G-PROTEINS IN N.I.D.D.M. AND HYPERTENSION

by

ALASTAIR ROBERT McLELLAN

M.B. Ch.B.(Glasgow), M.R.C.P.(U.K.)

Thesis presented for degree of Doctor of Medicine

to the University of Glasgow.

from

M.R.C. Blood Pressure Unit,

Western Infirmary,

Glasgow G11 6NT.

January 1991.

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

Table of Contents

List of tables p.8
List of figures p.11
Publications p.15
Abbreviations p.17
Acknowledgements p.19
Summary p.21
1. Introduction p.29
1.1. Historical background. p.29
1.2. The properties and mechanisms of action of G-proteins p.30
1.2.1. G-proteins are receptor-effector coupling proteins p.31
1.2.2. G-proteins-evolution and distribution p.33
1.2.3. G-proteins share common subunit structure and function p.34
1.2.4. G-proteins share features of an intrinsic GTPase cycle p.38
1.2.5. G-proteins act as substrates for ADP ribosylation by bacterial toxins p.38
1.3. How do G proteins work? p.38
1.4. Receptor-G-protein / G-protein-effector associations p.42
1.5. Regulation of G-proteins p.42
1.6. G-proteins in human and experimental models of disease p.45

1.6.1. G-protein abnormalities in human disease p.45
1.6.2. G-protein abnormalities in experimental models of disease p.49
1.7. Aims of this thesis p.53
2. Materials and Methods p.57
2.1. Materials p.57
2.2. Experimental animals p.59
2.3. Membrane preparations p.61
2.3.1. Human platelet plasma membrane preparation p.61
2.3.2. Plasma membrane preparations derived from animal tissues: rat liver cell plasma membrane preparation p.61
2.3.3. Rat myocardial plasma membrane preparation p.63
2.3.4. Rat brain plasma membrane preparation p.63
2.3.5. Rat renal cortical plasma membrane preparation p.64
2.3.6. Rat mesenteric and VSMC plasma membrane preparation p.65
2.4. Western Blotting p.65
2.4.1. Preparation of Western Blots p.65
2.4.2. G-proteins identified in plasma membrane preparations p.68
2.5. Cell Culture : VSMC p.72
2.6. Measurement of protein concentration p.73

2.7. 5' Nucleotidase activity p.73
2.8. Functional assessment of G-proteins p.74
2.8.1. Measurement of adenylyl cyclase activity p.74
2.8.2. Ligands used to study adenylyl cyclase activity..... p.77
2.8.3. Forskolin..... p.77
2.8.4. Other agents used in studies of G-proteins..... p.79
2.8.5. GTPase activity..... p.81
2.9. Other analytes..... p.84
2.10. Statistics p.84
3. G-proteins in NIDDM: a study of platelet plasma membranes p.85
3.1. Insulin and G-proteins p.85
3.2. The role of G-proteins in insulin's action p.85
3.3. G-proteins and experimental diabetes mellitus..... p.87
3.3.1. Experimental models of insulin dependent diabetes mellitus p.87
3.3.2. Experimental and human NIDDM..... p.92
3.4. Study of G-protein function and levels in platelets of NIDDM p.93
3.4.1. Introduction..... p.93
3.4.2. Subjects p.94
3.4.3. Materials & methods..... p.95
3.4.4. Results..... p.97
3.4.5. Discussion p.104

4. G-proteins in Essential Hypertension : a study of platelet plasma membranes..... p.110

4.1. Introduction to Chapters 4 & 5..... p.110

4.1.1. Essential hypertension and diabetes mellitus..... p.110

4.1.2. The role of adenylyl cyclase in blood pressure regulation..... p.113

4.2. G-proteins in essential hypertension..... p.115

4.2.1. Subjects..... p.117

4.2.2. Methods: Adenylyl cyclase studies p.118

4.2.3. Methods: immunoblotting studies p.119

4.3. Results..... p.121

4.4. Discussion..... p.127

5. G-proteins in experimental hypertension: a study of SHR myocardial, renal cortical and hepatic plasma membranes..... p.133

5.1. Introduction..... p.133

5.1.1.Experimental hypertension: cAMP and adenylyl cyclase..... p.134

5.1.2. cAMP (tissue) levels in experimental hypertension..... p.137

5.1.3. Adenylyl cyclase activity in experimental hypertension: studies in vascular tissues..... p.138

5.2. Materials and methods..... p.139

5.3. Results..... p.142

5.4. Discussion..... p.152

5.5. Conclusion..... p.162

6. Glucocorticoid hypertension: effect of dexamethasone treatment of rats on G-protein levels and/or function in brain, renal cortex, myocardium and vascular tissue p.166

6.1. General introduction to chapters 6 & 7..... p.166

6.1.1. Glucocorticoids and essential hypertension..... p.166

6.1.2. Steroids and hypertension..... p.167

6.1.3. Glucocorticoid hypertension..... p.167

6.1.4. Features of glucocorticoid hypertension..... p.168

6.1.5. The pathogenesis of glucocorticoid hypertension p.168

6.1.6. Glucocorticoids and plasma volume p.168

6.1.7. Glucocorticoids and the renin-angiotensin system..... p.168

6.1.8. Glucocorticoids and prostaglandin synthesis p.169

6.1.9. Glucocorticoids and vascular reactivity..... p.171

6.2. Introduction to chapter 6: glucocorticoids and experimental hypertension..... p.175

6.3. Materials and methods p.177

6.4. Results p.179

6.5. Discussion..... p.184

7. Glucocorticoid hypertension: effect of dexamethasone on G-protein levels and function in VSMC..... p.193

7.1. Introduction: glucocorticoids and adenylyl cyclase activity p.193
7.2. Material and methods..... p.194
7.3. Results p.195
7.4. Discussion p.200
8.Conclusions p.206
9.References..... p.216
10.Appendices..... p.266

List of Tables

Table 1 Properties shared by G-proteins..... p.31

Table 2 G-protein subunits p.35

Table 3 G-protein changes in experimental diabetes mellitus p.52

Table 4 Antibodies used to identify G-proteins: peptide sequence identified and antibody specificity p.58

Table 5 G-proteins identified in membrane preparations..... p70 & 206

Table 6 Ligands used in studies of adenylyl cyclase and G-proteins: sites of action in the receptor-G-protein-AC catalytic unit complex p.80

Table 7 Characteristics of diabetic and control subjects p.97

Table 8 Correlations (Spearman Rank) between measures of G-protein function or levels of G-protein subunits and fasting blood glucose concentrations p.101

Table 9 Characteristics of essential hypertensive and control subjects p.120

Table 10 Adenylyl cyclase activity in experimental hypertension: A: myocardium, B: aorta, C: mesenteric vasculature ..... p. 135/6

Table 11 Comparison of blood pressure, body and organ weights in a typical group of WKY, SHR and Wistar rats p.141

Table 12 Adenylyl cyclase studies in myocardial plasma membranes: effect of angiotensin II on forskolin and PGE1 stimulated cyclase p.151

Table 13 G-protein levels assessed by Western blotting in renal cortical plasma membranes from WKY, SHR and Wistar rats. p.151

Table 14 Adenylyl cyclase studies in livers from SHR and Wistar rats. p.152

Table 15 Details of one (of three) group of Sprague Dawley rats studied- showing effects of dexamethasone 5µg/d for 14d. p.178

Table 16 5'-Nucleotidase activities of tissues obtained from Sprague Dawley rats treated *in vivo* with dexamethasone 5µg/d for 14d p.179

Table 17 Levels of G-protein subunits in mesenteric vasculature from DEX-treated and control rats. p.184

Table 18 Adenylyl cyclase activities in plasma membranes derived from myocardium and mesenteric vasculature p.186

Table 19 Effect of adrenalectomy on levels of G-protein subunits *in vivo* p.189

List of Figures

Figure 1 Examples of ligands which are coupled to the adenylyl cyclase and the phosphoinositide pathways via G-proteins (Gs, Gi and 'Gp')..... p.32

Figure 2 How G-proteins function..... p.40

Figure 3 Demonstration of Gsα subunits in a range of plasma membrane preparations..... p.69

Figure 4 Relationship between CPM v (125 I 'second antibody' against SG1 labelled Gi2) and amount of protein loaded (rat myocardium). A: autoradiograph B: graph obtained p.71

Figure 5 Time course of adenylyl cyclase enzyme activity in rat myocardium-showing linear time course over 25min..... p.77

Figure 6 Ligand-stimulated GTPase activities in human platelet..... p.83

Figure 7 Adenylyl cyclase activity in platelet membranes from controls and NIDDM: results expressed as 'fold stimulation' of ACA by forskolin and PGE1 over 'basal' activity.. p.99

Figure 8 Inhibition of forskolin- and PGE1- stimulated ACA by adrenaline (& propranolol) in platelet membranes from control and NIDDM: results expressed as 'percentage inhibition'..... p.100

Figure 9 Immunoblotting studies in platelet membranes from NIDDM and controls showing

typical blots (R panel) and relative abundance (graph-L panel)(derived from densitometric scanning of blots) of A. Giα2, B. Giα3 and Gsα. A panel of Coomassie Blue stained gel is included (D)..... p.102/3

Figure 10 Adenylyl cyclase activities in platelet membranes derived from essential hypertensives and normal controls-expressed as absolute activites..... p.122

Figure 11 Adenylyl cyclase activities in platelet membranes derived from essential hypertensives and normal controls-expressed as 'percentage change' from forskolin-stimulated activity..... p.123

Figure 12 Platelet membrane Gsα subunit immunoblot data from essential hypertensive and control subjects..... p.124

Figure 13 Platelet membrane Giα2 subunit immunoblot data from essential hypertensive and control subjects..... p.125

Figure 14 Platelet membrane ß subunit immunoblot data from essential hypertensive and control subjects..... p.126

Figure 15 Adenylyl cyclase studies in myocardial plasma membranes. Comparison of WKY, SHR and Wistar rats. A: absolute data, B: data expressed as 'percentage change' over 'basal' determined in the presence of Mn2+..... p.143/4

Figure 16 Adenylyl cyclase studies in renal cortical plasma membranes. Comparison of WKY, SHR and Wistar rats. A: absolute data, B: data expressed as 'percentage change' over 'basal' determined in the presence of Mn2+..... p.145/6

Figure 17 Gsα subunit immunoblot data from myocardial plasma membranes: comparison of WKY, SHR and Wistar rats..... p.148

Figure 18 Giα2 subunit immunoblot data from myocardial plasma membranes: comparison of WKY, SHR and Wistar rats..... p.149

Figure 19 Goα subunit immunoblot data from myocardial plasma membranes: comparison of WKY, SHR and Wistar rats..... p.150

Figure 20 Effect of *in vivo* dexamethasone (5µg/d for 14d) on G-protein levels in rat brain.... p.180

Figure 21 Effect of *in vivo* dexamethasone (5µg/d for 14d) on G-protein levels in rat myocardium..... p.181

Figure 22 Effect of *in vivo* dexamethasone (5µg/d for 14d) on G-protein levels in rat renal cortex..... p.182

Figure 23 Effect of *in vivo* dexamethasone (5µg/d for 14d) on G-protein levels in rat liver p.183

Figure 24 Adenylyl cyclase activities in plasma membranes derived from renal cortex of dexamethasone-treated (5µg/d for 14d) and from control rats..... p.185

Figure 25 Adenylyl cyclase activities in plasma membranes from dexamethasone-treated (10nM for 24h) and from control VSMC p.196

Figure 26 Effect of 10nM dexamethasone (for 24h) on levels of Gsα subunits in cultured VSMC..... p.197

Figure 27 Effect of 10nM dexamethasone (for 24h) on levels of Giα2 subunits in cultured VSMC..... p.198

Figure 28 Effect of 10nM dexamethasone (for 24h) on levels of Giα3 subunits in cultured VSMC p.199

Publications & Abstracts

Papers and abstracts published in association with this work:

McLellan, A.R. & Connell, J.M.C. (1988) Diabetes and hypertension. Current Opinion in Cardiology, 3, 666-674.

McLellan, A.R. & Connell, J.M.C. (1989) Hypertension in the diabetic and elderly populations. Current Opinion in Cardiology, 3, 677-686.

Livingstone, C., McLellan, A.R., McGregor, M.A., Wilson, A., Connell, J.M.C., Small, M., Milligan, G., Paterson, K.R. & Houslay, M.D. (1991) Altered G-protein expression and adenylate cyclase activity in platelets of non-insulin dependent diabetes (NIDDM) subjects. Biochimica et Biophysica Acta (in press).

Connell, J.M.C. & McLellan, A.R. (1991) Hypertension, insulin and atherogenesis. Journal of Cardiovascular Pharmacology (in press).

McLellan, A.R. & Connell, J.M.C. (1991) G-proteins, cell signalling and hypertension. ACE Reports (in press).

McLellan, A.R., Clark, C.J., Houslay, M.D., Milligan, G. & Connell, J.M.C. (1990). G-protein levels and function in human and experimental hypertension. Journal of Hypertension, 8, 1060 abstract.

McLellan, A.R., Tawil, S., Lyall, F., Milligan, G., Connell, J.M.C. & Kenyon, C.J. (1990) Are G-proteins involved in glucocorticoid hypertension? Journal of Endocrinology, 127, suppl. abstract 146

Papers in preparation:

McLellan, A.R., Houslay, M.D., Milligan, G. & Connell, J.M.C. G-protein levels and function in platelet membranes of essential hypertensives.

McLellan, A.R., Houslay, M.D., Milligan, G. & Connell, J.M.C. G-protein levels and function in myocardial plasma membranes: a comparison of SHR, WKY and Wistar species.

McLellan, A.R., Tawil, S., Lyall, F., Milligan, G., Connell, J.M.C. & Kenyon, C.J. Effect of dexamethasone on G-protein levels and function in myocardium, brain, renal cortex and mesenteric vasculature of rats with glucocorticoid hypertension.

McLellan, A.R., Tawil, S., Lyall, F., Milligan, G., Connell, J.M.C. & Kenyon, C.J. Effect of dexamethasone on G-protein levels and function in cultured vascular smooth muscle cells.

Abbreviations

AC(A)	adenylyl cyclase (activity)
ATP	adenosine triphosphate
cAMP	3',5'-cyclic adenosine monophosphate
cGMP	3',5'-cyclic guanosine monphosphate
Con	control
DAG	diacylglycerol
DEX	dexamethasone (treated)
DM	diabetes mellitus
DTT	dithriothrietol
EDTA	ethylenediaminetetraacetic acid
EH	essential hypertension
GppNHp	guanylylimidodiphosphate
G-protein	guanine-nucleotide-binding-regulatory-protein
GTP	guanosine triphosphate
IBMX	3-isobutyl-1-methylxanthine
IDDM	insulin dependent diabetes mellitus
IP	inositol phosphate
5'-ND(A)	5'-Nucleotidase (activity)
NIDDM	non-insulin dependent diabetes mellitus
PDE	phosphodiesterase
PPI	phosphoinositol phosphodiesterase
SD	Sprague Dawley (rat)

SDS-PAGE	sodium dodecyl sulphate poly-acrylamide gel electrophoresis.
sem	standard error of the mean
SHR	spontaneously hypertensive rat
STZ	streptozotocin
TCA	trichloroacetic acid
Tris	tris(hydroyxmethyl)aminomethane
VSMC	vascular smooth muscle cells (cultured)

Acknowledgements

The work presented in this thesis was performed in the Medical Research Council Blood Pressure Unit, Western Infirmary, Glasgow from 1988-1991. I am especially grateful to Dr J.M.C. Connell (Consultant Physician, MRC BPU) who provided advice, encouragement and fruitful discussion as this work evolved. I must also acknowledge stimulating discussion provided by a number of staff members of the MRC BPU , including Dr F. Lyall, Dr C. J. Kenyon and the Director, Dr A. F. Lever.

The work would not have been possible without the cooperation of Dr G. Milligan and Prof. M.D. Houslay (Molecular Pharmacology Group, Dept. of Biochemistry, University of Glasgow) in whose laboratories I was taught the skills and techniques which were necessary for this research. I am grateful for the stimulating discussion with and guidance from Dr F. R. McKenzie, formerly a student studying for his doctorate under Dr G. Milligan.

All the laboratory studies described were performed exclusively by the author, with the exception of cyclase assays and Western blots described in chapter 3(these were performed by Mr A. Wilson (who tragically died 1990) and Miss A. MacGregor, Biochemistry Dept., University of Glasgow) and cell culture (described chapter 7) which was performed by Dr F. Lyall and Miss S. Tawil of the MRC BPU (Miss Tawil also provided assistance in preparing immunoblots for the study of cultured VSMC (ch.7). However, all other work described, including recruitment of patients & controls for human NIDDM (ch.3) and

hypertension studies (ch.4), all the laboratory work of the human essential hypertension study (ch.4), all the laboratory work for the studies in the SHR model of hypertension (ch.5), and for the studies in glucocortiocoid hypertension (ch. 6 & 7) was performed by myself. In addition, manuscript preparation, photography, autoradiography and illustrations were exclusively by the author. Access to essential hypertensive subjects was provided by Dr J.M.C. Connell, Dr J.J. Brown (MRC BPU) and the Glasgow Blood Pressure clinic and to NIDDM by Dr M.Small (Consultant Physician, Gartnavel General Hospital) and Dr K. Paterson (Consultant Physician, Glasgow Royal Infirmay). I am grateful to the many members of staff of the MRC BPU and of the Biochemistry Dept., University of Glasgow and to the spouses of many of our patients who acted as normal controls for the studies described in chapters 3 & 4.

Finally, I must acknowledge the tremendous support and tolerance of my wife, Sheila, without whose help (despite providing two major distractions by giving birth to our daughters, Kirsty and Morag!) this work would have been impossible.

A. R. McLellan 1991

Summary

G-proteins are guanine-nucleotide-binding regulatory proteins which couple receptors to effector mechanisms; G-protein associated 'second messengers' include cAMP and the phospho-inositides, while other G-proteins are coupled to calcium and potassium channels. The expanding G-protein family (all of which share a common trimeric structure) currently includes at least12 distinct G-proteins some of which can be identified in plasma membranes using specific antibodies (Western immuno-blotting): antibodies are available against several α subunits (Gsα, Giα1, Giα2, Giα3 and Goα)(which possess receptor and effector binding sites) as well as against ß subunits. Function has not yet been ascribed to all G-proteins, but Gsα and Giα2 are concerned with the dual regulation (respectively stimulating and inhibiting) of adenylyl cyclase (AC) catalytic unit activity (A).

G-protein abnormalities have been implicated in the patho-genesis of several human and experimental disease processes. Resistance to hormone action (specifically to hormones coupling to Gsα) is a feature of the human disease pseudohypopara-thyroidism, and is attributable to abnormal Gsα. Tissue specific alterations of G-proteins have also previously been demons-trated in the STZ model of IDDM where reduced expression, levels and function of hepatocyte Gi may be related to the insulin resistance seen in STZ-DM hepatocytes. Similar changes of Gi function have also been reported in hepatocytes from the Zucker rat model of obesity which also features insulin

resistance: this raises the possibility that abnormalities of G-proteins may be implicated in diseases characterised by insulin resistance.

Insulin resistance and hyperinsulinaemia are features of certain human diseases including NIDDM (chapter 3) and essential hypertension (EH) (chapter 4) and studies were performed in platelets from human NIDDM and EH to ascertain whether common G-protein abnormalities might be present in these conditions which might lend support to the concept of a common underlying pathophysiology. Platelets represent a homogeneous tissue from which purified plasma membranes can be readily prepared, are implicated in morbidity accompanying NIDDM and EH and exhibit abnormalities of function (assessed *in vitro*). G-protein levels were measured by semiquantitative immuno-blotting techniques (against subunits cited previously), while assessment of G-protein function was restricted to Gsα and Giα2, and was inferred from studies of ACA.

Platelets from 11 (6M) newly presenting NIDDM and 17 (12M) controls were studied in chapter 3. Platelets from NIDDM exhibited lower stimulation of ACA in response to forskolin and PGE1 (approx. 40% and 50% less stimulation, p=0.006 and p=0.001 respectively) while Giα2 (α2 receptor-activated and receptor-independent,via GppNHp) mediated inhibition of AC catalyitc unit was unaltered. Although altered catalytic unit activity may have contributed, it is probable that altered levels of G-protein subunits may also be relevant: reductions in the levels of $G_i\alpha$2(p=0.003), $G_S\alpha$ (p=0.02) and $G_i\alpha$3 (NS) were

demonstrated. It is possible that relative changes in $G_i\alpha 2$ and $G_s\alpha$ may have greater implications for functional change than alterations in either alone. The observation of reduced $G_i\alpha 2$ was similar to previous findings in hepatocyte membranes from the STZ-IDDM although contrasts with those in adipocytes where Gi3 has previously been shown to be increased. The observed reduction of G-protein levels in human platelets from NIDDM and hepatocytes from STZ-DM are consistent with the possibility that G-proteins may be implicated in diseases manifesting insulin resistance.

The study of human EH described in chapter 4, is based on the strong epidemiological and clinical links between NIDDM and EH, and the increasing evidence of shared pathophysiology on the basis of insulin resistance. Platelets from 14 EH with matched controls were studied. No changes in levels of G-proteins ($Gs\alpha$, $Gi\alpha 2$ and ß subunits) were seen. However, in contrast to the observations in NIDDM, studies of AC function identified greater PGE1 stimulated AC activites in hypertensive platelet membranes than controls (88.8% v 72% stimulation, $p=0.018$). Although unexplained by altered G-protein levels, this is consistent with the findings of previous workers and may have its physiological basis in protection of cells against Ca2+ overload (Resink et al. 1986).

Weak correlation was noted between plasma cholesterol concentration and relative amounts of $Gi\alpha 2$ ($z=-2.084$, $n=29$, $p=0.04$) when considering all EH and control subjects together.

(Correlations were also noted when considering NIDDM with control subjects, between plasma cholesterol concentration and extent of forskolin and PGE1 stimulation of adenylyl cyclase actvity). The explanation for this novel observation remains unclear, but does raise the possibility that abnormalities of G-protein levels could be related to abnormal lipids, providing an alternative explanation for changes in NIDDM and EH.

However, the data argue against alteration of G-proteins in hypertension by virtue of insulin resistance, but further studies would be desirable in metabolically active tissues and in arteriolar peripheral resistance vessels.

In chapter 5, G-protein levels and function were studied in vascular tissue (myocardium) from the spontaneously hypertensive rat (SHR). The SHR is a widely used model of genetic hypertension with many features in common with human EH including glucose intolerance/ insulin resistance. SHR myocardium is known to exhibit abnormalities of AC activation; reduced ß adrenergic activation of adenylyl cyclase has been reported.

Myocardial plasma membrane homogenates prepared from 11week SHR (weighing 255g) and (two control species) WKY and Wistar rats were studied (chapter 5). Differences in AC activity were seen in SHR when compared to WKY myocardium: WKY ACA was greater than SHR (up to 100% greater) under basal conditions and in the presence of fluoride, forskolin and a

number of ligands which couple to AC catalytic unit via Gs, including PGE1, glucagon and isoproterenol. However, SHR myocardial ACA was similar to activities in the other control group, Wistar rats (with exception of forskolin stimulated activity, where SHR was greater than Wistar).(A similar trend was observed when the data were expressed as 'percentage change' over 'basal' in the presence of Mn2+). Studies of Gi function failed to effect major inhibition of ACA. However, it seems likely that the differences in ACA may be attributable to species differences in amount of AC catalytic unit, rather than altered G-proten function. Immunoblotting studies excluded a contribution to these changes in ACA from altered levels of G-protein subunits; Gsα, Giα2 (and Goα) levels were similar in all three species..

Studies of renal cortical plasma membrane preps. failed to identify any differences in ACA (expressed as 'percentage change' over basal in presence of Mn2+), nor in Gsα, Giα1, Giα2, Goα and ß subunit levels.

These studies, however, emphasise the problems inherent in studies of the SHR. The absence of an isogenetic control species raises the distinct possibility that the differences observed in our studies may relate to the inherent genetic heterogeneity among the species studied: this problem cannot be circumvented. The inclusion in this study of the second control species (Wistar) highlights the dangers of the customary comparison between SHR and WKY species.

The studies in hypertension were extended to include assessment of G-proteins in the poorly understood entity of glucocorticoid hypertension (chapters 6 & 7). As humoral regulation of G-protein expression and function by gluco-corticoids has apparently been demonstrated in experimental animals and cell culture it seemed possible that G-protein changes could be implicated in this pathological state. Previous studies of glucocorticoid effect on the differential regulation of G-protein subunits are in general, based on administration of massive doses of glucocorticoid to adrenalectomised rats. In the study reported here, a model of glucocorticoid hypertension based on administration of rather more physiological concentrations (likely to still be in the 'pharmacological range') of the glucocorticoid, dexamethasone, was used. 5µg/d DEX infusion (s.c.)(to 180g Sprague Dawley rats) resulted in elevation of tail-cuff BP increased by 30mmHg (representing an increase of around 25%) without causing major restriction of weight gain. Immunoblotting studies for G-protein subunits failed to identify any relevant changes in brain, myocardium, renal cortex, liver and mesenteric vasculature. Our studies of ACA also failed to reveal any differences in ACA responses in renal cortical, myocardial and mesenteric plasma membranes. This contrasts with the *in vitro* work of chapter 7, and suggests that neither changes in G-protein function nor AC catalytic unit are implicated in the pathogenesis of glucocorticoid hyper-tension. Review of these results in the context of previous studies suggests that if glucocorticoids do have a role in regulating G-protein levels, then this may merely be a

permissive role, enabling other agents, possibly humoral, to exert their influence.

However, dexamethasone administration *in vitro* has been previously shown to increase ACA in vascular smooth muscle cells (from renal arteries)and in non-vascular cell lines. To ascertain whether G-proteins were implicated, cultured VSMC were studied: VSMC were obtained from (Sprague Dawley) rat mesenteric vascular tree and half the flasks were exposed to 10nM DEX for 24h (and 48h)(chapter 7). Basal ACA was increased about 50% in the presence of DEX. The differences persist and are independent of ligand mediated stimulation of catalytic unit via Gs (by isoproterenol and PGE1) and of direct, Gi mediated inhibition of catalytic unit by GppNHp (0.1nM), suggesting that AC catalytic unit, rather than the G-proteins which modulate its activity, is altered.

The results are compatible with possible alteration of AC catalytic unit; absence of alteration of the levels of Gsα, Gi2α, Gi3α and ß subunits in the membranes essentially excludes a role for DEX mediated alteration of levels or function of G-protein subunits in the observed changes in ACA.

• The studies of platelet membranes from NIDDM and EH thus exclude common pathophysiology based on alterations of G-proteins, although both conditions do feature abnormalities of ACA which may contribute to the altered platelet function observed in these diseases. Platelets from NIDDM (but not EH) do exhibit reduced levels of G-protein subunits which may

contribute to altered ACA, but these changes may be related to altered plasma membrane composition in diabetes mellitus.

• Species differences exist in ACA in myocardial plasma membrane preparations (comparing SHR, WKY and Wistar species). The validity of the customary comparison between SHR and WKY is challenged. Differences in ACA are not explained through altered G-protein levels, but may be due to differences in AC catalytic unit.

• Despite evidence implicating glucocorticoids in G-protein regulation, no changes in G-protein levels were seen in myocardium, brain, kidney and mesenteric artery in a well validated model of glucocorticoid hypertension. Glucocortiocoid exposure did enhance ACA in cultured VSMC but probably by altering AC catalytic unit, and not through changes in levels of G-protein subunits.

Chapter 1
Introduction

G-proteins are guanine nucleotide binding regulatory proteins which function as transmembrane signal transducers.

1.1. Historical background

The appreciation of the role of G-proteins in transmembrane signalling has its origins in three disparate observations made in the the early 1970s. Following the demonstration that GTP is a prerequisite for hormonal activation of adenylyl cyclase (AC) (Rodbell, Birnbaumer, Pohl & Krans 1971) it was subsequently shown that guanine nucleotides are able to alter ligand-receptor binding affinity in a manner which is agonist-specific (Maguire, Van Arsdale, & Gilman 1976). The third fundamental observation was the demonstration of catecholamine stimulated GTPase activity (in turkey erythrocyte membranes)(Cassel & Selinger 1976). These findings form the essentials of our current understanding of G-protein function (section 1.3. and figure 2). However, it was as recently as1980 that a 'transducing protein' was first isolated (Northup, Sternweis, Smigel et al. 1980) (using the cyc^- reconstitution system-see section 2.8.) and was shown to be a guanine-nucleotide-binding protein. This was definitive evidence in support of the hypothesis originally proposed by Rodbell and colleagues a decade previously (Birnbaumer, Pohl, Michiel, Krans & Rodbell 1970) that a regulatory protein couples receptors to AC. As will be described more fully, these findings

form the basis of our current understanding of G-proteins as receptor-effector coupling proteins. Although the bulk of the early work in this field was on the dual regulation of AC, the G-protein family has evolved over the last decade and it is clear that a number of other effector mechanisms are modulated by G-proteins; these include the inositol phosphate (IP) pathway (Cockcroft & Gomperts 1985) and calcium (Hescheler, Rosenthal, Trautwein & Schultz 1987)and potassium channels (Yatani, Codina, Brown & Birnbaumer 1986). Despite the current diversity of G-proteins-several properties are common to most G-proteins, and a detailed description of these facilitates an understanding of the initial definition and of the role and mechanisms of action of these "transmembrane signal trans-ducers".

1.2. The properties and mechanisms of action of G proteins

G-proteins are heterotrimeric proteins (see section 1.2.3.), comprising α, ß and γ subunits; each of these is encoded by a separate gene. Functional specificity is determined by the α subunit (section 1.2.3., table 2); for example, it is the α subunit of Gs which effects stimulation of AC catalytic unit (see section 1.3, figure 2). However, identical ß are shared by all members of the G-protein family (section 1.2.3.). Thus despite functional heterogeneity (by virtue of the α subunit), similar-ities do exist among G-proteins; these are summarised in table 1.

Table 1: Properties shared by G proteins:

1. **They are receptor-effector coupling proteins.**
2. **They are associated with the cytoplasmic surface of the plasma membrane.**
3. **They share a common subunit structure and function.**
4. **They possess GTPase enzyme activity.**
5. **They act as substrates for ADP ribosylation by bacterial toxins.**

1.2.1. G-proteins are receptor-effector coupling proteins.

Over 80 different G-protein mediated receptor-effector relationships have been described: ligands which bind to G-protein coupled receptors include biogenic amines, proteins and polypeptides, autocoids and neurotransmitters. Early studies of G-proteins concentrated on G-protein mediated modulation of AC-with cAMP as the second messenger system (effector); AC is under dual regulation by G_S and G_i-which respectively represent G-proteins which effect stimulation and inhibition of AC by a number of different ligands (see figure 1). The aim of this thesis is to examine the possible role of dysfunction of these G-proteins in non-insulin dependent diabetes mellitus and various types of hypertension; G-protein function has been studied by manipulation of AC activity using a number of agents which function via G_S and G_i.

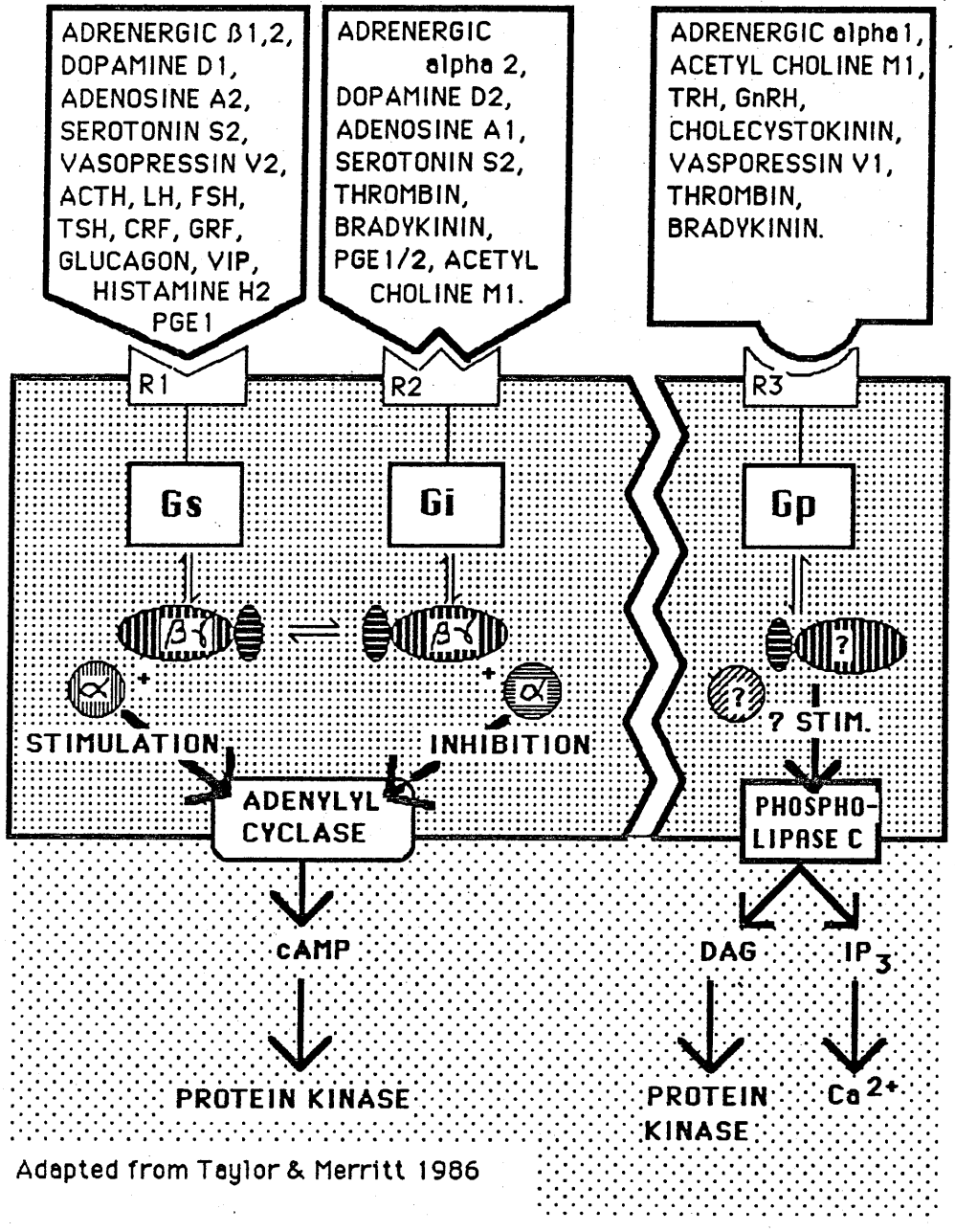


Fig. 1. Examples of ligands which are coupled to the adenylyl cyclase and the phosphoinositide pathways via G-proteins (Gs, Gi and 'Gp'). Ligands bind to receptors (R1-3) causing release of alpha subunits from the heterotrimeric G-proteins. Alpha subunits interact with effector & generate second messengers.

Other effector mechansims known to be regulated by G-proteins include the IP3 system, calcium and potassium channels and cGMP phosphodiesterase (see table 2). The G-protein regulation of the inositol triphosphate (IP3) system has been extensively studied but remains to be fully elucidated (figure 1); phosphatidylinositol 4,5-biphosphate (IP2) is catalysed to IP3 and diacylglycerol (DAG) by phosphoinositol phosphodiesterase (PPI-PDE) both of which act as second messengers. There is increasing evidence that IP3 and IP4 (a product of IP3 hydrolysis) are both required to mobilise intracellular calcium-which is how IP3 is thought to mediate its effects. Evidence that a G-protein couples PPI-PDE to receptors was provided by Cockcroft & Gomperts (1985)-but despite substantial evidence in support of the role of a G-protein (the G-protein has been termed 'G_p'), the DNA encoding this G-protein has hitherto has not been identified.

1.2.2. G-proteins-evolution and distribution.

G-proteins are ubiquitous in the animal kingdom. Teleogical studies have demonstrated the presence of similar G-protein subunits (see section 1.2.3.) for example $G_o\alpha$, in vertebrates (including mammals (rat), avians (pigeon), amphibians (frog), fish (trout), and reptiles (turtle)) and in invertebrates (such as molluscs (snail) and insects (locust)). In contrast, it has only been possibe to demonstrate $\beta\gamma$ complexes immunologically in vertebrates (Homburger, Brabet, Audigier et al.1987). (It is unclear whether homologous, but immunologically distinct $\beta\gamma$ complexes exist in these species.)

G-proteins are also ubiquitous within tissues (human and animal) although the relative preponderance of the various G-proteins may vary from tissue to tissue. They are in general, found within the plasma membranes of cells (in most membrane preparations several G-proteins are likely to be present) and are located at the cytoplasmic surface of the membrane where they are anchored by their amino terminus. Two recent reports have suggested, however, that G-proteins may also be associated with intracellular organelles-the Golgi stack (Melancon, Glick, Malhotra et al. 1987) and hepatic microsomes (Codina, Kimura & Kraus-Friedmann 1988). The functions of these G-proteins remain to be determined although it has been suggested at least in the Golgi stack that the G-proteins may subserve a role in transport through the complex.

1.2.3. G-proteins share common subunit structure and function

The G-proteins associated with transmembrane signal transduction are not the only proteins which have been identified which can bind and hydrolyse GTP; others include cytosolic initiation and elongation factors- two proteins involved in protein synthesis-and ras gene products. Furthermore coupling of receptors to effectors is not a function unique to G-proteins as the ras gene products (Barbacid 1987) (although this is controversial) are thought to share this function. However, the heterotrimeric structure of the G-proteins of transmembrane signal transduction sets them apart from all the other GTP-binding proteins. This heterotrimeric structure consists of α, β and γ

subunits (in order of decreasing molecular weight): the α subunit confers immunological and functional identity on the G-protein. Currently at least 9 distinct α subunits have been identified (each a unique gene product). cDNA studies have identified 4 G_i α subunits (numbered 1-4), and at least 4 different mRNA encoding G_Sα subunits] (see table 2).

Table 2: G-protein subunits.

SUBUNIT	Mr (kDa)	TOXIN	EFFECTOR
$G_{s}\alpha$	52	CT	AC-stimulation
$G_{s}\alpha$	44.5	CT	AC-stimulation
$G_{i}1\alpha$	40.4	PT	AC-inhibition
$G_{i}2\alpha$	40.5	PT	AC-inhibition
$G_{i}3\alpha$		PT	K+ channels
$G_{i}4\alpha$		PT	?AC-inhibition
$G_{o}\alpha$	39	PT	Ca^{2+} channels
$G_{t}1\alpha$	40	PT	cGMP PDE
$G_{t}2\alpha$	40.4	PT	?cGMP PDE CT
G_z		-	?
G(t)βx 2	37.4		
G(t)γ	8.4		

(After Casey & Gilman 1988)

αsubunits:

The G-protein α subunits have molecular weights ranging from 39kDa-52kDa. Amongst the recognised α subunits there is substantial, if variable, amino acid sequence homology; 95% homology has been reported for $G_i1\alpha$ and $G_i2\alpha$. However, lesser degrees of homology exist between $G_o\alpha$ and $G_i\alpha$ (68%), $G_i\alpha$ and $G_S\alpha$ (about 50%) and $G_o\alpha$ and $G_S\alpha$ (50%)(Gilman 1987). In addition

to the heterogeneity which defines the major G-protein classes, there are multiple subunit polypeptides within each class. While the different Gi subclasses represent distinct gene products the different forms of Gsα are due to alternative splicing of precursosr mRNA (Robishaw, Smigel & Gilman 1986).

Enzymatic cleavage of α subunits with trypsin and carboxy-peptidase A has enabled function to be ascribed to certain amino acid sequences e.g. tryptic cleavage of the $G_o\alpha$ subunits in the presence of guanosine 5'-(thio)triphosphate removes a 2 kDa peptide from the amino terminus which prevents the association of α subunits with the ß and γ units, but does not affect GTPase activity nor the Km for GTP (Neer, Pulsifer & Wolf 1988). ADP ribosylation by pertussis toxin (which is known to require the α.β.γ trimer) is however compromised. Digestion of the carboxyl terminus of α39 with carboxypeptidase A (Neer et al. 1988) similarly prevents ADP ribosylation by pertussis toxin, but has no effect on α.β.γ trimer formation. The receptor-contact site involved in receptor-G protein coupling has been identified in the cyc^- S49 lymphoma cell line which possesses a G_S which cannot be activated by hormone receptors and thus cannot transduce hormone signals: comparison of the cDNA and amino acid sequences of the $G_S.\alpha$ subunits from the cyc^- and the 'wild' cell lines has identified a point mutation near the carboxyl terminus of the α s polypeptide (proline for arginine) (Rall & Harris 1987). ADP ribosylation of G_i and G_t at a cysteine residue four from the carboxy-terminus prevents G-protein-receptor interaction (Van Dop, Yamanaka, & Steinberg et al.1984).

ß and γ subunits:

ß subunits prepared from G_S, G_t and G_i have identical amino acid composition and they are functionally interchangeable (Katada, Bokoch, Northup, Ui & Gilman 1984). However, there appear to be two genes encoding ß subunits (Gilman 1987): it is unclear whether these two genes account for a doublet which can be resolved on SDS-PAGE from the 35kDa beta subunits of G_S, G_O and G_i.

cDNA for G_t γ has been cloned and sequenced (Hurley, Fong, Teplow, Dreyer & Simon 1984). However, it is likely that different forms of γ subunit exist and these differences may contribute to the functional heterogeneity of different G-proteins: differences in the efficacies of the β-γ complexes of bovine brain G_i and bovine retinal (?G_t) subunits in inhibiting ACA in reconstituted phospholipid vesicles may be attributable to variability in the γ units as the β subunits appear to be immunologically identical (Cerione, Gierschik, Staniszewski et al. 1987).

It is artificial to consider ß and γ subunits as separate entities as they form a single functional unit. They remain closely associated and can only be separated by denaturation. As will be discussed (section 1.3.) the prime role of the βγ complex may be to 'mop up' free Gsα. However, it has been also been suggested that this complex may directly increase phospholipase A2 activity (Jelsema & Axelrod 1987).

1.2.4. G-proteins share features of an intrinsic GTPase cycle.

GTPase is an enzyme which cleaves the terminal phosphate from GTP. This enzyme, which is common to all α subunits, is an intergral component of the amino acid sequence of the α subunit, and is essential for G-protein function (see figure 2). Hydrolysis of GTP effects term-ination of the α subunit function, and permits reassociation of α with β-γ subunits and restores the G-protein to the dormant heterotrimeric form associated with GDP (vide infra).

1.2.5. G-proteins act as substrates for ADP ribosylation by bacterial toxins.

The pathophysiology of both whooping cough and cholera can be explained on the basis of systemic effects of secreted toxins which are known to function by altering G-protein activity. Pertussis toxin is the only known asparagine specific ADP ribosyl transferase and the con-sequent ADP ribosylation of G_i leads to subunit dissociation and loss of G_i function by impairing the ability of G_i to interact with receptor. The diarrhoea which characterises the effect of cholera toxin is due to ADP ribosyl-ation of $G_S\alpha$ - which results in activation (irreversible) of G_S. The susceptibilities of α subunits to ADP ribosylation by these two toxins are summarised in table 2.

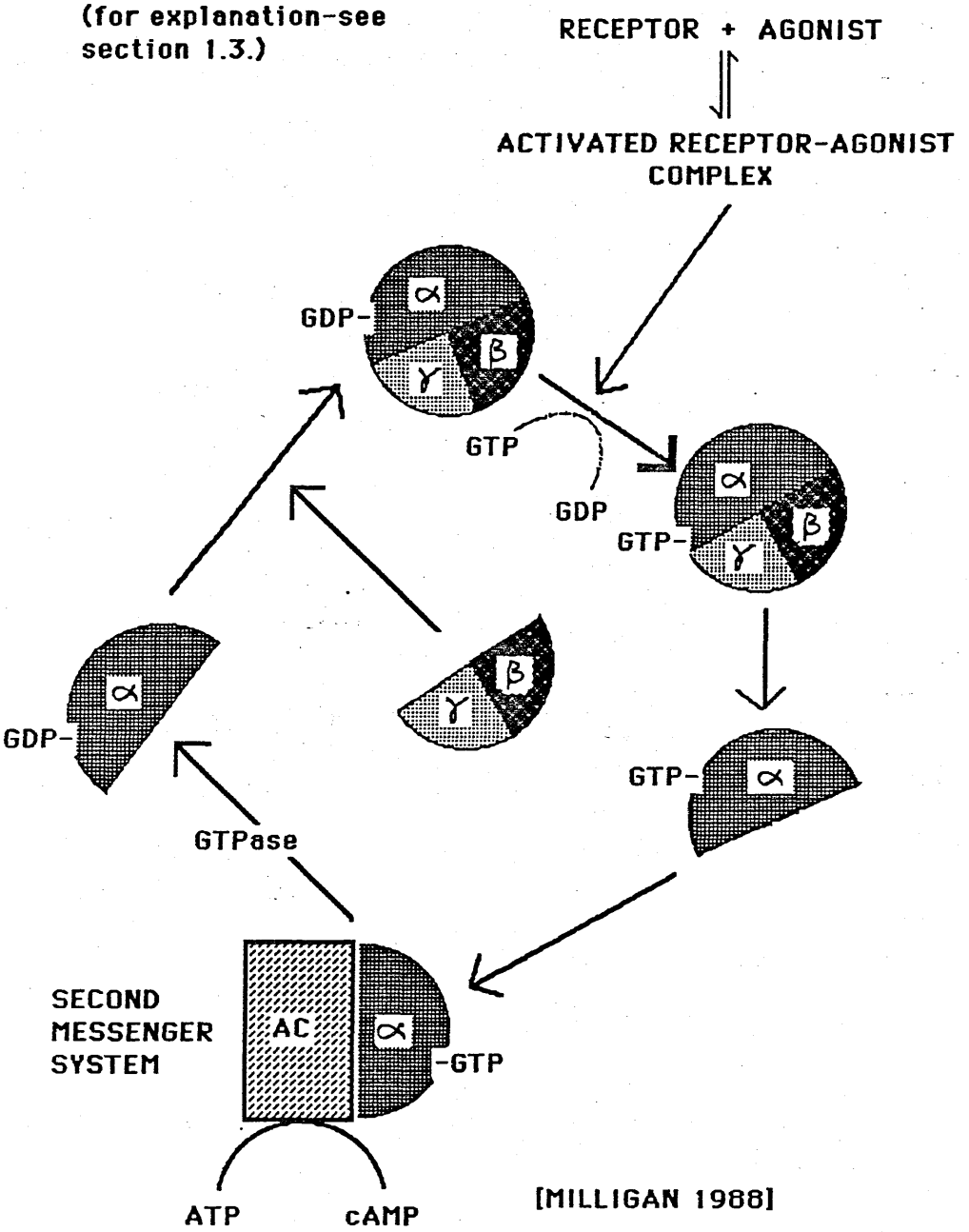
1.3. How do G proteins work? (Figure 2)

In the inactive state GDP is bound to the $\alpha.\beta.\gamma$ trimer. The cycle of activation is initiated by the binding of a ligand to a receptor

(examples of ligands associated with G-proteins are given in figure 1) which results in association with the G-protein ($\alpha.\beta.\gamma$ trimer). GTP then binds to the α subunit, displacing GDP. The α subunit (binding GTP) is then probably cleaved from the parent trimer, dissociating it from the $\beta.\gamma$ fraction (the subunit dissociation model for activation of G-proteins-see review Casey & Gilman 1988); this dissociation results in reduced affinity of the receptor for its bound ligand while the "free" α subunit-GTP can interact with the effector mechanism, for example, the cat-alytic subunit of AC. Hydrolysis of GTP to GDP allows $\alpha.\beta.\gamma$ reassociation. As will be seen later non-hydrolys-able GTP analogues such as GppNHp prevent reassociation of the subunits.

The mechanisms whereby dissociated α(-GTP) subunits effect activation of second messenger systems are becoming clearer; $G_S\alpha$ directly stimulates activity of the AC catalytic subunit. $G_i\alpha$ may effect inhibition of AC catalytic unit either by a direct action of $G_i\alpha$ or by liberating $\beta.\gamma$ subunits which "mop up" free $G_S\alpha$ subunits- relieving tonic stimulation of AC (the 'subunit exchange model') (Gilman 1984). Support for this theory is provided by the observation that addition of $\beta.\gamma$ subunits (from either bovine brain G_i or from retinal transducin) can effect 90% inhibition of guanine nucleotide stimulated cyclase in recons-tituted phospholipid vesicles which otherwise essentially contain only human erythrocyte G_S and bovine AC catalytic subunit (Cerione, Staniszewski, Gierschik et al. 1986; Cerione et al.1987). $\beta.\gamma$ subunits have been demonstrated to inhibit forskolin

Fig. 2. How G-proteins function (for explanation-see section 1.3.)



stimulated catalytic unit (without a requirement for guanine nucleotides)(Enomoto & Asakawa 1986) in addition to inhibition of G_S stimulated AC catalytic unit. Further evidence that $\beta.\gamma$ complexes may exert a direct inhibitory role on the catalytic subunit was provided by Katada and colleagues (Katada, Oinuma & Ui 1986) who showed additivity of cyclase inhibition between $\beta.\gamma$ complexes and GTPγS-bound-α 41 (raising the possibility therefore also of direct inhibition by α subunits).

How specificity of action is achieved within the current models of G-protein function remains to be fully elucidated. Although it is not impossible that there is strict coupling between a receptor and a number of G-protein molecules the consensus favours the existence of a mobile plasma membrane pool of G-proteins (the 'free dissociation model'); this model envisages that hormone receptors, $\alpha.\beta.\gamma$ trimers, dissociated subunits and effector molecules all float freely in the plasma membrane and that any subunit can theoretically bind any receptor or any effector provided it is of the appropriate structural specificity. Gilman (1987) proposes that coordinate regulation of transmembrane signalling pathways is possible against the background of the subunit dissociation model and with a $\beta.\gamma$ complex that is shared among different α subunits. The basic premise is that receptor mediated activation of a sufficient pool of G-protein will increase the concentration of $\beta.\gamma$ to a point where it will inhibit some pathways while release of αGTP can initiate other processes.

1.4. Receptor-G-protein/ G-protein-effector associations

Studies have been performed to assess the specificity of receptor-G-protein and of G-protein-effector interactions: There does appear to be more specificity of the latter processes than the former. Although activated receptors appear to exhibit a functional preference for a particular G-protein subtype-for example, $\alpha 2$ adrenergic receptors and G_i, a surprising degree of cross reactivity between receptors and G-proteins can be demonstrated experimentally such that $\alpha 2$ adrenergic receptors can also associate with G_o more readily than G_t (transducin), but both to lesser degrees than G_i. This presumably reflects the previously described homology which exists amongst G-proteins and which extends to their receptor binding domains. Greater specificity does, however, exist in the G-protein-effector relationship as, for example, only cGMP PDE can be activated by transducin. However, although $G_{s\alpha}$ can stimulate AC it can also activate dihydropyridine-sensitive Ca2+ channels; furthermore it seems likely that all splice variants of $G_{s\alpha}$ share these porperties (Mattera, Graziano, Yatani et al. 1989).

1.5. Regulation of G-proteins

Although the mechanisms controlling G-protein expression and function remain to be fully resolved, several regulatory processes have already been described: these include covalent modification, humoral regulation and downregulation associated with receptor desensitisation.

The known abilities of PT and CT to ADP ribosylate α subunits and modify their function raises the possibility that covalent modification of α subunits might be a mechansim by which G-protein function can be regulated. There is, however, only circumstantial evidence to support this concept. It has, for example, been demonstrated *in vitro*, that covalent modification of Gs can reduce AC activation (Perfilyeva, Skurat, Yurkova et al.1985). Two possible mechanisms of such regulation have been proposed-phosphorylation and ADP ribosylation. The existence of an enzyme system capable of effecting ADP ribosyl-ation *in vivo* has been demonstrated in human erythrocytes and adds credance to the concept: Tanuma and colleagues (1988) have shown that human erythrocytes contain an ADP ribosyltransferase which could catalyse ADP-ribosylation of a cysteine residue in $G_i\alpha$. Furthermore free $G_i\alpha$ subunits (but not G_i heterotrimer) have been demonstrated to be suitable substrates for phosphorylation by protein kinase C (Katada, Gilman, Watanabe, Bauer & Jakobs1985). Thus mechanisms capable of effecting covalent modification have been demonstrated.

G-protein levels and/ or function appear to be under hormonal control. Glucocorticoids (see chapters 6 & 7)(Rodan & Rodan 1986; Chang & Bourne 1987; Ros, Watkins, Rapiejko & Malbon 1989; Saito, Guitart, Hayward et al.1989) and thyroid hormones (Milligan, Spiegel, Unson & Saggerson 1987) have been shown in experimental animals and cell culture to regulate differentially G-protein subunits. The effects of glucocorticoids will be studied in detail later, but it remains unclear whether the role

of glucocorticoids is primary or merely permissive of the effects of other regulatory factors. Thyroid hormone deficiency (at least in rat adipose tissue) is associated with increased levels of $G_i\alpha$, $G_o\alpha$ and ß subunits; all these changes are reversible with thyroid hormone administration (Milligan 1987). Furthermore, insulin may have a role in regulating Gi function and levels. Loss of tonic Gi-mediated inhibition and attenuation of receptor mediated inhibition of ACA accompany induction of experimental IDDM (streptozotocin and alloxan diabetes in the rat) (Gawler, Milligan, Spiegel, Unson & Houslay 1987; Bushfield, Griffiths, Strassheim et al. 1990)(see section 1.6.2.): insulin administration restores these changes to normal. Clearly many metabolic changes accompany IDDM and it would be premature to ascribe G-protein changes specifically to changes in insulin concentration (although this possibility remains).

In addition to the regulatory roles of hormones on G-protein expression and /or levels (which may be mediated directly or possibly in association with receptor changes) G-protein levels are modulated by ligand-receptor binding and thus have key roles in receptor down-regulation / desensitisation. Heterologous desensitisation (that is when prolonged exposure to a ligand results in downregulation of a number of receptor types) has been shown to be related to alteration of G-protein subunits; exposure of rat adipocytes *in vivo* to PGE1 or phenylisopropyl adenosine (PIA)(an A1 adenosine receptor agonist) effects reduced responses to both hormones with concurrent 90% reduction of $G\alpha i1$ and $G\alpha i3$ in addition to 50% reduction of $G\alpha i2$

and ß subunits and without alteration of Gsα. By contrast, the homologous desensitisation induced by chronic exposure to nicotinic acid, is not associated with altered G-protein levels. Thus heterologous desensitisation is related to, and may be explained on the basis of, G-protein changes (Green, Milligan & Belt 1990).

G_o regulation has been studied in cultured neuroblastoma cells: chronic membrane depolarisation not only results in increase in muscarinic ACh receptor number but also increases G_o α subunit, without affecting G_iα or ß subunit quantities (Leutje & Nathanson 1988) adding further weight to the evidence that G-protein levels are intimately related to and partly determined by receptor function.

1.6. G-proteins in human and experimental models of disease

1.6.1. G-protein abnormalities in human disease

G-protein abnormalities have been demonstrated in the metabolic disease, pseudohypoparathyroidism and in a number of tumours-including some GH secreting adenomas causing acromegaly.

Albright's hereditary osteodystrophy (AHO)

AHO is an autosomal dominant condition which includes pseudohypoparathyroidism (PHP)(type 1a). The classical phenotype of AHO also includes a number of skeletal abnormalities including short stature, stocky build, round facies, brachydactyly and

subcutaneous ossification. A number of endocrine and metabolic abnormalities characterised by resistance of target organs to hormone action have been reported; these include pseudohypoparathyroidism, primary hypothyroidism and hypogonadism (Levine, Downs, Moses et al. 1983). The multi-organ hormone resistance has been attributed to a generalised deficiency of $G_S\alpha$ (both expression and function are reduced). The G-protein abnormality in PHP type 1a consists of a deficiency of G_S without change in G_i. Determined using reconstitution assays (reconstitution of G_S activity in cyc- membranes), G_S activities of 43% (Levine, Jap, Mauseth, Downs & Spiegel 1986)-79% (Saito, Akita, Fujita et al. 1986) normal have been reported in erythrocytes; similar findings have been reported in a number of other tissues including platelets (Farfel and Bourne 1980), erythrocyes (Levine, Downs, Singer et al. 1980), renal tissue (Downs, Levine, Drezner, Burch & Spiegel 1983) and cultured fibroblasts derived from affected subjects (Bourne, Kaslow, Brickman & Farfel 1981). Further evidence confirming the association of PHP type1a with reduced G_S is the finding of 50% reduction of mRNA for Gs in affected members of 6/8 pedigrees with the condition (Levine, Ahn, Klupt et al. 1988). The deficiency of $G_S\alpha$ appears to be due to single base substitution in the $G_S\alpha$ gene (Patten, Johns, Valle et al. 1990).

The pathogenesis of PHP may however be more complex: some kindreds with AHO include individuals who feature $G_S\alpha$ deficiency, and yet who have apparently normal hormone responsiveness (pseudopseudohypoparathyroidism)(Levine et

al.1986). A possible explanation for this perplexing paradox has been propsed by Spiegel (1990), who suggested that the variability in clinical expression may depend on modifying genes. He proposes the possibility that two different non-allelic genes may determine the expression of the disease; when inherited separately the disease is not manifest, but when inherited together the two genes may then cause hormone resistance.

Acromegaly:

Abnormal $G_S\alpha$ also characterises the abnormality underlying acromegaly, at least in some patients' GH secreting adenomas (Vallar, Spada & Giannattasio 1987; Landis, Masters, Spada et al.1989). cAMP functions as a second messenger for several trophic hormones (Rozengurt 1986) including GHRH (Billestrup, Swanson & Vale 1986). Vallar and colleagues have shown that some GH secreting adenomas charaterised by high secretory activity and high intracellular cAMP levels exhibit constitutive activation of $G_S\alpha$ (Vallar et al. 1987): the constitutive activation of $G_S\alpha$ is due to a mutation in the peptide sequence encoding GTPase activty such that GTPase is inhibited (Landis et al. 1989). Loss of this enzyme activity results in loss of the intrinsic switch mechanism which terminates G-protein function with consequent activation of $G_S\alpha$.

Cardiovascular disease:

G-protein abnormalities have been reported in plasma membranes from human myocardium (Feldman, Cates, Veazey et al. 1988; Neuman, Schmitz, Scholz et al. 1988; Bohm, Gierschik,

Schnabel & Erdmann 1990) and from peripheral blood lymphocytes(Horn, Corwin & Steinberg et al. 1988). Although the use of lymphocytes has been justified on the basis of some correlation between levels of Gsα in lymphocyte and myocardial membranes (Horn et al. 1988), contrasting changes in G-protein subunits have been reported in studies using lymphocytes: cholera toxin substrate (i.e. Gsα) has been reported to be reduced by 80% in lymphocyte membranes from patients with heart failure due to dilated cardiomyopathy (Horn et al. 1988), while in other studies substrates for both cholera toxin (Gsα) and pertussis toxins (including Giα (table 2)) were reported to be unaltered in cardiac failure (Maisel, Michel & Insel 1990) irrespective of whether it was due to cardiomyopathy or coronary artery disease (Michel, Maisel & Brodde 1990). Studies of G-protein function inferred from studies of ACA were similarly inconclusive.

However, G-protein studies in plasma membrane preparations derived from myocardium taken from patients with dilated cardiomyopathy (with donor heart controls) have proved more conclusive: Giα has been reported to be increased (around 36%) on the basis of ADP ribosylation studies with pertussis toxin (Feldman et al. 1988; Neuman et al. 1988; Bohm et al. 1990) and immunoblotting (Bohm et al. 1990). Gsα levels are not altered (Feldman et al. 1988). However, altered Giα was not confirmed in a study of myocardium from cardiac failure due to ischaemic heart disease (Bohm, Gierschik & Jakobs et al. 1990) raising the intruiging possibility that G-protein changes are more likely to be determined by the underlying pathology than the resulting

cardiac failure. Furthermore, it remains unclear currently whether the observed G-protein changes contribute to the reduced ß receptor-mediated inotropic responses which accompany chronic heart failure (Bristow, Hershberger, Port & Rasmussen 1989).

1.6.2. G-protein abnormalities in experimental models of disease

Hypothyroidism

Human and experimental hypothyroidism is associated with weight gain which is largely attributable to metabolic changes which include blunted responses to agents that stimulate cAMP accumulation and lipolysis (ß adrenergic agonists (Wahrenberg, Engfeldt, Arner, Wennlund & Ostman 1986), glucagon). In addition, responses to ligands which inhibit lipolysis and cAMP accumulation have generally been reported to be increased (adenosine, PGE1, nicotinic acid (Ohisalo & Stouffer 1979; Saggerson 1986). In the absence of major alterations in receptor number (α2 / ß in humans (Wahrenberg et al.1986; Ros, Northup & Malbon 1988) or AC activity (Armstrong, Stouffer, Van Inwegen, Thompson & Robison 1974) to account for the metabolic changes-the possible contribution from G-proteins has been studied. Using plasma membranes derived from adipocytes, hypothyroid rats have been shown to exhibit increased $G_{i}\alpha$ (based on ADP ribosylation studies (Malbon, Rapiejko & Mangano 1985) and on Western blots using specific antisera (Milligan et al. 1987)); increased $G_{i}\alpha$ in conjunction with elevated ß subunit levels (Milligan et al. 1987) could account for the observed

functional changes noted in experimental and human hypothyroidism. However, confirmation of similar alterations in G-proteins in human hypothyroidism (in contrast to the experimental model described above) is lacking. Studies based on plasma membranes from adipocytes taken from hypothyroid human subjects (post thyroidectomy for papillary carcinoma, but without thyroxine replacement for 4 weeks) failed to show any changes in G-protein levels: specifically Gi and Gs were unchanged (Ohisalo & Milligan 1989).

Obesity:

Obesity is associated with metabolic changes which contrast with those of hypothyroidism, viz. the sensitivity of adipocytes to inhibitory agents is reduced whereas sensitivity to ligands which promote lipolysis is increased (Ohisalo, Ranta & Huhtaniemi1986; Richelson 1988). The role of G-proteins has been studied in two experimental models-the ob/ob strain of obese mouse and the obese Zucker rat. In the former model Begin-Heick (1985) reported deficiency of functional G_i although a subsequent study by Greenberg and colleagues (Greenberg, Tatlor & Londos1987) failed to confirm this. Western blots for $G_{i\alpha}$ in hepatocyte plasma membranes from Zucker rats failed to show any change in $G_{i\alpha}$ levels although functional deficiency of $G_{i\alpha}$ was inferred from studies of GppNHp inhibition of AC (Houslay, Gawler, Milligan & Wilson 1989).

Ohisalo & Milligan (1989) performed similar studies in membranes derived from obese human subjects and although the

absolute levels of $G_s\alpha$ and $G_i\alpha$ were reduced the relative amounts of both were unaltered.

Thus controversy exists regarding the role of $G_i\alpha$ in obesity. There is agreement that $G_i\alpha$ levels in membranes from Zucker rats and obese human are not altered, but the possibility of deficiency of $G_i\alpha$ function remains and would be compatible with the biochemical changes discussed above.

Diabetes mellitus:

Houslay's group have shown that chemically induced insulin dependent diabetes mellitus in the rat is associated with altered G-protein expression and function (table 3) (Gawler et al. 1987; Bushfield et al. 1990; Bushfield, Griffiths, Murphy et al.1990; Srassheim, Milligan & Houslay 1990). These changes are common to both STZ and alloxan induced DM. The alterations are tissue specific, do not invariably reflect altered expression and are selective for particular G-protein α subunits.

STZ and alloxan diabetes are associated with a number of biochemical changes which include a marked reduction in plasma insulin concentrations with a consequent rise in blood glucose. However, both are also associated with development of apparent insulin resistance in hepatocytes and enhanced ability of glucagon to stimulate AC (Gawler et al. 1987; Bushfield et al. 1990). Changes in G-protein subunits are accompanied by altered G-protein function in hepatocytes and adipocytes; tonic inhibition of AC by Gi is lost while receptor mediated inhibition (via Gi) is attenuated in both tissues (Gawler et al.1987;

Bushfield et al. 1990). These changes are reversed by insulin administration.

Correlation of altered function with changes in G-protein subunits is currently only possible with the AC system ($G_S\alpha$

Table 3: G-protein changes in experimental diabetes mellitus.

TISSUE	$G_{i}1\alpha$	$G_{i}2\alpha$	$G_{i}3\alpha$	$G_s\alpha$	ß
HEPATOCYTES	-	↓ 1,2	↓ 1,2	↓ 1,2	
ADIPOCYTES	→ 1 ↑ 2	→ 1,2	↑ 1,2	→ 1,2	→ 2
KIDNEY HEART SKELETAL MUSCLE BRAIN	→ 2	→ 2	→ 2	→ 2	

KEY: 1= change in amount of G-protein assessed by western blotting
2= changes in mRNA encoding G-protein subunit

Data are dervied from the following references:

Gawler et al. (1987) Bushfield et al. (1990a)
Bushfield et al. (1990b) Srassheim et al.(1990)

effects stimulation while $G_{i}\alpha$ effects inhibition of AC catalytic unit). Loss of Gi function correlates with reduced amount of $G_{i}2\alpha$ cand reduced $G_{i}2\alpha$ mRNA. However, this plausible series of associated observations does not hold true for adipocytes where $G_{i}2\alpha$ levels and mRNA are unaltered. Post transcriptional modification of the G-protein subunits, for example by phospho-

rylation, could account for these observations. Whether this is effected by insulin (the insulin receptor tyrosyl kinase can phosphorylate the α subunit of Gi (Katada, Gilman, Watanabe, Bauer & Jakobs 1985) or by other hormones such as glucagon (Bushfield et al. 1990) remains unclear.

1.7. Aims of this thesis:

The aim of this thesis is to examine whether human non-insulin dependent diabetes mellitus and various types of hypertension (human and experimental) are associated with alterations of G-protein function or levels.

In chapter 3, human non-insulin dependent diabetics (who exhibit insulin resistance and hyperinsulinaemia) were studied to assess whether NIDDM is associated with G-protein abnormalities as seen in experimental STZ-IDDM. Platelets were used as a source of plasma membrane for study, partly because of their availability and because platelets from diabetics exhibit functional abnormalities for which the underlying mechanism remains to be elucidated.

Study of experimental models of obesity (Zucker rat)(Houslay et al. 1989) and of STZ/alloxan IDDM has raised the possibility that these two states which are characterised by insulin resistance (see above) may share a common G-protein abnormality viz., altered function (± levels) of Gi_2. Several human disease states also exhibit insulin resistance including obesity, NIDDM and essential hypertension (Ferrannini, Buzzigoli, Bonadonna et al.

1987): indeed there is growing evidence that insulin resistance and hyperinsulinaemia may be implicated in the pathogenesis of essential hypertension. In addition, strong clinical and epidemiological associations link diabetes mellitus (in particular NIDDM) and essential hypertension (for example, the prevalence of hypertension is increased in diabetes: 40-53% of patients presenting with NIDDM (age 25-65yrs) are hypertensive (BP>160/95) (United Kingdom Prospective Diabetes Study.1985) and after age adjustments, the prevalence of hypertension may actually be higher in IDDM(National Diabetes Data Group. 1985)). Thus, in chapter 4, G-protein levels and function in human essential hypertension (again using platelets) were assessed in order to study the possibility that EH might share similar abnormalities of function (or levels) of G-proteins (in particular Gi2) with experimental models of obesity and diabetes mellitus (by virtue of shared insulin resistance).

In chapter 5, studies of the spontaneously hypertensive rat (SHR), a commonly used model of genetic hypertension, are described. This model is particularly useful as it has many features in common with human EH including glucose intolerance/ insulin resistance. This experimental model of hypertension was used in order to study G-protein changes in myocardium (tissue which is not available from human subjects!) as there is substantial evidence of abnormalities of AC activation; reduced ß adrenergic activation of adenylyl cyclase has been widely reported (*vide infra* Amer, Gomoll, Perhach, Ferguson & McKinney 1974; Bhalla & Ashley 1978; Bhalla,

Sharma & Ramanathan 1980; Blumenthal, McConnaughey & Iams 1982; Sharma, Kemp, Gupta & Bhalla 1982; Kumano, Upsher & Khairallah 1983; Anand-Srivastava 1988). Thus, in addition to the possibility of G-protein changes in association with insulin resistance, the role of G-proteins in the observed changes in cyclase activities requires clarification.

In chapters 6 & 7 potential changes in G-proteins in glucocorticoid hypertension were studied. There is increasing evidence that glucocorticoids may directly differentially regulate G-protein subunits, at least in suprapharmacological doses (Rodan & Rodan 1986; Chang & Bourne 1987; Ros et al.1989; Saito et al.1989), and it is appropriate to examine whether these changes are relevant to glucocorticoid hypertension

Using an established animal model (Tonolo, Fraser, Connell & Kenyon 1988) of glucocorticoid hypertension the effect of glucocorticoid (dexamethasone (DEX)) administration for 14d (*in vivo*) on the function and levels of G-proteins in several organs with key roles in the regulation of blood pressure (chapter 6) was studied.

In chapter 7, the effects of *in vitro* DEX on G-protein function and levels in membranes derived from cultured VSMC were studied. The aim was to assess whether the increase in ACA seen in cultured VSMC (Yasunari, Kohno, Balmforth et al. 1989) in

response to DEX can be ascribed to alterations in G-protein subunits.

Chapter 2

Materials & Methods
& Preliminaries

2.1. Materials

All chemicals were supplied by Sigma (UK) Ltd. with the exception of guanosine-5'-triphosphate (GTP), guanylylimido-diphosphate (GppNHp), creatine kinase and creatine phosphate which were supplied by Boehringer Mannheim (West Germany).

Radioisotopes including α-32P ATP , 5',8-3H cAMP and γ 32P GTP were supplied by Amersham International plc (UK).

Antibodies to G-protein α subunits were prepared and kindly donated by Dr G. Milligan, Dept. of Biochemistry, University of Glasgow. Table 4 lists the antibodies used in these studies, the peptide sequences against which the antibodies were generated and the specificities of the resulting antibodies. The principle of antibody production is described for antisera CS1 and SG1, but similar methods were used to generate all the antibodies. Briefly, antiserum CS1 was produced in the New Zealand White rabbit, using a conjugate (conditions for coupling and use as described by Goldsmith, Gierschik, Milligan et al.1987) of keyhole limpet haemocyanin and a synthetic peptide (RMHLRQYELL) which corresponds to the C-terminal decapeptide of Gsα. Antiserum SG1 was raised in a similar fashion except that the synthetic peptide used (KENLKDCGLF) corresponds to the C-terminal decapeptide of rod transducin. This sequence differs from the equivalent region of Gi in only one substitution, and as for other antisera generated against this conjugate (Goldsmith

et al. 1987; Milligan et al. 1987), this antiserum identifies forms of Gi. As the distribution of transducin is limited to tissues that contain photoreceptors, this antiserum can be used to identify Gi in all other tissues. The antibodies bound to G-proteins (for example on a Western blot) were subsequently identified using one of two 'second antibodies' - horse radish peroxidase (HRP) labelled anti-rabbit-Ig (which was kindly supplied by the Scottish Antibody Production Unit, Law Hospital) and ^{125} I -labelled donkey antirabbit IgG which was obtained from Amersham International plc (UK).

Table 4: Antibodies used to identify G-proteins: peptide sequence identified and antibody specificity.

CODE	PEPTIDE SEQUENCE	NATIVE PEPTIDE	Ab SPECIFICITY
SG	KENLKDCGLF	TD 341-350	TD1,TD2,Gi1,Gi2
I1C	LDRIAQPNYI	Gi1 159-168	Gi1
LE3	CERYAQSDY	Gi2 160-169	Gi2
I3B	KNNLKECGLY	Gi3 345-354	Gi3
CS	RMHLRQYELL	Gs 385-394	Gs
ON	GCTLSAEERAALERSK	Go 1- 16	Go
BN	MSELDQLRQE	ß1 1-10	ß1,ß2

Peptide sequence based on single letter coding for amino acids.
TD = TRANSDUCIN
REFERENCES: Mitchell, Griffiths, Saggerson et al. 1989

2.2. Experimental animals

A number of genetic models of hypertension exist (reviewed by Yamori 1983). Although none exactly mirrors human EH, the spontaneously hypertensive rat (SHR) (developed in Japan, by Okamoto & Aoki (Okamoto & Aoki, 1963)) shares many features in common with EH, and is consequently widely used. The hypertension of human EH and of the SHR can be regarded as multifactorial in aetiology with a polygenic hereditary basis (the SHR, if anything has a stronger genetic basis than EH). As in EH, the hypertension of the SHR is not associated with volume overload, but develops and is maintained in association with increased peripheral vascular resistance. The factor initiating hypertension in the SHR may be neurogenic (promoting vasoconstriction) while (as in EH) later stages are characterised by structural and functional vascular changes: a number of membrane transport abnormalitites are shared by EH and SHR (reviewed by Yamori 1983). Both SHR (Mondon & Reaven 1988) and EH (Ferraninni et al. 1987) share the abnormalities of insulin resistance and hyperinsulinaemia which will be discussed later in greater detail (chapter 4). A further attraction of the SHR model is that it mimics the natural history of the human disease-in that both share a tendency to end organ damage (for example, myocardial hypertrophy) and to cardiovascular complications.

One potential weakness exists in the use of this particular model; there is controversy as to the most appropriate normotensive control. This problem stems from the original breeding programme. After the SHR species had been developed the

investigators (Okamoto & Aoki) subsequently appreciated the need for an appropriate control and returned to the original Wistar (outbred) species and selected and subsequently bred normotensive controls (WKY). The majority of the studies involving SHR cited in this thesis have used WKY control species. However, genetic heterogeneity has recently been shown not only between animal suppliers but also among WKY rats from a particular supplier, highlighting that the rats are not fully inbred (Kurtz et al. 1989). It has recently become apparent, however, that the outbred Wistar strain may at be least as appropriate a control as WKY, and for this reason, in this work both WKY and Wistar rats were used as control species for the studies involving SHR.

Animals were obtained from Charles River laboratories. In this thesis tissues were obtained from 10-11 week old SHR, Wistar and WKY rats in the study of rat myocardium. Sprague Dawley rats were used as donors of tissue and of cells for culture in the studies of the effects of dexamethasone (chapters 6 & 7) on G-protein expression.

The animals were housed in the local animal house under standard conditions of light and temperature. They were fed standard chow and free access to water was allowed. Further details of animal procedures are given in the appropriate methods sections; sacrifice was by cervical dislocation.

2.3. Membrane preparations

2.3.1. Human platelet plasma membrane preparation.

Platelets were prepared as described by MacIntyre and Pollock (1983). Blood was collected in 3.8% w/v trisodium citrate and centrifuged at1000g for 5 min. at room temperature to obtain a supernatant of platelet rich plasma. Following aspiration and further centrifugation at 30,000g for 15 min at 4°C the resulting pellet was resuspended in ice cold lysing buffer (10 mM-Tris/HCl buffer containing 1 mM EDTA, pH 7.4). Membranes were subsequently prepared from this pellet as described by Jakobs and colleagues (Jakobs, Lasch, Minuth, Aktories & Schultz 1982). The pellet was homogenised in lysing buffer with ten strokes of a motorised Teflon pestle/glass homogeniser and was then subjected to further centrifugation at 30,000g for 15 min. Following washing in lysing buffer and another centrifugation the final pellet was resuspended in 10 mM Tris/HCl buffer, pH 7.4 and frozen at -80°C.

2.3.2 Plasma membrane preparations derived from animal tissues: liver cell plasma membrane preparation.

Although liver is relatively homogeneous with hepatocytes accounting for more than 90% of adult liver mass, 40-50% of the actual cell population consists of other cells including erythrocytes, reticuloendothelial cells, Kupffer cells etc. Furthermore hepatocyte 'plasma membrane' comprises three distinct functional zones- the blood sinusoidal, the lateral and the bile canalicular regions. Membranes have been prepared in two ways- 'crude' membrane preparations prepared essentially by

differential centrifugation and 'purified' membrane preparations based on centrifugation through density gradients (Neville 1960; Neville 1967; Touster, Aronson, Dulaney & Hendrickson 1970). Several protocols representing variations on the latter theme are available and these are broadly designed to isolate lateral & bile canalicular membrane (Song, Rubin, Rifkind & Kappas 1969) or alternatively blood sinusoidal membrane (Touster et al. 1970)-but difficulties have arisen with low yield of membrane (Ray 1969) and with reproducibility between investigators (Touster et al. 1970). Glucagon-activated AC is primarily located in the blood sinusoidal membrane fraction while G-protein immunoreactivity appears to be greatest in the bile canalicular fraction. The hepatocyte preparation used here (Illiano and Cuatrecasas 1972) represents a compromise- being relatively simple to perform while offering a final membrane preparation which is enriched with plasma membrane; the essence of the method is differential centrifugation.

In brief,whole livers were excised from control and hyper-trensive rats and minced with a scalpel blade. The 'mince' was homogenised in ice cold 0.25M sucrose using a Polytron at 21,000 rpm (setting '6') for 90sec. After centrifugation for 10 min. at 600g, the supernatant was centrifuged at 12,000g for 30 min. This supernatant was adjusted with NaCl (0.1M) and MgSO4 (0.2mM) and centrifuged at 40,000g for 40 min. The pellet was then suspended in 10 mM TRIS (tris(hydroxymethyl)amino-methane-HCl) buffer pH 7.4, homogenised and centrifuged at 40,000g for 40 min. This step was repeated twice. The final

pellet was resuspended in TRIS buffer and stored in aliquots at -80°C.

2.3.3. Rat myocardial plasma membrane preparation:

Myocardial plasma membranes were prepared as described by Sharma and colleagues (Sharma, Kemp, Gupta & Bhalla (1982)). Hearts were removed as soon as possible after sacrifice of the rats. They were cleaned of loose connective tissue, fat and residual blood and were minced with a scalpel blade. The mince was washed twice with cold homogenising buffer [20mm Tris-HCl, pH 7.4, containing 0.25M sucrose, 1 mM EDTA, 1 mM MgCl2 and 1 mM DTT] and was then homogenised in 7-10 volumes of ice cold buffer using a 'Polytron' at setting '5' for 3 x 10 sec. with 1 minute interval between bursts. The homogenate was filtered through two layers of cheesecloth and centrifuged at 9000g for 10 min. The pellet was rehomogenised and centrifuged again. The two supernatants were combined and centrifuged at 37,000g for 30min. to yield membranes. The membrane fractions were washed once with 0.6M KCl in homogenising buffer followed by two washes with homogenising buffer to remove intrinsic guanine nucleotides and contractile proteins. The pellet was finally resuspended in a buffer containing 10mM Tris-HCl, 1mMDTT and 1mM MgCl2, pH7.4 and stored at -80°C.

2.3.4. Rat brain plasma membrane preparation:

Whole rat brains were excised and, after weighing, were homogenised in 10mM Tris/1mM EDTA pH 7.5 buffer with 20 strokes of a Potter-Elvejhem teflon-glass tissue homogeniser. This crude homogenate was centrifuged for 10 min. at 2,600 rpm

to remove nuclear and other dense material, and the supernatant containing the plasma membranes was recovered. The supernatant was then centrifuged at 40,000g for 20 min. to produce a membrane pellet which was resuspended and washed in Tris/EDTA buffer. After a further centrifugation (40,000rpm for 20 min.) the final pellet was suspended in buffer and stored in aliquots at -80°C.

2.3.5. Rat renal cortical plasma membrane preparation:
Rat renal cortical plasma membranes were produced according to a modification of Snavely and Insel's protocol (1982); the modification was drawn from the liver plasma membrane preparation of Illiano and Cuatrecasas (1972) and was incorporated to increase the purity of the final plasma membrane preparation. Whole kidneys were excised and the perinephric membrane and adjacent fat were removed. The kidneys were bisected longitudinally and the cortex was dissected from the renal pelvis and medulla. After weighing, the cortical tissue was homo-genised in 10 volumes of buffer [20mm Tris-HCl, pH 7.4, containing 0.25M sucrose, 1 mM EDTA, and 1 mM DTT with 10 strokes of a Potter-Elvejhem teflon-glass tissue homogeniser. The homogenate was centrifuged at 500g for 5 min. and the resulting supernatant was recovered; this was then centrfiuged at 12,000g for 30 min. and plasma membranes were recovered from the supernatant by centrifugation at 37,000g for 30 min. After a wash in homogenising buffer followed by further centrifugation at 37,000g for 30 min. the resulting plasma membrane pellet was suspended in 10mM Tris-HCl, 1mMDTT and 1 mM MgCl2, pH7.4 and stored in aliquots at -80°C.

2.3.6. Rat mesenteric & VSMC plasma membrane preparation:

The mesenteric vascular beds and aortas were dissected free of fat into ice cold homogenising buffer [10mM Tris/1mM EDTA]. They were homogenised using a 'Polytron' at setting'10' for 15 sec. The homogenate was then centrfiuged at 1000rpm for 10min. to remove the bulk of residual fat, and after a further 10 min. at 1500rpm the supernatant was recovered. Plasma membranes were recovered from this fraction by centrifugation at 37,000g for 30 min. The final pellet was suspended in 10 mM Tris and stored in aliquots at -80^{o}C.

2.4. Western Blotting

2.4.1. Preparation of Western Blots

The presence and amount of particular G-protein subunits was determined by Western blotting, using the antibodies described in (section 2.1.) Gel electrophoresis was performed according to the method of Laemmli (1970). Gels containing 10% acrylamide were prepared from a stock solution of 30% by weight of acrylamide and 0.8% N,N'-bis-methylene acrylamide. The final concentrations in the separation gel were as follows: 0.38M Tris-HCl (pH 8.8), 0.1% SDS and 3.3% glycerol. The gels were chemically polymerised by the addition 0.03% (by volume) of tetramethylethylenediamine (TEMED) and ammonium persulphate. 100 x 150 x 1.5 mm gels were prepared between glass plates. Stacking gels of 3% acrylamide, containing 0.12 M Tris-HCl (pH 6.8) and 0.1% SDS, polymerised by TEMED and ammonium persulphate were layered on top of the separation gel. The

electrode buffer contained 0.025M Tris, 0.19M glycine and 0.1% SDS.

In general, 100µg aliquots of plasma membrane protein were used unless otherwise stated. The protein was solubilised and precipitated by the addition of 2% deoxycholate and 24% TCA. Following resuspension of the protein in buffer containing 0.5M Tris, 2.4% SDS (w/v), 2.8% DTT,14.28% urea and Bromophenol blue dye, the samples were immersed in boiling water for 2 min. to ensure complete dissociation of the protein fractions (Maizel 1969). BRL (Bethesda Research Labs., USA) high MW protein standards were run in parallel with the samples under study. In addition, a 'standard' plasma membrane preparation was included on all blots to facilitate comparison of data from different blots.

The electrophoresis was run vertically in a BRL electrophoresis tank with a current of 15mA and voltage of 35 V until the Bromophenol blue dye front had reached the bottom of the gel (generally about 16 hr). The proteins were electrophoretically transferred to nitrocellulose paper (100V for 120 min) essentially as described by Towbin (Towbin, Staehelin & Gordon1979). After blocking with 5% "Marvel" (Cadbury Ltd, UK) in Tris-buffered saline (TBS) for 2h at 37°C, the blot was incubated with the first antibody in 1% gelatin in PBS overnight. The bound antibody was visualised either with horse radish peroxidase-labelled donkey, anti-rabbit IgG (developed using O-dianisidine)(as previously described by Milligan et al.1987) or with ^{125}I labelled anti-rabbit IgG which was suspended in PBS

containing1% gelatin with 0.2% Nonidet P40 (nonionic detergent). The radiolabelled 'second antibody' was visualised by autoradiography by exposure to Kodak XOMAT AR Film (Kodak Eastman Ltd) which was processed automatically in a Kodak XOMAT processor. The bands were subsequently cut out and counted on a NE1612 gamma counter (Nuclear Enterprises, EMI, UK).

The gels were retained and residual protein (which had not been transferred electrophoretically) was visualised by staining with Coomassie Blue (0.25% in 45% methanol/10% glacial acetic acid) with subsequent destaining in 45% methanol/10% glacial acetic acid.

Other methods are available for identification and quantitation of G-protein subunits. Covalent modification of G-proteins by ADP ribosylation has proved to be a useful means of identifying G-proteins. Cholera toxin and pertussis toxin (see section 1.2.5.) are essentially ADP-ribosyltransferases which catalyse the transfer of the ADP-ribose moiety from NAD^+ to the recipient α subunit. Cholera toxin mediated ADP ribosylation is a process specific for $G_s\alpha$. In contrast, pertussis toxin mediated ADP ribosylation is limited due to multiple substrates within membrane preparations- including G_i(1,2,3)(Suki et al.1987) and G_o (Itoh et al. 1986). Used extensively in the past to identify the presence of G-proteins, the problem of multiple substrates coupled with the availability of antibodies specific for particular α subunits has rendered Western blotting the prime means of identifying G-proteins.

Furthermore difficulties have arisen in achieving ADP-ribosylation in some systems-notably the human platelet. The problem may be partly circumvented by using detergent-(saponin) treated whole platelets- which allow entry of pertussis toxin through the membrane. In light of these difficulties use of specific antibodies, rather than ADP ribosylation studies, was the preferred method for identifying G-proteins within our membrane preparations.

2.4.2. G-proteins identified in plasma membrane preparations:

Figure 3 is a typical blot showing Gsα expression in a number of membrane preparations. The data obtained from all such exploratory blots are summarised in table 5.

It should be noted that none of the membrane preparations comprised highly purified plasma membrane-but rather they all represent plasma membrane enriched preparations. It would thus be invalid to draw conclusions form the apparent differences in the relative abundance of G-proteins in different tissues. However, comparisons can be made between similar plasma membrane types from different species as invariably the study preparations were prepared concurrently and according to the same protocol.

In order to make useful comparisons of G-protein levels in study and control membrane preparations, it was necessary first to identify which G-proteins were present in the membrane prep-

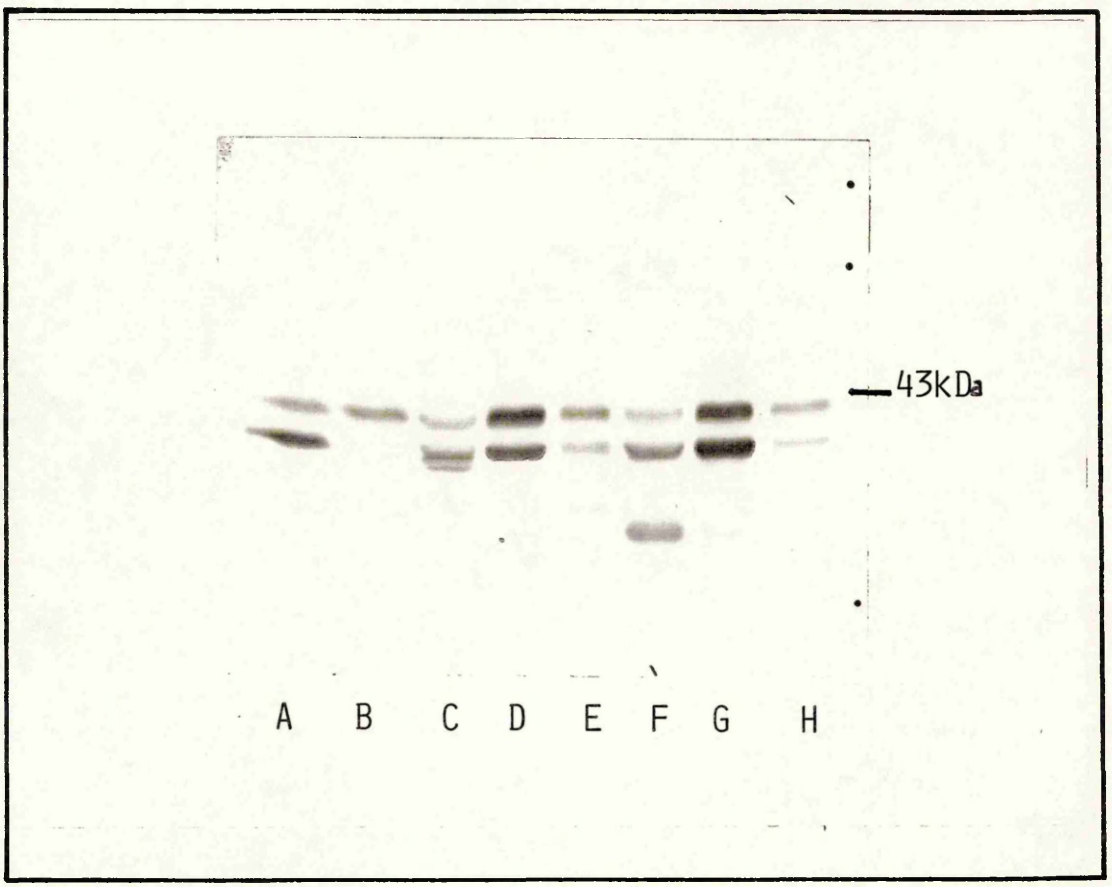


Fig. 3. Demonstration of Gsα subunits in a range of plasma membrane preparations. (100μg protein loaded).

A. nephroblastoma cell line
B. brain
C. liver
D. myocardium
E. adipocyte
F. renal cortex (A)
G. renal cortex (B)
H. mesenteric artery

Table 5: G-proteins expressed in membrane preparations

MEMBRANE PREP.	Gsα	Gi1	Gi2α	Gi3α	Goα	ß
PLATELET *	•		•	•		•
MYOCARDIUM	•		•	•	•	•
RENAL CORTEX	•	•	•		•	•
LIVER	•		•			
BRAIN	•	•	•		•	•
VSMC/MESENTERY	•		•	•		•

All membrane preparations studied were derived from rat species except platelet*- from humans.

Note: in all preparations Gsα was represented as 2 bands of around 40kDa and 42kDa (splice variants), although the relative abundance of these varied.

aration. For comparisons to be valid, identical amounts of membrane preparation were loaded (study and control preparations)(this was confirmed by determining protein concentration of the actual samples which would be loaded onto gels later in the day). It is assumed that the amount of antibody present is in excess of the available binding sites (G-protein, within preparation under study). Furthermore, to ensure that this was genuinely the case 'dose response' curves were constructed for each membrane preparation/ antibody used and the amount of protein to be loaded was determined from the linear portion of the curve (plot of CPM v μg protein loaded). A typical 'dose response' curve is shown-figure 4. (The amounts of plasma membrane protein derived from these studies and loaded on Western blots are cited in appropriate chapters.)

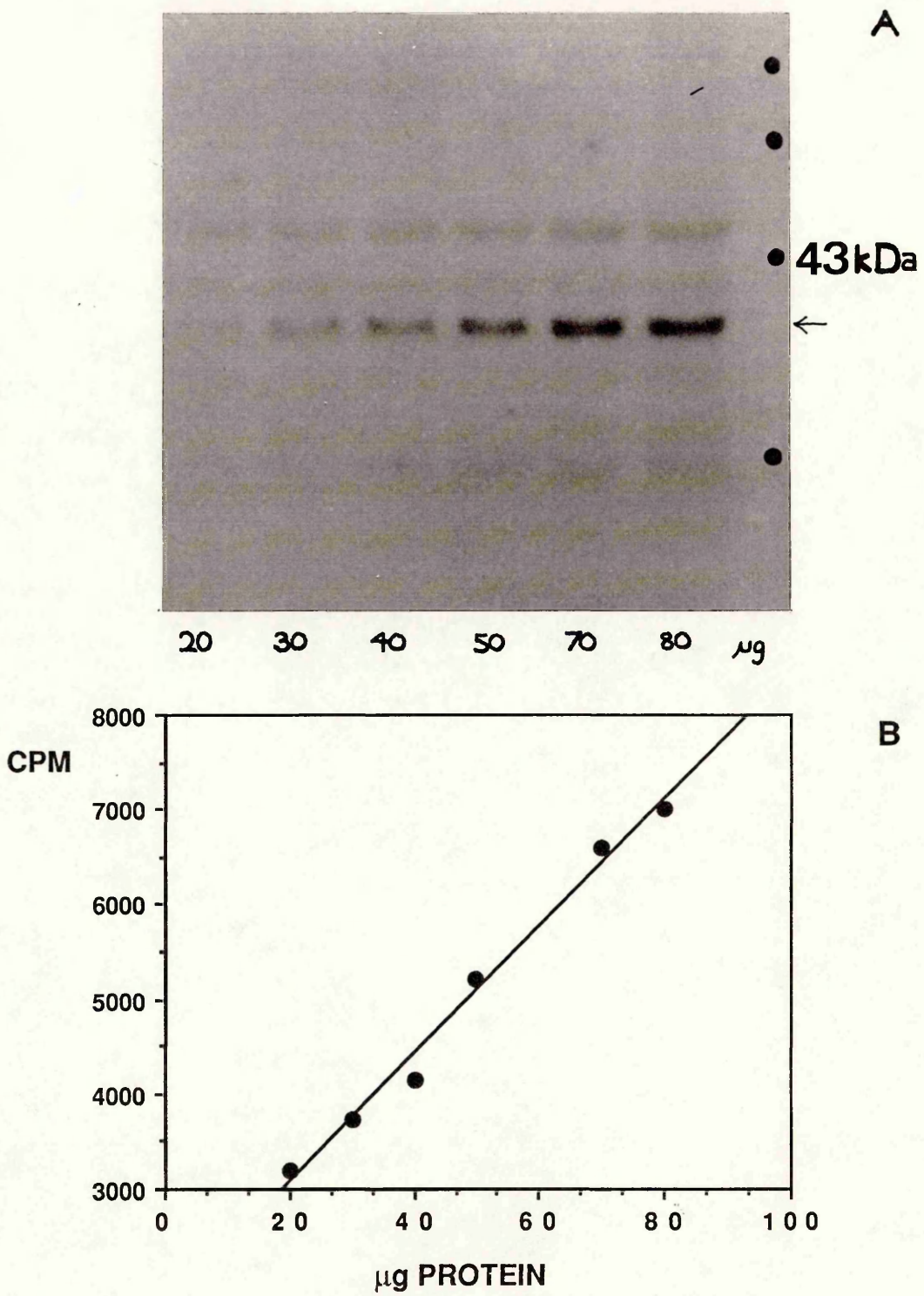


FIG.4.RELATIONSHIP BETWEEN CPM (125-I 'second antibody' against SG1 labelled Gi2) AND AMOUNT OF PROTEIN LOADED [RAT MYOCARDIUM]
A. REPRESENTS AUTORADIOGRAPH B. GRAPH OBTAINED

2.5. Cell Culture - VSMC.

VSMC culture (for subsequent G-protein studies by the author) was performed by Dr F. Lyall and Miss S. Tawil, MRC Blood Pressure Unit as described by Balmforth and colleagues (Balmforth, Lyall, Morton & Ball 1988). Briefly cells were isolated from the mesenteric vascular bed of 6-8 x 300-350g Sprague Dawley rats. Fat was removed by blunt dissection and the vascular tree was transferred to an enzyme dissociation mixture (10ml) of collagenase (1.25mg/ml), elastase (0.05 mg/ml) and soya bean trypsin inhibitor (0.1%) in Hank's balanced salt solution (HBSS). After incubation at 37°C for 10-15 min. the arteries were transferred to fresh HBSS and any remaining fat, adventitia and endothelial cells were removed by careful dissection. The cleaned arteries were cut into small pieces and transferred to fresh enzyme dissociation mixture and incubated at 37°C with periodic trituration until a single cell suspension was obtained. The cell suspension was centrifuged (5°C, 200g for 5 min.) and the pellet resuspended in Dulbecco's modified Eagle medium containing 10% foetal calf serum, 2mM L-glutamine, 100U/ml penicillin and 100µg/ml streptomycin. The dispersed cells were plated in a $25cm^2$ culture flask and maintained in a humidified atmosphere of 5% CO_2, 95% air at 37°C. Cells were subcultured as required up to 7th passage and were used for experiments when confluent.

Verification that the cultured cells were indeed VSMC was based on a number of investigations. These included electron microscopic morphology, absence of light microscopic staining for

endothelial cells, and the demonstration of cell contractility to angiotensin II (only feasible within the first 48h of culture).

Cell culture conditions and additions to culture medium are specified in chapter 7.

2.6. Measurement of protein concentration

Protein concentrations in the membrane preparations were measured colorimetrically using Peterson's modification (Peterson 1977) of the Lowry protocol (Lowry 1951) based on bovine serum albumin as standard.

2.7. 5' Nucleotidase Activity

The enzyme 5'-Nucleotidase (5ND) (EC 3.1.3.5) was used as a plasma membrane marker to trace recoveries and losses of membrane during the membrane preparative procedures. Although essentially an ectoenzyme, 5ND has been identified on intracellular membranes including endoplasmic reticular membranes (Widnell 1972) and Golgi membranes (Farquhar et al.1974). The enzyme hydrolyses 5' ribo-nucleotides to ribonucleosides and orthophosphate. 5ND activity was measured as described by Dixon & Purdom (1954); the phosphorus liberated during the 5'-nucleotidase hydrolysis of adenosine 5'-monophosphate is measured as an index of the enzyme's activity (the method allows correction for the hydrolysis of adenosine 5'-monophosphate by non-specific phosphatases by incorporating the determination of phosphorus released from glycerophosphate substrate). The phosphorus liberated is measured colorimetrically by the Fiske and Subbarow (1925) method.

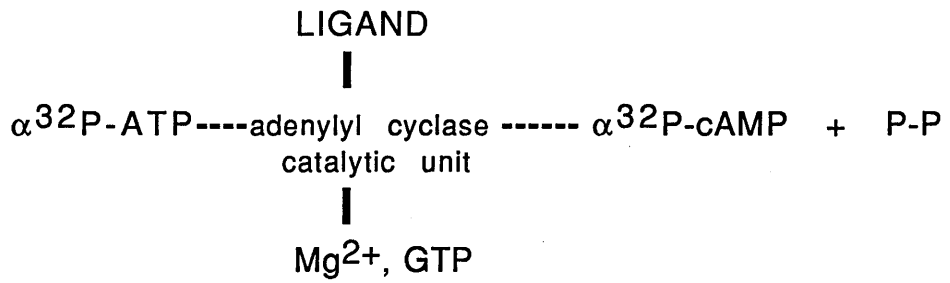
2.8. Functional assessment of G-proteins

Several methods are available to assess G-protein function. The most convenient of these is to measure changes in second messenger activity induced by ligands which either act directly on associated G-proteins (for example GTP and GppNHp) or which act on receptors which are coupled via G-proteins to the effector mechanism. The experiments described here are based on assessment of G-protein modulation of adenylyl cyclase activity- measured as cAMP production by the Salomon assay (see 2.8.1.).

Other methods are available and include the functional reconstitution of Gs activity using membrane extracts from cells under study in conjunction with cyc- S49 lymphoma membranes which lack an intrinsic Gs activity (genetic mutation prevents binding of Gs to AC catalytic unit). Alternatively, ADP ribosylation by cholera and pertussis toxins can be adapted to study G-protein function: pertussis toxin mediated ADP-ribosylation of Gi effects inhibition of Gi (i.e. net activation of effector) while cholera toxin mediated ADP ribosylation of Gs effects activation of Gs (i.e. net further activation of effector). This approach is clearly limited by the potential problems cited in the previous section. GTPase is an intrinsic component of the α subunit and its activity can be directly measured; as will be discussed later, this method also has its limitations (2.9.).

2.8.1. Measurement of adenylyl cyclase activity

Adenylyl cyclase activity was assessed according to the protocol of Salomon (1974) by measurement of the cAMP synthesised in the following reaction:



The method consists essentially of two phases. The reaction phase (detailed below) followed by the separation of the reaction products by sequential column chromatography on Dowex 50G and neutral alumina. Thus ^{32}P cAMP can be separated from ^{32}P ATP. The ^{32}P counts were corrected for column recovery (70-80%) using ^{3}H cAMP.

Two different reaction mixtures were used for incubations. Plasma membranes from (rat) myocardium, renal cortex, liver and mesenteric arteries were incubated in Mix A while membranes from platelets and VSMC were incubated in Mix B.

Mix A: Rat myocardium/ renal cortex /liver/mesenteric artery:
The reaction mix was essentially as described by Sharma et al. (1982) and included a phosphate regenerating system consisting of creatine phosphate (sodium salt) 20mM, 60U/ml creatine kinase and myokinase 40U/ml in addition to 25mM tris-acetate,pH 7.6, 5 mM MgCl2, 0.5mM ATP, 0.05 mM cAMP, 1mM DTT, 1mM EDTA and 0.5mM 3-isobutyl-1-methyl xanthine (a phosphodiesterase inhibitor). Guanine nucleotides were included at the specified concentrations, but unless otherwise stated the reaction mix included 10^{-5}M GTP. $\alpha^{32}P$ ATP was added to give 2

x 10^6 CPM/assay. Final assay volume was 50µl. All assays were performed in quadruplicate.

The reactions were started by the addition of membrane (generally 8µg membrane protein) suspended in 10mM tris pH7.4. Tubes were incubated at 30°C for 10 min.- a time shown to be in the linear portion of the time course of the reaction (figure 5).

The reactions were stopped by replacing in an ice bath and by the addition of 100µl of 'stopping solution' containing 2% SDS, 45mM ATP and 1.3mM cAMP. 50µl of ^{3}H-cAMP were added and the tubes were then placed in a 95°C water bath for 3 min. to solubilise all membrane protein. The contents of the tubes were then subjected to column chromatography (see above) after addition of 1 ml of water to each tube or were frozen at -20°C (without demonstrable loss of cAMP) until chromatography was performed. The final 6 ml eluate were collected directly into scintillation vials containing 12 ml. Ecoscint scintillation fluid (National Diagnostics, U.K.) and were counted in a Beckman LS1800 using a dual channel programme.

Mix B: Platelets and VSMC:

The assay mix for platelet membrane experiments was as described by Salomon (1979) and was similar to the above protocol with the exclusion of IBMX and myokinase, but with the inclusion of BSA 0.1mg/ml. Incubations were at 37°C for 15 min. which were again within the linear part of the time course of the reaction. The stopping solution and subsequent handling of the samples were exactly as shown for Mix A.

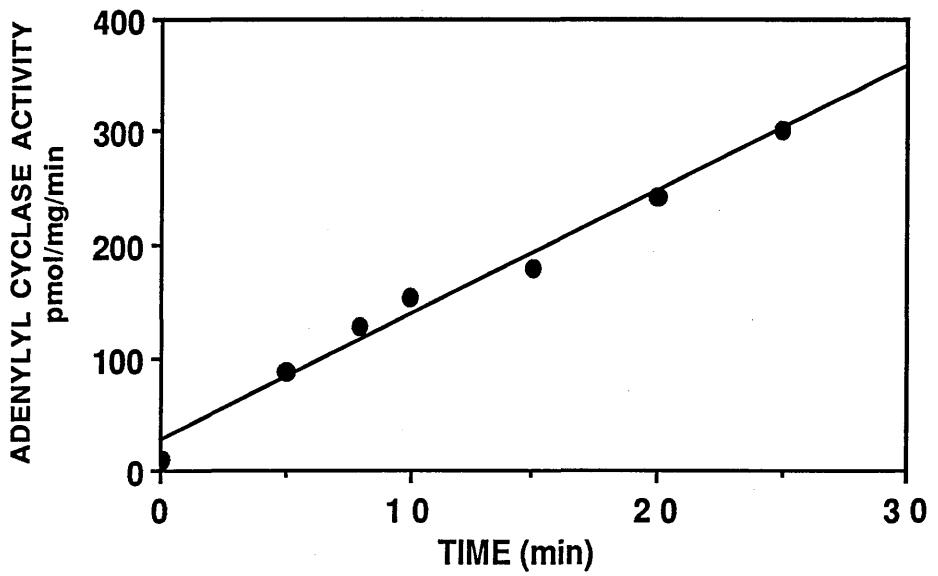


Figure 5. Time course of adenylyl cyclase enzyme activity in rat myocardium-showing linear time course over 25min.

The interassay coefficient of variation for this assay was around 10%.

2.8.2. Ligands used to study adenylyl cyclase activity (table 6)

2.8.3. Forskolin

Forskolin has been used in all studies of ACA described in this thesis; it has been used alone and in combination with ligands which stimulate or inhibit ACA. Forskolin, a derivative of the root of *Coleus Forskolii,* is a potent stimulant of adenylyl cyclase activity which serves to amplify basal or ligand stimulated AC. Since the discovery of this property in 1981

(Seamon, Padgett & Daly 1981), forskolin has been elevated from the realms of Indian herbal medicine to a useful tool in elucidating the properties and actions of AC and G-proteins. However, its ability to stimulate AC indiscriminately renders forskolin toxic on systemic administration; the prime effects in experimental animals are "cardiotonic", that is analagous to ß adrenergic effects (but not susceptible to ß blockade)- eliciting positive chronotropic and inotropic effects (dogs) but also vasorelaxation- resulting in reduced mean arterial resistance, peripheral resistance and left atrial pressure-and thus hypotension (Lindner, Dohadwalla & Bhattacharya 1978).

Forskolin is lipophilic and exhibits limited water solubility. 100µM forskolin has been widely used in studies of AC activity in platelet membranes (Insel, Stengel, Ferry & Hanoune1982; Awad, Johnson, Jakobs & Schultz 1983). It can activate AC in both membrane and intact cell preparations without activation or inhibition of other enzymes involved in cyclic nucleotide metabolism (including guanylate cyclase and cyclic nucleotide phosphodiesterases). Forskolin exhibits a rapid onset of activation of AC and the activation is reversible. The absolute magnitude of the activation ranges between 2-30 x basal levels of activity (in presence of Mg2+). The EC_{50} for activation of AC by forskolin is about 10µM. Stimulation of AC by forskolin increases V_{max} of the enzyme and in some preparations (including platelets) increases the Km of adenylyl cyclase for MgATP (platelets-from 20-80µM)(Awad et al. 1983). Forskolin has also been shown to alter the affinity of the enzyme for divalent cations and its substrate ATP.

Uncertainty exists as to the precise details of how forkolin effects stimulation of AC; it is probable, however, that forskolin interacts directly with the catalytic unit of cyclase (Seamon and Daly 1981)and probably also with Gsα. However, the activation is not dependent upon Gs or the Gs binding site (Seamon and Daly 1981) (demonstrated in cyc$^-$ S49 lymphoma cells)(cf activation of AC by hormones, NaF, guanine nucleotides and cholera toxin- all of which require Gs) or Gi (confirmed after ADP ribosylating membranes with pertussis toxin). However, the efficacy of forskolin in stimulating AC is affected by ligands which function via Gs or Gi. Ligands which function via Gs act generally in synergy with forskolin in increasing ACA. The interaction is associated with increased efficacy and/or potency of the agonist (and is probably dependent on the presence of guanine nucleotides). First seen in platelets, the EC50 for PGE1 stimulation of AC was not affected by 50μM forskolin, but maximal stimulation by PGE1 was increased 2 x (Insel et al.1982) (increased efficacy). In contrast in rat renal cortex basolateral membranes forskolin is associated with increased potency of PTH: the EC50 is reduced (5x) and the maximal stimulation of AC is increased (Martin et al. 1984). Inhibitory ligands (working via Gi) continue to effect inhibition albeit of enhanced basal cyclase in the presence of forskolin. This inhibition is independent of Gs, but requires the presence both of functional Gi (Aktories et al. 1983) and of GTP (Insel et al. 1982).

2.8.4. Other agents used in studies of G-protein function

Many other agents have been used in the study of G-protein function. Table 6 lists the agents used in the studies described

Table 6: Ligands used in studies of adenylyl cyclase and G-proteins: sites of action in the receptor (R)-G-protein(Gs or Gi)-catalytic unit (C) complex.

LIGAND	CONC.	R	Gs	Gi	C	REFERENCE
Mn2+	20mM				•	Ross & Gilman '82
STIMULATORY						
FORSKOLIN	10μM		•		•	see section2.8.3
NaF°	10mM		•	•	•	Insel et al. '82
PGE1	10μM	•				Lester et al. '82
ISOPROTERENOL	100μM	•				Birnbaumer et al'87
GLUCAGON	1μM	•				Birnbaumer et al'87
GppNHp	10μM		•			Houslay et al.'89
VASOPRESSIN	10nM	•				Birnbaumer et al'87
INHIBITORY						
ACETYL CHOLINE#	100μM	•				Hazeki & Ui '81
ANGIOTENSIN II	1μM	•				Cogan '90
ADRENALINE+	100μM	•				Birnbaumer et al'87
GppNHp	10nM			•		Houslay et al'89
NEURO-PEPTIDE Y	1μM	•				Kassis et al.'87

NOTES: NaF°: in practice it is AlF_4 which effects stimulation-the Al^{4+} is present in trace, but adequate amounts in NaF.

ACETYL CHOLINE#: used in conjunciton with the cholinesterase inhibitor PHYSOSTIGMINE (10μM). In addition, the poorly hydrolysed analogue of acetyl choline, CARBACHOLAMINE(100μM) was also used.

ADRENALINE+: adrenaline was used in conjucntion with 10μM propranolol-the net effect is alpha2 receptor activation.

Prime sites of action are indicated. NB 'downstream' consequences of events are not shown-for example if a G-protein activates Gs, catalytic unit will be activated as a consequence.

in this thesis, and indicates the probable site of action in the receptor-G-protein-catalytic unit complex. The methods sections in each chapter indicate which of the agents described have been used in any particular study.

2.8.5. GTPase activity.

GTPase activity is central to the function of G-proteins (figure 2): hydrolysis of GTP permits re-association of α with $\beta.\gamma$ units and thus 'switches off' the activation cycle. GTPase activity can be measured as an adjunct to functional assessment of G-proteins.

The principle of the assay is measurement of ^{32}Pi liberated from [$\gamma^{32}P$] GTP as described by Cassel and Selinger (1976). The reaction mixture (final volume 100µl) contained 0.5µM GTP pH 7.5, 1mM ATP, pH 7.5, 0.1mM App(NH)p, 1mM Ouabain, 0.1M NaCl, 5mM MgCl2, 2mM DTT, 0.1mM EDTA pH7.5, 20mM tris pH 7.5, 100 U creatine kinase, 10mM creatine phoshate and 50,000CPM gamma32P-GTP per assay tube. The reaction was initiated by addition of the membranes (8µg) and was terminated after 20 min incubation at 37^{o}C by placing in an ice bath and adding 0.9ml of a 5% suspension of activated charcoal in 20mM phosphoric acid. the tubes were then centrifuged at 2,500 rpm and 500µl of the supernatant was removed and counted on a Beckman LS1800 scintillation counter. Assays were performed in quadruplicate.

GTPase activities have previously been demonstrated satisfactorily and reproducibly in platelets (Houslay, Bojanic, Gawler, O'Hagan & Wilson 1986). In the preliminary studies for

this thesis GTPase activities were assessed in (normal) platelets. Platelet membranes were incubated as described both in the absence of added ligands ('basal') and in the presence of vasopressin (10nM), PGE1 (10μM) or adrenaline (100μM) (with propranolol (10μM)). The resulting GTPase activties are presented in figure 6.

This approach, however, has limitations; it has, for example, not been possible to demonstrate receptor stimulation of GTPase activity in all membrane preparations even where a G-protein coupled effector mechanism is known to exist. There are several possible explanations for this apparent paradox: 'basal' GTPase activity is a composite of all GTPase activities and the contribution to the total of a single ligand-coupled GTPase activity is likely to be very small depending on the relative preponderance of the G-proteins expressed in the membrane. Furthermore, the Km of G-protein related GTP hydrolysis is low. Thus ligand stimulated GTPase activity could be dwarfed by 'background' enzyme activty. The technique offers no major advantages over indirect assessment of function based on changes in the effector mechanism: it does however, offer the potential to assess G-proteins which are coulped to effectors other than adenylyl cyclase, for example, the phosphoinositide pathway. Although vasopressin did not induce activation of GTPase activity, a number of other ligands, for example thrombin, are known to exert effects through G'p' and can reproducibly and predictably activate GTPase activity.

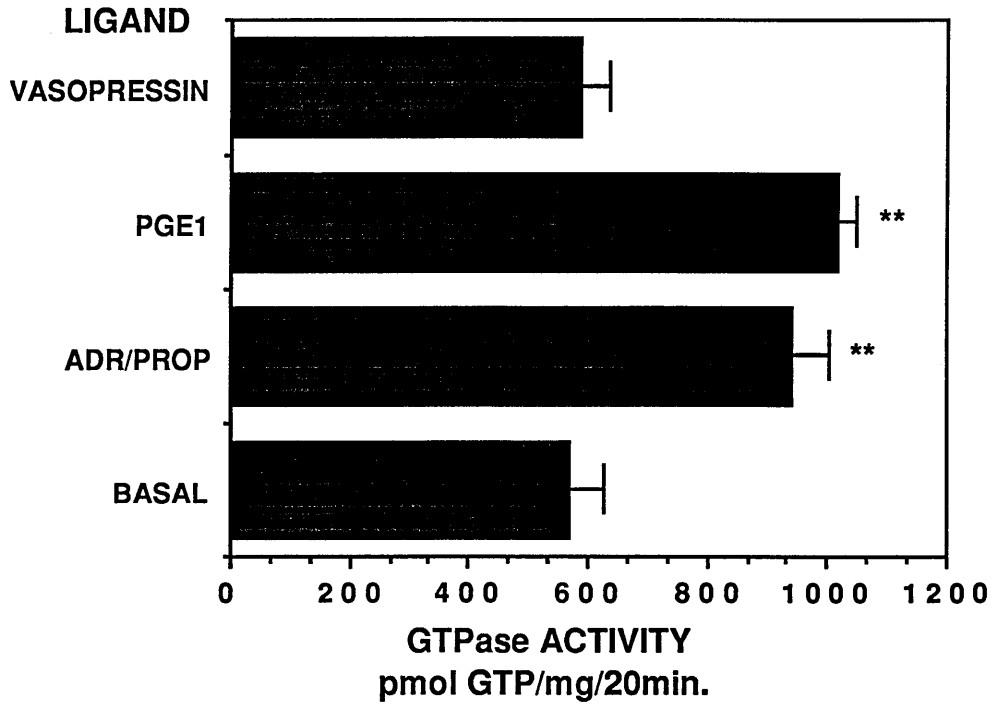


Figure 6. Ligand stimulated GTPase activities in human platelet (expressed as pmol/mg/ min.) (mean ± sem, n=12).
****p < 0.01 v basal.**

Comment: α2 adrenergic mediated AC inhibition, and PGE1- mediated AC stimulation are effected via Gi and Gs respectively. Thus both will activate GTPase activity (activation is intrinsic to G-protein function). Vasopressin may couple to the PPI pathway in platelets via G'p' and thus might be expected to activate GTPase activity; however, activation was not seen (see text for possible explanations).

In the light of these limitations GTPase activities as a means of assessing G-protein function were not studied further.

2.9. Other analytes

Serum urea, electrolytes and 'liver function tests' were determined on a SMAC II multichannel analyser (Technicon Corporation, Basingstoke, U.K.). HbA1 was measured by electro-endosmosis in agar gel, and bands were read at 420nm; coefficient of variation (C.V.) of assay was < 3.6% (ref. range: 4.9-7.8%). Blood glucose concentrations were measured by glucose oxidase method on a Beckman II glucose analyser (Beckman, Yellow Springs, Ohio); C.V. was 2%. Cholesterol (Henry 1974) and triglyceride (Bucolo & David 1973) were measured enzymatically using the Cobas Bio Centrifugal Analyser (Roche Diagnostica, Welwyn City,UK); C.Vs. of these assays were < 5%,reference ranges:3.1-6.5 and <2.1mmol/l respectively.

2.10. Statistics

Statistical analyses were performed on an Apple Macintosh Plus microcomputer using the Statview 512+ statistical programme, Brainpower Inc., USA. Nonparametric data are compared using Mann Whitney U tests and Wilcoxon Signed Ranks tests, correlations were calculated using Spearman Rank Correlations. Parametric data are compared using Student's t tests.

Chapter 3

G-proteins in NIDDM:
a study of platelet plasma membranes.

3.1. Insulin & G-proteins

Humoral regulation is one of several mechanisms controlling G-protein expression and function (section 1.5.) Glucocorticoids (see chapters 6 & 7)(Rodan & Rodan 1986; Chang & Bourne 1987; Ros et al.1989; Saito et al.1989) and thyroid hormones (Milligan et al. 1987) have been shown in experimental animals and cell culture to regulate G-protein subunits differentially. Furthermore, insulin may have a role in regulating Gi function and levels. Loss of tonic Gi-mediated inhibition and attenuation of receptor mediated inhibition of ACA accompany induction of experimental IDDM (streptozotocin and alloxan diabetes in the rat) (Gawler et al. 1987; Bushfield et al. 1990)(see section 1.6.2.); insulin administration restores these changes to normal. Thus insulin may have a direct role in regulating levels and/or function of certain G-protein subunits.

3.2. The role of G-proteins in insulin's action:

There is increasing evidence that G-protein(s) play a role in the action of insulin. Interaction of insulin with its receptors (which possess intrinsic tyrosyl kinase activity) results (directly or indirectly) in a number of changes in intracellular 'second messengers' including DAG, Ca2+ (Denton et al. 1986), cGMP (Denton et al. 1986) and inositol glycans (Low & Saltiel 1988). The activated insulin receptor also exerts an inhibitory influence on glucagon- and catecholamine- mediated elevations

in intracellular cAMP in hepatocytes (isolated membranes and intact hepatocytes) and adipocytes (Johnson, Das, Butcher & Fain 1972; Pilkis, Claus, Johnson & Park1975; Blackmore, Assimacopoulos-Jeannet, Chan & Exton 1979; Heyworth, Wallace & Houslay 1983). Houslay et al. (1983) have shown that insulin inhibits glucagon (1nM) stimulated AC in a dose dependent manner (no effect at 10^{-12}M increasing to maximum of 25% inhibition at 10^{-8}M insulin): the inhibition was shown to be dependent upon the presence of high concentrations of GTP. It is probable that two different mechanisms account for the ability of insulin to reduce cAMP levels- insulin activation of specific membrane bound phosphodiesterases (Heyworth et al.,1983; Houslay et al. 1983) and insulin mediated inhibition of AC (Hepp & Renner 1972; Illiano & Cuatrecasas 1972; Kiss 1978) (although support for this property is not universal (Pilkis et al. 1974; Bitensky, Gorman & Neufeld 1972). Houslay has demonstrated that insulin can activate liver plasma membrane cAMP PDE (Marchmont and Houslay 1980b, 1981; Houslay et al. 1983 a,b) and can also inhibit (hepatic) glucagon stimulated ACA; both effects appear to be mediated by G-proteins (Heyworth et al. 1983; Heyworth & Houslay 1983).

Although the G-protein which has been implicated in these actions of insulin has not been identified, there is some evidence to support the suggestion that G protein(s) may be involved. As described previously, the inhibition of glucagon stimulated AC insulin is a GTP-dependent process (Houslay et al. 1983). Insulin has been shown to stimulate GTPase activity (Gawler & Houslay 1987); Gawler & Houslay demonstrated a cholera toxin-sensitive

GTPase in human platelets , the activity of which was independent of (actually additive to) the GTPase of Gs (activated by PGE1), and of Gi (activated by adrenaline (+propranolol)) and of the putative Gp (activated by vasopressin). This apparent independence from recognised GTPase activities led to the suggestion of involvement of a putative 'Gins'. Despite its role in inhibition of AC it thus appears that the G-protein is not Gi (Gawler, Milligan & Houslay 1988); although it seems probable that another G-protein is directly involved it is not impossible that the AC modifying properties could involve alteration of Gs (?phosphorylation).

3.3. G-proteins and experimental diabetes mellitus

The experimental evidence regarding the possible role of G-proteins in the pathogenesis of diabetes relates to both experimental models of IDDM and NIDDM. This evidence will be reviewed: in both types of diabetes the net intracellular defect is deficiency of functioning insulin.

3.3.1. Experimental models of insulin dependent diabetes mellitus

IDDM can be induced chemically in rats by administration of streptozotocin (STZ) or alloxan. STZ-DM is the most extensively studied rat model of diabetes: STZ is a cytotoxic with specificity for pancreatic ß cells. At doses of 45-65 mg/kg i.p. STZ induces ketotic, insulin dependent diabetes within a few days; alloxan has similar effects. STZ and alloxan diabetes are associated with a number of biochemical changes which include (not surprisingly) marked fall in plasma insulin concentrations,

with rise in blood glucose with ketosis. However, both in addition, are associated with development of apparent insulin resistance in hepatocytes and enhanced ability of glucagon to stimulate AC (Gawler et al. 1987; Bushfield et al. 1990).

Liver

Given its central role in glucose metabolism, the liver is likely to be the most relevant tissue for study of AC and G-proteins in diabetes mellitus. Houslay's group have shown that chemically induced IDDM in the rat is associated with altered G-protein expression and function in hepatocyte membranes (table 3) (Gawler et al. 1987; Bushfield et al. 1990a; Bushfield et al. 1990b); reduced levels of $G_i2\alpha$, $G_i3\alpha$ and $G_s\alpha$ and their corresponding mRNAs have been reported. Changes in G-protein subunits are accompanied by altered G-protein function in hepatocytes; Gawler (Gawler et al.1987) reported loss of Gi function (inferred from AC studies on the basis of loss of GppNHp mediated inhibition (at low concentrations 10^{-8} to 10^{-10} M) and failure of pertussis toxin pretreatment (which inactivates Gi) to enhance glucagon stimulated ACA in diabetic hepatocyte membranes). Reduction of both basal and forskolin stimulated ACA were also reported. In essence, tonic inhibition of AC by Gi is lost while receptor mediated inhibition (via Gi) is attenuated in hepatocyte membranes (Gawler et al.1987) and these changes were consistent with the observed reduction of Gi immunoreactivity. The observed alterations of Gi function and levels could be reversed by insulin administration (Gawler et al.1987).

By contrast, the study of Lynch (Lynch et al. 1989) reported increased AC responses to GTP, NaF and glucagon; these changes were compatible with their evidence of increased Gi levels (by cholera toxin, immunoblotting and reconstitution studies). Lynch found no differences in Gi (ADP ribosylation by pertussis toxin and immunoblotting).

The conflicting results from studies of experimental IDDM demand explanation, and until such time no firm conclusions can be drawn on perturbations of G-protein function. It is of interest, however, that hepatocytes from the obese Zucker rat) which share hepatic insulin resistance with the STZ-DM model have also been shown by Houslay's group to exhibit functional deficiency of Gi (Houslay et al. 1989) raising the distinct possibility that insulin resistance may be related to altered function of Gi.

Adipose tissue

Houslay's group have also shown that chemically induced IDDM in the rat is also associated with altered G-protein function in adipocyte membranes (table 3) (Strassheim et al. 1990); loss of the tonic GTP-dependent inhibitory function of Gi (but not the receptor dependent inhibitory function) was observed in the absence of corresponding changes in the levels (nor of the mRNAs encoding) $G_{i}1\alpha$, $G_{i}2\alpha$, $G_{i}3\alpha$ ($G_{i}3\alpha$ actually increased), $G_{s}\alpha$ and ß subunits.

Vascular tissues- myocardium

The effect of experimental diabetes of between 5 days' and 4 months' duration has been studied. There appears to be a consensus in studies of greater than 8 weeks' duration that basal (Nishio, Kashiwagi, Kida et al.1988; Smith, Pierce & Dhalla 1974; Michel, Cros, McNeill & Serrano 1985) and forskolin (Nishio et al. 1988; Smith et al. 1984), NaF (Smith et al. 1984; Gizsche 1983; Chatelain, Gillat & Waelbroeck 1983), GppNHp (Smith et al. 1984; Gizsche 1983; Chatelain et al.1983), GTP (Srivastava & Anand-Srivastava 1985; Gizsche 1983; Chatelain et al. 1983) stimulated AC is unaltered. In contrast, diabetes of 5 days' duration has been reported to be associated with enhanced basal, but reduced forskolin and NaF stimulated AC suggesting that duration of diabetes may play an important role in determining responsiveness of AC (Srivastava & Anand-Srivastava 1985). The most notable change however, is reduced ß adrenergic stimulated AC (isoproterenol, epinephrine)(reduced by 10-56%) (Nishio et al. 1988; Smith et al. 1984; Srivastava & Anand-Srivastava 1985; Atkins, Dowell & Love 1985; Gizsche 1983; Sundaresan, Sharma, Gingold & Banerjee1984) although support has not been universal (Chatelain et al.1983; Atkins et al. 1985). Most studies which have included assessment of ß receptor number (Nishio et al. 1988; Smith et al. 1984; Sundaresan et al.1984) report substantial reduction of ß receptors (although again support has not been universal (Gizsche 1983)).

In summary, these studies in myocardium in the STZ-diabetes model can be summarised as showing no effect on AC catalytic unit nor on G-protein function. ß adrenergic agonist stimulated

AC is reduced and this appears to be attributable either wholly (Nishio et al. 1988; Smith et al. 1984; Michel et al. 1985) or in part to reduced ß receptor number. Functional uncoupling of ß receptor from catalytic unit may also contribute (Gizsche 1983). There is thus little evidence to support a major change in myocardial G-proteins in established STZ-DM. In contrast, the wide range of abnormalities demonstrated early (after just 5 days' diabetes)(that is reduction of AC responses mediated by both receptor -dependent and receptor-independent mechanisms) could be consistent with alterations of AC catalytic unit (but do not exclude the possibility of at least short lived alterations of G-protein levels or function). Only one study thus far has assessed levels of G-proteins in myocardium in STZ-DM; G-protein transcripts (rather than amounts of protein) were quantified-and no differences were found for $G_{i}1\alpha$, $G_{i}2\alpha$, $G_{i}3\alpha$ & $G_{s}\alpha$ (Bushfield et al. 1990b).

Other tissues

$G_{i}1\alpha$, $G_{i}2\alpha$, $G_{i}3\alpha$ & $G_{s}\alpha$ transcripts have also been measured in skeletal muscle, kidney and brain from STZ-DM and were found to be unchanged compared to non-diabetc controls.

Summary

From the various studies cited it is clear that the reported observations in experimental IDDM occur irrespective of the chemical agent used to induce DM (STZ or alloxan), are tissue specific, and when alterations of G-protein levels have been found (for example, in hepatocyte membranes) these do not invariably reflect altered expression and tend to be selective for

particular G-protein α subunits. Correlation between changes in G-protein function and levels is seen in hepatocyte membranes (Gawler et al. 1987) but is not invariable (c.f. adipocyte membranes-where $G_i2\alpha$ levels and mRNA are unaltered)(Strassheim et al. 1990) raising the possibility that other regulatory processes such as post transcriptional modification of the G-protein subunits, for example by phosphorylation may be operating. (Whether this is effected by insulin (the insulin receptor tyrosyl kinase can phosphorylate the α subunit of Gi (Katada, Gilman, Watanabe, Bauer & Jakobs 1985) or by other hormones such as glucagon (Bushfield et al. 1990) remains unclear).

3.3.2. Experimental & Human NIDDM

Some work has also been done in experimental and human NIDDM. In contrast to IDDM, experimental and human NIDDM are associated with increased insulin levels but resistance to the actions of insulin. Major changes have been reported in ACA in rat liver plasma membranes after 7-9 months of experimental NIDDM. Basal, NaF and glucagon-stimulated ACA is reduced (in the absence of insulin or glucagon receptor changes (Portha et al. 1983). This is consistent with reduced catalytic unit and may be independent of of G-protein change. Identical results have been found in a controlled study of ACA in liver from human (n=7) NIDDM (Arner et al. 1987).

3.4. Study of G-protein function and levels in platelets of NIDDM

3.4.1.Introduction

As tissues which are central to carbohydrate metabolism (including liver) are not readily available from human subjects, platelet plasma membranes were studied in this experiment. Platelets are a homogeneous tissue from which purified plasma membranes can be readily prepared (section 2.3.1.). The justification for use of platelets from diabetics for study is based on two premises: platelets from diabetics exhibit abnormal function (in vitro assessments) and secondly (and not necessarily unrelated to the first) platelets may be implicated in the development of vascular disease. Platelets and endothelium exhibit a functional interaction (for example, platelets release growth factors for endothelium)(Vanhoutte et al.1984) which may be implicated in the micro- and macro-vascular disease which frequently complicate diabetes mellitus. The prime functional change observed in diabetes mellitus is increased sensitivity to agonist induced aggregation, a change which may precede the appearance of clinically evident vascular disease.

The molecular basis of the altered platelet function in diabetes remains to be ascertained. Many possible candidate mechanisms exist including altered membrane signalling systems coupled to adenylyl cyclase and the phosphoinositide pathway. Other possibilities include altered membrane fluidity: reduced membrane fluidity has been reported in platelets from NIDDM.

The purpose of this study was to compare G-protein function (inferrred from adenylyl cyclase studies) and levels in platelets from NIDDM and non diabetic controls.

3.4.2. Subjects:

Subjects: 11 (6 male) untreated, newly presenting NIDDMs (table 7) were recruited from the Diabetic Outpatient clinics of Garnavel General Hospital, Glasgow and Glasgow Royal Infirmary. Diabetes mellitus was diagnosed on the basis of a standard 75g oral glucose tolerance test (OGTT) (fasting glucose 0' NIDDM v CONTROL: 12.0 ± 0.857 v 5.4 ± 0.2 mmol/l; 120' glucose 19.9 ± 1.3 v 5.5 ± 0.2 mmol/l). In addition,17 healthy controls (CON)(12 male) were recruited from among laboratory staff and from spouses of patients. All were studied according to a protocol which had been approved by the Hospital Ethical Committee. Subjects were studied after overnight fast. Blood pressure was measured after 10 min. sitting, with a standard mercury sphygmomanometer: (table 7) BP was normal within both groups, and did not differ between NIDDM and CON. Therafter 80 ml citrated (3.8%) blood were withdrawn: in addition to an OGTT, urea, electrolytes (U & E), liver function tests (LFT), plasma cholesterol, triglyceride were measured: all subjects had normal renal and hepatic function. Platelets rich plasma was obtained from the residue (about 70ml)from which plasma membranes were prepared as described (section 2.3.1.). Membrane aliquots were stored at -80^{o}C.

Membrane protein concentrations were measured by the modified Lowry protocol (described in section 2.6.).

3.4.3. Materials & Methods:

Adenylyl cyclase assays (ACA): ACA were performed according to the Salomon assay protocol as described (section 2.8.1.), but with modifications. Briefly, the reaction cocktail (final volume 100µl) contained creatine phosphate (sodium salt) 5mM, 50U/ml creatine kinase, 50µM ATP, 2mM $MgCl_2$, 0.1M EGTA, 1mM IBMX, 1mM DTT in 50mM triethanolamine HCl buffer pH7.4 and $\alpha^{32}P$ ATP was added to give 2 x 10^6 CPM/assay. The guanine nucleotides, GTP or GppNHp were included at final concentrations of 10^{-5}M and 10^{-7}M respectively. In addition to assessment of basal activities, ACA was measured in the presence of forskolin (10^{-5}M) and PGE1 (10^{-5}M). Gi function was inferred from studies of inhibition of ACA by GppNHp (10^{-7}M) and of inhibition of forskolin and PGE1 stimulated ACA by adrenaline (10^{-4}M), which in the presence of propranolol(10^{-5}M) effects inhibition via the Gi coupled α receptor. The concentrations of the ligands used had previously been shown to be saturating with respect to effect on AC activity. The reactions were started by the addition of membrane (5µg membrane protein) suspended in 10mM tris pH7.4. and tubes were incubated at 37°C for 15 min. Details of termination of enzyme activity and of the subsequent chromatographic separation of reaction products are as previously described (section 2.8.1.). Each assay was run in quadruplicate.

Immunoblotting studies for G-proteins: Immunoblots were performed under denaturing conditions on 10% gels (SDS-PAGE) (section 2.4.1.) as described with antibodies to Gsα, Giα2 and Giα3 subunits (respectively CS1, LE2 and I3B: details of preparation and specificity of these antibodies-see section 2.1.) Amounts of platelet membrane protein loaded to achieve optimal blots were derived from 'dose response' (that is CPM obtained against membrane protein loaded) curves to each of the antibodies; the amounts of protein used (60-100μg per lane) were determined from the linear portions of these curves. The antibody-labelled G-protein bands were visualised using ^{125}I-labelled second antibody; autoradiographs were prepared and were analysed by densitometric scanning using a Bio-Rad video densitometer. In addition, in some instances the ^{125}-I labelled bands were cut out and counted on a gamma counter, with appropriate background controls. Similar results were obtained using the two methods. To facilitate combined analysis of data obtained from different blots, a 'standard' platelet membrane preparation was included in all immunoblots and the counts from the G-protein bands were all expressed relative to standard, the 'standard' being ascribed the arbitary value of 100%.

Table 7: Characteristics of diabetic and control subjects. Data presented as mean ± sem.

	CONTROL	NIDDM	p
n	17 (12M)	11 (6 M)	
age (yr)	44.1 ± 2.8	51 ± 5	
weight (kg)	71.1 ± 1.6	84.1 ± 4.2	p < 0.01
SBP (mmHg)	126 ± 3.6	134 ± 7.2	
DBP	81 ± 2.9	87 ± 2.4	
HbA1 (%)	6.0 ± 0.1	10.2 ± 0.53	p < 0.0001

3.4.4. Results

Results of adenylyl cyclase studies and immunoblots are listed in appendix A. Complete data are not available from all subjects because amount of plasma membrane preparation available was limited.

Adenylyl cyclase studies:

Wide interindividual variation (approx. 4-5 fold) in ACA was noted under 'basal' conditions and irrespective of added ligands within CON and NIDDM study groups. No differences were observed between groups when comparing absolute ACA; for example, 'basal' ACA CON v NIDDM (mean ± sem) 120.2 ± 12.1 v 158.2 ± 20.9 pmol cAMP/mg/15min. Consequently, data were presented as 'fold stimulation' (PGE1 and forskolin) over 'basal'

or in the case of inhibitory ligands as 'percentage inhibition'. PGE1 and forskolin data are presented in fig.7. Fig.8 shows adrenaline (+ propranolol)(i.e. α 2 adrenergic) mediated inhibition of ACA stimulated by PGE1 and forskolin. GppNHp data (expressed as % inhibition of forskolin stimulated ACA) are only available for 7 CON and 6 NIDDM and show no difference between groups (29.7 ± 4.6 v 21.5 ± 4.7%).

Immunoblotting studies:

Fig.9. shows typical blots for Giα2, Giα3 and Gsα. In addition, a section of Coomassie Blue stained gel is included.

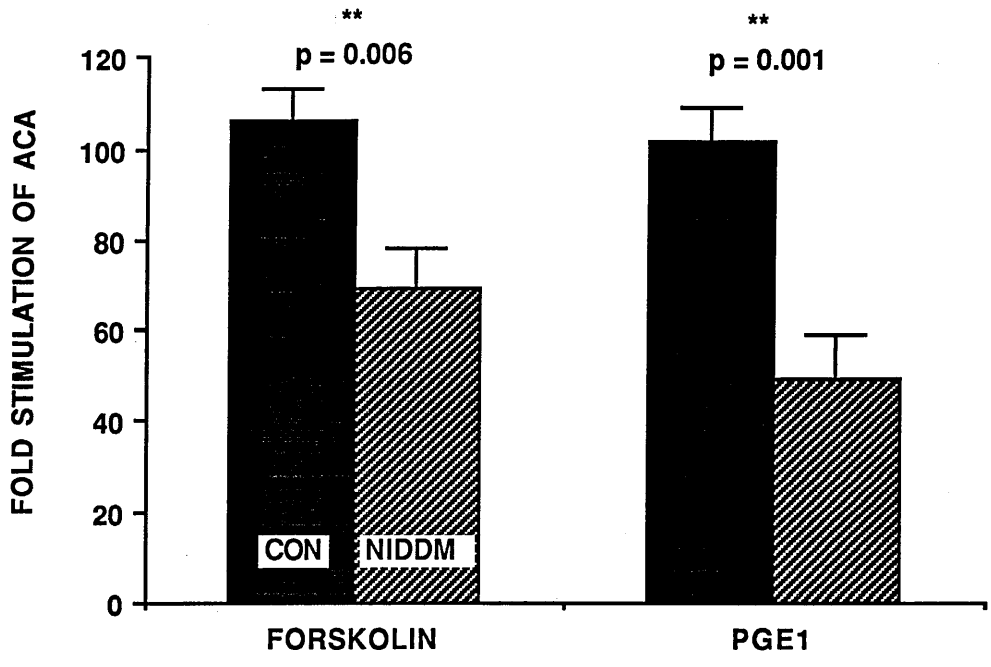


Fig.7. ACA in platelet membranes from CON and NIDDM. Results expressed as 'fold stimulation' of ACA by forskolin and PGE1 over 'basal activity'. (mean ± sem).

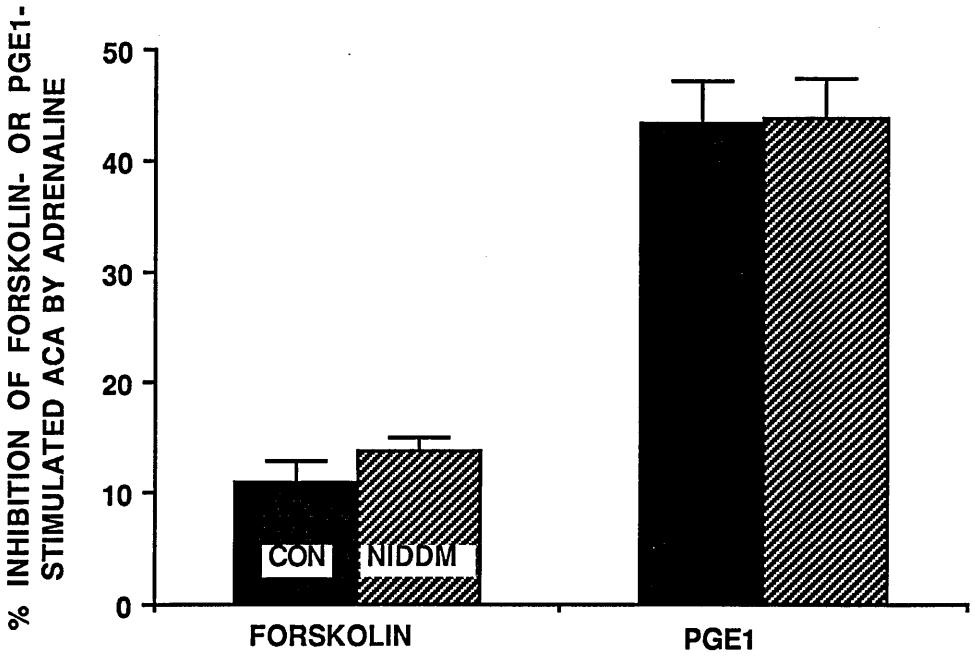


Fig.8. Inhibition of forskolin- and PGE1- stimulated ACA by adrenaline/propranolol (α2 adrenergic receptor activation) in platelet membranes from CON and NIDDM. Results expressed as '% inhibition'. (mean ± sem).

Other data:

Table 8: Correlations (Spearman Rank Correlation) between measures of G-protein function or levels of G-protein subunit and fasting blood glucose concentration. n= total number of data points (CON + NIDDM), z value- statistic used to assess the degree of correlation, with corresponding 'p value'.

CORRELATE	n	z value	p
'fold stim.' by forskolin	24	-2.535	0.01
'fold stim.' by PGE1	23	-2.461	0.014
Gs	17	-2.671	0.008
Gi2	22	-2.629	0.008
Gi3	19	-2.299	0.003

Other correlations were observed between forskolin-stimulated ACA and plasma cholesterol concentration (n=24, z=-2.784, p=0.005), between PGE1 stimulated ACA and plasma cholesterol concentration (n=23, z=-2.752, p=0.006) and between levels of Giα2 and body weight (kg)(n=22, z=-2.22, p=0.028).

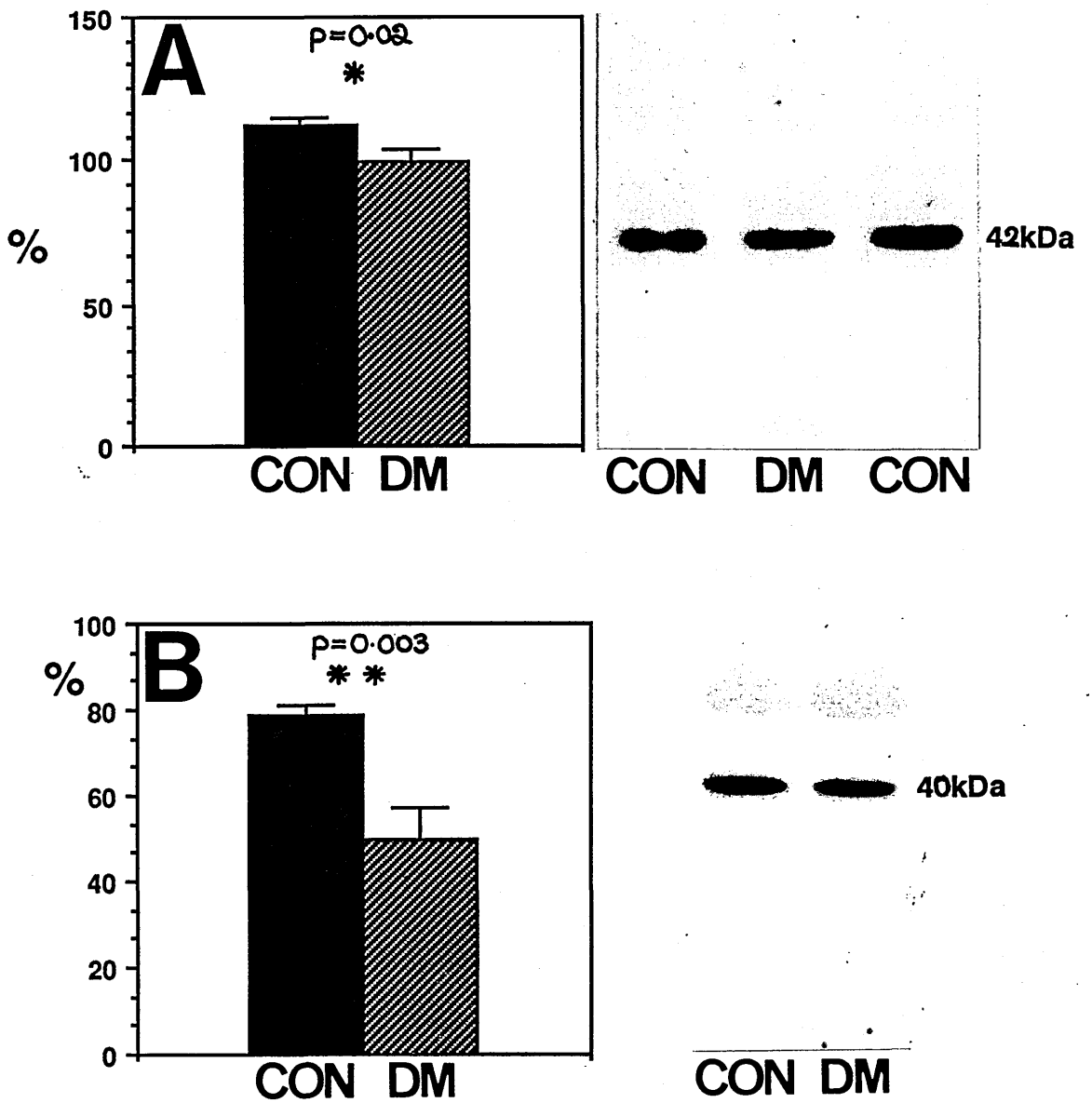
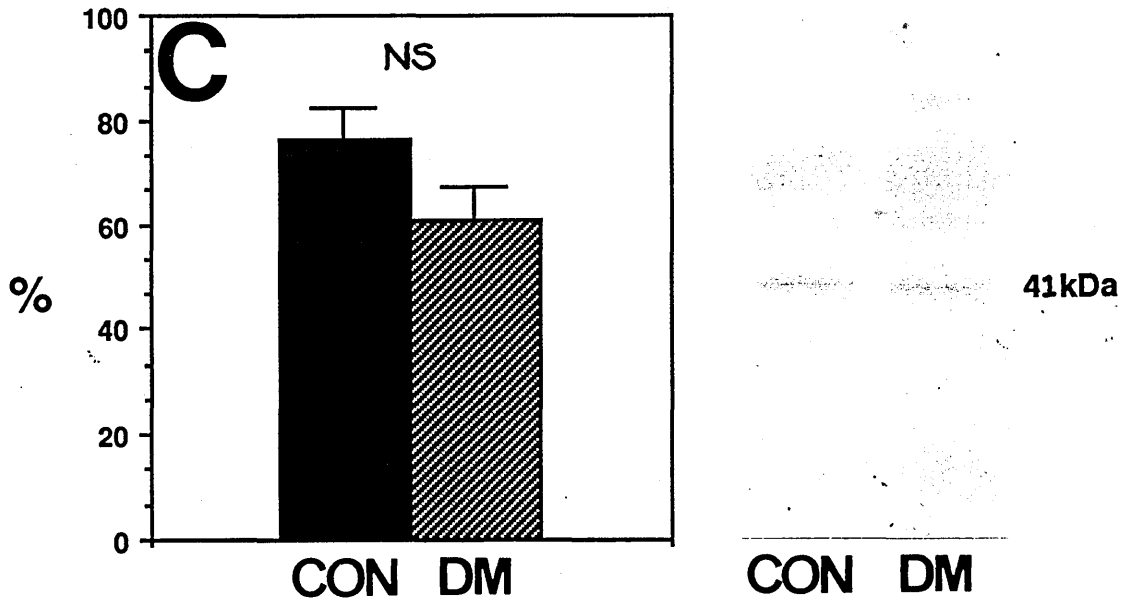
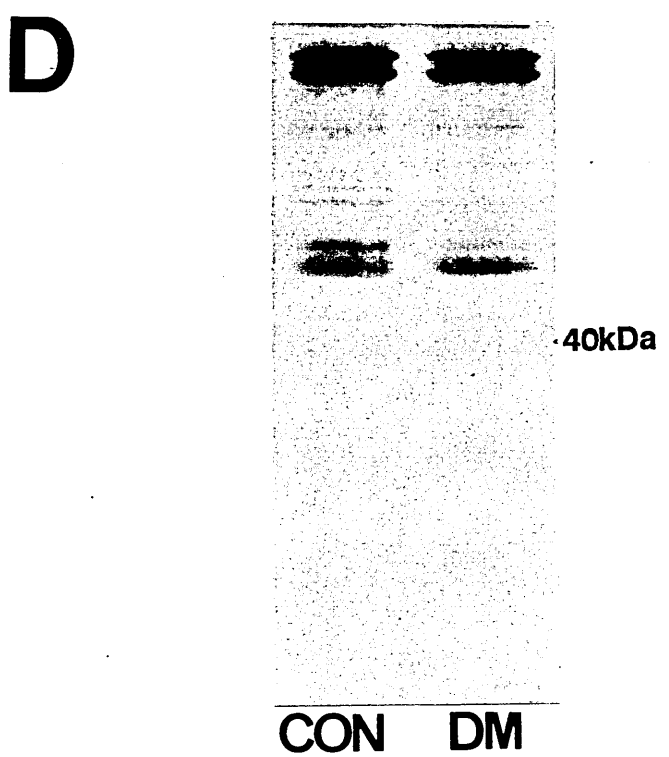


Fig. 9. Immunoblotting studies in platelet membranes from NIDDM and controls showing typical blots (R panel) and relative abundance (graph-L panel) (derived from densitometric scanning of blots) of (A) Gsα, (B) Giα2 and (C) Giα3.
A panel of Coomassie Blue stained gel is included (D).


C
NS
100
80
60
40
20
0
%
CON
DM
41kDa
CON
DM


D
40kDa
CON
DM

3.4.5. Discussion

α subunits of Gi2, Gi3 and Gs were identified in our platelet plasma membranes. ß subunits are also present (section 2.4.2. & Chapter 4) but were not assayed in this study. Lower levels of all three α subunits were identified in membranes from diabetic subjects compared to a group of controls of similar age; statistically significant reductions were observed in Gsα and Giα2 ($p=0.02$ and $p=0.003$ resp). This observation in human NIDDM, a condition characterised by insulin resistance (with associated hyperinsulinaemia) is similar to previous findings in hepatocyte membranes from the streptozotocin-induced diabetic rat model of insulin-dependent diabetes mellitus (Gawler et al. 1987; Bushfieldet al. 1990): tissue specific alterations of G-protein expression and mRNA are seen in STZ-DM (Griffiths et al. 1990; Bushfield et al. 1990). These findings, however, contrast with those in adipocytes where Gi3 has previously been shown to be increased (Strassheim et al.1990) and with a number of other vascular and non-vascular tissues where no changes in G-protein transcripts have been identified (see section 3.3.1). The observed reduction of G-protein levels in human platelets from NIDDM and hepatocytes from STZ-DM are consistent with the possibility that insulin may have a role in the regulation of G-proteins: despite elevated plasma levels of insulin in NIDDM, there will be a net intracellular deficiency of insulin, while STZ-DM is characterised by insulopenia due to pancreatic destruction. This hypothesis is supported indirectly by several correlations (table 8). Levels of Giα2, Giα3 and Gsα have all been shown to correlate inversely with fasting blood glucose concentration.

Functional characteristics of the various G-protein species remains to be fully elucidated. Gsα couples to and stimulates AC catalytic unit, but may also interact with dihydropyridine sensitive Ca^{2+} channels. There is increasing evidence that Giα2 mediates inhibition of AC (Bushfield et al. 1990; McKenzie & Milligan 1990; Simonds, Goldsmith, Codina, Unson & Spiegel (1989) while it appears that Giα2, Giα3 and Gsα may all share a role in the regulation of K^+ channel function (Birnbaumer, Abramowitz & Brown 1990). Current studies of G-protein function are restricted to indirect assessment of Giα2 and Gsα inferred from AC studies. No evidence of altered function of Giα2 was observed on the basis of studies with GppNHp (which effect AC inhibition directly via Gi) and with adrenaline (in conjunction with propranolol)(which binds to the α2 receptors which are coupled to AC catalytic unit via Gi). The study of GppNHp was limited by the small numbers studied (n=6 CON, n=6 NIDDM). As also noted in the study of human essential hypertension (chapter 4), marked interindividual variation in AC was observed under 'basal' conditions (and even in the presence of inhibitory and stimulatory ligands) in both groups. When forskolin and PGE1 responses were expressed as 'fold stimulation' over 'basal' cyclase, however, the NIDDM platelets exhibited significantly lower cyclase activities. Forskolin functions through catalytic unit and probably through Gsα while PGE1 receptors couple to AC catalytic unit via Gsα. Differences in activities assessed in the presence of these agents may reflect altered amounts of AC catalytic unit and/or reduction in net Gsα effect. Reduced Gsα effect could be due to reduction in Gsα levels or possibly alteration in Giα2: Gs ratio. Either possibility is consistent with

the immunoblotting studies. It was of interest that the extent of elevation of the levels of ACA achieved with forskolin and PGE1 correlated with fasting blood glucose concentration (table 8).

Although the findings of altered G-protein levels and function are consistent and could possibly be explained through altered intracellular insulin concentrations, other explanations exist. Artifactual contribution from erythrocyte membrane contamination in our platelet plasma membrane homogenate has largely been excluded by subjecting membrane preparations to analysis with a monoclonal antibody specific for (a 93 kDa species) Band 3 glycoprotein of human erythrocytes (kindly donated by Dr M.J. Tanner, Biochemistry, Bristol). Furthermore as interpretation of our AC assay results and G-protein immuno-blots are dependent upon correction for the amount of membrane protein loaded (identical amounts of study and control sample protein were used in all studies), intrinsic differences in membrane protein content could bias the results; however, it has previously been shown that diabetes is not associated with altered platelet membrane protein content (Winocour, Bryszweska, Watala et al. 1990). Altered forskolin and PGE1 stimulated ACA could result from altered membrane fluidity; membrane fluidity of diabetic platelets has previously been shown to be lower than normals (Winocour et al. 1990)and platelet ACA has previously been shown to be extremely sensitive to changes in membrane fluidity (Spence & Hously 1989). Furthermore the current studies do not preclude the possibilty that PGE1 receptor number could be altered in NIDDM and contribute to the observed lower levels of PGE1 stimulated

ACA in the diabetic group. However, such changes could not explain all the findings.

The study groups were similar with respect to age and blood pressure, but as might be anticipated, the NIDDMs were heavier (84.1 ± 4.2 v 71.1 ± 1.6 kg, $p<0.01$) and had higher plasma concentrations of cholesterol (6.6 ± 0.5 v 5.3 ± 0.2 mmol/l, $p<0.005$) and triglyceride (1.6 ± 0.1 v 0.9 ± 0.1 mmol/l, $p<0.0006$). Previous studies of a possible role of G-protein dysfunction in obesity in experimental models (obese Zucker rat: (Houslay et al. 1989; and ob/ob mouse: Begin-Heick 1985; Greenberg et al.1987) and have produced conflicting evidence. However, Ohisalo & Milligan (1989) studied adipocyte membranes obtained from obese humans and showed concomitant reduction of Gsα and Giα; they concluded, however, that the relative amounts of both were unaltered. Thus changes associated with obesity per se may have contributed to the observed differences between NIDDMs and controls. Obesity, like NIDDM, is a state of insulin resistance and could possibly share G-protein perturbations with diabetes mellitus and other conditions associated with insulin resistance. Thus if differences in weight contribute in some way to the differences in G-protein levels and ACA seen in the NIDDM (v CON) it may be inappropriate to regard obesity as an independent disease process. Some regard obesity and NIDDM (along with other entitites such as essential hypertension and hyperlipidaemia) as manifestations of a syndrome whose component diseases are characterised by insulin resistance (Reaven 1988). Consistent with this theory are correlations (inverse) between level of Giα2 and body weight ($n=22$, $z=-2.22$,

p=0.028), and between cholesterol and forskolin stimulated ACA (n=24,z=-2.784,p=0.005) and PGE1 stimulated ACA (n=23, z= -2.752, p=0.006).

In summary, platelets from subjects with NIDDM have been shown to have significantly reduced plasma membrane levels of $G_S\alpha$ and $G_i2\alpha$. PGE1 and forskolin stimulated ACAs were lower in diabetic platelets, while assessment of inhibitory G-protein function by GppNHp (limited interpretation due to small numbers) and via $\alpha 2$ receptor activation failed to show any differences. It is possible that reduced levels of AC catalytic unit may contribute to the differences in ACA although relative changes amongst the G-protein subunits are more likely to contribute to the reduced PGE1 and forskolin stimulated ACA seen in platelets from diabetics. A previous study of ACA in livers from NIDDM concluded that AC catalytic unit was reduced, and that this observation was likely to be independent of G-protein changes (Arner et al. 1987). We have shown unequivocally that levels of $G_S\alpha$ and $G_i2\alpha$ are reduced. These changes are rather similar to those reported in hepatocyte membranes from experimental IDDM (Gawler et al. 1987; Bushfield et al. 1990a; Bushfield et al. 1990a) but contrast with those in other tisses (table 3). The possibility exists that insulin resistance which is common to hepatocytes from STZ-DM (Bushfield et al. 199b) and to human NIDDM may contribute to the altered levels of G-proteins seen in this study.

The observed changes in ACA which may be due to altered G-protein levels (and/or altered levels of AC catalytic unit) are

likely to have implications for platelet function in NIDDM: cAMP has previously been shown to exert an inhibitory influence over platelet function (Mills 1982). It is thus probable that reduced cAMP generation in response to stimulatory ligands will be associated with a greater tendency to platelet activation and possibly contribute to the vascular disease which accounts for substantial morbidity in the diabetic population.

Chapter 4

G-proteins in Essential Hypertension: a study of platelet plasma membranes.

4.1. Introduction to Chapters 4 & 5:

4.1.1.Essential hypertension and diabetes mellitus.

Hypertension is not one distinct pathophysiologic entity, but may arise from a number of different aetiologies; these include essential hypertension (EH) (with diastolic and/or systolic hypertension), and hypertension which is secondary to a surgically treatable cause, for example, Cushing's syndrome or renal artery stenosis. However, EH is the most prevalent cause accounting for about 90% of hypertensives. Although its true pathophysiology remains uncertain, it is clear that there is a major genetic component.

Epidemiological studies have established strong links between EH and insulin resistance / hyperinsulinaemia. A relationship between insulin concentration and blood pressure has been demonstrated both in the obese (Christlieb, Krolewski, Warram & Soeldner1985; Rose, Yalow, Schweitzer & Schwartz 1986; Manicardi, Cameiilin, Bellodi et al.1986) and non-obese (Modan, Halkin, Almog et al. 1985). Hypertensive subjects are less glucose tolerant than normotensive controls (Voors, Radhakrishnamurthy, Srinavasas, Webber & Berenson 1981; Stamler, Rhomberg, Schoenberger et al. 1975; Florey, Uppal & Lowy 1976; Jarrett, Keen, Mccartney et al. 1978) and untreated hypertensives exhibit higher insulin levels than normotensives (Lucas, Estigarribia, Darga & Reaven 1985; Singer, Godicke,

Voigt, Hajdu & Weiss 1985; Modan, Halkin, Almog et al. et al. 1985; Manicardi, Camellini, Belloidi, Coscelli & Ferrannini 1986). Ferrannini and colleagues(Ferrannini, Buzzigoli, Bonadonna et al.1987) have provided the best evidence of an association between hyperinsulinaemia and hypertension with their demonstration that EH in the non-obese is an insulin resistant state: in their controlled study of non-obese essential hypertensive subjects with moderate to severe untreated hypertension, marked impairment of glucose uptake into the peripheral tissues was demonstrated - consistent with insulin resistance. Reduced glycolysis and glycogen synthesis were believed to account for the reduced tissue uptake of glucose. The insulin resistance in this group of EH appeared specifically to affect glucose metabolism and was thought to be located in the peripheral tissues-most likely in skeletal muscle. Although such observations do not establish a causal relationship between insulin resistance and hypertension, this possibility remains. Insulin resistance is likely to be a graded phenomenon-both quantitatively and qualitatively -although the pathophysiology at receptor or post receptor levels is not clear. Hyperinsulinaemia per se probably does not directly result in hypertension but may contribute indirectly through enhanced renal sodium reabsorption (Miller and Bogdonoff 1954), increased catecholamine secretion (Rowe, Young, Minaker et al. 1981), increased Na/K-ATPase activity (Ng and Hockaday 1986) or through its growth promoting action on smooth muscle (Lever 1986). Whether hyperinsulinaemia associated alteration of G-protein function can be added to this list will be assessed in chapters 4 & 5 of this thesis.

Insulin resistance and hyperinsulinaemia are not unique to EH, but are also found in obesity (Olefsky, Kolterman & Scarlett 1982) and NIDDM (DeFronzo & Ferrannini 1982). This has led to the concept of the existence of a syndrome ('syndrome X' (Reaven 1988; Foster 1989)) which includes the triad-NIDDM, obesity and EH but which includes other abnormalities, for example of plasma lipids. As the studies in hypertension described in this thesis have arisen from studies in diabetes and attempt to cast further light on the possible links between hypertension and diabetes the clinical links between these will be described in some detail.

The prevalence of hypertension is increased in diabetes: 40% of males and 53% females presenting with NIDDM (age 25-65yrs) are hypertensive (BP>160/95) (United Kingdom Prospective Diabetes Study. 1985). The prevalence of hypertension in IDDM appears to be even higher after age adjustments (National Diabetes Data Group. 1985)). Hypertension causes vascular disease and is the major risk factor in IDDM (Christlieb et al.1981) contributing to both microvascular (nephropathy) and macrovascular disease (ischaemic heart disease and cerebro-vascular disease) which account for the majority of deaths in IDDM (Feingold & Siperstein1985) and NIDDM (Dorman, Laporte, Kuller et al. 1984).

Possession of genetic markers for risk of developing EH (as inferred from parental blood pressure recordings (Viberti et al. 1987), family history of hypertension (Krolewski, Canessa, Warram et al.1988) and assessment of red blood cell (rbc)

sodium-lithium countertransport activity (Na-Li C A) (Krolewski et al.1988; Mangili, Bending, Scott et al. 1988) has been shown to be associated with increased prevalence of diabetic nephropathy (the relationships are stronger in subjects with poor glycaemic control(Krolewski et al.1988)) -a major cause of morbidity and mortality in diabetics although support for this association has not been universal. Family clustering of nephropathy in diabetic siblings has recently been noted, and although consistent with involvement of genetic factors such as hypertension, the role of common environmetal factors cannot be excluded (Seaquist, Goetz, Rich & Barbosa 1989). Whatever its role in the pathogenesis of nephropathy in the diabetic, hypertension is an invariable accompaniment of nephropathy (generally arising concurrently with the onset of microalbuminuria) and treatment of hypertension may result in attenuation of the rate of decline of renal function. (Mogensen 1982; Parving, Andersen, Smidt & Svendsen1983; review-McLellan & Connell 1988, 1989).

Diabetes mellitus may occur more frequently in EH than expected although this association has been complicated by possible diabetogenic effects of antihypertensive medication such as thiazide diuretics.

4.1.2. The role of adenylyl cyclase in blood pressure regulation

AC is a ubiquitous membrane-bound effector mechanism which has been studied extensively in hypertension. cAMP is one of

several 'second messengers' which control the contraction and relaxation of vascular smooth muscle; others include cGMP, inositol phosphates and Ca2+. However, aside from their key roles in determining vascular tone (Bar 1974), cyclic nucleotides are effectors in many other processes which govern blood pressure: they mediate inotropic and chronotropic effects in the myocardium (Katz, Tada & Kirchberger 1975; Keely, Lincoln & Corbin 1978), have key roles in the endocrine control of blood pressure (in particular by mediating the release and functioning of catecholamines (Ramanathan & Shibata 1974; Williams & Dluhy 1978) and renin secretion (Hauger-Klevene 1970; Winer, Chokshi & Kalkenhorst 1971). In vascular smooth muscle cAMP is not the only cyclic nucleotide with a major role in regulating vascular tone (Triner, Nahas, Vulliemoz et al. 1971); cGMP is also important (Goldberg, O'Dea & Haddox 1973; Amer 1974). Both cAMP and cGMP probably effect these changes through alteration of intracellular calcium level. In practice, the relative balance of concentrations of cAMP and cGMP is likely to be more important in governing vasomotor tone than either individually.

Consideration of cAMP in isolation would be inappropriate without appreciation of the AC system consisting of receptor/G-protein/ catalytic unit which is responsible for synthesis of cAMP and of the mechanisms by which cAMP couples to processes effecting vasomotor relaxation : cAMP effects changes via cAMP-dependent protein kinases (Greengard 1978; Cohen 1982) which phosphorylate target proteins. The effects of cAMP are in turn modulated at several steps in the chain of events;

enzymes can dephosphorylate the phosphorylated proteins (Cohen 1982) while cAMP itself is hydrolysed by cAMP phosphodiesterase. The work described in this thesis is based on measurement of ACA and thus of cAMP generated (as a means of assessing the dual regulation of the enzyme by G-proteins (Gs and Gi)) however, the existing evidence regarding the role of cyclic nucleotides in hypertension has been reviewed as a basis for discussion of the potential role of G-proteins in the abnormalities which have been reported.

4.2. G-proteins in human essential hypertension

Platelets are cell fragments which subserve the vital role of haemostasis through their abilities to aggregate (necessitating alteration of shape) and secrete ('degranulation'). Both processes require mutual interaction of cAMP and Ca^{2+}; cAMP and Ca^{2+} act antagonistically in the process of alteration of platelet shape while they act synergistically in platelet secretion. At a (simplified) biochemical level cAMP promotes platelet microsomal uptake of calcium (see review Tomlinson et al. 1985), while calcium (directly or through calmodulin) has been shown capable of inhibiting AC (Rodan & Feinstein 1976) and of activating cAMP phosphodiesterase (Cheung et al. 1975); in addition a dependence on calcium has been reported for hormone activation of many AC systems (Bradham and Cheung 1980) and this may be true for PGE1 activation of platelet adenylyl cyclase (Tremblay & Hamet 1984).

The usefulness of the platelet for studies in hypertension may extend beyond the biochemistry and physiology of clotting: resort has been made to platelets as a readily available source of homogeneous plasma membranes to study the mechanisms and effects of hypertension because of the lack of availability of human vascular tissue. The validity of the use of platelets is debatable but has previously been justified (Erne, Resink, Burgisser & Buhler1985) on the basis of several functional similarities to smooth muscle (Resink, Burgisser & Buhler 1986); platelets and smooth muscle share a number of receptors (including vasopressin and prostaglandin receptors), they share an adenylyl cyclase linked $\alpha 2$ adrenergic receptor system and, further-more, both exhibit hormonal regulation of AC by cAMP and calcium. Platelets show contractile properties on activation which are similar to the contraction of smooth muscle: in vivo there is a functional interaction between platelets and vascular smooth muscle as platelets are the source of a number of growth factors for smooth muscle (including PDGF) and contribute to arteriolar spasm through the release of vasoactive substances on activation by damaged endothelium (Vanhoutte et al. 1984) (which is more likely in vessels subject to hypertension).

Altered platelet function *in vitro* has been reported in human essential hypertension (Lentini and Bologna 1974; Poplawski, Skorulska & Niewiarowski 1968; Hamet, Skuherska, Pang & Tremblay1985) and these changes may relate to reported alterations of ACA (Brodde, Stuka, Dernuth et al. 1985; Erne et al. 1985; Resink et al. 1986). Similar functional changes in

platelets have been found in experimental hypertension (SHR) (Hamet, Fraysse & Franks 1978; Baudouin Legros, Dard & Guicheney 1986) where there is evidence to suggest that associated AC changes (Hamet et al 1978; Tremblay & Hamet 1984; Dard, Baudouin-Legros & Meyer 1987) may be at least partly due to altered G-protein function (Coquil and Brunelle 1989).

The purpose of this study was to examine whether platelets from untreated essential hypertensive subjects exhibit abnormal adenylyl cyclase activities and whether any such changes could be attributed to altered G-protein levels or function.

4.2.1.Subjects: 18 untreated EH subjects (table 9), recruited consecutively from the medical outpatient department and 21 healthy controls recruited from among laboratory staff and from spouses of patients were studied according to a protocol which had been approved by the Hospital Ethical Committee. Subjects were studied after over-night fast; following blood pressure measurement (after 10min. sitting, with a standard mercury sphygmomanometer), 80 ml citrated (3.8%) blood were withdrawn. Urea, electrolytes (U & E), liver function tests (LFT), plasma cholesterol, triglyceride were measured: all subjects had normal U&E and LFT. Platelets rich plasma was obtained from the residue (about 70ml)from which plasma membranes were prepared as described (section 2.3.1). Membrane aliquots were stored at -80^{o}C.

Membrane protein concentrations were measured by the modified Lowry protocol (described in section 2.5.).

It was not possible to complete both ACA and immunoblotting studies in all subjects due to limited quantities of platelet plasma membrane.

4.2.2.Adenylyl cyclase assays (ACA): Platelet membrane preparations from EH (n=14) and controls who had been matched as far as possible for age and sex were paired for assay. The characteristics of these subjects are summarised in table 9A. Control subjects were slightly but significantly younger than EH (40±3.1 v 47.1±4.1y, p=0.04). ACA were performed according to the Salomon assay protocol as described (section 2.8.1.). Briefly, the reaction cocktail (final volume 50µl) contained creatine phosphate (sodium salt) 5mM, 50U/ml creatine kinase, 25mM tris-acetate,pH 7.6, 5 mM magnesium acetate, 0.5mM ATP, 0.05 mM cAMP, 1mM DTT, BSA 0.1mg/ml and α^{32}P ATP was added to give 2 x 10^6 CPM/assay. The guanine nucleotides,GTP or GppNHp were included at final concentrations of 10^{-5}M and 10^{-7}M respectively. Apart from basal activities all studies (inhibitory and stimulatory) were performed in the presence of 10^{-5}M forskolin. Adrenaline, propranolol and PGE1 were used at final concentrations of 10^{-4}M,10^{-5}M and 10^{-5}M respectively, which had previously been shown to be saturating with respect to effect on AC activity. The reactions were started by the addition of membrane (5µg membrane protein) suspended in 10mM tris pH7.4. and tubes were incubated at 37°C for 15 min. Details of

termination of enzyme activity and of the subsequent chromatographic separation of reaction products are as previously described (section 2.8.1.). Each assay was run in quadruplicate. The interassay coefficient of variation of this assay was about 10%.

4.2.3. Immunoblotting studies for G-proteins:

Hypertensive (n=15)(table 9B) and control subjects were selected as before (although the control subjects were not only slightly younger (43.3±2.6 v 50.3± 3.7, p=0.01) but also slightly lighter (69.1±2.2 v 79.3±3.1kg,p=0.02). 10 pairs of subjects had both ACA and immunoblotting studies performed.

Immunoblots were performed under denaturing conditions on 10% gels (SDS-PAGE) (section 2.4.1.) as described with antibodies to Gsα, Giα2 and ß subunit (respectively CS1, SG1 and BN1: details of preparation and basis of use of these antibodies-see section 2.1.). Amounts of platelet membrane protein loaded to achieve optimal blots were derived from 'dose response' (that is CPM obtained against membrane protein loaded) curves to each of the antibodies; the amounts of protein used were determined from the linear portions of these curves. 100μg,100μg and 200μg membrane protein were loaded respectively for Gsα, Giα2 and ß subunit studies. The antibody-labelled G-protein bands were visualised using ^{125}I-labelled second antibody; following autoradio-graphy the bands were carefully cut out and counted on a gamma counter, with appropriate background controls. To facilitate combined analysis of data obtained from different blots, a 'standard' platelet

Table 9. Characteristics of essential hypertensive and control subjects.

	CONTROL	HYPERTENSIVE	SIGNIFICANCE
STUDY A			
n	14	14	
SEX	12 M	10 M	
AGE	40 ± 3.1	47.1 ± 4.1	P = 0.04
WEIGHT	69.3 ± 2.0	75.7 ± 2.8	N.S.
SBP	129.7 ±5.0	162.1 ± 5.6	P = 0.0007
DBP	80.3 ± 1.9	105.8 ± 3.1	P=0.0001
+VE FH HBP	5	5	
STUDY B			
n	15	15	
SEX	13 M	11 M	
AGE	43.3 ± 2.6	50.3 ± 3.7	P = 0.01
WEIGHT	69.1 ± 2.2	79.3 ± 3.1	P = 0.02
SBP	129.5 ± 4.6	160.6 ± 4.8	P = 0.0001
DBP	80.9 ± 2.4	102.4 ± 2.4	P=0.0001
+VE FH HBP	4	4	

membrane preparation was included in all immunoblots and the counts from the G-protein bands were all expressed relative to standard, the 'standard' being ascribed the arbitary value of one.

4.3. Results:

Adenylyl cyclase assays:

Data are presented in appendix B. Overall no differences were seen in adenylyl cyclase activities on comparing essential hypertensive membranes with those from normotensive controls under basal conditions (fig. 10). Similarly no differences were seen in the response to forskolin even when subject to Gi mediated inhibition (via $\alpha 2$ receptor activation or independently of receptor using GppNHp) and further stimulation by Gs (PGE1). However, as wide interindividual variations in cyclase activities were seen basally (5 fold variation) and in the presence of forskolin alone (3-4 fold variation), the data obtained in the presence of forskolin were expressed as 'percentage change' due to ligand from cyclase activity in the presence of forskolin alone (see fig. 11). Greater stimulation of adenylyl cyclase activity was observed in membranes from essential hypertensives (88.8 ± 3.8%) than control membranes (72± 6.5%) ($p=0.018$).

Immunoblots:

Data are presented in appendix C. The data obtained are shown in figs.12,13 & 14 . No differences were seen in blots exposed to antisera against$G_{s\alpha}$, $G_{i\alpha 2}$ and ß subunits. Even when the results were expressed as a ratio ($G_{i\alpha 2}$: $G_{s\alpha}$) no differences were seen (2.24 ± 0.44 v 1.75 ± 0.29). NB $G_{s\alpha}$ represented by 42kDa band (CS1 ab) (fig.12). SG1 ab identified 2 bands (fig.13), $G_{i\alpha 2}$ (band X) of 40kDa and a (?) phosphorylated variant of higher molecular weight (band Y). This band remains to be fully characterised.

When considering all subjects (hypertensives and controls) weak inverse correlations were found between plasma cholesterol

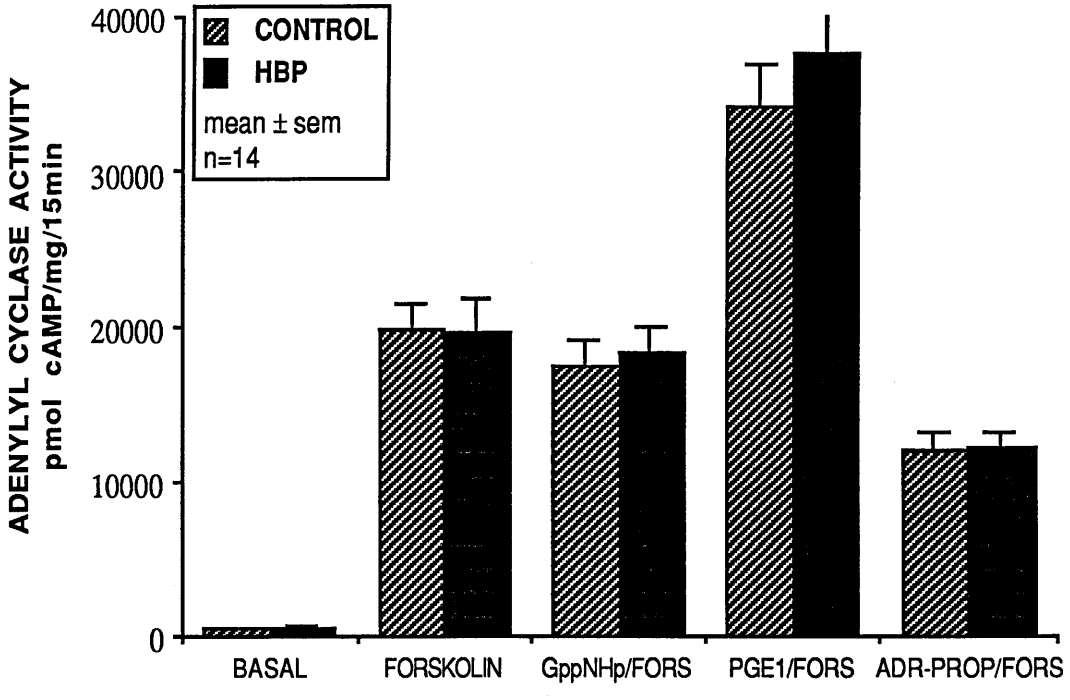


Fig. 10 Adenylyl cyclase activities in platelets from essential hypertensives and normal controls. ACA was measured under basal conditions and in the presence of forskolin ± a number of other ligands-indicated along abscissa.

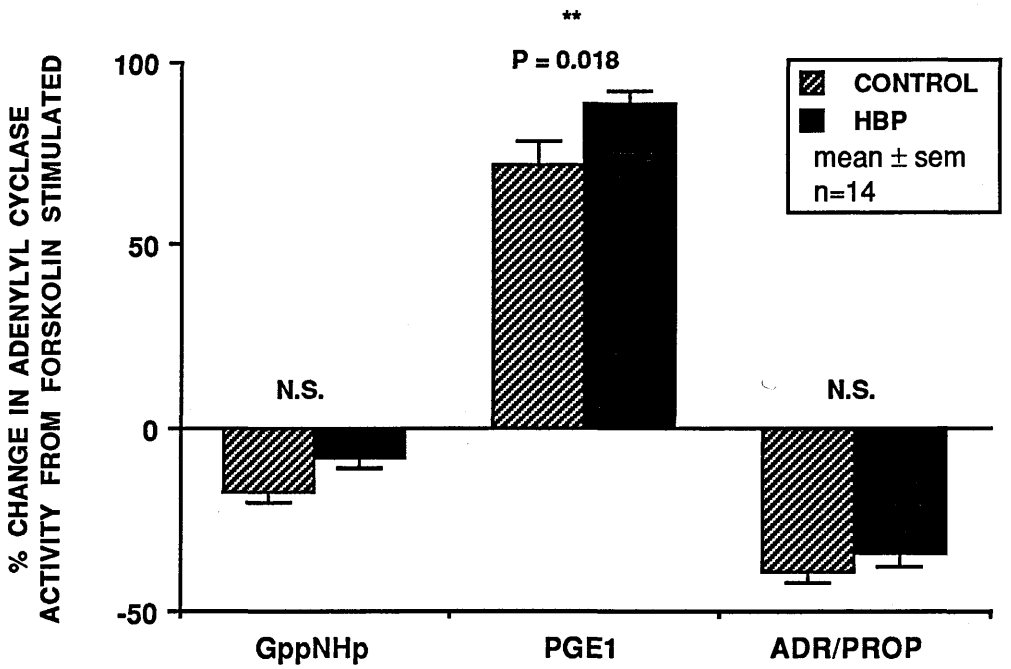


Fig.11. Adenylyl cyclase activities in platelets from essential hypertensives: results expressed as % change over forskolin-stimulated 'basal'.

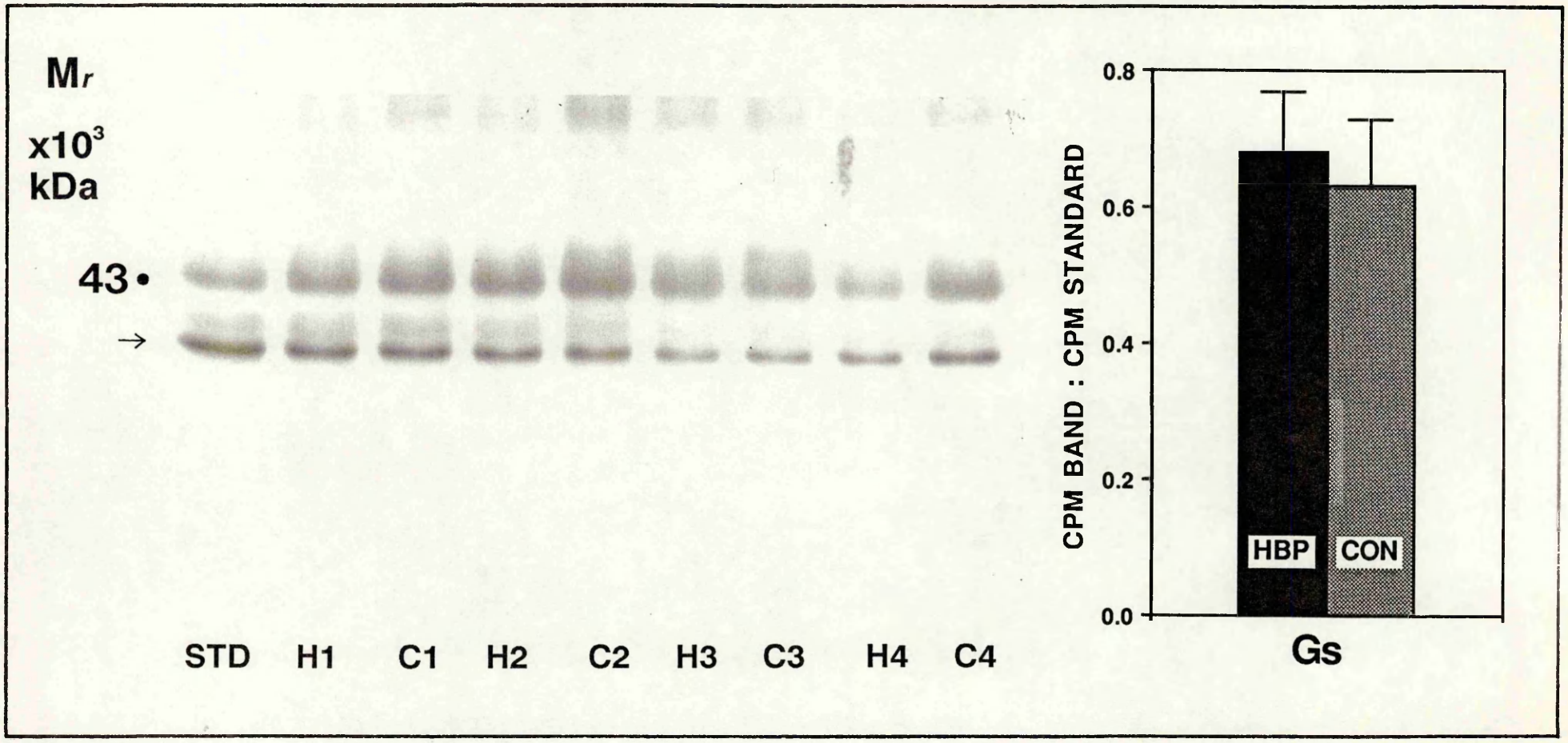


Fig.12. Platelet membrane Gsα subunit immunoblot data from essential hypertensive (HBP) (n=15) and control (CON) (n=15) subjects: (L panel-typical autoradiograph, with 4 CON (C),4 HBP (H) membrane preps., MW= molecular weight markers, STD= standard membrane prep.. R panel represents graph of cpm obtained by counting bands on gel, expressed relative to STD prep. for HBP and CON groups (mean ± sem)).

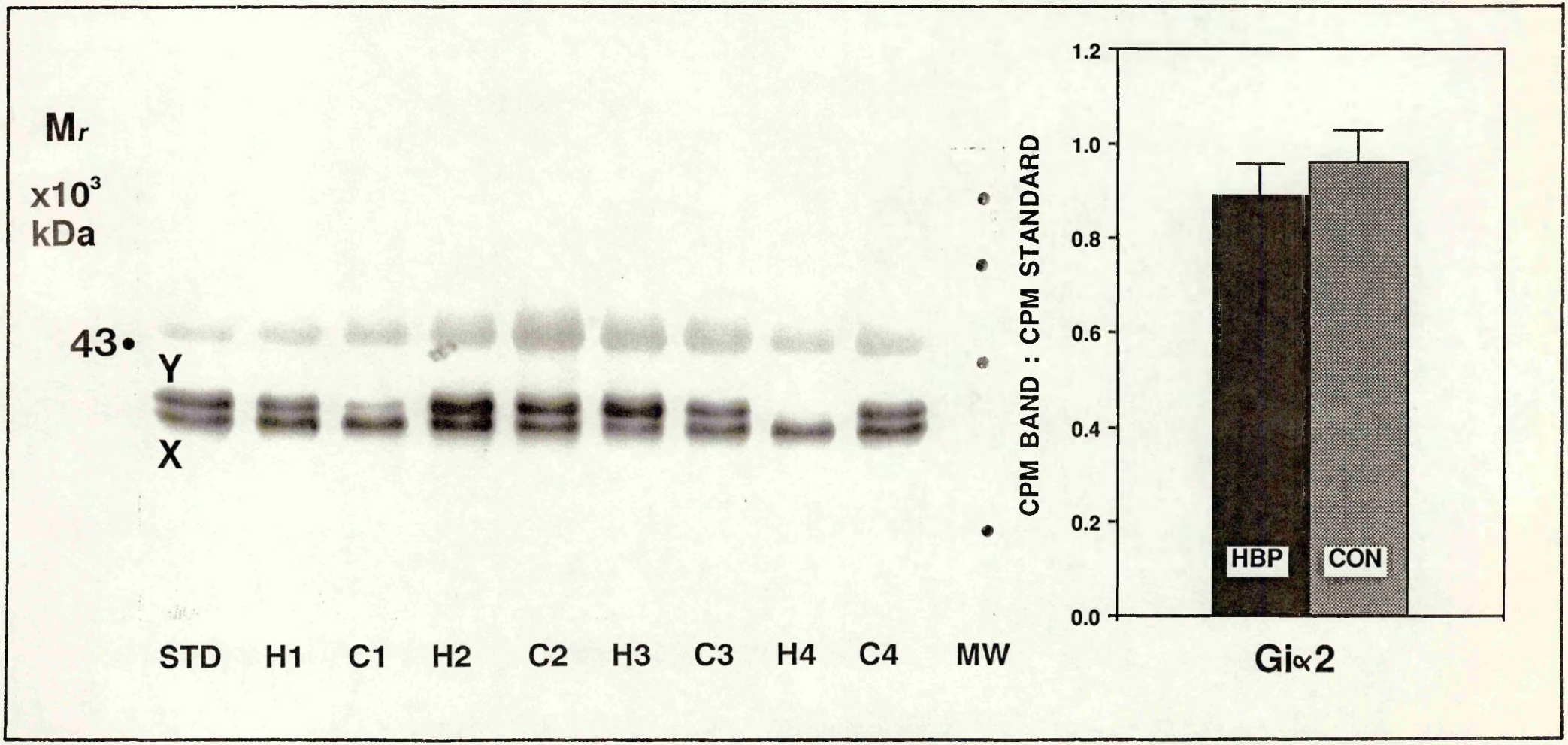


Fig.13. Platelet membrane Giα2 subunit immunoblot: essential hypertensives (HBP) v controls (CON). (Key-see fig. 12.)

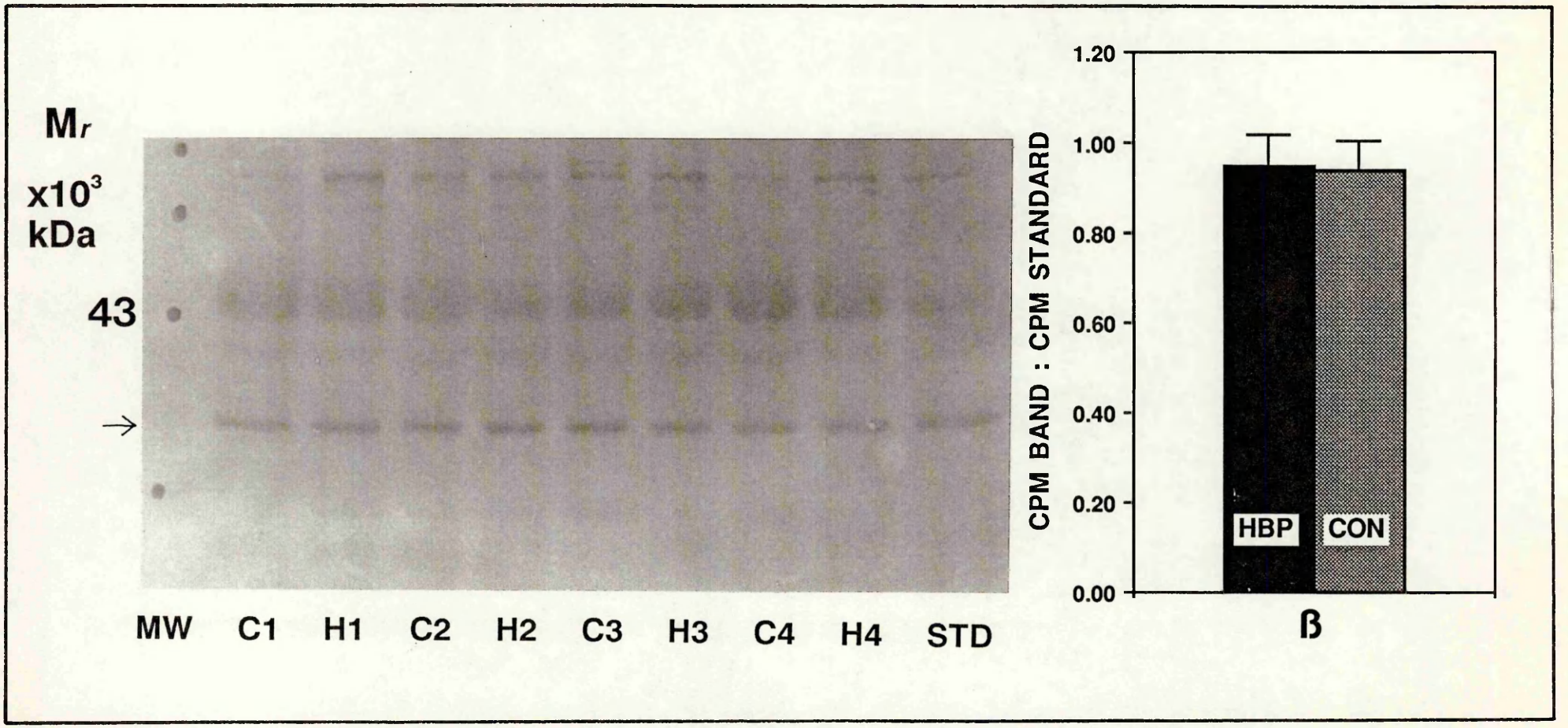


Fig.14. Platelet membrane ß subunit immunoblot: essential hypertensives (HBP) v controls (CON).(Key-see fig. 12.)

concentration and relative amounts of Giα2 (z=-2.084, n=29, p=0.04). When the 10 pairs of subjects who had both AC and immunoblotting studies were considered, the only significant correlation between relative abundance of G-proteins and AC function, was that between the amount of Gi2 (c.p.m. expressed relative to the standard membrane prep.) and ADR/PROP associated AC activity ; z=-2.071, n=20, p=0.04).

Hypertensive and controls with positive FH of hypertension were subsequently grouped together; thus study A then included 9 controls and 19 subjects and study B included 11 controls and 19 subjects respectively. The only difference identified in AC functional studies was in the derived ratio of % change from forskolin of PGE1: % change from forskolin of adrenaline/ propranolol; the ratio was higher in hypertensive subjects and those with positive FH than controls (p=0.036). No differences between groups were found in the immunoblotting studies.

4.4. Discussion:

The studies of ACA have shown that EH exhibit significantly greater PGE1 stimulated cyclase activity than controls (p=0.018). The absence of a correlation between either age or weight and the ACA obtained in these studies suggest that the age and weight differences observed between our hypertensive and control groups are unlikely to have influenced the results. Furthermore our observation is consistent with three previous studies which have shown similar enhancement of PGE1

stimulation of AC (Brodde et al. 1985; Erne et al. 1985; Resink et al. 1986) in essential hypertension.

This alteration of AC activity may at least in part account for the altered function of platelets reported in EH where increased platelet adhesion (Poplawski et al 1968) and altered aggregation (increased (Lentini & Bologna 1974) or reduced (Hamet et al. 1985)) have been found. (The divergent results from *in vitro* aggregation studies may reflect methodological differences (Hamet et al. 1978) and it is unclear how such studies relate to function in vivo.) It is of interest that enhanced PGE1 stimulation of AC activity has also been reported in platelets from experimental hypertension (SHR)(Hamet et al. 1978; Tremblay & Hamet 1984; Dard et al. 1978) although demonstration of this difference may require addition of extracellular calcium (Hamet et al. 1978). The biochemical explanation for the enhanced AC response to PGE1 may reside in altered number of PGE1 receptors, Gsα, Giα, amount of AC catalytic unit or in PGE1-Gsα, Gsα–catalytic unit coupling. In contrast to the study of Resink and colleagues (1986), no difference was observed in the ability of forskolin to stimulate AC activity; thus in the absence of changes in basal or forskolin stimulated cyclase activities it is unlikely that altered amount of AC catalytic unit could explain the findings.

Platelets from SHR may, in addition to altered PGE1 responsiveness, have abnormal Gi function suggested by reduced AC

inhibition by GppNHp (0.01-0.3μM)(Coquil and Brunelle 1989) and greater reduction of cAMP content in resonse to thrombin (Aktories and Jakobs 1984). Current models of Gi function propose that Gi may function as a donor of βγ units which serve to 'mop up' Gsα subunits and thus reduce AC activation. The converse situation could contribute to enhanced AC activation by a stimulatory ligand. The present studies in contrast to those in SHR platelets, however, show no differences in either receptor-independent activation of Gi, by GppNHp, or receptor driven AC inhibition (adrenaline/ propranolol-a combination which exclusively activates α2 adrenoreceptors) via Gi). The absence of significant differences in α2 receptor inhibited AC activity is consistent with studies which have reported similar α2 receptor numbers in platelets from controls and EH (Motulsky et al. 1983; Boon et al. 1983; Hollister, Onrot, Lonce, Nadeau & Robertson 1983), but is in contrast to those studies suggesting that EH may be associated with increased α2 receptor number (Kafka et al. 1979; Brodde et al. 1985). Resink et al. (1986), however, did report that EH showed greater sensitivty to adrenaline as an inhibitor of PGE1 stimulated AC activity.

To date no studies of PGE1 receptor number nor of the receptors' binding characteristics have been performed in EH and thus the explanation for the functional change in AC activty could reside either at PGE1 receptor number or G-protein coupling.

In spite of the observation of a weak inverse correlation Gi2α and adrenaline/propranolol associated AC activity

(p=0.04)(considering hypertensive and control subjects together) which is supportive of the evidence that Gi2α is implicated in receptor mediated AC inhibition, the immunoblotting studies of G-protein subunits failed to demonstrate any alteration of Gsα, Giα2 and ß subunits which could account for the functional differences observed between hypertensive and control groups.

Weak inverse correlation was also seen between plasma cholesterol and amount of Giα2 ,p=0.04); this raises the intruiging possibility that alterations of plasma membrane composition possibly associated with abnormal plasma lipid concentrations could govern the amount and type of G-proteins present and further studies are underway to examine whether abnormally elevated plasma lipid concentrations are assoc-iated with G-protein changes.

A positive family history (FH) of hypertension was obtained from 5 control subjects in study A and from 4 in study B. As FH is a marker for genetic risk of developing hypertension which could be relevant to any possible G-protein alteration (if any such change is involved in the pathogenesis of hypertension), the data were reanalysed. Although regrouping resulted in loss of the significance of the difference of PGE1 stimulation of AC activity the derived ratio of % change from forskolin of PGE1: % change from forskolin of adrenaline/ propranolol did prove to be higher in hypertensive subjects and those with positive FH than controls (p=0.036). No differences between groups were found in the immunoblotting studies.

The only other reported study of the role of G-proteins in EH was based on plasma membranes derived, not from platelets but from erythrocytes Farfel, Iaina, Eliahou & Cohen 1984). Human erythrocytes lack an inrinsic AC system but the membranes do express G-protein subunits; functional studies can, however, be performed by extracting G-proteins with detergent (Lubrol) and by reconstituting stimulable AC function in cyc- cells which lack a functioning Gs-cyclase system due to a mutation in the Gsα peptide sequence. No differences in (reconstituted) isoprenaline and guanosine-5'-O-(3-thiotriphosphate) stimulated adenylate cyclase activity were seen in a comparison with normal subjects (15) with EH(18). This study could be criticised on the basis of the use of erythrocyte membranes which have a substantially lesser claim to relevance to vascular smooth muscle than platelets and also on the heterogeneity of the hypertensive group; of the 18 hypertensive subjects only 8 were untreated (and had been off treatment for as short a period as 2 weeks) and 10 were receiving drug therapy at the time of study (2 propranolol, 4 propranolol/diuretic, 4 propranolol/diuretic/ vasodilator).

The current studies have shown that platelets from EH exhibit similar enhancement of AC response to PGE1 to that demonstrated by others in SHR, but may differ in response to GppNHp. It is apparent that SHR platelet membranes do not exhibit the same perturbations of ACA which have been reported in myocardial and other vascular tissues in experimental hypertension suggesting that in SHR there is unlikely to be a

ubiquitous abnormality of G-protein levels in plasma membranes in hypertension (see also chapter 5). There is no evidence of altered amounts of Gsα, Giα2 and ß in platelets from human EH. Any extrapolation of to human vascular tissue would be highly speculative, but it is probable that platelet membrane abnormalities are not representative of plasma membranes in general. The physiological relevance of altered platelet AC resonsiveness to PGE1 is probably based on the ability of cAMP to regulate intracellular Ca^{2+} (Zavoico & Feinstein 1984); as cytosolic free Ca^{2+} is significantly elevated in EH, it is suggested that enhanced PGE1 stimulation of adenylyl cyclase may serve as a negative feedback system to protect cells against Ca^{2+} overload (Resink et al. 1986). Further evidence supporting this hypothesis (le Quan Sang & Devynck 1986) is that 10^{-7}M PGE1 effects lowering of platelet Ca^{2+} in EH (although no effect was seen in control subjects.)

Altered G-protein levels appear not to explain the changes in PGE1 stimulation of ACA seen in platelet membranes from EH; however, further studies are required to elucidate the relative roles of changes in PGE1 receptor number and of altered PGE1 receptor-Gs coupling in this abnormality. The absence of major changes in G-protein levels in essential hypertension contrasts with the results obtained in the study of NIDDM (chapter 3). The data argue against alteration of G-proteins in hypertension by virtue of hyperinsulinaemia, but further study would be desirable in metabolically active tissues such as fat or liver and preferably in the arteriolar peripheral resistance vessels.

Chapter 5

G-proteins in Experimental Hypertension

a study of SHR myocardial, renal cortical and hepatic plasma membranes.

5.1. Introduction

The existence of several animal experimental models of hypertension (vide infra) permits study of possible causes and effects of hypertension; these models include the SHR, a model of genetically determined hypertension resembling human EH, and a number of renovascular hypertension models based on surgical induction of renal artery stenosis, for example the 2K,1C Goldblatt model. In addition, there are models of glucocorticoid hypertension (chapter 6), mineralocorticoid hypertension (the DOCA-salt model) and of hypertension attributable to central/ neural mechanisms-'stress hypertension' and 'neurogenic hypertension'. As already implied, hypertension represents the common outcome of a number of pathological processes: irrespective of aetiology, hypertension results in a number of well established functional and structural changes in affected organs which ultimately contribute to cardiovasular, cerebrovascular and renal morbidity and mortality. Experimental models express the same responses to hypertension, for example the development of myocardial hypertrophy, and thus identification of differences between hypertensive and control species must be interpreted with caution as to whether a change reflects a cause or effect of hypertension.

5.1.1. Experimental hypertension-cAMP and adenylyl cyclase.

Cyclic nucleotides and ACA have been studied in most models of experimental hypertension including the SHR (Amer 1973; Amer, Gomoll, Perhach, Ferguson & McKinney 1974; Triner, Valliemoz, Verosky & Manger 1975; Bhalla, Sharma & Ashley 1978; Bhalla & Ashley 1978, Bhalla, Sharma & Ramanathan 1980; Sharma, Kemp, Gupta & Bhalla 1982; Kumano, Upsher & Kairallah 1983; Anand-Srivastava 1988), renovascular hypertension (Goldblatt, 1K,1C and Grollman models) (Woodcock, Funder & Johnston 1978; Sharma et al.1982; Anand-Srivastava, Cantin & Genest 1983; Kumano et al. 1983; Kumano & Kairallah 1985), DOCA-salt (Amer et al. 1974; Woodcock et al.1979), neurogenic (Amer et al. 1974; Amer, Doba & Reis 1975) and stress hypertension (Amer et al. 1973,1974). There is no evidence currently to implicate abnormalities of cyclic nucleotides in the causation of hypertension which can with certainty preclude a cyclic nucleotide defect due to the presence of hypertension. Several vascular tissues have been studied (see table 10) including mesenteric arterial vessels (which are representative of resistance vessels), aorta and myocardium. Of these the mesenteric arteries are most likely to represent the site of greatest pathophysiological relevance in the aetiology of hypertension. Although the myocardium of hypertensive animals is unlikely to initiate hypertension, the hypertrophic changes in the heart consequent upon hypertension are well recognised both in human and experimental hypertension. Against this background it should be remembered that in addition to their possible roles in the

TABLE 10 ADENYLYL CYCLASE ACTIVITY IN EXPERIMENTAL HYPERTENSION

ARROWS DENOTE A.C ACTIVITY IN HYPERTENSIVE SPECIES (v CONTROL), LETTERS DENOTE REFERENCES

MYOCARDIUM

MODEL	BASAL A.C. ACTIVITY	ISOPROTERENOL STIM. A.C. ACTIVITY
SHR	↓ E,H → B,L	↓ B,E,H,I,N,L
RENOVASCULAR*	↓ I,L → G,M	↓ G,I,L
NEUROGENIC	→ C	↓ C
STRESS	→ B	↓ B
DOCA-SALT	↑ B → G ↓ K	↓ B,G,K

AORTA

MODEL	BASAL A.C. ACTIVITY	ISOPROTERENOL STIM. A.C. ACTIVITY
SHR	→ A,B,D,O ↓ N	↓ A,B,D,N,O
RENOVASCULAR	↓ G	↓ G
NEUROGENIC	→ C	↓ D
STRESS	→ A,B	↓ A,B
DOCA-SALT	↑ B	↓ B

TABLE 10 ADENYLYL CYCLASE ACTIVITY IN EXPERIMENTAL HYPERTENSION

MESENTERIC ARTERY

MODEL	BASAL A.C. ACTIVITY	ISOPROTERENOL STIM. A.C. ACTIVITY
SHR	→ B	↓ B
RENOVASCULAR		
NEUROGENIC		
STRESS	→ B	
DOCA-SALT	↑ B	

KEY TO REFERENCES:
A. Amer et al. 1973
B. Amer et al. 1974
C. Amer et al. 1975
D. Triner et al. 1975
E. Bhalla et al. 1978
F. Bhalla & Ashley 197
G. Woodcock et al. 197
H. Bhalla et al. 1980
I. Sharma et al. 1982
J. Blumenthal et al. 1982
K. Schutz et al. 1984
L. Kumano et al. 1983
M. Anand-Srivastava et al. 1983
N. Anand-Srivastava 1988
O. Bhalla & Sharma 1982

aetiology of and pathological response to hypertension there are inherent normal and physiological regional differences in cyclic nucleotides within the vascular tree where there is a general trend for basal adenylyl cyclase activity to increase towards the periphery (for example, enzyme activity in the coronary arteries is three times higher than in the upper aorta (Triner et al. 1971).

5.1.2. cAMP (tissue) levels in experimental hypertension.

cAMP levels have been quantified in the aorta and myocardium of the SHR (Amer 1973; Amer et al. 1974), stress hypertensive rats (Amer 1973; Amer et al .1974), DOCA-salt (Amer et al. 1974) and neurogenic hypertension (Amer et al. 1975). In all models except DOCA-salt hypertension (Amer et al. 1974) cAMP levels were generally significantly reduced compared to control species; Bhalla, however, using aortas from SHR & WKY reported no difference (Bhalla et al. 1978). Although those who reported reduction of cAMP ascribed it to increased cAMP phosphodiesterase (PDE) activity (Amer 1973;Amer et al.1974) several investigators have since demonstrated that cAMP PDE is either unchanged (Triner et al. 1985) or lower (Taylor & Shirachi 1977) in SHR vascular tissue. In practice an alteration of cAMP may be of greater pathological relevance when viewed in the context of concurrent cGMP changes which favour further elevation of the cGMP : cAMP ratio (Amer et al 1974,1975). Reduction of tissue cAMP has also been reported in the hearts of SHR (Amer et al. 1974) and neurogenic hypertensive species (Amer et al. 1975) but was not associated with significant change in the overall

cGMP:cAMP ratio. No significant cAMP changes were seen in hearts taken from the other models of hypertension.

5.1.3. Adenylyl cyclase activity in experimental hypertension: studies in vascular tissues.

Table 10 represents a summary of existing evidence. Studies of basal ACA have failed to identify consistent abnormalities both within a particular model of hypertension and when comparing different hypertensive models. The divergent results probably result from methodological differences, compounded by generally low but otherwise variable basal ACA in the different models. Amer et al. (1974) have, however, applied similar techniques concurrently to different vascular tissues from SHR, stress hypertension and DOCA-salt models and have shown similar trends in basal activities in relation to control species irrespective of the vascular tissue studied (myocardium, aorta or mesenteric artery); only in the DOCA salt model did they note any change from control, notably an increase of cyclase activity.

In marked contrast to these divergent observations is the remarkable constancy of reduced isoproterenol stimulated ACA (also expressed as a reduced 'sensitivity' to isoproterenol) which is common to all five models of hypertension, and which is demonstrable in all three of the vascular tissues studied (myocardium, aorta and mesenteric artery) (table 10).

Using plasma membranes derived from a number of tissues from SHR, WKY and Wistar rats several experiments were performed to address the following questions:

1. **Does SHR myocardium exhibit reduced stimulation of adenylyl cyclase in response to ligands other than isoproterenol?**
2. **Does non-vascular tissue (renal cortex and liver) from SHR exhibit the same trend in adenylyl cyclase responses as myocardium?**
3. **Do altered G-protein levels contribute to changes in adenylyl cyclase activity in SHR?**

5.2. Materials & Methods

11 week SHR with WKY and Wistar controls were studied. Because of uncertainty regarding the ideal control species in such experiments (see discussion section 2.2) the study includes two control groups (Wistar and WKY species).

Body weight and mean systolic BP were recorded. BP was measured in triplicate in warmed, unrestrained conscious rats by the tail cuff method (McAreavey et al. 1985) using a W & W Electronics recorder (Basle, Switzerland)(see table 11).

Methods:

Following sacrifice by cervical dislocation, the hearts, kidneys and livers were rapidly removed. Plasma membranes were prepared as described in sections 2.3.3., 2.3.4. and 2.3.2. respectively. Membrane aliquots were stored at -80^{o}C. Membrane protein concentrations were measured by the modified Lowry protocol (section 2.6.) and 5' Nucleotidase (5'ND) activities were measured (section 2.7.) to verify plasma membrane 'purity' of myocardial membranes.

Adenylyl cyclase studies:

Myocardial, renal cortical and hepatic plasma membrane ACA were measured using similar protocols (section 2.8.1). Enzyme reactions were performed in the presence or absence of forskolin 10^{-5}M. A number of ligands were used at concentrations previously shown to be saturating with respect to effect on ACA (see table 6); these included NaF (10mM), MnCl2 (20mM), PGE1 (10^{-5}M), acetyl choline (ACh)(10^{-4}M) used in conjunction with the cholinesterase inhibitor physostigmine (10^{-5}M), isoproterenol (10^{-4}M), glucagon (10^{-6}M), neuropeptide Y (10^{-6}M) and angiotensin II (10^{-6}M) .

The reactions were started by the addition of membrane (generally 8μg membrane protein) suspended in 10mM tris pH7.4. Tubes were incubated at 30°C for 10 min.- a time shown to be in the linear portion of the time course of the reaction.

Immunoblotting studies:

Immunoblotting studies for G-protein subunits were performed under denaturing conditions on10% gels (SDS-PAGE) as described in section 2.4.1. The G-protein subunits identified within the different plasma membrane fractions are summarised in table 5

The amounts of protein required to give clear reproducible blots with the different G-protein subunit antisera were derived from 'dose-response' curves (section 2.4.). Widely differing quantities of protein required to be loaded for the various preparations, presumably reflecting differences in purity of the preparations

Table 11. Comparison of BP, body and organ weights in a typical group of WKY, SHR & Wistar rats.

mean (sem)

	WKY	SHR	WISTAR
n	9	7	9
WEIGHT(g)(W)	238.3 (6·4)	255.3(4·2)	288.1(3·4)***
BP (mmHg)	142.2(3·2)***	212.9(3·5)	155.3(3·8)***
MYOCARDIAL WEIGHT(g)(M)	0.88(0·04)*	1.03(0·06)	0.97(0·03)
RENAL WEIGHT(g)(R)	1.79(0·06)	1.84(0·08)	2.05(0·04)**
M / W	3.71(0·18)	4.02(0·21)	3.37(0·18)**
R / W	7.51(0·1)	7.19(0·26)	7.13(0·12)

Note: Statistical comparisons with SHR data are shown:

*: p = 0.05, **: p = 0.01, ***: p = 0.0001.

derived from different tissues; for example, 80μg and 60μg membrane protein were loaded respectively for Gsα and Giα2 studies in myocardium, while around 200μg protein were required for similar studies in the hepatic membrane preparations. The antibody-labelled G-protein bands were visualised using ^{125}I-labelled second antibody and were counted on a gamma counter.

5.3. Results

The SHR rats studied were shown by tail cuff blood pressure measurements to have significantly higher BP than both WKY and Wistar control species (table 11). Body weights were different amongst the species; Wistar rats were heavier than SHR, which were slightly, but not significantly higher than WKY animals. Mean myocardial weight was noted to be heavier in SHR than WKY (when expressed as absolute weight (g)) and heavier than Wistar myocardium (organ weight expressed as fraction of body weight (x 1000)). By contrast SHR kidney mass expressed as absolute weight, or corrected for body weight was intermediate between control species.

Adenylyl cyclase studies.

Studies of basal AC activity (expressed as pmol cAMP/mg/min) in the myocardium revealed a trend (fig.15A); ACA in WKY was higher than SHR which in itself was higher than Wistar membranes. Despite the apparent differences, statistical significance was only achieved in the comparison between control species (WKY v Wistar). Similar trends in ACA were seen when the enzyme was assayed in the presence of a number of agents including NaF, PGE1, isoproterenol, glucagon and forskolin (fig. 15A). The greatest differences were observed between WKY and Wistar rats, while significantly greater stimulation of ACA was seen in WKY than SHR in the presence of NaF, PGE1, isoproterenol and forskolin (figure 15A); ACAs were higher in SHR than Wistar rats only in response to forskolin. By contrast, 20mM Mn^{2+} (which essentially uncouples AC from G-protein input) eliminated the differences amongst the species. When the data

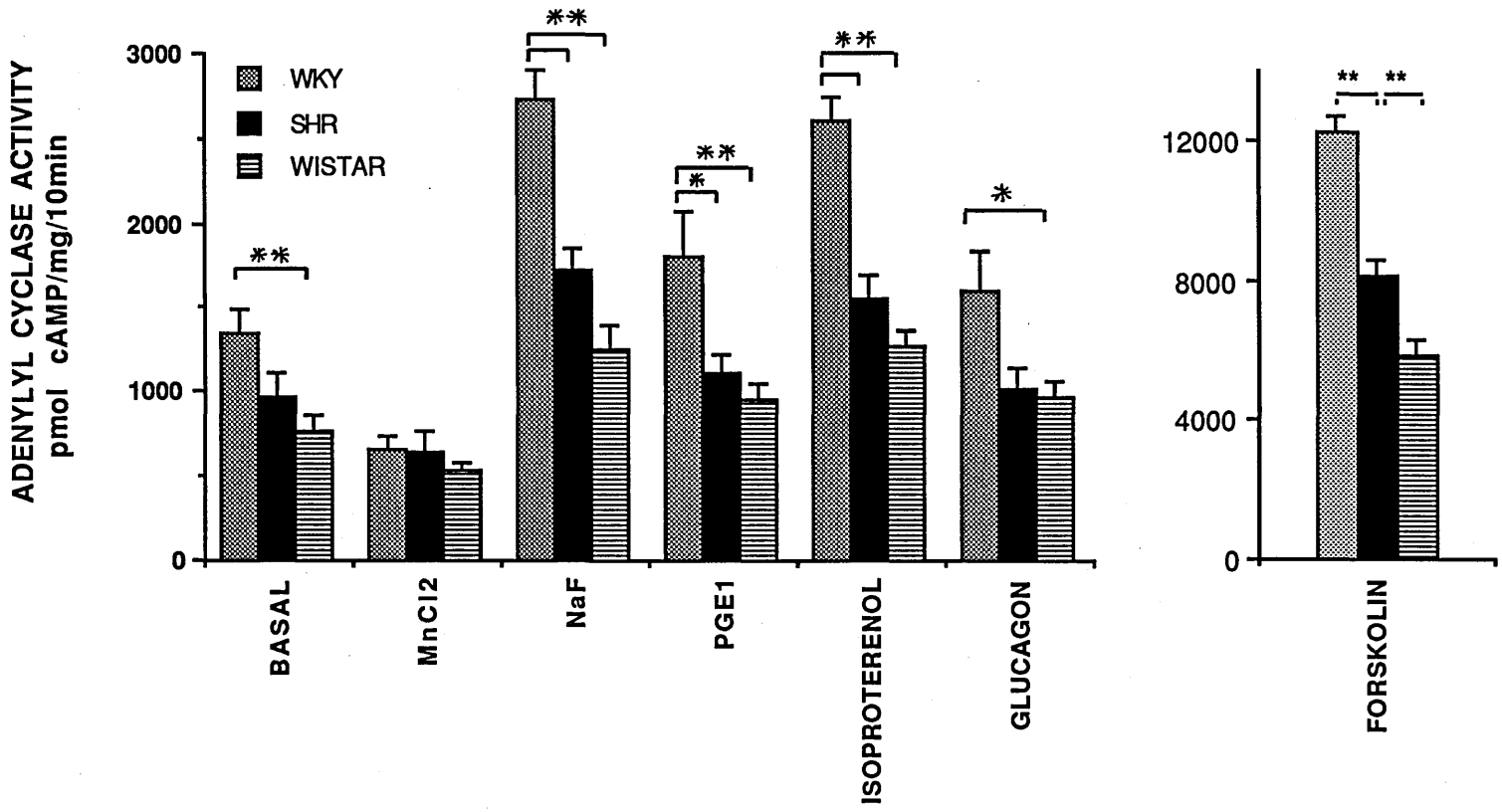


FIG 15A. **Adenylyl cyclase studies in myocardial plasma membranes. Comparison of WKY, SHR and Wistar rats. Data expressed as absolute activities. (mean ± sem,7 exp.)**
***: 0.006 < p < 0.05 ****: p < 0.006**

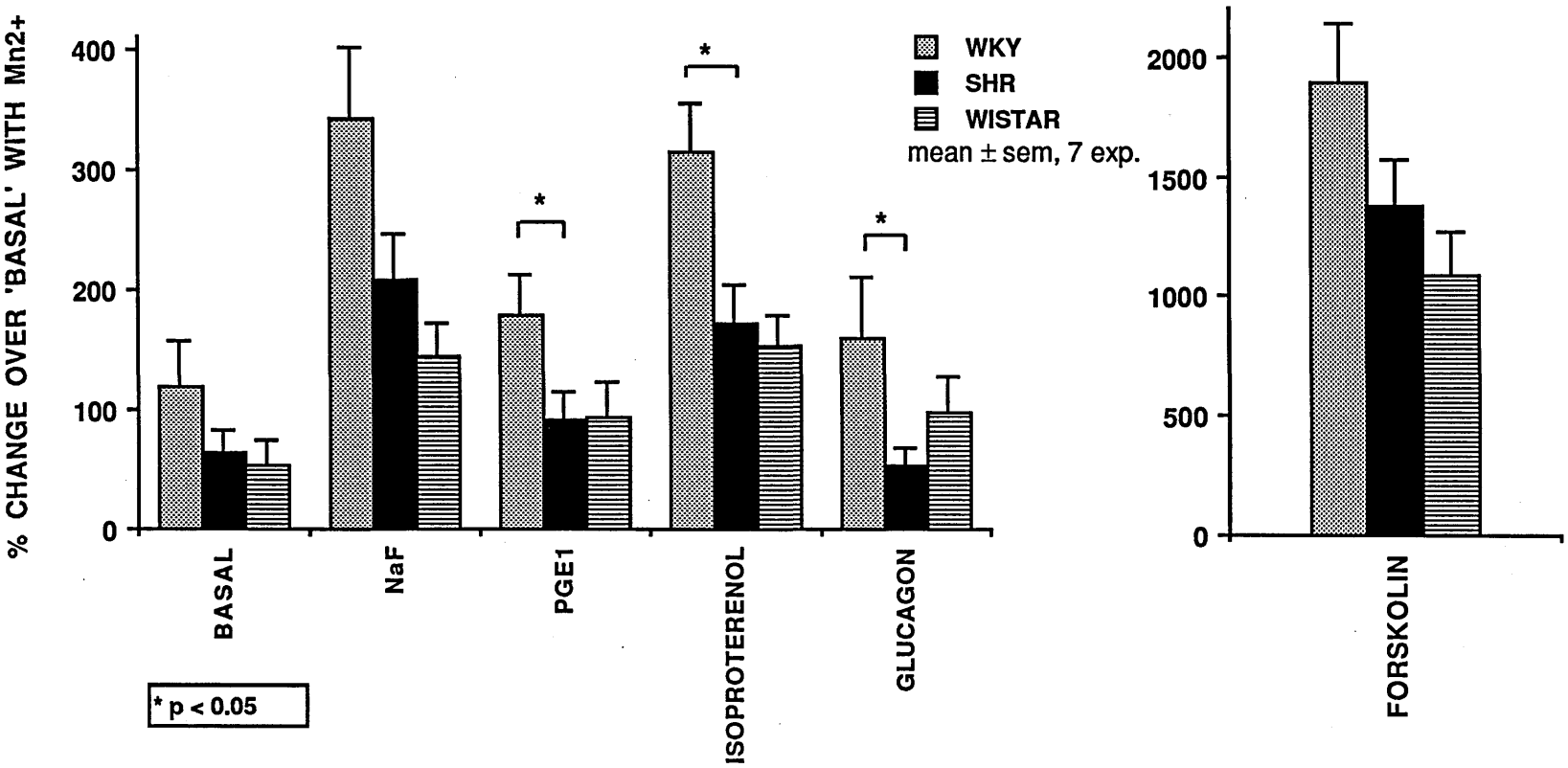


Fig.15B. Adenylyl cyclase studies in myocardial plasma membranes. Comparison of WKY, SHR and Wistar rats. Data expressed as percentage change from 'basal' determined in presence of Mn2+.(mean ± sem, 7 exp.)

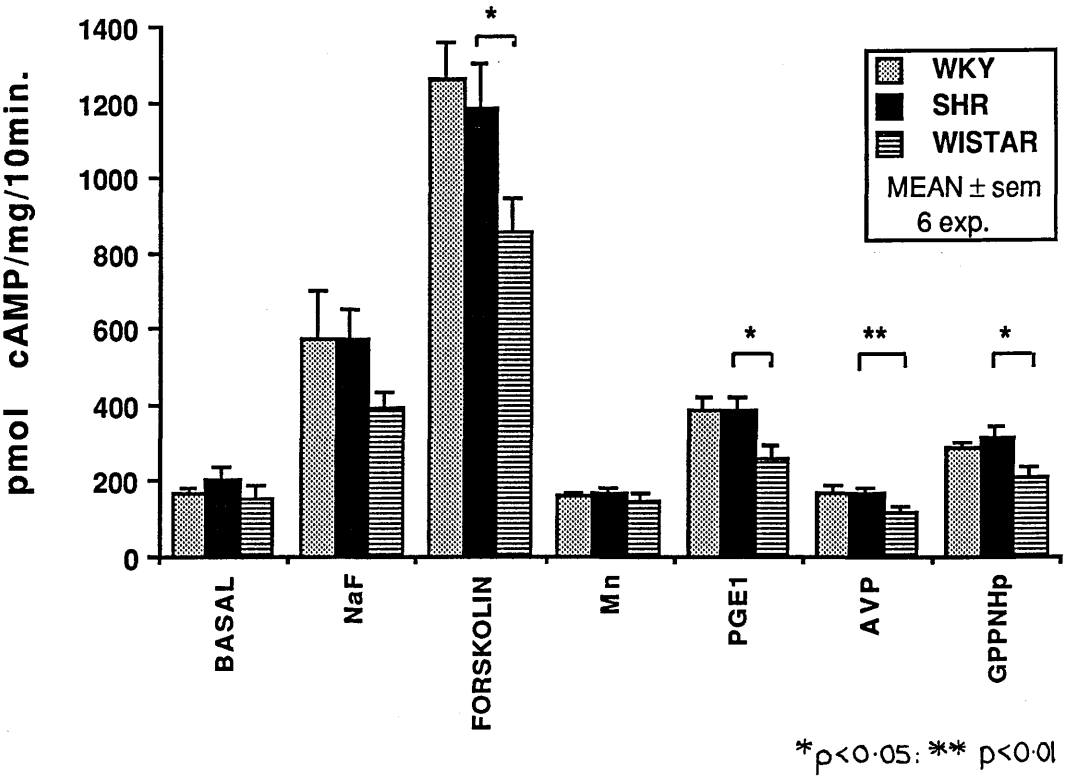


Fig.16A: Adenylyl cyclase studies in renal cortical plasma membranes. Comparison of WKY, SHR and Wistar species. Data expressed as absolute activities, mean ± sem of 6 exp.

were expressed as 'percentage change from 'basal' ('basal' measured in the presence of Mn2+'), the previously observed trend persisted (fig.15B); myocardial AC activities from WKY were much higher than from SHR and Wistar species. Statistically significant differences (WKY v SHR/Wistar) were seen only when ACA was assayed in the presence of ligands whose receptors couple to AC catalytic unit via Gs. However, (despite failing to achieve statistical significance) the same trend was seen even in receptor independent mechanisms (forskolin and NaF)(fig. 15B).

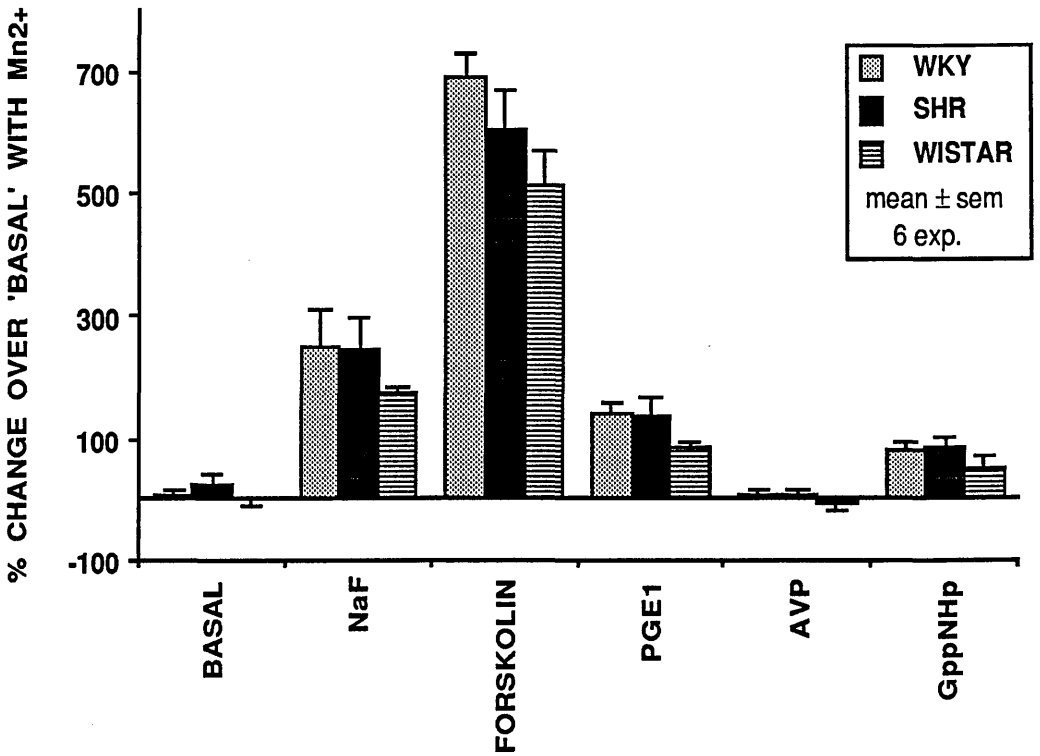


Fig.16B: Adenylyl cyclase studies in renal cortical plasma membranes. Comparison of WKY, SHR and Wistar species. Data expressed as 'percentage change from 'basal' measured in presence of $Mn2^+$, mean ± sem of 6 exp.

Minimal inhibition of ACA was achieved despite assaying ACA in the presence of several ligands which couple to catalytic unit via Gi. No more than 10% inhibition of forskolin stimulated ACA was observed in response to ligands including acetyl choline (10^{-4}M) (in the presence of physostigmine(10^{-5}M)), angiotensin II(10^{-6}M)(see table 12 and Figure 15A) neuropeptide Y(10^{-6}M) (data similar to table 12, but not shown). Furthermore, no

differences in the extent of inhibition (expressed as 'percentage change') were seen when comparing species.

ACA studies in plasma membranes derived from renal cortex (fig. 16) show no differences in either basal or stimulated ACA when comparing WKY v SHR species; ACA were, however, higher in SHR than Wistar rats when assayed in the presence of forskolin, PGE1, AVP and GppNHp. These differences were completely eliminated when expressing the data as 'percentage change from 'basal' in the presence of Mn2+'(fig. 16B).

ACAs were studied in hepatocytes from SHR and Wistar species only (table 14): no differences were seen.

Immunoblotting studies: Gsα, Giα2 and Goα immunoblots of myocardial membranes are shown in figs. 17-19; no differences were observed. Immunoblotting data from renal cortical membranes are presented in table 13, and again show no differences in the levels of G-protein subunits.

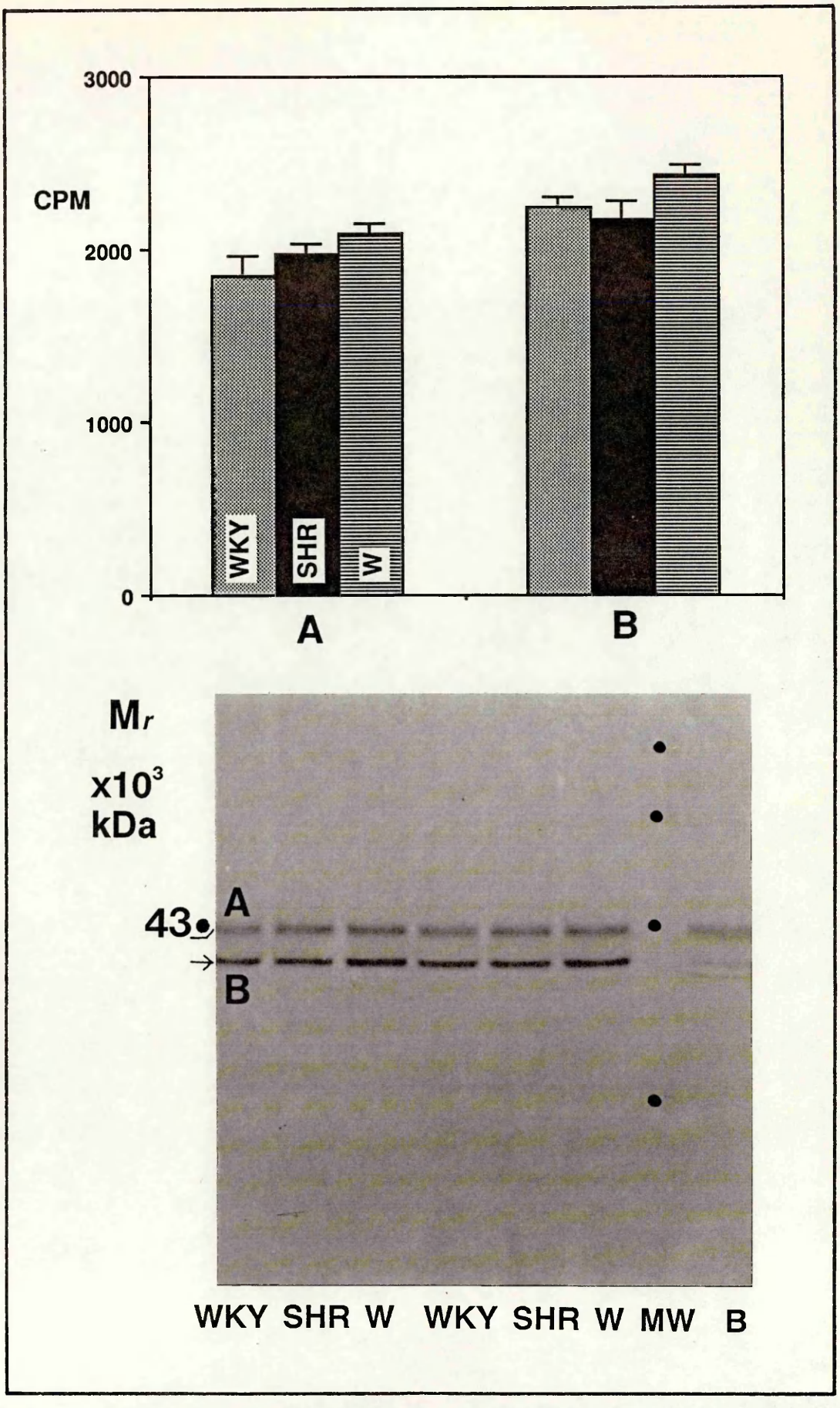


Fig.17. Gsα subunit immunoblot data from myocardial plasma membranes from WKY, SHR and Wistar rats. Lower—typical blot, showing 2 lanes each for sample preps., MW=molecular weight standards, B='standard' membrane prep.(brain). Upper—graph obtained by expressing data as cpm obtained by counting 125I-labelled bands from blot.(mean ± sem).

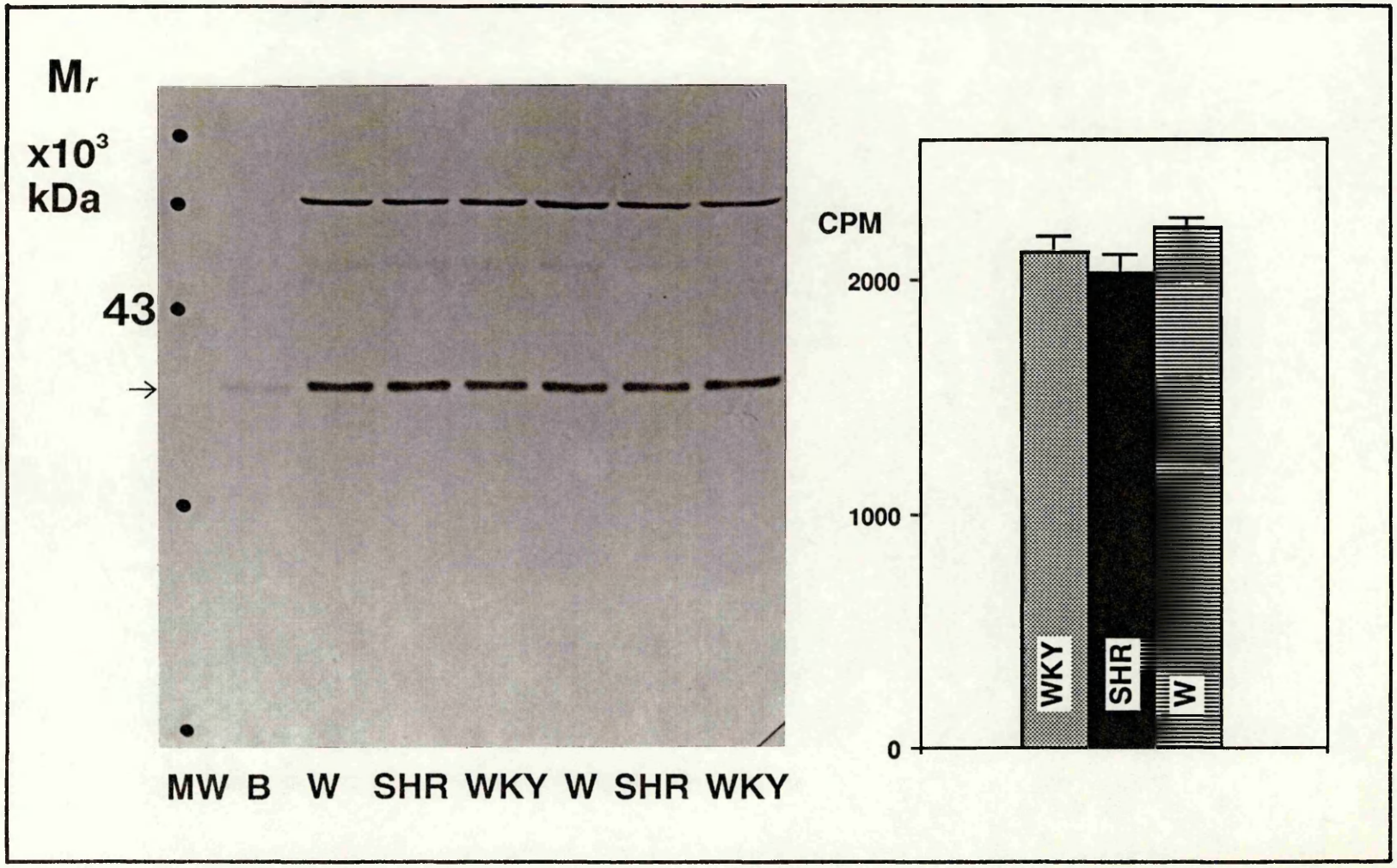


Fig.18. Giα2 subunit immunoblot data from myocardial plasma membranes from WKY, SHR and Wistar rats. (key as for fig. 17).

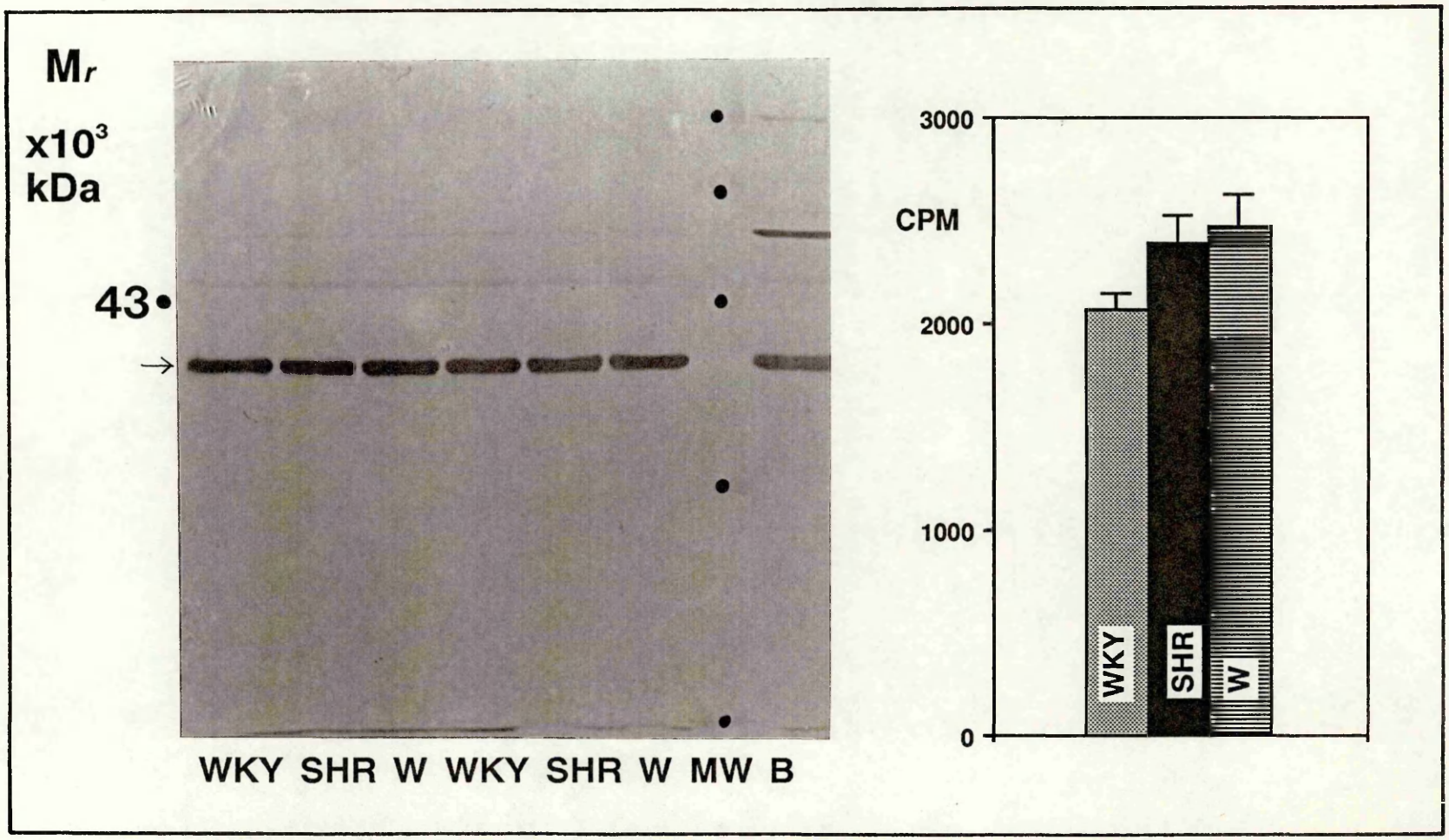


Fig.19. Goα subunit immunoblot data from myocardial plasma membranes from WKY, SHR and Wistar rats (key as for fig. 17).

Table:12 AC studies in myocardial plasma membranes. Effect of AII on forskolin and PGE1 stimulated cyclase.

	WKY	SHR	WISTAR
FORSKOLIN	846.6 ± 42.4	804.8 ± 61.7	497.2 ± 47.7
FORSKOLIN / AII	778.6 ± 42.3	780.2 ± 57.2	464.2 ± 43.4
PGE1	179.2 ± 20.9	180.2 ± 25.8	114.4 ± 15.5
PGE1/AII	196 ± 20.4	148.2 ± 32.4	98.2 ± 28.8

(data represent mean ± sem of 3 expt., expressed as pmol cAMP/mg/10min.)

Table 13: G-protein levels assessed by Western blotting in renal cortical plasma membranes from WKY, SHR & Wistar rats. Data expressed as mean ± sem of cpm;n = number of experiments.

G-protein subunit	WKY	SHR	WISTAR	n
Gsα42kDa	2243 ± 59	2253 ± 160	2209 ± 147	4
Gsα40kDa	2531 ± 67	2536 ± 136	2491 ± 147	4
Gi1α	440 ± 14	476 ± 14	536 ± 49	4
Gi2α	2916 ± 540	2618 ± 34	2454 ± 269	4
Goα	1115 ± 101	1169 ± 96	1068 ± 42	4
ß	524 ± 20	493 ± 21	524 ± 26	4

5'ND activities: (assays only performed once): Myocardium: WKY, 480.8; SHR, 462.6; Wistar 456.4 μg phosphorus/h/mg membrane protein.

Table 14. Adenylyl cyclase studies in liver from SHR and Wistar rats (data represent mean ± sem of results from 5-8 expt., each with 4 replicates; cyclase data as pmol cAMP/mg / 10min)).

ASSAY CONDITIONS -LIGAND STUDIED	SHR	WISTAR
BASAL	102.9 ± 22	91.2 ± 16.7
NaF	441.7 ± 60.3	505.8 ± 72.9
MnCl2	179.1 ± 32	112.7 ± 24.1
FORSKOLIN	785.3 ± 101.5	640.8 ± 96
GLUCAGON	537.4 ± 49.8	691.2 ± 60.1
ISOPROTERENOL	136.2 ± 48.1	104.1 ± 15.8

5.4. Discussion.

The SHR rats had significantly higher BP than both WKY and Wistar control species (table 11) and mean myocardial weight was noted to be heavier in SHR than WKY (when expressed as absolute weight (g)) and heavier than Wistar myocardium (organ weight expressed as fraction of body weight (x 1000)) suggesting a degree of myocardial hypertrophy in the SHR.

Studies of basal AC activity (expressed as pmol cAMP/mg/min) were significantly higher only when comparing WKY and Wistar rats. SHR ACAs differed from WKY (significanlty lower) when assayed in the presence of a number of agents including NaF, PGE1, isoproterenol, and forskolin (fig. 15A), while comparison between SHR and Wistar controls showed essentially no differences with the sole exception of higher ACA in SHR in the presence of forskolin. 20mM Mn2+ (which essentially uncouples AC from G-protein input) eliminated the differences amongst the species: this suggests that the amounts of AC catalytic unit are unlikely to differ greatly amongst the species. When the data were expressed as 'percentage change from 'basal', measured in the presence of Mn2+' the previously observed trend persisted; myocardial AC activities from WKY were much higher than from SHR and Wistar species (fig.15B). Thus although interspecies differences exist, the 'abnormal' species appears to be WKY, as SHR responses were virtually identical to those seen in the Wistar membranes. Statistically significant differences (WKY v SHR/Wistar) were seen only when ACA was assayed in the presence of ligands whose receptors couple to AC catalytic unit via Gs. However, (despite failing to achieve statistical significance) the same trend was seen even in receptor independent mechanisms (forskolin and NaF)(fig. 15B) adding support to the possibility that changes in Gs function and/or levels could explain the higher ACA seen in WKY species.

Why no more than about 10% inhibition of forskolin stimulated ACA could be achieved despite using a wide range of established inhibitory ligands (including acetyl choline, angiotensin II and

neuropeptide Y remains unclear. However, it may be related to the inclusion of Mg2+ in the buffer used to prepare the myocardial plasma membrane homogenate . While the results obtained with inhibitory ligands tend to exclude a major difference in Gi function amongst the species, this may not truely reflect the situation *in vivo* .

Comparison of SHR and WKY myocardial ACAs obtained in the current studies under basal conditions shows results similar to those of previous workers (Bhalla et al. 1978,1980)(table 10). Furthermore, the observed reduction of response of SHR myocardium to isoproterenol stimulation (compared to WKY)(fig. 15) is entirely consistent with many previous reports (Amer et al.1974; Bhalla et al. 1978,1980; Blumenthal, McConnaughey & Iams 1982; Sharma et al. 1982; Kumano et al. 1983; Anand-Srivastava 1988; Matsumori, Ohyanagi, Kawamoto, Shibata & Iwasaki 1989).

Reduced receptor-mediated stimulation of cyclase in response to adenosine, epinephrine, dopamine and glucagon has previously been reported in the SHR(Anand-Srivastava 1988) and to this list can be added the reduction of AC stimulation by PGE1. Reduced NaF/forskolin induced activation of cyclase (expressed as % change from basal) has been reported previously by Anand-Srivastava et al. (1988) but not by Kumano et al. (1983)(who reported no change).

Mechanisms of altered AC activity in SHR myocardium

Levels of Gsα, Giα2 and ß(cpm WKY, SHR, Wistar: 525 ± 15.4, 517.8± 32.1, 578.1 ± 24.2 (mean ± sem, n=3) the G-proteins implicated in regulation of ACA) and Goα did not differ among SHR, Wistar and WKY myocardial plasma membrane preparations (fig. 17,18,19); thus alterations of the levels of the G-proteins which modulate acitivty of AC catalytic unit do not account for the differences in ACAs. This is consistent with a previous study (Murakami, Katada & Yasuda 1987), which compared SHR and WKY myocardium and which showed no difference in the amount of Gs as determined by cholera toxin-catalysed ADP ribosylation, nor in amounts of Gi by pertussis toxin catalysed ADP ribosylation and the amount of ßγ subunit (antibody).

In view of the reduced responsiveness of AC in vascular tissues to ß adrenergic stimulation reported here and by others (table 10) in *in vitro* studies it is not surprising that there are functional consequences: the myocardium of hypertensive animals is known to exhibit reduced inotropic (Saragoca & Tarazi 1981) and chronotropic responses to adrenergic stimulation (Fujiwara, Kuchii & Shibata 1972; Cohen & Berkowitz 1975). In addition aortic muscle strips show less tendency to relax in response to isoproterenol (Cohen & Berkowitz 1975). Although changes in ß-adrenergic receptors have been reported and may contribute, it is likely that the altered catecholamine sens-itivity is multifactorial. ß adrenergic receptor characteristics have been studied in the SHR. ß-receptor numbers have been reported to be unchanged (Bhalla et al. 1980; Blumenthal et al.

1982; Murakami et al. 1987) or decreased (Limas & Limas 1978; Robberecht et al. 1981;Kumano et al 1983;Will-Shahab, Kutter & Warbanow 1986) in the SHR. One factor contributing to the disparity in results may be that there is evidence of altered cellular distribution of ß receptors- both with age (surface ß receptors decrease with age) and in SHR compared to WKY (Matsumori et al 1989); although total ß receptors are unchanged the surface ß receptors may be reduced in SHR. Receptor affinity was either not altered (Limas & Limas 1978; Murakami et al. 1987) or reduced (Bhalla et al. 1980): the reasons for these disparate results is unknown and although further clarification is required the possibility of the existence in hypertensive hearts of altered G-protein function (such as impairment of coupling of receptor to G-proteins) is consistent with reduced affinity in the context of unchanged receptor numbers as shown by Bhalla et al. (1980).

As myocardial hypertrophy is an inevitable consequence of experimental (as well as human) hypertension and can occur as early as 1-2 weeks after induction of experimental hypertension (Kumano & Kairallah 1985), studies of myocardium from hypertensive species must acknowledge that hypertrophy per se could cause changes independent of any effects due to or contributing to the underlying hypertension.

Studies of the evolution of blood pressure changes in the SHR show divergence of blood pressure (BP) tracings as recorded by the tail cuff method after about 5 weeks of age (the time at which tail cuff BP recordings become feasible). Extrapolation of

such blood pressure curves back to younger ages led to the assumption that the BP curves converge and this hypothesis engendered the concept of the 'prehypertensive' SHR; such a 'prehypertensive' state could have potential in allowing elucidation of factors relevant to the pathogenesis of hypertension in the absence of the hypertension phenotype. For example, ß adrenergic (isoproterenol) stimulated cyclase activity in myocardial membranes from 'prehypertensive' SHR has been shown to be increased compared to WKY. Furthermore, as the rats age the isoproterenol-stimulated cyclase activity declines to lower values-half the original activity at100 days and in absolute terms a level of activity below that in WKY of similar age (Blumenthal et al. 1982). Increased ACA was proposed to be a possible factor in the pathogensis of hypertension. However, the concept of the 'prehypertensive' animal may be flawed as recent evidence based on intraarterial blood pressure measurements in these young animals suggests that even at 3 weeks of age significant differences in BP recordings already exist (Morton, J.J., Personal communication).

Does myocardial hypertrophy per se cause specific changes in adenylyl cyclase activity?

The studies herein(table 11) suggest that SHRs do exhibit a degree of myocardial hypertrophy, at least compared to WKY. Correction of myocardial mass for differences in total body weight tends to reduce (but not eliminate) the difference between SHR and WKY. As SHR and Wistar membranes behaved similarly in assays of ACA it seems unlikely that hypertrophy is the sole explanation for the observed differences. However, the

similarity in mean myocardial weights between the SHR and Wistar rats does not exclude a contribution to altered ACA associated with myocardial mass per se (possbly associated with altered amount or composition of plasma membranes).

Our studies of 5'ND activity(a plasma membrane marker) tend to exclude major differences in plasma membrane concentration within the plasma membrane preparations which might cause artifactual differences in our immunoblotting and ACA studies.

It is impossible to dissociate myocardial hypertrophy completely from hypertension. However, myocardial hypertrophy in the context of systemic hypertension(SHR) has been compared to hypertrophy due to an aortic coarctation (proximal ligature); in the absence of genetic risk of developing hypertension, identical changes in cyclase activities were reported. Both exhibited reduced epinephrine stimulated adenylyl cyclae activity (Will-Shahab et al.1986) which was ascribed to the reduction of cardiac ß receptors (30-35% reduction). However, other studies have suggested that myocardial hypertrophy resulting from different models of hypertension may be associated with different alterations in the ß-adrenergic-receptor-G-protein-cyclase system (Kumano & Khairallah 1984). Further evidence of this was provided by study of the effects of angiotensin II (which mediates renovascular hypertension) and epinephrine (used because there is some evidence implicating catecholamines in hypertension of SHR-Williams et al. 1976) infusions (subcutaneously) in rats (Kumano & Khairallah 1984); both groups exhibited hypertension and myocardial hypertrophy.

However, although the myocardial plasma membrane from the epinephrine treated group had lower basal and ligand (isoproterenol/glucagon/forskolin /GppNHp) stimulated cyclase activities the membranes from the AII treated group showed no differences in cyclase activity (neither basally nor in response to ligands) (Kumano & Khairallah 1984). The overall pattern of results in our experiments is similar to that observed by Kumano & Khairallah in response to infused epinephrine, possibly suggesting an explanation for our findings.

Changes in other vascular tissues of the SHR.

Changes in basal and ligand stimulated cyclase which are identical to those in myocardium have been reported in the aorta of the SHR (Bhalla & Sharma 1982; Anand-Srivastava et al.1988). In studies which have assessed vascular physiology and its vascular correlates noradrenaline (ß) mediated relaxation of precontracted femoral vascular smooth muscle strips has been reported to be reduced in SHR (Asano, Masuzawa & Matsuda 1988a; Asano, Masuzawa, Matsuda & Asano 1988b): elegant elucidation of of the site of biochemical abnormality by Asano & colleagues suggests that reduced Gs function may be the prime determinant of these changes (*vide infra*) (Asano et al. 1988b).

Studies in non-vascular tissues: adenylyl cyclase activity in non vascular tissues of the SHR.

Changes in ACA may be more widely distributed than the vasculature allowing dissociation of the effects of hypertension or of its underlying determinants. The kidney contributes fundamentally to the mechansims of hypertension in genetically

hypertensive rats and is a major determinant of blood pressure as shown in renal transplant studies of these models (Bianchi et al. 1974). Liver has been studied as it represents a tissue without a direct role in BP regulation and which is not subject to the hypertrophic response to hypertension.

Plasma membranes derived from renal cortex (fig. 16) and liver (table 14) show no differences overall in basal, NaF, MnCl2, forskolin, glucagon and isoproterenol stimulated AC activity when expressed as absolute levels of activity. A number of non-vascular tissues from SHR have been studied by others; these include platelets, adipocytes (Chiappe de Cingolani 1988) and kidney (Umemura, Smyth & Pettinger 1985). Reduced PGE1 and PGE2 stimulation of ACA was found in renal cortical plasma membranes from SHR (v WKY) and although the responses to PTH, AVP, isoproterenol, forskolin, GppNHp and MnCl2 were unaltered, NaF stimulation of ACA was also reduced (in contrast to the current observations). In the single study of adipocyte function from SHR, reduced lipolysis in response to noradrenaline was reported (Chiappe de Cingolani et al. 1988).

Hypertension of different aetiologies-genetic and reno-vascular hypertension:

Study of acquired hypertension, for example the 2K,1C Goldblatt model of renal hypertension (analagous to renal artery stenosis) allows dissociation of the effects of hypertension from possible underlying genetically determined membrane defects which might predispose to hypertension. In contrast to the SHR, and in

common with the human condition, the Goldblatt hypertension model is essentially hyperreninaemic hypertension (at least in its early stages). In common with the SHR, the RHR exhibits reduced myocardial adenylyl cyclase response to ß agonists (Sharma et al.1982; Anand-Srivastava et al. 1983; Kumano et al 1983) and although basal or forskolin stimulated cyclase activities may be unaltered (Anand-Srivastava et al. 1983; Kumano et al 1983) or reduced (Sharma et al.1982; Kumano et al 1983; Anand-Srivastava et al. 1983) stimulation of cyclase via receptors (dopamine and adenosine) and activation of Gs/catalytic unit (NaF) appears to be reduced (Sharma et al.1982; Anand-Srivastava et al. 1983; Kumano et al 1983). Receptor changes have been reported. As in the SHR, divergent results have been reported; ß receptors may be increased (Kumano et al 1983) or reduced (Sharma et al.1982), and in addition, glucagon receptors may be reduced (Kumano et al. 1985).

Reduced G-protein activity may accompany both types of hypertension (Kumano et al 1983;Kumano et al. 1985; Asano et al 1988), suggesting an association with blood pressure change per se. Of interest is the observation in RHR that several changes may contribute to reduced ACA. The earliest change, occurring as early as 1-2 weeks after induction of hypertension (Kumano & Khairallah 1985), is reduction in G-protein activity (associated with unaltered catalytic unit activity (Kumano et al 1983)), followed subsequently by a decline in catalytic unit activity (Kumano & Khairallah 1985)(a change established at around 10 weeks) as a consequence presumably of sustained hypertension.

Downregulation of ß receptors may occur in both SHR and RHR (*vide infra*) possibly in association with uncoupling of G-protein from receptor in experimental hypertension but it remains unclear whether these changes are a consequence of excessive local or systemic catecholamines or whether the alterations in receptor characteristics are implicated in the pathogenesis of hypertension (Stiles, Caron & Lefkowitz 1984). Woodcock and Johnston (1980) have however demonstrated in the Goldblatt RHR model that receptor changes are tissue specific (reduced alpha and beta receptor concentrations occur only in myocardial membranes and not in renal or pulmonary membranes) (supporting current findings of differences in AC between SHR myocardium and liver) suggesting that a specific effect of enhanced sympathetic drive is more probable than a systemic increase in circulating catecholamines.

5.5. Conclusion

It has been demonstrated in this chapter that functional changes in ACA occur in SHR myocardium compared to WKY, but not Wistar control species. These changes are characterised essentially by relatively reduced stimulation of AC by agents that act on Gs or ligands whose receptors couple to AC via Gs. However, identical changes are seen in Wistar membranes. Alterations in the receptors per se cannot be excluded but concurrent reductions of responses to NaF and forskolin suggest that catalytic unit and/ or Gs or their mutual coupling may be abnormal. Murakami et al. (1987) previously showed altered Gs function in SHR myocardium (Gs function is significantly lower)

using a reconstitution assay based on G-protein cholate extracts from SHR. Neither Murakami using ADP ribosylation studies nor ourselves using specific antibodies could demonstrate any differences in amounts of Gi or Gs which could account for the functional differences. Similar changes have been reported in vascular smooth muscle plasma membranes from aorta (Anand-Srivastava 1988) and other vessels. No changes in ACA were seen in the current studies of non-vascular tissues (liver and renal cortical plasma membranes) from the three species. Thus a ubiquitous, perhaps genetically determined, abnormality of membrane function in SHR cannot be implicated. There is however, a surprising degree of similarity between the AC activities in rat myocardium in response to epinephrine infusion and those seen in SHR myocardium raising the possibility that the process of heterologous desensitisation which accounts for the former may also explain the latter.

Desensitisation has been catagorised into 'homologous' and 'heterologous' (Su et al. 1980). The former is agonist specific and is characterised by reduced responsiveness to a ligand due to reduction in its binding sites (Mukherjee et al. 1975) and/or receptor-catalytic unit coupling (Mukherjee & Lefkowitz 1976). The latter is a complex process characterised by reduced response to a number of hormones and other activators of AC after chronic exposure to an agent. Several mechanisms may contribute to heterologous desensitisation as demonstrated by Tanif & Harbon (1987), who characterised the heterologous desensitisation observed in the rat uterine AC during gestation

(presumably in response to steroid hormones): heterologous desensitisation was mediated by altered function and altered levels of G-proteins as well as altered receptor function. Thus if heterologous desensitisation is invoked to explain the observed changes in AC in SHR myocardium compared to WKY which are consistent with altered G-protein function, this begs the question as to its cause. The SHR is characterised by a number of physiological and biochemical abnormalities (Yamori 1983); it is possible that increased activity of the sympathetic nervous system which has a key role in inititiating hypertension and which is reflected in increased plasma noradrenaline (Nakamura & Nakamura 1979) and increased sypathetic neuronal discharges (Judy, Watanabe, Murphy, Aprison & Yu 1979) could play a role in much the same way as adrenaline infusion has been shown to induce a state of heterologous desensitisation (Kumano & Khairallah 1984). However, if this mechanism were operating then greater differences in ACA might be expected between SHR and Wistar controls. The reasons for the differences between WKY and Wistar rats is not clear. The current studies, however, do emphasise the problems inherent in studies of the SHR. The absence of an isogenetic control species raises the distinct possibility that the differences observed in our studies may relate to the inherent genetic heterogeneity among the species studied: this problem cannot be circumvented. The inclusion in our study of the second control species (Wistar) highlights the dangers of the customary comparison between SHR and WKY species. Further studies are planned in the Milan rat-another model of genetic hypertension for which an isogenetic control

exists, to ascertain whether our observed changes in ACA are seen in this model also.

Chapter 6

Glucocorticoid hypertension: effect of dexamethasone treatment of rats on G-protein levels and/or function in brain, renal cortex, myocardium and vascular tissue.

6.1.General Introduction to Chapters 6 & 7.

6.1.1. Glucocorticoids and essential hypertension:

Human essential hypertension precludes, by definition, a major underlying role for steroid abnormalities in its pathogenesis. Early studies which sought to link EH to steroid abnormalities through the identification of abnormal accumulation of unusual steroids or steroid metabolites have proved equivocal: 18-hydroxysteroids and 19nordeoxycorticosterone have previously been reported to be elevated in EH although subsequent studies have failed to confirm this (Griffing, Dale, Holbrook & Melby 1985; Gomez-Sanchez, Holland & Upcavage1985). Enhanced aldosterone secretion in response to AII (Kisch, Dluhy & Williams 1976) occurs in a minority of EH (Dluhy, Bavli, Leung et al.1979) and probably reflects an abnormality of the renin, angiotensin system rather than a primary adrenal phenomenon (Wisgerhof & Brown 1979). The most promising support to date for a role for glucocorticoids in the pathogenesis of EH was provided by Watt and colleagues (Watt, Harrap, Foy et al. 1990) who reported that adolescent offspring of parents whose blood pressure is at the upper end of the range had significantly higher plasma cortisol than in the adolescent offspring of children whose parents have lower blood pressure (without necessarily

being abnormal when the disorder is fully established). However, this finding contrasts with previous work showing that hypertensives and their first degree relatives exhibit reduced mid-afternoon plasma cortisol concentrations and reduced cortisol binding capacity (unchanged free cortisol) (Nowaczynski, Murakami, Wilkins & Levin 1986). There is thus some evidence to support a role for glucocorticoids in the pathogenesis of essential hypertension but at present the case remains 'not proven'.

6.1.2. Steroids and hypertension

Four classes of steroid hormones have been shown to produce hypertension (see review Coghlan, Butkus, Denton et al. 1980): androgens, oestrogens, mineralocorticoids and glucocorticoids. Androgens (in high doses) inhibit 11 hydroxylation which permits an ACTH driven excess of deoxycoricosterone which causes hypertension through its mineralocorticoid actions. Oestrogens primarily cause elevation of blood pressure through increased levels of angiotensin II consequent upon elevation of renin substrate. Mineralocorticoids cause hypertension through salt and water retention.

6.1.3. Glucocorticoid hypertension

Glucocorticoid hypertension, although the commonest cause of steroid hypertension in clinical practice, is the least understood. The association of glucocorticoids with hypertension is however well established; more than 70% patients with Cushing's syndrome have hypertension (Plotz, Knowlton & Ragan 1952; Raker, Cope & Ackerman 1964; Orth & Liddle 1971) The

hypertension is independent of ACTH, and is rather less frequent in recipients of steroid therapy (prevalence 5-25%) where there is less likely to be contribution to blood pressure elevation from adrenal mineralocorticoids: its incidence is however dependent upon type and dosage of the steroid used and is higher in association with impaired renal function (David, Grieco & Cushman 1970).

6.1.4. Features of glucocorticoid hypertension

Certain features of glucocorticoid hypertension are well established: the onset of glucocorticoid hypertension is of rapid-onset (blood pressure elevation demonstrable at 24h) and is induced by receptor dependent mechanisms (as administration of steroids with antiglucocorticoid properties can prevent or reverse the early phase of glucocorticoid hypertension (Grunfeld, Eloy, Movra et al. 1985)).

6.1.5. The pathogenesis of glucocorticoid hypertension

It is doubtful whether any single mechanism explains the pathogenesis of glucocorticoid hypertension. However, a number of glucocorticoid induced changes have been observed *in vivo* and may contribute; these include alterations in plasma volume, the renin-angiotensin system, prostaglandin synthesis and in vascular reactivity.

6.1.6. Glucocorticoids and plasma volume

Glucocorticoid-mediated plasma volume change has been implicated in the pathogenesis of glucocorticoid hypertension;

glucocorticoids effect an increase in plasma volume by causing shift of water from intracellular to extracellular compartments. However, while this change undoubtedly occurs it is unlikely to cause the hypertension as blood pressure elevation precedes plasma volume change (Haack, Mohring, Petri & Hackenthal 1977): whereas blood pressure elevation is demonstrable at 24h, plasma volume elevation is a late (possibly beyond day 5) event. In fact plasma volume actually falls early (Grunfeld et al. 1985) at a stage when hypertension is already established. Although said to be independent of Na^+ status (Knowlton et al. 1952; Haack et al. 1977), uncertainty regarding the role of sodium status has been increased by species differences.

6.1.7. Glucocorticoids and the renin-angiotensin system

Glucocorticoid excess may stimulate the renin-angiotensin system by increasing plasma renin substrate (Krakoff, Nicolis & Amsel 1975); angiotensinogen synthesis by liver is increased by glucocorticoids and reduced by adrenalectomy (Hasegawa, Nasjletti, Rice & Masson 1973).

6.1.8. Glucocorticoids and prostaglandin synthesis

Glucocorticoids cause major changes in prostaglandin synthesis; however, contrasting effects are seen *in vitro* and *in vivo* . Glucocorticoids can prevent prostaglandin synthesis in a number of *in vitro* systems including cultured cells (Cloix, Colard, Rothhut & Russo-Marie 1983) and whole tissue preparations (for example rat renal papillary slices & aorta (Jeremy & Dandorra

1986). Inhibition of prostaglandin synthesis is mediated by glucocorticoid-induced synthesis of lipocortin (Wallner, Mattaliano, Hession et al. 1986) a protein which inhibits phospholipase A2 (phospholipase A2 catalyses the hydrolysis of phosphatidylcholine to lysophosphatidylcholine and arachidonic acid); inhibition of phospholipase A2 interferes with prostaglandin generation by reducing the availability of fatty acid substrates (from phospholipids) (Flower & Blackwell 1979, Hirata, Schiffman, Venkatasubranian, salomon & Axelrod 1980).

Exposure to glucocorticoids *in vivo* may however, involve more complex mechanisms than the induction of lipocortin synthesis as increased urinary excretion of prostaglandins (for example PGE2) have generally (but not invariably (Handa, Kondo, Suzuki & Saruta 1983) been reported in animal and human studies; although lipocortin may be induced *in vivo* other factors may be more important in determining the net effect on prostaglandin synthesis, for example, increased free fatty acid availability, increased prostaglandin synthesis or reduced 15HO prostaglandin generation, where the net effect is enhanced production of prostaglandins.

Glucocorticoid induced alterations of prostaglandin synthesis could contribute to changes in vascular physiology: glucocorticoids may reduce prostacyclin synthesis in vessel walls in ex vivo studies of abdominal aorta; such a mechanism could at least partly explain (by attenuating the effects of vasoconstrictors) increases vascular response to noradrenaline or angiotensin II seen experimentally and clinically.

6.1.9. Glucocorticoids and vascular reactivity

The regulation of blood pressure by direct action of corticosteroids on blood vessel walls was first hypothesised by Tobian & Binion (1952). The recent demonstration of glucocorticoid receptors in arterial vessels from rats (Meyer & Nichols 1981), rabbits (Kornel, Kanamarlapudi, Travers et al. 1982) and humans (Scott, Lawrence, Nguyen & Meyer 1987) is consistent with this concept, but begs the question as to the mechanism of action. There is, however, substantial evidence to implicate glucocorticoids in the regulation of pressor responsiveness which raises the possibility at least of a pathological role for glucocorticoids in the aetiology of hypertension. The evidence linking glucocorticoids to pressor responsiveness will be reviewed briefly.

Increased vascular reactivity to infused pressor substances has been observed in patients with Cushing's syndrome (Mendlowitz, Gitlow & Naftchi 1958). Furthermore similar effects can be demonstrated in humans and in a number of animal species under experimental conditions in response to several pressor agents including adrenaline, noradrenaline, isoproterenol, phenylephrine, vasopressin (AVP), angiotensin II (AII) and KCl.

Pressor responsiveness to adrenaline and noradrenaline is increased within a few minutes of glucocorticoid administration to humans (Reis 1960), dogs (Besse & Bass 1966) and rats (Kalsner 1969) using conjunctival vascular bed (*in vivo*)(Reis 1960), and aortic strip (Fowler & Chou 1961; Kalsner 1969) preparations.

Longer term exposure of rats (Russo, Fraser & Kenyon 1990; Schomig, Luth, Dietz & Gross 1976; Krakoff, Selvadurai & Sutter 1975; Iijima & Malik 1985; Handa, Kondo, Suzuki & Saruta 1984), dogs (Lefer, Manwarring & Verrier 1966) and humans (Whitworth, Connell, Lever & Fraser 1986; Sudhir, Jennings, Esler et al. 1989) to DEX (Russo et al.1990; Iijima & Malik 1985; Handa et al. 1984), corticosterone (Schomig et al. 1976), methylpredinsolone (Krakoff et al. 1975) and hydrocortisone (Whitworth et al. 1986; Sudhir et al. 1989) for between two (Handa et al. 1984) and 14d (Russo et al. 1990; Krakoff et al. 1975; Iijima & Malik 1985) induces enhanced pressor responsiveness to noradrenaline (Russo et al. 1990; Schomig et al. 1976; Handa et al. 1984), AII (Krakoff et al. 1975), AVP (Iijima & Malik 1985) and phenylephrine (Whitworth et al.1986). It is noteworthy, however, that responses to all pressor agents were not necessarily enhanced concurrently (for example, Russo et al. 1990; Schomig et al. 1976; Krakoff et al. 1975; Iijima & Malik 1985).

It is thus clear that the effect is not restricted to any particular glucocorticoid (although may not necessarily occur with all glucocorticoids (Krakoff et al. 1975; Elijowitch & Krakoff 1980; Kohlman, Ribeiro, Marson, Sarogoca & Ramos 1981) and is common to several species including humans. The alteration in pressor responsiveness occurs rapidly and in response to several pressor agents (but not necessarily concurrently). However, most studies (but not all-Russo et al.1990) have studied the effects of high 'suprapharmacological' concentrations. As will be discussed further later, gluco-

corticoids may exhibit mineralocorticoid effects when used in high doses; as mineralocorticoids can independently alter vascular reactivity, difficulty may arise in differentiating the mineralocorticoid from the glucocortiocid contribution to the altered vascular reactivty. However, Russo et al. (1990) have shown changes in vascular responsiveness in a rat model pretreated with DEX 2µg/d for 14d: this is similar to the model used in our in vivo studies and represents a more physiological dosage of glucocorticoid at which mineralocorticoid actions are less likely.

The possible relevance of altered vascular reactivty to the pathogenesis of glucocorticoid hypertension has been raised by the observation that the enhanced pressor response to noradrenaline precedes the development of hypertension in DEX treated rats (Handa et al.1984). However, it is unclear whether the degree to which glucocorticoids can enhance pressor responsiveness in human studies could be relevant to the pathogenesis of hypertension (Whitworth et al. 1986) Pressor response in steroid induced hypertension in man).

Several mechanisms have been proposed to account for the glucocorticoid mediated changes in vascular reactivity. Handa and colleagues (1984) showed that indomethacin administration could block the glucocorticoid associated change in pressor responsiveness raising the possibility that altered prostaglandin synthesis could be relevant. Supported by the previous observation that alteration of prostaglandins (specifically, noradrenaline-mediated stimulation of PGE2) reduces vascular

reactivity (Malik, Ryan & McGiff 1976; Armstrong, Thirsk, Biol & Salmon 1979; Lippton, Chapnick, Hyman, Kadowitz 1979; Jackson & Campbell 1980) the action of glucocorticoids to inhibit prostaglandin synthesis (vide supra) has been implicated in the mechanism by which glucocorticoids alter vascular reactivity. Despite the observation that PGI2 administration abolishes the increase in hind limb vascular reactivity to noradrenaline produced by corticosterone in the rat (Rascher, Dietz, Schomig, Burkart & Gross 1980) the studies of Iijima & Malik (1985) cast doubt on a significant contribution of altered prostaglandin levels to the enhancement of the vasoconstrictor response to AVP; Iijima & Malik (1985) demonstrated that dexamethasone attenuated the rises of prostaglandins (6ketoPGF1alpha & PGE2) in response to noradrenaline, AVP and AII but were able to show altered vascular reactivity only to AVP.

Kalsner (1969) proposed alternatively the glucocorticoid effect was mediated by inhibtion of catecholamine hydrolysis (by COMT). Other possble mechanisms could account for the glucocorticoid-mediated change in vascular reactivity including structural change in vessel wall (although this seems unlikely given that changes occur as early as 15min. after exposure to glucocorticoid (Kalsner 1969)), electrolyte changes, alteration of the Na/K pump and altered membrane permeability. In chapters 6 & 7 the possible role for glucocorticoid mediated changes in G-proteins in altered vascular responsiveness and in glucocorticoid hypertension will be studied in detail.

6.2. Introduction to chapter 6

Glucocorticoids and experimental hypertension:

The elucidation of the possible physiological role of glucocorticoids in regulating G-protein function or levels has been hampered by the lack of a definitive model in which glucocorticoid effects can be exclusively and specifically studied *in vivo*. However, two approaches have been adopted to address this problem. The effects of glucocorticoids have been inferred from studies of adrenalectomy (vide infra), followed by glucocorticoid replacement. In addition, a number of glucocorticoids have been administered to otherwise normal rats with successful induction of hypertension; these include cortisone (Knowlton & Loeb 1957), corticosterone (Haack et al.1977), methylprednisolone (Krakoff, Selvadurai & Sutter 1975) and dexamethasone (Suzuki et al.1982). Irrespective of the glucocorticoid used however, there is a risk that the resulting elevation of blood pressure could result, not from the direct action of the glucocorticoid but from ancillary mineralocorticoid properties (sodium and fluid retention with expansion of ECV and body weight gain); the potency of these non-glucocorticoid effects varies among the glucocorticoids and with the dosage used but may be prominent given the common practice to administer very high, 'supra-pharmacological' doses of these glucocorticoids.

The preferred model depends on glucocorticoid administration to animals with intact adrenal glands. Only one previous study has examined the effects of *in vivo* glucocorticoid on G-protein levels in a particular tissue; Saito et al. (1989) showed that 100mg corticosterone (the predominant glucocorticoid in rats)

administered subcutaneously over 7d (to Sprague Dawley rats) resulted in 40% elevation of Gsα and 15% reduction of Giα (with similar changes in the corresponding mRNAs) in brain which raises the possibility of a role for glucocorticoids in G-protein regulation. Corticosterone, however, is not the ideal glucocorticoid for elucidating mechanisms of glucocorticoid hypertension as it exhibits weak sodium retaining properties; this mineralocorticoid action probably accounts for the plasma and extracellular fluid volume expansion (with renin suppression) and fall in plasma potassium which accompanies high dose corticosterone administration (for example, 10mg/ rat /day) (Haack et al. 1977).

Full details of the model used for the following *in vivo* studies of glucocorticoid hypertension have been previously described (Tonolo et al. 1988). This model allows assessment of the *in vivo* effects of relatively low doses of exogenous glucocorticoids (likely to be high physiological/ low pharmacological doses) administered to animals with intact adrenal glands; briefly, it is based on the administration (by mini-osmotic pump-see methods) to rats of dexamethasone, a gluco-corticoid with minimal mineralocorticoid receptor activty (Bia et al. 1982). Dexamethasone causes a dose related rise in blood pressure with failure of weight gain (the change in body weight correlates inversely with dexamethasone dosage). At the dosage of dexamethasone used in this study (5μg/day), a 20mmHg increment in SBP and body weight gain of around 40% (v 100% control) would be anticipated, without significant mineralocorticoid effects as reflected in plasma renin, aldosterone,

sodium and potassium (none of which is altered) (Tonolo et al. 1988). 5μg/day dexamethasone represents a substantially lower dose of glucocorticoid than previously used (doses of up to 375x greater have been given (reviewed by Tonolo et al. 1988)) and yet results in hypertension with minimal effective weight loss (actually failure to gain weight to the extent of untreated rats). A theoretical concern is that the substantial weight loss and the underlying catabolic state which accompany higher doses could *potentially* have adverse effects on plasma membrane function and composition and thus could possibly cause artifactual changes in G-protein expression and/or function.

The purpose of this study was to assess whether induction of glucocorticoid hypertension (using nearer physiological concentrations of the glucocorticoid, dexamethasone, in vivo) was associated with evidence of either altered G-protein function or levels in a number of organs implicated in the control of blood pressure.

6.3. Materials and Methods

Three groups of adult male 180g Sprague Dawley rats were studied (each group consisted of 6 treated & 6 control rats). Dexamethasone (Decadron, Merck, Sharp and Dohme, Hoddesden, Hertfordshire, UK) was administered in 0.154 mol/l NaCl through mini-osmotic pumps (Alzet Model 2002, Palo Alto, California, USA) which were implanted subcutaneously. The pumps released 5μg/d DEX over 14d (verified by previous studies (Tonolo et al. 1988) Control rats received vehicle alone.

Body weight and mean systolic BP were recorded. BP was measured in triplicate in warmed, unrestrained conscious rats by the tail cuff method (McAreavey et al. 1985) using a W & W Electronics recorder (Basle, Switzerland)(see table 15).

Table 15. Details of one (of three) group of Sprague-Dawley rats studied-showing effects of dexamethasone 5μg/d for 14d.

	CON	DEX
n	6	6
INITIAL WT. (g)	186 ± 4	186 ± 4
WT. GAIN (g)	6.5 ± 0.2	3.1 ± 0.3**
FINAL BP (mmHg)	147 ± 4	175 ± 5**
GLUCOSE (mmol/l)	3.4 ± 0.2	3.1 ± 0.2
total organ mass		
HEART (g)	5.53	5.16
KIDNEY (g)	5.98	5.29
BRAIN (g)	9.65	10.56

(mean ± sem) ** $p<0.0001$

After 14d DEX the animals were sacrificed by cervical dislocation-heparinised blood was collected for glucose estimation (2.9.)(table 15). Brains, hearts, kidneys and mesenteric vascular beds were recovered on ice and plasma membrane homogenates were prepared as described (sections 2.3.4., 2.3.3., 2.3.5. & 2.3.6.). G-protein function was assessed

indirectly by studies of ACA (section 2.8., mix A).

G-protein levels by Western blotting (2.4.1.) using the antibodies listed in table 4 (with exception of antiserum against Gi3, as exploratory blots had failed to identify this G-protein in any of the membrane preparations studied).

Protein concentrations within the plasma membrane homogenates were measured by the modified Lowry protocol (section 2.6.) and 5' nucleotidase activity was assessed within the various membrane fractions as a limited marker of altered membrane composition (section 2.7.)(see table 16).

Table:16. 5' Nucleotidase activities of tissues obtained from SD rats treated *in vivo* with DEX 5μg/d for 14d.

MEMBRANE PREP.	CON	DEX
BRAIN	45.6	39.8
LIVER	164.4	199.1
MYOCARDIUM	566.8	473.9
RENAL CORTEX	462.1	465.5

6.4. Results:

Immunoblotting data (with typical blots) for the various G-protein subunits identified in the plasma membrane preparations studied are shown if figs. 20 (brain), 21 (myocardium), 22 (renal

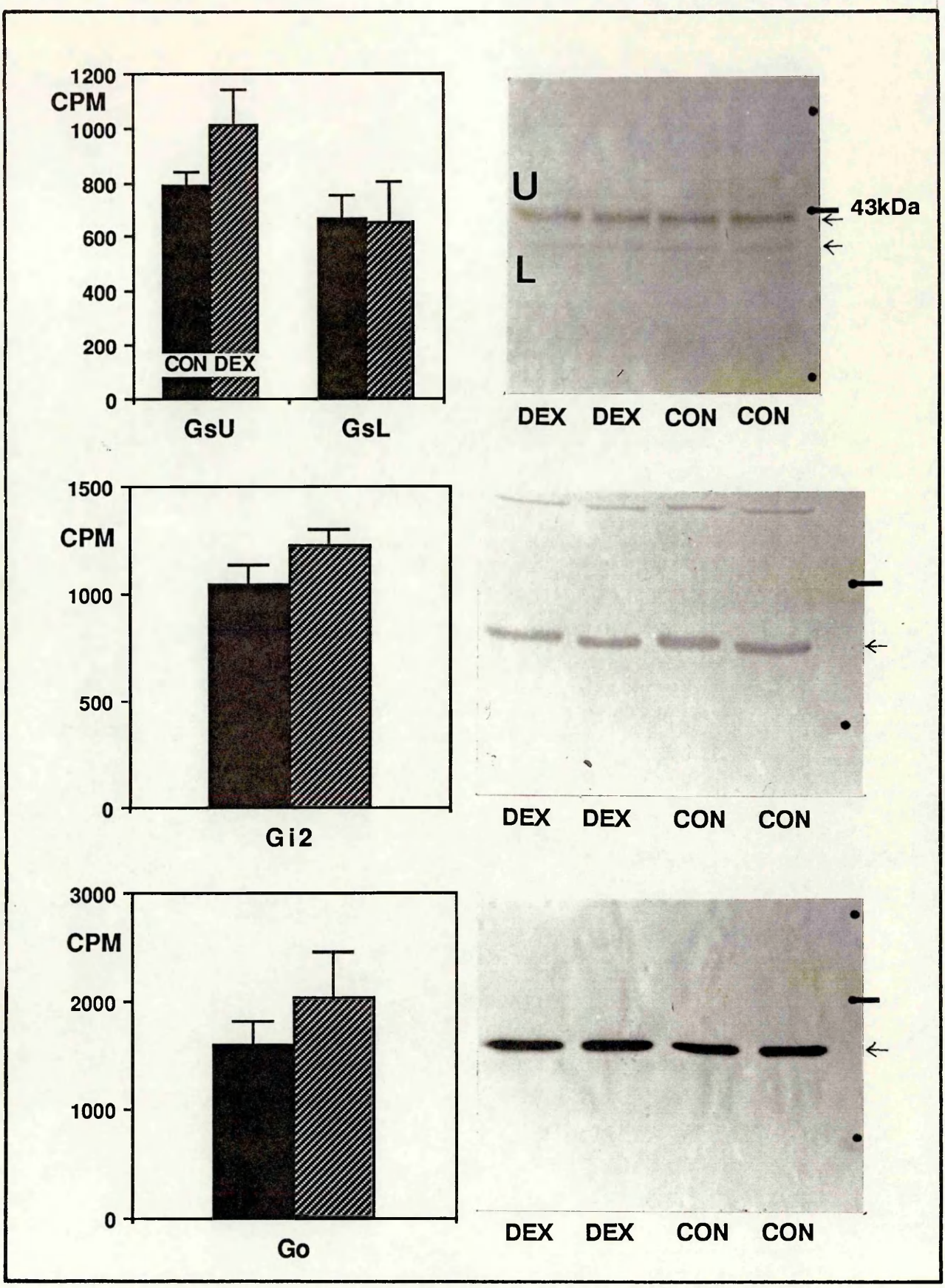


Fig.20. Effect of in vivo dexamethasone (5μg/d for 14d) on G-protein levels Gsα, Giα2 and Goαin rat brain. (mean ± sem, 6exp.) DEX=dexamethasone treated, CON=control. L panels=graphs of cpm from 125I labelled bands (mean ± sem). R panels=typical blots.

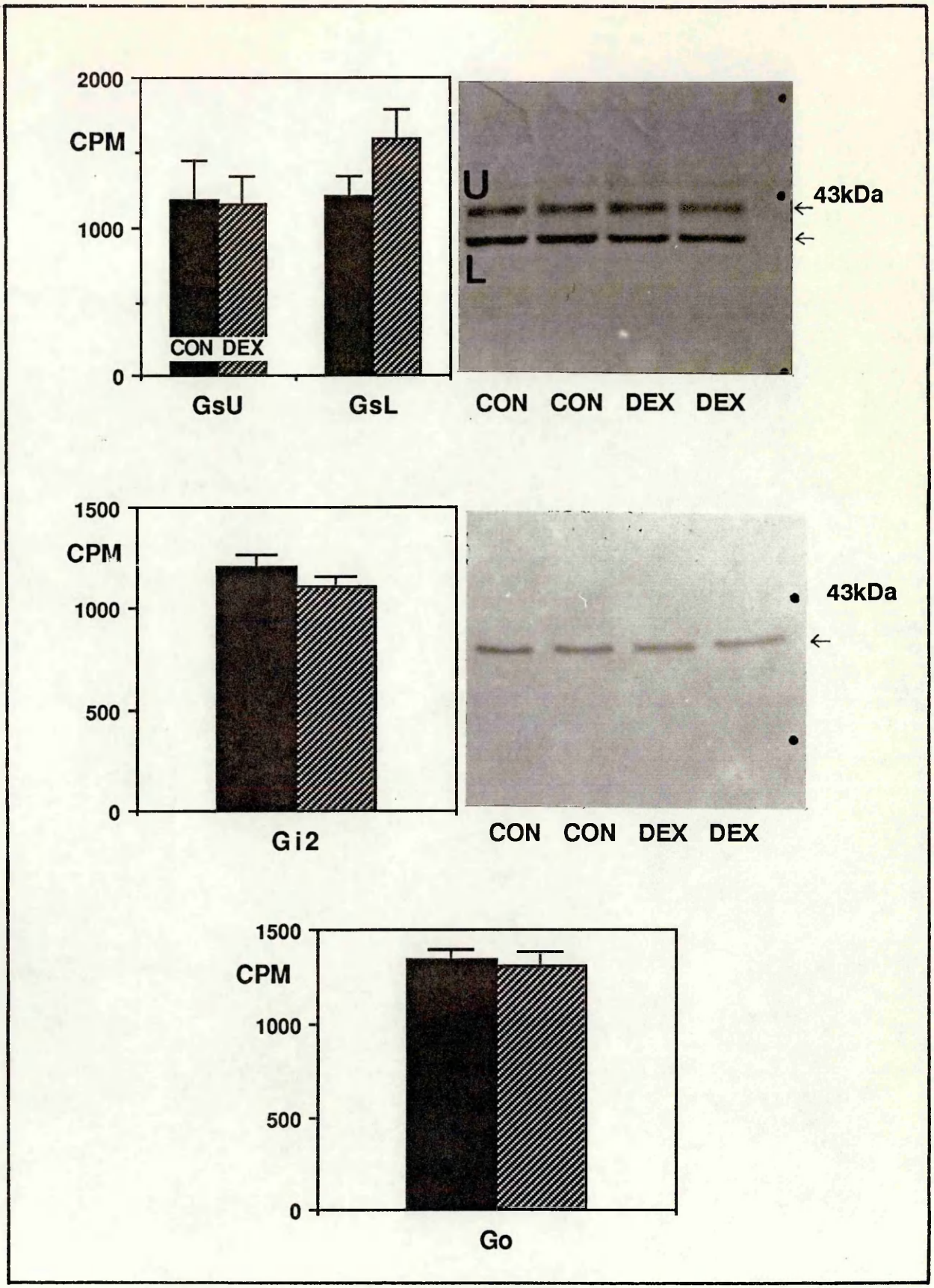


Fig.21. Effect of in vivo dexamethasone (5μg/d for 14d) on G-protein levels Gsα, Giα2 and Goαin rat myocardium.(mean ± sem, 6exp.) DEX=dexamethasone treated,CON=control. L panels=graphs of cpm from 125I-labelled bands (mean ± sem). R panels= typical blots.

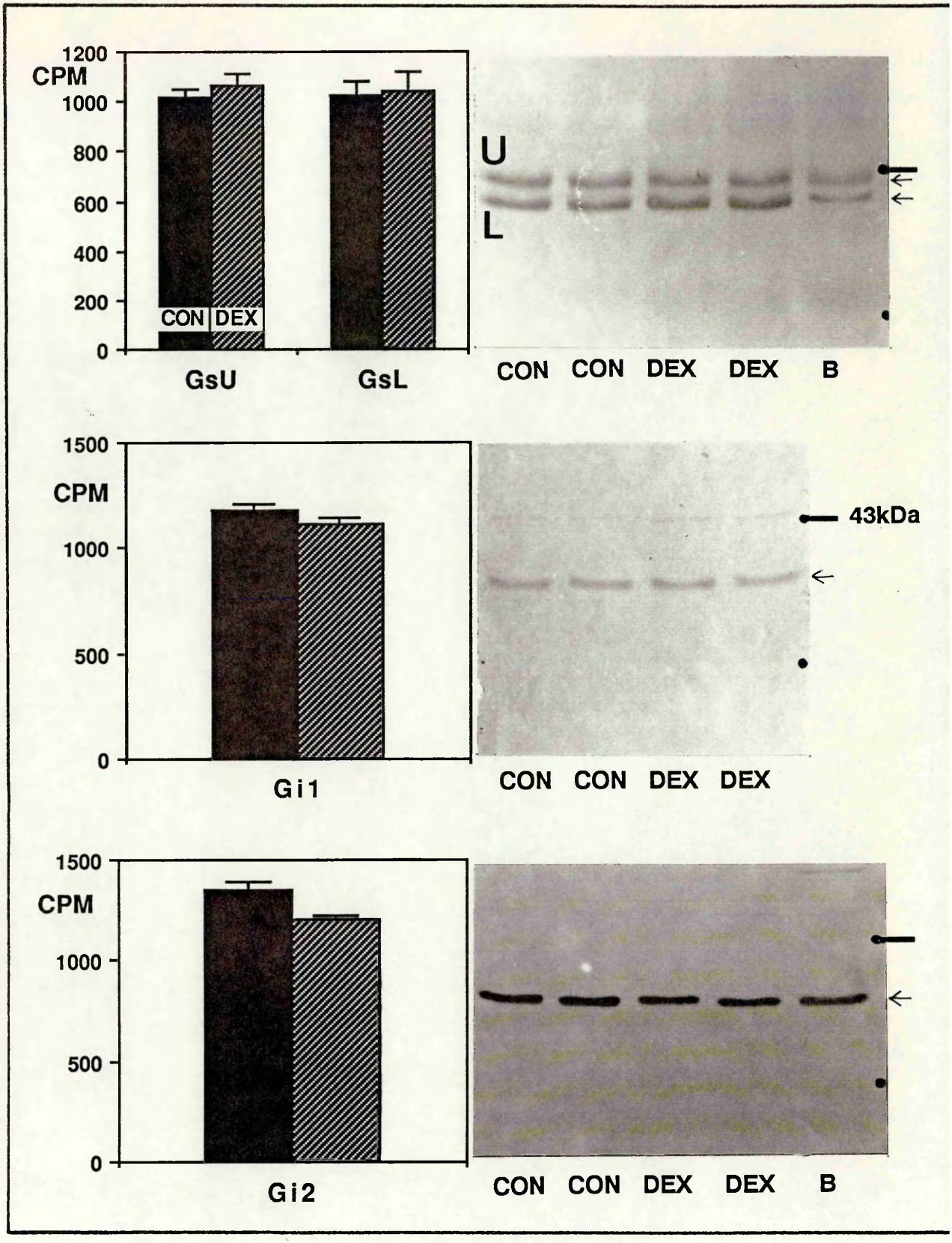


Fig.22. Effect of in vivo dexamethasone (5μg/d for 14d) on G-protein levels Gsα, Giα1 and Giα2 in rat renal cortical plasma membranes.(mean ± sem, 4-6exp.) DEX=dexamethasone treated, CON=control. L panels=graphs of cpm from 125I-labelled bands (mean ± sem). R panels=typical blots.

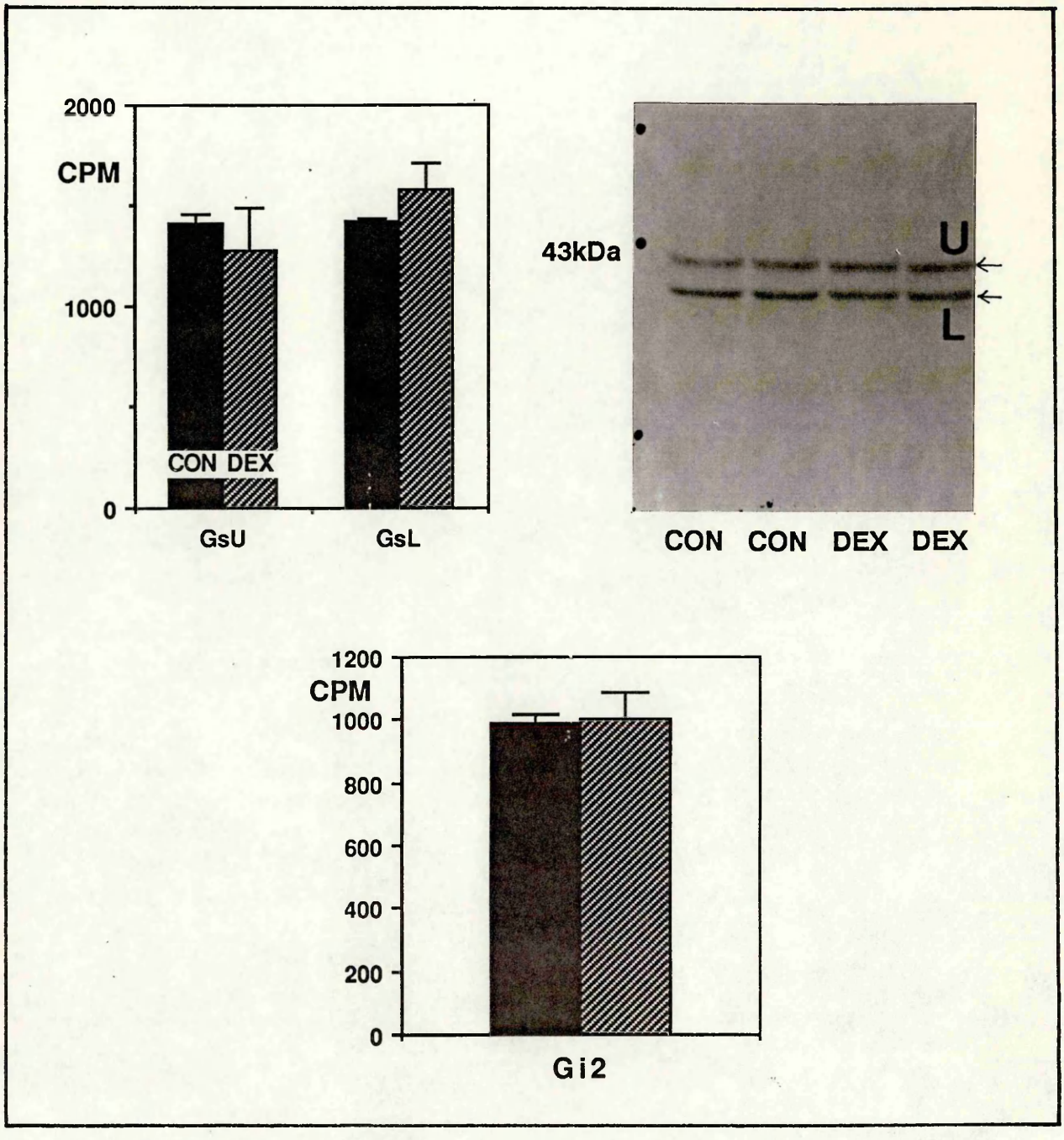


Fig.23. Effect of in vivo dexamethasone (5μg/d for 14d) on G-protein levels Gsαand Giα2 in rat liver plasma membranes.(mean ± sem, 6exp.) DEX= dexamethasone treated, CON=control. L panels= graphs of cpm from 125I-labelled bands (mean ± sem). R panels=typical blots.

cortex) and 23 (liver). No significant differences in G-protein levels were seen in any of these blots of membranes derived from 4 organs; DEX treated ß subunits were studied only in myocardial plasma membranes and were shown to be slightly reduced (CPM: 722 ± 35 v 940 ± 40 (control) n=4 exp.). Western blot data for mesenteric vasculature are shown in table 17.

Table 17: Levels of G-protein subunits (determined from cpm, expressed as mean ± sem of data) in mesenteric vasculature from DEX-treated and control rats.

G-PROTEIN SUBUNIT	CON	DEX	NO. EXP.
Gs 42 kDa	1652 ± 23	1573 ± 33	6
Gs 40 kDa	834 ± 68	816 ± 54	6
Gi2	565 ± 35	671 ± 44	4

Adenylyl cyclase data are available from renal cortical plasma membranes (fig. 24) and from myocardial and mesenteric plasma membranes (table 18).

6.5. Discussion

The experimental model of glucocorticoid hypertension responded to 5µg/d DEX infusion (s.c.) in a predictable, reproducible manner (Tonolo et al. 1988): tail-cuff BP increased by 30mmHg- representing an increase of around 25% with minimal compromise of weight gain (table 15). Blood glucose concentration was unaffected by DEX exposure.

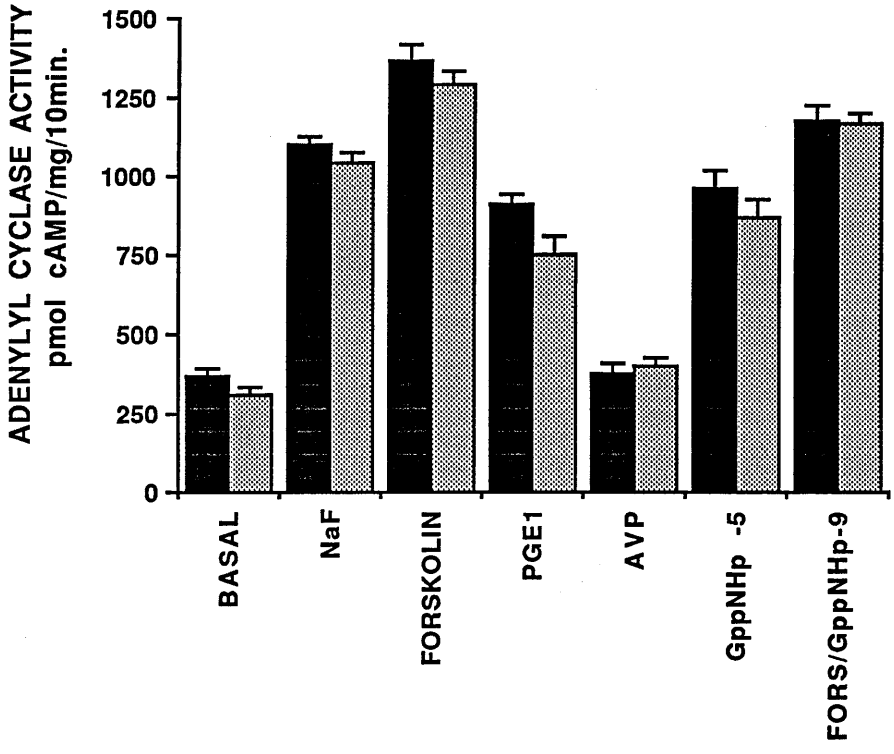


Fig.24. Adenylyl cyclase activities in plasma membranes derived from renal cortex of DEX (5μg/d for 14d) treated rats (STIPPLED BARS) and control (SOLID BARS). Data expressed as absolute cyclase activities, mean ± sem of 3 experiments each wih 4 replicates. Assay conditions and ligands studied are indicated along abscissa.

Table 18: Adenylyl cyclase activities in plasma membranes derived from myocardium and from mesenteric vasculature. ACA represented as pmol cAMP/mg/10 min., mean ± sem of 3 exp. (each with 4 replicates).

MYOCARDIUM LIGAND	CON	DEX
BASAL	412.5 ± 67.2	367.4 ± 16.3
MnCl2	433.6 ± 54.8	394.7 ± 36.4
NaF	1317.3 ± 172.8	1121.4 ± 126.1
FORSKOLIN	6177.6 ± 387.5	5879.5 ± 276.4
ISOPROTERENOL	1158.1 ± 136.4	987 ± 136.3
MESENTERY LIGAND	**CON**	**DEX**
BASAL	610.4 ± 61.8	604.7 ± 65.4
FORSKOLIN	3158.4 ± 97.5	2986.5 ± 110.6
ISOPROTERENOL	1107.8 ± 78.8	985.6 ± 80.7

Myocardial weight was reduced v CON suggesting that myocardial hypertrophy had not developed during the period of study.

Immunoblotting studies for G-protein subunits failed to identify any relevant changes in brain, myocardium, renal cortex, liver and mesenteric vasculature (figs. 20-23, table 17). These organs were studied as they represent the organs with roles in BP regulation; liver was included because of its central role in metabolism. None of the membrane preparations is a highly purified preparation; the myocardial, renal cortical and hepatic preps. were partially purified by differential centrifugation, while the preparations derived from brain and mesentery are likely to be even less purified. It is possible that the relative impurity of the membranes studied, perhaps by including either relativley small amounts of plasma membrane or by including plasma membrane fragments derived from other (contaminating) cell types could have reduced the specificity of the assays. Only slight differences were seen in 5'ND activities (measured only once per membrane prep.) and there was no overall trend in activities amongst the four tissues in which this was measured (table 16) excluding an artifactual contribution to our results from glucocorticoid mediated changes in plasma membrane content of our study samples.

ACA studies in renal cortical, myocardial and mesenteric plasma membranes showed that DEX did not affect enzyme activity.

The current results show important differences from those of previous workers. Only one previous study has examined the effects of *in vivo* glucocorticoid on G-protein levels in a particular tissue, in animals which have not been adrenal-ectomised; Saito et al. (1989) showed that 100mg cortico-

sterone (the predominant glucocorticoid in rats) administered subcutaneously over 7d (to Sprague Dawley rats) resulted in 40% elevation of Gsα and 15% reduction of Giα (this latter change was not apparently statistically significant)(with similar changes in the corresponding mRNAs) in brain which raises the possibility of a role for glucocorticoids in G-protein regulation. Corticosterone, how-ever, is not the ideal glucocorticoid for elucidating mechanisms of glucocorticoid hypertension as it exhibits weak sodium retaining properties; this mineralo-corticoid action probably accounts for the plasma and extra-cellular fluid volume expansion (with renin suppression) and fall in plasma potassium which accompanies high dose cortico-sterone administration (for example, 10mg/ rat /day) (Haack et al. 1977).

The bulk of studies assessing the effect of glucocorticoids have been in animals previously subjected to adrenalectomy. The reported effects of adrenalectomy on G-proteins are summarised in table 19.

Clearly adrenalectomy effects changes in G-protein subunits which vary depending on the particular subunit and on the tissue studied.

Glucocorticoid administration to adrenalectomised rats generally restores G-protein levels to those of the intact animal (Saito, Guitart, Hayward et al. 1989; Haigh, Jones & Milligan 1990). However, it does appear that DEX exerts differential effects on G-protein subunit expression and may

control other processes which modulate G-protein levels. Ros and colleagues (1989) showed that 4d DEX at a dose of 0.4mg/d restored levels

Table 19: Effect of adrenalectomy on levels of G-protein subunits *in vivo*.

TISSUE	Gsα	Giα	Goα	ß	REFERENCE
ADIPOSE		↓[1]	↓[1]		De Mazancourt et al. '89
ADIPOSE	↓[3,4]			↓[2,4]	Ros et al. '89
BRAIN	↓[3,4]	↑[4]			Salto et al. '89
LIVER	↓[3]	↑[1]	↑[1]		Garcia-Sainz et al. '89
AORTA	↑[3]	↓[3]	→[3]	→[3]	Haigh et al. '90

Note:
Method of assessment of G-proteins
1. Pertussis toxin-ADP ribosylation of 40-41kDa substrate= Gi + Go.
2. Western Blots with specific antisera
3. Cholera toxin-ADP ribosylation
4. Based on mRNA studies

of Gs in adipose tissue from adrenalectomised rats to those of the intact animal without apparent change in Gsα mRNA, while ß subunit levels were increased to final levels some 30% higher than in the intact animal in parallel with alterations of mRNA. Haigh et al. (1990) showed that DEX administration to adrenalectomised animals in vivo essentially restored Gsα (which had

been increased 210% by adrenalectomy) and Giα (53% reduction with adrenalectomy) to control levels: ß subunit was reported to be increased by DEX (levels unaltered by adrenalectomy). However, in Haigh's study doses of DEX 20x those used here were administered to the animals. Haigh reported that although adrenalectomy did not alter ß subunit levels in aorta, DEX increased the amount of this subunit by 90%. Their assessment was made on the basis of densitometric assessment of blots rather than direct counting of radiolabelled second antibody; furthermore in our hands antibody raised against ß subunit invariably produced the poorest blots of all those prepared-probably due to low concentrations of this subunit in all preps. (despite generally using 4-5x more protein per blot) that confirmation of this finding by other groups is required. ß subunit was only assessed in myocardium from our hypertensive rats and was shown to be lower, if anything (CPM: DEX-722 ± 35 v CON-940 ± 40) in the DEX treated group.

Adrenalectomy is, however, associated with multiple endocrine and metabolic effects; these include glucocorticoid, mineralocorticoid and catecholamine deficiencies. Although death from mineralocorticoid insufficiency can be prevented by maintenance on exogenous saline, experimental adrenalectomy is nonetheless associated with numerous metabolic derangements which could at least in theory contribute to changes in G-proteins. The potential influence of such confounding factors (which may stem not from glucocorticoid deficiency but from loss of catecholamines or of the mutual interaction between catechol-amines and glucocorticoids) are highlighted by a brief

review of the effects of adrenalectomy on the adrenergic-adenylyl cyclase system. Adrenalectomy is associated with pleotropic effects on the ß receptors (depending on the tissue studied; ß receptor number may be increased in liver (Guellaen, Yates-Aggerbeck, Vauquelin, Strosberg & Hanoune 1978), decreased in lungs (Mano, Akbarzadeh & Townley 1979) and fat or unaltered in myocardium (Davies, DeLean & Lefkowitz 1981). cAMP accumulation or ACA associated with catecholamines may be reduced (myocardium (Davies et al. 1981; Phorchirasilp & Melangkasombut 1982) adipose tissue (Allen & Beck 1972; Exton, Friedman, Hee-Aik et al.1972; Lamberts, Timmenmans, Krammer-Blankestijn & Birkenhager 1975) or increased (liver (Chan, Blackmore, Steiner & Exton 1979; Exton et al.1972; Bitensky et al. 1970; Leray, Chambaut, Perrenoud & Hanoune 1973) and brain (Mobley & Suler1980; Roberts & Bloom 1981). The physiological relevance of the subsequent administration of 'suprapharmacological' doses of glucocorticoids to such a system is debatable as major catabolic effects including negative sodium balance and weight loss (due to muscle and fat catabolism)(Haack et al. 1977; Handa et al. 1984) are inevitable at these high doses. The obvious clinical correlate of this model (which in practice bears little similarity) is the patient with Addison's disease in whom adrenal medullary function is preserved and for whom glucocorticoid replacement doses are restricted to 'near physiological' levels.

On the basis of adrenalectomy studies it is possible to conclude that glucocorticoids may have a role in regulating G-proten levels- but given the limitations of the model it would be

difficult to conclude that this amounts to anything more than a permissive role.

The current studies of ACA failed to reveal any differences in ACA responses in renal cortical (fig. 24), myocardial and mesenteric plasma membranes (table 19). This contrasts with the in vitro work which will be reviewed in chapter 7, and suggests that neither changes in G-protein function nor AC catalytic unit are implicated in the pathogenesis of glucocorticoid hypertension.

In conclusion, induction of hypertension in the rat, with dexamethasone concentrations which are nearer physiological glucocorticoid levels than used generally, is not associated with alterations in G-protein subunit levels (those which can currently be identified) nor G-protein function. Furthermore, no evidence of altered ACA in renal cortical, myocardial, nor mesenteric plasma membranes was found.

Chapter 7

Glucocorticoid hypertension: effect of dexamethasone on G-protein levels and function in VSMC.

7.1. Introduction

Glucocorticoids and adenylyl cyclase activity:

Dexamethasone (DEX) administration *in vitro* has been shown to increase adenylyl cyclase activity in vascular smooth muscle cells (VSMC)(from renal arteries)(Yasunari, Kohno, Balmforth et al.1989) and non-vascular cell lines including 132N1 astracytoma cells (Foster & Perkins 1977), cultured NRK fibroblasts (Johnson & Jaworski 1983), osteoblast cell line ROS 17/2.8 (Rizzoli, von-Tscharner & Fleisch1986, Rodan & Rodan 1986) and GH3 pituitary tumour cells (Chang & Bourne 1987). As will be discussed later, glucocorticoids can potentially modulate the adenylyl cyclase-second messenger pathway at several levels: there is increasing evidence that glucocorticoids can alter G-protein levels *in vitro* , in GH3 (rat pituitary cell line)(Chang & Bourne 1987) and the ROS 17/2.8 cells (Rodan & Rodan 1986). In both systems glucocorticoids (DEX 150nM for 3d or 30 nM for 4d) caused similar changes in Gs; cholera toxin substrate (Gs) was increased (40-70%). Contrasting effects of DEX on pertussis toxin substrate were reported (no change (Chang & Bourne 1987) and 20-50% increase (Rodan & Rodan 1986). In the former system corresponding changes were seen in G-protein mRNA species. Furthermore concurrent enhancement of ACA was observed (vide infra) although as discussed later altered G-proteins may not be the sole explanation for these changes. The

ability of cycloheximide to prevent changes in G-protein mRNA species (Chang & Bourne 1987) suggests that the glucocorticoid effect on G-proteins is mediated through altered protein synthesis.

The purpose of this study is to reassess the effects of glucocorticoids on cultured VSMC in vitro (these cells are representative of those from arteriolar resistance vessels) and to ascertain whether AC changes are associated with alterations in G-protein levels.

7.2. Materials and Methods

VSMC were isolated from the mesenteric vascular bed of 300-350g Spargue Dawley rats and cultured as described in section 2.4. Cells were subcultured as required up to 7th passage and were used for experiemnts when confluent. After exposure of cells to 10nM DEX or vehicle for 24-48h, plasma membrane homogenates were prepared as described (section 2.3.6.). A similar experiment was conducted after 48h pretreatment with anticortisol antibody (kindly donated by SAPU, Law Hosptial, Carluke) to ascertain whether the ambient glucocorticoid concentration within the culture medium (foetal calf serum) might tend to reduce the apparent effect of exogenous gluco-cortiocid exposure.

G-protein function was assessed indirectly by studies of ACA (section 2.8.1, mix A). ACA was studied under basal conditions and in the presence of MnCl2 (20mM), NaF (10mM), PGE1 (10-5M), Isoproterenol (10-4M), forskolin (10-5 M) and forskolin/GppNHp

(0.1nM). Experiments were conducted both in the presence of, and without IBMX (10mM).

G-protein levels were measured by Western blotting (2.4.1.) using the antibodies listed in table 4 (with exception of antisera against Gi1 and Go), as exploratory blots had failed to identify these G-proteins in the membrane preparations studied).

Protein concentrations within the plasma membrane homogenates were measured by the modified Lowry protocol (section 2.6.) and 5' nucleotidase activity was assessed (section 2.7.) within the various membrane fractions as a limited marker of altered membrane composition.

7.3. Results

Adenylyl cyclase activitites are shown in fig. 25. Exposure of VSMC to 10nM DEX for 24h was associated with significantly greater ACA, under basal conditions and in the presence of PGE1, isoproterenol and forskolin; the trend for DEX treated cells to exhibit higher ACA was also seen in the presence of MnCl2 and NaF, although the differences did not achieve statistical significance. Similar cyclase results were obtained after DEX exposure for 48h. Inclusion of anticortisol antibody for 48h prior to DEX exposure made little difference to the differential in cyclase activities between DEX and control membranes. In addition, although AC activities were slightly increased in both control and DEX treated cells, no difference was observed in the net effect of DEX (i.e. DEX exposure was still associated with

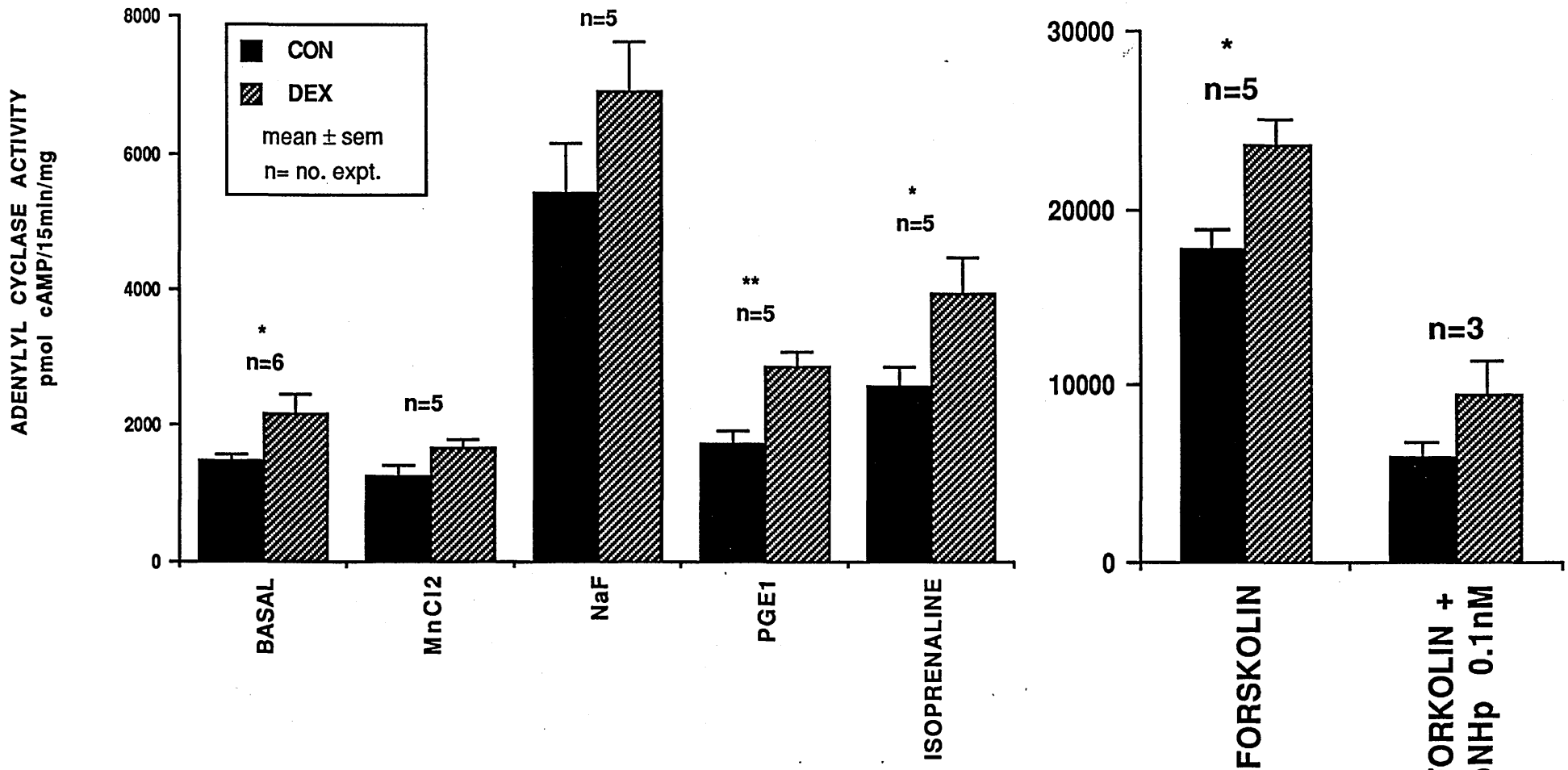


Fig.25. Adenylyl cyclase activities in plasma membranes from DEX-treated (10nM for 24h) and CON VSMC. Assay conditions and ligand studied are indicated along abscissa. * $p<0.05$ ** $p<0.01$

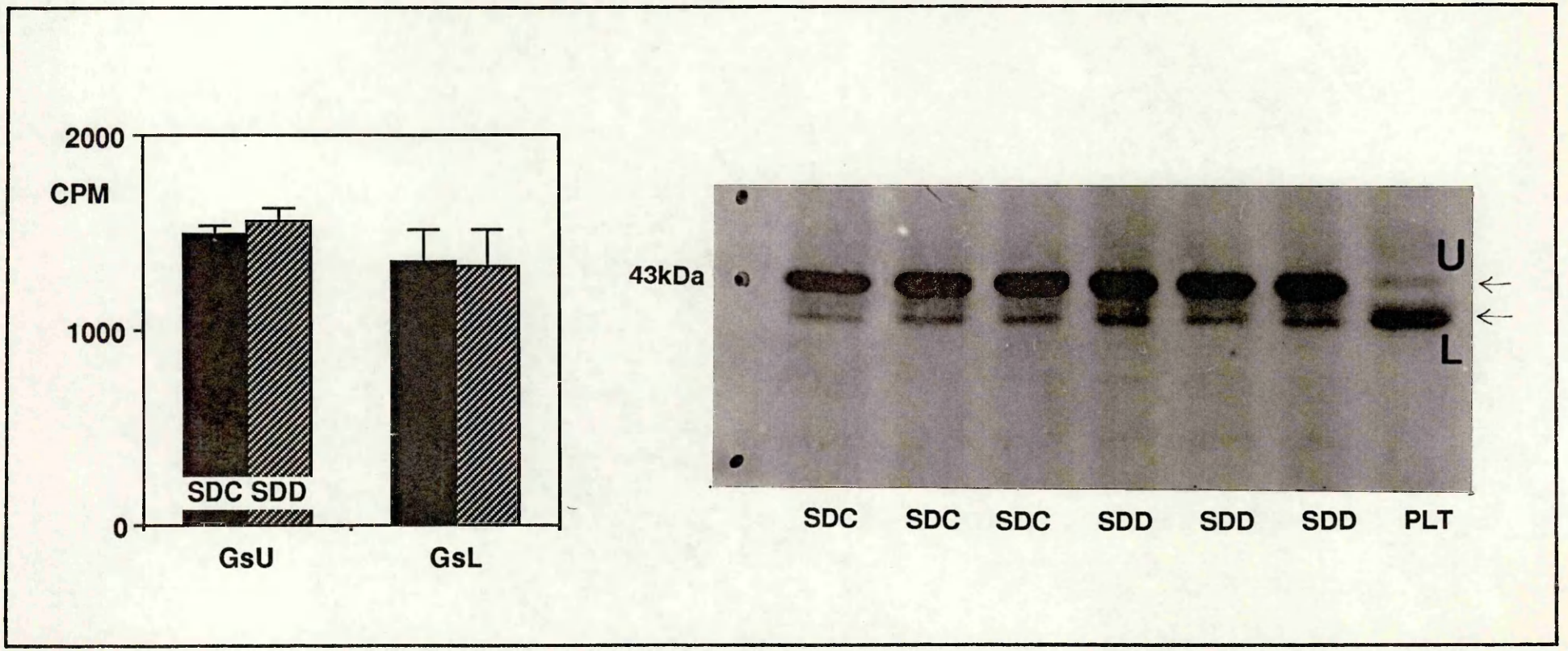


Fig.26. Effect of 10nM DEX (for 24h) on levels of Gsα subunits in cultured VSMC. (mean ± sem, 8 exp.). SDC=CON, SDD=DEX treated. PLT=standard membrane prep. (human platelet). L panel= graph obtained of cpm, R panel= typical autoradiograph.

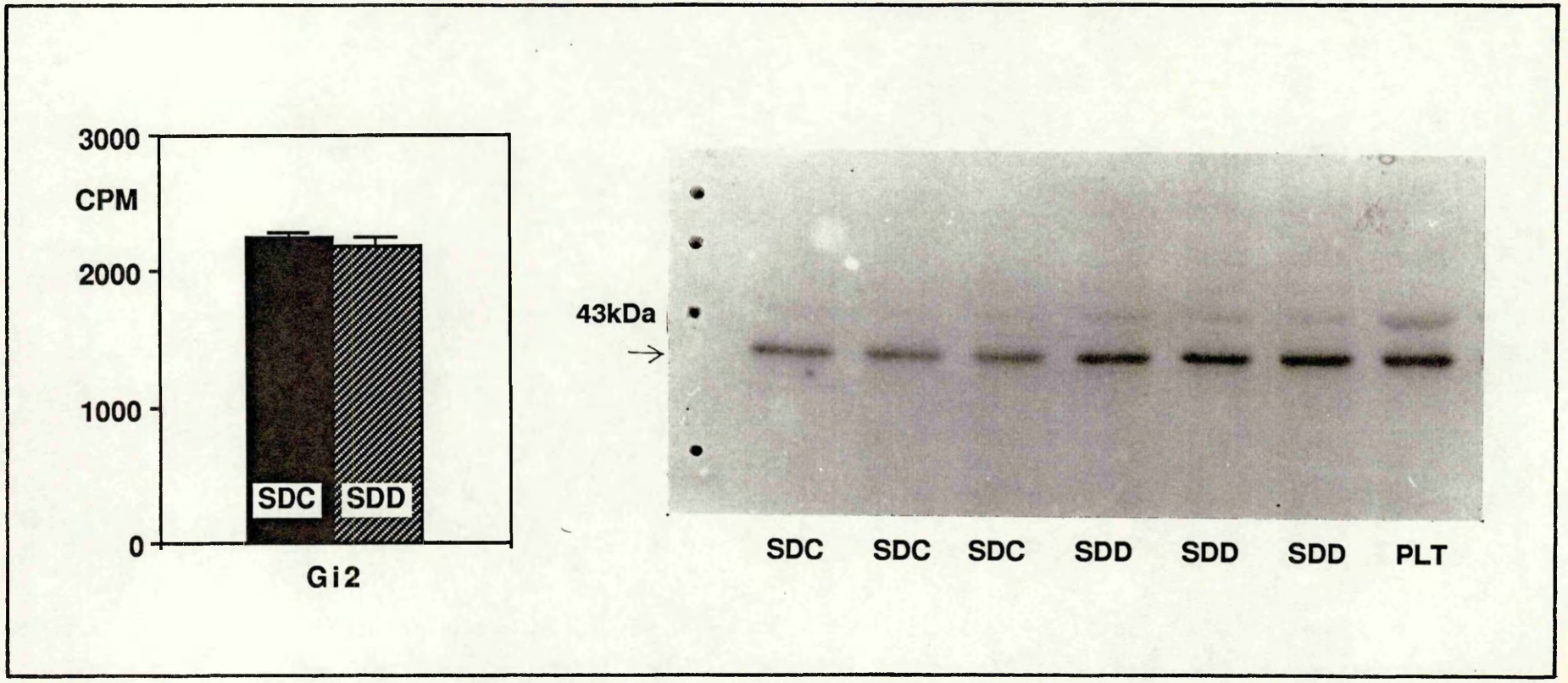


Fig.27. Effect of 10nM DEX (for 24h) on levels of Giα2 subunits in cultured VSMC. (mean ± sem, 8 exp.). SDC=CON, SDD=DEX treated. PLT=standard membrane prep. (human platelet). L panel= graph obtained of cpm, R panel= typical autoradiograph.

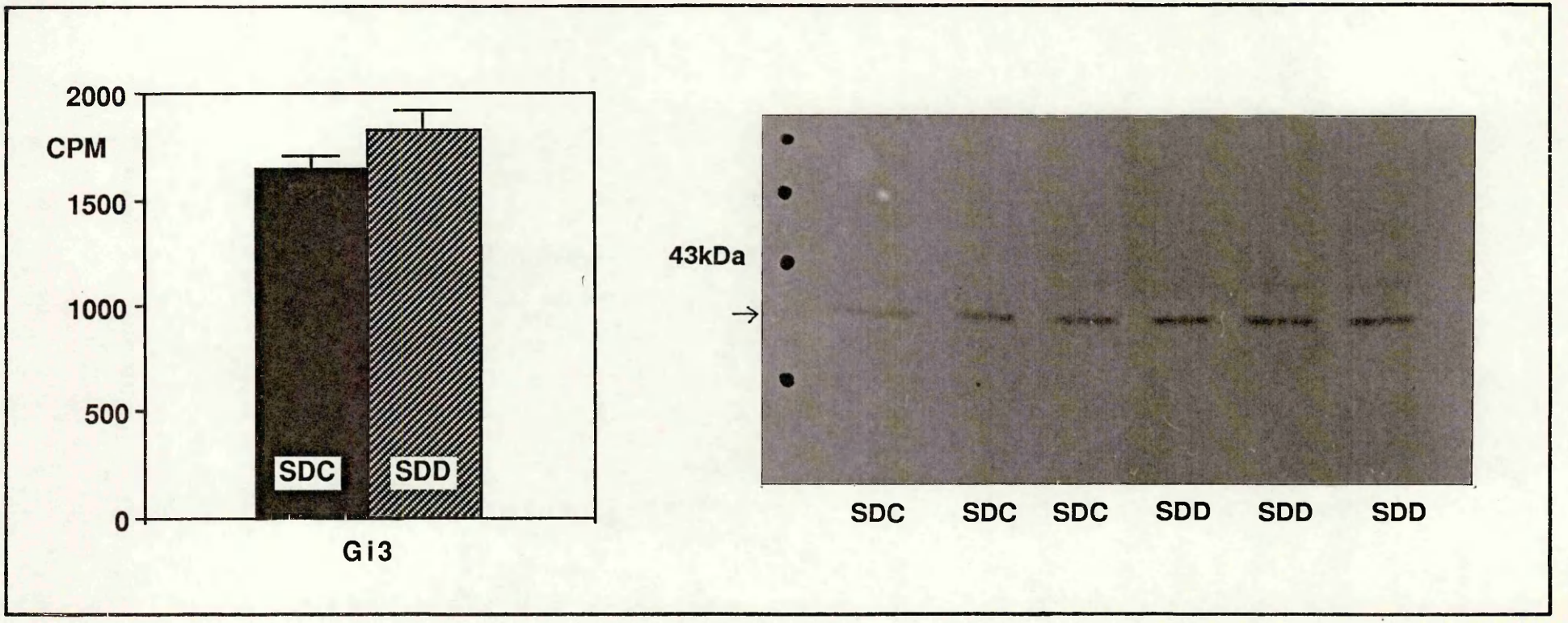


Fig.28. Effect of 10nM DEX (for 24h) on levels of Giα3 subunits in cultured VSMC. (mean ± sem, 8 exp.). SDC=CON, SDD=DEX treated. L panel= graph obtained of cpm, R panel= typical autoradiograph.

higher enzyme activities) on ACA when the cAMP phospho-diesterase inhibitor, IBMX,was included.

Data obtained from the Western blot studies are shown in figs. 26-28.

5' nucleotidase activitites were identical (0.84 Shinowara Units) in DEX treated and control cells.

7.4. Discussion

10nM DEX for 24h (and 48h) caused an increase in ACA (fig. 25) under basal conditions and irrespective of ligand added to the assay mix. Basal ACA was increased about 50%. The inclusion of 20mM MnCl2 did not eliminate the difference in cyclase activities between treated and control cells (although the difference was not statistically significant, it is likely to be relevant) suggesting that the major difference between DEX treated and control cells may relate to activity or levels of AC catalytic unit. The differences persist and are independent of ligand mediated stimulation of catalytic unit via Gs (by iso-proterenol and PGE1) and of direct, Gi mediated inhibition of catalytic unit by GppNHp (0.1nM), lending support to the suggestion that catalytic unit is altered rather than the G-proteins which modulate the activity of catalytic unit.

These results are consistent with those previously reported in DEX treated vascular smooth muscle cells (VSMC)(from renal arteries) (Yasunari et al. 1989) and non-vascular cell lines including 132N1 astracytoma cells (Foster & Perkins 1977),

cultured NRK fibroblasts (Johnson & Jaworski 1983), osteoblast cell line ROS 17/2.8 (Rizzoli et al. 1986, Rodan & Rodan 1986) and GH3 pituitary tumour cells (Chang & Bourne 1987). These previous studies have shown distinct changes over a wide range of concentrations of DEX (from 1nM to 1 μM), with exposure for between 48h and 6d. However, the time course of the effect of DEX on ACA has previously been studied in detail; changes have been reported as early as 6h, reaching a maximum at 24h without further increase up to 72h in VSMC (Yasunari et al. 1989). In the current studies, little change in ACA was noted when comparing ACA at 48h v 24h, and the longer period of exposure to DEX did not alter the net effect attributable to DEX itself.

DEX increased basal ACA by about 50% in cultured VSMC. Previous studies have shown DEX to enhance cyclase activity to a widely differing extent in different in vitro systems; basal ACA has been enhanced between 17% (Rizzoli et al. 1986) and 200% (Yasunari et al. 1989). In addition, consistent with our results, DEX has been observed to enhance ACA responses to a number of stimulatory agents including guanine nucleotides, forskolin, NaF, isoproterenol, dopamine and PTH; increases of between 50% (Rizzoli et al. 1986) and 600% (Foster and Perkins 1977) have been seen. Virtually all studies have shown a uniform stimulation of ACA by DEX virtually irrespective of the ligand studied but exceptions have been reported: Foster & Perkins (1977) observed that DEX enhanced PGE1 stimulation of ACA without enhancement of isoproterenol action and Johnson &

Jaworski (1983) showed that DEX reduced NaF stimulated ACA without alteration of baal activity.

Enhancement of ACA by DEX has been reported irrespective of whether ACA was assayed in membrane homogenates (Chang & Bourne 1987; Rodan & Rodan 1986; Rizzoli et al. 1986) or in whole cells (Yasunari et al. 1989; Foster & Perkins 1977; Rodan & Rodan 1986) and whether assessment was based on measurement of ACA (Chang & Bourne 1987; Rodan & Rodan 1986; Rizzoli et al. 1986; Yasunari et al 1989; Foster & Perkins 1977) or cAMP content (Johnson & Jaworski 1983; Rizzoli et al. 1986).

The inclusion of a phosphodiesterase inhibitor in the assay mix was not a prerequisite for demonstrating the effect of DEX; inclusion of IBMX made no difference to the net effect of DEX on ACA. However, previous workers have shown that the changes were abolished by administration of inhibitors of protein synthesis such as cycloheximide (Yasunari et al. 1989; Foster & Perkins 1977).

Other factors which may influence the increment of ACA observed following DEX exposure in vitro include the time of study after passage of the cells; Foster & Perkins (1977) demonstrated that PGE1 stimulated ACA was 2 fold greater on day 1, 6 fold greater on day 3 and 2 fold greater on day 8. All cells used in our studies were studied at either 7th or 8th passage, and DEX and control cells were grown concurrently. Membrane preparations from DEX treated and control cells were also prepared concurrently.

Dexamethasone is the glucocorticoid which has been studied most widely: the bulk of studies report use of dexamethasone in 'suprapharmacological doses' of around 10^{-7}M (Foster & Perkins 1977; Johnson & Jaworski 1983; Rizzoli et al. 1986; Chang & Bourne 1987). Yasunari (Yasunari et al. 1989) used concentrations of dexamethasone up to 10^{-6}M while Rodan & Rodan (1986) used 30nM dexamethasone in their culture medium to attain 'physiological levels' of steroid. 10nM DEX was used in the current studies. Inclusion of anticortisol antibody for 48h prior to DEX exposure made no difference to the obtained ACA suggesting that the glucocorticoid present within the FCS did not tend to have a major action in reducing the apparent effect of exogenous glucocorticoid.

The interpretation of these data is complex. The results are compatible with possible alteration of AC catalytic unit and this conclusion is in agreement with two previous groups of workers (Rizzoli et al. 1986, Chang & Bourne 1987). However, this explanation is not universally accepted (Johnson & Jaworski 1983). The changes may be independent of Gs (at least in osteoblast cell line ROS 17/2.8- as suggested by a reconstitution assay) (Rizzoli et al. 1986) but two groups have implicated glucocorticoid mediated increased in Gs to the enhanced ACA (Rodan & Rodan 1986; Chang & Bourne 1987).

The studies identified Gsα, Gi2α, Gi3α and ß subunits in membranes derived from VSMC (figs 26-28). Western blotting for these subunits excluded a role for DEX mediated alteration of the levels of G-protein subunits in the cyclase changes (fig. 25).

In addition to the data shown, no differences were seen in the amount of ß subunit present.

In addition to the multiple possible sites in the AC system at which DEX might exert its effects, glucocorticoids are known to exhibit a number of other influences on cAMP and its subsequent actions. Although it is clear that the effects of DEX discussed previously are not a consequence of enhanced cAMP phospho-diesterase (PDE) activity, previous workers have shown that high concentrations of glucocorticoids (specifically cortisol at concentrations of ≥ 2 μM) can inhibit (testicular and beef heart) cAMP PDE (Schmidtke, Wienker, Flugel & Engel 1976) (Manganiello & Vaughan 1973; Chen & Feldman 1978). Gluco-corticoids have also been shown to induce cAMP dependent kinase activity (Lamberts, Timmermans, Krammer-Blankestijn 1975). Whatever the ultimate fate of cAMP generated in DEX treated cells these studies support an effect of DEX on increasing levels and/or activity of AC cataytic unit. Other factors may have contributed to the observed changes in ACA; gluco-corticoids have previously been shown to increase ß receptor numbers in human neutrophils (Davies & Lefkowitz 1980), in rat lung (Mano et al. 1979) and in cultured human lung cells (Fraser & Venter 1980). In addition, glucocorticoid mediated alteration of G-protein coupling to receptors (without necessarily affecting levels of G-protein subunits)(Davies & Lefkowitz 1984; Haigh & Jones 1990) has previously been reported but the current AC studies suggest that neither of these mechansims is particularly prominent in our experimental system. As cAMP mediates vasodilatation, it is unlikely that the

current observation *in vitro* is of relevance to the pathogenesis of glucocorticoid hypertension.

Chapter 8
Conclusions

G-proteins are transmembrane signal transducers which couple receptors to effector mechansims. They are ubiquitous in human and animal tissues, and are located in the plasma membranes of cells. The current studies, based on Western (immuno-)blotting have identified a number of G-protein subunits (table 5) within plasma membranes derived from human platelets, and from a number of animal tissues including myocardium, renal cortex, liver, brain and mesenteric arterioles.

Table 5: G-proteins expressed in membrane preparations

MEMBRANE PREP.	Gsα	Giα1	Giα2	Giα3	Goα	ß
PLATELET *	•		•	•		•
MYOCARDIUM	•		•		•	•
RENAL CORTEX	•	•	•		•	•
LIVER	•		•			
BRAIN	•		•		•	
VSMC/MESENTERY	•		•	•		•

All membrane preparations studied were derived from rat species except platelet*-from humans.

In addition to immunoblotting studies to identify the presence of specific G-protein subunits, the function of $G_{i}2\alpha$ and $G_{s}\alpha$ subunits which mediate inhibition and stimulation of adenylyl cyclase was assessed.

G-protein abnormalities have previously been demonstrated both in human disease (pseudohypoparathyroidism and in some GH secreting adenomas causing acromegaly) and in experimental animal models of disease, for example STZ-IDDM and obesity. Houslay's group have shown that chemically induced IDDM in the rat is associated with altered G-protein expression and function (table 3) (Gawler et al. 1987; Bushfield et al. 1990a; Bushfield et al.1990b; Srassheim et al.1990). The alterations are tissue specific, do not invariably reflect altered expression and are selective for particular G-protein α subunits. Hepatocytes from STZ-IDDM manifest insulin resistance and enhanced ability of glucagon to stimulate AC (Gawler et al. 1987; Bushfield et al. 1990) and have been shown to exhibit loss of tonic inhibition of AC by Gi and attenuation of receptor mediated inhibition (via Gi)(Gawler et al.1987; Bushfield et al. 1990); Gi levels are also reduced (Gawler et al.1987). These alterations of G-protein levels and function are reversible with insulin administration. Similar changes of Gi function may be seen in hepatocytes from the Zucker rat, a model of obesity which also features insulin resistance raising the possibility that abnormalities of G-proteins may be implicated in diseases characterised by insulin resistance. Insulin resistance and hyperinsulinaemia are features of some human diseases including obesity (Olefsky, Kolterman & Scarlett 1982), NIDDM (DeFronzo & Ferrannini 1982) and essential hypertension (Ferrannini et al.1987) and studies were performed in platelets from human NIDDM and essential hypertensives to ascertain whether G-protein abnormalities might be present and lend support to the concept of a common pathophysiology (by virtue of insulin availability

determining G-protein function). Platelets represent a homogeneous tissue from which purified plasma membranes can be readily prepared and exhibit abnormalities of function both in NIDDM and EH.

In the study of platelets from NIDDM, significant reductions in the levels of $G_S\alpha$ and $G_i2\alpha$ were observed in NIDDM compared to controls. This result was similar to previous findings in hepatocyte membranes from the STZ-IDDM (Gawler et al. 1987; Bushfieldet al. 1990) although contrast with those in adipocytes where Gi3 has previously been shown to be increased (Strassheim et al.1990) and with a number of other vascular and non-vascular tissues where no changes in G-protein transcripts have been identified (see section 3.3.1). The observed reduction of G-protein levels in human platelets from NIDDM and hepatocytes from STZ-DM are consistent with the possibility that insulin may have a role in the regulation of G-proteins: despite elevated plasma levels of insulin in NIDDM, there will be a net intracellular deficiency of insulin, while STZ-DM is characterised by insulopenia due to pancreatic destruction. This hypothesis is supported indirectly by several correlations (table 8). Levels of $Gi\alpha2$, $Gi\alpha3$ and $Gs\alpha$ have all been shown to correlate inversely with fasting blood glucose concentration.

No evidence of altered function of $Gi\alpha2$ was seen in our diabetic platelets on the basis of studies with GppNHp which effect AC inhibition directly via Gi and with adrenaline (in conjunction with propranolol) which binds to the $\alpha2$ receptors which are coupled to AC catalytic unit via Gi. The study of GppNHp was

limited by the small numbers studied (n=6 CON, n=6 NIDDM). When forskolin and PGE1 responses were expressed as 'fold stimulation' over 'basal' cyclase, the NIDDM platelets exhibited significantly lower cyclase activities which may reflect altered amounts of AC catalytic unit and/or reduction in net Gsα effect. Reduced Gsα effect could be due to reduction in Gsα levels or possibly alteration in Giα2: Gs ratio. Either possibility is consistent with the immunoblotting studies.

The possibility exists that reduced intracellular insulin concentrations consequent upon insulin resistance which is common to hepatocytes from STZ-DM (Bushfield et al. 1990b) and to human NIDDM may contribute to the altered levels of G-proteins seen in this study. It would be useful to extend the current study to include a group of newly presenting IDDM to assess the effects of insulin deficiency in human subjects on G-protein levels and function.

The study in human EH is based on the strong epidemiological and clinical links between NIDDM and EH, and the increasing evidence of at least some overlap in pathophysiology on the basis of insulin resistance (Ferrannini et al. 1987). Given the abnormalities of G-proteins demonstrated in NIDDM, platelets from EH (which also exhibit functional abnormalities) were subjected to similar assessment of G-protein levels and function. However, no changes in levels of G-proteins were seen and in contrast to the observations in NIDDM, the studies of AC function identified greater PGE1 stimulated AC activites in hyper-tensive platelet membranes than controls (p=0.018). This

is consistent with the findings of previous workers and may have its physiological basis in protection of cells against Ca2+ overload (Resink et al. 1986).

Overall, no major changes were found in G-protein levels in essential hypertension, in contrast to the results obtained in our group of NIDDM The data argue against alteration of G-proteins in hypertension by virtue of hyperinsulinaemia, but further study would be desirable in metabolically active tissues such as fat or liver.

However, an interesting, if weak correlation was noted between plasma cholesterol concentration and relative amounts of Giα2 ($z=-2.084$, $n=29$, $p=0.04$) when considering all EH and control subjects together; this observation will be investigated further in a formal study of G-protein levels in hyperlipidaemic conditions.

In chapter 5, one of the most widely used models of genetic hypertension- the spontaneously hypertensive rat (SHR) was studied. This model is particularly useful as it has many features in common with human EH including glucose intolerance/ insulin resistance. This experimental model of hypertension was used in order to study G-protein changes in myocardium (tissue which is not available from human subjects) as there is substantial evidence of abnormalities of AC activation (reduced ß adrenergic activation of adenylyl cyclase has been widely reported). Differences in ACA were seen in SHR

myocardium compared to WKY, but not Wistar control species. These changes are characterised essentially by relatively reduced stimulation of AC by agents that act on Gs or ligands whose receptors couple to AC via Gs. However, identical changes are seen in Wistar membranes. Alterations in the receptors per se cannot be excluded but concurrent reductions of responses to NaF and forskolin suggest that catalytic unit and/ or Gs or their mutual coupling may be abnormal. No differences in amounts of Gi or Gs were identified which could account for the functional differences. Furthermore, no changes in ACA were seen in the studies of non-vascular tissues (liver and renal cortical plasma membranes) from the three species. Thus, a ubiquitous, perhaps genetically determined, abnormality of membrane function in SHR cannot be implicated. There is however, a surprising degree of similarity between the AC activities in rat myocardium reported by others in response to epinephrine infusion and those seen in the studies of SHR myocardium raising the possibility that the process of heterologous desensitisation which accounts for the former may also explain the latter.

These studies, however, emphasise the problems inherent in studies of the SHR. The absence of an isogenetic control species raises the distinct possibility that the observed differences may relate to the inherent genetic heterogeneity among the species studied: this problem cannot be circumvented. The inclusion in the studies of the second control species (Wistar) highlights the dangers of the customary comparison between SHR and WKY species. Further studies are planned in the Milan

rat-another model of genetic hypertension for which an isogenetic control exists, to ascertain whether the observed changes in ACA are seen in this model also.

The studies of G-proteins in hypertension were extended using the poorly understood entity of glucocorticoid hypertension. As humoral regulation of G-protein expression and function (section 1.5.) by glucocorticoids (see chapters 6 & 7)(Rodan & Rodan 1986; Chang & Bourne 1987; Ros et al.1989; Saito et al.1989) has apparently been demonstrated in experimental animals and cell culture it seemed possble that G-protein changes could be implicated in this pathological state. Previous studies of glucocorticoid effect on the differential regulation of G-protein subunits have generally been based on administration of massive doses of glucocorticoid to adrenalectomised animals: however, in contrast, in the current studies a model of glucocorticoid hypertension based on rather more physiological concentrations (likely to still be in the 'pharmacological range') of the glucocorticoid, dexamethasone was used (Tonolo et al. 1988). With administration of 5μg/d DEX infusion (s.c.) elevation of tail-cuff BP by 30mmHg-representing an increase of around 25% with minimal compromise of weight gain (table 15) was achieved. Immunoblotting studies for G-protein subunits failed to identify any relevant changes in brain, myocardium, renal cortex, liver and mesenteric vasculature. Studies of ACA failed to reveal any differences in ACA responses in renal cortical (fig. 24), myocardial and mesenteric plasma membranes (table 18). This contrasts with the *in vitro* work reviewed in chapter 7, and suggests that neither changes in G-protein function nor AC

catalytic unit are implicated in the pathogenesis of glucocorticoid hypertension

The results show important differences from those of previous workers and on the basis of previous adrenalectomy studies it is possible to conclude that glucocorticoids may have a role in regulating G-proten levels- but given the limitations of the adrenalectomy model it would be difficult to conclude that this amounts to anything more than a permissive role. It seems clear however, that glucocorticoids are not implicated in the pathogenesis of glucocorticoid hypertension.

As dexamethasone administration *in vitro* has been previously shown to increase adenylyl cyclase activity in vascular smooth muscle cells (from renal arteries)(Yasunari et al.1989) and non-vascular cell lines plasma membranes from VSMC treated similarly with DEX were studied *in vitro* to assess whether G-protein changes could be implicated in the observed changes. The findings were in agreement with those of Yasunari et al. (1989) viz., 10nM DEX for 24h (and 48h) effected an increase in ACA(fig. 25) under basal conditions and irrespective of ligand added to the assay mix. Basal ACA was increased about 50%. The differences persist and are independent of ligand mediated stimulation of catalytic unit via Gs (by isoproterenol and PGE1) and of direct, Gi mediated inhibition of catalytic unit by GppNHp (0.1nM), lending support to the suggestion that catalytic unit is altered rather than the G-proteins which modulate the activity of catalytic unit.

The results are compatible with possible alteration of AC catalytic unit and this conclusion is in agreement with two previous groups of workers (Rizzoli et al. 1986, Chang & Bourne 1987). However, this explanation is not universally accepted (Johnson & Jaworski 1983). The changes may be independent of Gs (at least in osteoblast cell line ROS 17/2.8- as suggested by a reconstitution assay) (Rizzoli et al. 1986) but two groups have implicated glucocorticoid mediated increased in Gs to the enhanced ACA (Rodan & Rodan 1986; Chang & Bourne 1987). The current studies identified Gsα, Gi2α, Gi3α and ß subunits in membranes derived from VSMC (figs 26-28). Western blotting for these subunits excluded a role for DEX mediated alteration of the levels of G-protein subunits in the cyclase changes (fig. 25). In addition to the data shown, no differences in the amount of ß subunit was seen.

In addition to the multiple possible sites in the AC system at which DEX might exert its effects, glucocorticoids are known to exhibit a number of other influences on cAMP and its subsequent effectors including cAMP dependent kinase activity (Lamberts, Timmermans, Krammer-Blankestijn 1975). Whatever the ultimate fate of cAMP generated in DEX treated cells the current studies support an effect of DEX on increasing levels and/or activity of AC cataytic unit although other factors may have contributed to the observed changes in ACA including altered receptor numbers. However, as cAMP mediates vasodilatation, it is unlikely that this observation *in vitro* is of relevance to the pathogenesis of glucocorticoid hypertension.

The major findings of this thesis can be summarised:

• The studies of platelet membranes from NIDDM and EH exclude common pathophysiology based on alterations of G-proteins, although both conditions do feature abnormalities of ACA which may contribute to the altered platelet function observed seen in these diseases. Platelets from NIDDM (but not EH) do exhibit reduced levels of G-protein subunits which may contribute to the altered ACA, but these changes may be related to altered plasma membrane composition in diabetes mellitus.

• Species differences exist in ACA in myocardial plasma membrane preparations (comparing SHR, WKY and Wistar species). The validity of the customary comparison between SHR and WKY is challenged. Differences in ACA are not explained through altered G-protein levels, but may be due to differences in AC catalytic unit.

• Despite evidence implicating glucocorticoids in G-protein regulation, no changes in G-protein levels were seen in myocardium, brain, kidney and mesenteric artery in a validated model of glucocorticoid hypertension. Glucocortiocoid exposure did enhance ACA in cultured VSMC but probably by altering AC catalytic unit, and not through changes in the levels of G-protein subunits.

References

Allen, D.O. & Beck, R.R. (1972) Alterations in lipolysis, adenylate cyclase and adenosine 3',5'-monophosphate levels in isolated fat cells following adrenalectomy. Endocrinology , 91, 504-510.

Amer, M.S. (1973) Cyclic adenosine monophosphate and hypertension in rats. Science ,179, 807-9.

Amer, M.S. (1974) Cyclic guanosine 3',5' monophosphate and gall bladder contraction. Gastroenterology, 67, 333-7.

Amer, M.S., Gomoll, A.W., Perhach, J.L., Ferguson, H.C. & McKinney, G.R. (1974) Aberrations of cyclic nucleotide metabolism in the hearts and vessels of hypertensive rats. Proceedings of the National Academy of Sciences of the United States of America, 71, 4930-34.

Amer, M.S., Doba, N. & Reis, D.J. (1975) Changes in cyclic nucleotide metabolism in aorta and heart of neurogenically hypertensive rats: possible trigger mechanisms of hypertension. Proceedings of the National Academy of Sciences of the United States of America, 72, 2135-39.

Anand-Srivastava, M.B., Cantin, M. & Genest, J. (1983) Altered hormone sensitivity of adenylate cyclase in myocardial sarcolemma of renal hypertensive rats. Biochemical Pharmacology, 32, 2857- 62.

Anand-Srivastava, M.B. (1988) Altered responsiveness of adenylate cyclase to adenosine and other agents in the myocardial sarcolemma and aorta of spontaneously

hypertensive rats. Biochemical Pharmacology , 37, 3017-22.

Armstrong, K.J., Stouffer, J.E., Van Inwegen, R.G., Thompson, W.J. & Robison, A.G.(1974) Effect of thyroid hormone deficiency on cyclic adenosine 3':5' monophosphate and control of lipolysis in fat cells. Journal of Biological Chemistry, 249, 4226-4231.

Armstrong, T.M., Thirsk, G., Biol, M.I. & Salmon, J.A. (1979) Effects of prostacyclin (PGI2), 6 0x0-PGF1α and PGE2 on sympathetic nerve function in mesenteric arteries and veins of the rabbit in vitro. Hypertension ,1, 309-315.

Arner, P., Einarsson, K., Ewerth, S. & Livingston, J.N. (1978) Altered action of glucagon on human liver in type 2 (non-insulin-dependent) diabetes mellitus. Diabetologia, 30,323-6.

Asano, M., Masuzawa, K. & Matsuda, T. (1988a) Evidence for reduced beta-receptor coupling to adenylate cyclase in femoral arteries from spontaneously hypertensive rats. British Journal of Pharmacology ,94, 73-86.

Asano, M., Masuzawa, K., Matsuda, T. & Asano, T. (1988b) Reduced function of the stimulatory GTP-binding protein in beta adrenoreceptor-adenylate cyclase system of femoral arteries isolated from spontaneously hypertensive rats. Journal of Pharmacology and Experimental Therapeutics, 246,709-18.

Atkins, F.L., Dowell, R.T. & Love, S. (1985) Beta-adrenergic receptors, adenylate cyclase activity, and cardiac

dysfunction in the diabetic rat. Journal of Cardiovascular Pharmacology, 7, 66-70.

Awad, J.A., Johnson, R.A., Jakobs, K.H. & Schultz, G. (1983) Interactions of forskolin and adenylate cyclase: Effects on substrate kinetics and protection against inactivation by heat and N-ethylmaleimide. Journal of Biological Chemistry, 258, 2960-2965.

Balmforth, A.J., Lyall, F., Morton, J.I. & Ball, S.G. (1988) Cultured mesenteric vascular smooth muscle cells express dopamine DA1-receptors.European Journal of Pharmacology ,155, 305-308.

Bar, H.-P. (1974)Cyclic nucleotides and smooth muscle. In Advances in cyclic nucleotide research, eds. Greengard, P. & Robison,G.A. Raven Press, New york. Vol. 4 pp 195-238.

Barbacid, M. (1987) *ras* genes. Annual Review of Biochemistry, 56 ,779-828.

Baudouin-Legros, M., Dard, B. & Guicheney, P.(1986) Hyperreactivity of platelets from spontaneously hypertensive rats. Hypertension , 8, 694-699.

Begin-Heick, N. (1985) Absence of inhibitory effect of guanine nucleotides on adenylate cyclase activity in white adipocyte membranes of the ob/ob mouse. Effect of the ob gene. Journal of Biological Chemistry, 260, 6187-6193.

Besse, J.C. & Bass, A.D.(1966) Potentiation by hydrocortisone of responses to catecholamines in vascular smooth

muscle. Journal of Pharmacology and Experimental Therapeutics, 154, 224-238.

Bhalla, R.C. & Ashley, T. (1978a) Altered function of adenylate cyclase in the myocardium of spontaneously hypertensive rat. Biochemical Pharmacology ,27,1967-71.

Bhalla, R.C., Sharma, R.V. & Ashley, T. (1978b) Adenylate cyclase activity in myocardium of spontaneously hypertensive rat: effect of endogenous factors and solubilisation. Biochemical and Biophysical Research Communications , 82, 273-80.

Bhalla, R.C., Sharma, R.V. & Ramanathan, S. (1980) Ontogenetic development of isoproterenol subsensitivity of myocardial adenylate cyclase and ß adrenergic receptors in spontaneously hypertensive rats. Biochimica et Biophysica Acta, 632, 497-506.

Bhalla, R.C. & Sharma, R.V. (1982) Characteristics of hormone stimulated adenylate cyclase in vascular smooth muscle: altered activity in spontaneously hypertensive rat. Blood Vessels ,19, 109-16.

Bia, F.J., Tyler, K.A. & De Fronzo, R.A.(1982) the effect of dexamethasone on renal electrolyte excretion in the adrenalectomised rat. Endocrinology, 111, 882-8.

Bianchi, G., Fox, U, Di Francesco, D., Giovanneti, A.M. & Pagetti, D. (1974) Blood pressure changes produced by kidney cross- transplantation between spontaneously hypertensive rats and normotensive rats. Clinical Science & Molecular Medicine, 47,435-448.

Billestrup, N., Swanson, L.W. & Vale, W. (1986) Growth hormone releasing factor stimulates proliferation of somatotrphs in vitro. Proceedings of the National Academy of Sciences of the United States of America, 83, 6854-6857.

Birnbaumer, L., Pohl, S.L., Michiel, H., Krans, J. & Rodbell, M. (1970) The actions of hormones on the adenyl cyclase system. Advances in Biochemical Psychopharmacology, 3, 185-208.

Birnbaumer, L., Codina, J., Mattera, R., Yatani, A., Schere, N., Toro, M.-J. & Brown, A.M. (1987) Signal transduction by G-proteins. Kidney International, 32, S14 -S37.

Birnbaumer, L., Abramowitz, J. & Brown, A.M. (1990) Receptor-effector coupling by G-proteins. Biochimica et Biophysica Acta, 1031, 163-224.

Bitensky, M.W., Gorman, R.E. & Neufeld, A.H. (1972) Selective effects of insulin on hepatic epinephrine responsive adenylyl cyclase activity. Endocrinology, 90,1331-5

Blackmore, P.F., Assimacopoulos-Jeannet, F., Chan, T.M. & Exton, J.H. (1979) Studies on alpha adrenergic activation of glucose output. Insulin inhibition of alpha adrenergic and glucagon actions in normal and calcium depleted hepatocytes. Journal of Biological Chemistry, 254, 2828-2834.

Blumenthal, S.J., McConnaughey, M.M. & Iams, S.G. (1982) Myocardial adrenergic receptors and adenylate cyclase in the developing spontaneously hypertensive rat. Clinical and Experimental Hypertension , 4, 883-888.

Bohm, M., Gierschik, P., Schnabel, P & Erdmann, E. (1990) Myocardial beta adrenoreceptors and inhibitory G-proteins in myocardial biopsies and in explanted hearts from patients with dilated cardiomyopathy. Cardioscience, 2, 109-117.

Bohm, M., Gierschik, P., Jakobs, K.-H., Pieske, B., Schnabel, P., Ungerer, M. & Erdmann, E. (1990). Increase of Giα in human hearts with dilated but not ischaemic cardio-myopathy. Circulation, 82, 1249-1265.

Boon, N.A., Elliot, J.M., Davies, C.L., Conway, F.J., Jones, J.V., Graeme-Smith, D.G. & Sleight, P. (1983) Platelet α adrenoreceptors in borderline and established essential hypertension. Clinical Science, 65, 207-208.

Bourne, H.R., Kaslow, H.R., Brickman, A.S. & Farfel, Z. (1981) Fibroblast defect in pseudohypoparathyroidism type 1: reduced activity of receptor-cyclase coupling protein. Journal of Clinical Endocrinology and Metabolism, 53, 636-640.

Bradham, L.S. & Cheung, W.Y. (1980) Calmodulin dependent adenylate cyclase. In Calcium and cell function, ed. W.Y. Cheung, Academic Press, New York, 1, 109-125.

Bristow, M.R., Kantrowitz, N.E., Ginsburg, R. & Fowler, M.R. (1985) Beta-adrenergic function in heart muscle disease and heart failure. Journal of Molecular and Cellular Cardiology, 17, 41-52.

Brodde, O.-E., Stuka, N., Dernuth, V., Fesel, R., Bergerhaven, J., Daul, A. & Bock, K.D. (1985) Alpha and beta receptors in circulating blood cells of essential hypertensive

patients. Clinical and Experimental Theory and Practice , A7,1135-1150.

Bucolo, G. & David, H. (1973) Quantitative determination of serum triglycerides by the use of enzymes. Clinical Chemistry, 19, 476-482.

Bushfield, M., Griffiths, S.L., Murphy, G.J., Pyne, N.J., Knowler, J.T., Milligan, G., Parker, P.J., Mollner, S. & Houslay, M.D. (1990a)Diabetes induced alterations in the expression, functioning and phosphorylation state of the inhibitory guanine nucleotide regulatory protein, Gi2 in hepatocytes.Biochemical Journal, 271, 365-372.

Bushfield, M., Griffiths, S.L., Strassheim, D., Tan, E., Shakur, Y., Lavan, B. & Houslay, M.D. (1990b) Guanine nucleotide binding proteins in diabetes and insulin resistant states.Biochemical Society Symposia, 56, 137-154.

Bushfield, M., Murphy, G.J., Lavan, B.E., Parker, P.J.,Hruby, V.J., Milligan, G. & Houslay, M.D. (1990c) Hormonal regulation of Gi2 alpha subunit phosphorylation in intact hepatocyes.Biochemical Journal, 268, 449-457.

Casey, P.J. & Gilman, A.G. (1988) G protein involvement in receptor-effector coupling. Journal of Biological Chemistry, 263, 2577-2580.

Cassel, D. & Selinger, Z. (1976) Catecholamine stimulated GTPase activity in turkey erythrocytes. Biochimica et Biophysica Acta ,452, 538-51.

Cerione, R.A., Staniszewski, C., Gierschik, P., Codina, J., Somers, R.L., Birnbaumer, L., Spiegel, A.M., Caron, M.G.

& Lefkowitz, R. J . (1986) Mechanism of guanine nucleotide regulatory protein-mediated inhibition of adenylate cyclase. Studies with isolated subunits of transducin in a reconstituted system. Journal of Biological Chemistry, 261, 9514-20.

Cerione, R.A., Gierschik, P., Staniszewski,C., Benovic,J.L., Codina, J., Somers, R.,Birnbaumer, L., Spiegel, A.M., Lefkowitz, R.J. & Caron, M.G. (1987) Functional differences in the beta gamma complexes of transducin and the inhibitory guanine nucleotide regulatory protein. Biochemistry, 26, 1485-91.

Chan, T.M., Blackmore, P.F., Steiner, K.E. & Exton, J.H. (1979) Effects of adrenalectomy on hormone action on hepatic glucose metabolism. Journal of Biochemistry, 254, 2428-2433.

Chang, F.-H. & Bourne, H.R. (1987) Dexamethasone increases adenylyl cyclase activity and expression of the alpha-subunit of Gs in GH3 cells. Endocrinology ,121,1711-1715.

Chatelain, P., Gillet, L., Waelbroeck, M., Camus, J.C., Robberecht, P. & Christophe, J. (1983) Selective alteration of secretin-stimulated cardiac adenylate cyclase in streptozotocin-diabetic rats. Hormone and Metabolic Research,15, 620-2.

Chen, T.L. & Feldman, D. (1978) Glucocorticoid potentiation of the adenosine 3',5'-monophosphate response to parathyroid hormone in cultured rat bone cells. Endocrinology, 102, 589-596.

Cheung, W Y., Bradham, L.S., Lynch, T.J., Lin, Y.M. & Tallant, E.A. (1975)Protein activator of cyclic 3',5' nucleotide phosphodiesterase of bovine and rat brain also activates its adenylate cyclase. Biochemical and Biophysical Research Communications, 66, 1055-1062.

Chiappe-de-Cingolani, G.E. (1988) Adipocyte responsiveness to norepinephrine in spontaneously hypertensive rats. Metabolism,37, 318-22.

Christlieb, A.R., Krolewski, A.S., Warram, J.H. & Soeldner, J.S.(1985) Is insulin the link between hypertension and obesity. Hypertension ,7, Suppl.2, II-54-57.

Cloix, J.F., Colard, O., Rothhut, B. & Russo-Marie, F. (1983) et al. (1983) Characterisation and partial purification of renocortins': two polypeptides formed in renal cells causing the anti-phospholipase-like action of gluco-corticoids. British Journal of Pharmacology, 79, 312-321.

Cockcroft, S. & Gomperts, B.D. (1985) Role of guanine nucleotide binding protein in the activation of poly-phosphoinositide phosphodiesterase. Nature, 314, 534-536.

Codina, J., Kimura, S., Kraus-Friedmann, N. (1988) Demonstration of the presence of G-proteins in hepatic microsomal fraction. Biochemical and Biophysical Research Communications, 150, 848-52.

Cogan, M.G. Angiotensin II: a powerful controller of sodium transport in the early proximal tubule. Hypertension, 15, 451-458.

Coghlan, J.P., Butkus, A., Denton, D.A., Graham, W.F., Humphrey, T.J., Scoggins, B.A. & Whitworth, J.A. (1980) Steroid receptors and hypertension. Circulation Research, 46, I-88-I-93.

Cohen, M.L. & Berkowitz, B.A. (1976) Decreased vascular relaxation in hypertension. Journal of Pharmacology and Experimental Therapeutics ,196, 396-406.

Cohen, P. (1982) The role of protein phosphorylation in neural and hormonal control of cellular activity. Nature, 296, 613-619.

Coquil, J.-F. & Brunelle, G. (1989) Adenylate cyclase of platelets from spontaneously hypertensive rats: reduction of inhibition by GTP. Biochemical and Biophysical Research Communications ,162, 1265-1271.

Dard, B., Baudouin-Legros, M., Meyer, P. (1987) Cyclic AMP content and activation state of platelets from spontaneously hypertensive rats. Journal of Hypertension ,5, S137-139.

David, S.D., Grieco, M.H. & Cushman, P. (1970) Adrenal glucocorticoids after twenty years. A review of their relevant clinical consequences. Journal of Chronic Diseases, 22, 637-642.

Davies, A.O. & Lefkowitz, R.J. (1980) Corticosteroid-induced differential regulation of ß adrenergic receptors in circulating human polymorphonuclear leukocytes and mononuclear leukocytes. Journal of Clinical Endocrinology and Metabolism,51, 599- 605.

Davies, A.O., DeLean, A. & Lefkowitz, R.J. (1981) Myocardial ß adrenergic receptors from adrenalectomised rats: impaired formation of high affinity agonist-receptor complexes. Endocrinology, 108, 720-722.

Davies, A.O. & Lefkowitz, R.J. (1984) Regulation of ß adrenergic receptors by steroid hormones. Annual Review of Physiology,46, 119-130.

Dixon, T.F. & Purdom, M. (1954) Serum 5'-nucleotidase. Journal of Clinical Pathology, 7, 341-343.

Dluhy, R.G., Bavli, S.Z., Leung, F.K., Solomon, H.S., Moore, T.J., Hollenberg, N.K. & Williams, G.H. (1979) Abnormal adrenal responsiveness and angiotensin II dependency in high renin essential hypertension. Journal of Clinical Investigation, 64, 1270-1276.

DeFronzo, R.A. & Ferrannini, E. (1982) The pathogenesis of non-insulin dependent diabetes: an update. Medicine (Batimore) , 61, 125-40.

De Mazancourt, P., Lacasa, D., Giot, J. & Giudicelli, Y. (1989) Role of adenosine 3',5' monophosphate and the R1-receptor Gi-coupled adenylate cyclase inhibitory pathway in the mechansim whereby adrenalectomy increases the adenosine antilipolytic effect in rat fat cells. Endocrinology, 124, 1131-1139.

Denton, R.M.(1986) Early events in insulin actions. Advances in Cyclic Nuceotide and Protein Phosphorylation Research, 20,295-341.

Dorman, J.S., Laporte, R.E., Kuller, L.H., Cruickshanks, K.J., Orchard, T.J., Wagener, D.K., Becker, D.J., Cavender, D.E. & Drash, A.L. (1984) The Pittsburgh insulin dependent

diabetes mellitus (IDDM) morbidity and mortality study. Diabetes ,33, 271-276.

Downs, R.W., Levine, M.A., Drezner, M.K., Burch, W.M. & Allen, M.S. (1982) Deficient adenylate cyclase regulatory protein in renal membranes from a patient with pseudohypoparathyroidism. Journal of Clinical Investigation, 71, 231-235.

Elijovich, F.& Krakoff, L.R. (1980) Effect of converting enzyme inhibition in the rat. American Journal of Physiology, 238, H844-H848.

Enomoto, K. & Asakawa, T. (1986) Inhibition of catalytic unit of adenylate cyclase and activation of GTPase of Ni protein by beta gamma-subunits of GTP-binding proteins. FEBS Letters, 202, 63-8.

Erne, P., Resink, T.J., Burgisser, E. & Buhler, F.R. (1985) Platelets and hypertension. Journal of Cardiovascular Pharmacology ,7, S103-8.

Exton, J.H., Friedmann, N., Wong, E.H., Brineaux, J.P., Corbin, J.D. & Park, C.R. (1972) Interaction of glucocortiocids with glucagon and epinephrine in the control of gluco-neogenesis and glycogenolysis in liver and lipolysis in adipose tissue.Journal of Biological Chemistry, 247, 3579-3588.

Farfel, Z. & Bourne, H.R. (1980) Deficient activity of receptor-cyclase coupling protein in platelets of patients with pseudohypoparathyroidsim. Journal of Clinical Endocrinology and Metabolism , 51,1202-1204.

Farfel, Z., Iaina, A., Eliahou, H. & Cohen, Z. (1984) Receptor-cyclase coupling protein in erythrocytes of patients

with essential hypertension. Clinical Science , 67, 111-15.

Farquhar, M.G., Bergeron, J.J.M. & Palade, G.E.(1974) Cyto-chemistry of Golgi fractions prepared from rat liver. Journal of Cell Biology ,60, 8-25.

Feingold, K.R. & Siperstein, M.D. (1985) Diabetic vascular disease: In: Stollerman, G.H., Harrington, W.J., Lamont, J.T., Leonard, J.J., Siperstein, M.D. (Eds.) Advances in Internal Medicine. Vol.31. Chicago: Year Book Medical; Publishers, Inc. pp 309-40.

Feldman, A.M., Cates, A.E., Veazey, W.B.,Hershberger, R.E., Bristow, M.R., Baughman, K.L., Baumgartner, W.A. & Van Dop, C. (1988)Increase of the 40,000 mol. wt. pertussis toxin substrate (G-protein) in the failing human heart. Journal of Clinical Investigation, 82, 189-197.

Ferrannini, E., Buzzigoli, G., Bonadonna, R., Giorico, M.A., Oleggini, M., Graziadei, L., Pedrinelli, R., Brandi, L. & Bevilacqua, S. (1987) Insulin resistance in essential hypertension. New England Journal of Medicine, 317, 350-356.

Fiske, C.H. & Subbarow, Y. (1925).The colorimetric deter-mination of phosphorus. Journal of Biological Chemistry, 66, 375-400.

Florey, C. du V., Uppal, S. & Clowy, C. (1976). Relation between blood pressure, weight and plasma sugar and serum insulin in school children aged 9-12years in Westland, Holland. British Medical Journal, 1, 1368-1371.

Flower, R.J. & Blackwell, G.J. (1979) Antiinflammatory steroids induce biosynthesis of phospholipase A2 inhibitor which prevents prostaglandin generation. Nature ,278, 456-459.

Foster, D.W. (1989) Insulin resistance-a secret killer? New England Journal of Medicine, 320,733-734.

Foster, S.J. & Perkins, J.P. (1977) Glucocorticoids increase the responsiveness of cells in culture to prostaglandin E1. Proceedings of the National Academy of Sciences of the United States of America, 74, 4816-4820.

Fowler, N.O. & Chou, H.F. (1961) Potentiation of smooth muscle contraction by adrenal steroids. Circulation Research,19, 153.

Fraser, C.M. & Venter, J.C. (1980) The synthesis of ß adrenergic receptors in cultured human lung cells: induction by glucocorticoids. Biochemical and Biophysical Research Communications ,94, 390-397.

Fujiwara, M., Kuchin, M. & Shibata, S. (1972) Differences of cardiac reactivity between spontaneously hypertensive and normotensive rats. European Journal of Pharmacology,19, 1-11.

Garcia-Sainz, J.A., Huerta-Bahena, M.E. & Malbon, C.C.(1989) Hepatocyte beta-adrenergic responsiveness and guanine nucleotide binding regulatory proteins. American Journal of Physiology, 256, C384- C389.

Gawler, D. & Houslay, M.D.(1987) Insulin stimulates a novel GTPase activity in human platelets. FEBS Letters, 216, 94-98.

Gawler, D., Milligan, G., Spiegel, A.M., Unson, C.G. & Houslay, M.D. (1987) Abolition of the expression of the inhibitory guanine nucleotide regulatory protein Gi activity in diabetes. Nature, 327: 229-232.

Gawler, D., Milligan, G. & Houslay, M.D. (1988) Treatment of streptozotocin-diabetic rats with metformin restores the ability of insulin to inhibit adenylate cyclase activity and demonstrates that insulin does not exert this action through the inhibitory guanine nucleotide regulatory protein Gi. Biochemical Journal, 249, 537-42.

Gilman, A.G. (1984) G proteins and the dual control of adenylate cyclase. Cell, 36, 577-579.

Gilman, A.G. (1987) G proteins: transducers of receptor generated signals. Annual Reviews of Biochemistry, 56, 615-49.

Gizsche, O. (1983) the adrenergic beta-receptor adenylate cyclase system in heart and lymphocytes from streptozotocin-diabetic rats. In vivo and in vitro evidence for a desensitized myocardial beta-receptor. Diabetes, 32, 1110-6.

Goldberg, N.D., O'Dea, R.F. & Haddox, M.K.(1973). Cyclic AMP. In Advances in cyclic nucleotide research, eds. Greengard, P. & Robison, G.A. Raven Press, New york. Vol. 3 pp 195-238.

Goldsmith, P., Gierschik, P., Milligan, G., Unson, C.G., Vinitsky, R.,Malech, H.L. & 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.

Gomez-Sanchez, C.E., Holland, O.B. & Upcavage, R. (1985) Urinary free 19 nor-deoxycorticosterone and its relation to systemic arterial blood pressure in normotensive and hypertensive subjects. Journal of Clinical Endocrinology and Metabolism, 56, 99-103.

Grandt, R., Aktories, K. & Jakobs, K.H. (1986) Evidence for two GTPases activated by thrombin in membranes of human platelets. Biochemical Journal, 273, 669-674.

Green, A., Milligan, G. & Belt, S.E. (1990) Gi down-regulation as a mechanism for heterologous desensitisation in adipocytes. Journal of Biological Chemistry (in press).

Greenberg, A.S., Taylor, S.I. & Londos, C. (1987) Presence of a functional inhibitory GTP-binding regulatory component, Gi, linked to adenylate cyclase in adipocytes of ob/ob mice. Journal of Biological Chemistry, 262, 4564-8.

Greengard, P. (1978) Phosphorylated proteins as physiological effectors. Science, 199, 146-152.

Griffing, G.T., Dale, S.L., Holbrook, M.M. & Melby, J.C. (1985) The regulation of urinary free 19 nordeoxycorticosterone and its relation to systemic arterial pressure in normotensive and hypertensive subjects. Journal of Clinical Endocrinology and Metabolism, 56, 99-103.

Griffiths, S.L., Knowler, J.T. & Houslay, M.D. (1990) Diabetes induced changes in guanine nuceotide regulatory protein mRNA detected using synthetic oligonuceotide

probes. European Journal of Biochemistry, 193, 367-374.

Grunfeld, J.P., Eloy, L., Moura, A.M., Ganeval, F.W., Ramos-Frendo, B. & Worcel, M. (1985) Effects of antiglucocorticoids on glucocorticoid hypertension in the rat. Hypertension, 7, 292-299.

Guellaen, G., Yates-Aggerbeck, M., Vauquelin, G., Strosberg, D. & Hanoune, J. (1978) Characterisation of the [3H] dihydroergocryptine of the α adrenergic receptor of hepatic plasma membranes: comparison with the ß adrenergic receptor in normal and adrenalectomised rats. Journal of Biological Chemistry, 253, 1114-1120.

Haack, D., Mohring, J., Petri, M. & Hackenthal, E. (1977) Comparative study on development of corticosterone and DOCA hypertension in rats. American Journal of Physiology , 233, F403-F411.

Haigh, R.M. & Jones, C.T. (1990) The effect of glucocorticoids on alpha1-adrenergic receptor binding in vascular smooth muscle. Journal of Molecular Endocrinology (in press).

Haigh, R.M., Jones, C.T. & Milligan, G. (1990) Glucocorticoids regulate the amount of G-proteins in rat aorta. Journal of Molecular Endocrinology, 5, 185-188.

Hamet, P., Fraysse, J. & Franks, D.J. (1978) Cyclic nucleotides and aggregation in platelets of spontaneously hypertensive rats. Circulation Research, 43, 583-591.

Hamet, P., Skuherska, R., Pang, S.C. & Tremblay, J. (1985) Abnormalities of platelet function in hypertension and diabetes. Hypertension ,7, II-135-42.

Handa, M., Kondo, K., Suzuki, H. &Saruta, T. (1984) Dexamethasone hypertension in rats: role of prostaglandins and pressor sensitivity to norepinephrine. Hypertension , 6, 236-241.

Handa, M., Kondo, K., Suzuki, H. &Saruta, T. (1983) Urinary prostaglandin E2 and kallilrein excretion in glucocorticoid hypertension in rats. Clinical Science, 65, 37-42.

Hasegawa, H., Nasjletti, A., Rice, K. & Masson, G.M.C. (1973) Role of pituitary and adrenals in the regulation of plasma angiotensinogen. American Journal of Physiology ,225,1-6.

Hauger-Klevene, J.H. (1970) ACTH, cyclic AMP, dexamethasone and actinomycin D effect on renin release. Acta Physiologica of Latin America.,20, 373-381.

Hazeki, O. & Ui ,M. (1981) Modification by islet-activating protein of receptor mediated regulation of cyclic AMP accumulation in isolated rat heart cells.Journal of Biological Chemistry, 256: 2856-2862.

Hepp, K.D. & Renner, R.(1972) Insulin action of the adenylyl cyclase system: antagonism to activatioby lipolytic hormones. FEBS Letters,20, 191-4.

Hescheler, J., Rosenthal, W., Trautwein, W. & Schultz, G. (1987) The GTP-binding protein, No, regulates neuronal calcium channels. Nature, 325, 445-447.

Heyworth, C.M. & 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., Wallace, A.V. & Houslay, M.D.(1983) Insulin and glucagon regulate the activation of two distinct membrane bound cyclic AMP phosphodiesterases in hepatocytes. Biochemical Journal, 214, 99-110.

Hirata, F., Schiffmann, E., Venkatasubramanian, K., Salomon, D.& Axelrod, J. (1980) A phospholipase A2 inhibitory protein in rabbit neutrophils induced by gluco-corticoids. Proceedings of the National Acadademy of Science of the United States of America,77, 2533-2536.

Hollister, A.S., Onrot, J., Lonce, S., Nadeau, J.H. & Robertson, D.(1986) Plasma catecholamine modulation of alpha 2 adrenoreceptor agonist affinity and sensitivity in normotensive and hypertensive human platelets. Journal of Clinical Investigation , 77, 1416-21.

Homburger, V., Brabet, P., Audigier, Y., Pantaloni, C., Bockaert, J. & Rouot, B. (1987) Immunological localization of the GTP-binding protein Go in different tissues of vertebrates and invertebrates. Molecular Pharmacology ,31, 313-9.

Horn, E.M., Corwin, S.J., Steinberg, S.F., Chow, Y.K., Neuberg, G.W., Cannon, P.J., Powers, E.R. & Bilezikian, J.P. (1988) Reduced lymphocyte stimulatory guanine nucleotide regulatory protein and ß adrenergic receptors in congestive cardiac failure and reversal with angio-

tensin converting enzyme inhibitor therapy. Circulation, 78, 1373-1379.

Houslay, M.D., Bojanic, D., Gawler, D., O'Hagan, S. & Wilson, A. (1986)thrombin, unlike vasopressin appears to stimulate two distinct guanine nucleotide regulatory proteins in human platelets. Biochemical Journal,238, 109-113.

Houslay, M.D., Gawler, D.J., Milligan, G. & Wilson, A. (1989) Multiple defects occur in the guanine nucleotide regulatory protein system in liver plasma membranes of obese (fa/fa) but not lean (Fa/Fa) Zucker rats: loss of functional Gi and abnormal Gs function. Cellular Signalling , 1, 9-22.

Hurley, J.B., Fong, H.K.W., Teplow, D.B., Dreyer, W.J. & Simon, M.I. (1984) Isolation and characterisation of a cDNA clone for the γ subunit of retinal transducin. Proceedings of the National Academy of Science of the United States of America ,81, 6948-52.

Iijima, F. & Malik, K.U. (1985) Mesenteric vascular reactivty in dexamethasone-treated hypertensive rats. Hypertension ,7, 783-790.

Illiano, G. & Cuatrecasas, P. (1972) Modulation of adenylate cyclase activity in liver and fat cell membranes by insulin. Science, 175, 906-908.

Insel, P.A., Stengel, D., Ferry, N. & Hanoune, J. (1982) Regulation of adenylate cyclase of human platelets by forskolin. Journal of Biological Chemistry, 257, 7485-7490.

Itoh, H., Kozasa, T., Nagata, S., Nakamura, S., Katada, T., Ui, M., Iwai, S., Ohtsuka, E., Kawasaki, H., Suzuki, K. & Itoh, Y. (1986) Molecular cloning and sequence determination of cDNAs for α subunits of the guaninie nucleotide binding proteins Gs, Gi and Go from rat brain. Proceedings of the National Academy of Science of the United States of America, 83, 3776-3780.

Jackson, E.K. & Campbell, W.B. (1980) The in situ blood perfused rat mesentery: a model for assessing modulation of adrenergic neurotransmission. European Journal of Pharmacology, 66, 217-229.

Jakobs, K.H., Lasch, P., Minuth, M., Aktories, K. & Schultz, G. (1982) Uncoupling of alpha adrenoreceptor-mediated inhibition of human platelet adenylate cyclase by N-Ethylmaleamide Journal of Biological Chemistry, 257, 2829-2833.

Jarrett, R.J., Keen, H., Mccartney, M., Fuller, J.H., Hamilton, P.J.S., Reid, D.D. & Rose, G. (1978) Glucose tolerance and blood pressure in two population samples: their relation to diabetes mellitus and hypertension. International Journal of Epidemiology, 7, 15-24.

Jelsema, C.L & Axelrod, J. (1987) Stimulation of phospho-lipase A2 activity in bovine rod outer segments by the beta gamma subunits of transducin and its inhibition by the alpha subunit. Proceedings of the National Academy of Science of the United States of America, 84, 3623-7.

Itoh, H., Kozasa, T., Nagata, S., Nakamura, S., Katada, T., Ui, M., Iwai, S., Ohtsuka, E., Kawasaki, H., Suzuki, K. & Itoh, Y. (1986) Molecular cloning and sequence determination of cDNAs for α subunits of the guaninie nucleotide binding proteins Gs, Gi and Go from rat brain. Proceedings of the National Academy of Science of the United States of America, 83, 3776-3780.

Jackson, E.K. & Campbell, W.B. (1980) The in situ blood perfused rat mesentery: a model for assessing modulation of adrenergic neurotransmission. European Journal of Pharmacology, 66, 217-229.

Jakobs, K.H., Lasch, P., Minuth, M., Aktories, K. & Schultz, G. (1982) Uncoupling of alpha adrenoreceptor-mediated inhibition of human platelet adenylate cyclase by N-Ethylmaleamide Journal of Biological Chemistry, 257, 2829-2833.

Jarrett, R.J., Keen, H., Mccartney, M., Fuller, J.H., Hamilton, P.J.S., Reid, D.D. & Rose, G. (1978) Glucose tolerance and blood pressure in two population samples: their relation to diabetes mellitus and hypertension. International Journal of Epidemiology, 7, 15-24.

Jelsema, C.L & Axelrod, J. (1987) Stimulation of phospholipase A2 activity in bovine rod outer segments by the beta gamma subunits of transducin and its inhibition by the alpha subunit. Proceedings of the National Academy of Science of the United States of America, 84, 3623-7.

Jeremy, J.Y. & Dandona, P. (1986) Inhibition by hydrocortisone of prostacyclin synthesis by rat aorta and its reversal with RU 486. Endocrinology, 119, 661-5.

Johnson, G.S. &Jaworski, C.J. (1983) Glucocorticoids increase GTP-dependent adenylate cyclase activity in cultured fibroblasts. Journal of Steroid Biochemistry, 16, 245-264.

Johnson, M.E.M., Das, N.M., Butcher, F.R. & Fain, J.N. (1972) The regulation of gluconeogenesis in isolated rat liver cells by glucagon, insulin, dibutyryl cyclic adenosine monophosphate and fatty acids. Journal of Biological Chemistry, 247, 3229-3235.

Judy, W.V., Watanabe, A.M., Murphy, W.R., Aprison, B.S. & Yu, P.L. (1979) Sympathetic nerve activity and blood pressure in normotensive black-cross rats genetically related to the spontaneously hypertensive rat. Hypertension, 1, 598-604

Kafka, M.S., Lake, C.R., Gullner, H.G., Tallman, J.F., Bartter, F.C. & Fujita, T. (1989) Adrenergic receptor function is different in male and female patients with essential hypertension. Clinical and Experimental Hypertension, 1, 613-627.

Kalsner, S. (1969) Mechanism of hydrocortisone potentiation of response to epinephrine and noradrenaline in rabbit aorta. Circulation Research, 24, 383-395.

Kassis, S., Olasmaa, M., Terenius, L. & Fishman, P.H. (1987) Neuropeptide Y inhibits cardiac adenylate cyclase through a pertussis toxin-sensitive G protein. Journal of Biological Chemistry, 262, 3429-3431.

Katada, T., Bokoch, G.M., Northup, J.K., Ui, M. & Gilman, A.G. (1984) The inhibitory guanine nucleotide binding regulatory component of adenylate cyclase. Journal of Biologial Chemistry, 259, 3568-77.

Katada, T., Gilman, A.G., Watanabe, Y., Bauer, S. & Jakobs, K.H. (1985) Protein kinase C phosphorylates the inhibitory guanine nucleotide regulatory component and apparently suppresses its function in hormonal inhibition of adeylyl cyclase. European Journal of Biochemistry, 151, 431-437.

Katada, T., Oinuma, M. & Ui, M. (1986) Mechanisms of the inhibtion of the catalytic activity of adenylate cyclase by the guanine nucleotide-binding proteins serving as the substrate of islet-activating protein, pertussis toxin. Journal of Biologial Chemistry, 261, 5215-21.

Katz, A.M., Tada, M. & Kirchberger, M.A.(1975) Control of calcium transport in the myocardium by the cAMP-protein kinase system. Advances in Cyclic Nucleotide Research, 5, 453-472.

Keely, S.L., Lincoln, T.M. & Corbin, J.D. (1978) Interaction of acetyl choline and epinephrine on heart cAMP-dependent protein kinase. Journal of Physiology (Lond.),234, 432-8.

Kisch, E.S., Dluhy, R.G. & Williams, G.H.(1976) Enhanced aldosterone response to angiotensin II in human hypertension. Circulation Research, 38, 502-505.

Kiss, Z. (1978) Inhibition of the glucagon stimulated adenylate cyclase activity by insulin. FEBS Letters, 92, 29-32.

Knowlton,,A.I., Loeb, E.N., Stoerk, H.C. & Heffernan, J.F. (1952) Induction of arterial hypertension in normal and adrenalectomised rats given cortisone acetate. Journal of Experimental Medicine,96, 187-205.

Knowlton, A.L. & Loeb, E.N. (1957) Depletion of carcass potassium in rats made hypertensive with desoxy-corticosterone acetate (DCA) and with cortisone. Journal of Clinical Investigation, 36, 1295-1300

Kohlmann, O., Ribeiro, A.B., Marson, O., Saragoca, M.A. & Ramos, O.L.(1981) Methylprednisolone induced hypertension: role for the autonomic and renin angiotensin systems. Hypertension, 3 (suppl. II):II107-II 111

Kornel, L., Kanamarlapudi, N., Travers, T., Taff, D.J., Patel, N., Chen, C., Baum, R.M. & Raynor, W.J. (1982) Studies on high affinitybinding of mineraloand glucocorticoids in rabbit aortacytosol. Journal of Steroid Biochemistry, 16, 245-264.

Krakoff, L., Nicolis, G. & Amsel, B.(1975) Pathogenesis of hypertension in Cushing's syndrome. American Journal of Medicine, 58, 216-220.

Krakoff, L.R., Selvadurai, R. & Sutter, E. (1975) Effect of methylprednisolone upon arterial pressure and the renin-angiotensin system in the rat. American Journal of Physiology ,228, 613-617.

Krolewski, A.S., Canessa, M., Warram, J.H., Laffel, L.M.B., Christlieb, A.R., Knowler, W.C. & Rand, L.I. (1988) Predisposition to hypertension and susceptibility to renal disease in insulindependent diabetes mellitus. New England Journal ofMedicine, 318, 140-145.

Kumano, K., Upsher, M.E. & Khairallah, P.A.(1983) Beta adrenergic receptor response coupling in hypertrophied hearts. Hypertension ,5, I-175-83.

Kumano, K. & Khairallah, P.A.(1984) Adenylate cyclase activity in rat myocardium following chronic infusions of angiotensin II and epinephrine. Journal of Cardiovascular Pharmacology, 6, 756-61.

Kumano, K. & Khairallah, P.A.(1985) Adenylate cyclase activity during development and reversal of cardiac hypertrophy. Journal of Molecular and Cellular Cardiology ,17, 537-48.

Kurtz, T.W., Montano, M., Chan, L. & Kabra, P.(1989) Molecular evidence of genetic heterogeneity in Wistar-Kyoto rats: implications for research with the spontaneously hypertensive rat. Hypertension, 13,188-192.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227, 680-685.

Lamberts, S.W., Timmermans, H.A.T., Krammer-Blankestijn, M. & Birkenhager, J.C.(1975) The mechansim of the potentiating effect of glucocorticoids on catecholamine induced lipolysis.Metabolism Clinical and Experimental ,24, 681-689.

Landis, C.A., Masters, S.B., Spada, A., Pace, A.M., Bourne, H.R. & Vallar, L. (1989) GTPase inhibiting mutations activate the α chain of Gs and stimulate adenylyl cyclase in human pituitary tumours. Nature, 340, 692-696.

Lefer, A.M., Manwarring, J.L. & Verrier, R.L. (1966) Effects of corticosteroids on the cardiovascular responses to angiotensin and norepinephrine. Journal of Pharmacology and Experimental Therapeutics,154, 83-91.

Lentini, S. & Bologna, E. (1974) Hypertension and platelet aggrgation. In Platelet aggregation and drugs, ed. L. Caprino, E.C. Rossie. London, Academic Press, 1974, pp 63-76

Le Quan Sang, K.H. & Devynck, M.A. (1986) Increased platelet cytosolic free calcium concentration in essential hypertension. Journal of Hypertension, 4, 567-574.

Leray, F., Chambaut, A.-M., Perrenoud, M.-L. & Hanoune, J. (1973) Adenylate cyclase activity of rat liver plasma membranes: hormonal stimulations and effect of adrenalectomy. European Journal of Biochemistry, 38, 185-192.

Lester, H.A., Steer, M.L. & Levitzki, A. (1982) Prostaglandin-stimulated GTP hydrolysis associated with activation of adenylate cyclase in human platelet membranes. Proceedingsof the National Academy of Sciences of the United States of America , 79, 719-723.

Leutje, C. W. & Nathanson, N.M.(1988) Chronic membrane depolarization regulates the level of the guanine nucleotide binding protein Go alpha in cultured

neuronal cells. Journal of Neurochemistry, 50, 1775-82.

Lever, A.F. (1986) Slow pressor mechanisms in hypertension: a role for hypertrophy of resistance vessels. Journal of Hypertension , 4, 515-24.

Levine, M.A., Downs, R.W.,Singer, M., Marx, S.J., Aurbach, G.D. & Spiegel, A.M. (1980) Deficient activity of guanine nucleotide regulatory protein in erythrocytes from patients with pseudohypoparathyroidsim. Biochemical and Biophysical Research Commununications, 94, 1913-24.

Levine, M.A., Downs, R.W., Moses, A.M., Breslau, N.A., Marx, S.J., Lasker, R.D., Rizzoli, R.E., Aurbach, G.D. & Spiegel, A.M. (1983) Resistance to multiple hormones in patients with pseudohypoparathyroidism: association with multiple deficient activity of guanine nucleotide regulatory protein. American Journal of Medicine ,74, 545-6.

Levine, M.A., Jap, T.-S., Mauseth, R.S., Downs, R.W. & Spiegel, A.M. (1986) Activity of the stimulatory guanine nucleotide binding protein is reduced in erythrocyes from patients with pseudohypoparathyroidism and pseudo-pseudohypoparathyroidism: biochemical, endocrine, and genetic analysis of Albright's hereditary osteodystrophy in six kindreds. Journal of Clinical Endocrinology and Metabolism, 62, 497-502.

Levine, M.A., Ahn, T.G., Klupt, S.F., Kaufman, K.D., Smallwood, P.M., Bourne,H.R., Sullivan, K.A. & Van-Dop, C. (1988) Genetic deficiency of the alpha subunit of the guanine

nucleotide-binding protein Gs as the molecular basis for Albrightheredítary osteodystrophy. Proceedings of the National Academy of Sciences of the United States of America, 85, 617-21.

Limas, C., Limas, C.J. (1978). Reduced number of ß adrenergic receptors in the myocardium of spontaneously hypertensive rats. Biochemical and Biophysical Research Communications, 83, 710-4.

Lindner, E., Dohadwalla, A.N. & Bhattacharya, B.K. (1978) Positive inotropic and blood pressure lowering activty of a diterpene derivative isolated from *Coleus Forskohlii* : Forskolin. Arzneim. Forsch. 1978; 28:284-289.

Lippton, H.L., Chapnick, B.M., Hyman, A.L. & Kadowitz, P.J. (1979)Inhibition of vasoconstrictor responses by prostacyclin (PGI2) in the feline mesenteric vascular bed. Archives Internationales de Pharmacodynamie et de Therapie ,241, 214-223.

Low, M.G. & Saltiel, A.R. (1988) Structural and functional roles of glycosylphosphatidylinositol in membranes. Science , 239, 268-275.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry ,193, 265-275.

Lucas, Estigarribia, Darga & Reaven (1985) Insulin and hypertension in obesity. Hypertension, 7, 702-706.

Lynch, C.J., Blackmore, P.F., Johnson, E.H., Wange, R.L., Krone, P.K. & Exton, J.H. (1989) Guanine nucleotide binding regulatory proteins and adenylte cyclase in livers of

streptozotocin-and BB/Wor-diabetic rats. Immuno-detection of Gs and Gi with antisera prepared against synthetic peptides. Journal of Clinical Investigation, 83, 2050-62.

MacIntyre, D.E. & Pollock, W.K. (1983) Platelet-activating factorstimulates phosphatidylinositol turnover in human platelets. Biochemical Journal, 212, 433-437.

McKenzie, F.R. & Milligan, G. (1990) Delta opioid receptor mediated inhibition of adenylate cyclase is transduced specifically by the guanine-nucleotide-binding-protein Gi2. Biochemical Journal, 267, 391-398.

McLellan, A.R. & Connell, J.M.C. (1988) Diabetes and hyper-tension.Current Opinion in Cardiology, 3, 666-674.

McLellan, A.R. & Connell, J.M.C. (1989) Hypertension in the diabetic and elderly populations. Current Opinion in Cardiology, 4, 677-686.

Maguire, M.E., Van Arsdale, P.M. & Gilman, A.G. (1976) An agonist specific effect of guanine nucleotides on binding to the beta adrenergic receptor. Molecular Pharmacology, 12, 335-9.

Maisel, A.S., Michel, M.C., Insel, P.A., Ennis, C., Ziegler, M.G. & Phillips, C. (1990) Pertussis toxin treatment of whole blood: a novel approach to assess G-protein function in congestive heart failure. Circulation, 81, 1198-1204.

Maizel, J.V. (1969) Fundamental techniques of virology. Ed Habel, K. & Salzman, N.P. Academic Press, New York, Ch 32, 334-362.

Malbon, C.C., Rapiejko, P.J. & Mangano, T.(1985) Fat cell adenylate cyclase system. Enhanced inhibition by

adenosine and GTP in the hypothyroid rat. Journal of Biological Chemistry , 260, 2558-2564.

Malik, K.U., Ryan, P. & McGiff, J.C.(1976) Modification by prostaglandins E1 and E2, indomethacin and arachidonic acid of the vasoconstrictor responses of the isolated perfused rabbit and rat mesenteric arteries to adrenergic stimuli. Circulation Research., 39, 163-168.

Manganiello, V. & Vaughan, M. (1972) An effect of dexamethasone on adenosine 3'-5'-monophosphate content and adenosine 3'-5'-cells. Journal of Clinical Investigation ,51,2763-2767.

Manganiello, V. & Vaughan, M. (1973) An effect of insulin on cyclic adenosine 3',5' monophosphate phosphodiesterase activity in fat cells. Journal of Biological Chemistry, 248, 7164 -7170.

Mangili, R., Bending, J.J., Scott, G., Li, L.K., Gupta, A. & Viberti, G.C.(1988) Increased sodium-lithium countertransport activity in red cells of patients with insulin dependent diabetes mellitus and nephropathy. New England Journal of Medicine ,318,146-150.

Manicardi, V., Camellini, L., Bellodi, G., Coscelli, C. & Ferrannini, E.(1986) Evidence for an association of high blood pressure and hyperinsulinaemia in obese man. Journal of Clinical Endocrinology and Metabolism, 62,1302- 8.

Mano, K., Akbarzadeh, A. & Townley, R.G. (1979) Effect of hydrocortisone on beta-adrenergic receptors in lung membranes. Life Sciences, 25, 1925-30.

Marchmont, R.J. & Houslay, M.D. (1980) Insulin triggers cyclic AMP dependent activation and phosphorylation of a plasma membrane cyclic AMP phosphodiesterase. Nature, 286, 904-906.

Marchmont, R.J. & Houslay, M.D. (1981) Characterisation of the phosphorylated form of the insulin stimulated cyclic AMP phosphodiesterase from fat liver plasma membranes. Biochemical Journal, 195, 653-660.

Matsumori, Y., Ohyanagi, M., Kawamoto, H., Shibata, R. & Iwasaki, T. (1989) Intracellular distribution of cardiac beta-adrenoreceptors in SHR and WKY. Japanese Circirculation Journal , 53,113-20.

Mattera, R., Graziano, M.P., Yatani, A., Graf, R., Codina, J., Gilman, A.G., Birnabaumer, L. & Brown, A.M. (1989) Bacterially synthesised splice variants of the α subunit of the G protein Gs activate both adenylyl cyclase and dihydropyridine-sensitive calcium channels. Science, 243,804-807.

Melancon, P., Glick, B. S., Malhotra, V., Weidman, P.J., Serafini, T.,Gleason, M.L., Orci, L. & Rothman, J.E. (1987) Involvement of GTP-binding "G" proteins in transport through the Golgistack. Cell, 24, 1053-62.

Mendlowitz, M., Gitlow, S. & Naftchi, N. (1958) Work of digital vasoconstriction produced by infused norepinephrine in Cushing's syndrome. Journal of Applied Physiology. 13,252-256.

Meyer, W.J. & Nichols, N.R. (1981)Mineralocorticoid binding in cultured smooth muscle cells and fibroblasts from rat

aorta. Journal of Steroid Biochemistry, 14, 1157-1168.

Michel, A., Cros, G.H., McNeill, J.H. & Serrano, J.J. (1985) Cardiac adenylate cyclase activity in streptozotocin-treated rats after 4 months of diabetes: impairment of epinephrine and glucagon stimulation. Life Science, 37, 2067-75.

Michel, M.C., Maisel, A.S. & Brodde, O.E. (1990) Mitigation of ß1 and or ß2 adrenoreceptor function in human heart failure. British Journal of Clinical Pharmacology, 30, 375-425.

Miller, J.H. and Bogdonoff, M.D. (1954) Antidiuresis with administration of insulin. Journal of Applied Physiology, 6, 509-12.

Milligan, G., Spiegel, A.M., Unson, C.G. & Saggerson, E.D. (1987) Chemically induced hypothyroidism produces elevated amounts of the alpha subunit of the inhibitory guanine nucleotide binding protein (Gi)and the beta subunit commonto all G-proteins. Biochemical Journal , 247, 223-7.

Milligan, G. (1988) Techniques used in the identification and analysis of function of pertussis toxin-sensitive guanine nucleotide binding proteins. Biochemical Journal, 255, 1-13.

Mills, D.C.B. (1982) The role of cyclic nucleotides in platelets. Handbook of Experimental Pharmacology, 58, 723-761.

Mitchell, F.M., Griffiths, S.L., Saggerson, E.D., Houslay, M.D., Knowler, J.T.& Milligan, G. (1989) Guaninie-nucleo-

tide- binding proteins expressed in rat white adipose tissue. BiochemicalJournal,262, 403-408.

Modan, M., Halkin, H., Almog, S, Lusky,A., Eshkol, A., Shefi, M., Shitril, M. & Fuchs, Z. (1985) A link between hypertension, obesity and glucose intolerance. Journal of Clinical Investigation,75, 809-817.

Mogensen (1982) Long term antihypertensive treatment inhibiting progression of diabetic nephropathy. British Medical Journal,285, 685-8.,

Mondon, C.E. & Reaven, G.M. (1988) Evidence of abnormalities of insulin metabolism in rats with spontaneous hypertension. Metabolism, 37, 303-305.

Motulsky, H.J., O'Connor, D.T. & Insel, P.A.(1983) Platelet α2 adrenergic receptors in treated and untreated essential hypertension. Clinical Science, 64, 265-272.

Mukherjee, C., Caron, M.G. & Lefkowitz, R.J. (1975) Catecholamine induced subsensitivity of adenylate cyclase associated with loss of ß-adrenergic binding sites. Proceedings of the National Academy of Science of the United States of America, 72, 1945-49.

Mukherjee, C. & Lefkowitz, R.J. (1976) Desensitisation of ß adrenergic agonists in a cell free system; resensitisation by guanosine 5'-(ß,gamma-imino)triphosphate and other purine nucleotides. Proceedings of the National Academy of Science of the United States of America, 73:1494-98.

Murakami, T., Katada, T. & Yasuda, H. (1987) Reduction in the activity of the stimulatory guanine nucleotide-binding protein in the myocardium of spontaneously hyper-

tensive rats. Journal of Molecular and Cellular Cardiology ,19, 199-208.

Nakamura, K. & Nakamura,K. (1979) Role of brainstem and spinal noradrenergic neurones in the development and maintenanceof hypertension in spontaneously hypertensive rats. Naunyn Schiedebergs Arch Pharmacol, 305, 127-133.

National Diabetes Data Group. Summary (1985), In: National Diabetes Data Group (ed.) Diabetes in America, publication 85-1468. US Dept. of Health & Human Services Ch.1, pp1-6.

Neer, E. J., Pulsifer, L. & Wolf, L.G. (1988) The amino terminus of G-protein alpha subunits is required for interaction with beta gamma. Journal of Biological Chemistry, 263, 8996-70.

Neuman, J., Schmitz, W., Scholz, H., Von Meyerinck, L., Doring, V. & Kalmar, P. (1988) Increase in myocardial Gi-proteins in heart failure. Lancet, ii, 936-937.

Neville, D.M. (1960) The isolation of a cell membrane fraction from rat liver. Journal of Biophysical and Biochemical Cytology, 8, 413.

Neville, D.M. (1968) Isolation of an organ specific protein antigen from cell surface membrane of rat liver. Biochimica et Biophysica Acta, 154 ,540-546.

Ng, L.L. & Hockaday, T.D.R. (1986) The leucocyte sodium pump in healthy and obese subjects: the association of insulin with its activty. Clinical Endocrinology, 25, 383-392.

Nishio, Y., Kashiwagi, A., Kida, Y., Kodama, M., Abe, N., Saeki, Y. & Shigeta, Y. (1988) Deficiency of cardiac beta adrenergic receptor in streptozocin-induced diabetic rats. Diabetes, 37, 1181-7.

Northup,J.K., Sternweis,P.C., Smigel, M.D., Schleifer, L.S., Ross, E.M. & Gilman, A.G.(1980) Purification of the regulatory compnent of adenylate cyclase. Proceedings of the National Academy of Science of the United States of America ,77, 6516-6520.

Nowaczynski, W., Murakami, T., Wilkins, G.E. & Levin, J. (1986) Association of decreased plasma cortisol and cortico-steroid binding capacity in patients with essential hypertension and their first degree relatives. Journal of Hypertension, 4, S616-S619.

Ohisalo, J.J. & Stouffer, J.E. (1979) Adenosine, thyroid status and regulation of lipolysis. Biochemical Journal, 178, 249-251.

Ohisalo, J.J., Ranta, S. & Huhtaniemi, I.T. (1986) Attenuated adenosine R-site effect in adipocytes in obesity. Metabolism Clinical and Experimental, 35, 143-146.

Ohisalo, J.J. and Milligan, G. (1989) Guanine nucleotide binding proteins Gi and Gs in fat cells from normal, hypo-thyroid and obese human subjects. Biochemical Journal, 260, 843-847.

Okamoto, K. & Aoki, K.(1963) Development of a strain of spontaneously hypertensive rats. Japanese Circulation Journal , 27, 282-293.

Olefsky, J.M., Kolterman, O.G. & Scarlett, J.A. (1982) Insulin action and resistance in obesity and non insulin

dependent type II diabetes mellitus. American Journal of Physiology , 243, E15-30.

Orth, D.N. & Liddle, G.W. (1971) Results of treatment in 108 patients with Cushing's syndrome. New England Journal of Medicine, 285, 243-247.

Parving, H.-H., Andersen, A.R., Smidt, U.M. & Svendsen, P.A.(1983) Early aggressive antihypertensive treatment reduces rate of decline in kidney function in diabetic nephropathy. Lancet , i,1175-9.

Patten, J.L., Johns, D.R., Valle, D., Eil, C., Gruppuso, P.A., Steele, G., Smallwood, P.M. & Levine, M.A. (1990) Mutation in the gene encoding the stimulatory G protein of adenylate cyclase in Albright's Herediatry Osteodystrophy. New England Journal of Medicine, 322, 1412-1419.

Perfilyeva, E.A., Skurat, A.V., Yurkova, M.S., Khropov, Yu.V., Bulargina, T.V. & Severin, E.S, (1985)N-Chloro-acetylhydrazone of oxo-GTP irreversibly inhibits the activating function of GTP-binding protein coupled with adenylate cyclase. Biochemistry International, 11, 1-9.

Peterson , G.L. (1977) A simplification of the protein assay method of Lowry et al. which is more generally applicable. Analytical Biochemistry, 83, 346-356.

Phornchirasilp, S. & Matangkasombut, O.P. (1982) Role of adrenal steroids in the cardiac cAMP response to isoprenaline in the rat. Hormone and Metabolic Research, 14, 207-9.

Pilkis, S.J., Exton, J.H., Johnson, R.A. & 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. & 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.

Plotz, C.M., Knowlton, A.T. & Ragan, C. (1952) The natural history of Cushing's syndrome. American Journal of Medicine, 13, 597- 614.

Poplawski, A., Skorulska, M. & Niewiarowski, S. (1968) Increased platelet adhesiveness in hypertensive cardiovascular disease. Journal of Atherosclerosis Research, 8, 721-723.

Portha, B., Chamras, H., Broer, Y., Picon, L. & Rosselin, G. (1983)Decreased glucagon-stimulated cyclic AMP production by isolated liver cells of rats with type 2 diabetes. Molecular and Cellular Endocrinology., 32, 3-26.

Raker, J.W., Cope, O. & Ackerman, P. (1964) Surgical experience with the treatment of hypertension of Cushing's syndrome. American Journal of Surgery, 107, 153-158.

Rall, T. & Harris, B.A. (1987) Identification of the lesion in the stimulatory GTP-binding protein of the uncoupled S49 lymphoma. FEBS Letters , 224, 365-71.

Ramanathan, S., & Shibata, S. (1974) Cyclic AMP in blood vessels of spontaneously hypertensive rat. Blood Vessels,11,312-8.

Rascher, W., Dietz, R., Schomig, A., Burkart, G. & Gross, F. (1980) Reversal of corticosterone-induced super-sensitivity of vascular smooth muscle to noradr-enaline by arachidonic acid and prostacyclin. European Journal of Pharmacology, 68, 267-273.

Ray, T.K. (1969) A modified method for the isolation of the plasma membrane from rat liver. Biochimica et Biophysica Acta. 196,1-9.

Reaven, G.M.(1988) Banting Lecture 1988: role of insulin resistance in human disease. Diabetes, 37, 1595-607.

Reis, D.J. (1960) Potentiation of the vasoconstrictor action of topical norepinephrine on the human bulbar conjunctival vessels after topical application of certain adrenocortical hormones. Journal of Clinical Endocrinology and Metabolism, 20, 446.

Resink, T.J., Burgisser, E. & Buhler, F.R. (1986) Enhanced platelet cAMP response to prostaglandin E1 in essential hypertension. Hypertension ,8 , 662-8.

Richelson, B. (1988) Prostaglandin E2 action and binding in human adipocytes: effects of sex, age and obesity. Metabolism Clinical and Experimental, 37, 268-275.

Rizzoli, R., von-Tscharner, V. & Fleisch, H. (1986) Increase of adenylate cyclase catalytic-unit activity by dexa-methasone in rat osteoblast-like cells. Biochemical Journal, 237, 447-54.

Robberecht, P., Winand, J., Chatelain, P., Poloczek, P., Camus, J.C., DeNeff, P. & Christophe, J. (1981) Comparison of ß receptors and the adenylate cyclase system with muscarinic receptors and guanylate cyclase activities in the heart of spontaneously hypertensive rats. Biochemical Pharmacology, 30, 385-92.

Robishaw, J.D., Smigel, M.D. & Gilman, A.G.(1986) Molecular basis for two forms of the G-protein that stimulates adenylate cyclase. Journal of Biological Chemistry, 261, 9587-9590

Rodan, G.A. & Feinstein, M.B. (1976) Interrelationships between Ca2+ and adenylate and guanylate cyclases in the control of platelet secretion and aggregation. Proceedings of the National Academy of Science of the United States of America, 73, 1829-1833.

Rodan, S.B. & Rodan, G.A. (1986) Dexamethasone effects on ß adrenergic receptors and adenylate cyclase regulatory proteins Gs and Gi in ROS 17/2.8 cells. Endocrinology, 118,2510-2518.

Rodbell, M., Birnbaumer, L., Pohl, S.L. & Krans, H.M.J. (1971) The glucagon-sensitive adenyl cyclase system in plasma membranes of rat liver. V. An obligatory role of guanyl nucleotides in glucagon action. Journal of Biological Chemistry ,246,1877-82.

Ros. M., Northup, J.K. & Malbon, C.C. (1988) Steady-state levels of G-proteins and beta-adrenergic receptors in rat fat cells. Permissive effects of thyroid hormones. Journal of Biological Chemistry, 263, 4362-8.

Ros, M., Northup, J.K.& Malbon, C.C. (1989) Adipocyte G proteins and adenylate cyclase. Effects of adrenalectomy. Biochemical Journal , 257, 737-44.

Ros, M., Watkins, D.C., Rapiejko, P.J. & Malbon, C.C. (1989) Glucocorticoids modulate mRNA levels for G-protein ß-subunits. Biochemical Journal. , 260, 271-5.

Rose, H.G., Yalow, R.S., Schweitzer, P. & Schwartz, E. (1986) Insulin as a potential factor influencing blood pressure in amputees. Hypertension, 8, 793-800.

Ross, E.M. & Gilman, A.G. (1980) Biochemical properties of hormone sensitive adenylate cyclase.Annual Reviews of Biochemistry, 49, 533-564.

Rozengurt, E. (1986) Early signals in the mitogenic response. Science, 234, 161-166.

Rowe, J.W., Young, J.B., Minaker, K.L., Stevens, A.I., Palotta, J. & Landsberg, L. (1981) Effect of insulin and glucose infusions on sympathetic nervous system activity in normal man. Diabetes , 30, 219-25.

Russo, D., Fraser, R., Kenyon, C.J. (1990) Increaed sensitivity to norepinephrine in glucocorticoid-treated rats: the effects of indomethacin and desipramine. Journal of Hypertension, 8, 827-833.

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.F., Duman, R.S. & Nestler, E.J. (1989) Corticosterone differentially regulates the expression of Gs alpha and Gi alpha messenger RNA and protein in the rat cerebral cortex.

Proceedings of the National Academy of Science of the United States of America, 86, 3906-10.

Saito, T.,Akita, Y., Fujita, H., Furukawa, Y., Tsuchiva, Y., Yasuda, T., Yamamoto, M., Kitagawa, T., Nakagawa, Y., Takehiro, A., Fujita, T., Kodama, S. & Kuzuya, T.(1986) Stimulatory guanine nucleotide binding protein activity in the erythrocyte membrane of patients with pseudohypoparathyroidism type 1 and related disorders. Acta Endocrinologica ,111, 507-515.

Salomon, Y. (1979) Adenylate cyclase assay. Advances in Cyclic Nucleotide Research, ed. G. Brooker, P. Greengard & G.A.Robison. Raven Press, New York, 10, 35-55.

Schmidtke, J., Wienker, Th., Flugel, M., Engel,W. (1976) In vitro inhibition of cyclic AMP phosphodiesterase by cortisol. Nature , 262, 593-594.

Schomig, A., Luth, B., Dietz, R. & Gross, F. (1976) Changes in vascular smooth muscle sensitivity to vasoconstrictor agents induced by corticosteroids, adrenalectomy and differing salt intake in rats. Clinical Science and Molecular Medicine , 51, 61S-63S.

Schutz, W., Steurer, G., Tuisl, E. & Kraupp, O. (1984). The cardiac and brain microvessel adenylate cyclase system in deoxycorticosterone acetate hypertensive rats. Journal of Cardiovascular Research, 6, 325-330.

Scott,A.B., Lawrence, B., Nguyen, H.H. & Meyer, J.W. (1987) Aldosterone and dexamethasone binding in human arterial smooth muscle cells. Journal of Hypertension., 5, 739-744.

Seamon, K.B., Padgett, W. & Daly, J.W. (1981) Forskolin-unique diterpene activator of adenylate cyclase in membranes and intact cells. Proceedings of the National Academy of Science of the United States of America, 78, 3363-7.

Seamon, K.B.,and Daly, J.W. (1981) Activation of adenylate cyclase by the diterpene forskolin does not require the guanine nucleotide regulatory protein. Journal of Biological Chemistry, 256, 9799-9801.

Seaquist, E.R., Goetz, F.C., Rich, S. & Barbosa, J. (1989) Familial clustering of diabetic kidney disease: evidence for genetic susceptibility to diabetic nephropathy. New England Journal of Medicine, 320, 1161-5.

Sharma, R.V., Kemp, D.B., Gupta, R.C. & Bhalla, R.C (1982). Properties of adenylate cyclase in the cardiovascular tissues of renal hypertensive rats. Journal of Cardiovascular Pharmacology, 4, 622-8.

Simmonds, W.F., Goldsmith, P.K., Codina, J., Unson, C. & Spiegel, A.M. (1989) Gi2 mediates α2 adrenergic inhibition of adenylyl cyclase in platelet membranes: in situ identification with Gα c-terminal antibodies. Proceedings of the National Academy of Sciences of the United States of America,86, 7809-7813.

Singer, P., Godicke, W., Voigt,S., Hajdu, I. & Weiss,M. (1985) Postprandial hyperinsulinaemia in patients with mild hypertension. Hypertension, 7, 182-186.

Smith, C.I., Pierce, G.N. & Dhalla, N.S. (1984) Alterations in adenylate cyclase activity due to streptozotocin-

induced diabetic cardiomyopathy. Life Sciences, 34, 1223-30.

Snavely, M.D. & Insel, P.A.(1982) Characterisation of alpha-adrenergic receptor subtypes in the rat renal cortex. Molecular Pharmacology, 22, 532-546.

Song, C.S., Rubin, W., Rifkind, A.B. & Kappas, A. (1969) Plasma membranes of the rat liver: isolation and enzymatic characterisation of a fraction rich in bile canaliculi. Journal of Cell Biology ,41,124-132.

Spence, S. & Houslay, M.D. (1989) The local anaesthetic benzylalcohol attenuates the α2 adrenoreceptor mediated inhibition of human platelet adenylate cyclase activty when stimulated by prostaglandin E1, but not when stimulated by forskolin.Biochemical Journal, 264, 483-488.

Spiegel, A.M. (1990) Albright's Hereditary Osteodystrophy and defective G proteins. New England Journal ofMedicine, 322, 1461-1462.

Srivastava, A.K. & Anand-Srivastava, M.B.(1985) Strepto-zotocin-induced diabetes and hormone sensitivity of adenylate cyclase in rat myocardial sarcolemma, aorta and liver. Biochemical Pharmacology, 34, 2013-7.

Stamler, J., Rhomberg, P. & Schoenberger, J.A. (1975) Multivariate analysis of the relationship of seven variables to blood pressure: findings of the Chicago Heart Association detection project in industry, 1967-72. Journal of Chronic Disease, 28, 527-48.

Steer, M.L. & Wood, A. (1979) Regulation of human platelet adenylate cyclase by epinephrine, prostaglandin E1,

and guanine nucleotides. Journal of Biological Chemistry, 254, 10791-10797.

Stiles, G.L., Caron, M.G. & Lefkowitz, R.J. (1984) ß-adrenergic receptors: biochemical mechanisms of physiological regulation. Physiology Reviews, 64, 661-743.

Strassheim, D., Milligan, G. & Houslay, M.D. (1990) Diabetes abolishes the GTP-dependent, but not the receptor dependent inhibitory function of the inhibitory guanine nucleotide binding regulatory protein (Gi) on adipocyte adenylate cyclase activty. Biochemical Journal, 266, 521-526.

Su, Y.A., Harden, T.K., & Perkins, J.P. (1980) Catecholamine specific desensitisation of adenylate cyclase: evidence for a multistep process. Journal of Biological Chemistry, 255, 7410-9.

Sudhir, K., Jennings, G.L., Esler, M.D., Korner, P.I., Blombery, P.A., Lambert, G.W., Scoggins, B. & Whitworth, J.A. (1989)Hydrocortisone-induced hypertension in humans: pressor responsiveness and sympathetic function. Hypertension,13, 416-421.

Suki, W.N., Abramowitz, J., Mattera, R., Codina, J. & Birnbaumer, L. (1987) The human genome encodes at least three non-allellic G-protiens with αλπηα i-type subunits. FEBS Letters, 220, 187-192.

Sullivan, K. A., Miller, R.T., Masters, S.B., Beiderman, B., Heideman, W. & Bourne, H.R. (1987) Identification of receptor contact site involved in receptor-G protein coupling. Nature , 330, 758-60.

Sundaresan, P.R., Sharma, V.K., Gingold, S.I. & Banerjee, S.P. (1984) Decreased beta-adrenergic receptors in rat heart in streptozotocin-induced diabetes: role of thyroid hormones. Endocrinology ,114, 1358- 63.

Suzuki, H., Handa, M., Kondo, K. & Saruta, T.(1982) Role of renin, angiotensin system in glucocortiocid hypertension in rats.American Journal of Physiology, 243, E48-E51.

Tanif, Z. & Harbon, S. (1987) Heterologous regulations of cAMP responses in pregnant rat myometrium. Evolution from a stimulatory to an inhibitory prostaglandin E2 and prostacyclin effect. Molecular Pharmacology , 32, 249-257.

Tanuma, S., Kawashima, K., Endo, H. (1988) Eukaryotic mono (ADP-ribosyl) transferase that ADP ribosylates GTP-binding regulatory Gi protein. Journal of Biological Chemistry, 263, 5485-9.

Taylor, J.E. & Shirachi, D.Y. (1977) Cyclic nucleotide phospho-diesterase activity in cardiac and vascular smooth muscle of the spontaneously hypertensive rat. Proceedings of the Western Pharmacological Society, 20, 307-10.

Taylor, C.E. & Merritt, J.E. (1986) Receptor coupling to polyphosphoinositide turnover-a parallel with the adenylate cyclase system. Trends in Pharmacological Science, 7, 238-242.

Tobian, L. & Binion, J.T. (1952) Tissue cations and water in arterial hypertension. Circulation, 5, 754-758.

Tomlinson, S., MacNeil, S. & Brown, B.L. (1985) Calcium, Cyclic AMP and hormone action. Clinical Endocrinology, 23, 595-610.

Tonolo, G., Fraser, R., Connell, J.M.C. & Kenyon, J.C.(1988) Chronic low dose infusion of dexamethasone in rats: effects on blood pressure, body weight and plasma atrial natriuretic peptide. Journal of Hypertension, 6, 25-31.

Touster, O., Aronson, N.N., Dulaney, J.T. & Hendrickson, H. (1970)Isolation of rat liver plasma membranes. Journal of Cell Biology, 47, 604-618.

Towbin, H., Staehelin, T. & Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proceedings of the National Academy of Science of the United States of America 76, 4350-4354.

Tremblay, J. & Hamet, P.(1984) Calcium-dependent proteolytic stimulation of adenylate cyclase in platelets from spontaneously hypertensive rats. Metabolism ,33, 689-95.

Triner, L., Nahas, G.G., Vulliemoz, Y, Overweg, N.I.A., Verosky, M., Habif, D.V. & Ngai, S.H. (1971) Cyclic AMP and smooth muscle function. Annals of the New York Academy of Science ,185, 458-76.

Triner, L., Valliemoz, Y., Verosky, M. & Manger, W.M. (1975) Cyclic adenosine monophosphate and vascular

reactivity in spontaneously hypertensive rats. Biochemical Pharmacology 1975, 24, 743-5.

Umemura, S., Smyth, D.D. & Pettinger, W.A. (1985) Defective renal adenylate cyclase response to prostaglandin E2 in spontaneously hypertensive rats. Journal of Hypertension, 3,159-165.

United Kingdom Prospective Diabetes Study. (1985) Prevalence of hypertension and hypertensive therapy in patients with newly diagnosed diabetes. Hypertension , 7, II 8-II 13

Vallar, L., Spada, A. & Giannattasio, G. (1987) Altered Gs and adenylate cyclase activity in human GH-secreting pituitary adenomas. Nature, 330, 566-568.

Van Dop, C., Yamanaka, G., Steinberg, F., Sekura, R.D., Manclark, C.R., Stryer, L. & Bourne, H.R.(1984). ADP ribosylation of transducin by pertussis toxin blocks the light stimulated hydrolysis of GTP and cGMP in retinal photoreceptors. Journal of Biological Chemistry, 259, 23-25.

Vanhoutte, P.M., Cohen, R.A. & Van Nuelen, J.M. (1984) Serotonin and arterial vessels. Journal of Cardio-vascular Pharmacology, 6 (suppl.2),S421-428.

Viberti, G.C., Keen, H. & Wiseman, M.J.(1987) Raised arterial pressure in parents of proteinuric insulin dependent diabetics. British Medical Journal, 295, 515-7.

Voors, A.W., Radhakrishnamurthy, B., Srinivasan, S.R., Webber, L.S. & Berenson, G.S. (1981) Plasma glucose level related to blood pressure in 272 children, ages 7-15

years sampled from a total biracial population. American Journal of Epidemiology,113, 347-356.

Wahrenberg, H., Engfeldt, P., Arner, P., Wennlund, A. & Ostman, J. (1986) Adrenergic regulation of lipolysis in human adipocytes: findings in hyper- and hypothyroidism. Journal of Clinical Endocrinology and Metabolism,63, 631-638.

Wallner, B.P., Mattaliano,R.J., Hession, C., Cate, R.L., Tizard, R., Sinclair, L.K., Foeller, C., Chow, E.P. Browning, J.L. & Ramachandran, K.L. et al. (1986) Cloning and expression of human lipocortin, a phospholipase A2 inhibitor with potential antiinflammatory activty. Nature, 320, 77-81.

Watt, G.C.M., Harrap, S.B., Foy, C.J.W., Holton, D.W., Edwards, H.V., Davidson, R., Conner, J.M. & Fraser, R. (1991) Application of the four corners approach to identification of genetic determinants of blood pressure (in press).

Whitworth, J.A., Connell, J.M.C., Lever, A.F., Fraser, R (1986) Pressor responsiveness in steroid induced hypertension in man. Clinical and Experimental Pharmacology and Physiology, 13, 353-8.

Widnell, C.C. (1972) Cytochemical localisation of 5'-Nucleotidase in subcellular fractions isolated from rat liver. Journal of Cell Biology, 52, 542-558.

Williams, J.V., Watanabe, A.M., Henry, O.P., Besch, H.R., Murphy, W.R. & Hockel, G.M. (1976) Sympathetic nerve activty: role in regulation of blood pressure in the spont-

aneously hypertensive rat. Circulation Research, 38 (suppl. 1), II 21-II 28.

Will-Shahab, L., Kutter, I. & Warbanow,W. (1986) Signal transfer in cardiac muscle. Alteration of the beta-adrenoreceptor adenylate cyclase system in the hypertrophied myocardium. Biomedica Biochimica Acta 45, S199-204.

Winer, N., Chokshi, D.S. & Walkenhorst, W.G. (1971) Effects of cyclicAMP sympathomimetic amines and adrenergic receptor antagonists on renin secretion. Circulation Research, 29, 239-248.

Winocour, P.D., Bryszweska, M., Watala, C., Rand, M.L., Epand, R.M., Kinlough-Rathbone, P.L., Packham, M.A. & Fraser Mustard, J. (1990) Reduced membrane fluidity in platelets from diabetic patients. Diabetes, 39, 241-244.

Wisgerof, M. & Brown, R.D. (1978) Increased adrenal sensitivity to angiotensin II in low renin essential hypertension. Journal of Clinical Investigation, 61, 1456-1462.

Woodcock, E.A., Funder, J.W. & Johnston, C.I. (1979) Decreased cardiac ß adrenergic receptors in deoxycortico-sterone-salt and renal hypertensive rats. Circulation Research, 45, 560-65.

Woodcock, E.A. & Johnston, C.I. (1980) Changes in tissue alpha-and beta- adrenergic receptors in renal hyper-tension in the rat. Hypertension, 2, 156-161.

Wybenga, D.R. & Inkpen, J.A. (1974) Lipids: cholesterol and cholesterol esters. In: Clinical Chemistry Principles &

Techniques, 2nd edition, ed: R.J. Henry, D.C. Connor, J.W. Winkelman. Harper & Row, New York,pp 1428-1449.

Yamori, Y. (1983) Physiopathology of the various strains of spontaneously hypertensive rats. In: Hypertension: physiopathology and treatment. Eds: Genest, J., Kuchel, O., Hamet, P., Cantin, M. McGraw Hill, New York. Ch.36, pp556-581

Yasunari, K., Kohno, M., Balmforth, A., Murakawa, K., Yokokawa, K.,Kurihara, N. & Takeda, T. (1989) Glucocorticoids and dopamine-1 receptors on vascular smooth muscle cells. Hypertension,13, 575-81.

Yatani, A., Codina, J., Brown, A.M. & Birnbaumer, L. (1986) Direct activation of mammalian atrial muscarinic K+ channels by a human erythrocye pertussis toxin sensitive G-protein, G_K. Science, 235, 207-211.

Zavoico, G.B. & Feinstein, M.B. (1984) Cytoplasmic Ca2+ in platelets is controlled by cyclic AMP: antagonism between stimulation and inhibition of adenylate cyclase, Biochemical and Biophysical Research Communications, 120 , 579-585.

APPENDIX A

	STUDY NO.	CATEGORY	SEX	AGE	WEIGHT (kg)	SBP(mmHg)	DBP(mmHg)	Na+(mmol/l)	K+(mmol/l)	Urea(mmol/l)	Cr(μmol/l)	HbA (%)	Cholesterol(mmol/l)
1	C24	NORMAL	MALE	28.9	65.2	116.0	78.0	141.0	3.9	4.4	95.0	6.2	4.9
2	C26	NORMAL	MALE	32.0	74.0	118.0	70.0	142.0	4.5	6.0	99.0	5.5	5.9
3	C18	NORMAL	MALE	40.0	70.0	120.0	80.0	141.0	4.1	5.3	93.0	6.1	4.9
4	C14	NORMAL	FEMALE	44.1	59.9	108.0	66.0	139.0	3.7	4.2	82.0	5.7	4.9
5	C16	NORMAL	MALE	46.7	73.5	134.0	100.0	142.0	3.8	4.4	93.0	5.9	4.6
6	C17	NORMAL	MALE	48.9	77.7	118.0	62.0	141.0	4.1	4.9	102.0	6.0	6.0
7	C25	NORMAL	MALE	51.4	83.3	128.0	92.0	141.0	3.8	5.5	107.0	5.8	5.4
8	C23	NORMAL	MALE	51.6	60.8	124.0	90.0	141.0	4.1	4.4	73.0	5.3	5.2
9	C22	NORMAL	MALE	59.8	70.0	174.0	104.0	140.0	4.9	3.6	88.0	6.6	6.3
10	C9	NORMAL	MALE	22.5	76.5	122.0	84.0	139.0	4.4	3.6	68.0	5.6	4.1
11	C42	NORMAL	FEMALE	59.2	72.4	116.0	70.0	141.0	4.1	5.2	74.0	6.7	5.8
12	C31	NORMAL	MALE	49.4	82.8	128.0	90.0	141.0	4.2	4.8	84.0	5.6	6.8
13	C34	NORMAL	FEMALE	46.8	68.1	140.0	78.0	143.0	4.0	5.7	57.0	6.2	5.8
14	C41	NORMAL	FEMALE	50.9	65.4	140.0	90.0	140.0	3.9	8.0	64.0	5.9	4.8
15	C36	NORMAL	MALE	52.5	70.0	124.0	80.0	141.0	4.6	4.9	88.0	6.1	4.6
16	C1	NORMAL	FEMALE	23.1	69.0	118.0	70.0	143.0	3.9	3.7	70.0	5.9	4.1
17	C27	NORMAL	MALE	42.7	69.3	118.0	78.0	•	•	6.3	90.0	7.6	5.4
18	A14	NIDDM	MALE	33.5	96.5	118.0	86.0	140.0	4.0	4.2	70.0	•	10.5
19	A15	NIDDM	MALE	29.1	75.0	130.0	82.0	138.0	3.9	6.3	94.0	9.8	5.9
20	A16	NIDDM	MALE	43.7	85.3	120.0	89.0	141.0	3.6	8.4	67.0	10.4	6.1
21	A19	NIDDM	FEMALE	33.5	93.9	130.0	76.0	134.0	4.0	3.8	75.0	12.0	•
22	A22	NIDDM	FEMALE	57.0	103.4	128.0	88.0	138.0	4.2	4.2	70.0	•	5.6
23	A25	NIDDM	MALE	76.0	78.5	150.0	86.0	138.0	4.4	5.5	95.0	12.7	6.3
24	A28	NIDDM	MALE	64.0	•	124.0	88.0	142.0	4.0	5.5	70.0	8.6	5.1
25	A35	NIDDM	FEMALE	53.5	66.2	146.0	92.0	•	•	•	•	9.4	7.7
26	A2	NIDDM	MALE	37.0	71.2	120.0	80.0	140.0	4.5	3.5	85.0	•	5.8
27	A31	NIDDM	FEMALE	71.3	99.0	128.0	100.0	140.0	4.4	5.2	64.0	8.6	6.5
28	A34	NIDDM	FEMALE	62.4	72.0	180.0	90.0	141.0	3.9	5.6	90.0	9.8	6.5

A

	Triglyceride(mmol/l)	Glucose 0'(mmol/l)	Gi2	Gi3	Gs	PGE stim %	FSK stim %	%inhib Ad on F	% inhib Ad on PGE1
1	.9	7.4	83.0	60.0	103.0	71.0	97.0	7.0	46.0
2	.7	5.2	90.0	90.0	•	84.0	93.0	11.0	28.0
3	.7	6.1	78.0	79.0	•	127.0	98.0	1.0	13.0
4	.6	6.6	73.0	•	•	92.0	113.0	9.0	26.0
5	1.1	5.5	77.0	58.0	113.0	138.0	119.0	14.0	50.0
6	1.2	4.9	61.0	90.0	•	117.0	102.0	17.0	51.0
7	1.0	5.7	73.0	86.0	121.0	161.0	136.0	9.0	48.0
8	.6	5.0	75.0	64.0	113.0	119.0	140.0	9.0	54.0
9	.6	5.2	82.0	75.0	•	73.0	108.0	6.0	51.0
10	1.0	5.0	•	•	95.0	•	•	•	•
11	1.7	5.2	94.0	•	•	105.0	148.0	29.0	24.0
12	1.5	5.7	85.0	42.0	111.0	57.0	42.0	18.0	54.0
13	.6	5.0	84.0	82.0	•	•	82.0	•	•
14	.7	4.9	72.0	114.0	122.0	67.0	114.0	6.0	51.0
15	.6	5.2	•	•	110.0	•	•	•	•
16	.6	4.1	•	•	120.0	99.0	98.0	12.0	52.0
17	1.8	5.2	•	•	•	108.0	104.0	4.0	59.0
18	•	13.9	46.0	70.0	92.0	52.0	56.0	15.0	58.0
19	1.4	10.6	57.0	63.0	•	70.0	76.0	10.0	43.0
20	1.9	12.6	32.0	58.0	106.0	•	78.0	13.0	•
21	•	18.0	9.0	•	78.0	93.0	52.0	12.0	36.0
22	2.0	•	71.0	•	100.0	49.0	91.0	15.0	49.0
23	1.9	11.5	22.0	67.0	100.0	69.0	99.0	19.0	35.0
24	1.3	10.5	86.0	49.0	88.0	87.0	119.0	10.0	58.0
25	1.5	11.4	•	•	112.0	17.7	•	•	•
26	1.4	13.7	44.0	36.0	•	12.0	18.0	21.0	28.0
27	1.3	9.5	51.0	45.0	112.0	15.0	53.0	7.0	42.0
28	2.0	8.5	77.0	98.0	107.0	30.0	51.0	15.0	46.0

APPENDIX B

	STUDY NO.	CATEGORY	SEX	AGE.	WEIGHT(kg)	SBP(mmHg)	DBP(mmHg)	Na+(mmol/l)	K+(mmol/l)	urea(mmol/l)	Cr(μmol/l)	HbA1(%)	fructosamine.
1	C33	NORMAL	MALE	43.6	85.1	150.0	86.0	143.0	4.0	3.4	77.0	6.8	2.5
2	C34	NORMAL	FEMALE	46.8	68.1	140.0	78.0	143.0	4.0	5.7	57.0	6.2	2.6
3	C3	NORMAL	MALE	25.5	63.0	113.0	79.0	136.0	4.0	4.7	93.0	6.6	2.4
4	C35	NORMAL	MALE	30.8	65.0	120.0	80.0	143.0	4.1	3.5	76.0	6.9	2.4
5	C29	NORMAL	MALE	45.1	72.4	134.0	82.0	140.0	4.3	6.5	84.0	6.6	2.7
6	C20	NORMAL	MALE	48.0	73.1	115.0	82.0	138.0	4.3	3.3	77.0	6.9	2.2
7	C25	NORMAL	MALE	51.4	83.3	129.0	88.0	141.0	3.8	5.5	107.0	5.8	2.8
8	C24	NORMAL	MALE	28.9	65.2	116.0	77.0	141.0	3.9	4.4	95.0	6.2	2.2
9	C10	NORMAL	FEMALE	29.4	65.5	126.0	77.0	142.0	4.1	4.7	99.0	5.2	2.2
10	C22	NORMAL	MALE	59.8	70.0	174.0	85.0	140.0	4.9	3.6	88.0	6.6	2.4
11	C23	NORMAL	MALE	51.6	60.8	124.0	90.0	141.0	4.1	4.4	73.0	5.3	2.3
12	C15	NORMAL	MALE	45.4	71.6	153.0	88.0	141.0	4.2	6.3	91.0	6.0	2.5
13	C19	NORMAL	MALE	25.9	63.7	115.0	64.0	141.0	4.4	5.5	88.0	6.0	•
14	C21	NORMAL	MALE	27.1	63.3	106.0	69.0	140.0	4.1	5.5	92.0	5.3	2.3
15	H10	HBP	MALE	46.7	73.5	134.0	100.0	142.0	3.8	4.4	93.0	5.9	2.4
16	H9	HBP	FEMALE	42.4	71.5	180.0	102.0	138.0	4.0	4.3	84.0	5.5	2.3
17	H1	HBP	MALE	24.8	75.3	140.0	100.0	145.0	3.8	5.6	84.0	5.2	2.2
18	H2	HBP	MALE	58.1	59.2	186.0	100.0	139.0	4.9	5.2	97.0	7.7	2.0
19	H3	HBP	MALE	44.1	75.2	154.0	100.0	144.0	4.1	4.9	82.0	5.2	1.9
20	H4	HBP	MALE	64.3	75.8	146.0	98.0	138.0	4.0	6.1	78.0	•	3.2
21	H5	HBP	MALE	61.9	59.0	190.0	118.0	138.0	4.0	2.3	75.0	5.8	1.7
22	H14	HBP	MALE	26.5	69.8	170.0	110.0	141.0	3.9	3.2	59.0	5.4	2.5
23	H6	HBP	MALE	60.5	79.0	192.0	128.0	142.0	3.6	4.7	132.0	•	•
24	H18	HBP	MALE	55.8	90.3	170.0	130.0	138.0	4.7	6.7	167.0	8.8	2.1
25	H17	HBP	FEMALE	69.7	71.8	178.0	96.0	144.0	4.3	7.0	67.0	6.7	2.2
26	H19	HBP	MALE	47.5	85.0	146.0	102.0	140.0	4.2	5.2	90.0	•	•
27	H16	HBP	FEMALE	31.9	99.0	138.0	98.0	139.0	3.7	3.0	57.0	•	•
28	H15	HBP	FEMALE	25.0	75.3	145.0	96.0	140.0	3.7	4.9	55.0	•	•

B

	cholesterol(mmol/l)	triglyceride(mmol/l)	hbp-present/FH	Gi2 v std	Gs v std	Gi2:Gs ratio	BASAL ACA	FORSKOLIN.	GppNHp+FORS	PGE1+FORS	ADR/PROP+FORS
1	5.1	.7	NO	1.184	1.169	1.013	590.6	23704.3	23220.8	44517.0	13910.1
2	5.8	.6	NO	•	•	•	391.9	15959.5	15310.2	29707.5	8776.6
3	3.5	.6	NO	•	•	•	406.8	17533.1	13693.1	28058.9	11574.2
4	3.0	.4	YES	•	•	•	245.3	15590.5	11826.1	19773.2	8641.1
5	5.5	.6	YES	1.085	.352	3.084	492.7	17768.0	15569.2	33766.6	9469.8
6	5.8	1.6	NO	1.067	.165	6.464	1037.6	34190.5	28271.1	53349.9	21279.7
7	5.4	1.0	NO	•	•	•	883.0	27425.1	21850.0	53126.5	12066.8
8	4.9	.9	YES	•	•	•	129.0	10801.7	8565.2	18785.1	6047.8
9	4.8	.5	YES	.914	.310	2.949	397.6	21885.7	32023.4	38775.9	14553.9
10	6.3	.6	NO	.971	.993	.978	380.2	17795.7	17593.0	32387.0	11385.1
11	5.2	.6	NO	.475	.679	.700	394.9	21630.3	15726.4	38288.9	15726.4
12	8.4	3.1	NO	.893	.187	4.774	415.9	18580.4	12140.6	22143.9	15348.3
13	3.8	.5	NO	.957	.638	1.500	1161.3	20581.9	15194.1	35905.5	11398.0
14	5.2	1.6	YES	1.448	.845	1.714	311.2	14456.4	12714.6	29440.8	8584.2
15	4.6	1.1	YES	.660	.669	.987	710.8	29569.2	28072.0	58167.8	15310.0
16	•	•	YES	.946	.485	1.950	675.6	38050.5	35781.2	79194.8	17116.6
17	5.8	1.6	YES	.991	.652	1.520	950.8	31759.3	24557.3	52864.6	17683.0
18	7.0	1.7	YES	.911	.238	3.828	434.8	15053.6	13523.7	29659.2	10617.2
19	3.8	.3	YES	1.151	1.049	1.098	526.7	19201.2	16371.6	35078.9	9979.3
20	•	•	YES	•	•	•	549.8	18240.6	14650.4	32675.8	11241.7
21	5.0	1.7	YES	1.217	1.181	1.030	1296.9	21102.5	17613.2	46062.4	13217.5
22	4.5	.8	YES	•	•	•	572.8	15088.2	13010.7	26347.9	11010.7
23	4.8	1.2	YES	•	•	•	591.2	16385.0	19555.7	31607.1	10283.8
24	3.1	.6	YES	1.373	1.190	1.154	256.9	10716.1	10345.2	18826.2	8222.7
25	6.2	1.1	YES	.477	.622	.767	281.8	11636.4	12986.5	20850.2	10650.9
26	5.2	2.9	YES	.655	.154	4.254	433.8	18814.5	17863.3	34340.6	14544.0
27	3.9	.9	YES	1.092	.443	2.465	469.5	12202.5	13031.9	23753.9	10593.1
28	4.7	.9	YES	.948	1.012	.937	393.5	17955.3	17075.4	34926.3	11070.0

B

	%ch fors v basal	%ch GppNHp v fors	%ch PGE1 v FORS	%ch adr/prop v fors
1	3913.4	-2.0	87.8	-41.3
2	3972.4	-4.1	86.1	-45.0
3	4209.7	-21.9	60.0	-34.0
4	6255.7	-24.1	26.8	-44.6
5	3505.9	-12.4	90.0	-46.7
6	3195.1	-17.3	56.0	-37.8
7	3005.9	-20.3	93.7	-56.0
8	8274.6	-20.7	73.9	-44.0
9	5405.1	•	77.2	-33.5
10	4580.7	-1.1	82.0	-36.0
11	5377.0	-27.3	77.0	-27.3
12	4367.9	-34.7	19.2	-17.4
13	1672.3	-26.2	74.5	-44.6
14	4545.0	-12.0	103.7	-40.6
15	4060.0	-5.1	96.7	-48.2
16	5531.7	-6.0	108.1	-55.0
17	3240.2	-22.7	66.5	-44.3
18	3361.8	-10.2	97.0	-29.5
19	3545.7	-14.7	82.7	-48.0
20	3217.7	-19.7	79.1	-38.4
21	1527.2	-16.5	118.3	-37.4
22	2533.9	-13.8	74.6	-27.0
23	2671.7	•	92.9	-37.2
24	4071.8	-3.5	75.7	-23.3
25	4028.7	11.6	79.2	-8.5
26	4237.0	-5.1	82.5	-22.7
27	2499.2	6.8	94.7	-13.2
28	4463.4	-4.9	94.5	-38.3

APPENDIX C

	STUDY NUMBER	CATEGORY	SEX	AGE	WEIGHT	SBP	DBP	Cholesterol (mmol/l)	Triglyceride (mmol/l)	Gi2	Gs	Gi2:Gs ratio	ß subunit
1	C38	NORMAL	MALE	37.7	64.5	120	84	6.5	.8	1.309	1.496	.875	•
2	C33	NORMAL	MALE	43.6	85.1	150	86	5.1	.7	1.184	1.169	1.013	1.419
3	C22	NORMAL	MALE	59.8	70.0	174	85	6.3	.6	.971	.993	.978	.642
4	C21	NORMAL	MALE	27.1	63.3	106	69	5.2	1.6	1.448	.845	1.714	1.012
5	C41	NORMAL	FEMALE	50.9	65.4	140	90	4.8	.7	1.134	.405	2.800	1.091
6	C15	NORMAL	MALE	45.4	71.6	153	88	8.4	3.1	.893	.187	4.774	•
7	C10	NORMAL	FEMALE	29.4	65.5	126	77	4.8	.5	.914	.310	2.949	.876
8	C19	NORMAL	MALE	25.9	63.7	115	64	3.8	.5	.957	.638	1.500	1.039
9	C17	NORMAL	MALE	48.9	77.7	118	62	6.0	1.2	.824	.603	1.366	1.090
10	C31	NORMAL	MALE	49.4	82.8	128	90	6.8	1.5	.566	.514	1.101	1.029
11	C37	NORMAL	MALE	38.7	52.9	132	88	5.6	1.2	.665	.836	.795	.919
12	C23	NORMAL	MALE	51.6	60.8	124	90	5.2	.6	.475	.679	.700	.606
13	C36	NORMAL	MALE	52.5	70.0	124	81	4.6	.6	1.104	.302	3.655	.856
14	C20	NORMAL	MALE	48.0	73.1	115	82	5.8	1.6	1.067	.165	6.464	.718
15	C18	NORMAL	MALE	40.0	70.0	119	79	4.9	.7	.922	.311	2.964	•
16	H18	HBP	MALE	55.8	90.3	170	130	3.1	.6	1.373	1.190	1.154	•
17	H3	HBP	MALE	44.1	75.2	154	100	3.8	.3	1.151	1.049	1.098	1.133
18	H5	HBP	MALE	61.9	59.0	190	118	5.0	1.7	1.217	1.181	1.030	.753
19	H15	HBP	FEMALE	25.0	75.3	146	96	4.7	.9	.948	1.012	.937	1.048
20	H2	HBP	MALE	58.1	59.2	186	100	7.0	1.7	.911	.238	3.828	.894
21	H19	HBP	MALE	47.5	85.0	146	102	5.2	2.9	.655	.154	4.254	1.361
22	H16	HBP	FEMALE	31.9	99.0	138	98	3.9	.9	1.092	.443	2.465	.655
23	H1	HBP	MALE	24.8	75.3	140	100	5.8	1.6	.991	.652	1.520	1.116
24	H10	HBP	MALE	46.7	73.5	134	100	4.6	1.1	.660	.669	.987	.676
25	H20	HBP	MALE	58.6	87.1	168	97	6.4	1.1	.675	.896	.753	.685
26	H17	HBP	FEMALE	69.7	71.8	178	96	6.2	1.1	.477	.622	.767	.859
27	H24	HBP	MALE	65.0	97.0	146	96	6.8	2.2	.557	.911	.612	1.251
28	H23	HBP	MALE	62.2	84.4	172	98	7.1	3.6	.669	.287	2.331	.979
29	H21	HBP	MALE	60.6	86.0	164	98	7.2	•	1.063	.413	2.574	•
30	H9	HBP	FEMALE	42.4	71.5	180	102	•	•	.946	.485	1.950	•