more detailed investigation we cannot conclude to what extent, if any, these various mechanisms contribute to insulin's sensitising effect.

In addition to its positive effect on maximal stimulatory responses observed for all three agonists the increased activation of adenylyl cyclase by lower concentrations of PGE<sub>1</sub> and GppNHp after insulin exposure suggested an additional action of insulin at a more proximal site in the system. An alteration in the number or affinity of prostanoid receptors could explain the change in PGE<sub>1</sub> response. Indeed it would be reasonable to suggest that insulin might positively affect receptor expression given its effects on differentiation noted in other cell types and sensitisation to PGE<sub>1</sub> action in platelets (Kahn and Sinha, 1990). An increase in PGE<sub>1</sub> binding affinity however, would not explain the higher affinity response to GppNHp whose activation of cyclase is independent of the receptor. This response suggested a positive effect of insulin at the level of the G-protein. This possibility is considered below.

The reduction in cholera toxin-labelling of G<sub>s</sub>- $\alpha$  after insulin exposure may suggest a decrease in the proportion of dissociated, GTP-bound G<sub>s</sub>- $\alpha$  as this is the form which is the substrate for cholera toxin (Northup *et al.*, 1980). As discussed in section 1.4.2 insulin has been proposed to influence guanine nucleotide binding by a functional interaction with the G-protein system. Whilst insulin by means of such an interaction with G<sub>s</sub> could potentially influence the amount of GTP binding and thence the dissociation equilibrium of G<sub>s</sub>, we would have expected to observe a decrease rather than an increase in adenylyl cyclase activity, as dissociated G<sub>s</sub>- $\alpha$  is the active form. If insulin is indeed having a negative effect at this proximal site, its influence must be outweighed by the considerable stimulatory effect of the hormone at the catalytic subunit of adenylyl cyclase. This explanation for the reduction in cholera toxin labelling of G<sub>s</sub>- $\alpha$  does not however explain the observed decrease in K<sub>0.5</sub> for GppNHp which implies a positive rather than a negative effect of insulin on agonist activation of



cyclase. An alternative explanation to accommodate this finding is that the reduction in cholera toxin labelling may not reflect any influence of insulin on the  $G_s$  dissociation equilibrium, but rather a covalent modification or conformational change in  $G_s$  in such a manner as to enhance the activity of the protein favouring GppNHp stimulation of the system and additionally rendering it less susceptible to toxin action.

$G_i$ - $\alpha$  was also a poorer substrate for pertussis toxin after insulin exposure though we cannot say to what extent the reduced labelling was due to functional changes in specific polypeptides as  $G_i$ -2 $\alpha$  and  $G_i$ -3 $\alpha$ , the two  $G_i$  subtypes expressed in P9 cells are not resolved by the gel system used. If the dissociation state of the G-protein is responsible for the change in labelling (Rothenberg and Kahn, 1988) this implies, as mentioned above, that there is less  $G_i$  in the trimeric (inactive) form, as the holomer is the toxin substrate. This is certainly consistent with the higher adenylyl cyclase activity after insulin exposure though it appears from the data presented in Chapter 3, that a reduction in  $G_i$  input would have little consequence for stimulatory cyclase functioning in P9 cells. Sensitisation to stimulatory agonists after insulin exposure of P9 cells cannot then, as has been proposed for hepatocytes from diabetic animals (Bushfield *et al.*, 1990b), be due to removal of tonic  $G_i$  input into the catalytic subunit of adenylyl cyclase. As discussed above for  $G_s$ , the reduction in toxin labelling of  $G_i$  may be caused by mechanisms other than an alteration in the dissociation equilibrium of the G-protein.

Whatever the mechanism of insulin's action in influencing toxin labelling, it speaks for a functional interaction between insulin signalling and the G-protein system either directly, as discussed in section 1.5 through interaction with the insulin or IGF-1 receptor, or indirectly, as a consequence of increased cell growth. One could speculate that a similar mechanism may underlie the impairment of toxin labelling observed in diabetic states (section 1.5.2.1). This could be further investigated by carrying out

studies like those of Rothenberg and Kahn (1988), to determine whether insulin can directly effect toxin labelling in isolated P9 cell plasma membranes. It seems likely that this reduction in toxin labelling might have consequences for cyclase functioning and be at least partly responsible for the functional changes observed here in plasma membrane adenylyl cyclase activity.

From the measurement of [<sup>3</sup>H]-Thymidine uptake it is clear that both insulin and IGF-1 are at best poor mitogens in P9 cells. This means that that increased plasma membrane adenylyl cyclase activity rather than cell growth must be responsible for most of insulin's low affinity effect in enhancing whole cell cAMP responses to stimulatory agonists. P9 cells must express at least the IGF-1 receptor, as IGF-1 has less than 1% of the potency of insulin at the insulin receptor (Baxter and Williams, 1983) but gives a similar response to insulin here. Insulin is recognised to exert low affinity effects on cell growth through the IGF-1 receptor (Florini *et al.*, 1991) and this is in line with its ability to cause maximal growth stimulation at supraphysiological concentrations, between 20nM and 1µM. Insulin then may be acting here at low affinity through the IGF-1 receptor but could in addition be acting through its own receptor. In a few cases insulin has been reported to increase [<sup>3</sup>H]-Thymidine incorporation into DNA by acting through the high affinity insulin receptor (Koontz and Iwahashi, 1981, Massague *et al.*, 1982, Taub *et al.*, 1987). However, this was clearly not the case in P9 cells where supraphysiological concentrations were required for even a small stimulation of [<sup>3</sup>H]-Thymidine incorporation. The situation in P9 cells then, resembles that in fibroblasts, where insulin under normal conditions is a weak mitogen (Burgering *et al.*, 1991), as fibroblasts contain a relatively small number of insulin receptors as compared to receptors for IGF-1 (Rechler and Nissley, 1985). Evidence of higher affinity insulin responsiveness and therefore expression of insulin receptors in P9 cells is given in Chapter 5.

Given the low affinity nature of this insulin response, it may represent a response signalling growth through the IGF-1 receptor. An important factor to bear in mind here is that all the cells used for experiment were serum-starved in order to remove the influence of other growth factors and quiesce the cells prior to agonist stimulation. Control cells exposed to serum-free medium alone would already be suffering from an absence of growth factors for 16 hours at the time of experiment whilst the insulin-exposed cells would be facing a more normal situation, viz. the presence of a growth factor. It might be more appropriate then to consider these data in terms of response down-regulation on serum deprivation, rather than up-regulation on growth factor exposure. Adenylyl cyclase and receptor expression may depend on the presence of serum. Indeed, serum growth factors are probably required for maintaining second messenger system integrity and integrity of the cells in general as the cells fail to adhere or grow if subcultured into serum-free medium. Insulin then, may exert a tonic control on cyclase expression (whose effect is noted only by its absence) rather than a positive effect in its own right. One could speculate that the removal of this tonic control, whether artificially as here or in disease states, may cause defects to arise in the second messenger system. From this argument it is clear that it is difficult to decide in what state, with respect to serum exposure, the cells are best studied.

This suggestion is in line with the findings of Johnson *et al.* (1990), who noted that exposing human astrocytoma (1321N1) cells to serum caused sensitisation of cAMP accumulation in response to the agonists forskolin, isoproterenol and PGE<sub>1</sub>. The sensitisation was dose-dependent, reversible on serum removal and resulted from an increase in the maximum, rather than from a change in potency in the agonists. The authors were able to demonstrate the effect on membranes derived from the cells and concluded that it was due to an increase in the activity of the adenylyl cyclase catalytic subunit. Interestingly, the PK-C inhibitor staurosporine inhibited this serum-induced sensitisation and down-regulation of PK-C abolished the ability of serum to

subsequently induce sensitisation. The authors were however, unable to identify the serum component responsible for sensitisation. Serum-induced sensitisation of adenylyl cyclase has been noted in a number of other cell types including human lymphoma cells, rat osteosarcoma cells and rat glioma cells (Toews and Arneson-Rotert, 1990). A component of blood plasma increased hormone stimulated adenylyl cyclase activity in human mononuclear leucocytes (Motulsky *et al.*, 1987). This phenomenon may therefore be widespread and it will be interesting to discover whether, in line with the work done here on P9 cells, insulin turns out to be a factor responsible for sensitisation.

From this work it is clear that large changes in adenylyl cyclase signalling can occur after insulin exposure and in the absence of any change in G-protein expression. This is interesting in view of the many G-protein defects observed in animal models of diabetes where there is either a lack of insulin or insulin resistance and insulin itself presumably has some influence on G-protein expression and function. It is likely that insulin itself cannot influence genes for these proteins in P9 cells. Presumably their levels are inextricably linked to the level of cell differentiation which is uninfluenced by insulin, as discussed in Chapter 3.

**Table 4.1      Agonist-stimulated adenylyl cyclase activity in plasma membranes from P9 cells exposed to insulin (0.5 $\mu$ M, 16hours).**

Confluent P9 cells were exposed to serum-free medium alone (-ins) or containing insulin, 0.5 $\mu$ M (+ins). After 16 hours medium was removed and cells harvested as described in section 2.6. Plasma membranes were prepared and assayed for agonist-stimulated adenylyl cyclase activity in order to determine maximum responses and  $K_{0.5}$  values. Stimulation parameters for the agonists were obtained from the dose response curves shown in Figs. 4.1 to 4.3. In each case results are mean $\pm$ SEM for triplicate observations obtained in one experiment. Similar results were obtained in another experiment in each case.

**Table 4.1**

Agonist	-ins		+ins	
	Maximum stimulation (pmols/mg/min)	K <sub>0.5</sub> (M)	Maximum stimulation (pmols/mg/min)	K <sub>0.5</sub> (M)
PGE <sub>1</sub>	129+/-29	9.4 x 10 <sup>-6</sup>	276+/-44	5.4 x 10 <sup>-6</sup>
GppNHp	97+/-4	2.2 x 10 <sup>-6</sup>	143+/-7.2	1.2 x 10 <sup>-6</sup>
Forskolin	153+/-29	2.4 x 10 <sup>-5</sup>	375+/-11	2.4 x 10 <sup>-5</sup>

**Table 4.2**      **Quantitative comparison of toxin labelling and G-protein levels in plasma membranes from P9 cells exposed to insulin (0.5 $\mu$ M, 16 hours)**

P9 cells were exposed to serum-free medium alone or containing insulin, 0.5 $\mu$ M for 16 hours, harvested and membranes prepared. Membranes were then ADP-ribosylated as described in section 2.12., using pertussis toxin and cholera toxin. Proteins were resolved using SDS PAGE and gels autoradiographed as described in section 2.10. G-protein levels were compared in these membranes as described in the legend to Fig. 4.11. All blots and autoradiographs were scanned densitometrically as described in section 2.10 and results from Figs 4.10 and 4.11 presented as the percentage alteration in level of the signal after insulin exposure. Data are mean $\pm$ SEM for three separate experiments.

**Table 4.2**

Insulin-induced change in  
toxin labelling (%)

Gi- $\alpha$	-73 $\pm$ 14
Gs- $\alpha$ (45kDa)	-56 $\pm$ 9
Gs- $\alpha$ (42kDa)	-58 $\pm$ 7

Insulin-induced change in  
G-protein level (%)

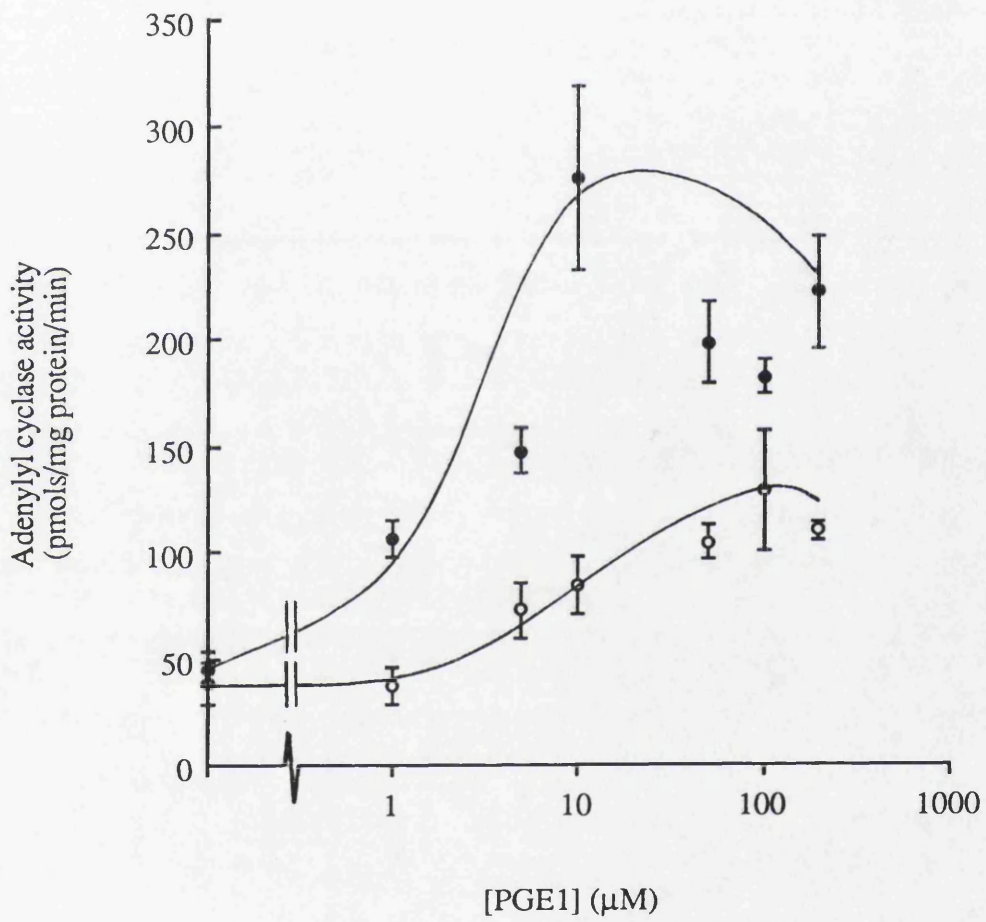
Gi-2 $\alpha$	+18 $\pm$ 15
Gi-3 $\alpha$	+29 $\pm$ 26
Gs- $\alpha$ (45kDa)	+10 $\pm$ 1
Gs- $\alpha$ (42kDa)	-6 $\pm$ 29
G $\beta$	-6 $\pm$ 6



**Fig. 4.1**      **Effect of increasing concentrations of PGE<sub>1</sub> on adenylyl cyclase activity in plasma membranes from control P9 cells and cells exposed to insulin (0.5μM, 16 hours).**

Confluent P9 cells were exposed to serum-free medium alone (open circles) or containing insulin, 0.5μM (closed circles). After 16 hours, medium was removed, the monolayer washed with buffer and cells harvested as described in section 2.6. Plasma membranes were prepared and assayed for agonist stimulated adenylyl cyclase activity in the presence of increasing concentrations of PGE<sub>1</sub> as detailed in section 2.13. Stimulation parameters are given in Table 4.1. Results are mean±SEM for triplicate observations obtained in one experiment. Similar results were obtained in another experiment.

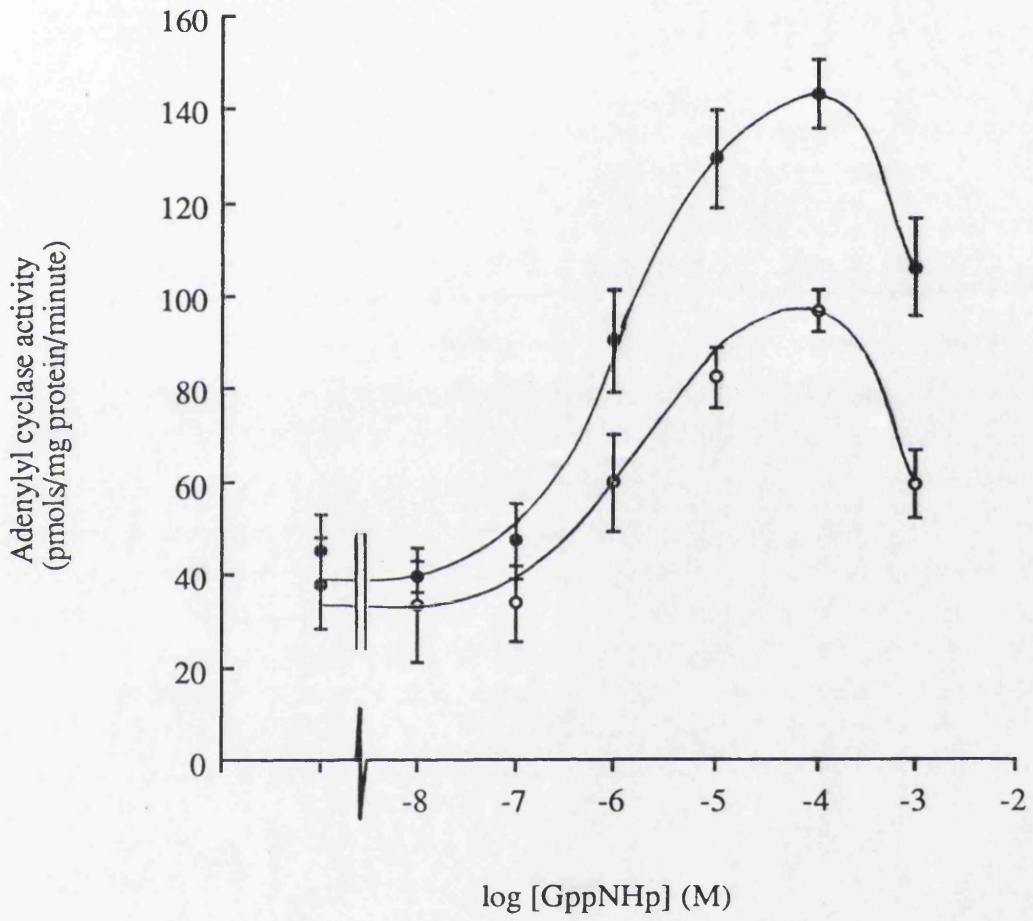
**Figure 4.1**



**Fig. 4.2**      **Effect of increasing concentrations of GppNHp on adenylyl cyclase activity in plasma membranes from control P9 cells and cells exposed to insulin (0.5 $\mu$ M, 16 hours).**

Plasma membranes from control P9 cells (open circles) and P9 cells exposed to insulin (0.5 $\mu$ M, 16 hours) (closed circles) as in the legend to Fig. 4.1 were assayed for adenylyl cyclase activity in the presence of increasing concentrations of GppNHp. Stimulation parameters are given in Table 4.1. Results are mean $\pm$ SEM for triplicate observations obtained in one experiment. Similar results were obtained in another experiment.

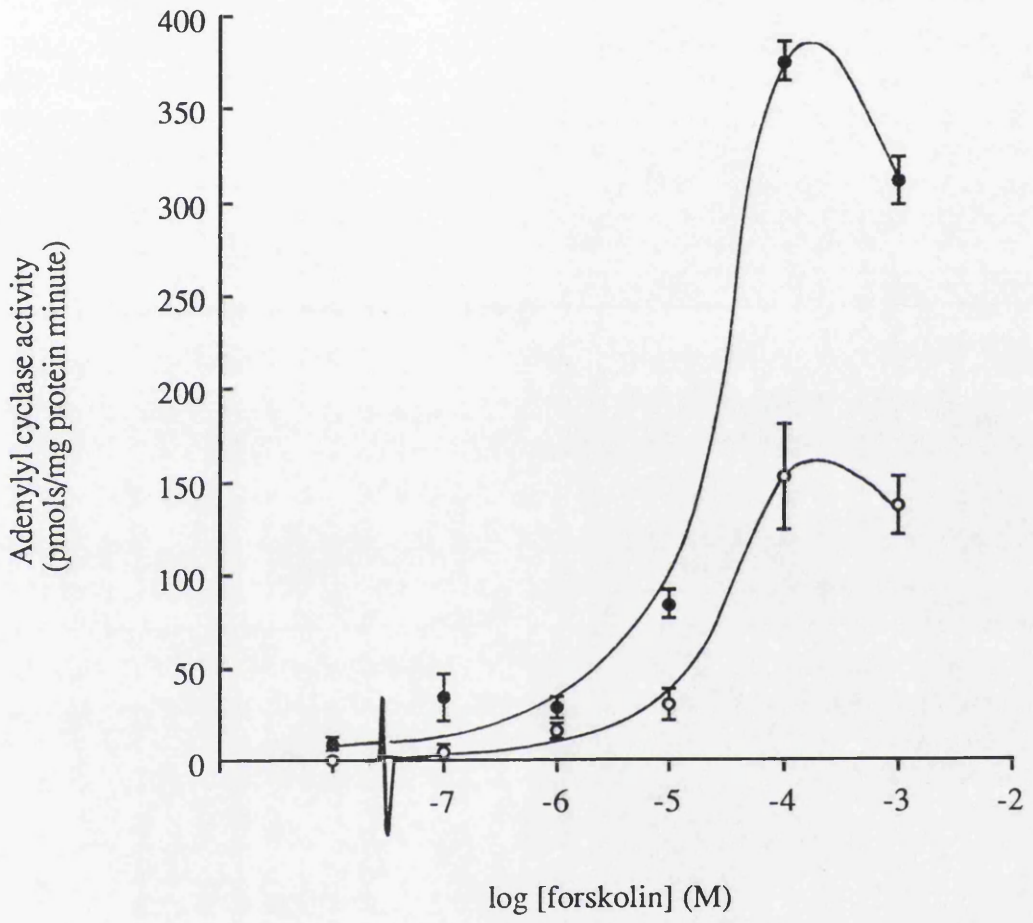
**Figure 4.2**



**Fig. 4.3**      **Effect of increasing concentrations of forskolin on adenylyl cyclase activity in plasma membranes from control P9 cells and cells exposed to insulin (0.5 $\mu$ M, 16 hours).**

Plasma membranes from control P9 cells (open circles) and cells exposed to insulin (0.5 $\mu$ M, 16 hours) (closed circles) as in the legend to Fig. 4.1 were assayed for adenylyl cyclase activity in the presence of increasing concentrations of forskolin. Stimulation parameters are given in Table 4.1. Results are mean  $\pm$  SEM for triplicate observations obtained in one experiment. Similar results were obtained in another experiment.

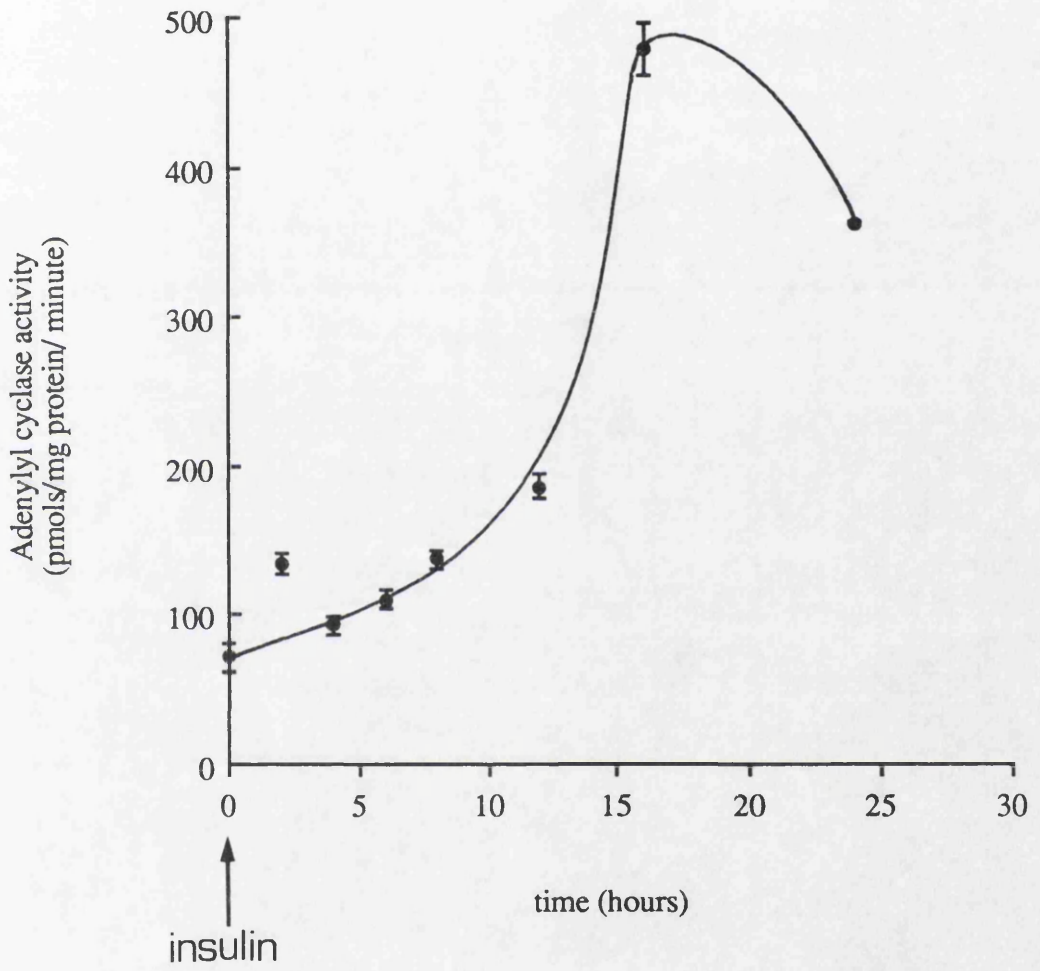
**Figure 4.3**



**Fig. 4.4**      **Time course for change in forskolin-stimulated plasma membrane adenylyl cyclase activity after addition of insulin (0.5 $\mu$ M) to P9 cells.**

Confluent P9 cells were exposed to insulin, 0.5 $\mu$ M as described in the legend to Fig. 4.3. and harvested at intervals. Membranes were then prepared and assayed for adenylyl cyclase activity in the presence of forskolin (10<sup>-4</sup>M) as described in section 2.13. Results are mean $\pm$ SEM for triplicate observations obtained in one experiment. Similar results were obtained in another experiment.

**Figure 4.4**

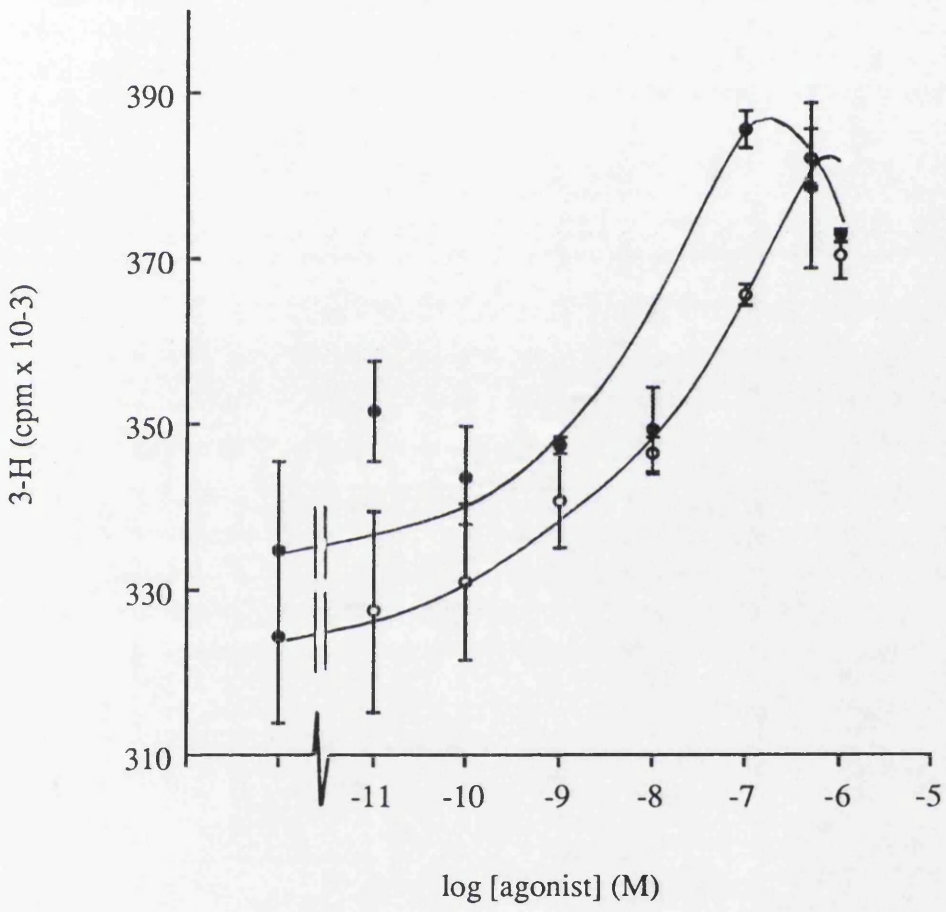




**Fig. 4.5**      **Uptake of [<sup>3</sup>H]-Thymidine by P9 cells in response to insulin and IGF-1.**

Confluent P9 cells were exposed to serum-free medium containing [<sup>3</sup>H]-Thymidine and increasing concentrations of insulin (open circles) or IGF-1 (closed circles) as described in section 2.16. Results are mean $\pm$ SEM for triplicate observations obtained in one experiment. Insulin, at 0.5 $\mu$ M caused a maximum increase in uptake of 18 $\pm$ 1% (CV) with a K<sub>0.5</sub> value of 2.3 x 10<sup>-8</sup>M. IGF-1, at 10<sup>-7</sup>M gave a maximum increase in uptake of 15 $\pm$ 1% (CV) with a K<sub>0.5</sub> value of 2.0 x 10<sup>-8</sup>M.

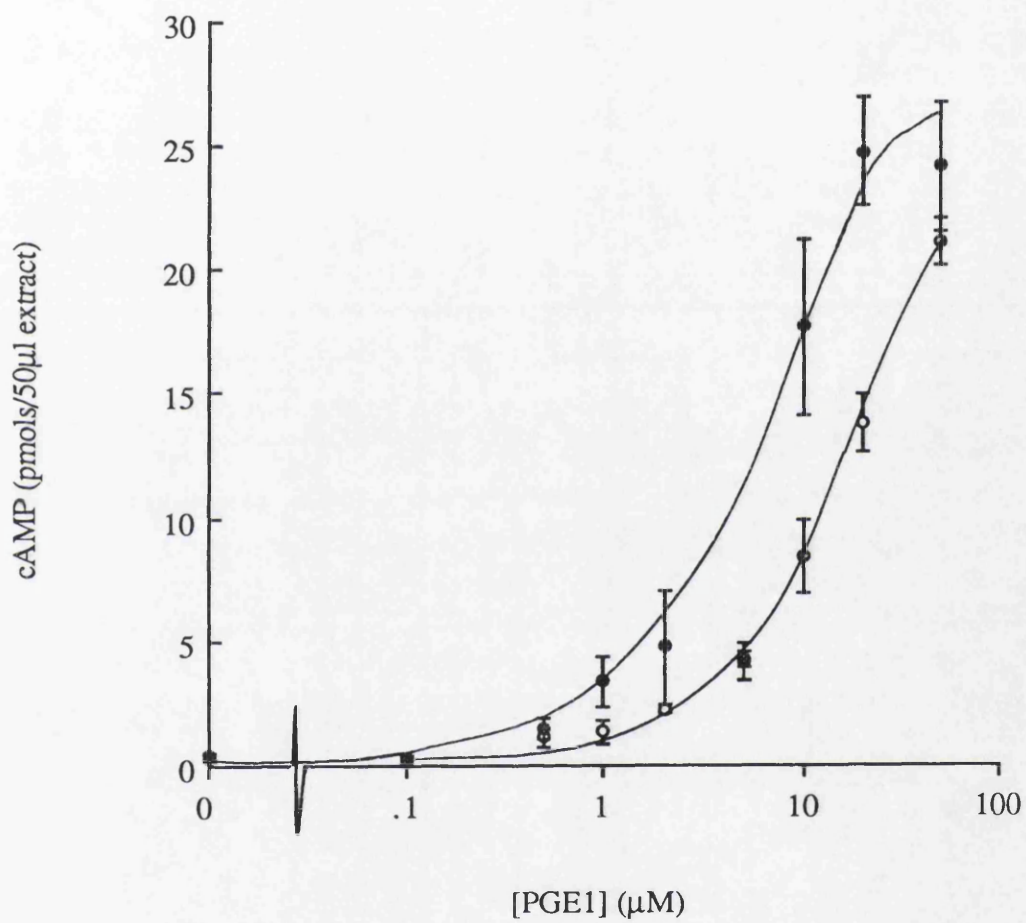
Figure 4.5



**Fig.4.6                    Effect of increasing concentrations of PGE<sub>1</sub> on P9 cell intracellular cAMP after exposure to insulin (0.5μM, 16 hours).**

P9 cells were exposed to serum-free medium alone (open circles) or containing insulin 0.5μM (closed circles). After 16 hours, cells were challenged for 2 minutes with increasing concentrations of PGE<sub>1</sub> and cAMP extracted and assayed as described in section 2.14. Maximum control response (at 5 x 10<sup>-5</sup>M) was 21±1 pmols/50μl extract with a K<sub>0.5</sub> for PGE<sub>1</sub> of 1.35 x 10<sup>-5</sup>M. Maximum response in insulin exposed cells (at 2 x 10<sup>-5</sup>M) was 25±3 with a K<sub>0.5</sub> for PGE<sub>1</sub> of 8.2 x 10<sup>-6</sup>M. Results are mean±SEM for triplicate observations obtained in one experiment. Similar results were obtained in another experiment.

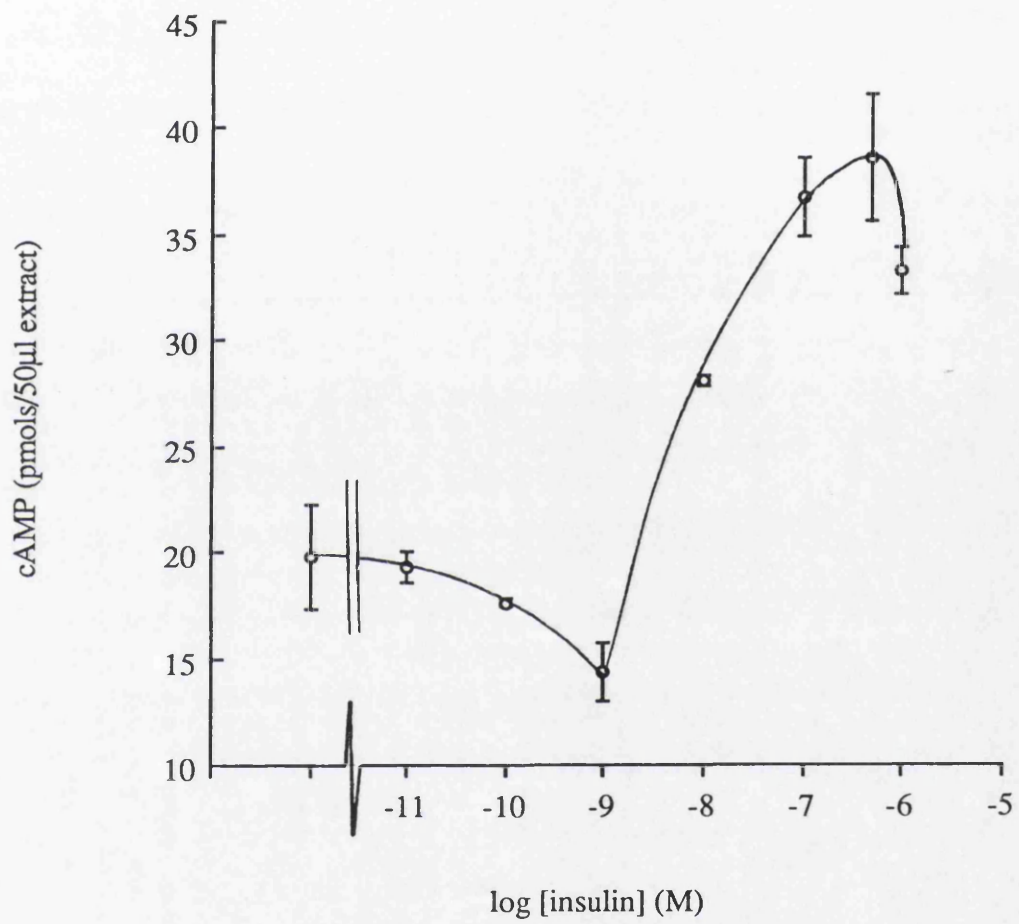
**Figure 4.6**



**Fig. 4.7      Effect of increasing concentrations of insulin  
(16 hours exposure) on PGE<sub>1</sub>-elevated  
intracellular cAMP in P9 cells.**

Confluent P9 cells were incubated with serum-free medium containing increasing concentrations of insulin (0-10<sup>-6</sup>M). After 16 hours, cells were challenged for 2 minutes with PGE<sub>1</sub> (10<sup>-5</sup>M) and cAMP extracted and assayed as described in section 2.14. Enhancement of the PGE<sub>1</sub> response by 0.5μM insulin was 1.95±0.15 fold, with a K<sub>0.5</sub> value for insulin of 1.35 x 10<sup>-8</sup>M. Results are mean±SEM for triplicate observations performed in one experiment. Similar results were obtained in three other experiments.

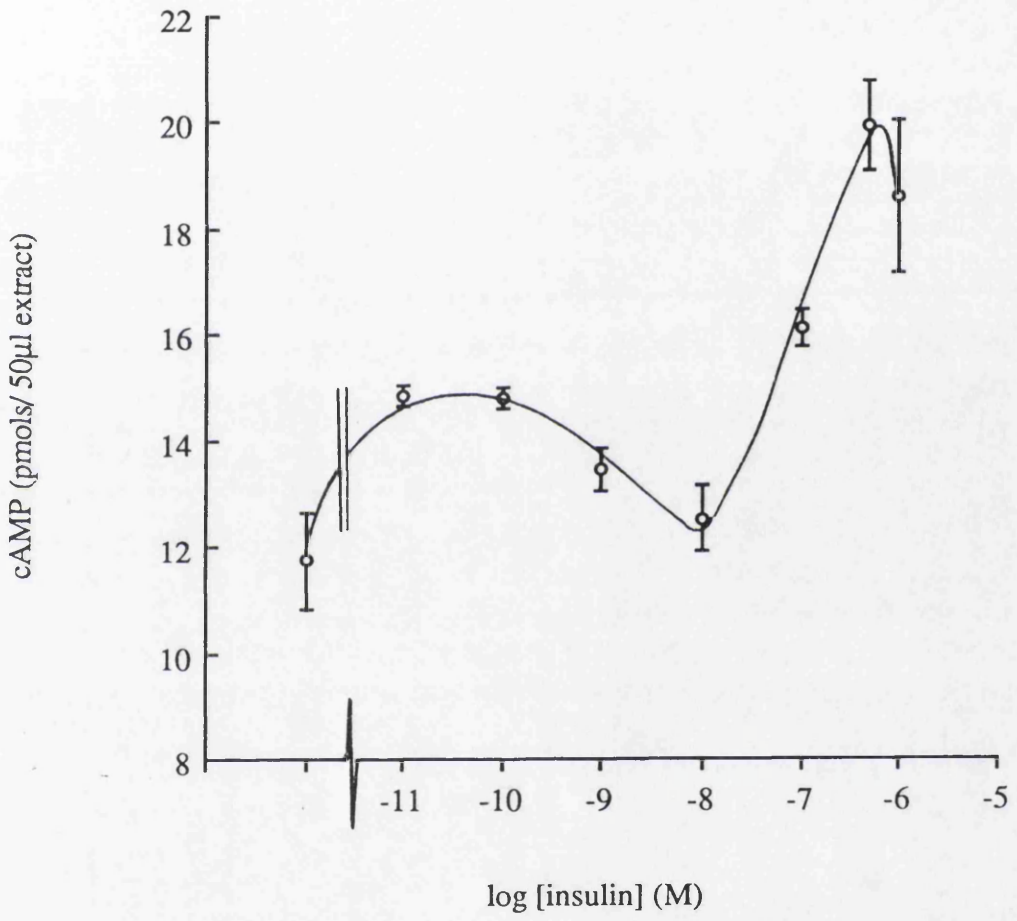
**Figure 4.7**



**Fig. 4.8**      **Effect of increasing concentrations of insulin**  
**(16 hours exposure) on forskolin-elevated**  
**intracellular cAMP in P9 cells.**

Confluent P9 cells were incubated with serum-free medium containing increasing concentrations of insulin ( $0$ - $10^{-6}$ M). After 16 hours, cells were challenged for 2 minutes with forskolin ( $10^{-5}$ M) and cAMP extracted and assayed as described in section 2.14. Enhancement of the forskolin response by  $0.5\mu$ M insulin was  $1.70\pm 0.13$  fold, with a  $K_{0.5}$  value for insulin of  $9.2 \times 10^{-8}$ M. Results are mean $\pm$ SEM for triplicate observations obtained in one experiment. Similar results were obtained three other experiments.

**Figure 4.8**

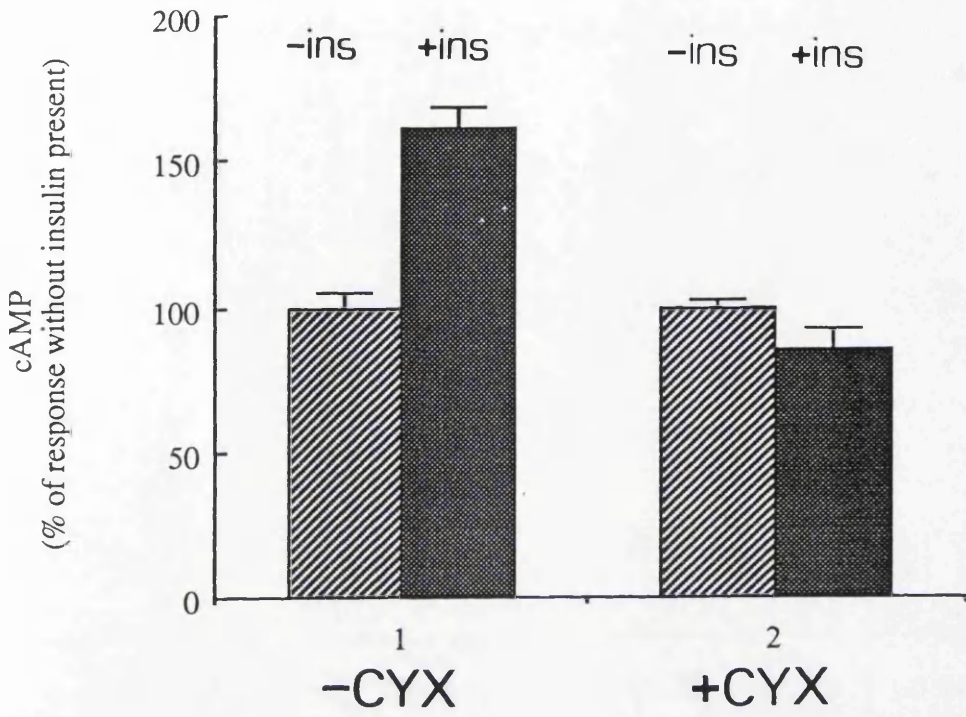




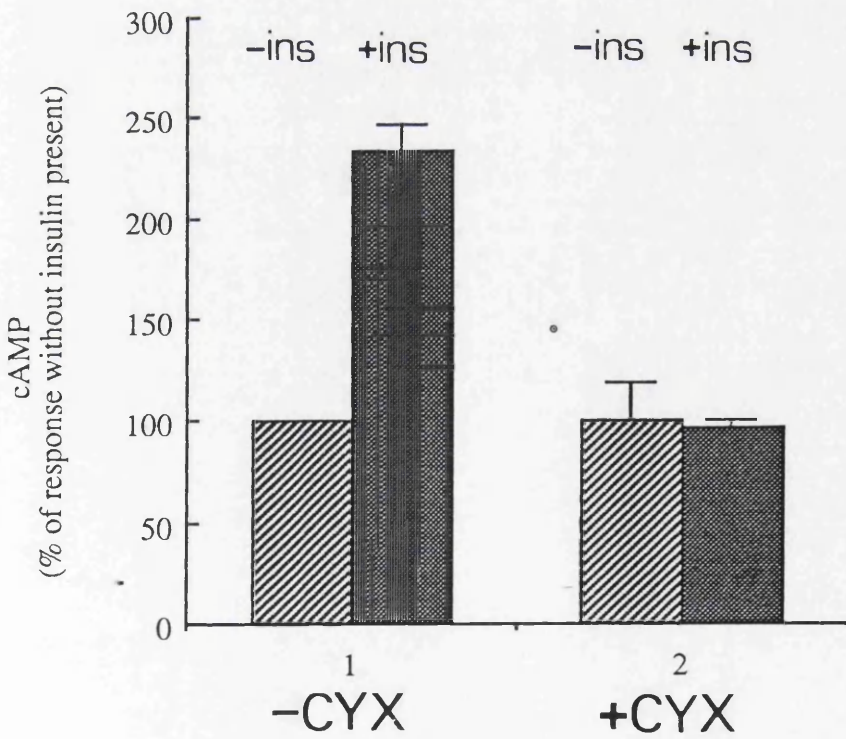
**Fig. 4.9      Effect of cycloheximide pre-treatment on insulin's modulation of PGE<sub>1</sub>- and forskolin-elevated intracellular cAMP in P9 cells.**

P9 cells were incubated with serum-free medium alone (column 1) or containing cycloheximide, 1 $\mu$ M (column 2). Cells were incubated with (hatched bars) or with (black bars) insulin, 0.5 $\mu$ M. After 16 hours, cells were challenged with PGE<sub>1</sub> (10<sup>-5</sup>M for 2 minutes) in Fig. 4.9a and forskolin (10<sup>-5</sup>M for 2 minutes) in Fig. 4.9b. Cyclic AMP was extracted and assayed as described in section 2.14. Data are expressed in each case as a percentage of the value without insulin present. Results are mean $\pm$ SEM for triplicate observations obtained in one experiment in each case. Similar results were obtained in two other experiments in the case of PGE<sub>1</sub>, and one other experiment in the case of forskolin.

**Figure 4.9(a)**



**Figure 4.9(b)**

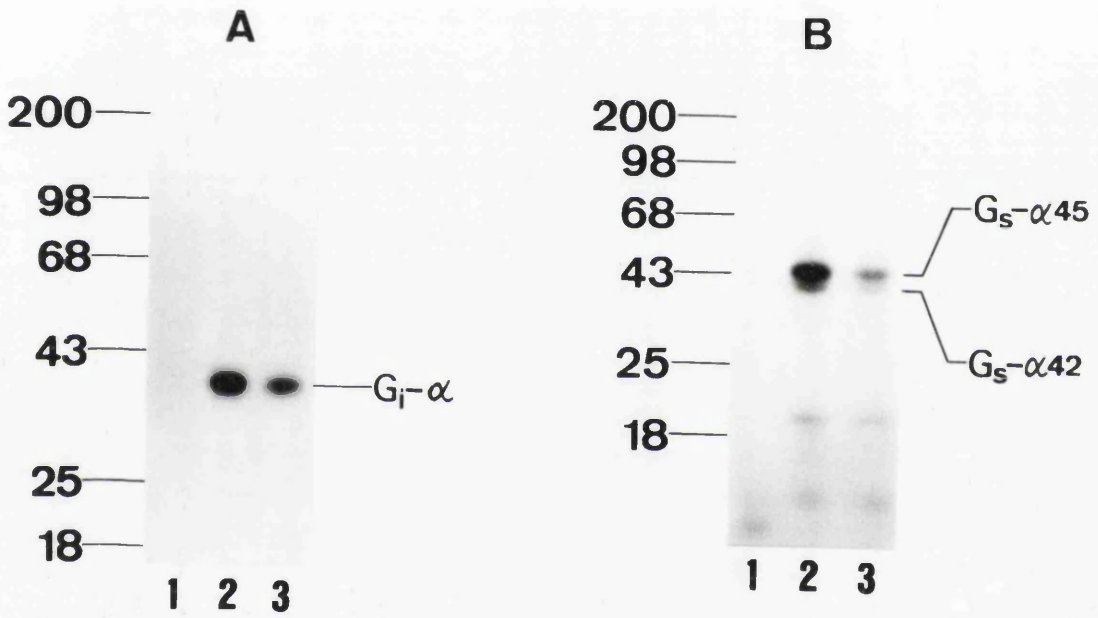


**Fig. 4.10      Toxin-catalysed ADP-ribosylation of  
G-proteins in plasma membranes from P9 cells  
exposed to insulin (0.5 $\mu$ M, 16 hours).**

P9 cells were exposed to serum-free medium alone (lane 2), or containing insulin, 0.5 $\mu$ M (lane 3) for 16 hours, harvested and plasma membranes prepared as in Figs. 4.1 to 4.3. Membranes were then ADP-ribosylated as described in section 2.12 using pertussis toxin (panel A) and cholera toxin (panel B). Membranes were also subjected to the same treatment in the absence of toxins (lane 1). Proteins were resolved and gels stained, dried, autoradiographed and scanned as described in section 2.10. Densitometric data are shown in Table 4.2. Results shown are from one experiment. Similar results were obtained in two other experiments.

**Figure 4.10**

$M_r \times 10^{-3}$

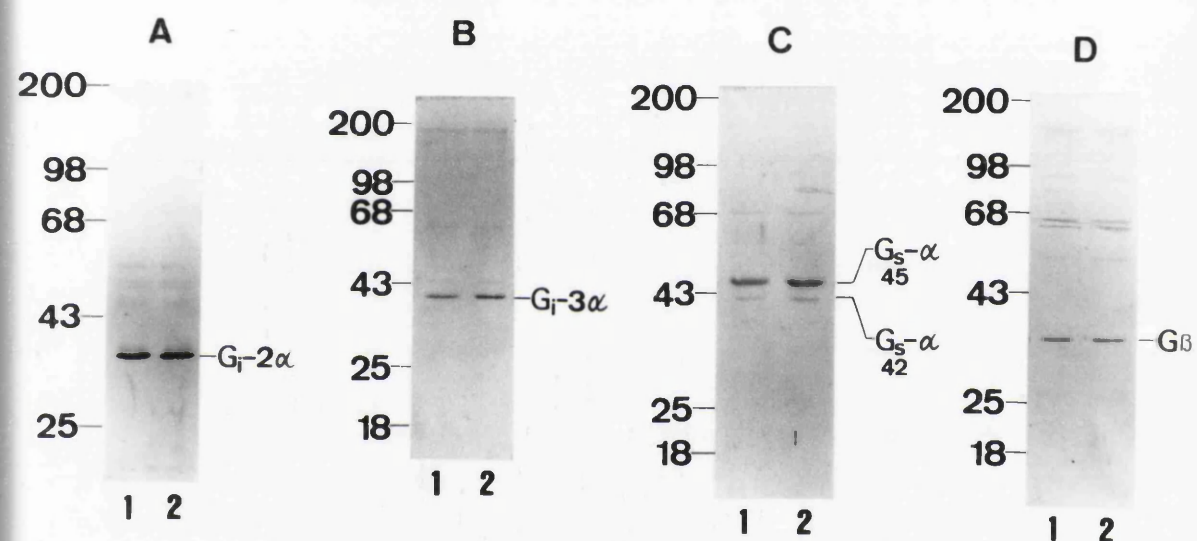


**Fig. 4.11**      **G-protein levels in plasma membranes from P9 cells exposed to insulin (0.5 $\mu$ M, 16 hours).**

Control P9 cells (lane 1) and cells exposed to insulin, 0.5 $\mu$ M for 16 hours (lane 2) were harvested and membranes prepared as described in Figure 4.1. Samples of membrane protein (100 $\mu$ g) were resolved, transferred to nitrocellulose paper and probed with antisera as described in section 2.11. The antisera used were 1432 (panel A), I3C (panel B), CS1 (panel C) and BN3 (panel D). Blots were scanned densitometrically and results presented in Table 4.2. Data shown are from one experiment. Similar results were obtained in two other experiments.

**Figure 4.11**

$M_r \times 10^{-3}$



## Chapter 5

### High affinity effects of insulin on adenylyl cyclase signalling in P9 cells

## 5.1 Introduction

In the previous chapter we investigated low affinity effects of insulin on adenylyl cyclase signalling in P9 cells. These effects, because of the high concentrations of hormone required to achieve them, are probably more relevant to growth factor action on the cells than events taking place through insulin receptor activation. If the P9 line is to be used as a model system for studying insulin action we need evidence of higher affinity insulin responsiveness. Such information may provide insight into the role of the high affinity insulin receptor in cyclase regulation. Furthermore, with regard to the data presented in Chapter 3 it would be interesting to know to what extent, if any, insulin responsiveness in the cells is comparable with that in rat hepatocytes. Here we investigated the effect of insulin (1nM) on agonist-elevated intact cell cAMP, plasma membrane adenylyl cyclase activity and G-protein levels in P9 cells.



## 5.2 Results

On examining the effects of insulin exposure on agonist-elevated intracellular cAMP, it was noted that exposure of cells to lower concentrations of insulin for 16 hours resulted in an impairment of the response to PGE<sub>1</sub> (Fig. 5.1) and elevated the response to forskolin (Fig. 5.2). Both effects of insulin on these responses were still observed in the presence of IBMX and with cycloheximide pre-treatment. The remaining experiments in this chapter were devoted to investigating these high affinity actions of insulin.

Incubation of P9 cells with insulin (1nM) in the same manner as before, caused a shift to the right of the PGE<sub>1</sub> dose response curve and, in addition, reduced the maximum agonist response (Fig. 5.3). No such shift in dose-dependence was observed when the agonist forskolin was used to elevate intracellular cAMP (Fig. 5.4). Pre-treatment of P9 cells with pertussis toxin had no effect on insulin's ability to impair the PGE<sub>1</sub> response (Fig. 5.5).

An investigation of the time course for the effect revealed that in insulin-treated cells, as opposed to cells treated with serum-free medium alone, there was a biphasic time course for insulin's action on the PGE<sub>1</sub> response. Insulin acutely enhanced the response which reached a maximum after 22 minutes and thereafter declined reaching a minimum after 10 hours (Fig. 5.6). The acute effect of insulin on the PGE<sub>1</sub> response in P9 cells is discussed later in this section.

In order to further examine insulin's action on the PGE<sub>1</sub> response, it was decided to observe whether the effect could be demonstrated on plasma membranes from insulin-exposed cells, as the stability or otherwise of the effect could provide clues as to its cause (Fig. 5.7 and Table 5.1). At high GTP concentrations, the PGE<sub>1</sub>

response was on average smaller in plasma membranes from insulin-exposed cells. There was no difference in the magnitude of the inhibitory phase, whether or not cells had been exposed to insulin. (PGE<sub>1</sub> was used here in addition to GTP in order to maximise the responses and give smaller percentage errors, so that the respective adenylyl cyclase activities could be compared more reliably). Inhibition by GppNHp however, was not observed in plasma membranes from insulin-exposed cells. For example, in a typical experiment, stimulation of adenylyl cyclase by GppNHp (10<sup>-4</sup>M) in membranes from insulin-exposed cells gave an activity of 381±7 pmols/mg protein/min (mean±SEM for triplicate observations) and an activity of 346±34 pmols/mg protein/min in control membranes. We must exercise caution in comparing adenylyl activities between different membrane preparations, especially when seeking a relatively small impairment of the response, as we are here. Nevertheless, it does appear that that in the presence of high GTP concentrations insulin's inhibition is stable and can be demonstrated in plasma membranes from treated cells.

The ability of a glucagon/forskolin combination to elevate P9 cell intracellular cAMP over the level achieved by forskolin alone was slightly by exposure of cells to insulin (1nM), but this reduction in response was not statistically significant (Fig. 5.12a).

Insulin's acute enhancement of the PGE<sub>1</sub> response was found to be dose-dependent for insulin (Fig. 5.8) and observed both in the presence and absence of IBMX. In contrast to the inhibition produced by the 16 hour insulin exposure, this acute effect was not seen on plasma membranes made from insulin-treated cells (Fig. 5.9 and Table 5.1), nor was there any enhancement of PGE<sub>1</sub>-stimulated plasma membrane adenylyl cyclase activity observed when membranes were challenged directly with PGE<sub>1</sub> together with insulin. (Fig. 5.10 and Table 5.1). Membranes were prepared in the presence of phosphatase inhibitors.

Experiments designed to examine whether insulin was also able to affect acutely intracellular cAMP elevated by forskolin, yielded at best a response which was small compared to the effect of insulin on the PGE<sub>1</sub> response and dose dependence could not be established (Fig. 5.11).

Whilst insulin (1nM) acutely and significantly enhanced the response to PGE<sub>1</sub> in intact P9 cells, it had no effect on the glucagon response (Fig. 5.12b). Insulin, at a concentration of 1μM, slightly enhanced the response to forskolin alone and abolished any further response on addition of glucagon to the cells (Fig. 5.12c).

On exposure of P9 cells to insulin (1nM), there was no significant change in toxin labelling of G<sub>s</sub>-α or G<sub>i</sub>-α to suggest any functional change in G-proteins as observed when using high concentrations of insulin (Fig. 5.13 and Table 5.2). Nor was there any alteration in plasma membrane levels of any of the G-protein α subunits or G-protein β subunit to account for insulin's action on the response (Fig. 5.14 and Table 5.2).

### 5.3 Discussion

In this chapter the action of insulin to modulate agonist-stimulated adenylyl cyclase activity in P9 cells was examined. The data indicated that insulin, at a concentration of 1nM, impaired the ability of PGE<sub>1</sub> to elevate acutely intracellular cAMP over a period of hours. This effect is unlikely to be due to activation of cAMP phosphodiesterase activity as pre-treatment of cells with IBMX failed to block insulin's action. Given that inhibition was only observed on the response to PGE<sub>1</sub> and not to forskolin this action of insulin was clearly exerted at a site in the signalling system proximal to the catalytic subunit of adenylyl cyclase and occurred at sufficiently low concentrations to suggest that it was mediated through high affinity insulin receptors.

Protein synthesis was not involved in this action as the effect was not blocked by cycloheximide pre-treatment. Indeed, the percentage inhibition was larger in the presence of cycloheximide. This is possibly because in the absence of cycloheximide insulin was having a small stimulatory effect on cell growth and adenylyl cyclase expression which was partially masking its functional impairment of the response to PGE<sub>1</sub>. On removing any effect of the hormone on protein synthesis, only its functional influence on the system remains intact.

The inhibition, although occurring at the level of the membrane was not brought about by any change in G-protein levels. Nor did it appear to be mediated by G<sub>i</sub> on adenylyl cyclase as pre-incubation of cells with pertussis toxin at a concentration adequate to ADP-ribosylate most of the G<sub>i</sub> present in the cells, had no effect on insulin's impairment of the PGE<sub>1</sub> response. The inability of insulin's effect to be abolished by pertussis toxin pre-treatment in P9 cells distinguishes it from insulin's effect in hepatocytes (Heyworth *et al.*, 1986) and excludes the involvement of an established or insulin-specific toxin substrate in insulin's action. That the magnitude

of the inhibitory phase of the GTP dose response curve was unaltered in membranes from insulin-exposed cells or in the acute presence of insulin, is further evidence that insulin's effect was unrelated to  $G_i$  function. The situation in P9 cells is different from that in rat hepatocyte plasma membranes where a dose-dependent inhibition of adenylyl cyclase activity by insulin was demonstrated in the presence of GTP (Heyworth and Houslay, 1983).

The site of insulin's action was at the level of the membrane and independent of protein synthesis, G-protein levels,  $G_i$  or cAMP phosphodiesterase activation. How then, did insulin bring about this effect? The dependence of the effect on PGE<sub>1</sub> concentration and its inability to be overcome by high concentrations of the agonist strongly suggest that the site of insulin's action was at the prostanoid receptor, either by a change in receptor affinity or by down-regulating the number of cell surface receptors. This explanation is compatible with the long time course observed for the effect which implies an adaptive change of the cells such as a change in protein distribution rather than a functional effect such as phosphorylation which we would expect to be rapid.

It was possible to demonstrate that insulin's inhibition of the PGE<sub>1</sub> response was retained in membranes from insulin-exposed cells, providing evidence in favour of a stable reduction in receptor number or affinity. The attenuation of PGE<sub>1</sub> response was observed on membranes and was apparently GTP dependent. In rat hepatocytes, GTP dependence for insulin's inhibition of cyclase was proposed to suggest the existence of an insulin-specific G-protein (Heyworth and Houslay, 1983). However, the authors quoted a  $K_{0.5}$  for the effect of  $3\mu\text{M}$  GTP, whereas here a significant inhibition in the presence of GTP was observed only at  $10^{-4}\text{M}$  with no effect at low concentrations such that a  $K_i$  value could not be calculated accurately. A possible explanation here is that saturating GTP is required to achieve an optimal agonist response thereby making

apparent the inhibition, itself exerted at a more proximal site in the signalling system.

An action of insulin to reduce the number of cell surface prostanoid receptors could be achieved either by reduced synthesis or accelerated degradation of the receptor in response to insulin or sequestration of the receptor at a subcellular site. Certainly there is extensive literature on insulin's ability to affect expression of G-protein-coupled receptors in cells. An impairment of  $\alpha_2$ -adrenergic receptor number for example, in response to insulin in the nM range has been observed in the HT29 (human adenocarcinoma) cell line (Devedjian *et al.*, 1991). As cycloheximide treatment had no influence on insulin's action, the authors proposed a decrease in transcription of the receptor as underlying this particular effect.

Interestingly, exposure to 1nM insulin, as with exposure to the high concentration, caused no change in G-protein levels in P9 cells. Agonist-induced changes in G-protein expression are largely restricted to heterologous desensitisation of agonists acting directly through G-protein linked receptors. In studies on receptor down-regulation, after prolonged exposure to PGE<sub>1</sub> in NG 108-15 cells, the prostanoid receptor was shown to co-downregulate with G<sub>s</sub>- $\alpha$ , indicating that the two processes were co-ordinated (Adie *et al.*, 1992). Clearly insulin, if acting in P9 cells by down-regulating the prostanoid receptor, is capable of doing so independently of any action on G<sub>s</sub>, as G-protein levels in P9 cells were not altered by any form of insulin exposure.

No evidence was provided for any change in G-protein function as contributing to insulin's high affinity effect on the PGE<sub>1</sub> response. GppNHp stimulation of adenylyl cyclase unlike the receptor-mediated response was not observed in membranes, demonstrating that insulin's effect on the PGE<sub>1</sub> response occurred proximal to coupling with G<sub>s</sub>. However, we have only examined G<sub>s</sub> function in membranes and not in the

intact cell. Although it remains possible that prolonged insulin exposure could influence the degree of GTP binding to G-proteins and thereby influence coupling, such an influence would not necessarily survive the process of membrane preparation, in which the hormone and endogenous GTP are removed. Thus, we cannot exclude an additional influence by insulin on coupling between the receptor and G<sub>s</sub>, as contributing to insulin's effect.

That insulin, over the same time period as the experiments with PGE<sub>1</sub>, appears to have little effect if any on glucagon responsiveness, suggests that this action is receptor-specific for the PGE<sub>1</sub> receptor in P9 cells. We must be cautious in our interpretation however, given the small size of the glucagon response in P9 cells and that its demonstration requires the presence of the exogenous agonist forskolin. The small size of the glucagon response over that of forskolin, means that a reduction in its magnitude, in order to be considered statistically significant, would require abolition of the entire response (given the size of the experimental errors arising from the cAMP assay and elsewhere in the experiment). Clearly, if the situation with the glucagon receptor parallels that of the PGE<sub>1</sub> receptor, abolition of the response would not occur, as the latter response was only impaired by some 25% under the same conditions. It may well be that insulin is having a similar effect on both stimulatory responses but that the limitations of the methodology make it impossible to conclude this with certainty.

Another modulating influence of insulin to consider is the possibility of the insulin receptor phosphorylating and thereby reducing the affinity of the prostanoid receptor. Phosphorylation of the receptor or any other component of the adenylyl cyclase signalling system could potentially occur either directly, as a result of tyrosine kinase activation, or indirectly following PK-C activation serving either to uncouple it from G<sub>s</sub> or as a signal for its internalisation. PK-C is known to be activated in certain cell types in response to insulin which can generate diacylglycerol by hydrolysis of

inositol glycolipids (Saltiel and Cuatrecasas, 1986) and there is evidence for activated PK-C influencing adenylyl cyclase activity at all levels of the second messenger system (Houslay, 1991b). However, phosphorylation events in response to insulin are generally rapid i.e. within minutes (Knutson, 1991), whereas this effect of insulin in P9 cells was only apparent after hours. Furthermore, lipid signalling has not been examined in P9 cells so it is not known whether insulin is capable of generating such a second messenger and thereby activating PK-C.  $G_i$  phosphorylation cannot be implicated as  $G_i$  appears to have little if any input into cyclase functioning in P9 cells and its inactivation has no influence on insulin's action. The only other site proximal to the adenylyl cyclase catalytic subunit which is a potential site of regulation is  $G_s$ . However,  $G_s$ - $\alpha$  is not known to be phosphorylated in response to insulin, either by the purified receptor tyrosine kinase (Krupinski *et al.*, 1988) or by insulin exposure of intact hepatocytes (Houslay, 1991b).

The absence of any impairment of the forskolin response by  $10^{-9}$ M insulin was shown not to be a consequence of the concentration of forskolin used and suggested insulin's site of action on the PGE<sub>1</sub> response to be proximal to the adenylyl cyclase catalytic subunit. The ability of lower concentrations of insulin, after a 16 hour period, to enhance the response to forskolin, suggested an insulin receptor-mediated effect on the catalytic subunit of adenylyl cyclase. It is more difficult to explain the mechanism of this high affinity effect of insulin on forskolin responsiveness in the cells, as we have less information regarding the effect. Like insulin's effect on the response to PGE<sub>1</sub> it was not dependent on protein synthesis or phosphodiesterase activity. However, the lower concentrations of insulin required for the effect on the forskolin response suggest a different mode of action from that on the PGE<sub>1</sub> response. A physical effect such as insulin's high affinity action on membrane fluidity could be implicated here, enhancing the enzymic activity of adenylyl cyclase. The functioning of adenylyl cyclase signalling system has been shown elsewhere to be influenced by membrane fluidity, for example



the  $\beta$ -adrenergic signalling system (Salesse *et al.*, 1982) and glucagon-stimulated adenylyl cyclase in rat liver (Dipple and Houslay, 1978).

Unlike exposure to 0.5 $\mu$ M insulin, exposure to 1nM insulin had little effect on toxin labelling of G-protein  $\alpha$  subunits. Furthermore, if insulin's impairment of the PGE<sub>1</sub> response were mediated through an alteration in G-protein activity ie. a change in the subunit dissociation equilibrium, we might have expected to find an alteration in labelling for the reasons discussed in Chapter 4. That there is no change in labelling lends further credence to the view that insulin's high affinity action is at a site distinct from G<sub>s</sub>.

Insulin's effect of acutely enhancing the ability of PGE<sub>1</sub> to elevate intracellular P9 cell cAMP levels was clearly also exerted at the level of the membrane. Various mechanisms can be proposed for this action. These include recruitment of PGE<sub>1</sub> receptors from a subcellular site, a change in physical properties of the plasma membrane, production of an intracellular second messenger or interaction with a G-protein in a manner enhancing coupling. The rapidity of insulin's acute effect on the PGE<sub>1</sub> response favours a functional interaction exerted by insulin at the level of the plasma membrane and makes receptor synthesis or synthesis of an associated protein unlikely causes of the enhanced response. That the effect is not stable on membrane preparation, nor repeated by challenging isolated membranes with the two agonists makes receptor recruitment unlikely, since insulin's ability to enhance acutely platelet PGE<sub>1</sub>-stimulated adenylyl cyclase activity by this mechanism was still observed on membranes from insulin challenged platelets (Kahn and Sinha, 1990). However, one could still propose that receptor recruitment occurs but that the time period after insulin removal and before membrane preparation (ie. during harvesting and washing of the cells) is adequate to allow reversal of the effect by receptor internalisation. This theory is appealing, as it means that insulin would be having both its high affinity effects at the

same site i.e. an initial acute enhancement by increasing plasma membrane receptor number and an ensuing desensitisation of the response by reduction in receptor number.

The acute action of insulin on the glucagon response was different from its action on the response to PGE<sub>1</sub>. This response specificity for the effect of 1 μM insulin implies different effects on the prostanoid and glucagon receptors, stimulatory and inhibitory respectively in which intact cell integrity is required either to provide a soluble protein substrate or components needed for mediator generation.

Although there was no change in plasma membrane GTP-stimulated adenylyl cyclase after insulin exposure suggesting no influence of insulin on coupling to G<sub>s</sub>, we would as above, not necessarily expect enhanced coupling to be a stable phenomenon. Similarly, when isolated membranes were challenged with both PGE<sub>1</sub> and GTP, no enhancement by insulin was observed, regardless of the GTP concentration. Clearly, isolated membranes do not possess all the components required for this effect of insulin. The possibility of a soluble mediator being involved in this action of insulin should be considered as such a mediator could easily be removed during the process of membrane preparation. Such a mediator may either not be generated by isolated membranes or be unable to exert its effects in this situation because of removal of for example, a soluble protein kinase required for its action.

On speculating why insulin could not produce any effect in isolated membranes in the adenylyl cyclase assay it should also be noted that a high concentration of cAMP was present in the assay to act as a [<sup>32</sup>P]-cAMP 'trap'. It has been suggested that cAMP acting via PK-A can cause phosphorylation and inactivation of the insulin receptor (Tanti *et al.*, 1987). This could potentially account for our inability to observe any effect of insulin when assaying cyclase activity in isolated membranes.

Any effect of insulin on signalling is likely to be produced downstream of receptor tyrosine kinase activation. Phosphorylation responses to insulin are generally stable to cell extraction in the presence of phosphatase inhibitors (Sale and Smith, 1989). As phosphatase inhibitors were present during cell breakage, phosphorylation of a protein dephosphorylated during the membrane preparation can be excluded as the mechanism of insulin's action. However, soluble protein substrates would clearly be lost during membrane preparation and clearly be unable to exert any effect on adenylyl cyclase during the assay. This seems the most likely explanation for the inability of insulin to directly affect membrane responses. In addition, phosphorylation in response to insulin is rapid and would precede any effect of insulin on mediator generation or receptor distribution. Whatever the mechanism of insulin's action here the integrity of the intact cell is clearly required.

**Table 5.1      Parameters for GTP/PGE<sub>1</sub>-stimulated adenylyl cyclase activity in P9 cell plasma membranes.**

P9 cells were exposed to serum-free medium alone (-ins) or containing insulin (+ins) 1nM for 16 hours (a) or 1nM for 10 minutes (b), then membranes prepared and assayed for adenylyl cyclase activity in the presence of increasing concentrations of GTP (0-10<sup>-3</sup>M) and a constant concentration of PGE<sub>1</sub> (10<sup>-5</sup>M). Dose response curves are shown in Figs. 5.7 and 5.9 respectively. In (c) plasma membranes from untreated cells were assayed similarly but in the absence (-ins) and presence (+ins) of insulin, 1μM. The dose response curve is shown in Fig. 5.10. Maximal stimulation (mean±SEM for triplicate observations in one experiment) and K<sub>0.5</sub> values were obtained. Percentage inhibition by 10<sup>-3</sup>M GTP was calculated and given as mean±%CV for triplicate observations in one experiment. Similar results were obtained in two other experiments.

**Table 5.1**

	Maximum stimulation (pmols/mg/min)	K <sub>0.5</sub> (M)	% inhibition by 10 <sup>-3</sup> M GTP
(a) -ins	759±29	3.5 x 10 <sup>-7</sup>	46±3.1
+ins (1nM, 16h)	632±18	7.0 x 10 <sup>-8</sup>	47±5.0
(b) -ins	596±4	2.2 x 10 <sup>-7</sup>	36±4.2
+ins (1nM, 10')	575±16	2.9 x 10 <sup>-7</sup>	36±4.0
(c) -ins	621±4	6.6 x 10 <sup>-7</sup>	30±2.0
+ins (1μM, 10')	569±10	4.0 x 10 <sup>-7</sup>	38±2.2

**Table 5.2**      **Quantitative comparison of toxin labelling and G-protein levels in plasma membranes after exposure of P9 cells to insulin (1nM, 16hours)**

P9 cells were exposed for 16 hours to serum-free medium alone or containing insulin, 1nM, harvested and membranes subjected to toxin labelling and immunoblotting as described in the legends to Figs. 5.12 and 5.13 respectively. Bands from three experiments in each case were scanned and the insulin-induced alteration given as a percentage. All data are mean+/-SEM.

**Table 5.2**

Polypeptide	Insulin-induced change in toxin labelling
Gi- $\alpha$	+13+/-2.6
Gs- $\alpha$ (45kDa)	-19+/-11
Gs- $\alpha$ (42kDa)	-13+/-13

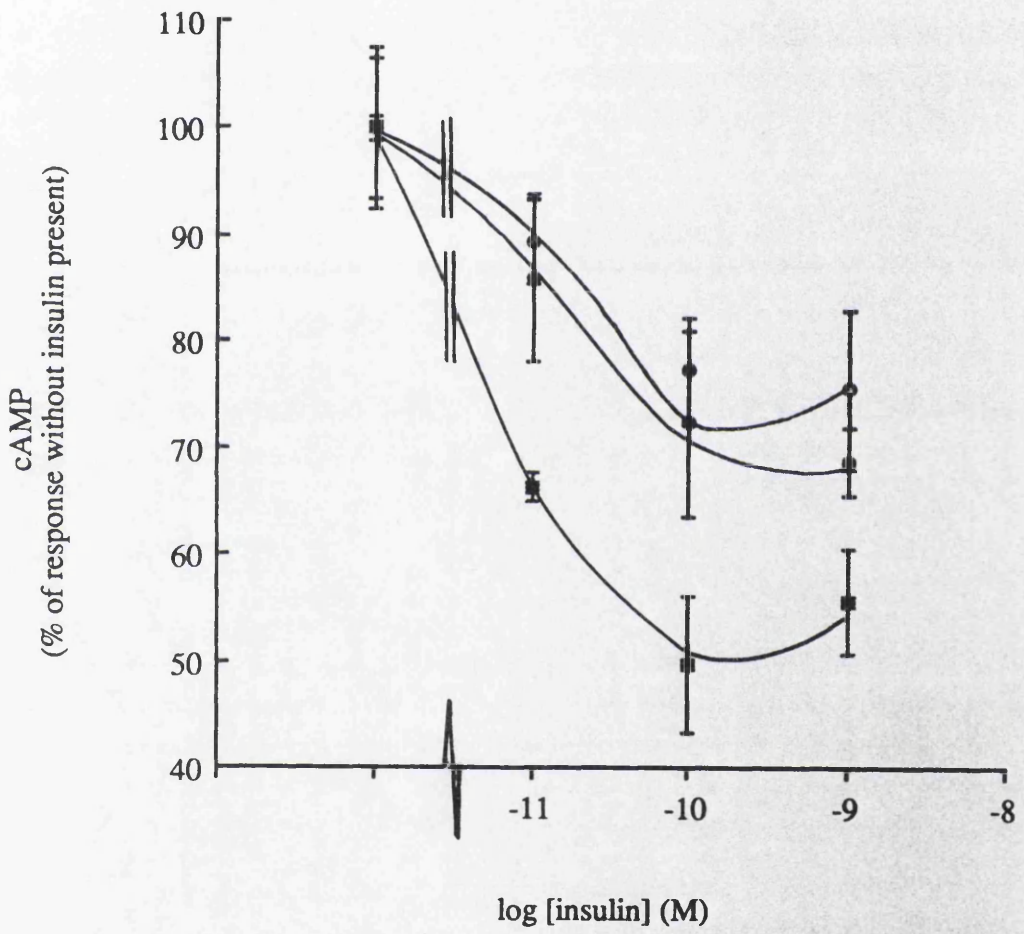
	Insulin-induced change in G-protein level
Gi-2 $\alpha$	+12+/-9.5
Gi-3 $\alpha$	+13+/-28
Gs- $\alpha$ (45kDa)	+11+/-8.0
Gs- $\alpha$ (42kDa)	-10+/-4.1
G $\beta$	+6.9+/-2.4

**Fig. 5.1**      **Effect of IBMX and cycloheximide pre-treatment on insulin's modulation of PGE<sub>1</sub>-elevated intracellular cAMP in P9 cells.**

P9 cells were incubated with serum-free medium containing insulin (from 0 to  $10^{-9}$ M) alone (open circles), with IBMX (1mM, 15 minute pre-incubation) (closed circles) and with cycloheximide (1 $\mu$ M, during the entire period of insulin exposure) (closed squares). After 16 hours cells were challenged with PGE<sub>1</sub> ( $10^{-5}$ M for 2 minutes) and cAMP extracted and assayed as described in section 2.14. In the absence of insulin, cAMP level was 9.7 $\pm$ 1.3 (control), 20 $\pm$ 1.3 (IBMX) and 16 $\pm$ 0.2 pmols/50 $\mu$ l extract (cycloheximide). K<sub>i</sub> values for insulin were  $1.7 \times 10^{-11}$ M (control),  $1.7 \times 10^{-11}$ M (IBMX) and  $5.3 \times 10^{-12}$ M (cycloheximide). Data are mean $\pm$ SEM for triplicate observations in one experiment. Similar results were obtained in two other experiments.



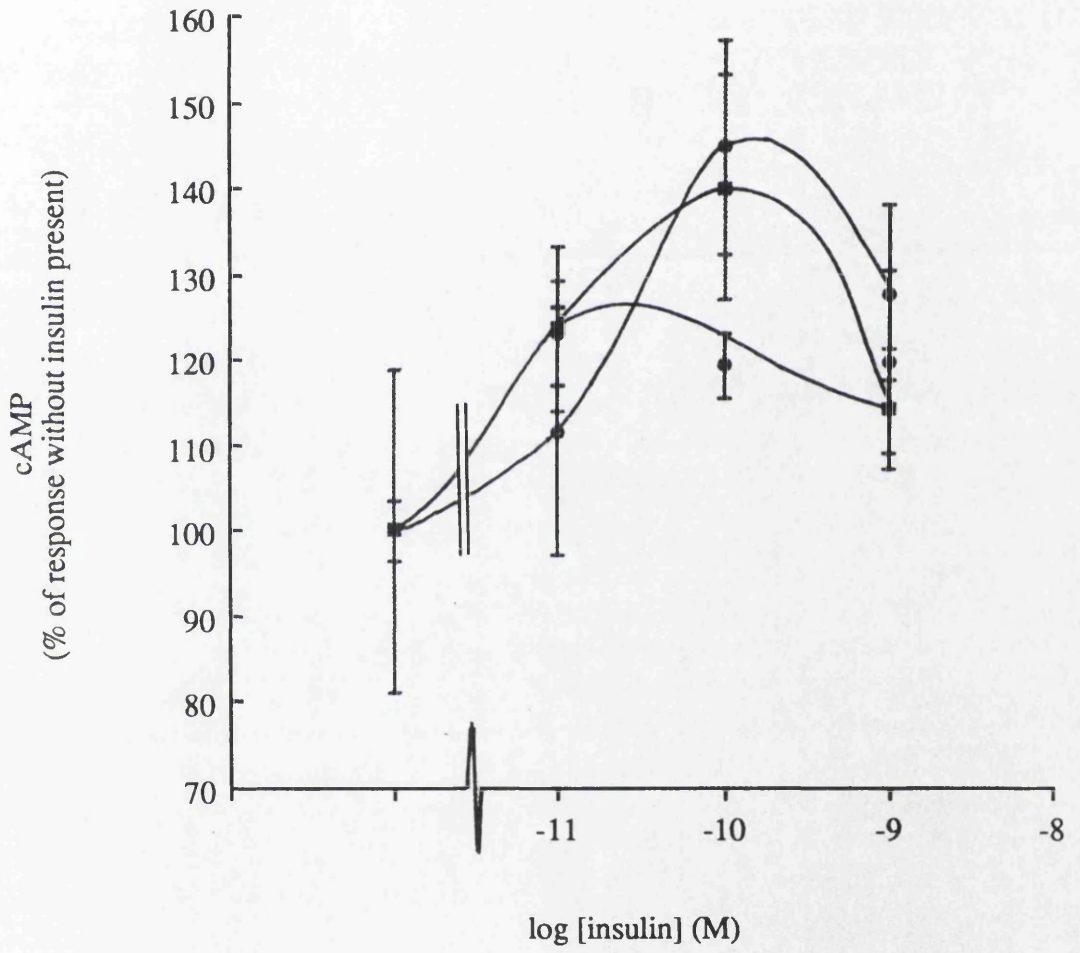
**Figure 5.1**



**Fig. 5.2**      **Effect of IBMX and cycloheximide pre-treatment on insulin's modulation of forskolin elevated intracellular cAMP in P9 cells.**

P9 cells were incubated with serum-free medium containing insulin (from 0 to  $10^{-9}$ M) alone (open circles), with IBMX (1mM, 15 minute pre-incubation) (closed circles) and with cycloheximide ( $1\mu$ M during the entire period of insulin exposure) (closed squares). After 16 hours cells were challenged with forskolin ( $10^{-5}$  for 2 minutes) and cAMP extracted and assayed as described in section 2.14. In the absence of insulin, cAMP level was  $9.8\pm 0.1$  pmols/ $50\mu$ l extract (control),  $16.6\pm 0.6$  (IBMX) and  $2.8\pm 0.5$  (cycloheximide).  $K_{0.5}$  values for insulin were  $2.2 \times 10^{-11}$ M (control),  $3.2 \times 10^{-12}$ M (IBMX) and  $7.0 \times 10^{-12}$ M (cycloheximide). Data are mean $\pm$ SEM for triplicate observations in one experiment. Similar results were obtained in another experiment.

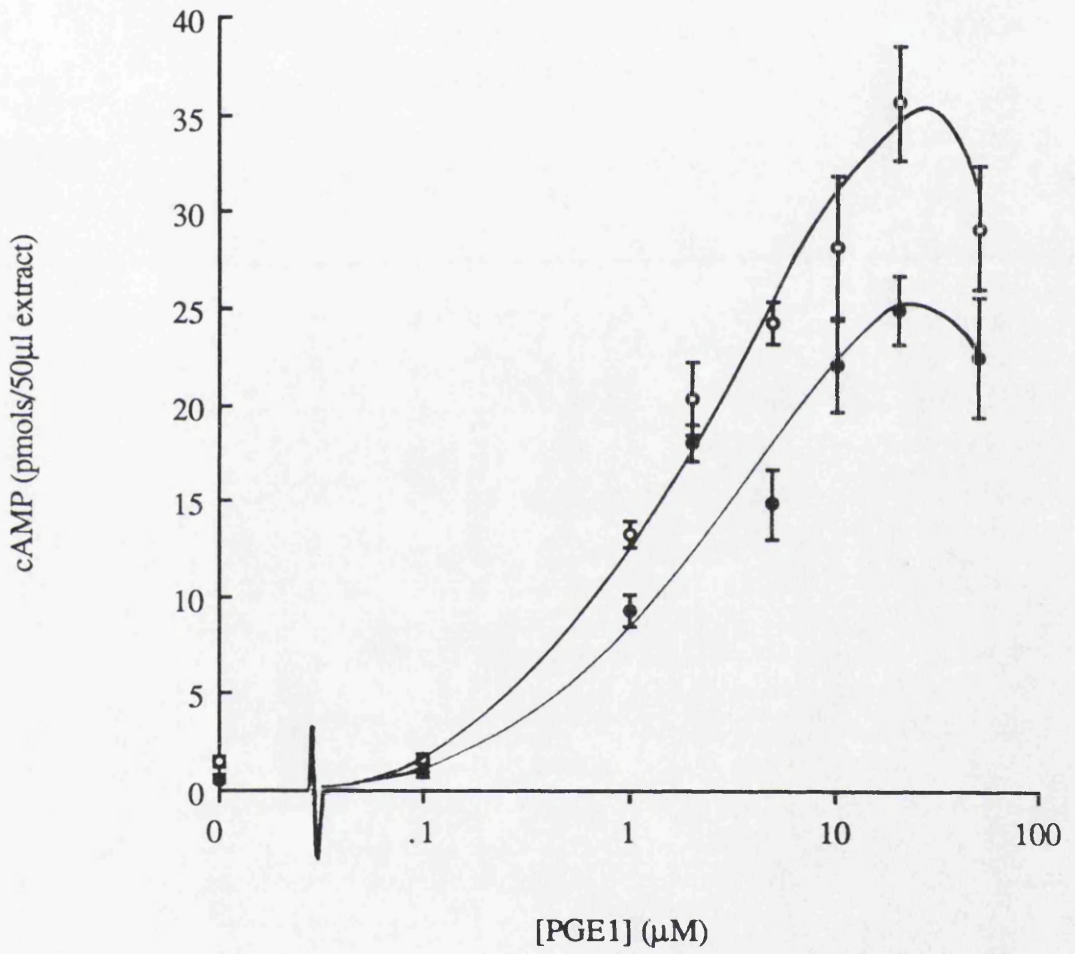
**Figure 5.2**



**Fig. 5.3**      **Elevation of P9 cell intracellular cAMP by increasing concentrations of PGE<sub>1</sub> after exposure to insulin (1nM, 16 hours).**

P9 cells were exposed to serum-free medium alone (open circles) or containing insulin, 1nM (closed circles). After 16 hours, cells were challenged with PGE<sub>1</sub> ( $0.5 \times 10^{-5}$ M for 2 minutes) and cAMP extracted and assayed as described in section 2.14. Maximal control response was  $36 \pm 3$  pmols/50 $\mu$ l extract with a K<sub>0.5</sub> for PGE<sub>1</sub> of  $1.5 \times 10^{-6}$ M. Maximal response after insulin exposure was  $25 \pm 2$  pmols/50 $\mu$ l extract with a K<sub>0.5</sub> value for PGE<sub>1</sub> of  $2.0 \times 10^{-6}$ M. Data are mean  $\pm$  SEM for triplicate observations in one experiment. Similar results were obtained in two other experiments and in one experiment in which cells were preincubated with IBMX (1mM, 15minutes).

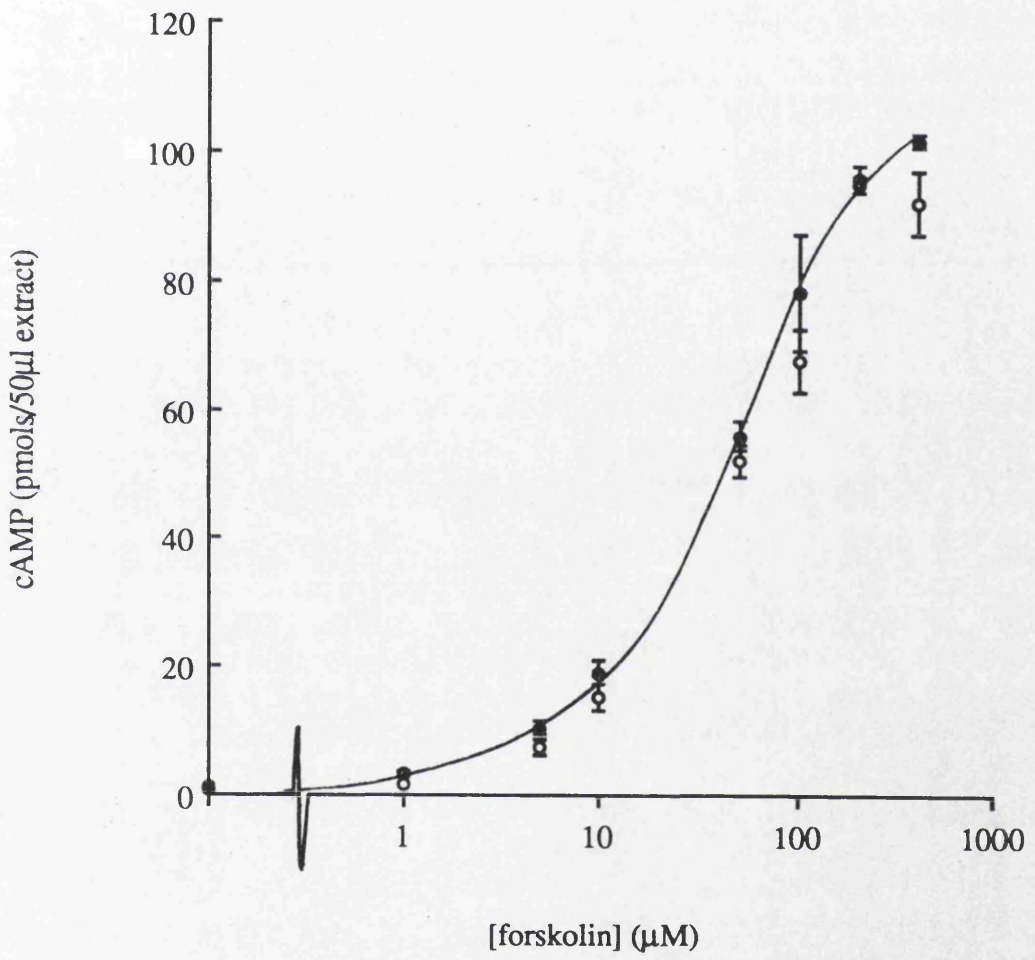
**Figure 5.3**



**Fig. 5.4      Elevation of P9 cell intracellular cAMP by  
increasing concentrations of forskolin after  
exposure to insulin (1nM, 16 hours)**

P9 cells were exposed to serum-free medium alone, (open circles) or containing insulin, 1nM (closed circles). After 16 hours, cells were challenged with forskolin ( $0-4 \times 10^{-4}$ M for 2 minutes) and cAMP extracted and assayed as described in section 2.14. Maximal control response was  $95 \pm 1$  (SEM) pmols/50 $\mu$ l extract with a  $K_{0.5}$  value for forskolin of  $4.2 \times 10^{-5}$ M. Maximal response after insulin exposure  $102 \pm 2$  (SEM) pmols/50 $\mu$ l extract with a  $K_{0.5}$  value for forskolin of  $4.1 \times 10^{-5}$ M. Data are mean $\pm$ -SEM for triplicate observations in one experiment. Similar results were obtained in another experiment.

**Figure 5.4**

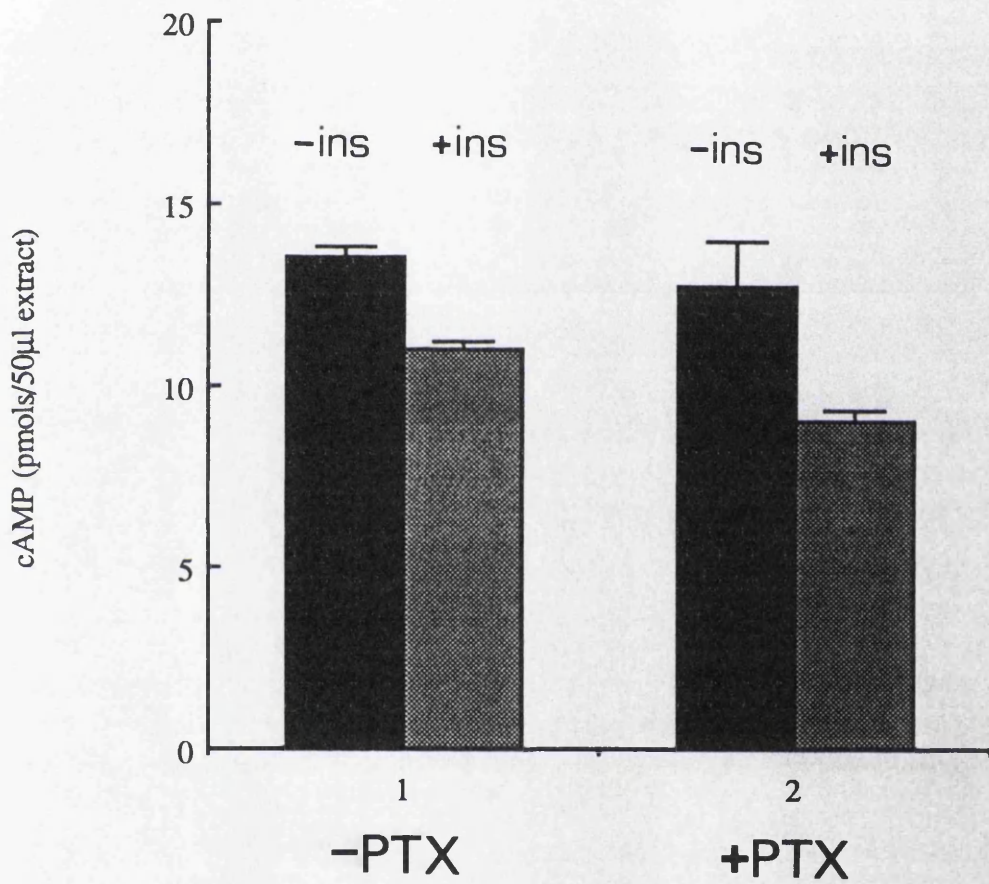


**Fig. 5.5      Effect of pertussis toxin pre-treatment on  
impairment of PGE<sub>1</sub>-elevated P9 cell  
intracellular cAMP by insulin (1nM, 16 hours).**

In column 1, P9 cells were exposed for 16 hours to serum-free medium alone (black bars), or containing insulin, 1nM (grey bars). In column 2, cells were similarly treated except that pertussis toxin, 100ng/ml was included during the last 2 hours of exposure to serum free medium. Cells were then challenged with PGE<sub>1</sub> (10<sup>-5</sup>M) for 2 minutes and cAMP extracted and assayed as described in section 2.14. Data are mean+/-SEM for triplicate observations in one experiment. Similar results were obtained in two other experiments.



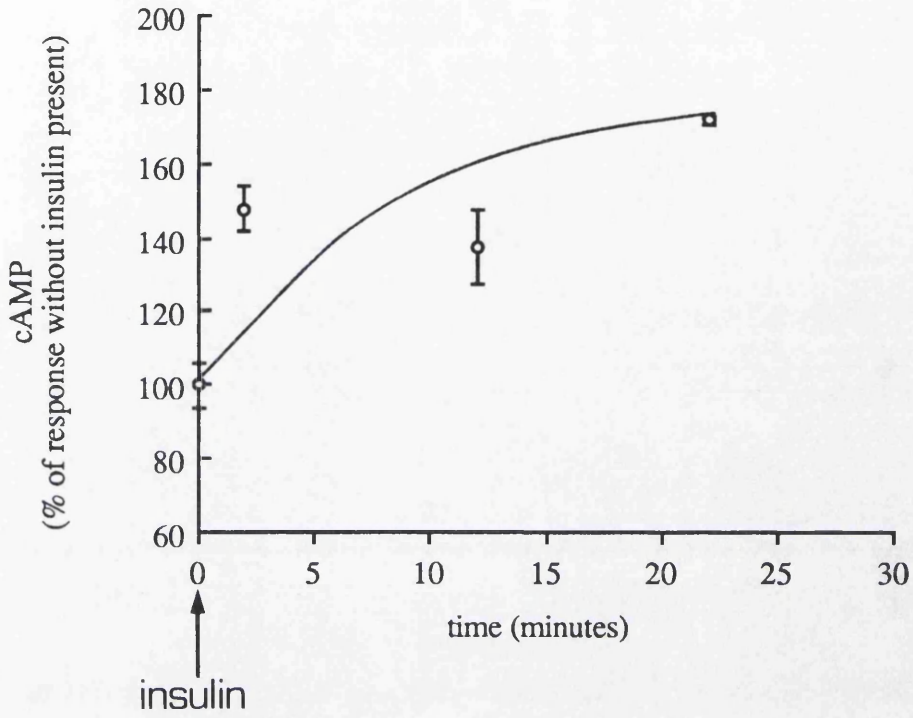
**Figure 5.5**



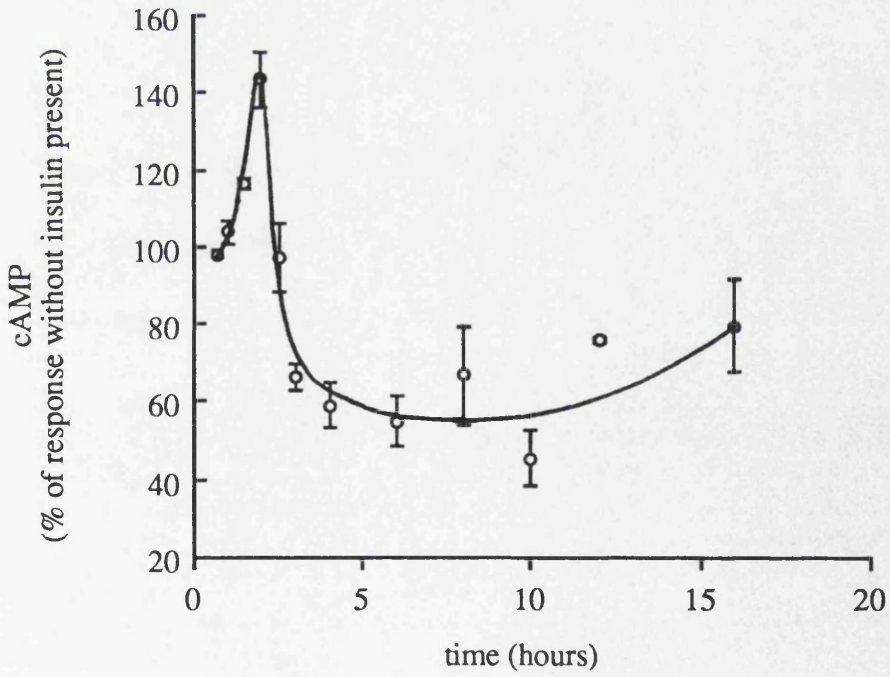
**Fig. 5.6      Time course for insulin's (1nM) modulation of  
PGE<sub>1</sub> elevated intracellular cAMP in P9 cells.**

P9 cells were incubated with serum-free medium alone (control) or containing insulin (1nM). At intervals cells were challenged with PGE<sub>1</sub> (10<sup>-5</sup>M for 2 minutes) and cAMP extracted and assayed as described in section 2.14. Results are plotted as the the response with insulin present as a percentage of the control response, in order to allow for any change in response over time, on exposure to serum-free medium alone. In Fig 5.6a. data are given for time points between zero and 22 minutes and in Fig. 5.6b. data for time points between 22 minutes and 16 hours. Enhancement of the response by insulin was 1.72±0.01 fold at 22 minutes (p < 0.001). The response at 10 hours was 45.5±0.8% of the control response (p < 0.05). Data are mean±SEM for triplicate observations in one experiment. Similar results were obtained in another experiment.

**Figure 5.6 (a)**



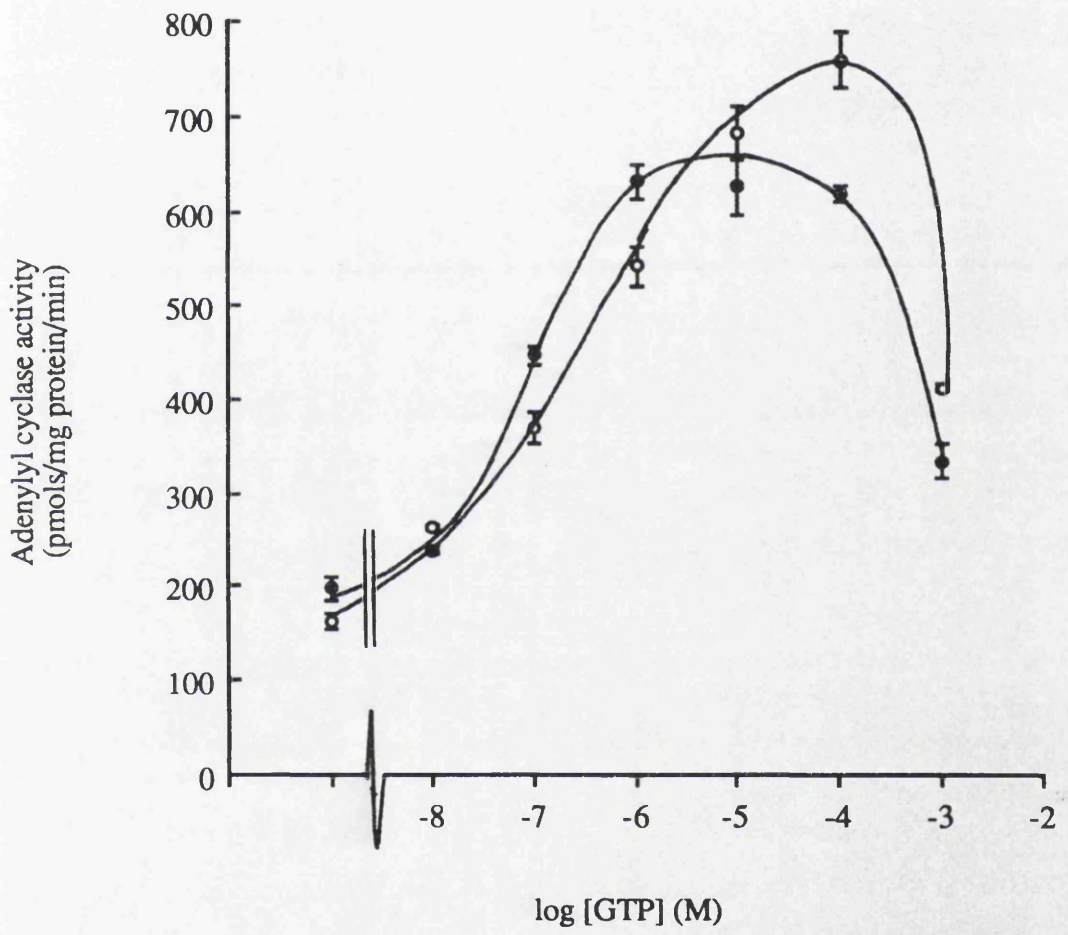
**Figure 5.6 (b)**



**Fig. 5.7**      **Effect of increasing concentrations of GTP on**  
**PGE<sub>1</sub>-stimulated adenylyl cyclase activity, after**  
**exposure of P9 cells to insulin (1nM, 16 hours).**

P9 cells were exposed for 16 hours to serum-free medium, alone (open circles) or containing insulin (1nM) (closed circles), harvested, membranes prepared and assayed for adenylyl cyclase activity in the presence of increasing concentrations of GTP (0-10<sup>-3</sup>M) and a constant concentration of PGE<sub>1</sub> (10<sup>-5</sup>M) as described in section 2.13. Adenylyl cyclase activity in the absence of insulin exposure was 162±9 and with insulin exposure was 198±13 pmols/mg protein /min. Stimulation parameters are given in Table 5.2. Data are mean±SEM for triplicate observations in one experiment. Similar results were obtained in two other experiments.

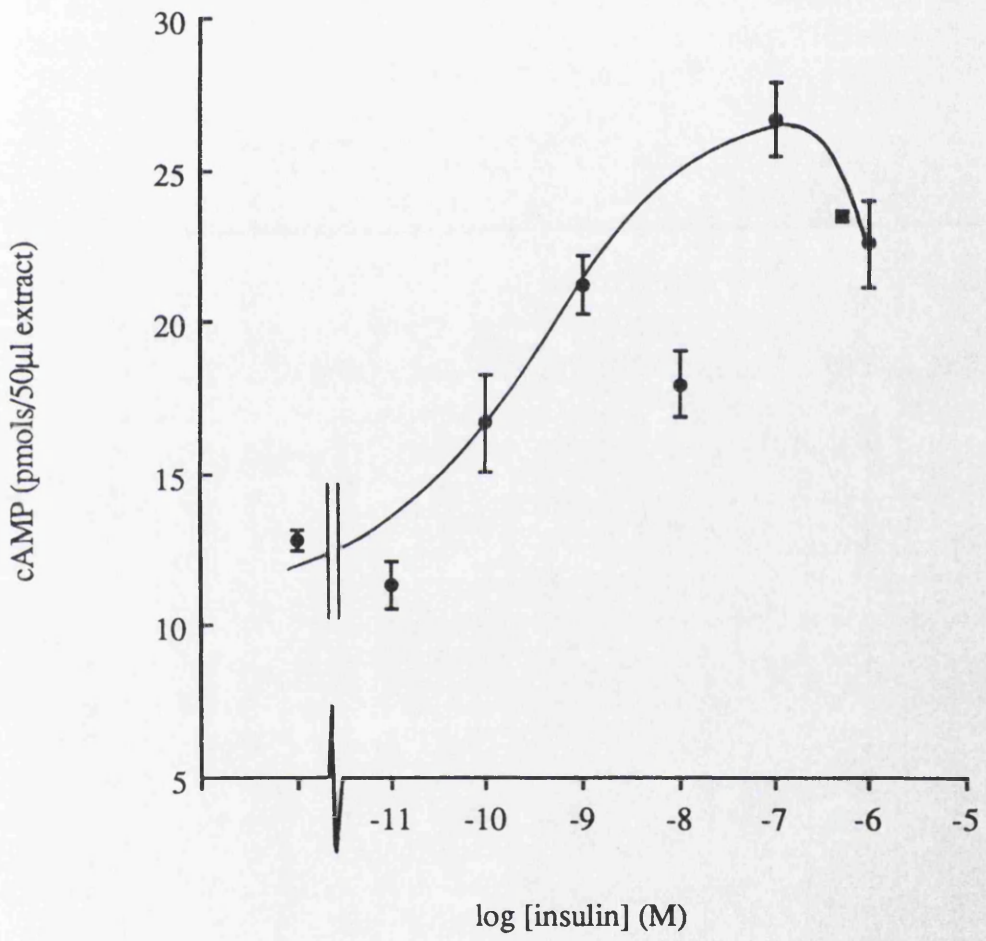
**Figure 5.7**



**Fig. 5.8**      **Effect of increasing concentrations of insulin**  
**(10 minutes exposure) on PGE<sub>1</sub>-elevated**  
**intracellular cAMP in P9 cells.**

P9 cells were washed with serum-free medium and exposed to insulin ( $0-10^{-6}$ M) for 10 minutes before challenge with PGE<sub>1</sub> ( $10^{-5}$ M for 2 minutes) and cAMP extracted and assayed as described in section 2.14. Maximal enhancement of the response by insulin was  $2.08 \pm 0.10$  (fold value without insulin present). The K<sub>0.5</sub> value for insulin was  $5.0 \times 10^{-10}$ M. Data are mean  $\pm$  SEM for triplicate obtained in one experiment. Similar results were obtained in two other experiments and in one experiment in which cells were pre-incubated with IBMX (1mM, 15 minutes).

**Figure 5.8**

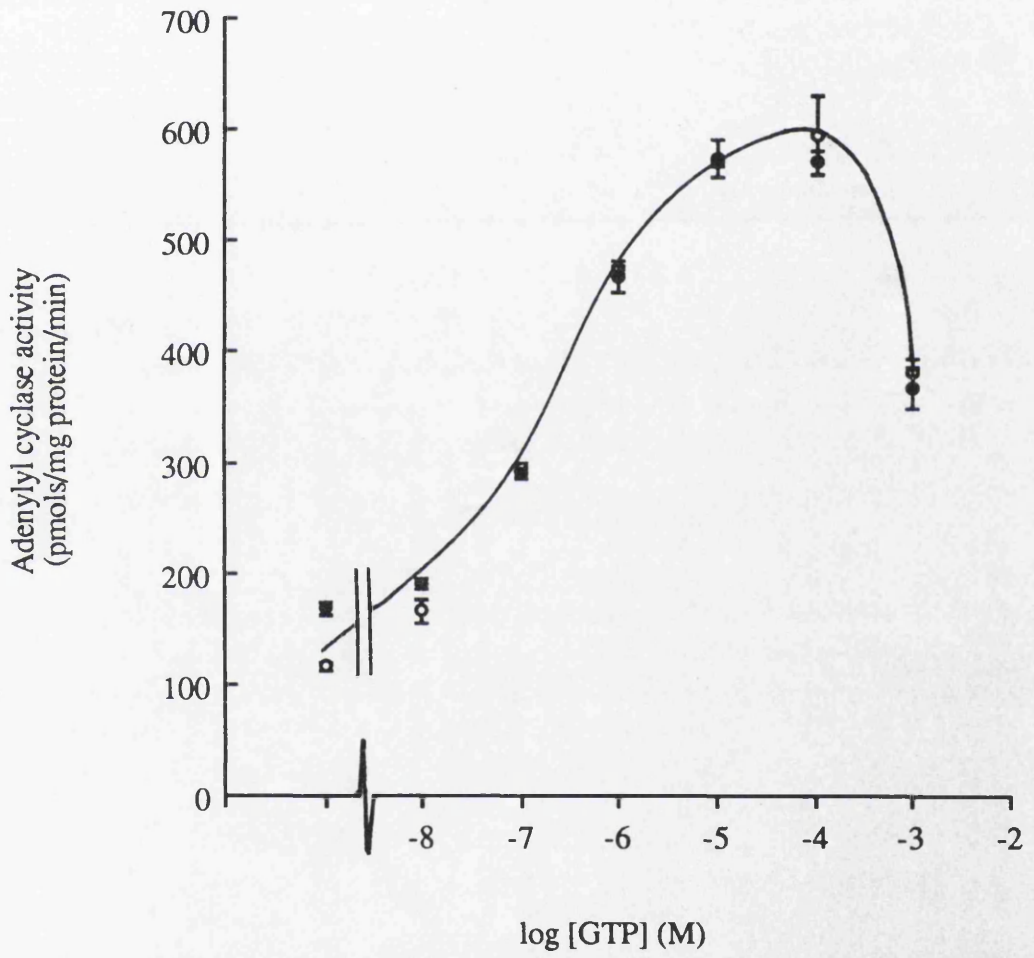


**Fig. 5.9      Effect of increasing concentrations of GTP on PGE<sub>1</sub>-stimulated adenylyl cyclase activity, after exposure of P9 cells to insulin (1nM, 10 minutes).**

P9 cells were exposed for 10 minutes to serum-free medium, alone (open circles) or containing insulin (1nM) (closed circles), harvested, membranes prepared and assayed for adenylyl cyclase activity in the presence of increasing concentrations of GTP (0-10<sup>-3</sup>M) and a constant concentration of PGE<sub>1</sub> (10<sup>-5</sup>M) as described in section 2.13. Adenylyl cyclase activity in the absence of insulin exposure was 115±3 and with insulin exposure was 168±6 pmols/mg protein/min. Stimulation parameters are given in Table 5.2. Data are mean±SEM for triplicate observations in one experiment. Similar results were obtained in two other experiments.



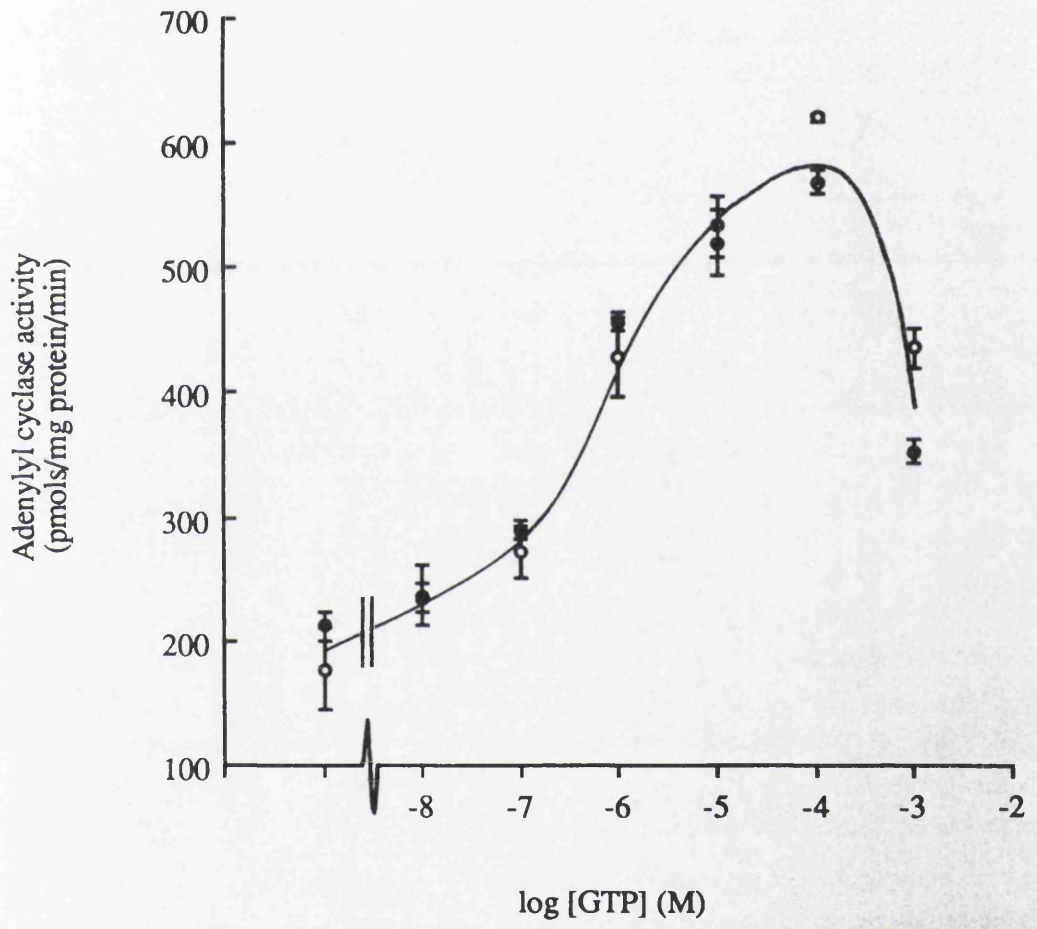
**Figure 5.9**



**Fig. 5.10      Effect of increasing concentrations of GTP on PGE<sub>1</sub>-stimulated adenylyl cyclase activity in the presence of insulin.**

P9 cells were harvested, membranes prepared and assayed for adenylyl cyclase activity in the presence of increasing concentrations of GTP (from 0 to  $10^{-3}$ M) and a constant concentration of PGE<sub>1</sub> with (closed circles) or without (open circles) insulin ( $10^{-6}$ M) present in the assay. Adenylyl cyclase activity without insulin present was  $177 \pm 33$  and with insulin was  $212 \pm 12$  pmols/mg protein /min. Stimulation parameters are given in Table 5.2. Data are mean  $\pm$  SEM for triplicate observations in one experiment. Similar results were obtained in two other experiments.

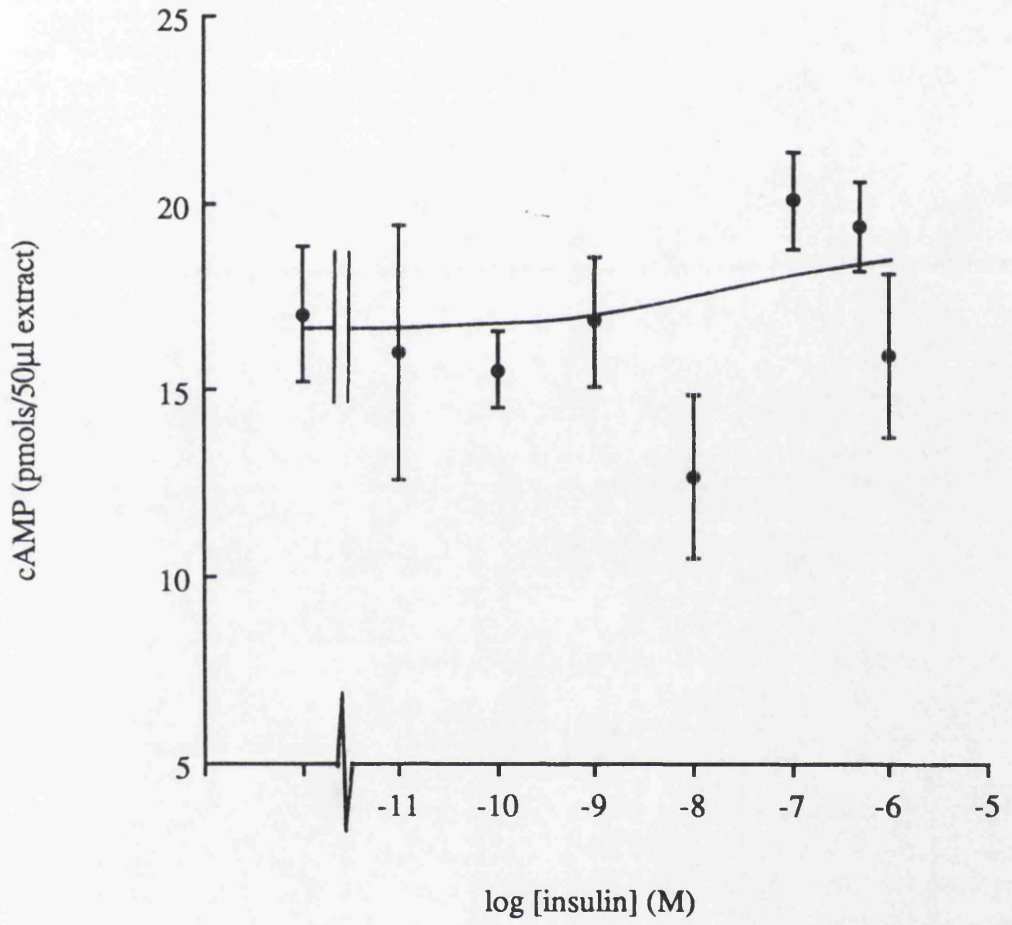
**Figure 5.10**



**Fig. 5.11      Effect of increasing concentrations of insulin  
(10 minutes exposure) on forskolin-elevated  
intracellular cAMP in P9 cells.**

P9 cells were washed with serum-free medium, exposed to insulin at increasing concentrations for 10 minutes in each case then challenged with forskolin ( $10^{-5}\text{M}$  for 2 minutes). Cyclic AMP was extracted and assayed as described in section 2.14. Data are mean $\pm$ SEM for triplicate observations in one experiment. Similar results were obtained in another experiment.

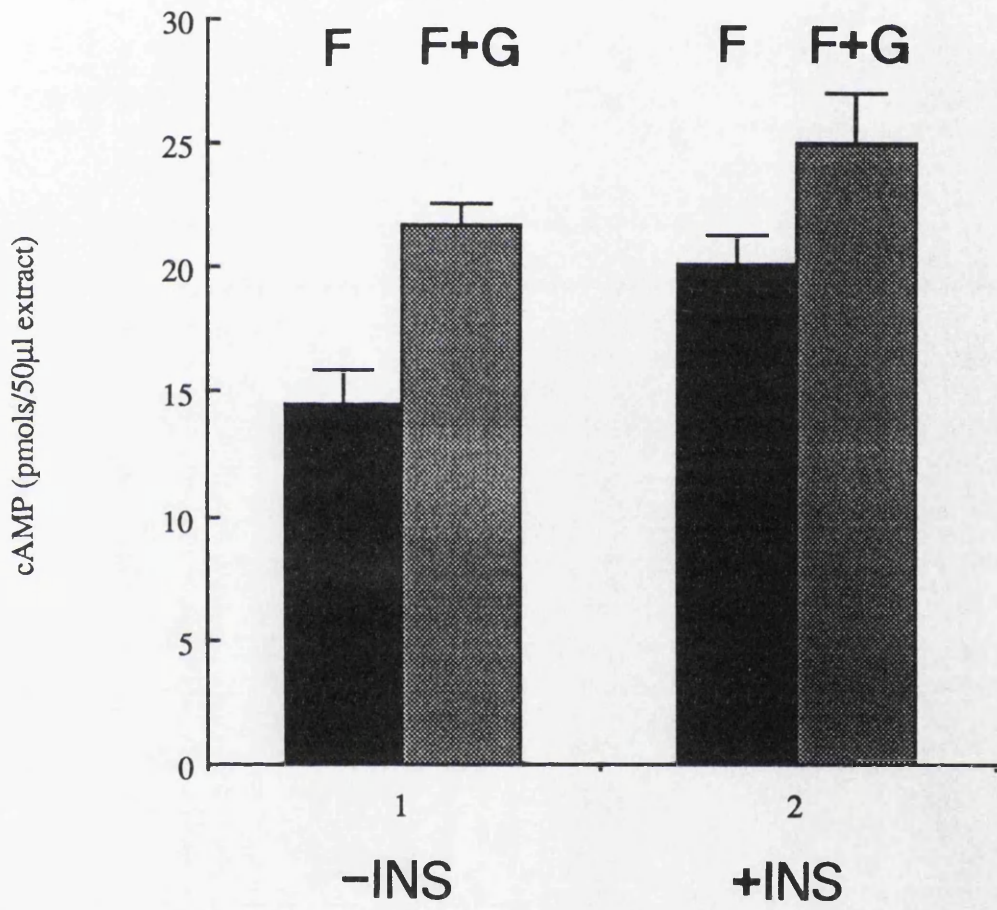
**Figure 5.11**



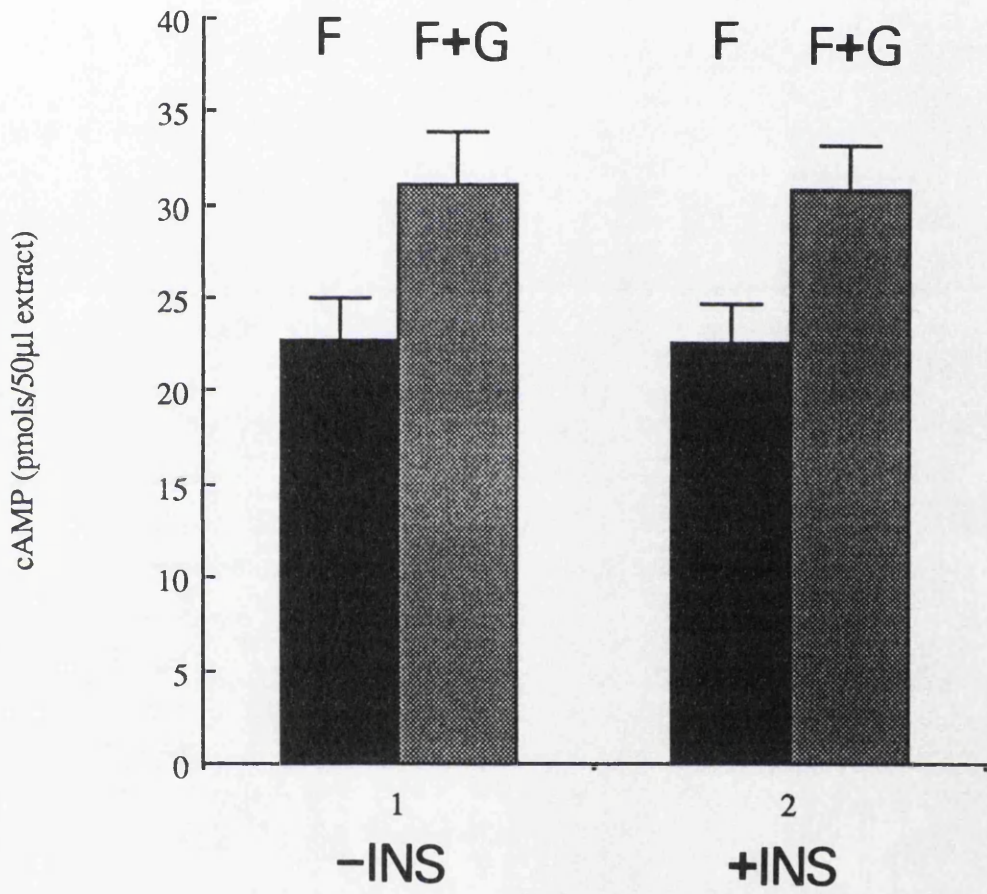
**Fig. 5.12      Effect of insulin exposure on glucagon- and forskolin-elevated intracellular cAMP in P9 cells.**

P9 cells were exposed to serum-free medium alone (column 1) or containing insulin (column 2), 1nM for 16 hours (Fig 5.12a.), 1nM for 10 minutes (Fig 5.12b.) or 1 $\mu$ M for 10 minutes (Fig 5.12c.) then challenged with forskolin ( $10^{-5}$ M) for 2 minutes (black bars) or with forskolin ( $10^{-5}$ M) and glucagon ( $10^{-8}$ M) combined for 2 minutes (grey bars). Cyclic AMP was extracted and assayed as described in section 2.14. Data are mean $\pm$ -SEM for triplicate observations in one experiment. Similar results were obtained in two other experiments and one experiment in which cells were pre-incubated with IBMX (1mM, 15 minutes).

**Figure 5.12 (a)**

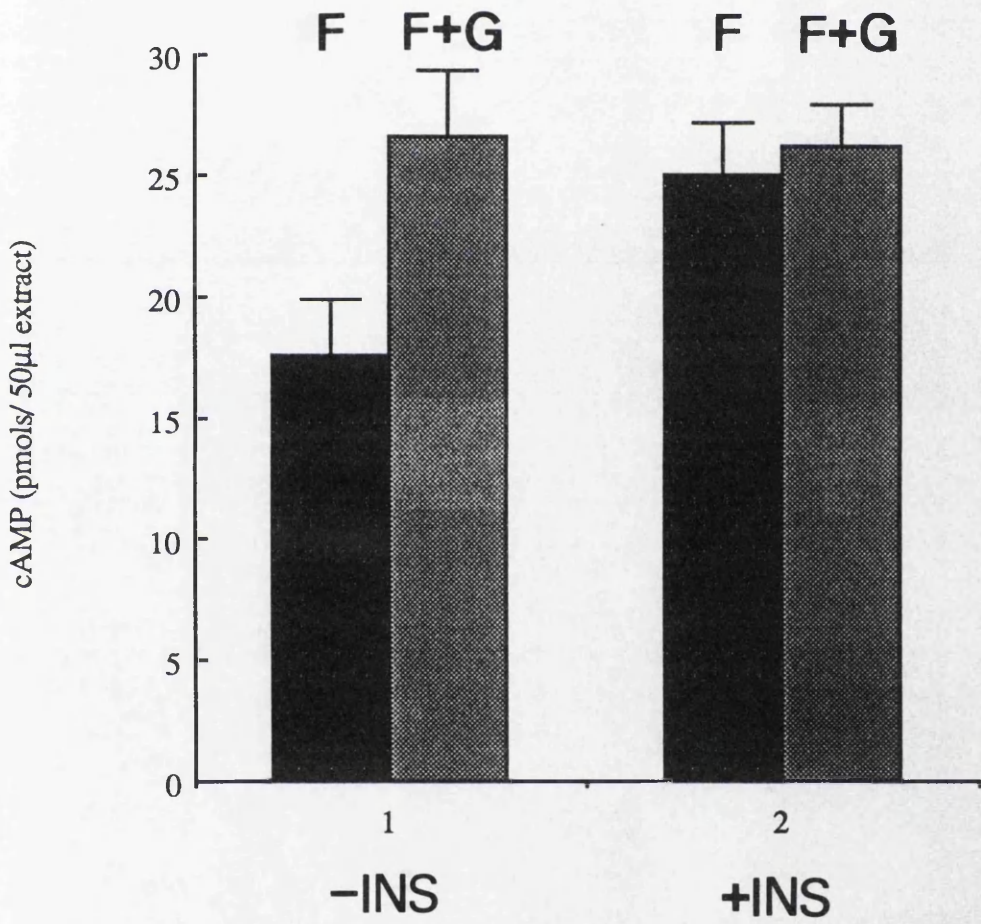


**Figure 5.12 (b)**





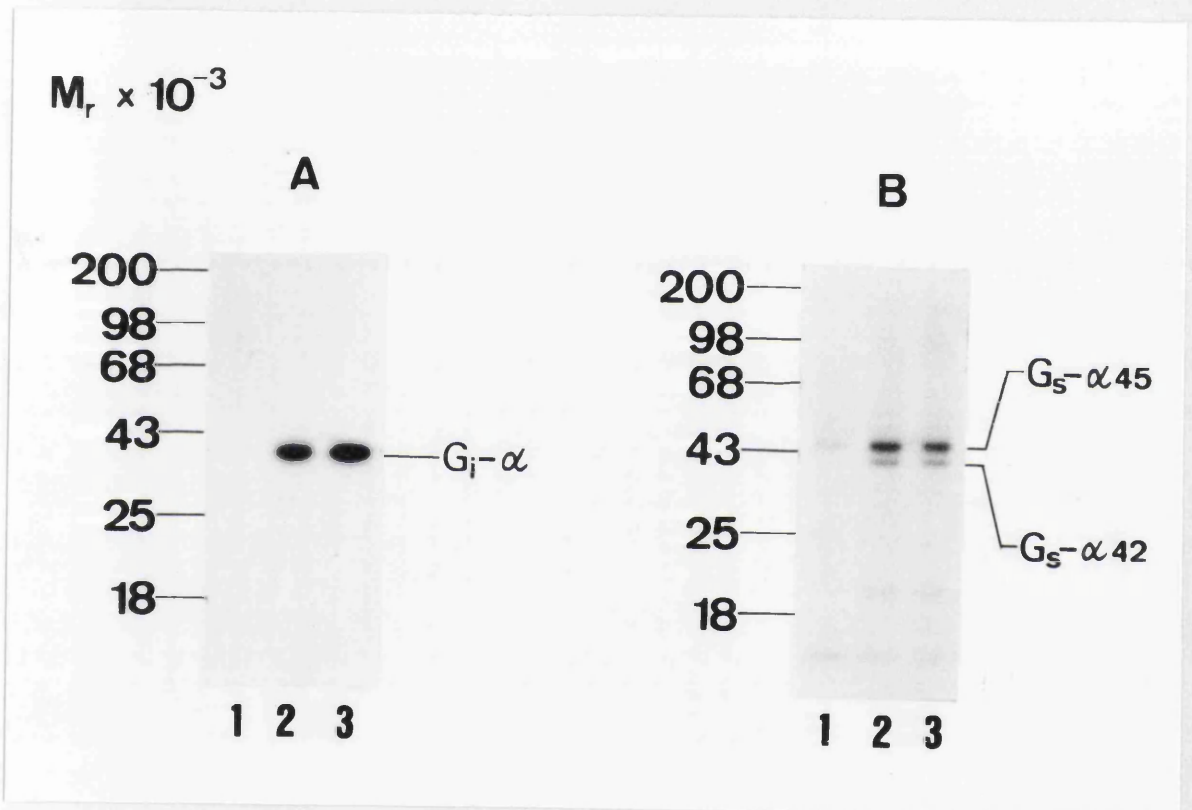
**Figure 5.12 (c)**



**Fig. 5.13      Effect of insulin exposure (1nM, 16 hours) on toxin labelling of G-proteins in P9 cell plasma membranes.**

P9 cells were exposed for 16 hours to serum-free medium alone (lane 1) or containing insulin, 1nM (lane 2) then harvested and plasma membranes prepared and subjected to toxin labelling in the presence of pertussis toxin (panel A) and cholera toxin (panel B) as described in section 2.12. Samples were resolved and gels dried, autoradiographed and scanned as described in section 2.10. There was no labelling in the absence of toxins. Densitometric data are presented in Table 5.1. Data shown are from one experiment. Similar results were obtained in two other experiments.

**Figure 5.13**

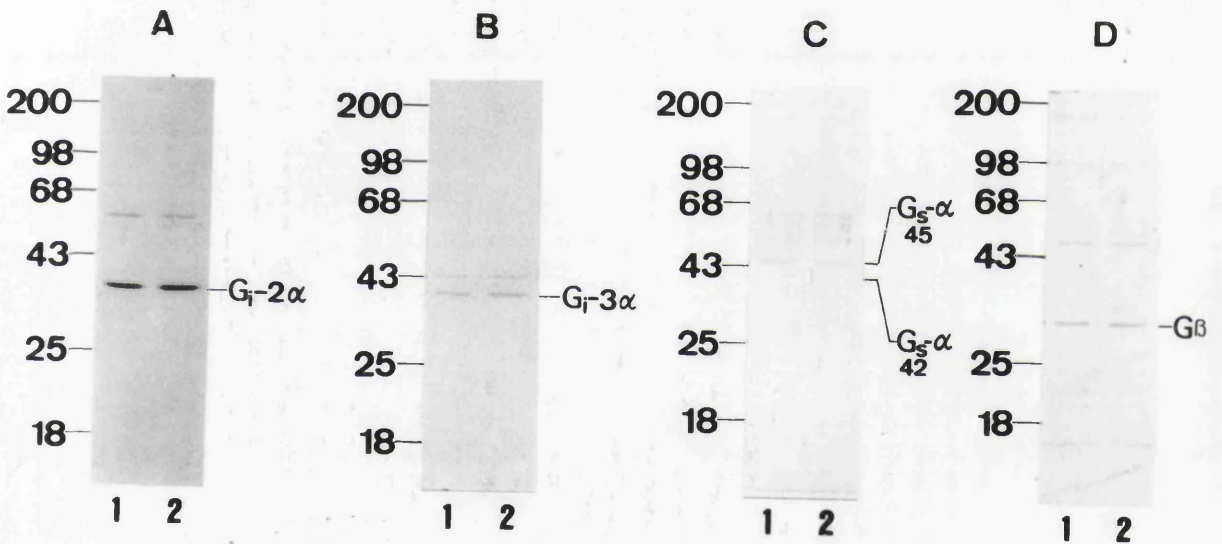


**Fig. 5.14      Effect of insulin exposure (1nM, 16 hours) on  
G-protein levels in P9 cell plasma membranes.**

P9 cells were exposed for 16 hours to serum-free medium alone (lane 1) or containing insulin, 1nM (lane 2) then harvested and plasma membranes prepared. Samples of membrane protein (100 $\mu$ g) were resolved using SDS PAGE, transferred onto nitrocellulose paper and probed with antisera as described in section 2.11. The antisera used were 1432 (panel A), I3C (panel B), CS1 (panel C) and BN3 (panel D). Blots were scanned densitometrically and results presented in 5.1. Data shown are from one experiment. Similar results were obtained in two other experiments.

**Figure 5.14**

$M_r \times 10^{-3}$



## **Chapter 6**

**G-protein expression and adenylyl cyclase activity in  
mononuclear leucocytes from type 2 diabetic subjects**

## 6.1 Introduction

As discussed in section 1.5.2, various studies have shown alterations in G-protein expression and function in animal models of type 1 and type 2 diabetes. This is a phenomenon which has been relatively poorly studied in humans mainly because of the difficulty in obtaining adequate amounts of insulin-sensitive tissues (*viz.* liver, adipose tissue and muscle) for study. In addition, the findings from studies carried out in small samples particularly of type 2 diabetic patients can be difficult to interpret because of the heterogeneous nature of the disorder itself and other factors which may contribute to the changes observed eg. the effects of any therapy (medication or other) and the presence of co-existing disorders which must be taken into account. Insulin's target tissues are the most useful for studies of signal transduction in diabetes as it is in these tissues that insulin's regulation of metabolism is best understood and any defects found will presumably have important consequences for the metabolic status of these tissues. However, only small amounts can be obtained by sampling under local anaesthetic, which limits the number of measurements that can be made. Larger amounts can be obtained at time of elective surgery although any diabetic undergoing elective surgery is likely to have their condition well controlled by anti-diabetic therapy. Consequently, many studies have used blood cells from diabetic subjects to examine signalling and other aspects of cell function rather than using insulin-sensitive tissues, as these samples are readily obtained in sufficient quantities for analysis.

Leucocyte function is of particular relevance to diabetes, as both type 1 and type 2 diabetics are known to have a predisposition to infection which accounts for a considerable amount of morbidity and mortality associated with the disease (Ganda, 1983) although the reasons for this reduction in immune function are still unclear. There are various reports from animal models of diabetes of abnormalities in both humoral (Fletcher-McGruder *et al.*, 1984) and cell-mediated (Pasko *et al.*, 1981)

immunity. As there is evidence for activation of both lymphocytes (Roper *et al.*, 1990) and neutrophils (Reibman *et al.*, 1990) involving G-protein-mediated signal transduction, it is possible that defective cyclase regulation may contribute to the impairment of immune function in diabetes. Since defects noted in cyclase signalling in insulin sensitive tissues from animal models go some way to explaining the pathophysiology of the condition, leucocytes certainly merit examination to consider whether cyclase signalling may play a role in their altered physiology.

This study had initially aimed to examine G-protein expression and adenylyl cyclase activity in neutrophil plasma membranes to examine whether any change in the function in this signalling system might be contributing to the altered physiology of these cells in diabetic patients. Neutrophils have the advantage that assays of their function are readily available (Shah *et al.*, 1983) and neutrophils from diabetic subjects are known to have defective functioning (Ganda, 1983). However, despite the liberal use of protease inhibitors during membrane preparation, a problem with proteolysis of  $G_i$  was encountered, to a degree which would have prevented reliable quantification of this protein. This section of work was therefore abandoned. Consequently, the study was limited to examination of G-protein expression and adenylyl cyclase activity in mononuclear leucocyte plasma membranes the results of which are presented here.

Leucocytes for study were separated only into two main fractions, that containing granulocytes (about 66% of leucocytes in whole blood) *viz.* neutrophils (neutrophil polymorphonuclear leucocytes), basophils and eosinophils and a fraction containing mononuclear leucocytes *viz.* lymphocytes (30%), monocytes (1-3%) (Hunt, 1987). Further separation of leucocytes for specific functional studies can be achieved by density gradient centrifugation to remove monocytes and cell sorting to separate T (~70%) and B (~30%) lymphocytes but these techniques were not employed in this study. Whilst lymphocytes are predominant in the fraction studied, monocytes are also



present and contribute to the data obtained.

Type 2 diabetic patients were chosen for study as it is relatively easy to recruit either newly diagnosed patients, before commencement of treatment or previously diagnosed patients who are receiving no treatment (other than dietary) at the time of sampling. It was particularly important that the patients should be free from infection at the time of sampling, as T lymphocyte activation is known to be accompanied by increases in G<sub>s</sub> and G<sub>i</sub> expression and enhanced cAMP responsiveness to E series prostaglandins (Holter *et al.*, 1991). The investigation was designed as a pilot study to screen type 2 diabetics for gross changes in functioning of the second messenger system which could later be followed up if desired. In order to have adequate plasma membrane protein to carry out more detailed studies would have meant taking unreasonably large blood samples and unreasonable expense in terms of cyclase assay materials. Therefore due to the small amount of membrane protein available from each subject the study did not examine closely the effect on G<sub>i</sub> function or responses to a large range of agonists but rather, by examining a reasonably large number of patients, aimed to observe whether there were any marked changes in G-protein expression or agonist-stimulated adenylyl cyclase activity which could later be pursued.

## 6.2 Results

As shown in Table 6.1. all type 2 diabetic patients had plasma glucose levels diagnostic of diabetes (The hospital quote a reference range for plasma glucose of 2.8-6.0mmols/l). In addition, the 10 male diabetics had a mean HbA<sub>1c</sub> (glycosylated haemoglobin) of 9.4+/-2.8% (SEM) and the 10 female diabetics had a mean HbA<sub>1c</sub> of 10.8+/-4.2%. This measurement is indicative of persistently elevated blood glucose levels during a period of about three months before sampling. (Normally about 6% of the total haemoglobin is glycosylated). From these measurements it was clear that the subjects selected were indeed diabetic and suitable for study.

Experiments were initially carried out to find the range of leucocyte plasma membrane protein over which increasing protein concentration yielded increasing band density on immunoblotting (Figs. 6.1-6.3). Using the three antisera, 1432 for Gi-2 $\alpha$ , CS2 for G<sub>s</sub>- $\alpha$  and BN1 for G-protein  $\beta$  subunit, signals were obtained which increased up to 200 $\mu$ g of membrane protein. The 1432 antiserum is specific for Gi-2 $\alpha$  in mononuclear leucocytes as they, in common with neutrophils and platelets, do not express Gi-1 (Holter *et al.*, 1991). Probing blots with the CS2 antiserum yielded a band at 42kDa corresponding to the G<sub>s</sub>- $\alpha$  form expressed by leucocytes. Probing blots with the BN1 antiserum yielded a band at 35kDa corresponding to the G-protein  $\beta$  subunit.

Leucocyte plasma membranes from 20 control and 20 diabetic patients were then subjected to immunoblotting using these specific antisera (Figs. 6.4 and 6.5). In view of the limited membrane protein available, the amount of protein chosen for blotting was 100 $\mu$ g. No significant differences were observed in G-protein levels between leucocyte plasma membranes obtained from control and diabetic subjects in either the male or female groups. However, in the case of Gi-2 $\alpha$  alone, larger signals

on average were obtained from female than from male subjects by a factor of 1.7 in the control group and 1.4 in the diabetic group. This difference was only statistically significant ( $p < 0.05$ ) in the control group. There was no significant correlation between G-protein levels observed and the subjects age, except in the case of the female diabetics where there was a weak positive correlation between  $G_s\text{-}\alpha$  expression and age ( $r = 0.70$ ,  $p < 0.05$ ).

Adenylyl cyclase activities were compared in leucocyte plasma membranes from all control and diabetic subjects (Fig. 6.6). There was a reduction in the ability of PGE<sub>1</sub> to stimulate adenylyl cyclase in leucocyte plasma membranes from male ( $p < 0.05$ ) but not female diabetic subjects. There was however no significant difference in the fold stimulation of adenylyl cyclase activity over the activity observed with GTP alone by PGE<sub>1</sub> or forskolin. No differences were observed between control and diabetic groups for any of the other agonists used *viz.* GTP ( $10^{-4}\text{M}$ ), forskolin ( $10^{-4}\text{M}$ ) and forskolin and GTP combined at the same concentrations. However, considerable sex differences were noted in adenylyl cyclase activity. Lower cyclase activity was observed in leucocyte plasma membranes from female subjects, both control and diabetic, for all agonists used ( $p < 0.001$ ). On examining values for fold stimulation over the cyclase activity with GTP present, this sex difference was retained only in the case of the diabetic patients. No correlation was observed between age and adenylyl cyclase activity for any agonist used, in any of the subject groups.

### 6.3 Discussion

Plasma membranes from leucocytes were shown to contain Gi-2 $\alpha$ , G<sub>s</sub>- $\alpha$  (42kDa form) and G-protein  $\beta$  subunit. Holter *et al.* (1991) showed using their antiserum to G<sub>s</sub>- $\alpha$  that resting lymphocytes (the predominant cell type examined here) expressed a 42kDa form of G<sub>s</sub>- $\alpha$  and also gave a very faint signal at 47kDa, both of which increased upon cell activation. Our failure to detect this higher molecular weight form in plasma membranes from any of the subjects may reflect a lower sensitivity of our detection system or a difference in the specificity of our antiserum.

In the study comparing control and diabetic subjects yielded we found that there was a significant reduction in PGE<sub>1</sub>-stimulated adenylyl cyclase in leucocyte plasma membranes from male diabetics. In considering this finding we must bear in mind the reservations discussed in Chapter 5 when comparing cyclase activities between different membrane preparations. Indeed less difference was observed between the groups on comparing fold stimulation by the agonist which implies that the effect is less significant than the statistics on the raw data would suggest. However, a large number of membrane preparations were examined here which would tend to reduce the influence of such errors. The change can be proposed to be either at the level of the prostanoid receptor or at the level of coupling to G<sub>s</sub>. It was clearly not due to any change in G-protein expression as has been suggested as the cause of alteration in cyclase signalling in some animal models of diabetes. The absence of any difference between control and diabetic groups on measuring the response to GTP alone would suggest that the difference is at the level of the prostanoid receptor rather than at a more distal point in the system though the smaller responses in the presence of GTP alone may not have been large enough to make any change in G<sub>s</sub> function apparent. There was no evidence for a change in forskolin responsiveness to suggest an additional effect of the diabetic state at the adenylyl cyclase catalytic subunit.

As discussed in section 1.4.1.2, there is extensive evidence that E series prostaglandins have a role in regulation of immune responsiveness. Thus there is certainly reason to believe that the reduction in PGE<sub>1</sub>-stimulated adenylyl cyclase observed in the male subjects might have consequences for the immune functioning of these leucocytes. However, the absence of any similar change in female patients makes one cautious in interpreting this result as male type 2 diabetics are no more or less prone to infection than female diabetics. This finding is particularly interesting in light of the work of Kahn and Sinha (1990) which suggested that at least in platelets, PGE<sub>1</sub> and insulin may modulate each others function by influencing binding of these agonists to their respective receptors. Perhaps a similar mechanism is at work in leucocytes where a reduction of insulin's influence in diabetes manifests itself in the adenylyl cyclase signalling system as a reduction in PGE<sub>1</sub> responsiveness. Unlike the findings in platelets however, this change presumably has no important consequences for cell physiology and simply represents an epiphenomenon associated with the diabetic state, although it would be interesting to substantiate this by measurements of lymphocyte immune functioning.

As a similar reduction in PGE<sub>1</sub> responsiveness has been observed in platelets from male type 2 diabetics (Livingstone *et al.*, 1991) it is interesting to speculate that males may have a genetic predisposition to this effect of diabetes on cyclase signalling. Perhaps the reduction in PGE<sub>1</sub> responsiveness represents a widespread phenomenon associated with insulin resistance. If this is the case, then in adipose tissue where the prostanoid receptor couples to G<sub>i</sub>, a reduction in PGE<sub>1</sub> responsiveness would tend to enhance intracellular cAMP accumulation in response to stimulatory agonists and therefore promote lipolysis. Indeed, a decreased antilipolytic response to PGE<sub>1</sub> noted in adipocytes from diabetic rats has been proposed to result from a decrease in G<sub>i</sub> function (Green and Johnson, 1991). This would be worthwhile to consider in any subsequent study undertaken on human adipose tissue or any other tissues from diabetic subjects.

One study carried out on adipocytes from type 1 diabetic subjects showed no change in G-protein expression (Ohisalo *et al.*, 1989). However, the study was carried out on only a small number of patients and did not examine adenylyl cyclase activity in the membrane preparations. Until extensive studies have been done on insulin sensitive tissues from both type 1 and type 2 diabetic subjects, it is not possible to say how closely the situation in humans resembles that in animal models.

In view of the size of the sex differences observed in both control and diabetic groups and that they were observed both on cyclase activity and  $G_i-2\alpha$  levels, it seems unlikely that the changes were artifacts. The spread of ages between the two groups selected was reasonably similar so we cannot suggest that the sex differences are accounted for by an uneven comparison of age groups. In addition, all samples were processed in an identical manner. A study by Bouvier *et al.* (1991) on pituitary G-protein expression in rats noted that oestrogen treatment caused a significant reduction in G-protein levels with the exception of  $G_i-1\alpha$  and the 42kDa form of  $G_s-\alpha$ . Levels of these polypeptides varied during phases of the oestrous cycle and levels of  $G_o-\alpha$ ,  $G_i-3\alpha$ ,  $G\beta$  and the 47kDa form of  $G_s-\alpha$  were significantly lower in female than in male rats. These changes were not observed in corpus striatum showing that they were tissue specific. It is clear that ovarian hormones can influence G-protein levels and may account for sex differences in expression observed in animals. Here however, the majority of the female subjects were post-menopausal, implicating genetic factors rather than ovarian hormones in the aetiology of the sex differences in G-protein expression. It is interesting to speculate that the higher level of  $G_i-2\alpha$  expression in cells from female subjects may be responsible for the lower adenylyl cyclase activity observed with all agonists as this is the  $G_i-\alpha$  subtype thought to be responsible for adenylyl cyclase inhibition. Presumably these sex differences in the second messenger system have no effect on physiological functioning of the cells. In this regard it would be interesting to know in detail the roles of particular G-protein subtypes in leucocyte

function and the precise levels required for normal functioning. In addition it would be interesting to follow up this finding by studying G-protein expression in other tissues from both sexes.

Age differences in adenylyl cyclase functioning have been reported. Krall *et al* (1981) noted an increase in plasma catecholamine levels in elderly subjects and a reduction in lymphocyte adenylyl cyclase responsiveness. Abrass and Scarpace (1982) obtained similar results and concluded that the reduction in activity was at the catalytic subunit and that there was no change in G-protein function. However, their study only examined male subjects. Here we have observed no such correlation with age, although the study was not designed primarily to address this question and because of the nature of type 2 diabetes, the majority of subjects were elderly. In addition to the subjects sex, it may be that there is another factor which we have not taken into account, common to many of the subjects of one sex and responsible for the difference in cyclase regulation. Possibilities include body weight, dietary habits and self medication.

It is worthwhile to consider other follow-up clinical studies which could be done in the light of the findings obtained here from diabetic subjects. Firstly, in leucocytes it would be interesting to obtain adequate samples to examine the level of prostanoid receptor binding to see whether the impaired response to this agonist in male subjects represents a reduction in the level of prostanoid receptor or whether there is abnormal coupling between components of the signalling system. It would be interesting to follow up some of these patients after treatment, particularly if their clinical condition had improved and a normal blood glucose had been achieved for a prolonged period. All these studies would again require to be done on a reasonably large number of patients for the reasons discussed above.

Type 2 diabetes is associated with insulin resistance in peripheral tissues as a result of these subjects having supraphysiological levels of glucose despite normal or elevated insulin levels. In view of this it would be worthwhile, by measuring the plasma insulin level and pancreatic beta cell reserve, to observe whether the reduced PGE<sub>1</sub> stimulation of cyclase correlates with the degree of insulin resistance. The study on neutrophils could be repeated using an alternative form of membrane preparation and studies of whole cell cAMP elevation in response to agonist could be carried out on freshly isolated cells. In order to assess G-protein levels a chosen number of neutrophils from each patient could be solubilised directly in SDS to denature the proteases and samples subjected directly to immunoblotting.

The study could be extended to include type 1 diabetics, ideally patients who have been newly diagnosed and before the commencement of insulin treatment. These subjects have the advantage that they are of a younger age group and have fewer coincidental medical conditions although, for practical reasons, samples from these patients are more difficult to obtain for study. Thus there are many ways in which the work could be extended in order to clarify the effects of diabetes on cyclase signalling and the role of such defects in the pathology of the condition.



**Table 6.1      Plasma glucose measurements from control and type 2 diabetic subjects.**

Fasting plasma glucose was estimated in 20 control (10 male and 10 female) and 20 type 2 diabetic (10 male and 10 female) subjects. Age ranges of the subjects in each group are given in brackets. Data shown are mean+/-SEM for the 10 subjects in each group.

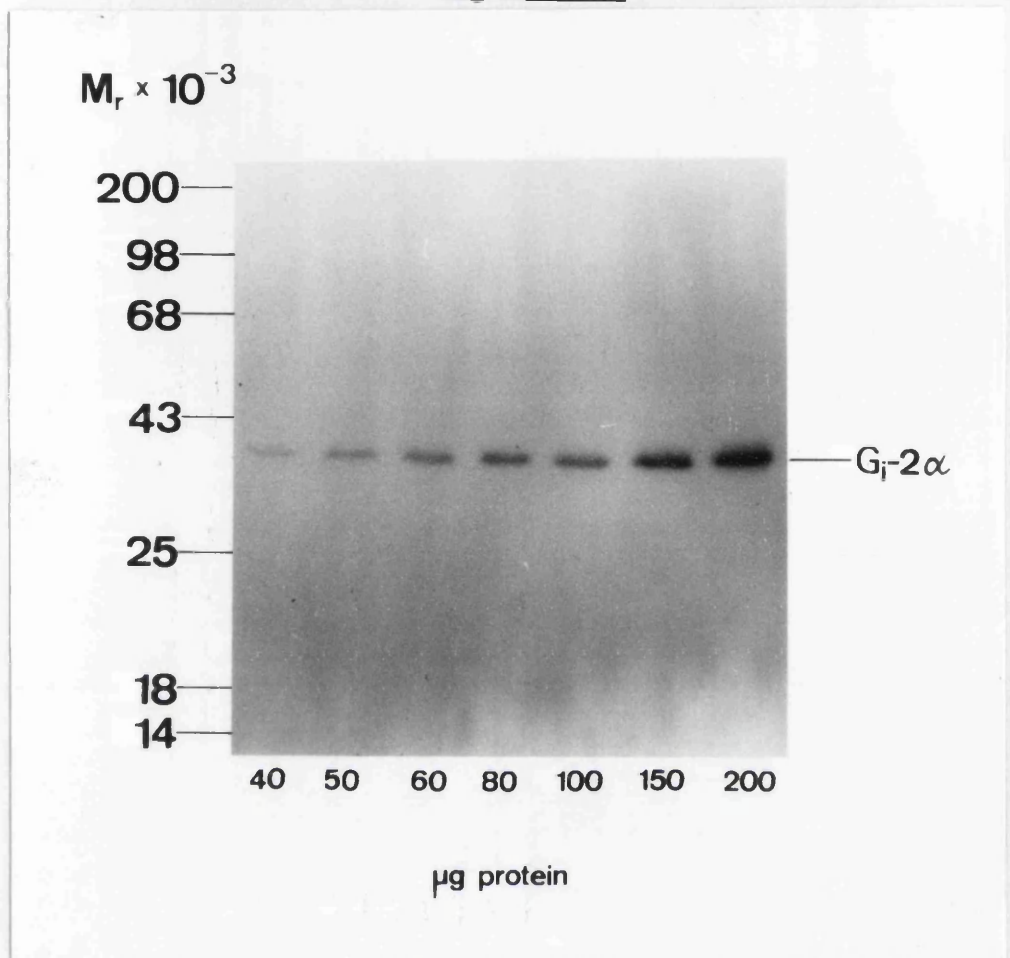
**Table 6.1**

Subject group	Plasma glucose level (mmols/l)
Male controls (35-74)	5.5+/-0.8
Male diabetics (30-73)	12.3+/-4.8
Female controls (38-72)	5.2+/-0.5
Female diabetics (40-77)	10.9+/-4.2

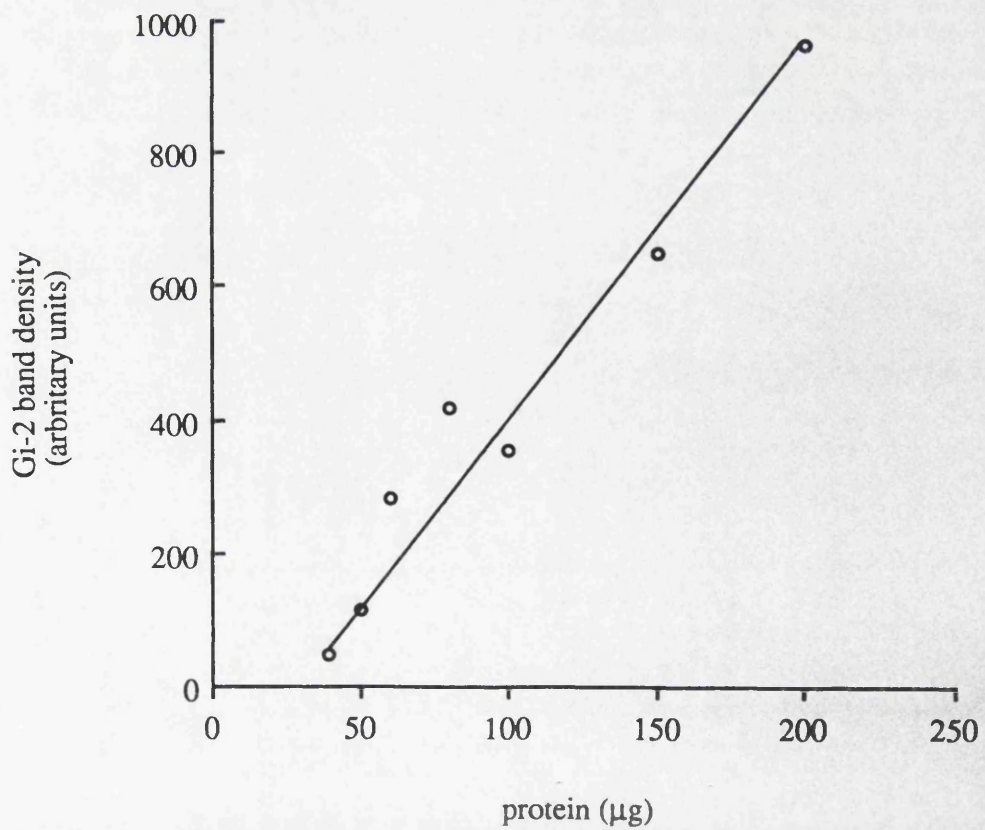
**Fig. 6.1**      **Immunological identification of Gi-2 $\alpha$  in mononuclear leucocyte plasma membranes.**

Samples of leucocyte plasma membrane protein (40-200 $\mu$ g) were TCA precipitated and resolved using SDS PAGE on a 10% acrylamide/0.27% bisacrylamide gel as described in section 2.10 then transferred onto nitrocellulose membrane as described in section 2.11. Immunoblotting was performed using a 1: 200 dilution of antiserum 1432. Detection of the primary antiserum was achieved using  $^{125}$ I-labelled anti-rabbit IgG (2 $\mu$ Ci/50ml) (Fig.6.1a). Bands were scanned densitometrically and results plotted graphically (Fig. 6.1b) using arbitrary units for band density. Data shown are from one experiment.

**Figure 6.1 (a)**



**Figure 6.1 (b)**



**Fig. 6.2**      **Immunological identification of G<sub>s</sub>-α in mononuclear leucocyte plasma membranes.**

Samples of leucocyte plasma membrane protein (40-200μg) were TCA precipitated, resolved and transblotted as described in the legend to Fig. 6.1, then G<sub>s</sub>-α detected using a 1: 200 dilution of the antiserum CS2 and <sup>125</sup>I-labelled anti-rabbit IgG (2μCi/ml) as second antibody (Fig. 6.2a). Bands were scanned densitometrically and results plotted graphically using arbitrary units for band density (Fig. 6.2b). Data shown are from one experiment.

Figure 6.2 (a)

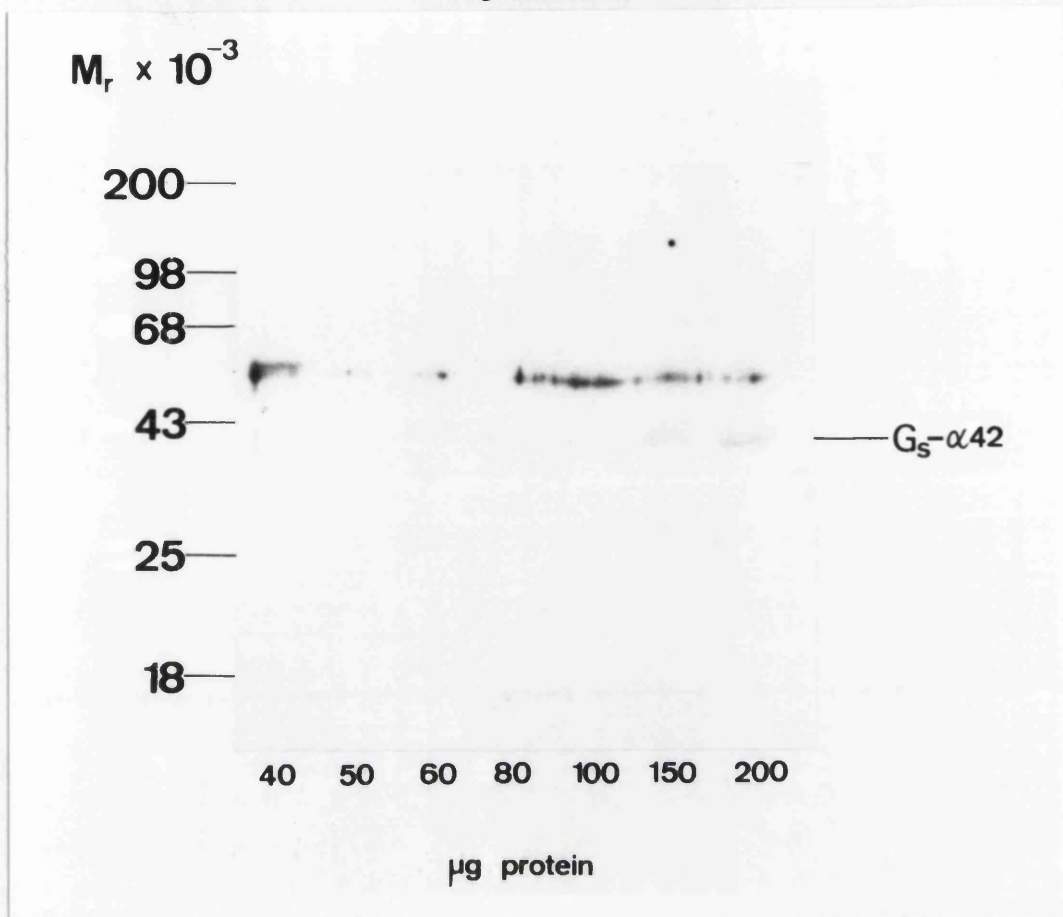
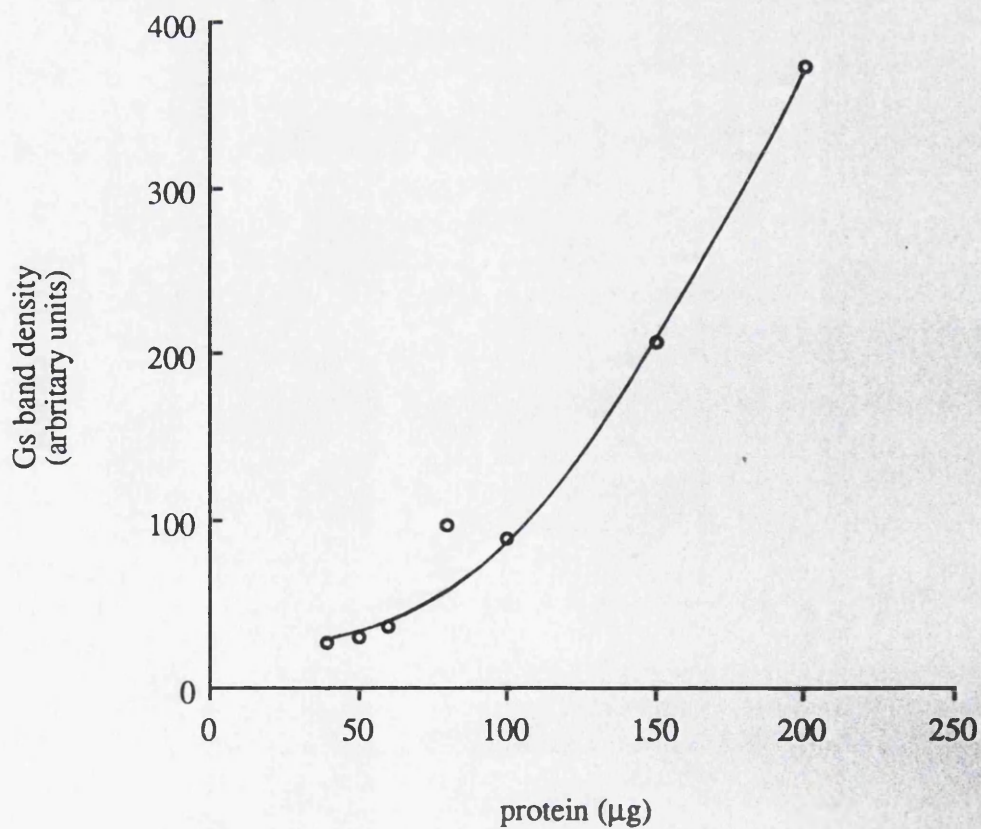


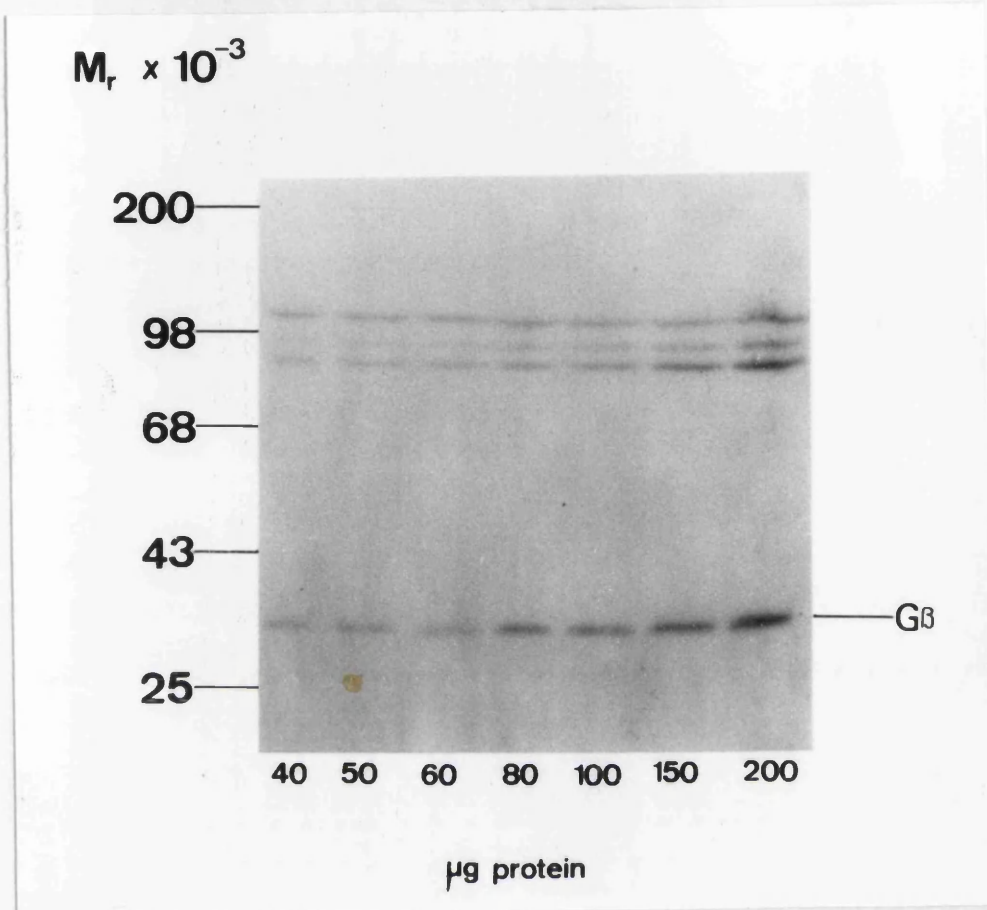
Figure 6.2 (b)



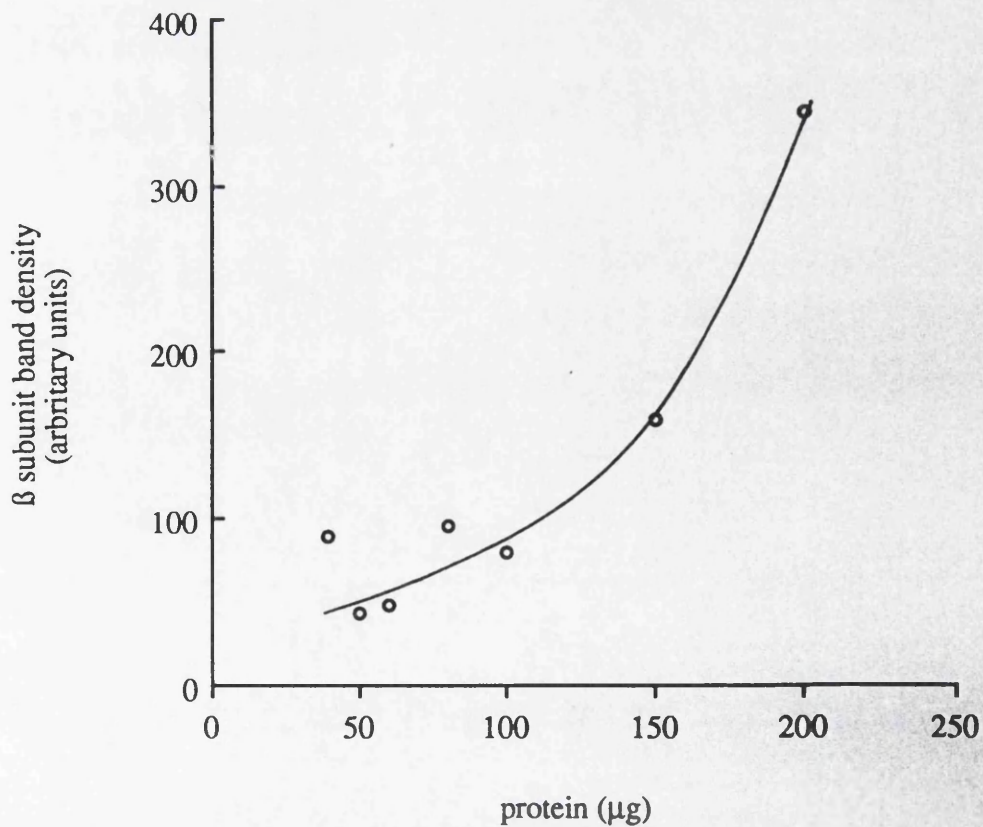
**Fig. 6.3**      **Immunological identification of G-protein  $\beta$  subunit in mononuclear leucocyte plasma membranes.**

Samples of leucocyte plasma membrane protein (40-200 $\mu$ g) were TCA precipitated, resolved and transblotted as described in the legend to Fig. 6.1, then G-protein  $\beta$  subunit detected using a 1: 200 dilution of the antiserum BN1 and  $^{125}$ I-labelled anti-rabbit IgG (2 $\mu$ Ci/ml) as second antibody (Fig. 6.3a). Bands were scanned densitometrically and results plotted graphically using arbitrary units for band density (Fig. 6.3b). Data shown are from one experiment.

**Figure 6.3 (a)**



**Figure 6.3 (b)**



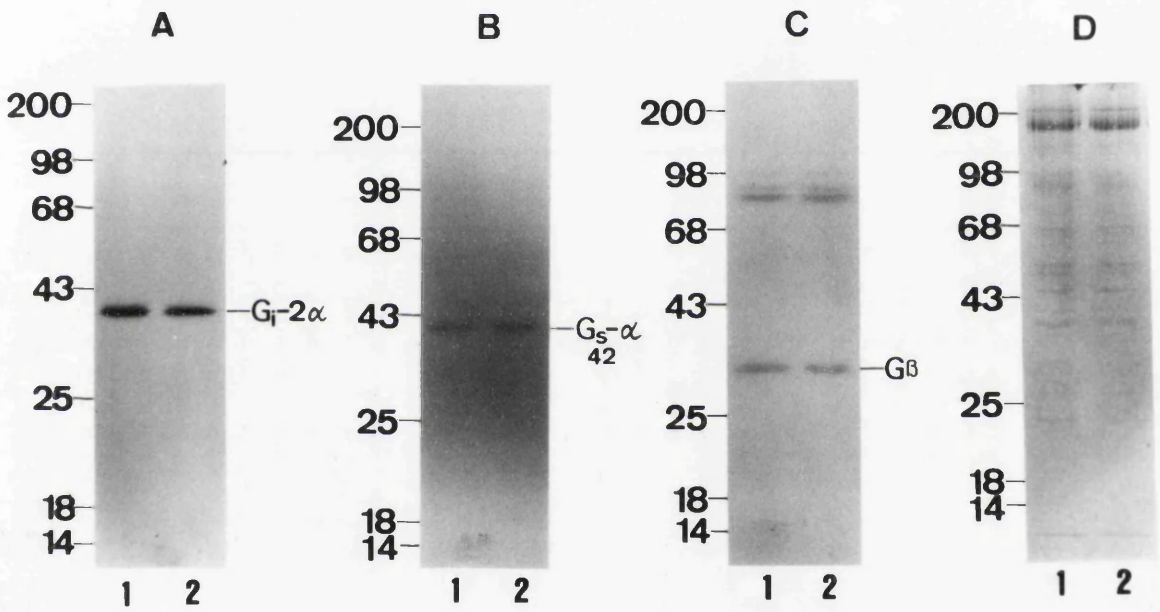


**Fig. 6.4**      **G-protein levels in mononuclear leucocyte plasma membranes from control and type 2 diabetic subjects.**

Samples of leucocyte plasma membrane protein (100 $\mu$ g) from control (lane 1) and diabetic (lane 2) subjects were TCA precipitated, resolved and transblotted as described in the legend to Fig. 6.1. Polypeptides were detected using the antisera 1432 (panel A), CS2 (panel B) and BN1 (panel C) and  $^{125}$ I-labelled anti-rabbit IgG (2 $\mu$ Ci/ml) as second antibody. All SDS gels were stained for protein to confirm equal protein loading in all lanes (panel D). Samples from a total of 20 control and 20 type 2 diabetic subjects were run in a similar manner. Autoradiographs were scanned densitometrically, band densities compared to an internal standard included on every blot and the results presented in Fig. 6.5.

**Figure 6.4**

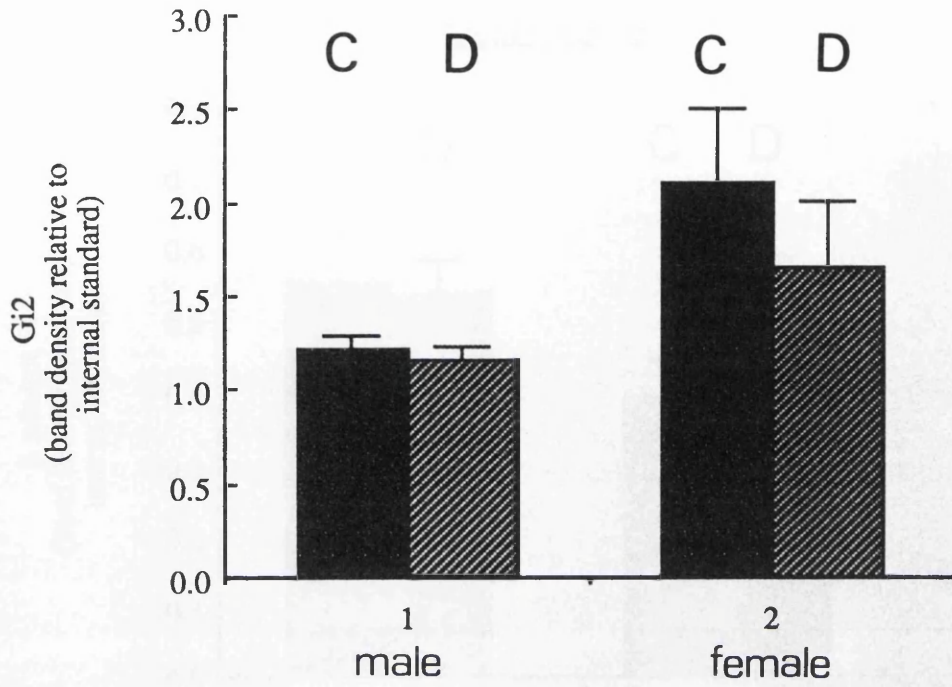
$M_r \times 10^{-3}$



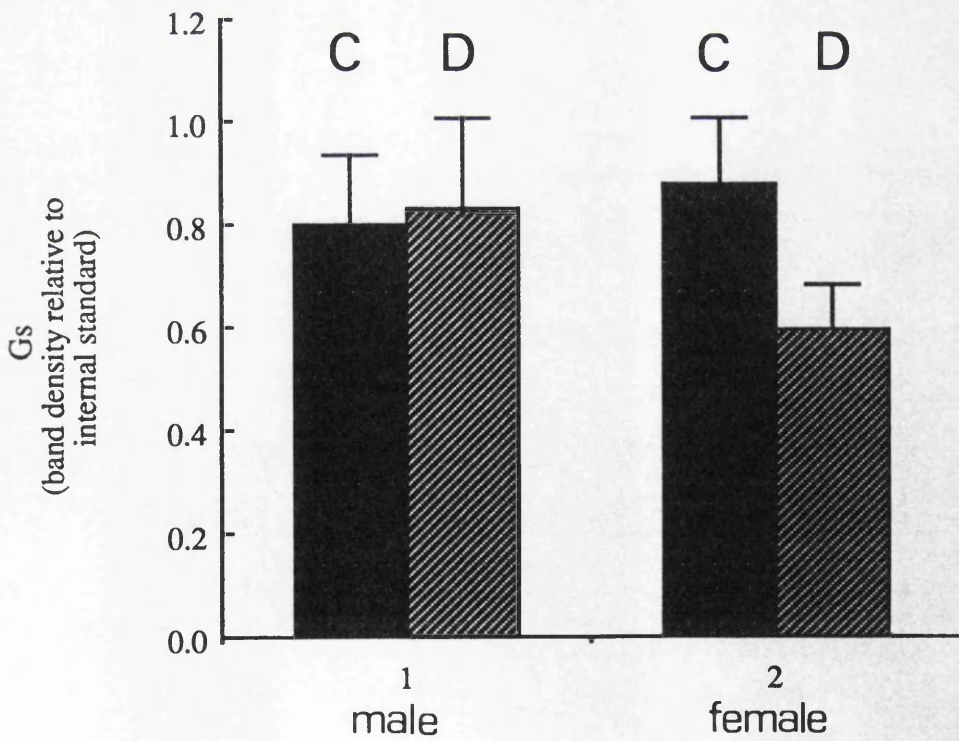
**Fig. 6.5                    Comparison of G-protein levels in  
mononuclear leucocyte plasma membranes from  
control and type 2 diabetic subjects.**

G-protein levels were examined in leucocyte plasma membranes from 10 control (black bars) and 10 type 2 diabetic subjects (hatched bars) of each sex, male (column 1) and female (column 2). Polypeptides were detected by immunoblotting using specific antisera as described in the legend to Fig. 6.4. Antisera used were 1432 to detect Gi-2 $\alpha$  (Fig. 6.5a), CS2 to detect Gs- $\alpha$  (Fig. 6.5b) and BN1 to detect G-protein  $\beta$  subunit (Fig. 6.5c). The second antiserum was <sup>125</sup>I-labelled anti-rabbit IgG. Autoradiographs were scanned densitometrically and all densities given relative to an internal standard included on every blot. Data are mean $\pm$ SEM for 10 subjects in each case. There were no significant differences between control or diabetic groups for any of the polypeptides detected, but control males showed significantly lower Gi-2 $\alpha$  levels than control females ( $p < 0.05$ ).

**Figure 6.5 (a)**



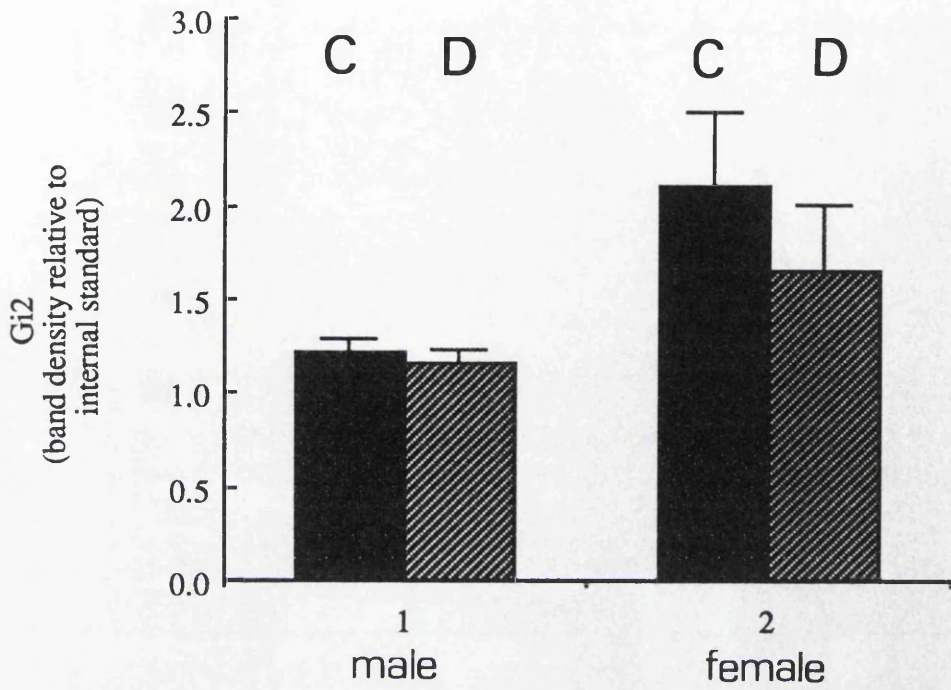
**Figure 6.5 (b)**



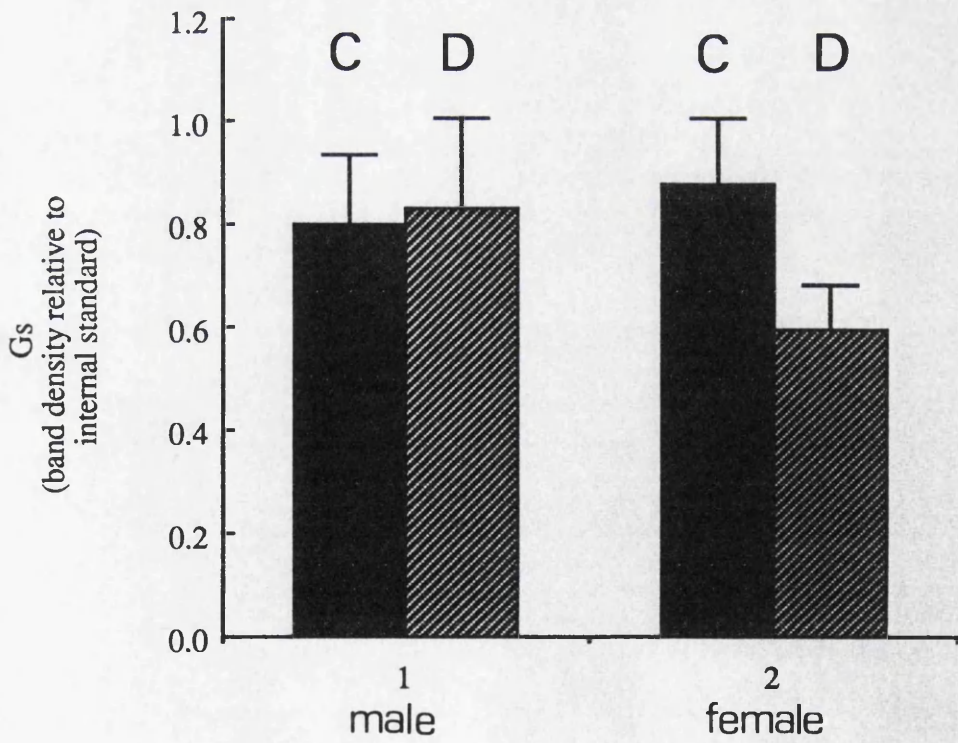
**Fig. 6.5                    Comparison of G-protein levels in  
mononuclear leucocyte plasma membranes from  
control and type 2 diabetic subjects.**

G-protein levels were examined in leucocyte plasma membranes from 10 control (black bars) and 10 type 2 diabetic subjects (hatched bars) of each sex, male (column 1) and female (column 2). Polypeptides were detected by immunoblotting using specific antisera as described in the legend to Fig. 6.4. Antisera used were 1432 to detect Gi-2 $\alpha$  (Fig. 6.5a), CS2 to detect Gs- $\alpha$  (Fig. 6.5b) and BN1 to detect G-protein  $\beta$  subunit (Fig. 6.5c). The second antiserum was <sup>125</sup>I-labelled anti-rabbit IgG. Autoradiographs were scanned densitometrically and all densities given relative to an internal standard included on every blot. Data are mean $\pm$ SEM for 10 subjects in each case. There were no significant differences between control or diabetic groups for any of the polypeptides detected, but control males showed significantly lower Gi-2 $\alpha$  levels than control females ( $p < 0.05$ ).

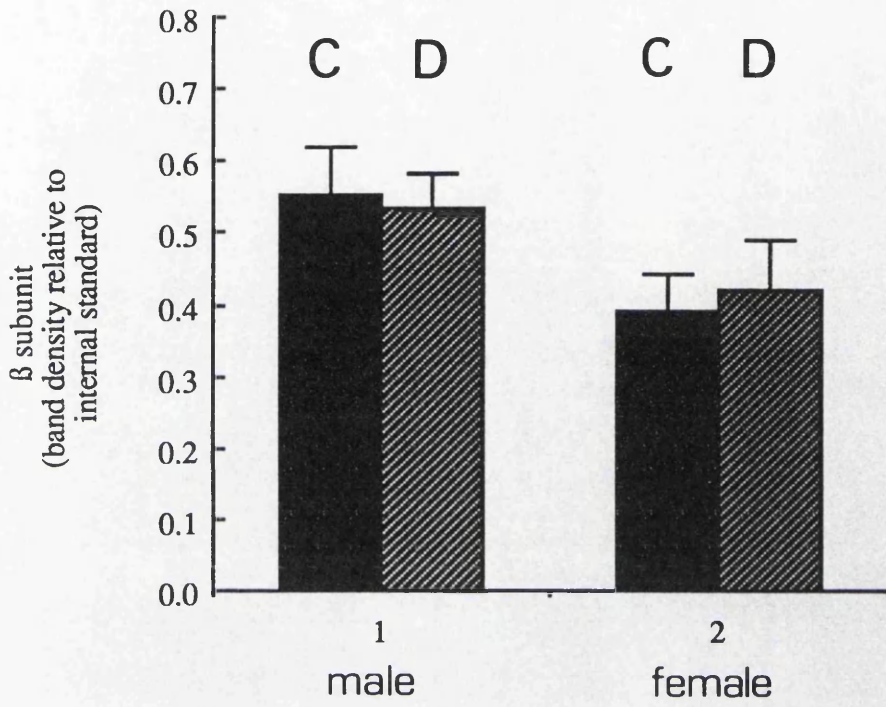
**Figure 6.5 (a)**



**Figure 6.5 (b)**



**Figure 6.5 (c)**



**Fig. 6.6      Comparison of adenylyl cyclase activities in mononuclear leucocyte plasma membranes from control and type 2 diabetic subjects.**

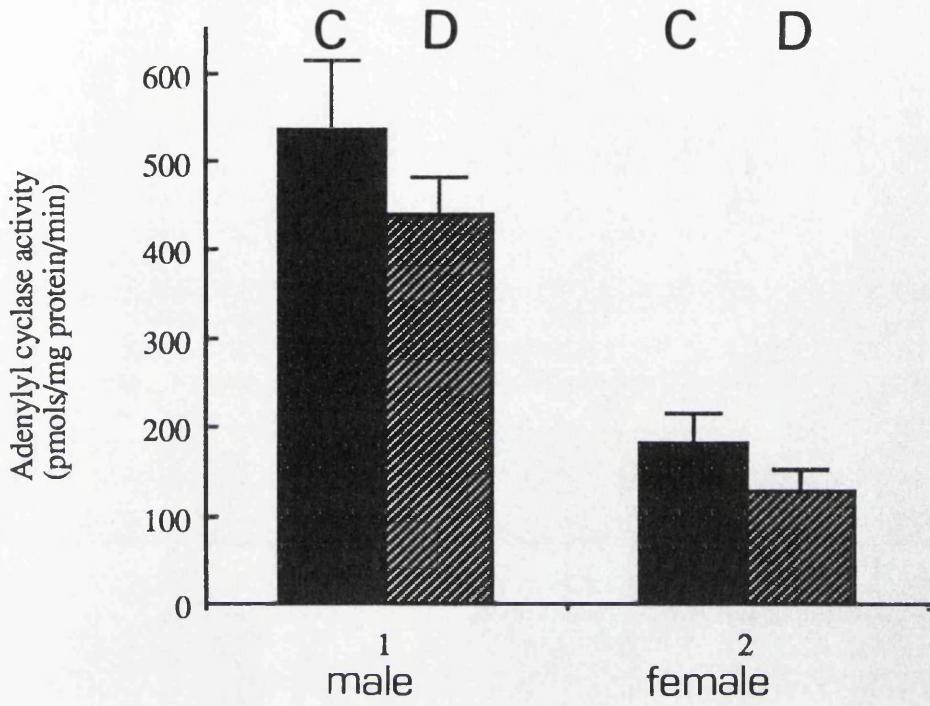
Adenylyl cyclase activity was measured in leucocyte plasma membranes from control (black bars) and type 2 diabetic subjects (hatched bars) of each sex, male (column 1) and female (column 2). Activities given are those stimulated with GTP ( $10^{-4}\text{M}$ ) (Fig. 6.6a), PGE<sub>1</sub> ( $10^{-4}\text{M}$ ) and GTP ( $10^{-4}\text{M}$ ) (Fig. 6.1b), forskolin ( $10^{-4}\text{M}$ ) alone (Fig.6.1c) and forskolin and GTP combined at the same concentrations (Fig.6.1d).

Fold values for PGE<sub>1</sub>/GTP stimulation over the level of adenylyl cyclase activity with GTP alone in Fig. 6.1b were: control males,  $2.81 \pm 1.57$ ; diabetic males,  $2.15 \pm 0.51$ ; control females  $2.91 \pm 2.35$ ; diabetic females,  $3.18 \pm 1.47$ . Fold values for forskolin/GTP stimulation over the level of adenylyl cyclase activity with GTP alone were: control males,  $2.80 \pm 1.08$ ; diabetic males,  $2.61 \pm 0.75$ ; control females,  $3.08 \pm 1.66$ ; diabetic females,  $4.22 \pm 1.87$ .

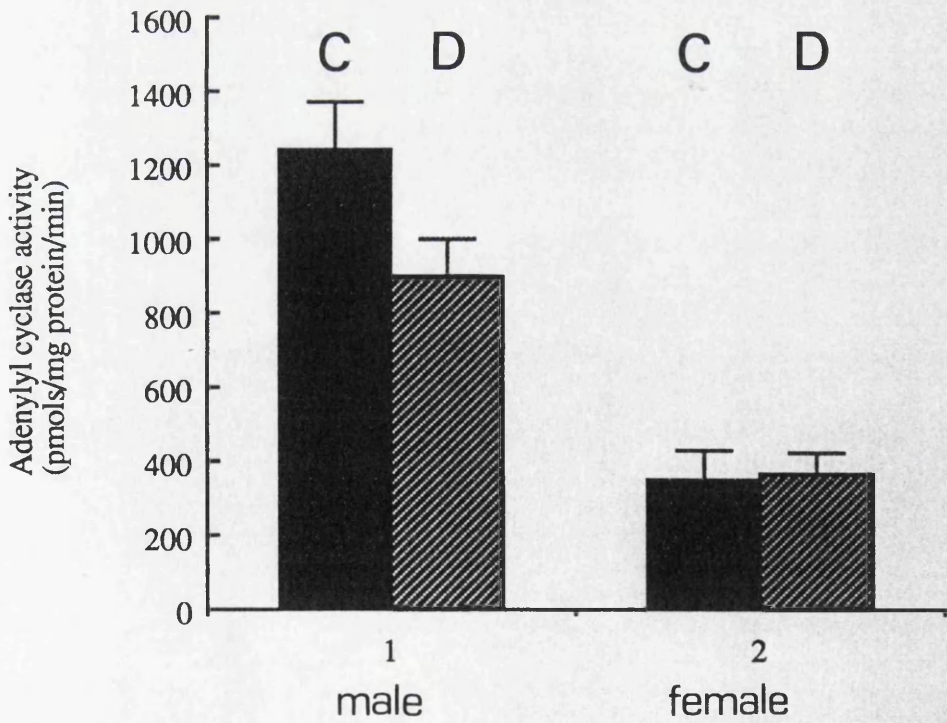
All data are mean $\pm$ SEM for assays performed in triplicate, for 10 patients in each group. There was a significant reduction in PGE<sub>1</sub>-stimulated adenylyl cyclase activity in leucocyte plasma membranes from male diabetic subjects as compared to their controls ( $p < 0.05$ ). No other significant differences were observed between control and diabetic data. Significantly lower agonist-stimulated adenylyl cyclase activity was observed in leucocyte plasma membranes from female subjects, in both control and diabetic groups, for all agonists employed ( $p < 0.001$ ).



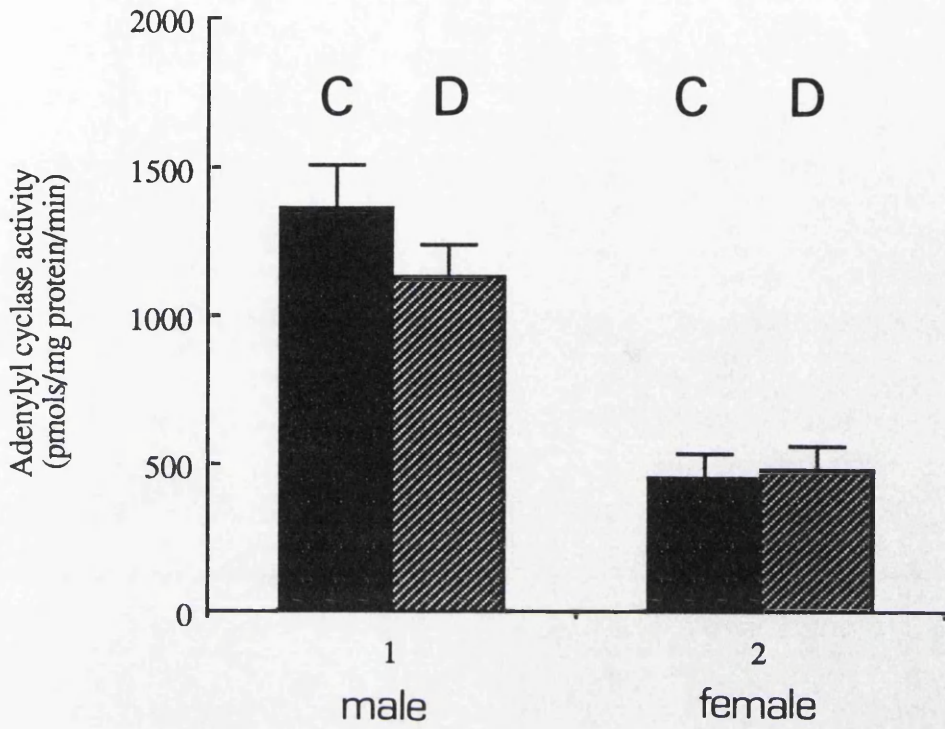
**Figure 6.6 (a)**



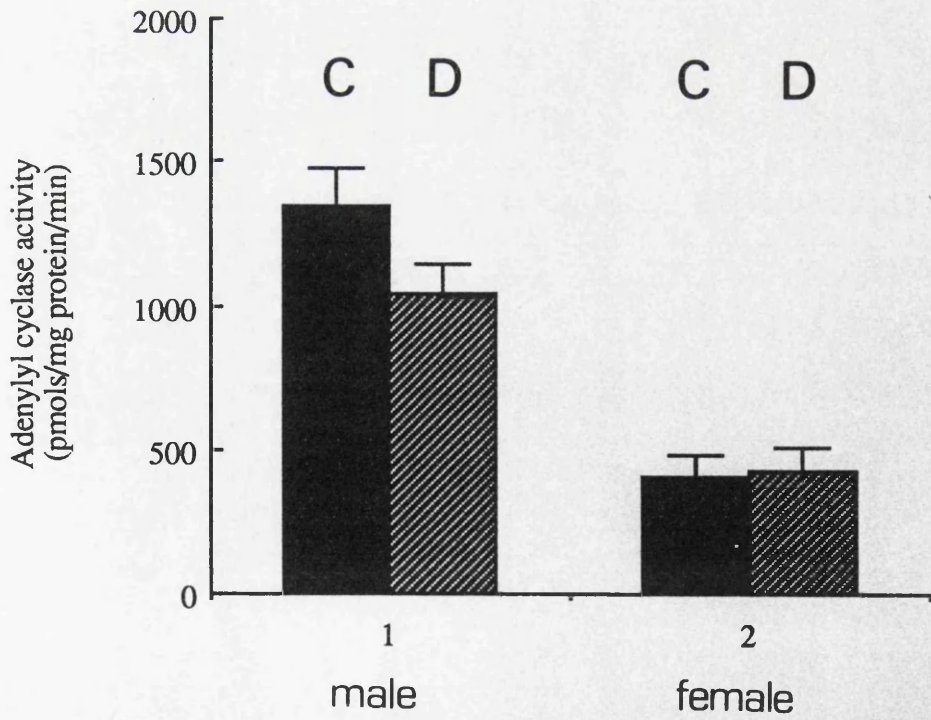
**Figure 6.6 (b)**



**Figure 6.6 (c)**



**Figure 6.6 (d)**



**Chapter 7**  
**General discussion**

It has been suggested from work on marker protein expression, that SV40-transfected hepatocytes, at least at low passage number, bear greater similarity to rat hepatocytes than to established hepatoma cell lines (Nairn *et al.*, 1990). SV40-transfected cells have also been shown to be genotypically more similar to native cells than other cell lines having a smaller degree of chromosomal damage than tumour cell lines (MacDonald *et al.*, 1991). The work presented here attempted to examine how closely one such line P9, resembled native hepatocytes in its adenylyl cyclase signalling and thereby to extend this comparison with native cells, in order to consider the merits of this potentially very useful cell line. Two main findings have arisen from the work. Firstly, that adenylyl cyclase signalling in the P9 cell line has probably diverged further from that of native hepatocytes than the soluble enzyme systems examined by other authors. Secondly, insulin's influence on the cells, both as a growth factor and in its acute modulation of cyclase activity is very different from its established actions on cyclase in hepatocytes. This means that as regards cyclase signalling, SV40-transfected cell lines are probably no more useful than the established hepatoma cell lines. Indeed the P9 line has the disadvantage that it cannot be passaged indefinitely, without phenotypic change in its signalling, unlike tumour cell lines. This finding was in agreement with those of Woodworth *et al.* (1988) who noted expression of hepatocyte-specific genes to change with time in culture. Some of the other implications of the work carried out here and possibilities for future study are discussed below.

Artificiality in cell culture systems is the price paid for being able to examine responses in isolated, proliferating cells. This work has centred on the response to PGE<sub>1</sub>, an agonist little used in hepatocytes. It would be interesting therefore to know more about insulin's effect on PGE<sub>1</sub> responsiveness in rat hepatocytes and find whether there is any further similarity between the two cell types. However, as discussed in sections 1.5.1 and 1.5.2 insulin's modulation of cyclase signalling

appears highly system-specific and it seems likely that P9 cells would be as divergent in this as in other aspects of their signalling. Speculation is difficult firstly as we are studying a facet of insulin's action which is far from understood in native hepatocytes and even less in artificial systems such as this. Secondly, in considering the relevance of these effects to *in vivo* hormone action, we must bear in mind that PGE<sub>1</sub> was used here in a highly artificial manner. It is normally present *in vivo* in the circulation at about 10<sup>-9</sup>M (Norman and Litwack, 1987) and is active locally at about 10<sup>-8</sup>M. Systemically it never reaches the high concentrations used here and would be toxic if it did so; whether insulin could influence the action of the low concentrations of PGE<sub>1</sub> present *in vivo* is more doubtful. It is possible that insulin's impairment of the ability of PGE<sub>1</sub> to elevate intracellular cAMP may, like agonist-induced receptor down-regulation, represent a form of cell memory to a stimulus, depending upon both the magnitude of the stimulus and duration of exposure. The lower agonist-stimulated cAMP levels after prolonged insulin exposure could, as in hepatocytes, influence the level of phosphorylation and activity of key enzymes or modulate the effects of these agonists on gene expression. An investigation into these factors in the cell line would put us in a better position to speculate on the role of insulin action in functioning of the cells. Whether or not the effects observed here are entirely artificial, they demonstrate that insulin can markedly modulate adenylyl cyclase signalling in the cell line, suggesting that crosstalk between the two signalling systems is present as a form of control. Mechanisms of crosstalk, like the structure of signalling components themselves, are likely to be similar in many cell types and therefore worthwhile investigating in artificial systems.

A full assessment of glucagon and PGE<sub>1</sub> binding in P9 cells as compared to rat hepatocytes would provide further information regarding the phenotypic similarities or otherwise between the two cell types. Indeed it would be revealing to carry out such studies and work similar to that done here on hepatoma cell lines. It is possible that

many of the findings would prove common to transformed and artificially immortalised cells. Although preliminary evidence has been presented here for the presence of insulin and IGF-1 receptors in the cells, the P9 line would in addition merit an examination of its insulin-binding characteristics. As both native hepatocytes and hepatoma cells are known to show insulin receptor down-regulation in response to insulin exposure, presumably the same would be true for SV40-transfected cells. If so, the cells could be used for studies on receptor expression, trafficking and signal generation by the insulin receptor. Furthermore, it would be interesting to know in this regard whether, like rat hepatocytes and hepatoma cell lines, the P9 line is truly insulin resistant after insulin exposure.

At the level of the G-protein, a study of G-protein phosphorylation in the cells could be rewarding, with respect to understanding more about  $G_i$  function in the cells and why inhibitory regulation of adenylyl cyclase should be different from that in native cells. The effect of phosphatase treatment of cell membranes on subsequent phosphate incorporation in response to agonist could be investigated. Removal of endogenously incorporated phosphate by phosphatase treatment might provide a means of demonstrating that  $G_i-2\alpha$  is already phosphorylated *in vivo* in P9 cells. Furthermore, the factors influencing expression of G-proteins and other signalling components merit investigation since the cells clearly show priority for alteration in expression of these components over other highly hepatocyte-specific proteins. This would tell us more about how the immortalisation process affects gene expression and thereby cell signalling.

The expression of PK-C isoforms would merit investigation, firstly as the kinase may have a role in insulin's low affinity sensitisation of cyclase responses, as discussed in Chapter 4 and secondly, as this kinase is thought to be involved in glucagon desensitisation and  $G_i-2$  phosphorylation in rat hepatocytes, both of which are profoundly altered in P9 cells, suggesting a lesion of PK-C.

It would be interesting finally, to know more about metabolic responses of the cells. If, like other SV40-immortalised hepatocytes, the cells contain a good complement of hepatocyte-specific proteins (including the complete pathway required for glutathione synthesis in one such cell line), then their metabolism and its regulation by insulin may yet prove fairly similar to that of hepatocytes, as may the expression and function of the soluble kinases acting as intermediates in insulin signalling. If so, the cell line would prove a useful asset to workers in this field. Furthermore, the variability in marker protein expression between cell lines, noted by the first workers to generate immortalised hepatocytes (Isom *et al.*, 1980) suggests that some may more closely resemble native hepatocytes than others and therefore demand closer scrutiny with a view to their use as model systems.

The heterogeneity noted between different cell lines derived in the same manner also suggests that the alterations in cyclase signalling in the P9 line will not necessarily apply to all cell lines and any prospective model system would clearly require to undergo thorough characterisation before use. The cells may yet also prove suitable as a model system for studying other second messenger systems or other cellular processes.

## References

- Abbracchio, M.P., Di Luca, M., Di Giulio, A.M., Catabeni, F., Teconi, B. and Gorio, A. (1989). Denervation and hyperinnervation in the nervous system of diabetic animals: functional alterations of G-proteins in diabetic encephalopathy. Journal of Neuroscience Research **24**, 517-523.
- Abraham, D.R., Hollingsworth, P.J., Smith, C.B., Jim, L., Zucker, L.B., Sobotka, P.A. and Vinik, A.I. (1986). Decreased  $\alpha_2$ -adrenergic receptors in platelet membranes from diabetic patients with autonomic neuropathy and orthostatic hypotension. Journal of Clinical Endocrinology and Metabolism **63**, 906-912.
- Abrass, M.D. and Scarpace, P.J. (1982). Catalytic unit of adenylate cyclase: reduced activity in aged human lymphocytes. Journal of Clinical Endocrinology and Metabolism **55**, 1026-1028.
- Adie, E., Mullaney, I., McKenzie, F.R. and Milligan, G. (1992). Concurrent down regulation of IP prostanoid receptor and the  $\alpha$  subunit of the stimulatory guanine nucleotide binding protein ( $G_s$ ) during prolonged exposure of neuroblastoma x glioma cells to prostanoid agonists-quantification and functional implications. Biochemical Journal **285**, 529-536.
- Alexander, M.D., Kowaloff, E.M., Witters, L.A., Denniky, D.T., and Avruch, J. (1979). Purification of a hepatic 123kDa hormone stimulated p peptide and its identification as ATP citrate lyase. Journal of Biological Chemistry **254**, 8052-8056.
- Ali, N., Milligan, G. and Evans, W.H. (1989). Distribution of G-proteins in rat liver plasma membrane domains and endocytic pathways. Biochemical Journal **261**, 905-912.
- Allen, R.E. and Boxhorn, L.K. (1989). Regulation of skeletal muscle satellite cell proliferation and differentiation by transforming growth factor-beta, insulin-like growth factor I and fibroblast growth factor. Journal of Cellular Physiology **138**, 311-315.



- Almira, E.C. and Misbin, R.I. (1989). Effects of insulin and streptozotocin diabetes on isoproterenol-stimulated cyclic AMP production in myocytes isolated from rat heart. Metabolism **38**, 102-103.
- Anderson, O., Gliemann, J. and Gammeltoft, S. (1977). Receptor binding and biological effects of insulin in human adipocytes. Diabetologia **13**, 589-593.
- Attali, B. and Vogel, Z. (1989). Long term opiate exposure leads to reduction of the  $\alpha$ -1 subunit of a GTP binding protein. Journal of Neurochemistry **53**, 1636-1639.
- Backer, J.M., Kahn, C.R. and White, M.F. (1989). Tyrosine phosphorylation of the insulin receptor during insulin-stimulated internalization in rat hepatoma cells. Journal of Biological Chemistry **264**, 1694-1701.
- Bakalyar, H.A. and Reed, R.R. (1990). Identification of a specialised adenylyl cyclase that may mediate odorant detection. Science **250**, 1403-1406.
- Barovsky, K., Pedone, C. and Brooker, G. (1984). Distinct mechanisms of forskolin-stimulated cyclic AMP accumulation and forskolin-potentiated hormone responses in C6-2B cells. Molecular Pharmacology **25**, 256-260.
- Bauer, P.H., Müller, S., Pozicha, M., Pippig, S., Obermaier, B., Helmreich, E.J.H. and Lohse, M. (1992). Phosducin is a protein kinase A-regulated G-protein regulator. Nature (Lond.) **358**, 73-76.
- Baxter, R.C. (1988). The insulin-like growth factors and their binding proteins. Comparative Biochemistry and Physiology **91B**, 229-235.
- Baxter, R.C. and Williams, P.F. (1983). Reciprocal modulation of insulin and insulin-like growth factor-I receptor affinity by calcium. Biochemical and Biophysical Research Communications **116**, 62-67.
- Beard, P. and Bruggmann, H. (1989). A transcription factor from simian virus 40 chromosomes which activates the viral late promoter in vitro. Journal of Virology **62**, 4296-4302.

- Beavo, J. (1990). Multiple phosphodiesterase isoenzymes: background, nomenclature and implications in 'Molecular pharmacology of cell regulation' pp3-15. (Beavo, J. and Houslay, M.D. eds.). John Wiley and Sons, Chichester.
- Becker, A.B. and Roth, R.A. (1990). Insulin receptor structure and function in normal and pathological conditions. Annual Review of Medicine **41**, 99-115.
- Begeot, M., Langlois, D. and Saez, J.M. (1989). Insulin-like growth factor-I and insulin increase the stimulatory guanine nucleotide binding protein ( $G_s$ ) in bovine adrenal cells. Molecular and Cellular Endocrinology **66**, 53-57.
- Begum, N., Sussman, K.E. and Draznin, B. (1991). Differential effects of diabetes on adipocyte and liver phosphotyrosine and phosphoserine phosphatase distribution. Diabetes **40**, 1620-1629.
- Berlin, I., Grimaldi, A., Bosquet, F. and Puech, A.J. (1986). Decreased  $\beta$ -adrenergic sensitivity in insulin dependent diabetic subjects. Journal of Clinical Endocrinology and Metabolism **63**, 262-265.
- Berry, M.N. and Friend, D.S. (1969). High yield preparation of isolated rat liver parenchymal cells. Journal of Cell Biology **43**, 506-520.
- Bhat, S.V., Bajwa, B.S., Dornauer, H. and De Souza, N.J. (1977). Structures and stereochemistry of new labdane diterpenoids from *Coleus forskohli*. Tetrahedron Letters **19**, 1669-1672.
- Bhathena, S.J., Voyles, N.R., Smith, S., Recant, L. (1978). Decreased glucagon receptors in diabetic rat hepatocytes. Journal of Clinical Investigation **61**, 1488-1497.
- Birnbaumer, L., Codina, J., Mattera, R., Cerione, R.A., Hildebrandt, J.D., Sunyer, T., Rojas, F.J., Caron, M.G, Lefkowitz, R.J. and Iyengar, R. (1985). Regulation of hormone receptors and adenylyl cyclases by guanine nucleotide binding N proteins. Recent Progress in Hormone Research **41**, 41-99.

- Birnbaumer, L. (1992). Receptor to effector signaling through G-proteins: Roles for  $\beta\gamma$  dimers as well as  $\alpha$  subunits. Cell **71**, 1069-1072.
- Bokoch, G.M. and Gilman, A.G. (1984). Inhibition of receptor mediated release of arachidonic acid by pertussis toxin. Cell **39**, 301-303.
- Bokoch, G.M. (1990). GTP binding proteins in phagocytic cells. Current Topics in Membrane Transport **35**, 65-101.
- Bokoch, G.M., Sklar, L.A. and Smolen, J.E. (1987). Guanine nucleotide regulatory proteins as transducers of receptor-stimulated neutrophil activation. International Journal of Tissue Reactivity **9**, 285-294.
- Bokoch, G.M., Bickford, K. and Bohl, B.P. (1988). Subcellular location and quantitation of the major neutrophil pertussis toxin substrate  $G_i$ . Journal of Cell Biology **106**, 1927-1936.
- Bourne, H.R. (1989). G-protein subunits-who carries what message? Nature (Lond.) **337**, 504-505.
- Bourne, H.R., Sanders, D.A. and McCormick, F. (1991). The GTPase superfamily: conserved structure and molecular mechanism. Nature (Lond.) **349**, 117-127.
- Bouvier, C., Lagacé, G. and Collu, R. (1991). G-protein modulation by estrogens. Molecular and Cellular Endocrinology **79**, 65-73.
- Bøyum, A. (1968). Isolation of mononuclear cells and granulocytes from human blood. Scandinavian Journal of Clinical and Laboratory Investigation **21** (suppl. 97), 1-109.
- Brass, E.P., Alford, C.A. and Garrity, M.J. (1988). Inhibition of glucagon-stimulated cyclic AMP accumulation and fatty acid oxidation by E series prostaglandins in rat hepatocytes. Biochimica et Biophysica Acta **930**, 122-126.

- Bray, P., Carter, A., Simons, C., Guo, V. and Rickett, C. (1986). Human cDNA clones for four species of Gs $\alpha$  signal transduction protein. Proceedings of the National Academy of Sciences of the U.S.A. **83**, 8893-8897.
- Brooker, G., Pedone, C. and Barovsky, K. (1983). Selective reduction of forskolin-stimulated cyclic AMP accumulation by inhibition of protein synthesis. Science **220**, 1169-1170.
- Brown, B.L., Ekins, R.P. and Albano, J.D.M. (1972). Saturation assay for cyclic AMP using endogenous binding protein. Advances in Cyclic Nucleotide Research **2**, 25-40.
- Brownlee, M. and Cerami, A. (1981). The biochemistry of the complications of diabetes mellitus. Annual Review of Biochemistry **50**, 385-432.
- Brownsey, R.N. and Denton, R.M. (1982). Evidence that insulin activates fat cell acetyl CoA carboxylase by increased phosphorylation at a specific site. Biochemical Journal **202**, 345-352.
- Bruneau, C., Hubert, P., Waksman, A., Beck, J.-P. and Staedel-Flaig, C. (1987). Modifications of cellular lipids induce cellular insulin resistance in cultured hepatoma cells. Biochimica et Biophysica Acta **928**, 297-304.
- Brunton, L.L. and Mayer, S.E. (1979). Extrusion of cyclic AMP from pigeon erythrocytes. Journal of Biological Chemistry **254**, 9714-9720.
- Bryszewska, M., Watala, C. and Torzecka, W. (1986). Changes in fluidity and composition of erythrocyte membranes and in composition of plasma lipids in type 1 diabetes. British Journal of Haematology **62**, 111-116.
- Burgering, B.M.T., Medema, R.H., Maassen, J.A., van de Wetering, M.L., van der Eb, A.J., McCormick, F. and Bos, J.L. (1991). Insulin stimulation of gene expression mediated by p21ras activation. E.M.B.O. Journal **10**, 1103-1109.

- Bushfield, M., Griffiths, S.L., Murphy, G.J., Pyne, N.J., Milligan, G., Parker, P.J., Mollner, S. and Houslay, M.D. (1990a). Diabetes-induced alterations in the expression, functioning and phosphorylation of the inhibitory guanine nucleotide regulatory protein Gi-2 in hepatocytes. Biochemical Journal **271**, 365-372.
- Bushfield, M., Murphy, G.J., Lavan, B.E., Parker, P.J., Hruby, V.J., Milligan, G. and Houslay, M.D. (1990b). Hormonal regulation of Gi-2  $\alpha$  subunit phosphorylation in intact hepatocytes. Biochemical Journal **268**, 449-457.
- Bushfield, M., Griffiths, S.L., Strassheim, D., Tang, E., Shakur, Y., Lavan, B.E. and Houslay, M.D. (1990c). Guanine nucleotide binding proteins in diabetes and insulin resistant states. Biochemical Society Symposia **56**, 137-154.
- Cantiello, H.F., Patenaude, C.R., Codina, J., Birnbaumer, L., Ausiello, D.A. (1990). Gi-3 $\alpha$  regulates epithelial Na<sup>+</sup> channels by activation of phospholipase A2 and lipoxygenase pathways. Journal of Biological Chemistry **265**, 21624-21628.
- Caro, J.F., Raju, M.S., Exton, J.H., Thakkar, J.K., Roy, L., Hale, J.C. and Pories, W.J. (1991). Journal of Biological Chemistry (suppl.) **15B**, 13.
- Cassel, D. and Selinger, Z. (1976). Catecholamine-stimulated GTPase activity in turkey erythrocyte membranes. Biochemica et Biophysica Acta **452**, 532-551.
- Chamras, H., Foucherau-Peron, M. and Rosselin, G. (1980). The effect of streptozotocin diabetes on the early steps of glucagon action in isolated rat liver cells. Diabetologia **19**, 74-80.
- Chang, F.H. and Bourne, H.R. (1987). Dexamethasone increases adenylyl cyclase activity and expression of the  $\alpha$  subunit of G<sub>s</sub> in GH3 cells. Endocrinology **121**, 1711-1715.
- Chou, J.Y. (1989). Differentiated mammalian cell lines immortalized by temperature sensitive tumour viruses. Molecular Endocrinology **3**, 1511-1514.

- Cobb, M.N. and Rosen, O.M. (1983). Description of a protein kinase derived from insulin treated 3T3-L1 cells that catalyses the phosphorylation of ribosomal protein S6 and casein. Journal of Biological Chemistry **258**, 12472-12481.
- Coleman, R.A. (1987). Methods in prostanoid receptor classification in 'Prostaglandins and related substances. a practical approach.' pp267-303 (Benedetto, G., McDonald-Gibson, R.G., Nigan, S. and Slater, T.F. eds.). I.R.L. Press, Oxford, Washington D.C.
- Connell, J.M.C., Ding, Y.-A., Fisher, B.M., Frier, B.M. and Semple, P.F. (1986). Reduced number of angiotensin-II receptors on platelets in insulin dependent diabetes. Clinical Science **71**, 217-220.
- Conti, M., Swinnen, J.V., Tsikalas, K.E. and Jin, S.L.C. (1991). Structure and regulation of the rat high affinity cyclic AMP phosphodiesterases: a family of closely related enzymes. Advances in Second Messenger and Phosphoprotein Research **25**, 87-99.
- Crettaz, M. and Kahn, C.R. (1976). Insulin receptor regulation and desensitization in rat hepatoma cells. Diabetes **33**, 477-485.
- Cryer, P.E. (1980). Physiology and pathophysiology of the human sympathoadrenal neuroendocrine system. New England Journal of Medicine **303**, 436-444.
- Czech, M.P. (1985). The nature and regulation of the insulin receptor: structure and function. Annual Review of Physiology **47**, 357-381.
- Darfler, F.J., Mahon, L.C., Koachman, A.M. and Insel, P.A. (1982). Stimulation by forskolin of intact S49 lymphoma cells involves the nucleotide regulatory protein of adenylate cyclase. Journal of Biological Chemistry **257**, 11901-11907.
- Davi, G., Rini, G.B., Aversa, M., Novo, S., Difede, G., Pinto, A., Notarbartolo, A. and Strano, A. (1982). Thromboxane B2 formation and platelet sensitivity to prostacyclin in insulin-dependent and insulin-independent diabetics. Thrombosis Research **26**, 359-370.

- Davis, A.O. and Lefkowitz, R.J. (1984). Regulation of the  $\beta$ -adrenergic receptor by steroid hormones. Annual Review of Physiology **46**, 119-130.
- Davis, H.W. and McDonald, J.M. (1990). Insulin receptor function is inhibited by guanosine 5'-[ $\gamma$ -thio] triphosphate. Biochemical Journal **270**, 401-407.
- Debant, A., Guerre-Millo, M., Le Marchand-Brustel, Y., Freychet, P., Lavau, M. and Van Obberghen, E. (1987). Insulin receptor kinase is hyperresponsive in adipocytes of young obese Zucker rats. American Journal of Physiology **252**, E273-E277.
- Debant, A., Clauser, E., Morgan, D.O., Ponzio, G., Filloux, C., Auzan, C., Contreres, J.O. and Rossi, B. (1990). Replacement of the insulin receptor tyrosine residues 1162-1163 does not alter the mitogenic effect of the hormone in 'Insulin and the cell membrane' pp73-79. (Klimes, I., Howard, B.V. and Kahn, C.R. eds.). Harwood Acad. Pub. London.
- Deutsch, P.J., Wan, C.F., Rosen, O.M. and Rubin, C.S. (1983). Latent insulin receptors and possible receptor precursors in 3T3-L1 adipocytes. Proceedings of the National Academy of Sciences of the U.S.A. **80**, 133-136.
- Devedjian, J.C., Fargues, M., Denis-Pouxviel, C., Daviand, D., Prats, H. and Paris, H. (1991). Regulation of the  $\alpha_{2A}$ -adrenergic receptor in the HT29 cell line: Effects of insulin and growth factors. Journal of Biological Chemistry **266**, 14359-14366.
- Dills, D.G., Moss, S.E., Klein, R. and Klein, B.E.K. (1991). Association of elevated insulin-like growth factor-I levels with increased retinopathy in late onset diabetes. Diabetes **40**, 1725-1730.
- Dipple, I. and Houslay, M.D. (1978). The activity of glucagon-stimulated adenylate cyclase from rat liver plasma membranes is modulated by the fluidity of its lipid environment. Biochemical Journal **174**, 179-190.
- Dutta-Roy, A.K. and Sinha, A.K. (1985). Binding of prostaglandin E1 to human erythrocyte plasma membranes. Biochimica et Biophysica Acta **812**, 671-678.

- Ebina, Y., Ellis, L., Jarnagin, K., Edery, M., Graf, L., Clauser, E., Ou, J.-H., Masiar, F., Kan, Y.W., Goldfine, I.D., Roth, R.A. and Rutter, R.J. (1985). Human insulin receptor cDNA- the structural basis for hormone-activated transmembrane signalling. Cell **40**, 747-758.
- Ebina, Y., Araki, E., Taira, M., Shimada, M., Mori, M., Craik, C.S., Siddle, K., Pierce, S.B., Roth, R.A. and Rutter, W.J. (1987). Replacement of lysine residue 1030 in the putative ATP binding region of the insulin receptor abolishes insulin- and antibody-stimulated glucose uptake and receptor kinase activity. Proceedings of the National Academy of Sciences of the U.S.A. **84**, 704-708.
- Efrat, S. and Hanahan, D. (1989). Evidence for threshold effects in transformation of pancreatic  $\beta$  cells by simian virus 40 large T antigen in transgenic mice. Current Topics in Microbiology and Immunology **144**, 89-95.
- Ellis, L., Clauser, E., Morgan, D.O., Edery, M., Roth, R.A. and Rutter, W.J. (1986). Replacement of insulin receptor tyrosine residues 1162 and 1163 compromises insulin-stimulated kinase activity and uptake of 2-deoxyglucose. Cell **45**, 721-732.
- EI-Maghrabi, M.R., Fox, E., Pilkis, J. and Pilkis, S.J. (1982). Cyclic AMP dependent phosphorylation of rat liver 6-phosphofructo-2-kinase / fructose 2,6- bisphosphatase. Biochemical and Biophysical Research Communications **106**, 794-802.
- Engelhard, V.H., Storm, D.T. and Glaser, M. (1978). Negative cooperativity exhibited by prostaglandin E1-stimulated adenylate cyclase in LM cells. Biochemistry **17**, 5304-5308.
- Fanning, E., Schneider, J., Arthur, A., Höss, A., Moarefi, I. and Madrow, S. (1989). Structure and function of simian virus 40 large T antigen-communication between functional domains. Current Topics in Microbiology and Immunology **144**, 9-19.
- Farias, R.N. (1987). Insulin-membrane interactions and membrane fluidity changes. Biochimica et Biophysica Acta **906**, 459-468.



- Feder, D., Im, M-J., Klein, H.W., Hekman, M., Holzhöfer, A., Dees, C., Levitski, A., Helmreich, E.J. and Pfeuffer, T. (1986). Reconstruction of  $\beta_1$ -adrenoceptor dependent adenylate cyclase from purified components. E.M.B.O. Journal **5**, 1509-1514.
- Federmann, A.D., Conklin, B.R., Schrader, K.A., Reed, R.R. and Bourne, H.L. (1992). Hormonal stimulation of adenylyl cyclase through  $G_i$  protein  $\beta\gamma$  subunits. Nature (Lond.) **356**, 159-161.
- Fiske, C.H. and Subbarow, Y. (1925). The colorimetric determination of phosphorus. Journal of Biological Chemistry **66**, 375-400.
- Fletcher-McGruder, B.L. and Gerritsen, G.C. (1984). Deficient humoral antibody response of the spontaneously diabetic Chinese hamster. Proceedings of the Society of Experimental Biology and Medicine **175**, 74-78.
- Florini, J.R., Ewton, D.Z. and Magri, K.M. (1991). Hormones, growth factors and myogenic differentiation. Annual Review of Physiology **53**, 201-206.
- Frame, M.C., Freshney, R.I., Vaughan, P.F.T., Graham, D.I. and Shaw, R. (1984). Interrelationship between differentiation and malignancy-associated properties in glioma. British Journal of Cancer **49**, 269-280.
- Freissmuth, M., Casey, P.J. and Gilman, A.G. (1989). G-proteins control diverse pathways of transmembrane signalling. F.A.S.E.B. Journal **3**, 2125-2131.
- Frey, J., Janes, M., Engelhardt, W., Afting, E.G., Geerds, C. and Möller, B. (1986).  $Fc\gamma$ -mediated changes in the plasma membrane potential induce prostaglandin release from human fibroblasts. European Journal of Biochemistry **158**, 85-89.
- Froesch, E.R. and Zapf, J. (1985). Insulin-like growth factors and insulin: comparative aspects. Diabetologia **28**, 485-493.
- Fujinaga, Y., Morozumi, N., Sato, K., Tokumitsu, Y., Fujinaga, K., Kondo, Y., Ui, M. and Fumikazu, O. (1989). A pertussis toxin-sensitive GTP binding protein plays a role in the Go-G1 transition of rat hepatocytes following establishment of primary culture. F.E.B.S. Letters **245**, 117-121.

- Galizzi, J.P., Carillat, H., Rousset, F., Ménétrier, C., de Vries, J.E. and Banchereau, J. (1988). Interferon- $\gamma$  and prostaglandin E<sub>2</sub> inhibit IL-4 induced expression of FcE/CD23 on B lymphocytes through different mechanisms, without altering binding of IL-4 to its receptor. Journal of Immunology **141**, 1982-1988.
- Ganda, O.P. (1983). Morbidity and mortality from diabetes: a look at preventable aspects. American Journal of Public Health **73**, 1156-1158.
- Gao, B. and Gilman, A.G. (1991). Cloning and expression of a widely distributed (Type IV) adenylyl cyclase. Proceedings of the National Academy of Sciences of the U.S.A. **88**, 10178-10182.
- Garber, A.J. (1980). The impact of streptozotocin-induced diabetes mellitus on cyclic nucleotide regulation of skeletal muscle amino acid metabolism in the rat. Journal of Clinical Investigation **65**, 478-487.
- Garrity, M.J., Brass, E.P. and Robertson, R.P. (1984). Kinetics of prostaglandin E metabolism in isolated hepatocytes. Biochimica et Biophysica Acta **796**, 136-141.
- Garrity, M.J., Brass, E.P. and Robertson, R.P. (1987). Prostaglandin E binding in isolated hepatocytes: regulation during fasting. Advances in Prostaglandin, Thromboxane and Leukotriene Research **17**, 686-690.
- Garrity, M.J., Reed M.M. and Brass, E. (1988). Coupling of hepatic prostaglandin receptors to adenylyl cyclase through a pertussis toxin-sensitive guanine nucleotide regulatory protein. Journal of Pharmacology and Experimental Therapeutics **248**, 979-983.
- Gawler, D.J. and Houslay, M.D. (1987). Insulin stimulates a novel GTPase in human platelets. F.E.B.S. Letters **216**, 94-98.
- Gawler, D.J., Milligan, G., Spiegel, A.M., Unson, C.G. and Houslay, M.D. (1987). Abolition of expression of inhibitory guanine nucleotide regulatory protein G<sub>i</sub> activity in diabetes. Nature (Lond.) **327**, 229-232.

- Gerich, J.E. (1990). Role of liver in the pathogenesis of type 2 diabetes. Advances in Second Messenger and Protein Phosphorylation Research **24**, 507-510.
- Gibbs, E.M., Allard, W.J. and Lienhard, G.E. (1986). The glucose transporter in 3T3-L1 adipocytes is phosphorylated in response to phorbol ester but not in response to insulin. Journal of Biological Chemistry **261**, 16597-16603.
- Gierschik, P., McLeish, K. and Jakobs, K.H. (1988). Regulation of G-protein mediated signal transfer by ions. Journal of Cardiovascular Pharmacology **12** (Suppl 5), S20-S24.
- Gilman, A.G. (1987). G-proteins- transducers of receptor generated signals. Annual Review of Biochemistry **56**, 615-649.
- Ginsberg, B.H., Chatterjee, P. and Yorek, M. (1990). Increased membrane fluidity is associated with increased sensitivity to insulin in 'Insulin and the cell membrane' pp313-330. (Klimes, I. Howard, B.V. and Kahn, C.R.). Harwood Acad. Pub. London.
- Goldsmith, P., Gierschik, P., Milligan, G., Unson, C.G., Vinitsky, R., Malech, H.L. and Spiegel, A.M. (1987). Antibodies directed against synthetic peptides distinguish between GTP binding proteins in neutrophil and brain. Journal of Biological Chemistry **262**, 14683-14688.
- Goodman, H.M. and Knobil, E. (1959). Mobilisation of fatty acids in normal and hypophsectomised rhesus monkeys. Proceedings of the Society of Experimental Biology and Medicine **100**, 195-197.
- Gordon, A.S., Mochly-Rosen, D. and Diamond, I. (1992). Alcoholism: a possible G-protein disorder in 'G-proteins. Signal transduction and disease' pp191-216. (Milligan, G. and Wakelam, M.J.O.eds.) Acad. Press. London.
- Gotzche, O. (1983). The  $\beta$ -adrenergic receptor adenylate cyclase system in heart and lymphocytes from streptozotocin diabetic rats. Diabetes **32**, 1110-1116.
- Granneman, J.G., Haverstick, D.M. and Chaudry, A. (1990). Relationship between  $G_s\alpha$  messenger ribonucleic acid and splice variants and the molecular forms of  $G_s\alpha$  protein in rat brown adipose tissue. Endocrinology **127**, 1596-1601.

- Granner, D.K. (1987). The molecular biology of insulin action on protein synthesis. Kidney International **32**, S82-S93.
- Graziano, M.P., Casey, P.J. and Gilman, A.G. (1987). Expression of cDNAs for G-proteins in E coli: two forms of G $\alpha$  stimulate adenylate cyclase. Journal of Biological Chemistry **262**, 11375-11381.
- Green, A. and Johnson, J.L. (1989). Evidence for altered expression of the GTP dependent regulatory proteins, G $s$  and G $i$  in adipocytes from aged rats. Biochemical Journal **258**, 607-610.
- Green, A. and Johnson, J.L. (1991). Evidence for impaired coupling of receptors to G $i$  protein in adipocytes from streptozotocin-induced diabetic rats. Diabetes **40**, 88-94.
- Greene, D.A., Lattimer, S.A., Sima, A.A.F. (1987). Sorbitol, phosphoinositides and sodium-potassium ATPase in the pathogenesis of diabetic complications. New England Journal of Medicine **316**, 599-606.
- Hadcock, J.R., Ros, M., Watkins, D.C. and Malbon, C.C. (1990). Cross regulation between G-protein mediated pathways. Journal of Biological Chemistry **265**, 14784-14790.
- Hadcock, J.R. and Malbon, C.C. (1992). Adrenal dysfunction and G-protein mediated pathways in 'G-proteins. Signal transduction and disease' pp109-127. (Milligan, G. and Wakelam, M.J.O. eds.) Acad. Press. London.
- Hadcock, J.R., Port, D.J., Gelman, M.S. and Malbon, C.C. (1992). Crosstalk between tyrosine kinase and G-protein linked receptors. Journal of Biological Chemistry **267**, 26017-26022.
- Halushka, P.V., Mayfield, R. and Colwell, J.A. (1985). Insulin and arachidonic acid metabolism in diabetes mellitus. Metabolism **34** (Suppl 1), 32-36.
- Häring, H., Kirsch, D., Obermaier, B., Ermel, B. and Machicao, F. (1986). Decreased tyrosine kinase activity of insulin receptor isolated from rat adipocytes rendered insulin resistant by catecholamine treatment in vivo. Biochemical Journal **234**, 59-66.

- Harnett, M. and Rigley, K. (1992). The role of G-proteins versus protein tyrosine kinases in the regulation of lymphocyte activation. Immunology Today **13**, 482-486.
- Hatada, E.N., McClain, D.A., Potter, E., Ullrich, A. and Olefsky, J.M. (1989). Effects of growth and insulin treatment on the levels of receptors and their mRNA in HEP G2 cells. Journal of Biological Chemistry **264**, 6741-6747.
- Hay, R.J. (1989). Preservation and characterisation in 'Animal cell culture. a practical approach' pp71-112 (Freshney, R.I. ed.). I.R.L. Press, Oxford.
- Hedo, J.A., Kahn, C.R., Hayashi, M., Yamada, K.M. and Kasuga, M. (1983). Internalization of insulin receptors. Evidence for a single polypeptide precursor of the two major subunits. Journal of Biological Chemistry **258**, 10020-10026.
- Heinen, E., Cormann, N., Braun, M., Kinet-Denoël, C., Vanderschelden, J. and Simar, L.J. (1986). Isolation of follicular dendritic cells from human tonsils and adenoids-analysis of prostaglandin secretion. Annals of the Pasteur Institute of Immunology **137D**, 369-382.
- Herberg, L. and Coleman, D.L. (1977). Laboratory animals exhibiting obesity and diabetes syndromes. Metabolism **26**, 59-99.
- Heyworth, C.M. and Houslay, M.D. (1983). Insulin exerts actions through a distinct species of guanine nucleotide regulatory protein: inhibition of adenylate cyclase. Biochemical Journal **214**, 547-552.
- Heyworth, C.M., Hanski, E. and Houslay, M.D. (1984). Islet activating protein blocks glucagon desensitisation in intact hepatocytes. Biochemical Journal **222**, 189-194.
- Heyworth, C.M., Grey, A.M., Wilson, S.R., Hanski, E. and Houslay, M.D. (1986). The action of islet activating protein (pertussis toxin) on insulin's ability to inhibit adenylate cyclase and activate cyclic AMP phosphodiesterases in hepatocytes. Biochemical Journal **235**, 145-149.

- Hickman, J. and McElduff, A. (1990). Insulin sensitises a cultured rat osteogenic sarcoma cell line to hormones which activate adenylate cyclase. Calcified Tissue International **46**, 401-405.
- Hildebrandt, J.D., Sekura, R.D., Codina, J., Iyengar, R. Manclark, C.R. and Birnbaumer, L. (1983). Stimulation and inhibition of adenylate cyclase is mediated by distinct regulatory proteins. Nature. (Lond.) **302**, 706-709.
- Hod, Y., Cook, J.S., Weldon, S.L., Short, T.M., Wynshaw-Boris, A. and Hanson, R.W. (1986). Differential expression of the genes for the mitochondrial and cytosolic forms of PEPCK. Annals of the New York Academy of Science **478**, 31-45.
- Holter, W., Spiegel, A.M., Howard, B.H., Weber, S. and Brann, M.R. (1991). Expression of GTP binding proteins and prostaglandin E2 receptor during human T cell activation. Cellular Immunology **134**, 287-295.
- Houslay, M.D. (1991a). Gi-2 is at the centre of an active phosphorylation / dephosphorylation cycle in hepatocytes: the fine tuning of stimulatory and inhibitory inputs into adenylyl cyclase in normal and diabetic states. Cellular Signalling **3**, 1-9.
- Houslay, M.D. (1991b). Crosstalk: a pivotal role for protein kinase C in modulating relationships between signalling pathways. European Journal of Biochemistry **195**, 9-27.
- Houslay, M.D. and Palmer, R.W. (1978). Changes in the form of Arrhenius plots of the activity of glucagon-stimulated adenylate cyclase and other hamster liver plasma membrane enzymes occurring during hibernation. Biochemical Journal **174**, 909-919.
- Houslay, M.D., Dipple, I. and Elliott, K.R.F. (1980). Guanosine 5'-triphosphate and guanosine 5'-[ $\beta$ -imido] triphosphate effect a collision coupling mechanism between the glucagon receptor and catalytic subunit of adenylate cyclase. Biochemical Journal **186**, 649-658.

- Houweling, A., van den Elsen, P.J. and van der Eb, A.J. (1980). Partial transformation of primary rat cells by the leftmost 4.5% fragment of adenovirus 5 DNA. Virology **105**, 537-550.
- Hughes, W.A., Brownsey, R.W. and Denton, R.M. (1980). Studies on the incorporation of [<sup>32</sup>P] phosphate into pyruvate dehydrogenase in intact rat fat cells. Biochemical Journal **192**, 469-481.
- Hunt, S.V. (1987). Preparation of lymphocytes and accessory cells in 'Lymphocytes, a practical approach' pp1-34. (Klaus, G.G.B. ed.) I.R.L. Press, Oxford.
- Illiano, G. and Cuatrecasas, P. (1972). Modulation of adenylate cyclase activity in liver and fat cell membranes by insulin. Science **175**, 906-908.
- Imai, Y., Rodan, S.B. and Rodan, G.A. (1988). Effects of retinoic acid on alkaline phosphatase messenger ribonucleic acid, catecholamine receptors and G-proteins in ROS 17/2.8 cells. Endocrinology **122**, 456-463.
- Ingebritsen, C.G., Hawelu-Johnson, C. and Ingebritsen, W.R. (1983). Alloxan-induced diabetes reduces  $\beta$ -adrenergic receptor number without affecting adenylate cyclase in rat ventricular membranes. Journal of Cardiovascular Pharmacology **5**, 454-461.
- Isom, H.C., Trevetha, M.J. and Taylor, J.M. (1980). Transformation of isolated rat hepatocytes with simian virus 40. Journal of Cell Biology **85**, 651-659.
- Ives, H.E. (1991). GTP binding proteins and growth factor signal transduction. Cellular Signalling **3**, 491-499.
- Jacobowitz, O., Chen, J., Premont, R.T. and Iyengar, R. (1993). Stimulation of specific types of G<sub>s</sub>-stimulated adenylyl cyclases by phorbol ester treatment. Journal of Biological Chemistry **268**, 3829-3832.
- Jat, P.S. and Sharp, P.A. (1986). Large T antigens of simian virus 40 and polyomavirus efficiently establish primary fibroblasts. Journal of Virology **59**, 746-750.

- Johnson, G.L., Kaslow, H.R. and Bourne, H.R. (1978). Genetic evidence that cholera toxin substrates are regulatory components of adenylate cyclase. Journal of Biological Chemistry **253**, 7120-7123.
- Johnson, M.L. and Venezia, C.M. (1980). Hormonal regulation of liver pyruvate kinase concentration and activity. Biochemistry **19**, 2191-2195.
- Johnson, R.A. and Toews, M.L. (1990). Protein kinase C activators sensitize cyclic AMP accumulation by intact 1321N1 human astrocytoma cells. Molecular Pharmacology **37**, 296-303.
- Johnson, R.A. and Salomon, Y. (1991). Assay of adenylyl cyclase catalytic activity. Methods in Enzymology **195**, 3-21.
- Johnson, R.A., Arneson-Rotert, L.J., Hoffman, J.M. and Toews, M.L. (1990). Serum-induced sensitization of cyclic AMP accumulation in 1321N1 human astrocytoma cells. Molecular Pharmacology **39**, 399-406.
- Jungermann, K. (1985). Metabolic zonation of liver parenchyma. Naturwissenschaften **72**, 76-84.
- Kahn, C.R. (1976). Membrane receptors for hormones and neurotransmitters. Journal of Cell Biology **70**, 261-286.
- Kahn, N.N. and Sinha, A.K. (1990). Stimulation of prostaglandin E<sub>1</sub> binding to human blood platelet membrane by insulin and the activation of adenylyl cyclase. Journal of Biological Chemistry **265**, 4976-4981.
- Karasik, A., Rothenberg, P.L., Yamada, K., White, M.F. and Kahn, C.R. (1990). Increased protein kinase C activity is linked to reduced insulin receptor phosphorylation in liver of starved rats. Journal of Biological Chemistry **265**, 10226-10231.
- Karlsson, A., Grunfeld, C., Kahn, C.R. and Roth, J. (1979). Regulation of insulin receptors and insulin responsiveness in 3T3-L1 fatty fibroblasts. Endocrinology **104**, 1383-1392.



- Kassis, S. and Fishman, P.H. (1982). Different mechanisms of desensitization of adenylyl cyclase by isoproterenol and prostaglandin E1 in human fibroblasts. Role of regulatory components in desensitization. Journal of Biological Chemistry **257**, 5312-5318.
- Kasuga, M., Karlson, F.A. and Kahn, C.R. (1982a). Insulin stimulates the phosphorylation of the 95kDa subunit of its own receptor. Science **215**, 185-187.
- Kasuga, M., Zick, Y., Blithe, D.L., Karlsson, F.A., Häring, H.U. and Kahn, C.R. (1982b). Insulin stimulates the phosphorylation of the  $\beta$  subunit of the insulin receptor. Formation of both phosphoserine and phosphotyrosine. Journal of Biological Chemistry **257**, 9891-9894.
- Katada, T. and Ui, M. (1982). Direct modulation of the membrane adenylate cyclase system by islet activating protein due to ADP-ribosylation of a membrane protein. Proceedings of the National Academy of Sciences of the U.S.A. **79**, 3129-3133.
- Katada, T., Bokoch, G.M., Smigel, M.D., Ui, M. and Gilman, A.G. (1984). The inhibitory guanine nucleotide binding regulatory component of adenylate cyclase: subunit dissociation and the inhibition of adenylate cyclase in S49 lymphoma  $cyc^-$  and wild type membranes. Journal of Biological Chemistry **259**, 3586-3595.
- Katada, T., Kusakabe, K., Oinuma, M. and Ui, M. (1987). A novel mechanism for the inhibition of adenylate cyclase via inhibitory G-proteins. Journal of Biological Chemistry **262**, 11897-11900.
- Katz, A., Wu, D. and Simon, M.I. (1992). Subunits  $\beta\gamma$  of heterotrimeric G-proteins activate  $\beta_2$  isoform of phospholipase C. Nature (Lond.) **360**, 686-689.
- Katz, N., Teutsch, H.F. and Sasse, D. and Jungermann, K. (1977). Heterogeneous distribution of glucose-6-phosphatase in microdissected periportal and perivenous rat liver tissue. F.E.B.S. Letters **76**, 226-230.

- Kavanaugh, W.M., Williams, L.T., Ives, H.E. and Coughlin, S.R. (1988). Serotonin-induced DNA synthesis in vascular smooth muscle cells involves a novel pertussis toxin sensitive pathway. Molecular Endocrinology **2**, 599-605.
- Kaziro, Y., Itoh, H., Kozasa, T., Nakafuku, M. and Satoh, T. (1991). Structure and function of signal transducing GTP binding proteins. Annual Review of Biochemistry **60**, 349-400.
- Kellerer, M., Obermaier-Kusser, B., Pröfrock, A., Schleicher, E., Seffer, E., Mushack, J., Ermel, B. and Häring, H-U. (1991). Insulin activates GTP binding to a 40kDa protein in fat cells. Biochemical Journal **276**, 103-108.
- Kelley Bentley, J. and Beavo, J.A. (1992). Regulation and function of cyclic nucleotides. Current Opinions in Cell Biology **4**, 233-240.
- Kikuchi, A., Ozawa, O., Kaibuchi, K., Katada, T., Ui, M. and Takai, Y. (1986). Direct evidence for involvement of a G-protein in chemotactic peptide stimulated formation of inositol bisphosphate and triphosphate in differentiated human leukaemic (HL-60) cells. Journal of Biological Chemistry **261**, 11558-11562.
- King, C.D. and Mayer, S.E. (1974). Inhibition of egress of adenosine 3'5' monophosphate from pigeon erythrocytes. Molecular Pharmacology **10**, 941-953.
- King, M.J. and Sale, G.S. (1990). Dephosphorylation of insulin receptor autophosphorylation sites by particulate and soluble phosphotyrosyl protein phosphatase. Biochemical Journal **266**, 251-259.
- Kleuss, C., Scherübl, H., Hescheler, J., Schultz, G. and Wittig, B. (1992). Different  $\beta$  subunits determine G-protein interaction with transmembrane receptors. Nature (Lond.) **358**, 424-426.
- Klip, A., Ramlal, T., Douen, A.G., Bilan, P.J. and Shorecki, K.L. (1988). Inhibition by forskolin of insulin-stimulated glucose transport in L6 muscle cells. Biochemical Journal **255**, 1023-1029.

- Knippers, R. (1989). The transforming protein of simian virus 40. Current Topics in Microbiology and Immunology **144**, 4-7.
- Knutson, V.P. (1991). Cellular trafficking and processing of the insulin receptor. F.A.S.E.B. Journal **5**, 2130-2138.
- Koch, C.A., Anderson, D., Moran, M.F., Ellis, C. and Pawson, T. (1991). SH2 and SH3 domains: elements that control interactions of cytoplasmic signalling proteins. Science **252**, 669-674.
- Koontz, J. and Iwahashi, M. (1981). Insulin as a potent specific growth factor in a rat hepatoma cell line. Science **211**, 947-949.
- Koski, G. and Klee, W.A. (1981). Opiates inhibit adenylate cyclase by stimulating GTP hydrolysis. Proceedings of the National Academy of Sciences of the U.S.A. **78**, 4185-4189.
- Kowluru, A., Kowluru, R.A. and Yamazaki, A. (1992). Functional alterations of G-proteins in diabetic rat retina: a possible explanation for the early visual abnormalities in diabetes mellitus. Diabetologia **35**, 624-631.
- Kozasa, T., Itoh, H., Tsukamoto, T. and Kaziro, Y. (1988). Isolation and characterisation of the human G $\alpha$  gene. Proceedings of the National Academy of Sciences of the U.S.A. **85**, 2081-2085.
- Kozka, I.J., Clark, A.E. and Holman, G.D. (1991). Chronic treatment with insulin selectively down-regulates cell surface GLUT 4 glucose transporters in 3T3-L1 adipocytes. Journal of Biological Chemistry **266**, 11726-11731.
- Krall, J.F., Connelly, M., Weisbart, R. and Tuck, M.L. (1981). Age-related elevation of plasma catecholamine concentration and reduced responsiveness of lymphocyte adenylate cyclase. Journal of Clinical Endocrinology and Metabolism **52**, 863-867.
- Krupinski, J., Rajaram, R., Lakonishok, M., Benovic, J.L. and Cerione, R.A. (1988). Insulin-dependent phosphorylation of GTP binding proteins in phospholipid vesicles. Journal of Biological Chemistry **263**, 12333-12341.

- Krupinski, J., Coussen, F., Bakalayar, H.A., Tang, W.J., Feinstein, P.G., Orth, K., Slaughter, C., Reed, R.R. and Gilman, A.G. (1989). Adenylyl cyclase amino acid sequence: possible channel or transporter-like structure. Science **244**, 1558-1564.
- Kurland, J.I. and Bockman, R. (1978). Prostaglandin E production by human blood monocytes and mouse peritoneal macrophages. Journal of Experimental Medicine **147**, 952-957.
- Kvanta, A., Nordstedt, C., van der Ploeg, I., Jondal, M. and Fredholm, B.B. (1989). CD3/T cell receptor coupling to a pertussis and cholera toxin insensitive G-protein. F.E.B.S. Letters **250**, 536-540.
- Laemmli, U.K. (1970). Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature (Lond.) **227**, 680-685.
- Landis, C.A., Masters, S.B., Spada, A., Pace, A.M., Bourne, H.R. and Vallar, L. (1989). GTPase inhibiting mutations activate the  $\alpha$  chain of  $G_s$  and stimulate adenylyl cyclase in pituitary tumours. Nature (Lond.) **340**, 692-696.
- Lanford, R.E., Wong, C. and Butel, J.S. (1985). Differential ability of a T antigen transport-defective mutant of simian virus 40 to transform primary and established rodent cells. Molecular and Cellular Biology **5**, 1043-1050.
- Langlois, D., Hinsch, K.D., Saez, J.M. and Begeot, M. (1990). Stimulatory effect of insulin and insulin-like growth factors on  $G_i$  proteins and angiotensin-II induced phosphoinositide breakdown in cultured bovine adrenal cells. Endocrinology **126**, 1867-1872.
- Larner, J., Romero, G., Kennington, A.S., Lilley, K., Kilgour, E., Zhang, C., Heimark, D., Gamez, G., Houston, D.B. and Huang, L.C. (1990). Duality of the mechanism of action of insulin. Advances in Second Messenger and Protein Phosphorylation Research **24**, 290-294.
- Lavan, B.E., Kuhné, M.R., Garner, C.W., Anderson, D., Reedijk, M., Pawson, T. and Lienhard, G.E. (1992). The association of insulin-elicited phosphotyrosine proteins with src homology 2 domains. Journal of Biological Chemistry **267**, 11631-11636.

- Le Marchand-Brustel, Y., Grémeaux, T., Ballotti, R. and Van Obberghen, E. (1985). Insulin receptor tyrosine kinase is defective in skeletal muscle of insulin resistant obese mice. Nature (Lond.) **315**, 676-679.
- Leung, D.Y.M., Key, L., Steinberg, J.J., Young, M.C., Von Deek, M., Wilkinson, R. and Geha, R.S. (1988). Increased in vitro bone resorption by monocytes in the hyperimmunoglobulin E syndrome. Journal of Immunology **140**, 84-88.
- Levitski, A. (1988). Transmembrane signalling to adenylyl cyclase in mammalian cells and in *Saccharomyces cerevisiae*. Trends in the Biochemical Sciences **13**, 298-301.
- Ligeti, E., Doussiene, J. and Vignais, P.V. (1988). Activation of the O<sup>2</sup>- generating oxidase in plasma membranes from bovine polymorphonuclear neutrophils by arachidonic acid, a cytosolic factor of protein nature and nonhydrolyzable analogues of GTP. Biochemistry **27**, 193-200.
- Linder, S. and Marshall, H. (1990). immortalization of primary cells by DNA tumour viruses. Experimental Cell Research **191**, 1-7.
- Livingston, D. and Bradley, M. (1987). The simian virus 40 large T antigen-a lot packed into a little. Molecular Biology and Medicine **4**, 63-80.
- Livingstone, C., McLellan, A.R., McGregor, M.A., Wilson, A., Connell, J.M., Small, M., Milligan, G., Paterson, K.R. and Houslay, M.D. (1991). Altered G-protein expression and adenylylase activity in platelets of non-insulin dependent diabetic (NIDDM) male subjects. Biochimica et Biophysica Acta **1096**, 127-133.
- Lloyd, C.E., Kalinyak, J.E., Hutson, S.M. and Jefferson, L.S. (1987). Stimulation of albumin gene transcription by insulin in primary cultures of rat hepatocytes. American Journal of Physiology **252**, C205-C214.
- Logothetis, D.E., Kurachi, Y., Galper, J. Neer, E.J. and Clapham, D.E. (1987). The  $\beta$  subunits of GTP binding proteins activate the muscarinic K<sup>+</sup> channel in heart. Nature (Lond.) **325**, 321-326.

- Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry **193**, 265-275.
- Luetje, C.W., Giershik, P., Milligan, G., Unson, C.G., Spiegel, A.M. and Nathanson, N.M. (1987). Tissue specific regulation of GTP binding proteins and muscarinic acetyl choline receptor levels during cardiac development. Biochemistry **26**, 4876-4884.
- Lynch, C.J., Blackmore, P.F., Johnson, E.H., Wange, R.L., Krone, P.K. and Exton, J.H. (1989). Guanine nucleotide binding regulatory proteins and adenylyl cyclase in livers of streptozotocin- and BB/Wor-diabetic rats. Journal of Clinical Investigation **83**, 2050-2062.
- Lynch, C.J and Exton, J.H. (1992). Alterations in G-protein mediated cell signalling in diabetes mellitus in 'G-proteins. Signal transduction and disease' pp87-108. (Milligan, G. and Wakelam, M.J.O. eds.). Acad. Press. London.
- Lyons, J., Landis, C.A., Harsh, G., Vallar, L., Grunewald, K., Feichtinger, H., Duh, Q.Y., Clark, O.H., Kawasaki, E., Bourne, H.R. and McCormick, F. (1990). Two G-protein oncogenes in human endocrine tumours. Science **249**, 655-659.
- MacDonald, C., Watts, P., Stuart, B., Kreuzburg-Duffy, U., Scott, D.M. and Kinne, R.K.H. (1991). Studies on the phenotype and karyotype of immortalised rabbit kidney epithelial cell lines. Experimental Cell Research **195**, 458-461.
- Martens, I., Nilsson, M., Magnusson, G. and Linder, S. (1988). Glucocorticoids facilitate the stable transformation of embryonal rat fibroblasts by a polyomavirus large T antigen-deficient mutant. Proceedings of the National Academy of Sciences of the U.S.A. **85**, 5571-5575.
- Martin, B.R. (1987). The mechanisms of action of insulin in 'Metabolic Regulation' pp144-169. Blackwell. London.

- Massague, J., Pilch, P.F. and Czech, M.P. (1981). Electrophoretic resolution of three major insulin receptor structures with unique subunit stoichiometries. Proceedings of the National Academy of Sciences of the U.S.A. **77**, 7137-7141.
- Massague, J., Blindermann, L.A. and Czech, M.P. (1982). The high affinity insulin receptor mediates growth stimulation in rat hepatoma cells. Journal of Biological Chemistry **257**, 13958-13963.
- Matlashewski, G., Schneider, J., Banks, L., Jones, N., Murray, A. and Crawford, L. (1987). Human papillomavirus type 16 DNA cooperates with activated ras in transforming primary cells. E.M.B.O. Journal **6**, 1741-1746.
- Mato, J. M. (1989). Insulin mediators revisited. Cellular Signalling **1**, 143-146.
- Mato, J.M., Kelly, K., Abler, A. and Jarett, L. (1987). Identification of a novel insulin sensitive glycopospholipid from H35 hepatoma cells. Journal of Biological Chemistry **262**, 2131-2137.
- Mattera, R., Yatani, A., Kirsch, G.E., Graf, R., Okabe, K., Olate, J., Codina, J., Brown, A.M. and Birnbaumer, L. (1989). Recombinant  $\alpha_3$  subunit of G-protein activates G $\kappa$  gated K $^+$  channels. Journal of Biological Chemistry **264**, 465-471.
- McArdle, H., Mullaney, I., Magee, A., Unson, C. and Milligan, G. (1988). GTP analogues cause release of the  $\alpha$  subunit of the GTP binding protein G $_o$  from the plasma membrane of NG108-15 cells. Biochemical and Biophysical Research Communications **152**, 243-251.
- McClue, S.J., Selzer, E., Freissmuth, M. and Milligan, G. (1992). Gi3 does not contribute to the inhibition of adenylyl cyclase when stimulation of an  $\alpha_2$  adrenergic receptor causes activation of both Gi2 and Gi3. Biochemical Journal **284**, 565-568.
- McFadzean, J., Mullaney, I., Brown, D.A. and Milligan, G. (1989). Antibodies to the GTP binding protein G $_o$  antagonize noradrenaline induced calcium current inhibition in NG108-15 hybrid cells. Neuron **3**, 177-182.

- McGowan, J.A., Strain, A.J. and Bucher, N.L.R. (1981). DNA synthesis in primary cultures of adult rat hepatocytes in a defined medium-effects of epidermal growth factor, insulin, glucagon and cyclic AMP. Journal of Cell Physiology **108**, 353-363.
- McKenzie, F.R. and Milligan, G. (1990). Prostaglandin E<sub>1</sub>-mediated cyclic AMP independent down regulation of G<sub>s</sub>α in neuroblastoma x glioma hybrid cells. Journal of Biological Chemistry **265**, 17084-17093.
- McNulty, E.E., Plevin, R. and Wakelam, M.J.O. (1990). Stimulation of the hydrolysis of phosphatidylinositol 4,5-bisphosphate and phosphatidylcholine by endothelin, a complete mitogen for Rat-1 fibroblasts. Biochemical Journal **272**, 761-766.
- Melin, B., Cherqui, G., Blivet, M.J., Caron, M., Lascols, O., Capeau, J. and Picard, J. (1990). Dual effect of metformin in cultured rat hepatocytes: potentiation of insulin action and prevention of insulin-induced insulin resistance. Metabolism **39**, 1089-1095.
- Milligan, G. (1990). Tissue distribution and subcellular location of guanine nucleotide binding proteins: implications for cell signalling. Cellular Signalling **1**, 411-419.
- Milligan, G. and Green, A. (1991). Agonist control of G-protein levels. Trends in the Pharmacological Sciences **12**, 207-209.
- Milligan, G., Streaty, R.A., Giershik, P., Spiegel, A.M. and Klee, W.A. (1987). Development of opiate receptors and GTP binding proteins in neonatal rat brain. Journal of Biological Chemistry **262**, 8626-8630.
- Mochly-Rosen, D., Chang, F-H, Cheever, M., Kim, I., Diamond, I. and Gordon, A.S. (1988). Chronic ethanol causes heterologous desensitization of receptors by reducing as RNA. Nature (Lond.) **333**, 848-850.
- Morgan, D.O. and Roth, R.A. (1987). Acute insulin action requires insulin receptor kinase activity- introduction of an inhibitory monoclonal antibody into mammalian cells blocks the rapid effects of insulin. Proceedings of the National Academy of Sciences of the U.S.A. **84**, 41-45.



- Mortensen, E.R., Drachman, J. and Guidotti, G. (1992). Guanosine nucleotides regulate hormone binding of insulin receptors. Biochemical Journal **281**, 735-743.
- Motulsky, H.J., Smith, D., Terman, B.I. and Feldman, R.D. (1987). Regulation of hormone stimulated cyclic AMP accumulation in intact human mononuclear leukocytes by blood plasma. Journal of Cyclic Nucleotide and Protein Phosphorylation Research **11**, 329-343.
- Mullaney, I., Magee, A.I., Unson, C.G. and Milligan, G. (1988). Differential regulation of the amounts of the guanine nucleotide binding proteins  $G_i$  and  $G_o$  in neuroblastoma x glioma hybrid cells in response to dibutyryl cyclic AMP. Biochemical Journal **256**, 649-656.
- Mumby, S.M., Kahn, R.A., Manning, D.R. and Gilman, A.G. (1986). Antisera of designed specificity for subunits of guanine nucleotide binding regulatory proteins. Proceedings of the National Academy of Sciences of the U.S.A. **83**, 265-269.
- Munnich, A., Marie, J., Reach, G., Vaulont, S., Simon M.P. and Kahn, A. (1984). In vivo hormonal control of L type pyruvate kinase gene expression. Journal of Biological Chemistry **259**, 10228-10231.
- Murphy, G.J., Gawler, G.J., Milligan, G., Wakelam, M.J.O., Pyne, N.J. and Houslay, M.D. (1989). Glucagon desensitization of adenylate cyclase and stimulation of inositol phospholipid metabolism does not involve the inhibitory guanine nucleotide regulatory protein  $G_i$  which is inactivated upon challenge of hepatocytes with glucagon. Biochemical Journal **259**, 191-197.
- Musk, P., Heyworth, C.M., Vallance, S.J. and Whetton, A.D. (1992). The role of G-proteins in the regulation of myeloid haemopoietic cell proliferation and transformation in 'G-proteins. Signal transduction and disease' pp217-240. (Milligan, G. and Wakelam, M.J.O. eds.). Acad. Press. London.
- Nagata, Y., Diamond, B. and Bloom, B.R. (1983). The generation of human monocyte / macrophage cell lines. Nature (Lond.) **306**, 597-599.

- Nairn, A., Willett, B., Grant, M.H., Scott, A. and MacDonald, C. (1990). Maintenance of differentiated function in cultured rat hepatocytes by transfection with viral DNA. Biochemical Society Transactions **18**, 1201-1202.
- Nakamura, T., Tomita, Y. and Ichihara, A. (1983). Density dependent growth control of adult rat hepatocytes in primary culture. Journal of Biochemistry **94**, 1029-1035.
- Neer, E.J., Echeverria, D. and Knox, S. (1980). Increase in the size of soluble brain adenylate cyclase with activation by guanosine 5'-( $\beta,\gamma$ -imido) triphosphate. Journal of Biological Chemistry **255**, 9782-9789.
- Neufeld, N.D., Ezrin, C., Corbo, L., Long, D. and Bush, M. (1986). Effects of caloric restriction and exercise on insulin receptors in obesity: association with changes in membrane lipids. Metabolism **35**, 580-587.
- Newbold, R.F., Overell, R.W. and Connell, J.R. (1982). Induction of immortality is an early event in malignant transformation of mammalian cells by carcinogens. Nature (Lond.) **299**, 633-635.
- Norman, A.W. and Litwack, G. (1987). Prostaglandins and leukotrienes in 'Hormones' pp645-680. Acad. Press Inc. San Diego.
- Northup, J.K., Sternweis, P.C., Smigel, M.D., Schleifer, L.S., Ross, E.M. and Gilman, A.G. (1980). Purification of the regulatory component of adenylate cyclase. Proceedings of the National Academy of Sciences of the U.S.A. **77**, 6516-6520.
- O'Brien, R., Houslay, M.D., Milligan, G. and Siddle, K. (1987). The insulin receptor tyrosine kinase phosphorylates holomeric forms of the guanine nucleotide regulatory proteins  $G_i$  and  $G_o$ . F.E.B.S. Letters **212**, 281-288.
- O'Brien, R. and Granner, D.K. (1991). Regulation of gene expression by insulin. Biochemical Journal **278**, 609-619.

- Offringa, R., Smits, A.M.M., Houweling, A., Bos, J.L. and van der Eb, A.J. (1988). Similar effects of adenovirus E1A and glucocorticoid hormones in the expression of the metalloprotease stromelysin. Nucleic Acids Research **16**, 10973-10984.
- Ohisalo, J.J., Vikman, H.L., Ranta, S., Houslay, M.D. and Milligan, G. (1989). Adipocyte plasma membrane Gi and Gs in insulin dependent diabetic patients. Biochemical Journal **264**, 289-292.
- Okamoto, M., White, M.F., Maron, R. and Kahn, C.R. (1986). Autophosphorylation and kinase activity of insulin receptor in diabetic rats. American Journal of Physiology **251**, E542-E550.
- Okamura, T., Sago, T. and Saito, K. (1988). Effect of prostaglandins and their analogues on hormone stimulated glycogenolysis in primary cultures of rat hepatocytes. Biochimica et Biophysica Acta **958**, 179-187.
- Orford, M., Mazurkiewicz, D., Milligan, G. and Saggerson, E.D. (1991). Abundance of the  $\alpha$  subunits of Gi1, Gi2 and Go in synaptosomal membranes from several regions of the rat brain is increased in hypothyroidism. Biochemical Journal **275**, 183-186.
- O'Shea, J.J., Urdahl, K.B., Luong, H.T., Chused, T.M., Samelson, L.E. and Klausner, R.D. (1987). Aluminium fluoride induces phosphatidylinositol turnover, elevation of cytosolic free calcium and phosphorylation of the T cell antigen receptor in murine T cells. Journal of Immunology **139**, 3463-3469.
- Pace, A.M., Wong, Y.H. and Bourne, A.R. (1991). A mutant  $\alpha$  subunit of Gi2 induces neoplastic transformation of Rat-1 cells. Proceedings of the National Academy of Sciences of the U.S.A. **88**, 7031-7035.
- Palmer, T., Taberner, P.V. and Houslay, M.D. (1992). Alterations in G-protein expression, Gi function and stimulatory receptors mediated regulation of adipocyte adenylyl cyclase in a model of insulin resistant diabetes with obesity. Cellular Signalling **4**, 365-377.

- Pang, D.T., Sharma, B.R., Shafer, J.A., White, M.F. and Kahn, C.R. (1985). Predominance of tyrosine phosphorylation of insulin receptors during the initial response of intact cells to insulin. Journal of Biological Chemistry **260**, 7131-7136.
- Pasko, K.L., Salvin, S.B. and Winkelstein, A. (1981). Mechanisms in the in vivo release of lymphokines- Responses in alloxan treated and genetically diabetic mice. Cellular Immunology **62**, 205-219.
- Pawson, T. and Gish, G.D. (1992). SH2 and SH3 domains: from structure to function. Cell **71**, 359-362.
- Petit, C.A., Gardes, M. and Feunteun, J. (1983). Immortalization of rodent embryo fibroblasts is maintained by the A gene. Virology **127**, 74-82.
- Petruzelli, L.M., Herrera, R., Arenas Garcia, R., Fernandez, R., Birnbaum, M.J. and Rosen, O.M. (1986). Isolation of a Drosophila genomic sequence homologous to the kinase domain of the human insulin receptor and detection of the phosphorylated Drosophila receptor with an antipeptide antibody. Proceedings of the National Academy of Sciences of the U.S.A. **83**, 4710-4714.
- Pfeiffer, A. and Schatz, H. (1992). Pathophysiological aspects of non-tyrosine kinase signal transduction. Hormone and Metabolic Research **24**, 219-224.
- Pfeuffer, T. (1991). Synthesis of forskolin-agarose affinity matrices. Methods in Enzymology **195**, 44-51.
- Phipps, R.P., Lee, D., Schad, V. and Warner, G.L. (1989). E series prostaglandins are potent growth inhibitors for some  $\beta$  lymphokines. European Journal of Immunology **19**, 995-1001.
- Pilkis, S.J. (1970). Hormonal control of glucokinase. Biochimica et Biophysica Acta **461**, 215-226.
- Pilkis, S.J., Exton, J.H., Johnson, R.A. and Park, C.R. (1974). Effects of glucagon on cyclic AMP and carbohydrate metabolism in livers from diabetic rats. Biochimica et Biophysica Acta **343**, 250-267.

- Pilkis, S.J., Claus, T.H., Johnson, R.A. and Park, C.R. (1975). Hormonal control of cyclic 3',5'-AMP levels and gluconeogenesis in isolated hepatocytes from fed rats. Journal of Biological Chemistry **250**, 6328-6336.
- Pobiner, B.F., Northup, J.K., Bauer, P.H, Fraser, E.D., Garrison, J.C. (1991). Inhibitory guanine nucleotide binding regulatory protein Gi3 can couple angiotensin II receptors to inhibition of adenylyl cyclase in hepatocytes. Molecular Pharmacology **40**, 156-167.
- Polakis, P.G., Uhing, R.J. and Snyderman, R. (1988). The formyl peptide chemoattractant receptor copurifies with a GTP binding protein containing a distinct 40kDa pertussis toxin substrate. Journal of Biological Chemistry **263**, 4969-4976.
- Premont, R.T., Chen, J., Ma, H-W., Ponnappalli, M. and Iyengar, R. (1992). Two members of a widely expressed subfamily of hormone stimulated adenylyl cyclases. Proceedings of the National Academy of Sciences of the U.S.A. **89**, 9809-9813.
- Pyne, N.J., Heyworth, C.M., Balfour, N.W. and Houslay, M.D. (1989a). Insulin affects the ability of Gi to be ADP-ribosylated but does not elicit its phosphorylation in intact hepatocytes. Biochemical and Biophysical Research Communications **165**, 251-256.
- Pyne, N.J., Cushley, W., Nimmo, H.G. and Houslay, M.D. (1989b). Insulin stimulates the tyrosyl phosphorylation and activation of the 52kDa peripheral plasma membrane cyclic AMP phosphodiesterase in intact hepatocytes. Biochemical Journal **264**, 897-904.
- Rall, T.W., Sutherland, E.W. and Berthet, J. (1967). The relationship of epinephrine and glucagon to liver phosphorylase. Effect of epinephrine and glucagon on the reactivation of phosphorylase in liver homogenates. Journal of Biological Chemistry **224**, 463-475.
- Rappaport, R.S. and Dodge, G.R. (1982). Prostaglandin E inhibits the production of human interleukin-2. Journal of Experimental Medicine **155**, 943-948.

- Ray, T.K., Dutta-Roy, A.K. and Sinha, A.K. (1986). Regulation of insulin receptor activity of human erythrocyte membranes by prostaglandin E. Biochimica et Biophysica Acta **856**, 421-427.
- Rechler, M.M. and Nissley, S.P. (1985). The nature and regulation of the receptors for insulin-like growth factors. Annual Review of Physiology **47**, 425-442.
- Reibman, J., Haines, K. and Weissmann, G. (1990). Alterations in cyclic nucleotides and the activation of neutrophils. Current Topics in Membrane Transport **35**, 399-424.
- Reithmann, C., Giershik, P., Sideropoulos, D., Werdan, K. and Jakobs, K.H. (1989). Mechanism of noradrenaline-induced heterologous desensitization of adenylyl cyclase stimulation in rat muscle cells: increase in the level of inhibitory G-protein  $\alpha$  subunits. European Journal of Pharmacology (Molecular Pharmacology) **172**, 211-221.
- Reithmann, C., Giershik, P., Werdan, K. and Jakobs, K.H. (1991). Role of inhibitory G-protein  $\alpha$  subunits in adenylyl cyclase desensitization. Molecular and Cellular Endocrinology **82**, C215-C221.
- Rice, K.M., Lienhard, G.E. and Garner, C.W. (1992). Insulin signalling through the protein phosphotyrosine pathway in 3T3-L1 adipocytes and insulin resistance. Diabetes **41**, (Suppl 1) 8A.
- Rich, K.A., Codina, J., Floyd, G., Sekura, R., Hildebrandt, J.D. and Iyengar, R. (1984). Glucagon-induced heterologous desensitization of the MDCK cell adenylyl cyclase. Journal of Biological Chemistry **259**, 7893-7901.
- Richman, R.A., Claus, T.H., Pilkis, S.J. and Friedman, D.L. (1976). Hormonal stimulation of DNA synthesis in primary cultures of adult rat hepatocytes. Increases in the apparent levels of the inhibitory regulator (Ni). Proceedings of the National Academy of Sciences of the U.S.A. **73**, 3589-3593.
- Robertson, R.P. and Chen, M. (1977). Reversal by methylsergide of inhibition of insulin secretion by prostaglandin E in the dog. Journal of Clinical Investigation **62**, 1014-1019.

- Robertson, R.P., Westcott, K.R., Strom, D.R. and Rice, M.G. (1980). Down regulation of in vivo prostaglandin E receptors and adenylate cyclase stimulation in rat liver plasma membranes. American Journal of Physiology **239**, E75-E80.
- Rocklin, R.E. and Thistle, L. (1986). Reduced prostaglandin E<sub>2</sub> receptors on atopic T lymphocytes. Cellular Immunology **99**, 294-299.
- Rodbell, M. (1980). The role of hormone receptors and GTP regulatory proteins in membrane transduction. Nature (Lond.) **284**, 17-22.
- Rodbell, M., Krans, H.M.J., Pohl, S.L. and Birnbaumer, L. (1971). The glucagon sensitive adenylyl cyclase system in plasma membranes of rat liver-effects of guanyl nucleotides on binding of <sup>125</sup>I-glucagon. Journal of Biological Chemistry **246**, 1872-1876.
- Roesler, W.J., Vandenbark, G.R. and Hanson, R.W. (1988). Cyclic AMP and the induction of eukaryotic gene transcription. Journal of Biological Chemistry **263**, 9063-9066.
- Rojas, F.J. and Birnbaumer, L. (1985). Assay for the glucagon receptor. Methods in Enzymology **109**, 3-20.
- Romero, G., Luttrell, L., Rogol, A., Zeller, K., Hewlett, E. and Lerner, J. (1988). Phosphatidylinositol-glycan anchors of membrane proteins: potential precursors of insulin mediators. Science **240**, 509-511.
- Roper, R.L., Conrad, D.H., Brown, D.M., Warner, G.L. and Phipps R.P. (1990). Prostaglandin E<sub>2</sub> promotes IL-4-induced IgE and IgG<sub>1</sub> synthesis. Journal of Immunology **145**, 2644-2651.
- Ros, M., Northup, J.K. and Malbon, C.C. (1989a). Adipocyte G-proteins and adenylyl cyclase: effects of adrenalectomy. Biochemical Journal **257**, 737-744.
- Ros, M., Watkins, D.C. Rapiejko, P.J. and Malbon, C.C. (1989b). Glucocorticoids modulate mRNA levels for G-protein  $\beta$  subunit. Biochemical Journal **260**, 271-275.

- Roth, R.A. (1988). Structure of the receptor for insulin-like growth factor-II: the puzzle amplified. Science **239**, 1269-1271.
- Roth, R.A. and Beaudoin, J. (1987). Phosphorylation of the purified insulin receptor by cyclic AMP kinase. Diabetes **36**, 123-126.
- Rothenberg, P.L. and Kahn, C.R. (1988). Insulin inhibits pertussis toxin-catalyzed ribosylation of G-proteins. Journal of Biological Chemistry **263**, 15546-15552.
- Sager, G., Johansen, O., and Mjøs, O.D. (1990). Effects of insulin on mononuclear leukocyte  $\beta$ -adrenoceptor density and adenylyl cyclase coupling. European Journal of Pharmacology (Molecular Pharmacology) **188**, 243-249.
- Saggerson, E.D. (1992). Thyroid disorders in 'G-proteins. Signal transduction and disease' pp157-190. (Milligan, G. and Wakelam, M.J.O. eds.). Acad. Press London.
- Saggerson, E.D. (1986). Sensitivity of adipocyte lipolysis to stimulatory and inhibitory agonists in hypothyroidism and starvation. Biochemical Journal **238**, 387-394.
- Saito, N., Guitart, X., Hayward, M., Tallman, J.M., Duman, R.S. and Nestler, E.J. (1989). Corticosterone differentially regulates the expression of  $G_s\alpha$  and  $G_i\alpha$  messenger RNA and protein in rat cerebral cortex. Proceedings of the National Academy of Sciences of the U.S.A. **86**, 3906-3910.
- Sale, E.M. and Denton, R.M. (1985). Adrenergic agents increase the phosphorylation of phosphofructokinase in isolated rat epididymal white adipose tissue. Biochemical Journal **232**, 905-910.
- Sale, G.J. and Smith, D.M. (1989). Serine phosphorylations triggered by the insulin receptor. Cellular Signalling **1**, 205-218.
- Salesse, R., Garnier, J., Letterrier, F., Daveloose, D. and Viret, J. (1982). Modulation of adenylate cyclase activity by the physical state of pigeon erythrocyte membrane-parallel drug induced changes in the bilayer fluidity and adenylate cyclase activity. Biochemistry **21**, 1581-1586.



- Saltiel, A.R. and Cuatrecasas, P. (1986). Insulin stimulates the generation from hepatic plasma membranes of modulators derived from an inositol glycolipid. Proceedings of the National Academy of Sciences of the U.S.A. **83**, 5793-5797.
- Sasaki, T. and Hasegawa-Sasaki, H. (1987). Activation of polyphosphoinositide phospholipase C by guanosine 5'-O-(3-thio) triphosphate and fluoroaluminate in membranes prepared from a human T cell leukaemia cell line JURKAT. E.E.B.S. Letters **218**, 87-92.
- Sato, N., Irie, M., Kajinuma, H. and Suzuki, K. (1989). Decreased glucagon binding and glucagon-stimulated lipolysis in adipocytes from streptozotocin diabetic rats. Acta Endocrinologica **121**, 705-713.
- Sattler, C.A., Michalopoulos, G., Sattler, G.L. and Pitot, H.C. (1978). Ultrastructure of adult rat hepatocytes cultured on floating collagen membranes. Cancer Research **38**, 1539-1549.
- Scheidtmann, K.H. (1989). SV40 large T antigen induces a protein kinase responsible for phosphorylation of the cellular protein p53. Current Topics in Microbiology and Immunology **144**, 85-88.
- Schimmel, R.J. (1976). Adenosine 3'5'cyclic monophosphate in adipose tissue of diabetic rats. Biochimica et Biophysica Acta **451**, 363-371.
- Schmid, C., Steiner, T. and Froesch, E.R. (1983). Preferential enhancement of myoblast differentiation by insulin-like growth factors (IGF-I and IGF-II) in primary cultures of chicken embryonic cells. E.E.B.S. Letters **161**, 117-121.
- Scott Thies, R., Molina, J.M., Ciaraldi, T.P., Freidenberg, G.R. and Olefsky, J.M. (1990). Insulin receptor autophosphorylation and endogenous substrate phosphorylation in human adipocytes from control, obese and NIDDM subjects. Diabetes **39**, 250-259.
- Seamon, K.B., Padgett, W. and Daly, J.W. (1981). Forskolin: unique diterpene activator of adenylate cyclase in membranes and intact cells. Proceedings of the National Academy of Sciences of the U.S.A. **78**, 3363-3367.

- Seamon, K.B. and Daly, J.W. (1983). Forskolin, cyclic AMP and cell physiology. Trends in the Pharmacological Sciences **4**, 120-123.
- Seamon, K.B. and Daly, J.W. (1986). Forskolin: its biological and chemical properties. Advances in Second Messenger and Protein Phosphorylation Research **20**, 1-150.
- Seuwen, K., Magnaldo, I. and Pouysségur, J. (1988). Serotonin stimulates DNA synthesis in fibroblasts acting through 5-HT<sub>1B</sub> receptors coupled to a G<sub>i</sub> protein. Nature (Lond.) **335**, 254-256.
- Seuwen, K., Magnaldo, I., Kobilka, B.K., Caron, M.G., Regan, J.W., Lefkowitz, R.J. and Pouysségur, J. (1990).  $\alpha_2$ -adrenergic agonists stimulate DNA synthesis in Chinese hamster lung fibroblasts transfected with a human  $\alpha_2$ -adrenergic receptor gene. Cell Regulation **1**, 445-451.
- Shah, S.V., Wallin, J.D. and Eilen, S.D. (1983). Chemiluminescence and superoxide anion production by leukocytes from diabetic patients. Journal of Clinical Endocrinology and Metabolism **57**, 402-409.
- Shamoon, H., Hendler, R. and Sherwin, R.S. (1980). Altered responsiveness to cortisol, epinephrine and glucagon in insulin-infused juvenile onset diabetics. Diabetes **29**, 284-291.
- Sheorain, V.S., Khatra, B.S. and Soderling, T.R. (1982). Hormonal regulation of skeletal muscle glycogen synthase through covalent phosphorylation. Federal Proceedings **41**, 2618-2622.
- Simkin, M.J., Jelinek, D.F. and Lipsky, P.E. (1987). Inhibition of human B cell responsiveness by prostaglandin E<sub>2</sub>. Journal of Immunology **138**, 1074-1081.
- Simmoteit, R.R., Schulzki, H.D., Palm, D., Mollner, S. and Pfeuffer, T. (1991). Chemical and functional analysis of components of adenylyl cyclase from human platelets treated with phorbol esters. F.E.B.S. Letters **249**, 189-194.
- Simon, M.I., Strathmann, M.P. and Gautam, N. (1991). Diversity of G-proteins in signal transduction. Science **252**, 802-808.

- Solomon, S.S., Steiner, M.S., Sanders, L. and Palazzolo, M.R. (1986). Spontaneously diabetic BB rat: studies of cyclic adenosine 3'5'-monophosphate phosphodiesterase and calmodulin. Endocrinology **119**, 1839-1844.
- Solomon, S.S., Sibley, S.D. and Cunningham, T.M. (1990). Growth hormone receptors are up-regulated in diabetic adipocytes. Endocrinology **127**, 1544-1546.
- Soman, V. and Felig, P. (1978). Glucagon binding and adenylate cyclase activity in liver membranes from untreated and insulin-treated diabetic rats. Journal of Clinical Investigation **61**, 552-560.
- Spandidos, D.A. and Wilkie, N.M. (1984). Malignant transformation of early passage rodent cells by a single mutated human oncogene. Nature (Lond.) **310**, 469-475.
- Spence, J.T. (1983). Levels of translatable mRNA coding for rat liver glucokinase. Journal of Biological Chemistry **258**, 9143-9146.
- Spiegel, A.M. (1992). G-proteins in cellular control. Current Opinions in Cell Biology **4**, 203-211.
- Srikant, C.B., Freeman, D., McCorkle, K. and Unger, R.H. (1977). Binding and biologic activity of glucagon in liver cell membranes of chronically hyperglucagonaemic rats. Journal of Biological Chemistry **252**, 7434-7436.
- Stadtmauer, L. and Rosen, O.M. (1986). Phosphorylation of synthetic insulin receptor peptides by the insulin receptor kinase and evidence that the preferred sequence containing tyrosine 1150 is phosphorylated in vivo. Journal of Biological Chemistry **261**, 10000-10006.
- Staedel, C., Bruneau, C., Le Marchand-Brustel, Y., Cremel, G. and Hubert, P. (1990). Insulin sensitivity and membrane lipid environment in hepatoma cells in 'Insulin and the cell membrane' pp355-371. (Klimes, I., Howard, B.V. and Kahn, C.R. eds.) Harwood Acad. Pub. London.

- Stanley, F. (1988). Stimulation of prolactin gene expression by insulin. Journal of Biological Chemistry **263**, 13444-13448.
- Stow, J.L., Almeida, J.B., Narula, N., Holtzman, E.J., Ercolani, L. and Ausiello, D.A. (1991). A heterotrimeric G-protein G $\alpha$ -3 in Golgi membranes regulates the secretion of a heparan sulphate proteoglycan in LLC-PK1 epithelial cells. Journal of Cell Biology **114**, 1113-1124.
- Stralfros, P., Bjorgell, P. and Belfrage, P. (1984). Hormonal regulation of hormone sensitive lipase in intact adipocytes: identification of phosphorylated sites and effects on the phosphorylation by lipolytic hormones and insulin. Proceedings of the National Academy of Sciences of the U.S.A. **81**, 3317-3321.
- Strassheim, D., Milligan, G. and Houslay, M.D. (1990). Diabetes abolishes the GTP dependent, but not the receptor dependent inhibitory function of the inhibitory guanine nucleotide binding regulatory protein G $_i$  on adipocyte adenylyl cyclase activity. Biochemical Journal **266**, 521-566.
- Strassheim, D., Palmer, T. and Houslay, M.D. (1991). Genetically acquired diabetes: adipocyte guanine nucleotide regulatory protein expression and adenylyl cyclase regulation. Biochimica et Biophysica Acta **1096**, 121-126.
- Straus, D.S. and Takemoto, C.D. (1987). Insulin negatively regulates albumin mRNA at the transcriptional and post-transcriptional level in rat hepatoma cells. Journal of Biological Chemistry **262**, 1955-1960.
- Strom, S.C. and Michalopoulos, G. (1982). Collagen as a substrate for cell growth and differentiation. Methods in Enzymology **82**, 544-555.
- Summers, S.T. and Cronin, M.J. (1986). Phorbol esters enhance basal and stimulated adenylyl cyclase activity in a pituitary cell line. Biochemical and Biophysical Research Communications **135**, 276-281.
- Sun, X.J., Rothenberg, P., Kahn, C.R., Backer, J.M., Araki, E., Wilden, P.A., Cahill, D.A., Goldstein, B.J. and White, M.F. (1991). Structure of the insulin receptor substrate IRS-1 defines a unique signal transduction protein. Nature (Lond.) **352**, 73-77.

- Sutherland, E.W., Rall, T.W., and Menon, T. (1962). Adenyl cyclase-distribution, preparation and properties. Journal of Biological Chemistry **237**, 1220-1227.
- Tabakoff, B., Hoffman, P.L., Lee, J.M., Saito, T., Willard, B. and DeLeon Jones, F. (1988). Differences in platelet enzyme activity between alcoholics and non-alcoholics. New England Journal of Medicine **318**, 134-139.
- Takayama, S., White, M.F., Lauris, V. and Kahn, C.R. (1984). Phorbol esters modulate insulin receptor phosphorylation and insulin action in cultured hepatoma cells. Proceedings of the National Academy of Sciences of the U.S.A. **81**, 7797-7801.
- Takayama, S., Stegman, E.W., Maron, R. and Kahn, C.R. (1988a). The effect of antiphosphotyrosine antibodies on insulin receptor phosphorylation and insulin response in rat hepatoma cells. Diabetes **35** (Suppl 1), 8A.
- Takayama, S., Kahn, C.R., Kubo, K. and Foley, J.E. (1988b). Alterations in insulin receptor autophosphorylation in insulin resistance-correlation with altered sensitivity to glucose transport and anti lipolysis to insulin. Endocrinology and Metabolism **66**, 992-999.
- Tamborlane, W.V., Sherwin, R.S., Koivisto, V., Hendler, R., Genel, M. and Felig, P. (1979). Normalization of the growth hormone and catecholamine response to exercise in juvenile onset diabetic subjects treated with a portable insulin infusion pump. Diabetes **28**, 785-788.
- Tanaka, K., Sato, M., Tomita, Y. and Ichihara, A. (1978). Biochemical studies on liver function in primary cultured hepatocytes of adult rats. Journal of Biological Chemistry **84**, 937-946.
- Tang, W.J. and Gilman, A.G. (1992). Adenylyl cyclases. Cell **70**, 869-872.
- Tanti, J.F., Grémeaux, T., Rochet, N., Van Obberghen, E. and Le Marchand-Brustel, Y. (1987). Effect of cyclic AMP dependent protein kinase on insulin receptor tyrosine kinase activity. Biochemical Journal **245**, 19-26.

- Taub, R., Roy, A., Dieter, R. and Koontz, J. (1987). Insulin as a growth factor in rat hepatoma cells-stimulation of protooncogene expression. Journal of Biological Chemistry **262**, 10893-10897.
- Taylor, C.W., Blakeley, D.M., Corps, A.N., Berridge, M.J. and Brown, K.D. (1988). Effects of pertussis toxin on growth factor-stimulated inositol phosphate formation and DNA synthesis in Swiss 3T3 cells. Biochemical Journal **249**, 917-920.
- Taylor, S.S. (1989). Cyclic AMP-dependent protein kinase: model for an enzyme family. Journal of Biological Chemistry **264**, 8443-8446.
- Todaro, G.J. and Green, H. (1963). Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. Journal of Cell Biology **17**, 299-313.
- Toews, M.L. and Arneson-Rotert, L.J. (1990). Serum-induced sensitization of cyclic AMP accumulation in C62B rat glioma cells. F.A.S.E.B. Journal **4**, A4962.
- Tovey, K.C., Oldham, K.G. and Whelan, J.A.M. (1974). A simple direct assay for cyclic AMP in plasma and other biological samples using an improved competitive protein binding technique. Clinica Chimica Acta **56**, 221-234.
- Towbin, H., Staehelin, T. and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proceedings of the National Academy of Sciences of the U.S.A. **76**, 4350-4354.
- Tsukada, Y., Hibi, N. and Ohkawa, K. (1985). Hormonal regulation during secretion of  $\alpha$ -fetoprotein in hepatoma cells grown on synthetic medium. Journal of Biological Chemistry **260**, 16316-16320.
- Uhing, R.J., Polakis, P.G. and Snyderman, R. (1987). Isolation of GTP binding proteins from HL-60 cells. Journal of Biological Chemistry **262**, 15575-15579.

- Ullrich, A., Bell, J.R., Clen, E.Y., Herrera, R., Petruzelli, L.M., Dull, T.J., Gray, A., Liao, Y.C., Tsubokawa, M., Mason, A., Seeburg, P.H., Grunfeld, C., Rosen, O.M. and Ramachandran, J. (1985). Human insulin receptor and its relationship to the tyrosine kinase family of oncogenes. Nature (Lond.) **313**, 756-761.
- Urasawa, K. and Insel, P.A. (1992). GTP binding proteins and cardiovascular disease in 'G-proteins. Signal transduction and disease' pp47-85. (Milligan, G. and Wakelam, M.J.O. eds.) Acad. Press London.
- Vallar, L., Spada, A. and Giannattasio, G. (1987). Altered G<sub>s</sub> and adenylate cyclase activity in human growth hormone-secreting pituitary adenomas. Nature (Lond.) **330**, 566-568.
- Van Obberghen, E., Le Marchand-Brustel, Y., Gammeltoft, S. and Ballotti, R. (1990). Insulin receptor: tyrosine kinase activity and insulin action in 'Insulin and the cell membrane' pp153-163. (Klimes, I., Howard, B.V. and Kahn, C.R. eds.) Harwood Acad. Pub. London.
- Vom Dahl, S., Hallbrucker, C., Lang, F., Gerok, W. and Häussinger, D. (1991). Regulation of liver cell volume and proteolysis by glucagon and insulin. Biochemical Journal **278**, 771-777.
- Wahrenberg, H., Lönnqvist, F., Engfeldt, P. and Arner, P. (1989). Abnormal action of catecholamines in lipolysis in adipocytes of type 1 diabetic patients treated with insulin. Diabetes **38**, 524-533.
- Walseth, T.F., Zhang, H-J., Olson, L.K., Schroeder, W.K. and Robertson, R.P. (1989). Increase in G<sub>s</sub> and cyclic AMP in HIT cells-evidence that the 45kDa  $\alpha$  subunit of G<sub>s</sub> has greater functional activity than the 52kDa  $\alpha$  subunit. Journal of Biological Chemistry **264**, 21106-21111.
- Watkins, D.C., Northup, J.K., Malbon, C.C. (1987). Regulation of G-protein levels in differentiation-altered ratio of  $\alpha$  to  $\beta$  subunits. Journal of Biological Chemistry **262**, 10651-10657.

- Watkins, D.C., Rapiejko, P.J., Ros, M., Wong, H.Y. and Malbon, C.C. (1989). G-protein mRNA levels during adipocyte development. Biochemical and Biophysical Research Communications **165**, 929-934.
- Weinstein, L.S., Spiegel, A.M. and Carter, A.D. (1988). Cloning and characterization of the human gene for the  $\alpha$  subunit of Gi2, a GTP binding signal transduction protein. E.E.B.S. Letters **232**, 333-340.
- White, M.F., Maron, R. and Kahn, C.R. (1985). The insulin receptor rapidly stimulates tyrosine phosphorylation of a Mr = 185000 protein in intact cells. Nature (Lond.) **318**, 183-186.
- White, M.F. and Kahn, C.R. (1986). The insulin receptor and tyrosine phosphorylation in 'The Enzymes' pp247-302.(Boyer, P.D. and Krebs, E.G. eds.). Acad. Press Inc. Orlando.
- White, M.F., Stegman, E.W., Dull, T.J., Ullrich, A. and Kahn, C.R. (1987). Characterization of an endogenous substrate of the insulin receptor in cultured cells. Journal of Biological Chemistry **262**, 9766-9777.
- White, M.F., Livingston, J.N., Backer, J.M., Dull, T.J., Ullrich, A. and Kahn, C.R. (1988). Mutation of the insulin receptor at tyrosine 960 inhibits signal transmission but does not affect its tyrosine kinase activity. Cell **54**, 641-649.
- Widstrom, A. (1977). Influence of indomethacin on glucose-induced insulin response in normal man-role of prostaglandins in the rapid insulin release. Hormone and Metabolic Research **9**, 172-175.
- Winocour, P.D., Bryszewska, M., Watala, C., Rand, M.L., Epanand, R.M., Kinlough-Rathbone, R.L., Packham, M.A. and Mustard, J.F. (1990). Reduced membrane fluidity in platelets from diabetic patients. Diabetes **39**, 241-244.
- Woodworth, C.D., Secott, T., and Isom, H.C. (1986). Transformation of rat hepatocytes by transfection with SV40 virus DNA to yield proliferating differentiated cells. Cancer Research **46**, 4018-4026.

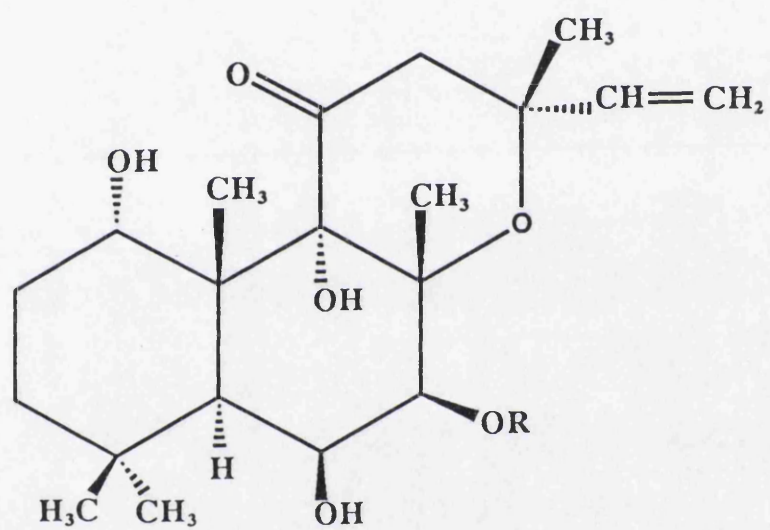


- Woodworth, C.D., Kreider, J.W., Mengel, L., Miller, T., Meng, Y.L. and Isom, H.C. (1988). Tumorigenicity of simian virus 40 hepatocyte cell lines-effect of in vitro and in vivo passage on expression of liver specific genes and oncogenes. Molecular and Cellular Biology **8**, 4492-4501.
- Yamamura, H., Lad, P.M. and Rodbell, M. (1977). GTP stimulates and inhibits adenylate cyclase in fat cell membranes through distinct regulatory processes. Journal of Biological Chemistry **252**, 7964-7966.
- Yip, C.C., Yeung, C.W.T. and Moule, M.L. (1978). Photoaffinity labelling of insulin receptor of rat adipocyte plasma membranes. Journal of Biological Chemistry **253**, 1743-1745.
- Yonezawa, K., Endemann, G., Kovacina, K.S., Chin, J.E. Stover, C. and Roth, R.A. (1990). Substrates of the insulin receptor kinase. Advances in Second Messenger and Protein Phosphorylation Research **24**, 266-272.
- Yu, K.-T. and Czech, M.P. (1984). Tyrosine phosphorylation of the insulin receptor  $\beta$  subunit activates the receptor-associated tyrosine kinase activity. Journal of Biological Chemistry **259**, 5277-5286.
- Zaagsma, J. and Nahorski, S.R. (1990). Is the  $\beta$ -adrenoceptor a prototype for the recently cloned atypical  $\beta_3$ -adrenoceptor? Trends in the Pharmacological Sciences **11**, 3-7.
- Zachary, I., Masters, S.B. and Bourne, H.R. (1990). Increased mitogenic responsiveness of Swiss 3T3 cells expressing constitutively active  $G_{s\alpha}$ . Biochemical and Biophysical Research Communications **168**, 1184-1193.
- Zapf, J., Waldvogel, M., Zumstein, P. and Froesch, E.R. (1978). Increased sensitivity to epinephrine of the cyclic AMP protein kinase system in adipose tissue of diabetic rats. E.E.B.S. Letters **94**, 43-46.
- Zhang, S.-R., Shi, Q.-H. and Ho, R.-J. (1983). Cyclic AMP-lowering mediator of insulin: generation, quantitation and properties. Journal of Biological Chemistry **258**, 6471-6476.

Zola, B.E., Miller, B., Stiles, G.L., Rao, P.S., Sonnenblick, E.H. and Fein, F.S.  
(1988). Heart rate control in diabetic rabbits-blunted response to  
isoproterenol. American Journal of Physiology **255**, E636-E641.

## **Appendix of chemical structures**

Figure A1 Forskolin



**Figure A2 GppNH<sub>2</sub>**

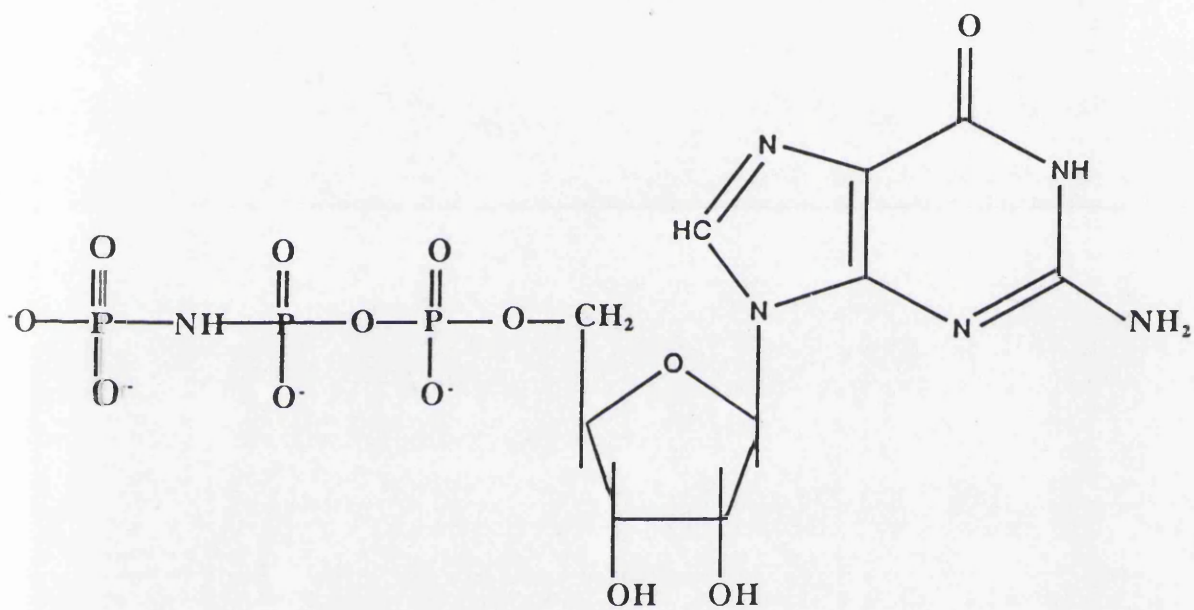
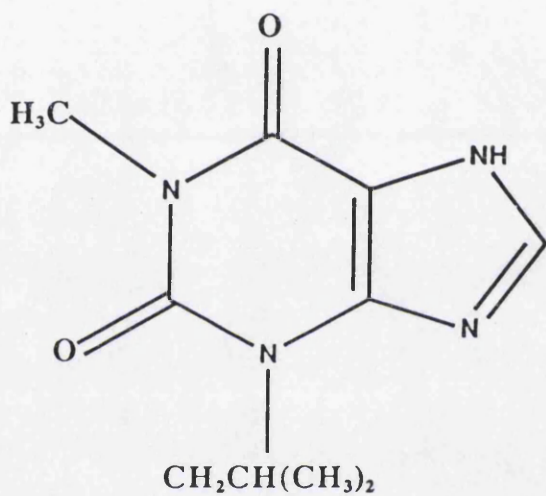


Figure A3 IBMX



**Figure A4 The E-series prostaglandins**

