VASCULAR G-PROTEINS IN GENETIC HYPERTENSION: LEVELS AND FUNCTION IN TWO RAT MODELS

by

CATHERINE JANE CLARK, B.Sc. (Hons.)

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to the University of Glasgow

MRC Blood Pressure Unit,
Western Infirmary, and
Department of Biochemistry,
University of Glasgow

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

Abstract

The cause of human essential hypertension is not known, but it is characterised by increased vascular smooth muscle (VSM) contractility. Similar changes are present in rodent models of genetic hypertension, which have been extensively studied in order to elucidate their pathogenetic mechanisms. The work of this thesis has utilised two such models, namely the spontaneously hypertensive rat (SHR) and the Milan hypertensive strain (MHS). Altered regulation of adenylyl cyclase, the enzyme which produces the second messenger and vasodilator, adenosine 3',5'-cyclic monophosphate (cAMP), has been reported in SHR vascular tissues; however, the roles of its guanine nucleotide regulatory proteins, G-proteins, (stimulatory, G_S, and inhibitory, G_i) in this abnormality have not been examined in detail. The regulation of adenylyl cyclase activity (ACA) has not been studied in any tissue from the MHS. Hence, it was of interest to investigate the regulation of ACA in VSM from the SHR and MHS, and examine the contribution from G_S and G_i to any abnormalities found. The pathogenetic mechanisms leading to hypertension in the SHR and MHS are probably different, and it was also of interest to compare the alterations in the regulation of ACA in the two models.

Altered regulation of ACA was shown in VSM from both hypertensive models. No significant differences were shown in the levels of the various G-protein subunits among SHR, Wistar-Kyoto (WKY) and Wistar VSM membranes. The function of G_i was also similar in these three strains; however, data suggest that the activity of G_S may be decreased in the SHR, and alterations in the level/activity of the adenylyl cyclase catalytic subunit may also exist in this strain.

The adenylyl cyclase system was studied in VSM from two age-groups of MHS rats. Significant reductions in the levels of $G_{S\alpha}$ (44 and 42kDa forms), $G_{i3\alpha}$ and the β-subunit were observed in both young (prehypertensive) and adult (hypertensive) MHS rats in comparison to age-matched controls. The function of G_i was similar in MHS and Milan normotensive strain (MNS) membranes from both age-groups. As G_i function was unchanged in the presence of a substantial reduction in the level of $G_{i3\alpha}$, it appears that G_{i3} does not contribute to the inhibition of adenylyl cyclase in these rat VSM membranes, and instead may participate in altered transmembrane ion fluxes in the MHS. It was observed that G_S function may also be

decreased in the MHS, and this may, in part, be explained by reduced levels of its α-subunits. Again, as in the SHR, data suggest that changes are likely in the level/activity of the catalytic subunit of adenylyl cyclase in the MHS.

The stimulatory responses of ACA to isoproterenol in the two hypertensive models were contrasting. In adult SHR membranes, a decreased response to isoproterenol was observed in comparison to controls. Membranes prepared from young MHS rats also exhibited a decreased stimulatory response to isoproterenol in comparison to age-matched controls; however, this effect was reversed with age, and an increased response to isoproterenol was measured in adult MHS membranes. This difference in response to isoproterenol with age was paralleled by appropriate alterations in β-adrenergic receptor number in the MHS.

It is unlikely that these alterations in isoproterenol response (and β-adrenergic receptor number) are primarily responsible for the hypertension in MHS rats, as if this were the case, the trend would be expected to be reversed, as in the SHR. A more likely explanation is that these alterations represent some form of compensatory mechanism to the increasing blood pressure and possible increased VSM contractility in the MHS. Alterations in isoproterenol response may play varying roles in the pathogenesis of hypertension in these models, being of primary importance in the SHR, but serving as a compensatory relief to increasing blood pressure in the MHS.

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Abbreviations

The abbreviations used in this thesis are defined below:

ACA	adenylyl cyclase activity
ADP	adenosine diphosphate
Ang II	angiotensin II
ATP	adenosine 5'-triphosphate
BSA	bovine serum albumin
cAMP	adenosine 3',5'-cyclic monophosphate
cDNA	complementary deoxyribonucleic acid
cGMP	guanosine 3',5'-cyclic monophosphate
[^{125}I]CYP	[^{125}I]-cyanopindolol
DG	diacylglycerol
DTT	dithiothreitol
EDTA	diaminoethanetetra-acetic acid disodium salt
EH	essential hypertension
GDP	guanosine diphosphate
GH	growth hormone
GHRH	growth hormone releasing hormone
G-protein	guanine nucleotide regulatory protein
G_s	stimulatory G-protein
G_i	inhibitory G-protein
G_o	"other" G-protein
GTP	guanosine-5'-triphosphate
GTPγS	guanosine 5'-[γ-thio]triphosphate
p(NH)ppG	guanosine 5'-[$\beta\gamma$-imido]triphosphate
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethane-sulphonic acid]
IP_3	1,4,5-trisphosphate
MHS	Milan hypertensive strain

MNS	Milan normotensive strain
mRNA	messenger ribonucleic acid
NIDDM	non-insulin dependent diabetes mellitus
PBS	phosphate-buffered saline
PGE_1	prostaglandin E_1
PHP	pseudohypoparathyroidism
PLC	phospholipase C
SBP	systolic blood pressure
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
S.E.	standard error of the mean
SHR	spontaneously hypertensive rat
SHRSP	stroke-prone spontaneously hypertensive rat
TBS	tris-buffered saline
TEMED	N,N,N',N'-tetramethylethylenediamine
VSM	vascular smooth muscle
VSMC	vascular smooth muscle cells
WKY	Wistar-Kyoto

Publications

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Chapter 1 Introduction

1.1 An introduction to guanine nucleotide regulatory proteins

1.1.1 Historical perspectives

(i) Formulation of second messenger hypothesis

Cellular activity is regulated by the concerted action of various hormones, neurotransmitters and growth factors. These ligands bind to specific receptors which traverse the plasma membrane; however, the mechanism by which the binding of a suitable ligand to a transmembrane receptor exerts an intracellular effect is unclear and is the subject of intense investigation. Data provided in 1957 by Rall *et al.* and Sutherland and Rall, led to the formulation of the second messenger hypothesis, whereby binding of a hormone (primary messenger) to specific plasma membrane receptors elicited the production of a second messenger, which was functionally active and able to control intracellular events. Rall *et al.* (1957) studied the relationship of epinephrine and glucagon to liver phosphorylase. The concentration of active phosphorylase in liver represents a balance between inactivation by liver phosphorylase phosphatase and reactivation by dephosphophosphorylase kinase (requires magnesium ions and adenosine 5'-triphosphate (ATP)). In cell-free liver homogenates, epinephrine and glucagon displace this balance in favour of the active phosphorylase. These investigators showed that the response of the homogenates to the hormones occurred in two stages. In the first stage, a particulate fraction of homogenates produced a heat-stable factor in the presence of the hormones; in the second stage, this factor stimulated the formation of liver phosphorylase in supernatant fractions of homogenates in which the hormones themselves were inactive. The heat-stable factor was identified as being an adenine ribonucleotide and was designated 3',5'-adenosine monophosphate (Sutherland and Rall, 1957). Further research identified 3',5'-adenosine monophosphate as a cyclic monophosphate which is now termed adenosine 3',5'-cyclic monophosphate (cAMP). The enzyme catalysing the production of cAMP from ATP is known as adenylyl cyclase.

(ii) GTP requirement

Rodbell and co-workers (1971) provided the first evidence for the requirement of guanosine-5'-triphosphate (GTP) in the hormonal regulation of adenylyl cyclase. It was observed that GTP enhanced the initial rate of basal and glucagon-stimulated adenylyl cyclase activity in rat liver plasma membranes, and bound at sites distinct from the glucagon binding sites. In addition, isoproterenol (a β-adrenoceptor agonist) and a poorly hydrolysable analogue of GTP, guanosine 5'-[βγ-imido]triphosphate (p(NH)ppG), activated frog erythrocyte adenylyl cyclase to a level much higher than the sum of the activities produced by the catecholamine and the synthetic nucleotide tested separately (Schramm and Rodbell, 1975). It was found that p(NH)ppG was a potent activator of adenylyl cyclase in the absence of hormone, and even in the absence of a functional hormone receptor. These data suggested that the hormone may be required only transiently to facilitate an initial interaction of the enzyme with guanylyl nucleotides. The involvement of a guanylyl nucleotide binding site was suggested by the persistent activation of adenylyl cyclase by p(NH)ppG, which was in contrast to the effect of GTP, which produced a transient activation. This guanylyl nucleotide binding site was postulated to be capable of binding and hydrolysing GTP. Evidence for this was presented by Cassel and Selinger (1976) who noted a 30-70% increase in GTP hydrolysis in turkey erythrocyte membranes in the presence of catecholamines and a low concentration of GTP. The elucidation by this group of a technique to measure high affinity GTPase activity in membrane preparations led to the proposal of a mechanism for the control of adenylyl cyclase, whereby the coupling of a stimulatory hormone to a specific receptor caused the exchange of guanosine diphosphate (GDP) for GTP on a regulatory protein. Transient activation of adenylyl cyclase was terminated by hydrolysis of GTP on the regulatory protein, as shown by treatment of turkey erythrocyte membranes with cholera toxin. The toxin caused an enhancement of basal and catecholamine-stimulated adenylyl cyclase activities, dependent on the presence of GTP, and an inhibition of the catecholamine-stimulated guanosinetriphosphatase activity (Cassel and Selinger, 1977). A regulatory protein, which is the site for GDP/GTP exchange, was isolated by Pfeuffer (1977) from pigeon erythrocytes and led to the concept that the transduction of

information from receptor to effector requires a GTP-binding protein.

(iii) Dual regulation of adenylyl cyclase

The possibility of hormonal inhibition of adenylyl cyclase, in addition to receptor-mediated stimulation of the enzyme, was discussed by Murad *et al.* (1962). This group observed a decrease in the formation of cAMP by heart adenylyl cyclase preparations from a number of species in the presence of acetylcholine and related compounds. Indeed, in 1975, Rodbell demonstrated inhibitory and stimulatory effects of p(NH)ppG on rat fat cells' adenylyl cyclase activity. The inhibitory effects of both p(NH)ppG and GTP were abolished, and stimulation occurred by increasing the concentration of magnesium ions and pH. Hence, it was postulated that adenylyl cyclase is under dual control by stimulatory and inhibitory guanine nucleotide regulatory proteins (G-proteins), termed N_S and N_i, respectively, by Rodbell (1980).

1.1.2 Identification of the stimulatory (G_S) and inhibitory (G_i) G-proteins

(i) G_S

It was shown by Gill and Meren (1978) that cholera toxin catalysed the transfer of adenosine diphosphate-ribose (ADP-ribose) to a number of membrane-bound proteins under circumstances that resulted in the activation of adenylyl cyclase in lysed pigeon erythrocytes. The most readily modified membrane protein (M_r 42,000) was the adenylyl cyclase-associated GTP-binding protein, G_S (previously termed N_S), and its modification by toxin was stimulated by guanine nucleotides. They observed that, in addition to adenylyl cyclase activity increasing in parallel with the addition of ADP-ribose to this protein, activity of the enzyme decreased in parallel with the subsequent reversal of ADP-ribosylation.

(ii) G_i

Pertussis toxin was shown to produce alterations in receptor-mediated control of cAMP production. In crude membrane preparations from rat C6 glioma cells, pertussis toxin

ADP-ribosylated a membrane protein with M_r=41,000, and GTP activation of membrane adenylyl cyclase was enhanced when membranes were incubated with pertussis toxin (Katada and Ui, 1982). These two actions of pertussis toxin were strictly paralleled by one another in magnitude. The release of tonic inhibition of adenylyl cyclase activity, paralleled by the modification of the 41kDa protein, thus identified this polypeptide as the putative, but previously unidentified, inhibitory G-protein, G_i (previously termed N_i), of the adenylyl cyclase cascade.

The functional effect of cholera toxin-catalysed ADP-ribosylation of the G_s α-subunit is to attenuate the ability of $G_s\alpha$ to hydrolyse GTP, thus producing an irreversibly activated G_s α-subunit, as shown by Birnbaumer *et al.* (1980) in rat liver plasma membranes. After treatment with cholera toxin, adenylyl cyclase becomes maximally activated and is no longer responsive to hormonal stimulation. In contrast, the functional effect of pertussis toxin-catalysed ADP-ribosylation of the G_i α-subunit is to prevent productive coupling between receptor and G-protein thus, receptor-mediated inhibition of adenylyl cyclase is attenuated after pretreatment with pertussis toxin. Burns *et al.* (1983) demonstrated that treatment of neuroblastoma-glioma (NG108-15) hybrid cells with pertussis toxin attenuated receptor-mediated inhibition of adenylyl cyclase by inhibiting opiate-stimulated GTPase.

1.1.3 Purification of G-proteins

Studies of mutants of the S49 lymphoma cell line (see section 1.2.4) provided evidence that receptor, G_s, and adenylyl cyclase were separate entities. Bourne *et al.* (1975) isolated a stable variant clone of mouse lymphosarcoma cells which was unresponsive to four different stimulators of adenylyl cyclase activity. This clone, cyc^-, was later shown to be deficient in G_s, providing a functional assay for the purification of G_s. The effects of β-adrenergic agonists and antagonists, characteristic of the wild-type S49 lymphoma cell, were reproduced by Ross and Gilman (1977) in a reconstituted system. Membranes of mouse L cells that contain adenylyl cyclase, but lack β-adrenergic receptors were solubilised with detergent and added to cyc^- membranes, resulting in the production of a catecholamine-sensitive adenylyl cyclase system.

G_s was purified to homogeneity by Northup *et al.* (1980) and was shown to reconstitute guanine nucleotide-, fluoride-, and

hormone-stimulated adenylyl cyclase activity in cyc$^-$ membranes. Purified G_S also recoupled hormonal stimulation of the enzyme in the uncoupled variant of S49. Preparations of pure G_S contained three polypeptides with approximate molecular weights of 52,000, 45,000 and 35,000Da. The α-subunits, of either 52 or 45kDa, corresponded to the guanine nucleotide binding site, as well as the substrate for cholera toxin-catalysed ADP-ribosylation (Cassel and Pfeuffer, 1978). A β-subunit (35kDa) and also a γ-subunit (8kDa) were found to be associated with G_S, and other G-proteins, including G_i.

A protein with 41,000 and 35,000Da subunits was purified from rabbit liver membranes as the predominant substrate for pertussis toxin. This dimer restored the inhibitory effects of guanine nucleotides and epinephrine on the adenylyl cyclase activity of pertussis toxin-treated human platelet membranes. It was shown that the subunits of the dimer dissociated in the presence of guanine nucleotide analogues or Al^{3+}, Mg^{2+}, and F^- ions, and that the 41kDa subunit had a high affinity binding site for guanine nucleotides. The characteristics of G_i-mediated inhibition of adenylyl cyclase have been studied in both S49 cyc$^-$ and wild type membranes. It has been suggested that dissociation of G_i is responsible for inhibitory regulation of adenylyl cyclase in both S49 cyc$^-$ and wild type membranes. In cyc$^-$ membranes, a major fraction of the inhibition appears to result from the action of the α-subunit of G_i; however, the β-subunit appears to mediate inhibition in wild type membranes, which contain functional G_S (the β-subunits of G_i and G_S are functionally indistinguishable, and such inhibition can be explained by reduction of the concentration of the free α-subunit of G_S, as a result of its interaction with the β-subunit of G_i (Katada *et al.*, 1984 a,b,c)).

1.1.4 Heterogeneity of G-proteins

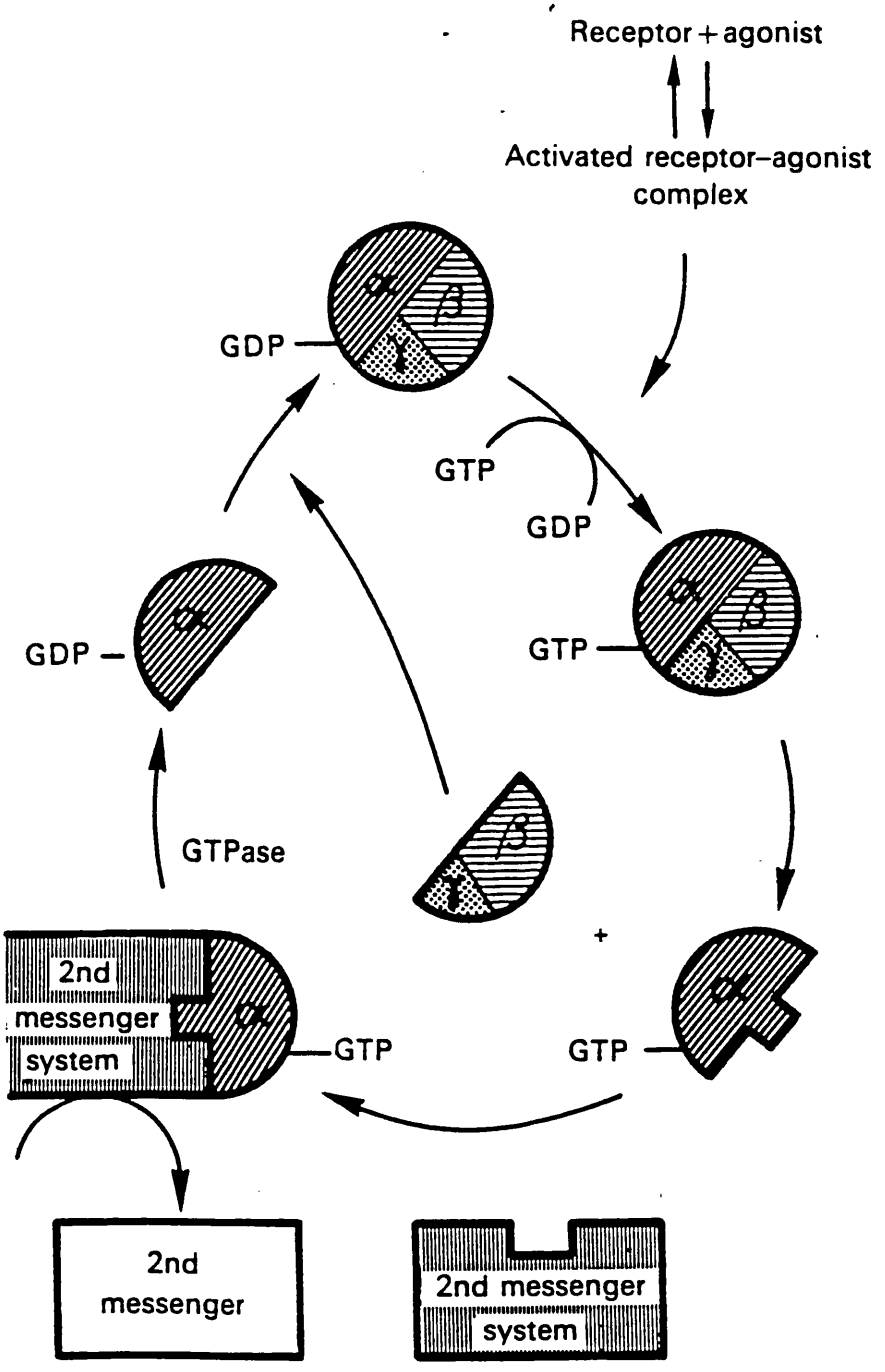
It has been demonstrated that more than a single pertussis toxin-sensitive G-protein exists. Purified preparations of pertussis toxin substrates from brain contained more than one polypeptide in the range of 39-41kDa, along with β,γ subunits. Subsequently, several additional heterotrimeric G-proteins have been identified and purified. Measurement of high-affinity guanine nucleotide binding in brain suggested that brain might be a rich source of GTP-binding proteins. This was confirmed by the purification of a

novel G-protein, termed "other" G-protein (G_O), which comprised approximately 1 to 2% of total brain membrane protein. It was thus, becoming apparent that the G-protein family was not restricted to G_S and G_i, and that additional heterogeneity was evident. The fact that G_O co-purified with G_i from brain, suggested that the increasing variety of G-proteins might share structural similarities (Milligan and Klee, 1985).

1.1.5 Mechanism of G-protein action

Studies on cholera toxin by Cassel and Selinger (1977) supported the view that the guanylyl nucleotide binding component of the adenylyl cyclase system plays a dual role. On the one hand, in the presence of hormone plus GTP it activates adenylyl cyclase; on the other hand, it functions in the hydrolysis of bound GTP and thus terminates the activation. Brandt and Ross (1986) utilised labelled GTP to measure the binding and hydrolysis of GTP and the release of GDP. Agonist-liganded receptor stimulated both the binding of GTP and the release of the GDP product, and both processes were formally catalytic with respect to receptor, in that several (up to 8) molecules of G_S were stimulated per molecule of receptor. An investigation involving the functional reconstitution of β-adrenergic receptors and G_S in phospholipid vesicles also identified catalytic activation of G_S by receptor (under optimal conditions, 5 to 6 molecules of G_S were activated per receptor) (Pedersen and Ross, 1982). A study examining the requirement for Mg^{2+} ions in the receptor activation of adenylyl cyclase postulated that the mechanism by which Mg^{2+} ions and receptors facilitate G_S activation involves dissociation of $G_{S\alpha}$ activated subunits (with guanine nucleotide bound to them) from G_S β,γ subunits (with receptor and Mg^{2+} ions bound to them) (Iyengar and Birnbaumer, 1982). The dissociated $G_{S\alpha}$-GTP is now in an activated state and may interact with a specific effector. This activation of $G_{S\alpha}$ is transient, and the bound GTP is hydrolysed to GDP by the α-subunit's intrinsic GTPase activity (Rodbell, 1980). The GDP-bound form of $G_{S\alpha}$ is now in an inactivated state and re-associates with its β,γ subunits. Hence, G_S undergoes a cyclical pattern of activation followed by a deactivation (Figure 1.1). This sequence of events applies not only to the mechanism of action of G_S, but to all G-proteins. Thus, G-proteins mediate a host of diverse signals including vision, olfaction, control of cell proliferation, ribosomal

Figure 1.1 The binding and hydrolysis of GTP in the activation and deactivation of G-proteins. G-proteins couple agonist-activated receptors to effectors, and as this process is of limited duration, the G-protein undergoes a cyclical pattern of activation followed by deactivation. This figure is a diagrammatic representation of the mechanism of G-protein action, and is reproduced from Milligan (1988).


Receptor + agonist
Activated receptor–agonist complex
GDP
GTP
GDP
GTP
GDP
GTPase
2nd messenger system
GTP
GTP
2nd messenger
2nd messenger system

protein synthesis and cellular regulation by an array of hormones and neurotransmitters, by similar activation/deactivation processes (Bourne, 1986).

G-proteins can be activated without the requirement for an agonist-occupied receptor. Poorly hydrolysable analogues of GTP, such as p(NH)ppG and guanosine 5'-[γ-thio]triphosphate (GTPγS), cause persistent activation of the G-protein (still requires magnesium). Also, fluoroaluminates (AlF_4^-) activate G-proteins if GDP is present in the GDP/GTP-binding site of the α-subunit. It was suggested that AlF_4^- is in contact with the GDP, and since there are striking structural similarities between AlF_4^- and PO_4^{3-}, it was proposed that AlF_4^- mimics the role of the γ-phosphate of GTP. Hence, the requirement for GDP-dissociation for G-protein activation is removed in the presence of AlF_4^- (Bigay *et al.*, 1985).

Masters *et al.* (1989) took advantage of mutational replacements of specific residues in the GTP-binding pocket of the 21kDa *ras* proteins ($p21^{ras}$) which reduce their GTPase activity to produce a variety of G-protein mutants. Mutated α chains of G_S were expressed in an α_S-deficient S49 mouse lymphoma cell line, cyc^-. A mutated α_S, in which leucine replaced glutamine 227, constitutively activated adenylyl cyclase and reduced the GTPase activity of G_S. There was a smaller reduction in GTPase activity in another mutant in which valine replaced glycine 49. This mutant α_S was a poor activator of adenylyl cyclase, probably due to an impairment of the mutant protein's ability to attain the active (GTP-bound) conformation. These results suggested that residues near glutamine 227 and glycine 49 of $G_S\alpha$ participate in the binding and hydrolysis of GTP.

There is evidence to suggest that the N-terminus of the α-subunit of G-proteins is important for interaction with β,γ subunits. The functional domains of $G_i1\alpha$ and $G_O\alpha$ have been studied using proteolysis by trypsin. In the presence of GTPγS, trypsin removed a 2kDa peptide from the N-terminus of these proteins. Tryptic cleavage did not affect the GTPase activity of the truncated molecules nor the apparent K_m for GTP. However, removal of the 2kDa amino-terminal peptide prevented association of the α-subunits with β,γ subunits, since the truncated α-subunits were no longer substrates for pertussis toxin (the apparent substrate for pertussis toxin-catalysed ADP-ribosylation is the $\alpha\beta\gamma$ heterotrimer) (Neer *et al.*, 1988).

1.1.6 Identification of receptor coupled to G-protein

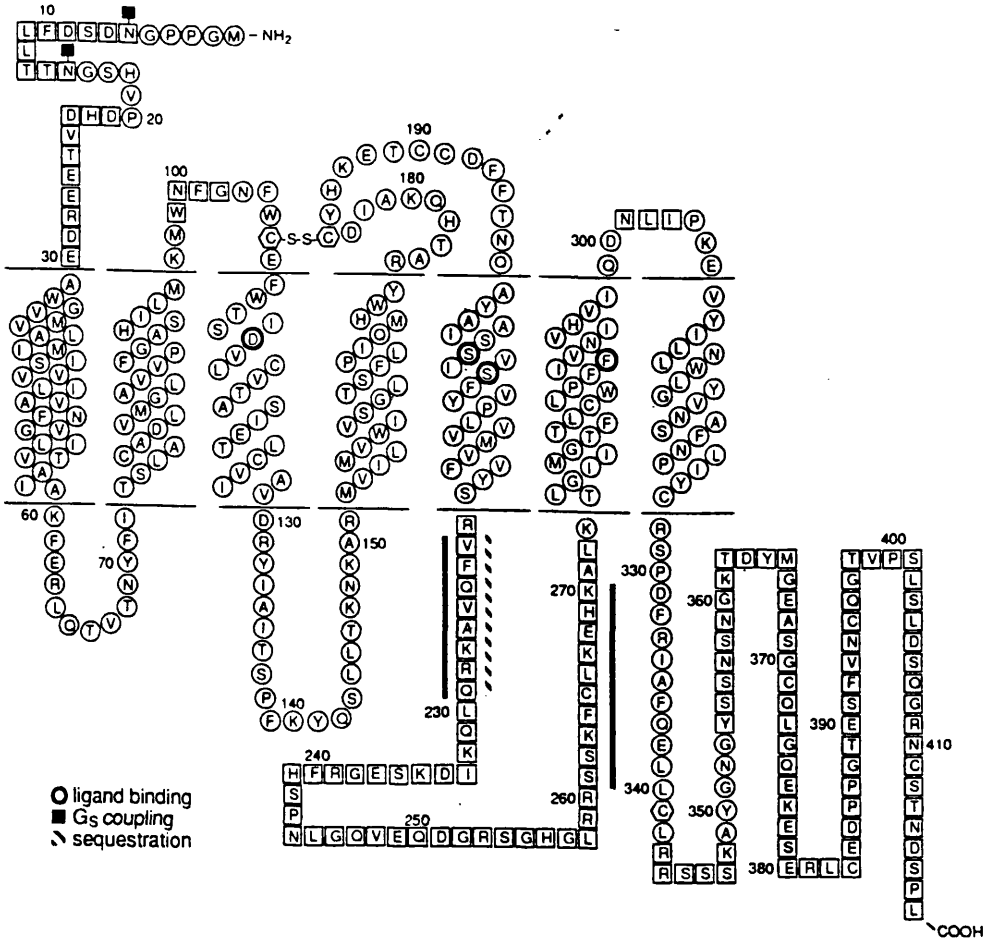
Receptors which function through G-protein activation show an absolute requirement for GTP, and the receptor-mediated response can be attenuated by pretreatment with either pertussis or cholera toxin. A study by De Lean *et al.* (1980) of radiolabelled agonist and antagonist interactions with the β-adrenergic receptor has revealed unique properties of agonist binding which may be indicative of the mechanism by which they activate the effector, adenylyl cyclase. It has been shown that, while competition curves for antagonists versus radiolabelled antagonists are "steep" with slope factors ("pseudo Hill coefficients") of 1, competition curves for agonists are "shallow" with slope factors less than 1. The shallow agonist competition curves are compatible with at least two states of the receptor in the absence of guanine nucleotides, whereas, antagonist binding to the same receptor is characterised by a single affinity form insensitive to guanine nucleotides. The formation of the high affinity state of the agonist-receptor complex is a prerequisite for its action. The transition or "shift" from the high to the low affinity state of the agonist-receptor complex, promoted by guanine nucleotides, is normally associated with an increase in agonist-induced adenylyl cyclase catalytic activity. De Lean *et al.* (1980) studying turkey and frog erythrocyte β-adrenergic receptor systems proposed a ternary complex model to explain agonist binding to the β-adrenergic receptor. In this model, the reversal of the agonist-promoted formation of the high affinity ternary complex HRX (H=hormone; R=receptor; X=nucleotide binding site) to the low affinity form HR is promoted by guanine nucleotides which interact with X and destabilise the HRX complex. The nucleotide-bound component X, freed from the ternary complex HRX, interacts with the effector, adenylyl cyclase, and results in the activation of the latter. This model provides a general scheme for the activation by agonists of adenylyl cyclase-coupled receptor systems and can be equally applied to inhibition of adenylyl cyclase activity by α_2-adrenergic receptor agonists and also to other systems where the effector might be different. Hence, if a receptor's affinity for agonist is altered in the presence of guanine nucleotides, then that receptor functions through activation of a G-protein.

The C-terminus of the α-subunit of G-proteins has been implicated in the interaction with receptor, as pertussis toxin catalyses the ADP-ribosylation of a residue close to the extreme

C-terminus of $G_{i}\alpha$, attenuating productive coupling between receptor and G-protein, while keeping intact the ability of G_i to dissociate in response to activation by guanine nucleotides (Katada *et al.*, 1986). More evidence for the C-terminus of $G\alpha$ interacting with receptor has come from sequencing of complementary deoxyribonucleic acid (cDNA). A variant of the S49 mouse lymphoma, UNC, is unable to synthesise cAMP in response to stimulation by β-adrenergic agonists or by prostaglandin E_1; however, basal and NaF-stimulated activities of adenylyl cyclase are normal, as are responses to p(NH)ppG and cholera toxin. There is a normal number of β-adrenergic receptors in these variant cell membranes; however, the interaction between the hormone-binding and the catalytic moieties of the adenylyl cyclase system is lost (Haga *et al.*, 1977). The cDNAs encoding $G_{S}\alpha$ in both UNC and wild type S49 cells were sequenced and revealed a point mutation in the UNC mutant $G_{S}\alpha$, replacing an arginine residue with a proline residue, six amino-acids from the C-terminus. Hence, a mutation at the C-terminus of $G_{S}\alpha$ resulted in the inability of G_S to interact with stimulatory receptors (Sullivan *et al.*, 1987).

Receptors play a crucial part in the conveyance of information from the outside to the inside of a cell. There are several categories of cell surface receptors including, growth factor receptors (which have an intrinsic tyrosine kinase activity); multisubunit receptors (which are ion channels); receptors which function as carrier proteins; and receptors which activate G-proteins. Receptors that mediate their actions by stimulating G-proteins share structural as well as functional similarities (Strader *et al.*, 1989). Hydropathy analysis of the primary sequences of these receptors has revealed the existence of seven hydrophobic stretches of 20-25 amino acids each, which are surrounded by eight hydrophilic regions of variable length. The transmembrane topology that has been proposed for the β-adrenergic receptor is shown in Figure 1.2. According to this model, each of the seven hydrophobic domains of the receptor forms a transmembrane α-helix, with the intervening hydrophilic loops exposed (alternately) intracellularly and extracellularly; the amino-terminal region is exposed on the extracellular face of the membrane and the carboxyl-terminal region on the cytoplasmic face. The regions of greatest sequence similarity lie within the putative transmembrane helices of the receptors, with the hydrophilic loop regions being more divergent. G-protein-linked receptors are glycoproteins with heterogeneous glycosylation patterns that

Figure 1.2 Model for the transmembrane topology of the β-adrenergic receptor. Horizontal lines represent the limits of the plasma membrane of the cell, with the region at the top of the page corresponding to the extracellular space and the region at the bottom representing the cytoplasm. Thus, according to this model, the receptor has seven transmembrane helices, with the amino terminus of the protein exposed extracellularly and the carboxyl terminus intracellularly. The amino acid residues represented by squares could be deleted from the protein without affecting ligand binding or protein folding, whereas the deletion of those residues shown in circles adversely affected either the folding or binding properties of the receptor. The residues shown in bold-faced circles are proposed to be involved in interactions with the ligand. The regions of the third intracellular loop delineated by the solid stripe were determined to be critical for G-protein coupling, whereas the region delineated by the broken stripe was required for agonist-mediated sequestration (reproduced from Strader *et al.*, 1989).


ligand binding
Gs coupling
sequestration
COOH

contribute to their anomalous migration on polyacrylamide gels. All of the receptors whose sequences are known have 1-3 consensus sites for N-linked glycosylation near the amino-terminus of the protein (Dohlman *et al.*, 1987). The functional significance of receptor glycosylation is unclear, but does not appear to be required for either ligand binding or functional coupling to G-proteins, although it clearly plays a role in the expression of the protein at the cell surface and in the trafficking of the receptor through the cell.

G-protein-linked receptors must interact with both their specific ligand and G-protein, and identifying the domains of a receptor responsible for such binding has been the basis of much research. Chabre (1987) proposed that G-proteins are cytoplasmic shuttles, localised on the membrane surface like the cytoskeleton. They interact transiently with receptors which are intrinsic membrane components and with effectors which are essentially peripheral. He suggested that their critical interactions are in the cytoplasm, not in the membrane; thus, they are more sensitive to the membrane surface charge and ionic environment than to the lipid milieu. Deletion mutagenesis directed at the sixth hydrophilic segment (third intracellular loop) of hamster β-adrenergic receptors expressed in mouse L cells, identified this region as being involved in receptor-mediated G_S coupling. In particular, regions of the β-adrenergic receptor encompassing amino acids 222-229 and 258-270, which are predicted to form amphiphilic helices, were suggested to play a role in the agonist-promoted activation of G_S (Strader *et al.*, 1987). A study of chimeric α_2-, β_2-adrenergic receptors (α_2- and β_2-adrenergic receptors have inhibitory and stimulatory effects, respectively, on the adenylyl cyclase system) showed that the fifth and sixth hydrophobic domains and the third intracellular loop were capable of conferring specificity for G_S coupling to the β_2-adrènergic receptor. It was also observed that the seventh hydrophobic domain of the α_2- and β_2-adrenergic receptors was a major determinant of both agonist and antagonist ligand binding specificity, and that several of the first five hydrophobic domains may contribute to agonist binding specificity (Kobilka *et al.*, 1988). However, research by O'Dowd *et al.* (1988), examining the effects of multiple mutations of amino acid sequences in each of the cytoplasmic domains of the human β_2-adrenergic receptor, identified regions comprising amino acid sequences in the C-terminal segment of the third intracellular loop and in the

N-terminal segment of the cytoplasmic tail as being critical contributors to formation of the binding site for G_S. Hence, the function of the three cytoplasmic loops and cytoplasmic tail common to each of the G-protein-coupled receptors has become the subject of intense interest and some controversy.

Receptor activation of G-proteins is a catalytic process, but unlike conventional enzymes, G-protein-linked receptors do not catalyse the formation or cleavage of covalent bonds. Instead, Wessling-Resnick *et al.* (1987) revealed through kinetic studies, the observation of allosterism in the molecular interaction between rhodopsin (photon receptor) and transducin. Equilibrium binding studies showed that oligomeric assemblies of these molecules provided the basis for positive cooperative behaviour, and this cooperativity may be widespread in nature concerning other G-protein/receptor interactions.

1.1.7 Mechanism of G-protein interaction with effector

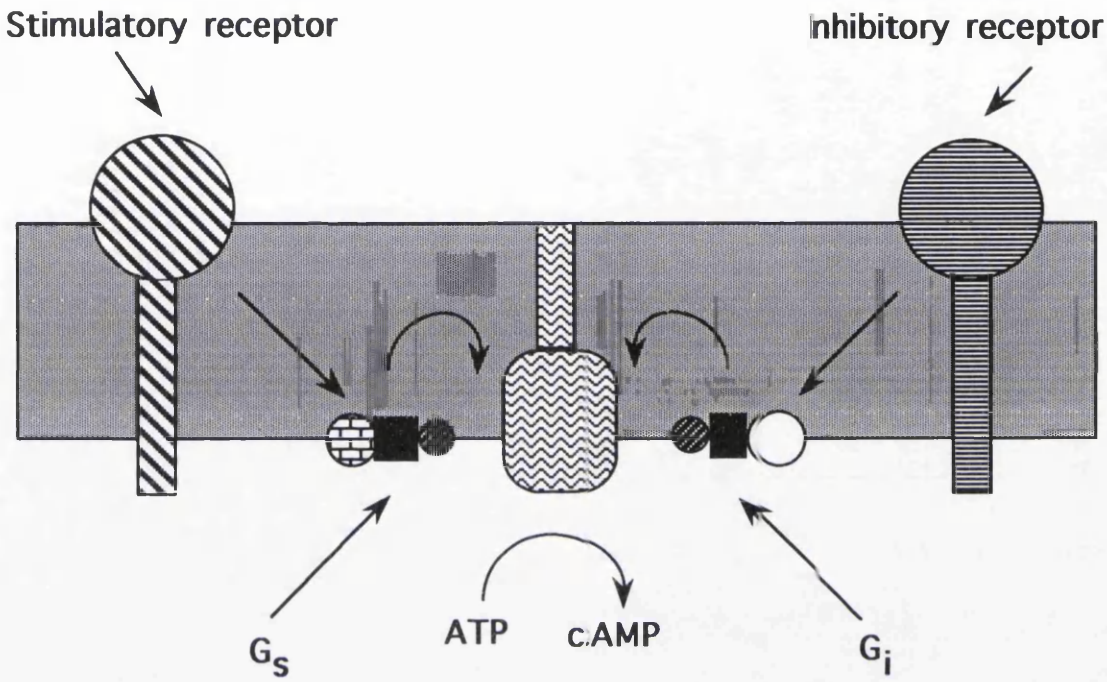
Adenylyl cyclase exists as multiple molecular species. At least one major form of the enzyme in brain is activated by calmodulin, probably directly. Most species are also stimulated directly by a diterpene, forskolin, isolated from the roots of the aromatic herb *Coleus forskohlii.* The enzyme appears to be a single polypeptide with a molecular weight of approximately 150,000Da; it also appears to be a glycoprotein. $GTP\gamma S$ and AlF_4^- activate G_S (which in turn activates adenylyl cyclase) by a similar mechanism involving subunit dissociation, and this stimulation of G_S by these agents is inhibited by $G\beta\gamma$. Inhibitory activity has been associated with $G_{i}\alpha$; however, in the presence of G_S, the primary source of inhibition is the G_i β,γ-subunit complex, which doesnot directly inhibit adenylyl cyclase, but interacts with and deactivates $G_{S}\alpha$ (Gilman, 1987). The presence of a low concentration of the G_i β,γ-subunit complex has also been observed to markedly inhibit Ca^{2+}-calmodulin-stimulated adenylyl cyclase activity in rat brain membranes, while essentially not affecting G_S- or forskolin-stimulated activity. The inhibition appeared to be competitive with calmodulin, suggesting that the inhibition of calmodulin-stimulated adenylyl cyclase is a result of direct interaction of β,γ-subunit complexes with calmodulin (Katada *et al.*, 1987).

The α-subunits of G_S and G_i link different sets of hormone receptors to stimulation and inhibition, respectively, of the same

effector enzyme, adenylyl cyclase (Figure 1.3). Masters *et al.* (1988) constructed a chimeric α_i/α_s cDNA that encoded a polypeptide composed of the amino terminal 60% (212 amino acids) of an α_i (α_i2) chain and the carboxyl terminal 40% (160 residues) of α_s. The cDNA was introduced via a retroviral vector into S49 cyc$^-$ cells, which lack endogenous α_s. Although less than half of the hybrid α-chain was derived from α_s, its ability to mediate β-adrenoceptor stimulation of adenylyl cyclase matched that of the normal α_s polypeptide expressed from the same retroviral vector in cyc$^-$ cells, indicating that carboxyl terminal amino acid sequences of α_s contain the structural features that are required for specificity of interactions with adenylyl cyclase, as well as with the hormone receptor. There is evidence indicating that the Gα carboxyl-terminus composes a part of the receptor-binding site or is a domain which is essential for controlling receptor activation of GDP/GTP exchange. Based on this evidence, Woon *et al.* (1989) substituted the $G_s\alpha$ carboxyl terminus with sequences predicted to have significantly different secondary structure from $G_s\alpha$, but which are known to be functional at the carboxyl terminus of other Gα-proteins. They designed a $G_s\alpha$ chimeric construct in which the last 38-carboxyl-terminal amino acids were replaced by the last 36-carboxyl-terminal residues of a $G_i\alpha$ protein. When this chimeric transcript was expressed in COS cells, cAMP levels were persistently elevated, resulting from a dominant, constitutively active G_s-like activity. Kinetic studies suggested that the chimeric G-protein may exhibit an enhanced rate of GDP-dissociation, implying that in addition to interacting with receptor, the C-terminal region of Gα may also play a role in the regulation of guanine nucleotide exchange.

A study utilising solubilised turkey erythrocyte membranes supports the view that G_s and the catalytic unit of adenylyl cyclase are tightly associated and do not come apart during the process of enzyme activation by the β-adrenergic receptor (Arad *et al.*, 1984). These authors proposed that the β-adrenergic receptor activation of adenylyl cyclase is by a simple "collision coupling" between the receptor and the tight complex of G_s and the catalytic unit of adenylyl cyclase. Functional studies with antibodies to the α_s carboxyl-terminal decapeptide by Simonds *et al.* (1989b) suggested that the C-terminal region of $G_s\alpha$ is not the site of interaction with effector. It was shown that these antibodies could immunoprecipitate adenylyl cyclase activity in detergent extracts

Figure 1.3 Dual control of adenylyl cyclase. Regulation of adenylyl cyclase is achieved by both stimulatory and inhibitory receptors. These processes are mediated via G_S and G_i, respectively, whose heterotrimeric nature is indicated. Participants of this signalling system interact via mobile collisions in the plane of the membrane.


Stimulatory receptor
Inhibitory receptor
G_s
ATP
cAMP
G_i

from GTPγS- or fluoride-preactivated bovine brain membranes; thus, binding of α_S to effector and carboxyl-terminal antibody was mutually compatible.

1.1.8 G-protein cloning and sequence homology

Sequence data from purified G-proteins allowed the cloning of complementary DNAs that encoded putative G-protein α-subunits. To date there are at least nine different classes of α-subunits. G_S and G_i are thought to be expressed ubiquitously, and are responsible for the regulation of adenylyl cyclase activity. Four types of α_S cDNA have been cloned that differ in nucleotide sequence in the region that corresponds to amino acid residues 71-88. S1 nuclease protection experiments revealed at least two forms of α_S messenger ribonucleic acid (mRNA), and alternative splicing of precursor RNA was proposed as the mechanism for generating four species of α_S mRNA (Bray *et al.*, 1986). Most cells contain two forms of the α-subunit of G_S; their apparent molecular weights being 45,000 and 52,000Da. Two cDNAs that correspond to distinct mRNAs for $G_S\alpha$ have been cloned from a bovine adrenal library and sequenced, revealing identical sequences except for a single stretch of 46 nucleotides in the coding region, where four are altered and 42 are deleted in the shorter cDNA. Expression of the two cDNAs in COS-m6 cells yielded protein products with apparent molecular weights of 45 and 52kDa, and Robishaw *et al.* (1986) proposed that the mRNAs encoding these proteins arise from a single gene by internal alternative RNA splicing. Mumby *et al.* (1986) raised antisera against purified subunits of G-proteins and against synthetic peptides that correspond to defined regions of G-proteins. Using an antiserum which recognises both the 52 and 45kDa forms of $G_S\alpha$, they observed wide variation in the ratios of the 52 and 45kDa $G_S\alpha$-subunits among various tissues tested by immunoblotting. The exact relationship of the two forms of this α-subunit remains unknown. There is evidence that the 45kDa α-subunit of G_S has greater functional activity than the 52kDa α-subunit (Walseth *et al.*, 1989). It has been observed that cAMP accumulation in response to forskolin, cholera toxin, or isoproterenol is dramatically increased in HIT T-15 cells, a clonal cell line of Syrian hamster pancreatic islet β cells, as a function of passage number. It was shown that although the amounts of the 52kDa form of $G_S\alpha$ and the 40kDa pertussis toxin substrate did not change with passage number, the

level of the 45kDa form of $G_S\alpha$ increased in the later passages. Extracts from HIT cell membranes from various passages were reconstituted in S49 cyc$^-$ membranes, and it was found that extracts derived from later passages reconstituted three to four times more adenylyl cyclase activity in cyc$^-$ membranes than extracts from earlier passages. These data suggested that functional differences exist between the G_S α-subunits, with the smaller 45kDa subunit being more efficacious in coupling to cAMP synthesis than the larger 52kDa subunit. However, a study by Graziano *et al.* (1987), which involved insertion of cDNAs that encode the two forms of $G_S\alpha$ into plasmid vectors for expression in *Escherichia coli*, showed that both forms of $G_S\alpha$ can reconstitute isoproterenol-, guanine nucleotide-, and fluoride-stimulated adenylyl cyclase activity in S49 cyc$^-$ cell membranes to approximately the same degree, suggesting that there is no functional difference between the two forms of $G_S\alpha$.

The activity of calcium channels has been examined in membrane patches excised from guinea pig cardiac myocytes and bovine cardiac sarcolemmal vesicles incorporated into planar lipid bilayers (Yatani *et al.*, 1987). It was shown that GTPγS independently prolonged the survival of excised calcium channels, as did $G_S\alpha$ purified from the plasma membranes of human erythrocytes. Thus, in addition to regulating calcium channels indirectly through activation of cytoplasmic kinases, G-proteins can regulate calcium channels directly.

In addition to investigating cDNA clones encoding $G_S\alpha$-subunits, Bray *et al.* (1987) obtained two cDNA clones from a λgt11 cDNA human brain library that correspond to $G_i\alpha$-subunits. The nucleotide sequence of human brain α_i is highly homologous to that of bovine brain α_i, and the predicted amino acid sequences are identical. However, it has been noted that human and bovine brain α_i cDNAs differ significantly from α_i cDNAs from human monocytes, rat glioma, and mouse macrophages in amino acid (88% homology) and nucleotide (71-75% homology) sequences (the nucleotide sequences of the 3' untranslated regions differ markedly). Hence, comparison of human brain and human monocyte α_i cDNAs suggested that the two types of human α_i are transcribed from separate genes. The nucleotide sequences of α_i cDNAs revealed that α_i genes are subject to strong selective pressure in the coding region and the 5' and 3' untranslated regions. Comparison of amino acid sequences predicted from α_i cDNAs suggest that α_i1 (from human brain, bovine

brain and bovine pituitary) and α_i2 (from human monocytes, rat C6 glioma cells and mouse macrophages) proteins may differ in function as well as in tissue distribution and abundance.

Studies in vertebrate olfactory tissue have indicated that certain smells stimulate adenylyl cyclase in a GTP-dependent manner. A rat olfactory cDNA library was screened with an oligonucleotide probe and 32 recombinant clones encoding five distinct types of G-protein α-subunits were isolated. The majority of the clones encoded $G_s\alpha$, while the remaining clones encoded $G_o\alpha$, $G_i1\alpha$, $G_i2\alpha$, and a novel species, $G_i3\alpha$. (To date, three sub-types of G_i have been isolated, namely G_i1, G_i2 and G_i3, which are all sensitive to ADP-ribosylation by pertussis toxin. G_i2 is universally distributed and has been implicated to have functional roles in the inhibition of adenylyl cyclase and in the stimulation of phospholipase C. G_i3 has an undefined distribution, but is thought to be expressed universally. There is evidence to suggest that this inhibitory G-protein is involved in the regulation of K^+ channels. G_i1 has a limited distribution, but high levels have been observed in brain tissue. The function of G_i1 is not yet clear (Milligan, 1989).) The predicted protein sequences of each of the rat cDNA clones have been aligned. It was found that $G_s\alpha$ is the most divergent, retaining approximately 40% identity with any of the other α-subunits. The α-subunits for G_o and the G_i-proteins share greater homology; $G_o\alpha$ shares 73% identity with $G_i1\alpha$, 68% with $G_i2\alpha$, and 70% with $G_i3\alpha$. $G_i2\alpha$ shares 88% identity with $G_i1\alpha$ and 85% identity with $G_i3\alpha$, while $G_i1\alpha$ and $G_i3\alpha$ are the most homologous with only 22 differences out of 354 residues (94% identity). The 18-amino acid stretch implicated in guanine nucleotide binding ($G_s\alpha$ 43-60) is perfectly conserved in all of the G-proteins, whereas the most divergent region among all of the G-proteins occurs between amino acid residues 65 and 150. Comparison of each of the predicted protein sequences from rat with those from other species indicated that minimal divergence occurred during speciation. Specifically, corresponding Gα-subunits across species share greater than 98% identity, whereas different Gα-subunits within a species share only 40-94% identity. Thus, protein sequence comparisons have indicated that individual Gα-subunits are more highly conserved across species than are any of the different Gα-subunits within a species (Jones and Reed, 1987).

In contrast to the diversity in the number of G-protein α-subunits, a similar degree of diversity regarding G-protein

β-subunits has not been observed. Levine *et al.* (1990) screened bovine and human retina cDNA libraries and isolated clones encoding three distinct types of G-protein β-subunit. β1-, β2- and β3-subunit cDNA clones were found to encode 36,000, 35,000 and 37,221Da forms of the β-subunit, respectively. The amino acid sequences of the three β-subunits are closely related; 83% identity between β1- and β3-subunits and 81% identity between β2- and β3-subunits. However, the 3'-untranslated regions of the three cDNAs show no significant homology. The N-terminal region contains the greatest diversity in amino acid sequence and predicted secondary structures in the β family of proteins, indicating that this area contains sites that confer specificity on the interactions between β proteins and α- or γ-subunits. The complete amino acid sequence of the γ-subunit of transducin from cattle retina has been reported (Ovchinnikov *et al.*, 1985). The polypeptide chain of the γ-subunit consists of 69 amino acid residues, with M_r 8008.7Da.

1.1.9 Regulation of G-protein levels

It is possible that aging may lead to altered expression of G-proteins. A study by Green and Johnson (1989) observed a 3-fold increase in the level of $G_{i}\alpha$, and a 2-fold increase in the level of $G_{s}\alpha$ (43kDa form only), when adipocyte membranes from young (2-month) and older (18-month) rats were compared. The levels of $G_{o}\alpha$, $G_{i}\alpha$ and the β-subunit were quantified in atria and ventricles during embryonic chicken cardiac development. Altered G-protein levels were noted during organ development, and these changes were shown to be associated with changes in G-protein-mediated regulation of adenylyl cyclase activity (Luetje *et al.*, 1987). A study by Silbert *et al.* (1990) implied that protein translation controls may be important determinants of G-protein α-subunit concentrations in biological membranes. They observed different degradation rates for $G_{o}\alpha$, but similar steady-state protein levels and approximately equal mRNA levels in GH_4 cells (rat pituitary cell line) and in primary rat cardiocytes, suggesting the rate of $G_{o}\alpha$ synthesis was different in GH_4 cells and cardiocytes.

Adrenal steroid hormones and catecholamines play central roles both in maintaining survival in times of stress and in regulating normal physiological responsiveness. Glucocorticoids regulate responsiveness of many different cells and tissues to hormones that act by stimulating adenylyl cyclase. Exposure to

steroid hormones may result in increases in receptor density, enhanced coupling, and enhanced adenylyl cyclase activity. If reductions in receptor density, coupling and adenylyl cyclase activity are induced through desensitisation, steroid hormones may act to return the receptor-cyclase system to a more normal state of sensitivity (Davies and Lefkowitz, 1984). In a rat pituitary cell line, GH_3, exposure to dexamethasone doubled the specific activity of adenylyl cyclase in membrane extracts. A 5-fold increase in RNA encoding the α-chain of G_S, as well as a 2-fold increase in membrane-bound α_S-polypeptide, were observed, and may, at least in part, contribute to the glucocorticoid-induced enhancement of adenylyl cyclase activity in these cells (Chang and Bourne, 1987). The results from a study by Saito *et al.* (1989) indicate that $G_{S}\alpha$ and $G_{i}\alpha$ are under the coordinated control of glucocorticoids in rat brain, and demonstrate that G-proteins are physiological targets of glucocorticoids *in vivo*. They found that chronic corticosterone administration to normal rats and adrenalectomised rats increased levels of $G_{S}\alpha$ immunoreactivity, mRNA, and ADP-ribosylation, but decreased levels of $G_{i}\alpha$ mRNA and tended to decrease levels of $G_{i}\alpha$ immunoreactivity. In contrast, levels of $G_{O}\alpha$ and the β-subunit immunoreactivity and mRNA were not influenced by corticosterone treatment. However, adrenalectomy, without corticosterone replacement, produced the opposite effects on $G_{S}\alpha$ and $G_{i}\alpha$ compared to sham-operated controls, indicating that these G-proteins are regulated by this class of steroid hormone under physiological conditions *in vivo*.

The regulation of G-proteins has been examined in 3T3-L1 cells during differentiation from a fibroblast-like to an adipocyte-like phenotype. $G_{S}\alpha$ levels and specific activity increased by approximately 6-fold and 3-fold, respectively, during differentiation. Both $G_{i}\alpha$ and $G_{O}\alpha$ levels increased by approximately 4-fold over this same period. In addition, the increase in the levels of the β-subunits (35 and 36kDa forms) was approximately 2-fold. Quantitative immunoblotting identified a decrease in the overall ratio of β- to α-subunits of G-proteins, from approximately 4.7 in fibroblasts to 2.5 in adipocytes, suggesting that in differentiation not only is the complexion of G-proteins altered, but more importantly, the relative amounts of α- to β-subunits are regulated. The increase in β-subunits was most pronounced during the early part of the time course of differentiation, when the amount of $G_{S}\alpha$/mg of membrane protein was more or less constant. Thus, the

change in the ratio of β- to $G_S\alpha$-subunits was greatest at this time and may affect, in a manner opposite to hormone activation, the availability of $G_S\alpha$ for GTP binding and adenylyl cyclase activation. It seems likely, therefore, that the increasing excess of β-subunits in 3T3-L1 cells is responsible for the attenuation of GTP-stimulated adenylyl cyclase activity (Watkins *et al.*, 1987).

1.2 Abnormalities of G-proteins in disease states

1.2.1 Pseudohypoparathyroidism

Pseudohypoparathyroidism (PHP) is a genetic disorder associated with a decreased responsiveness of hormones which function through G_S in order to activate adenylyl cyclase (Motulsky *et al.*, 1982). The erythrocytes of patients with PHP Type Ia have been shown to have decreased G_S activity, whereas those of patients with PHP Type Ib have normal G_S activity. This decreased G_S activity (measured by the ability to restore adenylyl cyclase activity to membranes prepared from S49 cyc^- cells) also occurs in the platelets of PHP Type Ia, but not of PHP Type Ib patients. G_i activity was unaltered in platelets from patients with PHP Type Ia, as observed by inhibition of adenylyl cyclase via the α_2-adrenoceptor.

Levine *et al.* (1988) analysed total RNA from cultured fibroblasts obtained from PHP Type Ia patients and observed an ~50% reduction in mRNA levels for $G_S\alpha$, but normal mRNA levels for $G_i\alpha$. These investigators were not able to show a clear correlation between the extent of reduction of mRNA for $G_S\alpha$ and either the extent of reduction of G_S activity in the cells or the clinical phenotype. Thus, the relevance of reduced G_S expression to this syndrome and the factors controlling G_S expression are far from clear.

1.2.2 Chronic ethanol administration

Acute exposure to ethanol leads to the stimulation of adenylyl cyclase activity, presumably due to its effects on increasing lipid fluidity (Whetton *et al.*, 1983). However, chronic exposure to ethanol attenuates adenylyl cyclase activity, as determined both in cell systems and in lymphocytes and platelets from alcoholic subjects. Part of this inhibitory effect may be due to the membrane lipid-mediated adaptive response to chronic ethanol administration.

This leads to increased levels of cholesterol incorporation into membranes, a lipid whose elevation in biological membranes can lead to the attenuation of adenylyl cyclase activity. However, it is clear that such a lipid-mediated effect cannot account for the magnitude of the diminished receptor-coupled adenylyl cyclase activity in cells treated chronically with ethanol.

In a study by Mochly-Rosen and colleagues (1988), it has been shown that chronic treatment of a neuroblastoma cell line (NG108-15) with ethanol led to the reduced expression of $G_{s}\alpha$. This was monitored by both reconstitution into cyc^- cells and by using a specific anti-peptide antibody. The lesion appeared to be due to decreased synthesis of $G_{s}\alpha$, rather than enhanced degradation, as the mRNA concentrations were also reduced by a comparable amount (~30%).

1.2.3 Growth hormone secreting pituitary adenomas

Secretion from somatotrophic cells is known to be controlled by alterations in the intracellular concentration of cAMP. A number of human growth hormone (GH)-secreting adenomas have been shown to have abnormally high intracellular concentrations of cAMP, and to show very high rates of secretion of GH in the absence of stimulation by growth hormone releasing hormone (GHRH). Indeed, in such cells, GHRH had little effect on both the intracellular concentration of cAMP and on the rate of GH secretion.

When the adenylyl cyclase activity of GH-secreting adenomas was studied in isolated membranes (Vallar *et al.*, 1987), no stimulation by GTP or by fluoride was observed. In contrast, adenylyl cyclase stimulation by Mg^{2+} was greatly increased, suggesting that either G_s or adenylyl cyclase, in these tumour cells, has adopted a highly (fully) activated conformation. It has been shown that the lesion is indeed due to the presence of a hyper-responsive form of G_s in these cells. Extraction of G_s from adenoma cell membranes and its subsequent incorporation into the membranes of cyc^- cells conferred the same hyper-responsiveness to Mg^{2+} on the adenylyl cyclase of cyc^- cells. Also, cholera toxin-treatment of these adenoma cells, whilst causing the ADP-ribosylation of $G_{s}\alpha$, did not elicit activation, presumably because G_s was already in an active conformation. Such altered G_s was not found in all hypersecreting adenomas, but was observed in a substantial group. Further work by this group (Landis *et al.*, 1989)

identified somatic mutations that inhibit the GTPase activity of $G_{S}\alpha$ in these tumours. Thus, the resulting activation of adenylyl cyclase bypasses the cells' normal requirement for trophic hormone.

1.2.4 S49 lymphoma cell line

A mutant, cyc^{-}, derived from this cell line provided the means for the first unequivocal demonstration of a G-protein, namely G_S. Plasma membranes from cyc^{-} cells offer a useful reconstitution system for G_S as the cyc^{-} cell line fails to express G_S whilst still expressing β-adrenoceptors and adenylyl cyclase (Gilman, 1987).

The underlying selection mechanism of mutants of this cell line is that elevation of intracellular cAMP levels are cytotoxic to these cells. A mutant cell line called UNC has an aberrant G_S which although capable of stimulating adenylyl cyclase when activated directly, using either NaF or a poorly hydrolysable GTP analogue, p(NH)ppG, could not mediate β-adrenoceptor stimulation of this enzyme, suggesting a lesion in the domain of $G_{S}\alpha$ which interacted with the β-adrenoceptor (Haga *et al.*, 1977). Consistent with this was the failure to observe guanine nucleotide-mediated effects on β-agonist binding to the β-adrenoceptor. Sequence analysis of the gene for $G_{S}\alpha$ from these cells has shown a single point mutation at the C-terminal end of this protein, giving credence to the suggestion that it is the C-terminal end of G-protein α-subunits which interacts with specific receptors.

Another mutant form of S49 cells which shows a lesion in G-protein mediated coupling has been identified, namely H21a (Bourne *et al.*, 1982). The adenylyl cyclase of these cells fail not only to respond to β-adrenoceptor agonist stimulation, but also to activation by ligands which interact with G_S directly, such as NaF, poorly hydrolysable GTP analogues and cholera toxin. However, guanine nucleotide analogues are capable of modifying β-agonist binding, indicating that the form of G_S expressed in these cells is capable of coupling to the β-adrenoceptor, but is incapable of coupling to adenylyl cyclase. Sequence analysis of the gene for $G_{S}\alpha$ from these cells has identified a single point mutation at residue 226, where glycine is replaced by alanine. The region where this mutation lies is highly conserved amongst the various G-proteins and thus seems an unlikely domain for providing the required specificity to direct $G_{S}\alpha$ to interact with a specific second messenger system i.e. adenylyl cyclase. However, it may be that this

highly conserved domain is responsible for transducing a conformational change between the GTP-binding domain and the domain, postulated to be at the N-terminal region of $G_{S}\alpha$, responsible for interacting directly with adenylyl cyclase. Experiments using tryptic proteolysis to probe for conformational changes induced by guanine nucleotides did show aberrations from the normal, indicating that this single mutation can alter the GTP-induced conformational changes in $G_{S}\alpha$ (Miller *et al.*, 1988).

1.2.5 Adrenalectomy

In the adipocytes of adrenalectomised rats there is an impaired lipolytic response to adrenaline (β-adrenoceptor) stimulation (Ros *et al.*, 1989). Although the number of β-adrenoceptors appears not to be reduced by adrenalectomy, and there is no change in the amount of adenylyl cyclase, as indicated by forskolin-stimulated adenylyl cyclase activity, there appears to be a marked reduction in G_{S}, as determined by the cholera toxin-catalysed ADP-ribosylation of $G_{S}\alpha$. A comparable reduction in G-protein β-subunit was also noted. Adrenalectomy may thus, reduce ligand-stimulated adenylyl cyclase activity by attenuating the expression of G_{S} in adipocytes.

1.2.6 Hypothyroidism

Hypothyroidism can be experimentally induced in rats by treatment with 6-propyl-2-thiouracil. In adipocytes from such hypothyroid animals, receptors which are linked to the inhibition of adenylyl cyclase show an increased responsiveness compared to that noted in control animals, suggesting that hypothyroidism might lead to an increase in the expression of G_{i} in adipocytes from these animals. Certainly, in rats this would appear to be the case, as hypothyroidism has been shown to lead to an enhanced labelling of the 40kDa α-subunit of G_{i}. Indeed, specific anti-peptide antibodies have been used to provide clear evidence of enhanced expression of $G_{i}\alpha$ in adipocyte membranes from hypothyroid animals (3-fold greater than controls). Such changes in the amount of $G_{i}\alpha$ appeared to be paralleled by alterations in β-subunit expression, which may indicate some co-ordination in the expression of these two proteins (Milligan *et al.*, 1987).

1.2.7 Heart failure

Changes in the intracellular concentration of cAMP are believed to play a fundamental role in the contractile response of cardiac tissue. Thus, β-adrenergic stimulation is seen to promote contraction, and selective inhibitors of cAMP-specific phosphodiesterases have the potential, albeit experimentally, for use in treating congestive heart failure. Reduced β-adrenergic responsiveness appears to be characteristic of the myocardium in congestive heart failure, and enhanced G_i function may offer one contributing factor (Ginsburg *et al.*, 1983). Reduced numbers of β-adrenergic receptors have been found in tissue obtained from failing hearts (Fowler *et al.*, 1986); however, whether this is sufficient to account for the loss of responsiveness is unclear. It has been shown that when turkey erythrocyte adenylyl cyclase is stimulated by β-adrenergic receptor agonists then ~90% of β-adrenoceptors have to be lost before any significant reduction in the ability of β-adrenoceptor agonists to activate adenylyl cyclase occurred (Tolkovsky and Levitzki, 1978).

Horn *et al.* (1988) examined the hypothesis stating that G_S, which couples the β-adrenergic receptor to adenylyl cyclase activity, is also decreased in congestive heart failure. In addition to the 40% decrease in lymphocyte β-adrenergic receptors in patients with congestive heart failure, they found an 80% decrease in levels of G_S compared with age- and sex-matched healthy control subjects. Myocardial G_S levels correlated significantly with lymphocyte G_S levels.

1.2.8 Diabetes and insulin-resistant states

(i) Animals - chemically-induced diabetes

Treatment of rats with either streptozotocin or alloxan elicits the destruction of the pancreatic β cells which secrete insulin. This induces diabetes and also causes insulin-resistance in liver and adipose tissues. Such an effect can be reversed by insulin therapy.

In hepatocytes and adipocytes from such diabetic rats there appear to be alterations in the levels and/or function of G_i. Levels of $G_i2\alpha$, $G_i3\alpha$ and the 42kDa, but not the 45kDa, form of $G_S\alpha$ were markedly decreased in hepatocyte membranes from diabetic animals. G-protein β-subunits in plasma membranes were unaffected by

diabetes induction. Immunoblot analysis showed that levels of the catalytic unit of adenylyl cyclase were increased in hepatocyte membranes upon induction of diabetes. Under basal conditions, $G_i2\alpha$ from hepatocytes of diabetic animals was found to be both phosphorylated to a greater extent than $G_i2\alpha$ isolated from hepatocytes of normal animals, and furthermore was resistant to any further phosphorylation. Treatment of isolated plasma membranes from normal, but not diabetic, animals with purified protein kinase C caused the phosphorylation of $G_i2\alpha$. Treatment of membranes from diabetic animals with alkaline phosphatase caused the dephosphorylation of $G_i2\alpha$ and rendered it susceptible to subsequent phosphorylation with protein kinase C. Low concentrations of the poorly hydrolysable GTP analogue, p(NH)ppG, inhibited adenylyl cyclase activity in hepatocyte plasma membranes from normal, but not diabetic, animals (Bushfield *et al.*, 1990a).

Adipocyte membranes from animals made diabetic with streptozotocin had their inhibitory functions of G_i abolished, as assessed by the inhibitory action of low concentrations of p(NH)ppG upon forskolin-stimulated adenylyl cyclase activity, and by the inhibitory action of high concentrations of GTP upon isoproterenol-stimulated adenylyl cyclase activity. In contrast, receptor-mediated inhibitory responses of G_i, effected by adenosine, prostaglandin E_2 or nicotinate, were either unchanged or even apparently more effective in membranes from diabetic animals. Induction of diabetes did not cause any change in the adipocyte plasma membrane levels of $G_i1\alpha$, $G_i2\alpha$ or of $G_s\alpha$, but elicited an increase in the level of $G_i3\alpha$. The induction of diabetes reduced the specific activity of adenylyl cyclase in adipocyte membranes and enhanced the stimulatory effect of isoproterenol. It is suggested that diabetes causes selective changes in the functioning of G_i in adipocyte membranes, which removes the tonic GTP-dependent inhibitory function of this G-protein (Strassheim *et al.*, 1990).

The factor which leads to the loss of G_i function remains to be established. Certainly, the fact that two very different compounds, namely alloxan and streptozotocin, elicited this effect, suggests that they did not exert any direct action. Indeed, this was confirmed by the fact that insulin therapy of streptozotocin diabetic animals restored G_i function.

(ii) Animals - genetically acquired diabetes and obesity

Altered adipocyte G-protein expression and adenylyl cyclase regulation have been observed in genetically acquired diabetic (db/db) mice (Strassheim *et al.*, 1991). Adipocyte membranes from diabetic animals showed marked elevations in the levels of $G_{i}1\alpha$, which were almost twice those found in membranes from their normal, lean littermates. In contrast, no apparent differences were noted in levels of $G_{i}2\alpha$, $G_{i}3\alpha$, $G_{s}\alpha$ (42 and 45kDa forms) and the β-subunit. Adenylyl cyclase activity was similar in membranes from both normal and diabetic animals under basal conditions and also when stimulated by optimal concentrations of either NaF or forskolin. However, the ability of isoproterenol, glucagon and secretin to stimulate adenylyl cyclase activity was greater in membranes from normal animals compared with membranes from diabetic animals. Receptor-mediated inhibition of adenylyl cyclase, as assessed using prostaglandin E_1 (PGE_1) and nicotinate, was similar using membranes from both sources, but phenylisopropyladenosine was a slightly more effective inhibitor in membranes from diabetic animals. Hence, a doubling in the expression of $G_{i}1$ appears to have little discernible effect upon the inhibitory regulation of adenylyl cyclase.

Obese (fa/fa) Zucker rats, unlike their lean (Fa/Fa) littermates, are hyperinsulinaemic and show tissue insulin-resistance although they are not classified as being diabetic, as no pronounced hyperglycaemia is exhibited. However, these genetically obese Zucker rats may be a better model of human non-insulin dependent diabetes than chemically-induced diabetic rats which have undergone pancreatic β cell destruction. Hepatocyte membranes from both lean and obese Zucker rats exhibited adenylyl cyclase activity that could be stimulated by glucagon, forskolin, NaF and elevated concentrations of p(NH)ppG. In membranes from lean animals, functional G_{i} was detected by the ability of low concentrations of p(NH)ppG to inhibit forskolin-activated adenylyl cyclase. In hepatocyte membranes from obese animals, no functional G_{i} activity was detected. Quantitative immunoblotting showed that hepatocyte plasma membranes from both lean and obese Zucker rats had similar amounts of $G_{i}\alpha$. Hence, it was suggested that liver plasma membranes from obese Zucker rats express an inactive G_{i} α-subunit. Thus, lesions in liver G_{i} functioning are seen in insulin-resistant obese rats and in alloxan- and

streptozotocin-induced diabetic rats, which also show resistance as regards the acute actions of insulin. Liver plasma membranes of obese animals also showed an impairment in the coupling of glucagon receptors to G_S-controlled adenylyl cyclase. Membranes from obese animals were shown to exhibit a reduced ability for a high concentration of p(NH)ppG to activate adenylyl cyclase. The use of ADP-ribosylation by cholera toxin to label the 43kDa and 52kDa forms of $G_{S\alpha}$ showed that a reduced labelling occurred using liver plasma membranes from obese animals. It is suggested that abnormalities in the levels of expression of primarily the 52kDa form of $G_{S\alpha}$ may give rise to the abnormal coupling between glucagon receptors and adenylyl cyclase in liver membranes from obese Zucker rats (Houslay *et al.*, 1989).

A study by Strassheim *et al.* (1992) investigated β-adrenergic regulation of adenylyl cyclase activity in heart tissue membranes from the genetically obese Zucker rat, the genetically obese CBA mouse and the genetically obese diabetic (db/db) mouse. Responsiveness to β-adrenergic stimulation was impaired in membranes from the obese Zucker rat, but not in the other models. Cardiac membranes from obese Zucker rats showed both reduced β-adrenergic receptor number and altered coupling between β-adrenergic receptors and G_S. In contrast, no alterations in either the levels of G_S or the functional interaction between this protein and the catalytic moiety of adenylyl cyclase were observed. Hence, dissimilar alterations in the control of adenylyl cyclase were observed in these three genetic models of obesity.

(iii) Humans - non-insulin dependent diabetes mellitus

There are two types of diabetes mellitus in humans, namely insulin dependent diabetes mellitus (Type I) and non-insulin dependent diabetes mellitus (Type II). Type I diabetes in humans arises from pancreatic destruction (similar to chemically-induced diabetic rats) resulting in low insulin levels. The levels and function of G-proteins have not been examined in humans with this type of diabetes. The cause of Type II diabetes in humans is unknown; however, patients with this type of diabetes are insulin resistant (similar to obese Zucker rats). The role of G-proteins in humans with Type II diabetes has been studied in platelets. Adenylyl cyclase activity and levels of G-proteins have been compared in platelets from normal and non-insulin dependent diabetic (NIDDM) male subjects (Livingstone

et al., 1991). No differences were noted in basal and NaF-stimulated adenylyl cyclase activities; however, the degree of stimulation achieved by both forskolin and prostaglandin E_1 was lower in platelet membranes from diabetic subjects compared with those from normal control subjects. Decreased α_2-adrenoceptor-mediated inhibition of prostaglandin E_1-stimulated adenylyl cyclase activity was evident in platelet membranes from diabetic subjects compared to controls. Analysis of G-protein α-subunits, using specific anti-peptide antisera, showed that platelets from all subjects exhibited the G_i2 and G_i3, but not the G_i1, forms of the inhibitory G-protein G_i, and all expressed the 42kDa species of $G_s\alpha$. Whilst platelets of diabetic subjects had levels of G_s which were comparable to those found in control subjects, their levels of G_i2 and G_i3 were some 49% and 75%, respectively, of those found in platelets from control subjects. It was suggested that changes in adenylyl cyclase functioning and G-protein expression may contribute to altered platelet functioning in NIDDM subjects.

(iv) Summary

In conclusion, it is evident from these data that diabetes, whether occurring in humans (Type II), genetically insulin-resistant obese rats or experimentally-induced diabetic rats, is associated with abnormal G_i function. Altered levels and/or activity of G_i have been noted in a range of tissues, namely hepatocytes, adipocytes and platelets. Although there are differences in the levels of the various sub-types of G_i in diabetic subjects with respect to controls, these differences vary depending on the model of human diabetes and tissue studied. It is apparent, however, that G_i function is in some way abolished in all of the systems studied.

1.3 Genetic hypertension

1.3.1 Human essential hypertension

In most patients with high blood pressure the cause is not known, and these subjects are said to have essential hypertension (EH). In the past years, studies have underlined the role of genetic factors in the development of hypertension in man, stimulating investigators to look for possible biochemical-functional alterations that could be considered as a direct phenotypic expression of underlying genetic

abnormalities. It is becoming increasingly clear that hypertension is a complex disease, in which the final phenotype "high blood pressure" or "vascular complications" can be reached through various genetic mechanisms, all interacting differently with a range of confounding environmental factors. It is, therefore, very difficult to dissect the various pathogenetic mechanisms caused by different intermediate phenotypes in a random mating population like the human one, without the aid of appropriate models such as the genetically hypertensive rat in which hypertension develops through fewer and better defined genetic mechanisms, with little influence from the environment (Cusi *et al.*, 1990). Clues to the pathogenesis of hypertension can be found by examining epidemiological associations of the disorder.

(i) Hypertension, obesity, and glucose intolerance

Hypertension, obesity, and glucose intolerance (impaired glucose tolerance and non-insulin dependent diabetes) are commonly associated in epidemiological studies, suggesting a common pathogenetic mechanism. Analysis of epidemiological data shows an impressive pattern of overlap among hypertension, diabetes, and obesity that amplifies with age. For example, it can be estimated that in the fifth decade of life, only some 15% of diabetic individuals are non-obese and normotensive, and, conversely, only approximately 20% of obese subjects have normal glucose tolerance and blood pressure, whereas only about one-third of the hypertensive population is free of both diabetes and obesity (Ferrannini *et al.*, 1990). Impaired glucose tolerance and obesity are characterised by hyperinsulinaemia, which reflects peripheral insulin resistance. Non-insulin dependent diabetes is also an insulin-resistant state, although absolute levels of insulin may be in the normal range or lower. Hyperinsulinaemia has been ascribed a pathogenetic role in hypertension via a range of pathogenic mechanisms, including increased renal sodium retention.

(ii) Cell membrane cation transport

Abnormalities of cell membrane cation transport have been demonstrated in obesity, non-insulin dependent diabetes and hypertension. These, as well as the obligatory linkage of cellular sodium efflux and potassium influx, and the regulatory role of

insulin in cell membrane cation transport, raise the possibility that such shifts in internal cation distribution are associated with insulin resistance and/or hyperinsulinaemia (Modan *et al.*, 1985). A recent study by Ohno *et al.* (1993) has shown that in young, lean normotensive offspring of essential hypertensives, insulin sensitivity correlates inversely with intracellular calcium concentration in platelets. Hence, impaired insulin sensitivity and disturbed calcium metabolism exist before the onset of hypertension, and their association may contribute to its development. Thus, numerous abnormalities in the function of plasma membranes have been described in patients with EH, some of which may be important in the development of hypertension. Losse *et al.* (1960) were the first investigators to report elevation of intracellular sodium in erythrocytes obtained from subjects with EH. They demonstrated increased net sodium flux across the erythrocyte membrane, suggesting cell permeability to sodium was increased in hypertensive patients (Wessels *et al.*, 1967). Increased cell-membrane permeability to sodium and the consequent rise in the intracellular sodium concentration would normally stimulate sodium efflux through stimulation of the energy-dependent ouabain-sensitive sodium pump. This was reported by Postnov *et al.* (1977).

Studies of white-cell electrolyte handling may more closely reflect the physiology of other nucleated cells. Increased intracellular sodium in these cells was suggested by work done by Edmondson *et al.* (1975). Ambrosioni *et al.* (1981) showed an increase in intracellular sodium in lymphocytes, which was directly correlated with blood pressure.

Two other carrier-mediated transport pathways have been investigated in erythrocytes from humans with EH and have been found to be abnormal. Frusemide-sensitive sodium-potassium co-transport was decreased in a group of patients with EH (Garay *et al.*, 1980), while sodium-lithium counter-transport was observed to be increased in a group of hypertensive subjects (Canessa *et al.*, 1980).

Since membrane ion transport is such a fundamental participant in smooth muscle excitation-contraction, it seems likely that these abnormalities are in some way related to the process which elevates blood pressure. Vascular smooth muscle contraction is mediated by an increase in ionised calcium in the cytoplasm in the region of the contractile protein (Bolton, 1979).

This change is produced by the entry of calcium ions into the cell, the effect of which is amplified by the release of calcium bound by the cell membrane and by intracellular stores. The increase in cell-membrane permeability to calcium is due to the opening of two types of ion channel. One opens in response to depolarisation produced by a change in sodium or potassium gradient across the membrane. Receptor-operated channels, on the other hand, respond to specific agonists by increasing cell-membrane permeability to calcium and to other ions. A degree of depolarisation may, therefore, result from agonist binding to receptors, with the resultant opening of potential-mediated as well as receptor-mediated channels. Two hypotheses have been put forward to link cation transport with the processes mediating smooth muscle contraction. One postulates physiological inhibition of transmembrane calcium movements, whilst the other postulates an intrinsic membrane abnormality.

Rather than incriminating a specific abnormality of one pathway in EH, a genetic membrane defect has been postulated (Canessa *et al.*, 1981 and Orlov *et al.*, 1982). Thus, in addition to abnormalities of sodium and potassium handling, red-cell membrane viscosity is increased (Orlov and Postnov, 1982) and calcium binding by red-cell membranes reduced in EH (Postnov *et al.*, 1979). If this is associated with an increase in free intracellular calcium either directly or as a result of decreased affinity for the ATP-dependent calcium extrusion pump, this would provide a basis for enhanced smooth muscle tone. If increased intracellular calcium, as a result of a decreased cell-membrane affinity for calcium, was responsible for enhanced smooth muscle contractility, the previously described changes in potassium and sodium handling could merely represent a marker for the primary abnormality. It is also possible, however, that they are more directly linked to calcium handling. Two properties of calcium may be relevant. Firstly, increasing intracellular calcium enhances red-cell sodium and potassium permeability (Romero and Whittam, 1971), and there is a close relationship between potassium loss from red-cell ghosts and intracellular calcium (Whittam, 1968 and Romero, 1976). Secondly, high external calcium concentrations stabilise smooth muscle cell membranes, reducing potassium efflux and inhibiting excitation (Rothstein, 1968 and Holloway & Bohr, 1973).

(iii) Role of G-proteins in altered cell membrane function

G-proteins are membrane proteins which provide an essential link between cell surface receptors and second messenger generating systems, such as adenylyl cyclase (Houslay, 1984). In addition, these proteins appear to regulate the function of a number of transmembrane ion channels (e.g. potassium (Yatani *et al.*, 1988) and calcium (Hescheler *et al.*, 1987) channels). Since alterations of G-protein activity would affect many aspects of cell membrane function, as demonstrated in diabetes mellitus (Gawler *et al.*, 1987) and obesity (Houslay *et al.*, 1989), it is of interest to examine whether such changes occur in human genetic hypertension. Hypertension-associated abnormalities of cAMP metabolism have been studied in human platelets (Resink *et al.*, 1986). Platelets from hypertensive subjects had an enhanced cAMP accumulation response to prostaglandin E_1 (two-fold increase in prostaglandin E_1 sensitivity). The degree of adenylyl cyclase activation in response to forskolin was greater in hypertensive than normotensive subjects, suggesting that the catalytic subunit of the enzyme is the rate-limiting step of this hormonal information transduction. Platelets from hypertensive subjects were more sensitive to epinephrine-induced inhibition of the stimulatory effects of prostaglandin E_1 on cAMP accumulation. This increased sensitivity to inhibition of adenylyl cyclase activity may contribute to enhanced stimulated vasoconstriction and an increased risk for coronary infarction and cerebrovascular accidents in hypertension. It was proposed that the enhanced adenylyl cyclase stimulation and cAMP accumulation response to prostaglandin E_1 in platelets from hypertensives reflect a potentiated negative feedback controlling mechanism. Such an adaptive mechanism may function to protect the platelets against calcium overload and to dampen their participation in hemostatic and thrombotic processes. A study by McLellan *et al.* (1990) examined the role of G-proteins in this abnormal platelet adenylyl cyclase activity (ACA) reported in humans with essential hypertension (Resink *et al.*, 1986). ACA under basal conditions and in the presence of forskolin were observed to be similar in the essential hypertensives and their controls. Activation of the inhibitory G-protein, G_i, with a low concentration of p(NH)ppG and adrenaline/propranolol did not differ between patients with EH and normotensive controls. However, greater stimulation of ACA in the presence of prostaglandin E_1 was seen in platelets from essential

hypertensives than in platelets from controls. The levels of G-protein subunits were measured, by immunoblotting using specific anti-peptide antisera, but no differences were found in the levels of $G_{s}\alpha$, $G_{i}2\ \alpha$ and the β-subunit in platelets from essential hypertensives and normotensive controls. Hence, this study confirmed abnormal ACA in platelets from patients with essential hypertension. Although no differences were found in the levels of the G-proteins concerned with the dual regulation of ACA, abnormal G-protein functioning may contribute to these abnormalities. Hence, abnormal regulation of ACA has been reported by different groups in platelets from humans with essential hypertension. There are no data regarding G-protein function in vascular smooth muscle from human hypertensives due to the difficulty in obtaining such tissue. However, animal models of human essential hypertension can provide relevant tissues to study. Genetically hypertensive rats are valuable models for such studies, as it is possible to have direct access to vascular tissues, and hypertension develops in these rats with little influence from the environment.

1.3.2 Rodent models of human essential hypertension

Almost all studies of membrane electrolyte transport have been carried out in the rat, and the majority have utilised genetic hypertension in an inbred strain, on the grounds that this may provide a better model for EH in man than models which require administration of mineralocorticoids or renal manipulations. The demonstration of similar changes in different tissues of genetically hypertensive rats and in patients with EH may throw light upon the mechanisms responsible for blood pressure elevation in both species.

There are several genetically hypertensive rat strains available internationally, including spontaneously hypertensive rats (SHR), stroke-prone spontaneously hypertensive rats (SHRSP), Dahl salt-sensitive strain, Lyon hypertensive rats and Milan hypertensive strain (MHS). The work of this thesis examines two animal models of human EH, the SHR (Chapters 3 and 4) and the MHS (Chapters 5 and 6). The SHR is insulin resistant, as are humans with EH, and the elevated blood pressures of both the SHR and the MHS (Dall'Aglio *et al.*, 1991) are associated with hyperinsulinaemia, as in the case of hypertensive humans.

A study by Horie *et al.* (1986) obtained comparable data from several hypertensive rat strains under the same experimental conditions. The SHRSP and SHR showed markedly higher blood pressure levels and earlier blood pressure rises in comparison with other hypertensive strains. (The MHS develops mild hypertension for the first two months after birth and shows no further augmentation of hypertension during the process of aging, so that the developmental course of hypertension is different from EH, which tends to increase gradually with age.) All hypertensive strains showed increases in left ventricular weight in proportion to blood pressure rises. Kidney weights were significantly decreased only in MHS compared with its control, while they were either unchanged or significantly greater in other hypertensive strains. These comparative data indicate the early involvement of cardiovascular structural alterations, especially left ventricular hypertrophy in the development of genetic hypertension in all rat strains, whether the hypertension is primary or secondary. It is proposed that renal functional insufficiency may contribute to the pathogenesis of hypertension in MHS with significantly reduced kidney sizes. These differences noted in SHRs and MHS suggest possible differences in the pathogenic mechanisms of genetic hypertension. Further details of the two models studied in this thesis are described below.

(i) The Okamoto spontaneously hypertensive rat (SHR)

The development of the SHR strain was started in 1959 by Okamoto and Aoki. The SHR strain, which is derived from Wistar-Kyoto (WKY) rats, is unique (in addition to the SHRSP) among the various hypertensive rat models because it not only develops hypertension spontaneously, but also hypertension-related complications without any additional inducements such as salt loading (Yamori, 1981). The SHR has severe hypertension and develops cardiovascular complications frequently and is, therefore, a valuable model for cardiovascular diseases in hypertension. Also, early development of hypertension in the SHR, in which genetic factors are concentrated by the long-term repetition of selective breeding, can also be noted in humans (confirmed by epidemiological and clinical studies) (Londe *et al.*, 1971 and Harlan *et al.*, 1979). Similarly, cardiac hypertrophy in hypertension develops early in the SHR, as well as in children or young adults with borderline or mild hypertension (Yamori *et al.*, 1979).

Blood pressure is determined by cardiac output and total peripheral resistance, and the former is related to circulating volume regulation by cardiac, renal, or adrenal functions, while the latter is based on either a functional vasopressor-depressor system or structural changes of resistance arteries. Volume factors seem to play no role in the development of hypertension in the SHR (Trippodo *et al.*, 1978), and consistent with these findings, cardiac output is normal or reduced in this model. On the other hand, total peripheral resistance is increased in the SHR, indicating that functional or structural factors increasing the total peripheral resistance are more important than volume factors in this hypertensive model. These findings appear to be similar to essential hypertension at its established stage.

a. Altered vascular structure

In this strain, hypertension is hemodynamically maintained by the increased total peripheral resistance, as mentioned previously, and it is now commonly accepted that structural vascular alterations substantially contribute to this increase in the total peripheral resistance (Folkow *et al.*, 1973). Such structural vascular alterations, measured as an increased wall thickness/lumen ratio and detected by hind limb perfusion experiments, can be commonly observed in a number of types of hypertension. Morphological analysis of structural vascular changes in the aorta clearly indicates that both hyperplasia and hypertrophy of smooth muscle cells, with concomitant increase in elastic component, are involved in the process of structural vascular alterations in adult SHRs with established hypertension. The structural changes reflect altered vascular smooth muscle cell (VSMC) growth and differentiation, and this process of adaptive metabolic change can be regarded as the common path to structural changes (Yamori, 1976). Therefore, the common mechanisms for increasing total peripheral resistance in hypertension are increased vascular contractility and structural vascular changes induced by accelerated vascular protein synthesis. Moreover, an enhanced intracellular water content in arterial walls appears to contribute to increased total peripheral vascular resistance (Cox, 1979), and may be related, as well as altered functional vascular reactivity, to alterations in receptors and cellular membranes in SHR erythrocytes, vascular smooth muscle, etc. In summary, the functional vascular alterations, primarily

caused by neural vasomotor activation and activation of cellular membrane mechanisms of vascular smooth muscle cell contraction, are involved in the initiation of spontaneous hypertension, and the structural vascular changes brought about by vascular protein synthesis, activated by the rise in blood pressure and increased neuronal input, may contribute to the further development or maintenance of hypertension.

b. Membrane cation transport abnormalities

Genetic analysis of blood pressure in the SHR has indicated that hypertension is a multifactorial disease caused by autosomal additive inheritance of at least three to five major genes (Tanase *et al.*, 1970), as similarly suggested for essential hypertension in men. Heritability or the degree of genetic determination of blood pressure is greater in the SHR than in human essential hypertension. Although no biochemical abnormalities have so far been proved to be causally related to the pathogenesis of genetic hypertension either in the SHR or in humans, studies employing the SHR as well as hypertensive men suggest that some biomembrane abnormalities commonly detected in erythrocytes, etc., may be involved in the cellular mechanism of genetic hypertension, and might also be utilised as genetic markers of hypertension (De Mendonca *et al.*, 1980 and Canessa *et al.*, 1980). Most studies of the erythrocyte and arterial wall maintained in artificial media have shown increased sodium-potassium permeability associated with increased turnover of these cations (Jones, 1974). Increased cell-membrane permeability to sodium and potassium would normally be associated with increased activity of (Na^+/K^+)-ATPase-mediated sodium pumping in SHRs. There is evidence for this in vascular smooth muscle from SHRs (Hermsmeyer, 1976). Webb and Bohr (1979) investigated the relaxation of vascular smooth muscle produced by depolarising the cell membrane with increased potassium concentration in the surrounding medium. Such relaxation was greater in SHRs than in normotensive controls, although the duration of relaxation was no different. The difference in response between SHRs and normotensive controls could be eliminated by ouabain. This was interpreted as indicating increased activity of the ouabain-sensitive sodium pump in SHRs, and is consistent with other studies indicating increased sodium and potassium permeability and the resulting stimulation of the electrogenic

sodium pump. Therefore, in the most widely studied genetic model of hypertension, the Okamoto SHR, cell-membrane permeability to sodium is increased and this is associated with increased active sodium pumping through (Na^+/K^+)-ATPase activity.

Calcium binding was observed to be decreased in SHR erythrocytes (Postnov *et al.*, 1979 and Devynck *et al.*, 1981), adipocytes (Postnov and Orlov, 1980), arterial wall (Wei *et al.*, 1976) and aortic smooth muscle (Zsoter *et al.*, 1977). SHR adipocyte cell-membrane fluidity was also observed to be decreased (Orlov *et al.*, 1982). Hence, similar changes regarding cation transport in humans with EH exist in SHRs, emphasising its relevance as an animal model of EH in man.

Abnormal regulation of ACA in vascular tissues (and non-vascular tissues) from the SHR has been demonstrated (see section 3.1 for details). However, the roles of adenylyl cyclase's regulatory G-proteins, G_S and G_i, in this abnormality have not been examined in detail. Adenylyl cyclase is under dual control, hence, the production of the second messenger and vasodilator, cAMP, can be stimulated and inhibited. Thus, increased vasoconstriction in the SHR may arise from a decreased stimulation and/or an increased inhibition of adenylyl cyclase activity. It is possible that a common membrane defect may be responsible for the changes in membrane cation transport, decreased membrane fluidity, and altered regulation of ACA in the SHR. The increased vascular smooth muscle (VSM) contractility and altered vascular structure in the SHR may also be related to a possible common membrane defect.

(ii) <u>The Milan hypertensive strain (MHS)</u>

The Milan hypertensive strain and the Milan normotensive strain (MNS) descend from two mating pairs of Wistar origin rats from an outbred colony, which had mean blood pressures of 164mmHg and 168mmHg. The hypertensive strain was obtained by breeding for high blood pressure and the control strain was obtained by breeding for normal blood pressure (Bianchi *et al.*, 1984).

The MHS is another useful animal model for studying the mechanisms that may be responsible for human essential hypertension. For example, Bianchi *et al.* (1984) measured different factors in the prehypertensive phases of both rats and humans and compared the results with those obtained for appropriate controls of the same age. They chose the prehypertensive phase in order to

detect changes that existed before the development of hypertension, and which cannot be secondary to the hypertension, *per se*. Moreover, during the time-interval between birth and the development of overt hypertension, the genetic factors responsible for hypertension may already have produced biochemical or physiological abnormalities. Due to the difficulty of defining a prehypertensive phase in human subjects, young normotensive subjects with two hypertensive parents were compared with subjects of the same age, but with two normotensive parents, on the assumption that, later in life, the former will develop hypertension more frequently (about 50%) than the latter (about 2%). This group observed that a number of features in the MHS are similar to those observed in human essential hypertension, with simliar changes in whole-kidney function (glomerular filtration rate and renal blood flow), morphology of the kidney and adrenal (by light or electron microscopy), 24 hour urinary electrolyte output, extracellular fluid volume, total and exchangeable body Na^+, plasma electrolytes, plasma and urine osmolarity, plasma aldosterone, and plasma catecholamines.

The key role of the kidney in this animal model of hypertension has been demonstrated. Studies of the effect of kidney cross-transplantation between adult MHS rats and adult MNS rats showed that the MHS kidney produced hypertension in MNS rats. Transplantation has also been carried out between MHS and MNS rats at one month of age, when the difference in donor blood pressure was slight and of short duration, to determine whether this pressor effect of the MHS kidney was primary in nature or secondary to renal injury due to prolonged perfusion at high blood pressure of the MHS donor kidney. The blood pressure measured 120 days after kidney transplantation into one-month-old MHS rats was 152±2mmHg in recipients of MHS kidneys and 130±5mmHg in recipients of MNS kidneys ($p<0.01$). Therefore, MHS kidneys have a greater pressor effect even when taken from young donors with almost equal blood pressure, demonstrating that the pressor effect of the MHS kidney exists prior to hypertension (Fox and Bianchi, 1976). In addition, the relative sodium retention in MHS rats, during the development of the blood pressure difference between the two strains, is in keeping with a renal origin of hypertension in MHS. To evaluate further the role of the kidney, whole kidney clearance and micropuncture techniques were used to study whole kidney and single nephron filtration rate and proximal fluid reabsorption in both strains before, during and after the development of hypertension in MHS.

Both single nephron and whole kidney glomerular filtration rates are significantly lower in young MHS rats before the development of hypertension, while in adult animals, whole kidney glomerular filtration rate is equal, and superficial single nephron filtration rate is higher in MHS rats (discrepancy explained by lower number of glomeruli in MHS kidney) (Bianchi and Baer, 1976).

Sodium transport across red cell membranes was measured with the red cell volume in prehypertensive MHS and MNS rats. Moreover, a genetic analysis involving crosses between the two strains and backcrosses of the first generation of hybrids with the two parental strains was carried out in an attempt to evaluate whether the red cell volume and kidney weight traits segregate together with the blood pressure in these hybrid generations (Bianchi, 1981). The results obtained demonstrated that the prehypertensive MHS have smaller red cell volume, lower intracellular Na^+ concentration, and faster outward Na^+ cotransport across their cell membranes. These differences in red cell function were consistent with the increased transtubular transport of ions and water in MHS kidneys and with the lower weight of these kidneys in spite of a similar number of nephrons. Moreover, in the hybrid generations there was an inverse correlation between the volume of the red cells in young rats or the weight of the kidney measured in the adult age, and the level of blood pressure reached by the same animal in the adult age. All these findings are consistent with the hypothesis that young prehypertensive MHS rats have a genetically determined faster transport of ions across their cell membranes, and that this abnormality is responsible for the differences in renal function between MHS and MNS rats.

Red blood cell abnormalities were apparent in lethally irradiated (MHSxMNS) F_1 hybrids which were recipients of bone marrow from MHS. In these rats, Na^+-K^+ cotransport was increased, and intracellular Na^+ content and cell volume were decreased (Bianchi *et al.*, 1985). Smaller volume and Na^+ content have also been demonstrated in MHS proximal tubular cells. Also, the Na^+ transport is faster and the Ca^{2+} ATPase at V_{max} is lower in these cells and in erythrocytes (Bianchi *et al.*, 1986). These results indicate that the MHS erythrocyte abnormalities may well be associated with the primary cause of spontaneous hypertension in rats, as erythrocyte abnormalities are genetically determined within the stem cells.

A study by Parenti *et al.* (1991) examined the (Na^+/K^+)-ATPase activity from the kidney cortex of MHS and MNS rats in active solubilised enzyme preparations and in isolated basolateral membrane vesicles. Kinetic analysis of the purified enzyme showed that the V_{max} value was significantly higher in MHS rats. The difference between MHS and MNS was not linked to a different number of sodium pumps, but was related to the activity of the enzyme. Using basolateral membrane vesicles, an increased ATP-dependent ouabain-sensitive sodium transport was also demonstrated in MHS rats. These results support the hypothesis that a higher tubular sodium reabsorption may be involved in the pathogenesis of hypertension in this rat strain.

A recent study by Ferrari *et al.* (1992) has investigated whether cytoplasmic or membrane skeleton abnormalities may be possible causes of faster $Na^+/K^+/Cl^-$ cotransport in MHS erythrocytes. It has been observed that the difference in $Na^+/K^+/Cl^-$ cotransport is no longer present in inside-out vesicles of erythrocyte membranes. Resealed ghosts retained the characteristics of the $Na^+/K^+/Cl^-$ cotransport of the original erythrocytes, suggesting that an abnormality in the membrane skeletal proteins may play a role in the different $Na^+/K^+/Cl^-$ cotransport modulation between MHS and MNS erythrocytes.

In summary, the MHS rat serves as an alternative and useful model for human essential hypertension. Although there is a range of data on cell ion flux abnormalities in this strain, there is little information on the regulation of second messenger generating systems, such as adenylyl cyclase, and no data on G-protein abnormalities.

1.4 Aims of study

1.4.1 Regulation of adenylyl cyclase activity in genetically hypertensive rat vasculature

Altered regulation of adenylyl cyclase activity has been demonstrated in SHR vascular tissues in comparison to normotensive controls (see section 3.1 for details), and may contribute to increased vascular reactivity in this rat model. These previous studies, however, did not concern themselves with the underlying mechanisms responsible for the altered regulation of this second messenger generating system. The roles of stimulatory and

inhibitory G-proteins of the adenylyl cyclase system were examined in vascular tissue from SHRs (Chapters 3 and 4), MHS (Chapters 5 and 6), and their appropriate controls, as abnormalities in the levels/function of these G-proteins have been observed in diabetes and other insulin-resistant states (see section 1.2.8), which share insulin-resistance and hyperinsulinaemia with genetic hypertension.

1.4.2 Common primary cause of hypertension in SHRs and MHS?

Membrane signal transduction was examined in vascular tissue from two rodent models of human EH. There is early involvement of cardiovascular structural alterations, especially left ventricular hypertrophy, in the developmental process of genetic hypertension in both SHRs and MHS; however, renal functional changes may contribute to the pathogenesis of hypertension in MHS (but not SHRs), which has significantly reduced kidney sizes (Horie *et al.*, 1986). These observations suggest possible differences in the pathogenic mechanisms responsible for genetic hypertension in SHRs and MHS. However, both models exhibit major changes in transmembrane ion fluxes, which may be of primary importance in the development of increased blood pressure; there are no data on the regulation of ACA in the MHS. It was, therefore, of interest to compare the role of G-proteins involved in the regulation of ACA in the two models of human EH, and to examine in detail the regulation of ACA in the MHS.

Chapter 2 Materials and Methods

2.1 Materials

All reagents employed were of the highest quality available and were obtained from the following suppliers:

2.1.1 General reagents

The following reagents were purchased from Sigma Chemical Company Ltd., Poole, Dorset, United Kingdom: soybean trypsin inhibitor, collagenase (type II), elastase (type IV), calcium chloride, tris (hydroxymethyl) aminomethane pH 7 to pH 9, cupric sulphate, sodium potassium tartrate, bovine serum albumin (fraction V), deoxycholic acid (sodium salt), alumina (type WN-3:neutral), magnesium acetate, adenosine 5'-triphosphate (A2383), adenosine 3',5'-cyclic monophosphate (A6885), dithiothreitol (DTT), imidazole, adenosine 5'-triphosphate (A3377), adenosine 3',5'-cyclic monophosphate (A9501), manganese chloride, forskolin, sodium fluoride, (-)-isoproterenol ((+)-bitartrate salt), (-)-epinephrine ((+)-bitartrate salt), DL-propranolol, carbamylcholine chloride (carbachol), 5-hydroxytryptamine, neuropeptide Y, prostaglandin E_1, acrylamide, N,N'-methylene-bis-acrylamide, N,N,N',N'-tetramethylethylenediamine (TEMED), glycine, urea, bromphenol blue, coomassie brilliant blue G, potassium phosphate (monobasic, anhydrous), thimerosal, nonidet P-40, o-dianisidine, N-[2-Hydroxyethyl]piperazine-N'-[2-ethane-sulphonic acid] (HEPES).

The following products were obtained from BDH Chemicals Ltd., Poole, Dorset, United Kingdom: hydrochloric acid, sodium dodecyl sulphate (SDS), Folin & Ciocalteu's phenol reagent, trichloroacetic acid, acetic acid (glacial), glycerol, ammonium persulphate, methanol, disodium hydrogen orthophosphate dihydrate, gelatine powder (approx. 150 bloom from pig skin), hydrogen peroxide solution, magnesium chloride, disposable plastic cuvettes.

The following chemicals were supplied by FSA Laboratory Supplies, Loughborough, United Kingdom: sucrose, diaminoethanetetra-acetic acid disodium salt (EDTA), sodium carbonate, sodium hydroxide, sodium chloride, potassium chloride.

The following products were purchased from Boehringer Mannheim UK (Diagnostics & Biochemicals) Ltd., East Sussex, United Kingdom: creatine phosphate, creatine kinase, guanosine-5'-triphosphate, guanosine 5'-[γ-thio]triphosphate, guanosine 5'-[βγ-imido]triphosphate.

The following were supplied by Bio-Rad, Hertfordshire, United Kingdom: cation exchange resin (dowex 50W-X4, 200-400 mesh, hydrogen form), columns (for adenylyl cyclase assay).

Bethesda Research Laboratories-Life Technologies, Inc., Paisley, United Kingdom supplied: prestained protein molecular weight standards (molecular weight range 14,300-200,000), 1.5mm spacers, vertical gel electrophoresis systems.

The Scottish Antibody Production Unit, Law Hospital, Carluke, Scotland provided: horseradish peroxidase-labelled donkey anti-rabbit IgG (polyclonal).

Anderman & Co. Ltd., Laboratories Supplies Division, Surrey, United Kingdom supplied: nitrocellulose (0.45μm).

National Diagnostics, Manville, New Jersey, USA supplied: ecoscint-A.

Pfizer, Sandwich, Kent, United Kingdom supplied: UK 14304.

2.1.2 Tissue culture products

The following tissue culture products were obtained from GIBCO BRL, Paisley, United Kingdom: Dulbecco's modified Eagle medium ((high glucose) with L-glutamine, with 4500mg/l D-glucose, without sodium pyruvate), minimum essential medium, penicillin-streptomycin solution, foetal calf serum (heat inactivated), horse serum (heat inactivated), tissue culture flasks (25cm^2, 80cm^2 and 175cm^2), 50ml centrifuge tubes.

ICN Flow Laboratories, High Wycombe, Buckinghamshire, United Kingdom supplied: L-glutamine.

The Department of Virology, University of Glasgow, Glasgow, Scotland supplied: trypsin (0.25% in tris saline (tris saline: NaCl 0.8%, KCl 0.038%, Na_2HPO_4 0.01%, dextrose 0.1%, tris 0.3%, phenol red 0.0015%, penicillin 100units/ml, streptomycin 0.01%, pH 7.7 with HCl)), versene (0.02% in phosphate-buffered saline consisting of NaCl 0.8%, KCl 0.02%, Na_2HPO_4 0.115%, KH_2PO_4 0.033%, diaminoethanetetra-acetic acid disodium salt 0.02%, phenol red 0.0015%, pH 7.12-7.3).

2.1.3 Radiochemicals

The following radioisotopes and products were purchased from Amersham International plc, Buckinghamshire, United Kingdom: [α^{32}P] adenosine 5'-triphosphate, [8-^{3}H] adenosine 3',5'-cyclic monophosphate, (-)-3-[^{125}I] iodocyanopindolol, [^{125}I]-Ig (anti-rabbit, whole Ab from donkey), hyperfilm-MP, hypercassette (18x24cm), hyperscreen intensifying screens (18x24cm), sensitize pre-flash unit.

2.2 Animals

Spontaneously hypertensive rats, Wistar-Kyoto rats and Wistar rats were purchased from two different suppliers, namely Charles River Ltd., Margate, Kent, United Kingdom and Harlan Olac Ltd., Oxon, United Kingdom. The rats arrived at 10 weeks of age and were sacrificed by a blow to the head within one week of arrival. Before sacrifice, the rats had their weights and systolic blood pressures (SBP) measured (tail-cuff method (McAreavey *et al.*, 1985)) using a Mettler PE 2000 balance (Fisons, Crawley, Sussex, United Kingdom) and a W+W electronic BP recorder 8006 (Kontron Electronics, Watford, United Kingdom), respectively. SBP were measured in conscious rats which were warmed at 37°C for 15 minutes and readings were made without anaesthesia by means of inflation of tail-cuff and a piezo-electric crystal detector connected to a blood pressure recorder. SBP were calculated from the mean of six recordings for each rat. The rats were fed on rat and mouse maintenance diet, allowed free access to H_2O and kept under constant light conditions.

SHRs and normotensive control rats were used in this study at 10-11 weeks of age, as by this age SHRs had significantly elevated blood pressures and established hypertension in comparison to

age-matched control rats. Figure 2.1 shows the systolic blood pressures of SHRs and WKY rats related to age. Data are mean±S.E. (standard error of the mean) of measurements made from eight rats of each strain.

Part of the work in this study involved the Milan hypertensive strain as an animal model of human essential hypertension. Vascular smooth muscle cells isolated from the thoracic aortas of MHS and MNS rats were kindly supplied by Dr. Patrizia Ferrari, Prassis Sigma-Tau Research Institute, Milan, Italy. VSMC were isolated from aortas of young rats (30 days old) and adult rats (60 days old). From Figure 2.2 it can be seen that young MHS rats have slightly, but significantly increased systolic blood pressures in comparison to age-matched MNS rats, while adult MHS rats have significantly elevated systolic blood pressures and are hypertensive compared to MNS rats of the same age.

2.3 Apparatus

Laminar flow hood (MDH, Andover, Hampshire, United Kingdom), CO_2 incubator (ASSAB Medicin AB, Stockholm, Sweden), microscope (Leitz Labovert, Wetzlar, Germany), 11x64mm polystyrene tubes, universals and plastic Pasteur pipettes (standard and fine tipped) (Alpha Laboratories Ltd., Hants., United Kingdom), millex-GS 0.22μm filter unit (Millipore Ltd., Herts., United Kingdom), 'polytron', kinematica, CH-6010 kriens-LU PT 10-35 (The Northern Media Supply Ltd., North Humberside, United Kingdom), Beckman DU-70 spectrophotometer (Beckman, Buckinghamshire, United Kingdom), scintillation vials (LIP, Shipley, West Yorkshire, United Kingdom), LKB Wallac 1217 rackbeta (liquid scintillation counter), glass plates, teflon combs, electrophoresis constant power supply ECPS 3000/150, LKB Bromma 2005 transphor power supply (Pharmacia Biosystems Ltd., Milton Keynes, United Kingdom), NE 1612 gamma counter (Nuclear Enterprises, EMI, United Kingdom), Titertek cell harvester (2 valve) (Flow Laboratories, High Wycombe, Buckinghamshire, United Kingdom), Whatman glass microfibre filters (Whatman International Ltd., Maidstone, Kent, United Kingdom), radio-immuno-assay disposable cups (Sarstedt Ltd., Leicester, United Kingdom).

Figure 2.1 Graph relating systolic blood pressure to age for SHRs and WKY rats. Systolic blood pressure measurements were initiated at 4 weeks of age and terminated at 20 weeks. Data are mean±S.E. for measurements taken from eight rats of each strain.

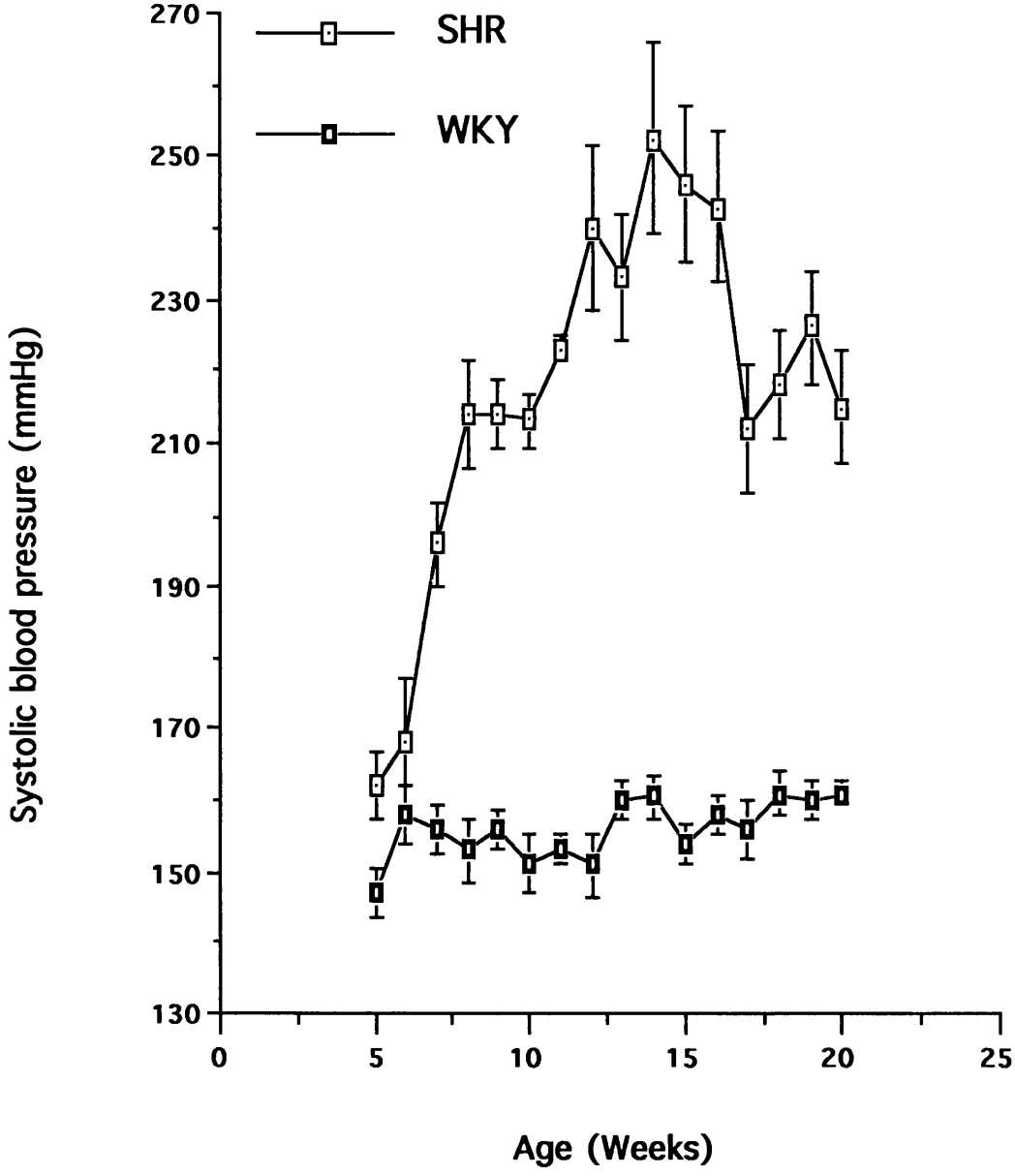
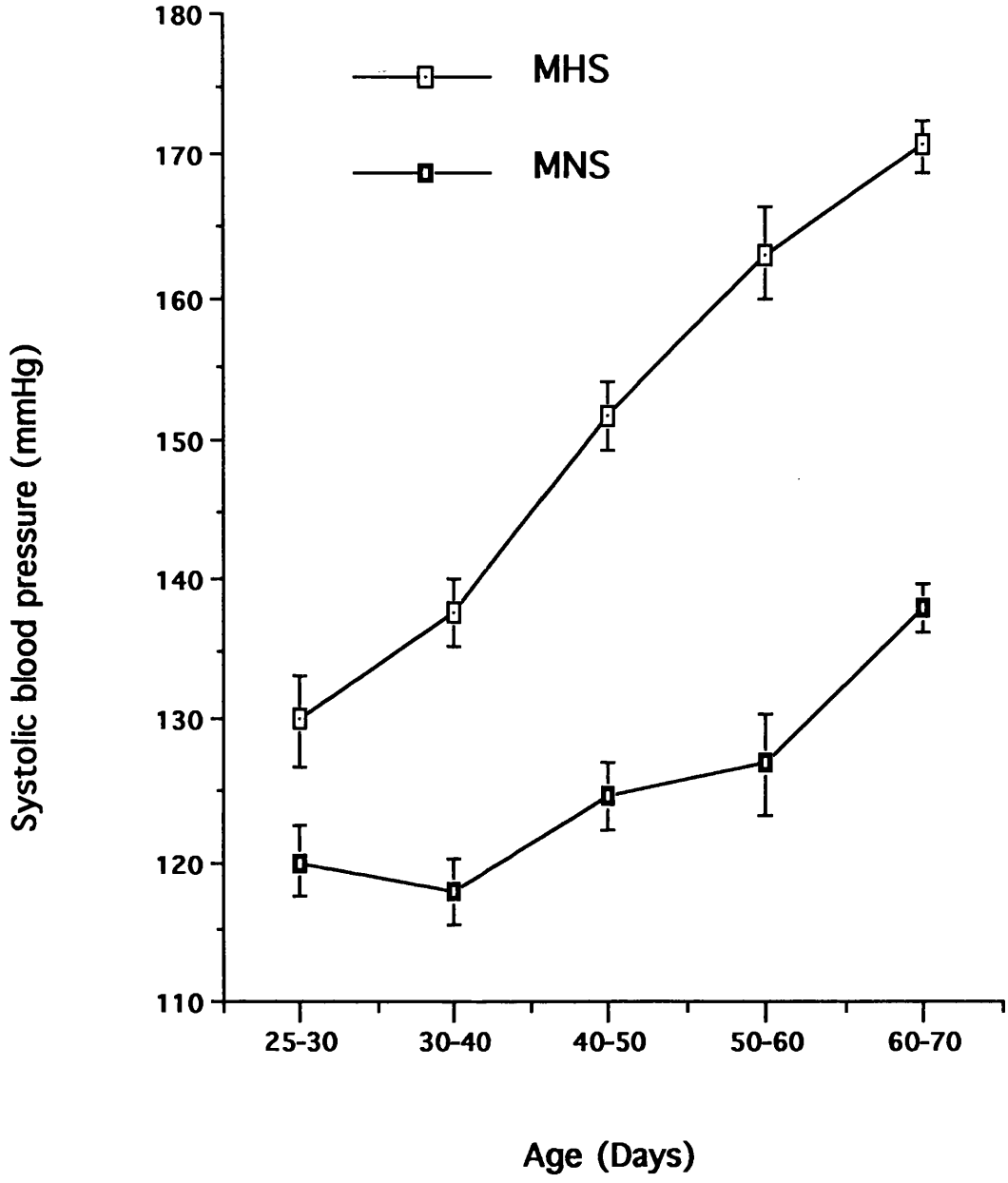

SHR
WKY
Systolic blood pressure (mmHg)
270
250
230
210
190
170
150
130
0
5
10
15
20
25
Age (Weeks)

Figure 2.2 Graph relating systolic blood pressure to age for MHS and MNS rats. Systolic blood pressure measurements were taken from weaning until two months of age. Blood pressure was measured at the tail in conscious rats. Data are mean±S.E. for measurements taken from six rats of each strain.


MHS
MNS
180
170
160
150
140
130
120
110
Systolic blood pressure (mmHg)
25-30
30-40
40-50
50-60
60-70
Age (Days)

2.4 General procedures

2.4.1 Glassware

All items of glassware were washed in solutions of the detergent decon 75 (Decon Laboratories Ltd., Hove, United Kingdom), rinsed thoroughly with tap water, then distilled water and dried in an oven at 37°C.

2.4.2 Micropipetting

Solution volumes in the range of 0.05-25.00μl were transferred accurately using Rainin EDP-plus electronic pipettes obtained from Rainin Instrument Co., Inc., Woburn, USA. Volumes in the range of 25-1000μl were transferred reproducibly using Finnpipettes purchased from Labsystems, Helsinki, Finland.

2.4.3 pH Measurement

Measurements of pH were performed using a digital pH/temperature meter obtained from Electronic Instruments Ltd. (United Kingdom). This apparatus was regularly standardised with a solution of pH 7 prepared from buffer tablets.

2.4.4 Centrifugation

Accelerations of 2,000xg were attained using a Damon/IEC division DPR-6000 centrifuge purchased from International Equipment Company (Bedfordshire, United Kingdom). Accelerations of 110,000xg were achieved using an LKB Bromma 2332 ultrospin 85 obtained from Pharmacia Biosystems Ltd. (Milton Keynes, United Kingdom).

2.5 Cell culture

2.5.1 Vascular smooth muscle cell preparation

Figure 2.3 is a photograph of a rat's intestine, illustrating the mesenteric artery. Mesenteric arteries were excised from six rats of each strain under sterile conditions, cleaned and transferred to a mixture (10ml) containing collagenase (1.25mg/ml), elastase

Figure 2.3 Photograph of a rat's intestine, illustrating the mesenteric artery.

(0.25mg/ml) and soybean trypsin inhibitor (0.05mg/ml). After incubation at 37°C for 5 minutes, any remaining fat, adventitia and endothelium were removed. The arteries were cut into small pieces (1-2mm), transferred to fresh enzyme mixture and incubated until a single cell suspension was obtained.

Cells isolated in this manner have previously undergone a series of tests to properly characterise them. Firstly, the method of isolation (i.e. first digestion) removes adventitia (fibroblasts). An antibody raised against Factor VIII (visualised by a fluorescent secondary antibody) was used to identify any endothelial cells in the cultures, but proved negative. Electron microscopy of the cells showed micropinocytotic vesicles, common to smooth muscle cells, and an abundance of myofilaments. Also, angiotensin II caused contraction of spindle-like cells (observed using time-lapse video) and measurements of angiotensin II receptor number on the cells after six passages were comparable to physiological measurements.

VSMC were isolated from thoracic aortas from MHS and MNS rats. Aortas were removed aseptically from four rats of each strain and age-group, cleaned of adherent connective tissue, and cut into small rings. The tissue was incubated in 12ml of minimum essential medium with 0.2mM $CaCl_2$, 1mg/ml of collagenase, 0.375mg/ml of soybean trypsin inhibitor, 0.125mg/ml of elastase and 2.0mg/ml of bovine serum albumin (BSA) for 90 minutes at 37°C in a revolving water bath. The partially digested tissue fragments were then collected with a coarse stainless steel mesh, resuspended in 20ml of minimum essential medium, titrated 10 times through a 14-g stainless steel cannula, and sieved through a 100μm mesh to separate the dispersed cells from undigested tissue fragments. The resulting cell suspension was centrifuged (200xg, 5 minutes) and then resuspended in 20ml of Dulbecco's modified Eagle medium supplemented with 10% calf serum and antibiotics (Brock *et al.*, 1985). Figure 2.4 is a photograph showing VSMC, isolated from Milan rat thoracic aortas.

2.5.2 Cell growth

The VSMC were grown in Dulbecco's modified Eagle medium containing 10% (v/v) foetal calf serum, 10% (v/v) horse serum, L-glutamine (2mM), penicillin (100U/ml) and streptomycin (100μg/ml) at 37°C in a humidified 5% CO_2 to 95% air atmosphere.

Figure 2.4 Photograph of VSMC isolated from Milan rat thoracic aortas.

2.5.3 Cell subculture

Once cells had grown to confluence, the growth medium was decanted and the cells washed with versene and then a mixture of versene/trypsin (4 volumes versene:1 volume trypsin). The cells were incubated at 37°C for a short time until all the cells had become detached from the surface of the flask. A volume of growth medium was added and a single-cell suspension attained by repeatedly triturating the cell solution with a fine-tipped Pasteur pipette. The cell solution was transferred to a larger flask (or divided among a greater number of flasks of the same size) and an appropriate volume of growth medium added.

2.6 Plasma membrane preparation

Production of plasma membranes directly from rat mesenteric arteries and VSMC was similar. VSMC (or finely chopped mesenteric arteries) were homogenised in ice-cold 10mM tris/1mM EDTA, pH 7.5 (250mM sucrose for arteries) using a 'polytron' at setting '5' (maximum speed for arteries) for 2x12 seconds. The homogenate was centrifuged at 2,000xg for 10 minutes at 4°C to remove unbroken cells, cellular debris and nuclei. The resulting supernatant was centrifuged at 110,000xg for 1 hour at 4°C to obtain a crude plasma membrane fraction which was resuspended in 10mM tris/HCl pH 7.5, aliquoted and stored at -70°C.

2.7 Protein determination

The protein concentrations in the plasma membrane preparations were measured colorimetrically using Peterson's modification (1977) of Lowry and co-workers' protocol (1951) based on bovine serum albumin as standard.
Stock Solutions:

Solution A	0.1% (w/v) copper sulphate in 0.2% (w/v) sodium potassium tartrate and 10% (w/v) sodium carbonate
Solution B	10% (w/v) sodium dodecyl sulphate
Solution C	0.8M sodium hydroxide
Solution D	0.15% (w/v) sodium deoxycholate
Solution E	72% (w/v) trichloroacetic acid

Just prior to use, stock solutions A, B and C were mixed with distilled H_2O in the following ratio A:B:C:H_2O, 1:1:1:1, to produce solution F.

Protein standards were prepared in duplicate (using a 1mg/ml solution of bovine serum albumin) to construct a standard curve for a maximum of 50μg of protein per sample. Unknowns were assayed in 10 and 15μl volumes in triplicate. Each sample volume was made up to 1ml with distilled H_2O, 100μl of solution D added and left to stand at room temperature for 10 minutes. 100μl of solution E was then added, the samples mixed and then centrifuged at 2,000xg for 15 minutes at room temperature. The supernatants were discarded and each sample pellet was resuspended in 1ml of distilled H_2O. 1ml of solution F was added, the samples mixed and left to stand at room temperature for 10 minutes. 0.5ml of a solution of Folin & Ciocalteu's phenol reagent and distilled H_2O (1 volume Folin & Ciocalteu's:5 volumes H_2O) was added to each sample, mixed, and left to stand at room temperature for 30 minutes. The absorbance of light by each sample was assessed spectrophotometrically at 750nm in a Beckman DU-70.

2.8 Adenylyl cyclase assay

Two step chromatography (Salomon) method

Adenylyl cyclase activity was measured by the method of Salomon (1979). In this assay, the formation of [^{32}P]cAMP from [α^{32}P]ATP was measured in the presence of GTP and magnesium ions.

2.8.1 Sample preparation

The reaction mix (final volume of 50μl) was essentially that described by Sharma *et al.* (1982) and included an ATP regenerating system consisting of creatine phosphate (5mM) and creatine kinase (50U/ml), in addition to tris acetate, pH 7.6 (25mM), magnesium acetate (5mM), ATP (0.5mM), cAMP (0.05mM), DTT (1mM), BSA (0.1mg/ml), GTP (0.01mM), [α^{32}P]ATP (1x10^{6}cpm/assay), 5μg of freshly thawed membrane protein and the ligand(s) of interest. Assay tubes were kept on ice at all times and the reaction started by removal to a 30°C water bath. After 15 minutes, the reaction was terminated by removal to ice and the addition of 100μl of a stopping solution, pH 7.5 consisting of sodium dodecyl sulphate (2%(w/v)),

ATP (45mM) and cAMP (1.3mM). 50μl of [^{3}H]cAMP (approx. 10,000cpm) was added to each tube prior to boiling for 3 minutes. 1ml of deionised water was then added to each sample, and the [^{32}P]cAMP content of each tube determined.

2.8.2 Preparation of dowex and alumina columns

The method used to quantify the amount of cAMP produced by each sample was identical to that of Salomon (1979) and involves the separation of cAMP from other nucleotides by dowex and then alumina chromatography. Dowex AG 50W-X4 (200-400 mesh) was washed repeatedly with deionised water until the effluent was colour-free. The dowex was mixed with deionised water to a slurry (1:1 v/v ratio) and 2ml samples of homogeneous slurry were added to glass wool-stoppered columns. The water was allowed to drain out and the columns filled with deionised water to allow all the beads to settle and form the column bed. The columns were then washed with 2ml of 1M HCl and stored at room temperature. Prior to use, each column was washed with 20ml of deionised water.

The alumina columns were prepared by the addition of 0.6g of dry neutral alumina to glass wool-stoppered columns. The columns were washed with 12ml of 1M imidazole buffer, pH 7.3, followed by 15ml of 0.1M imidazole buffer, pH 7.3 and stored at room temperature. On the day of use, each column was washed with 8ml of 0.1M imidazole buffer, pH 7.3.

2.8.3 Separation of cAMP on dowex columns

Prior to sample chromatography, the nucleotide elution profiles for each column were determined. This was performed by applying a mixture of [^{3}H]cAMP and [α^{32}P]ATP to the column and determining the elution volumes.

Assay blank systems were prepared containing approximately $1x10^4$ cpm [α^{32}P]ATP and $1x10^4$ cpm [^{3}H]cAMP standards. 1ml H_2O was added to each assay blank which was then decanted into a dowex column. The ATP and cAMP were eluted from the column by successive washes of the column with 0.5ml of water. Fractions were collected in vials containing 13ml of ecoscint and radioactivity determined by scintillation counting in a dual label programme. The elution volumes required to elute the cAMP from the dowex columns were then determined from a graph as shown (Figure

2.5). Typical recovery from the dowex columns was always between 70 and 80%.

The elution volume required to elute the cAMP from the alumina columns was determined as for the dowex columns. The cAMP was eluted from the alumina columns by successive washes of the columns with 0.5ml of 0.1M imidazole buffer. Recoveries were similar to that obtained for the dowex columns.

2.8.4 Determination of cAMP produced by membrane fractions

Samples (total volume 1.2ml) were added to prepared dowex columns and the ATP eluted with 1.5ml of water. 4ml of water was then added to the dowex columns and this eluate allowed to run directly onto the alumina columns. The cAMP fraction was eluted into vials containing 13ml of ecoscint, with 5ml of 0.1M imidazole buffer, pH 7.3.

The cAMP fractions obtained were counted on a dual label scintillation counting programme which automatically corrected for 'spillover' from each channel. The amount of cAMP produced by each sample was calculated by taking into account the recovery from each column, based on the recovery of the [^{3}H]cAMP internal standard, and corrected to 100%. Data was thus calculable in pmoles of cAMP produced per 15 minutes per mg of membrane protein.

2.8.5 Data analysis

Where appropriate, data were analysed for statistical significance using the Mann-Whitney U test for non-parametric data.

2.9 Gel electrophoresis

Gel electrophoresis was carried out according to the discontinuous system described by Laemmli (1970).

Figure 2.5 The elution profiles of ATP and cAMP standards from dowex AG 50W-X4 (hydrogen form) and neutral alumina columns. The elution profiles of [α^{32}P]ATP and [^{3}H]cAMP from dowex 50W-X4 (H^+ form) columns (Figure 2.5a) and the elution of [^{3}H]cAMP from neutral alumina columns (Figure 2.5b) were determined. Firstly, by the addition of a [^{3}H]cAMP standard ($1x10^4$ cpm) and an [α^{32}P]ATP standard ($1x10^4$ cpm) to a dowex column, followed by elution from the column by repeated addition of 0.5ml volumes of H_2O (Figure 2.5a). Secondly, an [α^{32}P]ATP standard ($1x10^4$ cpm) and a [^{3}H]cAMP standard ($1x10^4$ cpm) were added to a dowex column and eluted by the addition of 1.5ml and a further 4ml of H_2O, respectively. The [^{3}H]cAMP standard was eluted from the alumina column by repeated addition of 0.5ml volumes of 0.1M imidazole buffer, pH 7.3 (Figure 2.5b). The progress of both standards was monitored by counting 0.5ml fractions of eluate in a scintillation counter.

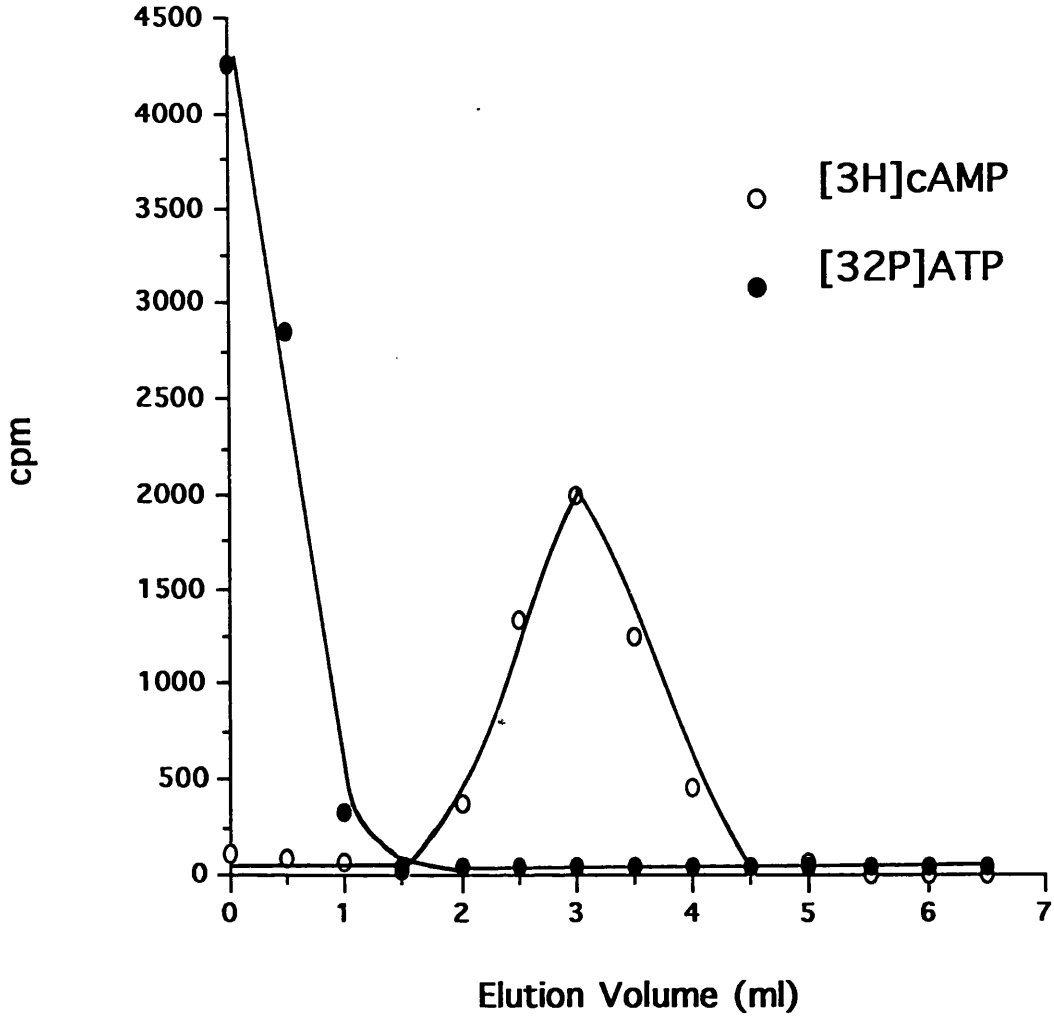

4500
4000
3500
3000
2500
2000
1500
1000
500
0
cpm
0
1
2
3
4
5
6
7
Elution Volume (ml)
[3H]cAMP
[32P]ATP

Figure 2.5a

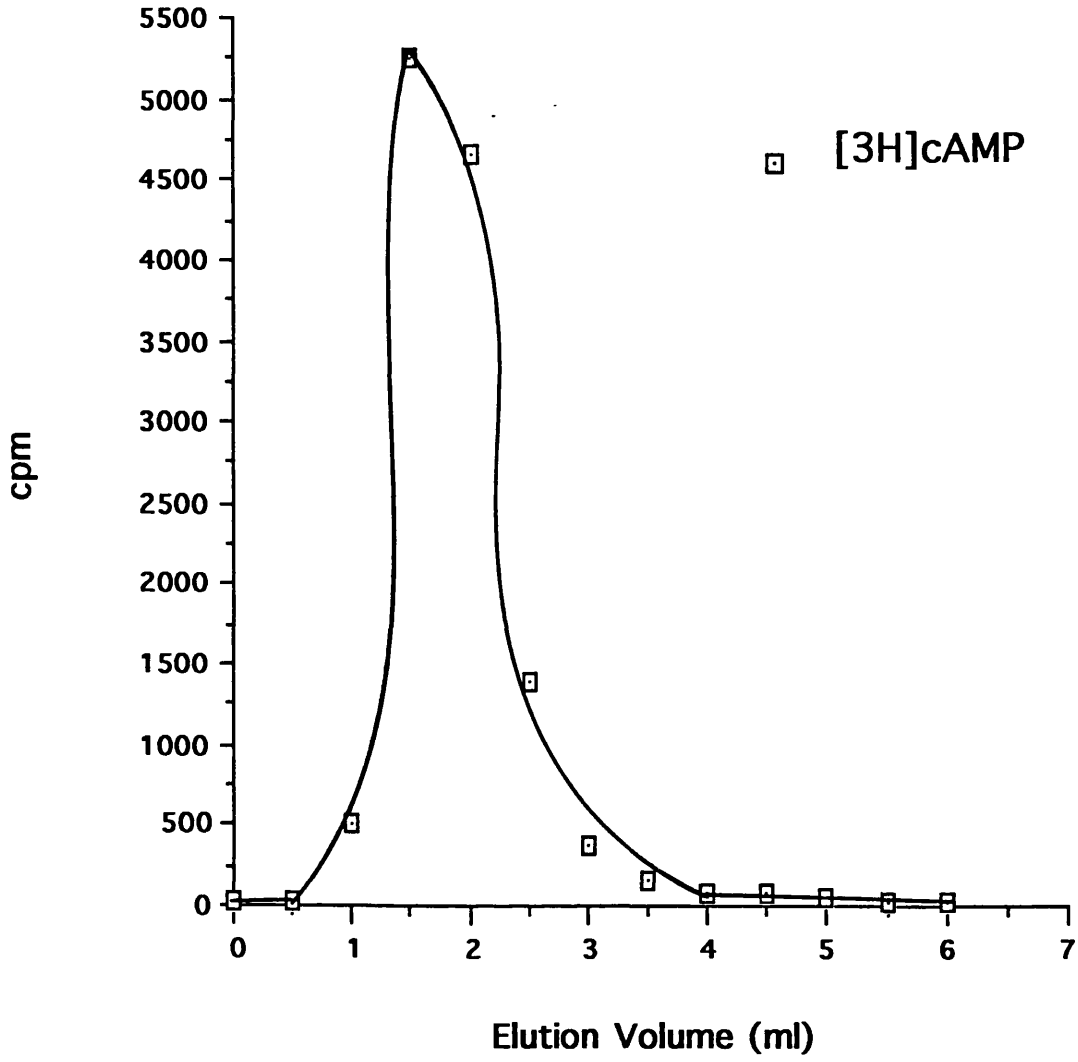

5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
cpm
[3H]cAMP
0
1
2
3
4
5
6
7
Elution Volume (ml)

Figure 2.5b

2.9.1 Resolving gel preparation

Stock Solutions (stored at 4°C):

Solution A	1.5M tris, 0.4% (v/v) SDS, pH 8.8 with HCl
Solution B	0.5M tris, 0.4% (v/v) SDS, pH 6.8 with HCl
Solution C	30% (w/v) acrylamide, 0.8% (w/v) N,N'-methylene-bis-acrylamide
Solution D	50% (v/v) glycerol
Solution E	10% (w/v) ammonium persulphate (made daily)
Solution F	TEMED

Reservoir buffer: 0.025M tris, 0.192M glycine, 0.1% (v/v) SDS.

10% (v/v) acrylamide/0.27% (v/v) N,N'-methylene-bis-acrylamide gels were prepared from the stock solutions as follows:

Solution	Volume (ml)
A	9
C	12
D	2.4
E	0.135
F	0.012
H_2O	12.3

The solution was immediately mixed and poured between two glass plates (200x200mm) with 1.5mm spacers. The gel was layered with 0.1% (v/v) SDS to exclude air, and left to set at room temperature for approximately 2 hours.

2.9.2 Stacking gel preparation

Stacking gels were prepared from the stock solutions as follows:

Solution	Volume (ml)
B	3.75
C	1.5
E	0.15
F	0.008
H_2O	9.7

The solution was immediately mixed, layered on top of the resolving gel and allowed to polymerise around a 10-well teflon comb.

Electrophoresis was performed overnight at 50V at room temperature (Pharmacia power supply).

2.9.3 Sample preparation

Protein precipitation

Samples were prepared for gel electrophoresis by sodium deoxycholate/trichloroacetic acid precipitation. 6.25μl of 2% (w/v) sodium deoxycholate was added to each sample, followed by 750μl of H_2O, and then 250μl of 24% (w/v) trichloroacetic acid. Samples were mixed then centrifuged at 12,000xg for 20 minutes at room temperature, after which time the supernatants were removed and the pellets dissolved in 20μl of 1M tris base followed by 20μl of Laemmli buffer (Laemmli, 1970) which consisted of 5M urea, 0.17M SDS, 0.4M DTT, 0.05M tris/HCl pH 8.0, 0.01% bromphenol blue. The samples were then boiled for 3 minutes (Maizel, 1969), ready for gel electrophoresis.

2.9.4 Gel protein staining

After electrophoresis, the gel was placed in a tray on a stirring table and covered in stain solution which consisted of 0.1% (w/v) coomassie blue in 45% (v/v) H_2O, 45% (v/v) methanol, 10% (v/v) acetic acid (glacial) for 2 hours. The stain solution was removed and replaced with destain solution (identical to stain solution, but lacking coomassie blue) until excess stain had been removed to leave a clear background, and proteins were apparent on the gel as discrete bands.

2.10 Western blotting

Proteins were separated under appropriate resolving conditions on SDS polyacrylamide gels overnight at 50V. The proteins were transferred to a nitrocellulose sheet for 2 hours at 1.5 Amps in an LKB transblot apparatus (Towbin *et al.*, 1979) with blotting buffer which consisted of 0.192M glycine, 0.025M tris, 20% (v/v) methanol. The sheet was then "blocked" for 2 hours at 37°C with 5% (w/v)

"marvel" (Premier Brands Ltd., Stafford, United Kingdom) in tris-buffered saline (TBS) consisting of 0.5M NaCl, 0.02M tris, pH 7.5. The "marvel" was washed off with distilled H_2O, and the nitrocellulose sheet incubated overnight at 37°C with the appropriate primary antiserum in 1% (w/v) gelatin, 0.2% (v/v) nonidet P-40 in phosphate-buffered saline (PBS) consisting of 2.68mM KCl, 1.47mM KH_2PO_4, 137mM NaCl, 8.31mM $Na_2HPO_4.2H_2O$. Next day, the antiserum was removed and the sheet subjected to thorough washing with distilled H_2O, then a 10 minute wash in PBS, 0.2% nonidet P-40. The blot was then incubated for 2 hours at 37°C with a second antibody (horseradish peroxidase-labelled anti-rabbit IgG). This antibody was then removed, and the sheet subjected to 10 minute washes in distilled H_2O then PBS, 0.2% nonidet P-40. The sheet was then washed thoroughly in PBS before being developed in 40ml of 10mM tris, pH 7.5 with 0.025% (w/v) ortho-dianisidine as substrate (Gawler *et al.*, 1987 and Bushfield *et al.*, 1990a). (Both first and second antibodies could be reused for up to four times, and were stored at 4°C using 0.004% (w/v) thimerosal as an anti-bacterial agent.)

The bands on the nitrocellulose sheets were quantified by incubation at 37°C for 2 hours with [^{125}I]-labelled anti-rabbit IgG. The [^{125}I]-IgG was removed and the sheet rinsed with PBS, 0.2% nonidet P-40. The sheet was then washed thoroughly with PBS, dried, and the bands subsequently cut out and counted using an NE 1612 gamma counter. (An alternative (and on occassions an additional) method of quantifying the bands on the nitrocellulose sheets was to subject the dried nitrocellulose sheets to autoradiography. Hyperfilm-MP was pre-exposed to an instantaneous flash of light using a sensitize pre-flash unit. The developed autoradiograph was then analysed by densitometric scanning using a Shimadzu dual-wavelength flying-spot scanner CS-9000 (VA Howe & Co., Banbury, Oxon, United Kingdom).)

2.11 Antibody production

All antisera used were generated against synthetic peptides, essentially as described by Goldsmith and colleagues (1987). Synthetic peptides were obtained from Biomac Ltd., University of Glasgow, Glasgow, Scotland.

3mg of the appropriate peptide and 10mg of keyhole limpet haemocyanin were dissolved slowly in 1ml of 0.1M phosphate buffer,

pH 7.0. 0.5ml of 21mM glutaraldehyde (also in 0.1M phosphate buffer, pH 7.0) was then added dropwise with stirring and the combined 1.5ml incubated overnight at room temperature.

The 1.5ml solution was mixed with an equal volume of complete Freund's adjuvant and briefly sonicated with a Branson 'soniprobe' (Type 7532B). 1ml aliquots of the resulting emulsion were injected in multiple subcutaneous sites in New Zealand white rabbits. Four weeks later, each animal received a booster immunisation with material identically prepared, except one half as much keyhole limpet haemocyanin and peptide were injected in incomplete Freund's adjuvant.

Bleeds were performed monthly with approximately 15ml taken from the ear artery and collected into a glass universal. Blood was left to clot overnight at 4°C and the plasma removed and centrifuged at 1,000xg in a Beckman TJ 6 for 10 minutes to pellet any remnants of the clot. The supernatants so produced were aliquoted into 250μl volumes and stored at -70°C until use.

A range of different antisera were produced in the manner described, as summarised in Table 2.1.

2.12 Antibody purification

Crude antisera were chromatographed on a 1.5x2cm column of protein-A-sepharose 4B. A 5ml volume of antiserum in glycine buffer (1.5M glycine, 3M NaCl, pH 8.9) was added to the column and allowed to equilibrate. The column was washed with glycine buffer until the eluate had an A_{280} of 0.0 and was then eluted with 0.1M citric acid, pH 4.0 into 2M tris/HCl pH 7.5. The eluted IgG fractions were dialysed overnight against 1,000 volumes of 10mM tris/HCl, 0.1mM EDTA, pH 7.5, and lyophilised. Just prior to use, samples were reconstituted to the required dilution with the same buffer.

2.13 Dose-responses for the anti-peptide antisera

Before the anti-peptide antisera were used routinely to quantify the levels of the different G-protein subunits in rat vascular tissue plasma membranes, dose-response curves were constructed for each primary antibody, plotting amount of membrane protein against amount of radioactivity incorporated. From the resulting graphs, a protein concentration within the linear part of the plot was chosen as the value of membrane protein to be loaded on the 10%

Antiserum	Peptide employed	G-protein sequence	Antiserum identifies
CS1	RMHLRQYELL	$G_s\alpha$ 385-394	$G_s\alpha$
SG2	KENLKDCGLF	Transducin α 341-350	Transducin α, $G_i1\alpha$, $G_i2\alpha$
I3C	KNNLKECGLY	$G_i3\alpha$ 345-354	$G_i3\alpha$
BN3	MSELDQLRQE	β1 (1-10)	β
CQ2	QLNLKEYNLV	$G_q\alpha$ 351-360 $G_{11}\alpha$ 350-359	$G_q\alpha$, $G_{11}\alpha$

Table 2.1 Specificities of anti-G-protein antisera.

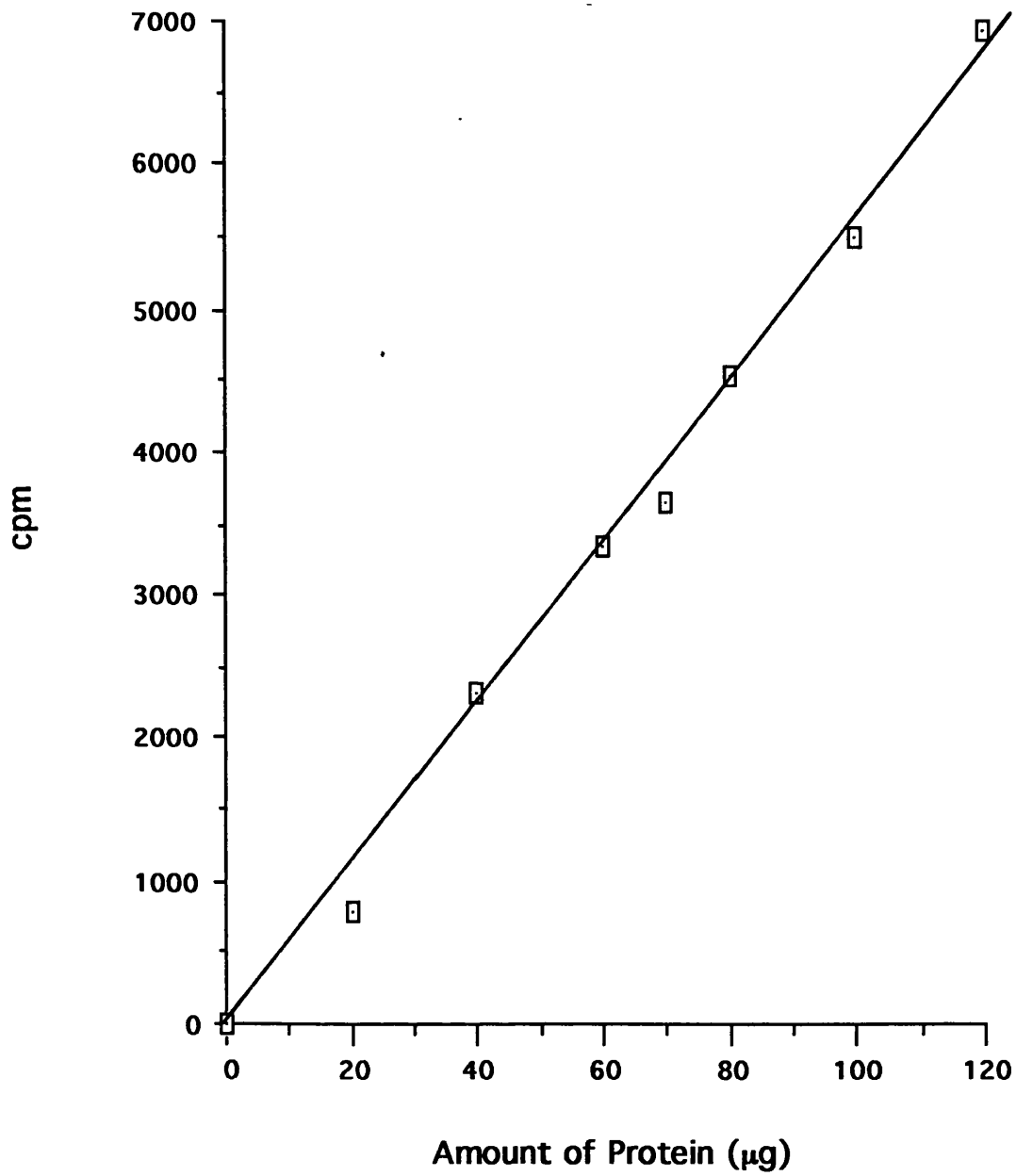
acrylamide/0.27% N,N'-methylene-bis-acrylamide gels. Figure 2.6 shows an example of a dose-response curve constructed using an anti-$G_i2\alpha$ antiserum (SG2) in combination with [^{125}I]-IgG. The amount of membrane protein chosen to be loaded on gels in order to quantify the levels of the G_i2 α-subunit in membranes was 60μg. Table 2.2 lists the results from the dose-response curves constructed for the other anti-peptide antisera.

2.14 Binding assays

Binding of [^{125}I]-cyanopindolol ([^{125}I]CYP) to membranes was performed in assays (250μl) with 15μg of membrane protein in 20mM HEPES, pH 8.0, containing 5mM $MgCl_2$ and 1mM EDTA (buffer A) for 45 minutes at 30°C over which time equilibrium was achieved. [^{125}I]CYP bound to the membranes was collected by vacuum filtration through Whatman GF/C filters, which were washed continually for 32 seconds with ice-cold buffer A. Non-specific binding was determined in parallel assays by the inclusion of 10μM propranolol. Blank values were determined by replacement of membrane protein with buffer. Non-specific binding was subtracted from total binding in order to determine specific binding.

Analysis of the specific binding data was performed using EBDA and LIGAND programmes from a collection of radioligand binding analysis programmes by G.A. McPherson (version 3.0), published and distributed by Elsevier-BIOSOFT, Cambridge, United Kingdom.

Figure 2.6 Dose-response for anti-$G_{i2}\alpha$ antiserum (SG2). The amount of membrane protein to be used in the quantification of G_{i2} α-subunit levels was chosen from the linear portion of the graph plotting protein concentration against radioactivity incorporated.


7000
6000
5000
4000
3000
2000
1000
0
cpm
0
20
40
60
80
100
120
Amount of Protein (µg)

Antiserum	Amount of membrane protein (μg)
CS1	50
SG2	60
I3C	50
BN3	50
CQ2	25

Table 2.2 Results of optimal quantities of membrane protein chosen from dose-responses for anti-peptide antisera.

Chapter 3 Comparison of vascular G-proteins in SHRs and Wistar rats

3.1 Introduction

The enzyme adenylyl cyclase plays an important role in determining vascular tone, generally by counteracting Ca^{2+}-induced vasoconstriction (Tomlinson *et al.*, 1985). Therefore, changes in ACA may be relevant to altered vascular reactivity in hypertension. Altered regulation of adenylyl cyclase has been reported in vascular tissues from the SHR. Reduced ACA in response to β-adrenergic stimulation has been demonstrated in mesenteric vasculature (Amer *et al.*, 1974), aorta (Amer *et al.*, 1974; Amer, 1973; Triner *et al.*, 1975; Bhalla and Sharma, 1982 and Anand-Srivastava, 1988) and myocardium (Amer *et al.*, 1974; Anand-Srivastava, 1988; Bhalla and Ashley, 1978; Bhalla *et al.*, 1980; Sharma *et al.*, 1982; Kumano *et al.*, 1983 and McLellan *et al.*, 1993) of the SHR. Thus, Amer *et al.* (1974) observed a significantly elevated intracellular guanosine 3',5'-cyclic monophosphate (cGMP):cAMP ratio in the aortas and mesenteric arteries from SHRs. This group also noted that ACA in both the aortas and hearts of the hypertensive animals was less sensitive to stimulation by the β-adrenergic stimulant, isoproterenol. Triner *et al.* (1975), using thoracic aortic strips, observed a higher basal ACA in SHRs, which was not significantly different from the value measured in WKY rats. NaF-stimulated ACA in SHRs was significantly higher, while the relaxing effect of isoproterenol at all concentrations on a dose-response curve was markedly smaller, as was the cAMP response. A study performed by Bhalla and Sharma (1982) showed no difference in the basal ACA in the caudal artery of SHRs as compared to WKY rats, but guanine nucleotide-, isoproterenol-, and fluoride-stimulated enzyme activity was significantly lower in SHRs. Anand-Srivastava (1988) studied ACA in the myocardial sarcolemma and aorta of SHRs and WKY controls. Basal enzyme activity was decreased in the SHR as was stimulation by isoproterenol, NaF, forskolin, GTP and p(NH)ppG. These studies reported altered regulation of ACA in SHR vascular tissues (in particular, reduced response to β-adrenergic stimulation), but did not examine the mechanism(s) underlying this abnormality.

In a study examining G_S function in vascular tissue from the SHR, Asano and colleagues (1988) suggested that defects in

relaxation of femoral arterial strips in response to β-adrenoceptor stimulation were due to altered function of a cholera toxin-sensitive G-protein i.e. G_S. Bhalla and co-workers (1980) suggested that sensitivity of the adenylyl cyclase enzyme to isoproterenol in SHR myocardial membrane preparations was decreased. Further studies by this group suggested that ACA and the number of β-adrenergic receptors in rat heart decrease with age and that in hypertension, a specific decrease in isoproterenol stimulation of cyclase appears at all stages of development. Kumano *et al.* (1983), also studying myocardial membranes, observed that ACA was unchanged in SHRs compared to WKY rats measured under basal conditions or following stimulation by NaF, a GTP analogue, $MnCl_2$, or forskolin. However, isoproterenol-stimulated cyclase activity in SHRs was decreased as was β-adrenergic receptor density, with no change in receptor affinity. A study by McLellan *et al.* (1993) assessed whether changes in G-protein expression and functioning were responsible for reduced β-adrenergic agonist-stimulated ACA in SHR myocardium. Similar experiments were performed in non-vascular tissue (renal cortex) to investigate whether any observed changes were part of a more widely distributed membrane defect. A reduced ACA in response to forskolin, NaF, PGE_1, glucagon and isoproterenol was observed in SHR myocardium compared with WKY. However, with the exception of forskolin-stimulated activity, which was greater in SHRs than in Wistar rats, SHR myocardial ACAs were similar to those in Wistar rat membranes. Immunoblotting studies showed similar levels of G-protein subunits ($G_S\alpha$, $G_i1\alpha$, $G_i2\alpha$, $G_i3\alpha$, $G_O\alpha$ and β) in all three strains. Studies of renal cortical plasma membranes failed to identify any differences in ACA or in G-protein subunit levels. Hence, SHRs, WKY rats and Wistar rats exhibited differences in myocardial ACA which were not seen in renal cortical plasma membranes. It was suggested that these differences were not related to differences in G-protein levels, and probably reflected strain differences in G_S function. Michel *et al.* (1993) also examined whether alterations in cardiac G-protein number or function, or both, were involved in the desensitisation of adenylyl cyclase responsiveness in the SHR. The amounts of immunodetectable $G_S\alpha$, $G_i\alpha$, $G_q\alpha$ and β-subunit did not differ significantly in SHR and WKY myocardial membranes. The function of $G_S\alpha$ was quantified by reconstitution of cardiac cholate extracts into cyc^- cell membranes with subsequent measurement of NaF-stimulated ACA. It was

observed that G_S function did not differ significantly in myocardial membranes from SHRs and WKY rats. As reduced stimulation of ACA in vascular smooth muscle might lead to altered vascular responsiveness to vasodilator ligands, these findings may be important in the pathogenesis of hypertension.

Reduced β-adrenergic stimulation in SHR vascular tissues may not be a consequence of just altered G_S levels/function and/or β-adrenoceptor number/affinity. In addition, abnormalities in the levels and/or function of G_i may exist. Reduced function of G_i has been reported in platelets from the SHR. Coquil and Brunelle (1989) compared the effects of p(NH)ppG on ACA of platelet membranes in SHRs and WKY rats. In the presence of forskolin, low concentrations of p(NH)ppG inhibited the enzyme activity in both strains, but the maximal level of inhibition was significantly lower in SHRs. The significance of this, and its relevance to vascular tissue remain unclear.

The SHR (and human essential hypertension) share a number of common pathophysiological features with diabetes mellitus, including resistance to the action of insulin and hyperinsulinaemia, and it is of interest that abnormal expression and function of G_i has also been reported in human non-insulin dependent diabetes mellitus and in animal models of diabetes. Livingstone *et al.* (1991) compared ACA and G-protein levels in platelets from normal and non-insulin dependent diabetic male subjects. Whilst no differences were noted in basal and NaF-stimulated ACA, the degree of stimulation achieved by both forskolin and PGE_1, was lower in diabetic subjects. Lower α_2-adrenoceptor-mediated inhibition of PGE_1-stimulated ACA in platelet membranes from diabetic subjects was evident. Whilst platelets of diabetic subjects had levels of $G_S\alpha$ which were comparable to those found in control subjects, their levels of $G_i2\alpha$ and $G_i3\alpha$ were reduced. Gawler and colleagues (1987) showed that experimentally-induced Type I diabetes led to the loss of expression of $G_i\alpha$ in rat liver. Strassheim *et al.* (1990) noted that adipocyte membranes from rats made diabetic with streptozotocin had their GTP-dependent, but not receptor-dependent inhibitory function of G_i on ACA abolished. This group also observed that induction of diabetes did not cause any change in the levels of $G_i1\alpha$, $G_i2\alpha$ and $G_S\alpha$ in adipocyte membranes, but elicited an increase in the level of $G_i3\alpha$. The induction of diabetes reduced the specific activity of adenylyl cyclase in adipocyte membranes and enhanced the stimulatory effect of isoproterenol. Further studies by Bushfield *et*

al. (1990a) using hepatocyte membranes from streptozotocin-diabetic animals showed markedly decreased levels of $G_{i}2\alpha$, $G_{i}3\alpha$ and the 42kDa, but not the 45kDa, form of $G_{s}\alpha$ compared with controls. In hepatocyte membranes, immunoblot analysis showed that levels of the catalytic unit of adenylyl cyclase were increased upon induction of diabetes. Under basal conditions, it was noted that $G_{i}2$ from hepatocytes of diabetic animals was both phosphorylated to a greater extent and resistant to any further phosphorylation. Low concentrations of p(NH)ppG inhibited ACA in hepatocyte membranes from normal, but not diabetic, animals. These studies reported altered regulation of ACA in human diabetes mellitus and animal models of the condition, with significant alterations in G_{i} levels and function.

It was, therefore, of interest to examine whether altered G-protein levels or function in VSM membranes from hypertensive animals might account for the abnormal regulation of adenylyl cyclase in response to β-adrenergic stimulation. It was also of interest to examine whether changes in G_{i} function, similar to those observed in human diabetes mellitus and animal models of the condition, might be present in vascular tissue from the SHR. G-protein levels and function were studied using plasma membranes prepared from mesenteric arteries. A similar set of experiments were performed using membranes prepared from vascular smooth muscle cells isolated from mesenteric arteries, to investigate whether any abnormalities in the regulation of ACA and/or G-protein levels/function, noted in mesenteric artery membranes, persisted in vascular tissue cultured out of its physiological environment.

The outbred Wistar strain, rather than Wistar-Kyoto rats, was chosen as the control group in this study, in view of evidence that the WKY rats obtainable from most commercial suppliers display marked genetic heterogeneity. Kurtz and Morris (1987) obtained WKY rats from two of the largest commercial suppliers in the United States and systematically measured the growth rate and blood pressure of these rats under identical physical and metabolic conditions. They found that WKY rats from one source differed from those of the other in both growth rate and blood pressure. In particular, DNA fingerprinting on genomic DNA of WKY rats showed evidence of genetic variability not only among WKY rats from two different breeding facilities, but also among WKY rats within a single breeding facility (Kurtz *et al.*, 1989 and Samani *et al.*, 1989). SHR and WKY strains from one source only shared approximately 50%

of their DNA fingerprint bands in common, providing evidence of extensive genetic polymorphism between these strains (St. Lezin *et al.*, 1992). Johnson *et al.* (1992) estimated the amount of genetic divergence between the SHR and WKY strains using a method of restriction fragment length polymorphism analysis. They found an extremely high amount of divergence, given the known origin of these two strains, which is comparable to the maximum divergence possible between unrelated humans.

3.2 Materials and Methods

3.2.1 Rats

Spontaneously hypertensive rats and Wistar rats were purchased from Charles River Ltd., Margate, Kent, United Kingdom. The rats were male and were sacrificed at 11 weeks of age. Prior to sacrifice, the rats had their systolic blood pressures and weights measured (see section 2.2 for details): SHR 208±2.7mmHg; Wistar 144±2.5mmHg, p=0.001 and SHR 258±1.9g; Wistar 286±2.7g, p=0.001, respectively. Measurements were made from ten rats of each strain and significance was assessed by *t*-test analysis.

3.2.2 Smooth muscle cell preparation and maintenance

VSMC were isolated by enzymic digestion from mesenteric arteries according to the procedure outlined in section 2.5.1. The cells were grown in culture through four passages by following the methods of sections 2.5.2 and 2.5.3.

3.2.3 Preparation of membranes

Production of plasma membranes from mesenteric arteries and VSMC is outlined in section 2.6.

3.2.4 Measurement of protein concentration

Protein concentrations in the membrane preparations were determined using the method outlined in section 2.7.

3.2.5 Adenylyl cyclase activity

The regulation of adenylyl cyclase activity was monitored by the method of Salomon (1979) outlined in sections 2.8.1 and 2.8.4.

3.2.6 Quantification of G-protein subunits

The levels of the G-protein subunits were quantified using a combination of SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (see section 2.9 for details) and immunoblotting with specific anti-peptide antisera (see section 2.10 for details).

3.3 Results

3.3.1 Regulation of adenylyl cyclase activity in mesenteric artery membranes

Adenylyl cyclase activity assays were performed using mesenteric artery plasma membranes isolated from SHRs and Wistar rats. The basal level of ACA in SHR membranes was significantly higher than that measured in Wistar: SHR 690±73 pmol cAMP/15min/mg; Wistar 445±52, p=0.0181 (Figure 3.1). In the presence of $MnCl_2$ ($2x10^{-2}M$), a higher ACA was again observed in SHR membranes; however, it was not significantly different from the value measured in Wistar membranes: SHR 717±109 pmol cAMP/15min/mg; Wistar 502±48 (Figure 3.1). (A high concentration of Mn^{2+} ions uncouples adenylyl cyclase from its regulatory G-proteins (Limbird *et al.*, 1979).)

The results of regulation of adenylyl cyclase have been expressed as % change from basal activity (measured in the absence and presence of Mn^{2+} ions) due to the differences observed in basal ACA, in the absence and presence of Mn^{2+} ions, in SHR and Wistar membranes. Figures 3.2(a) and 3.2(b) show the % change in ACA expressed relative to basal activity measured in the absence of Mn^{2+} ions in SHR and Wistar mesenteric artery membranes after the addition of forskolin ($10^{-4}M$), NaF ($10^{-2}M$) and isoproterenol ($10^{-4}M$), PGE_1 ($10^{-5}M$), respectively. There were significant differences in the extents of stimulation caused by the addition of NaF (SHR 324±35%; Wistar 782±87%, p=0.0019), isoproterenol (SHR 11±8%; Wistar 48±11%, p=0.0356) and PGE_1 (SHR 19±10%; Wistar 77±16%, p=0.0053), with a decreased response in SHR membranes. The response to forskolin was decreased in SHR membranes

Figure 3.1 Adenylyl cyclase activity in SHR and Wistar mesenteric artery membranes measured in the absence and presence of $MnCl_2$ ($2x10^{-2}M$). Data are mean±S.E. for eight experiments using different membrane preparations.

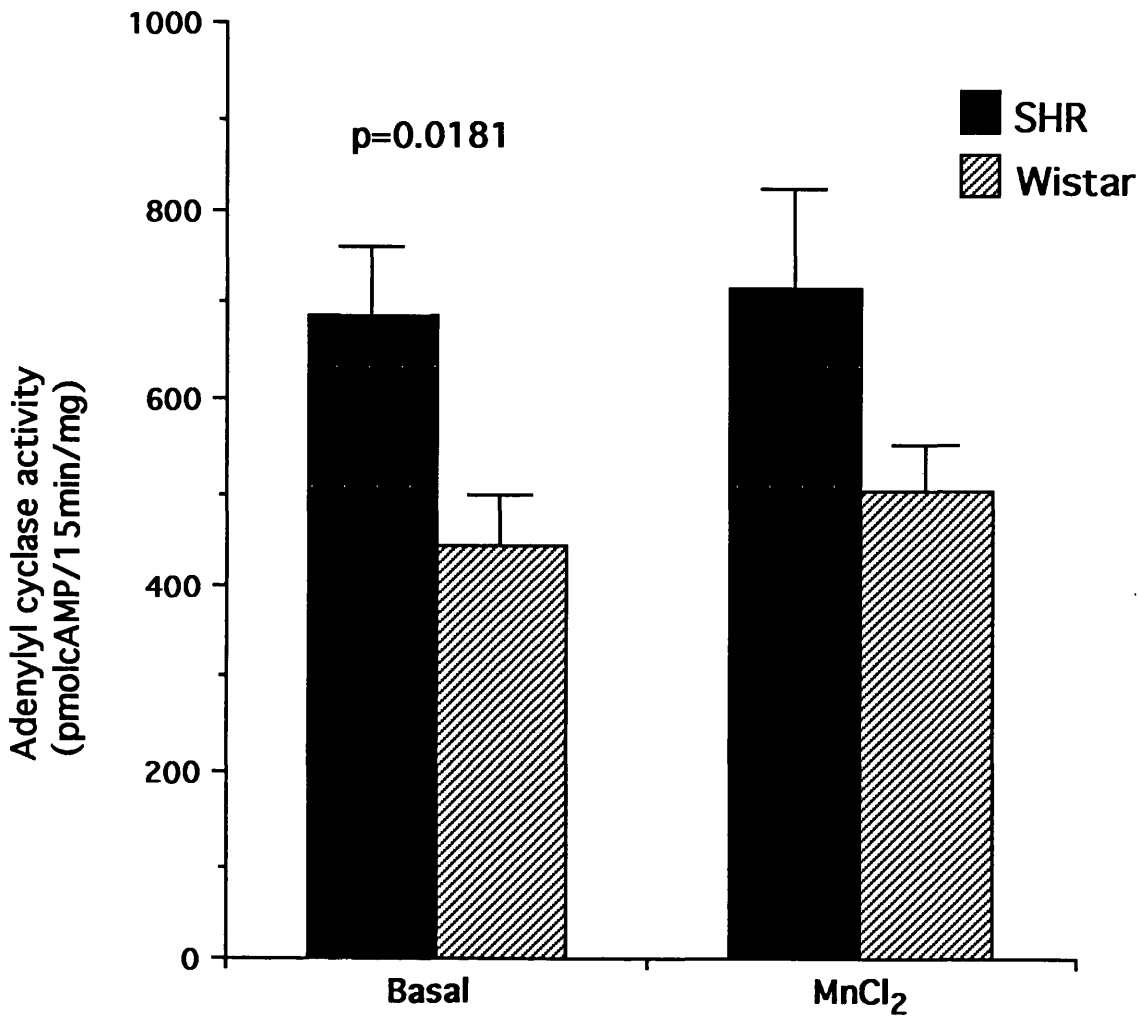

1000
800
600
400
200
0
p=0.0181
SHR
Wistar
Adenylyl cyclase activity
(pmolcAMP/15min/mg)
Basal
MnCl2

Figure 3.2 % Change in ACA expressed relative to basal activity measured in the absence of $MnCl_2$ in SHR and Wistar mesenteric artery membranes in the presence of (a) forskolin (10^{-4}M) and NaF (10^{-2}M) and (b) isoproterenol (10^{-4}M) and PGE_1 (10^{-5}M). Data are mean±S.E. for eight experiments using different membrane preparations.

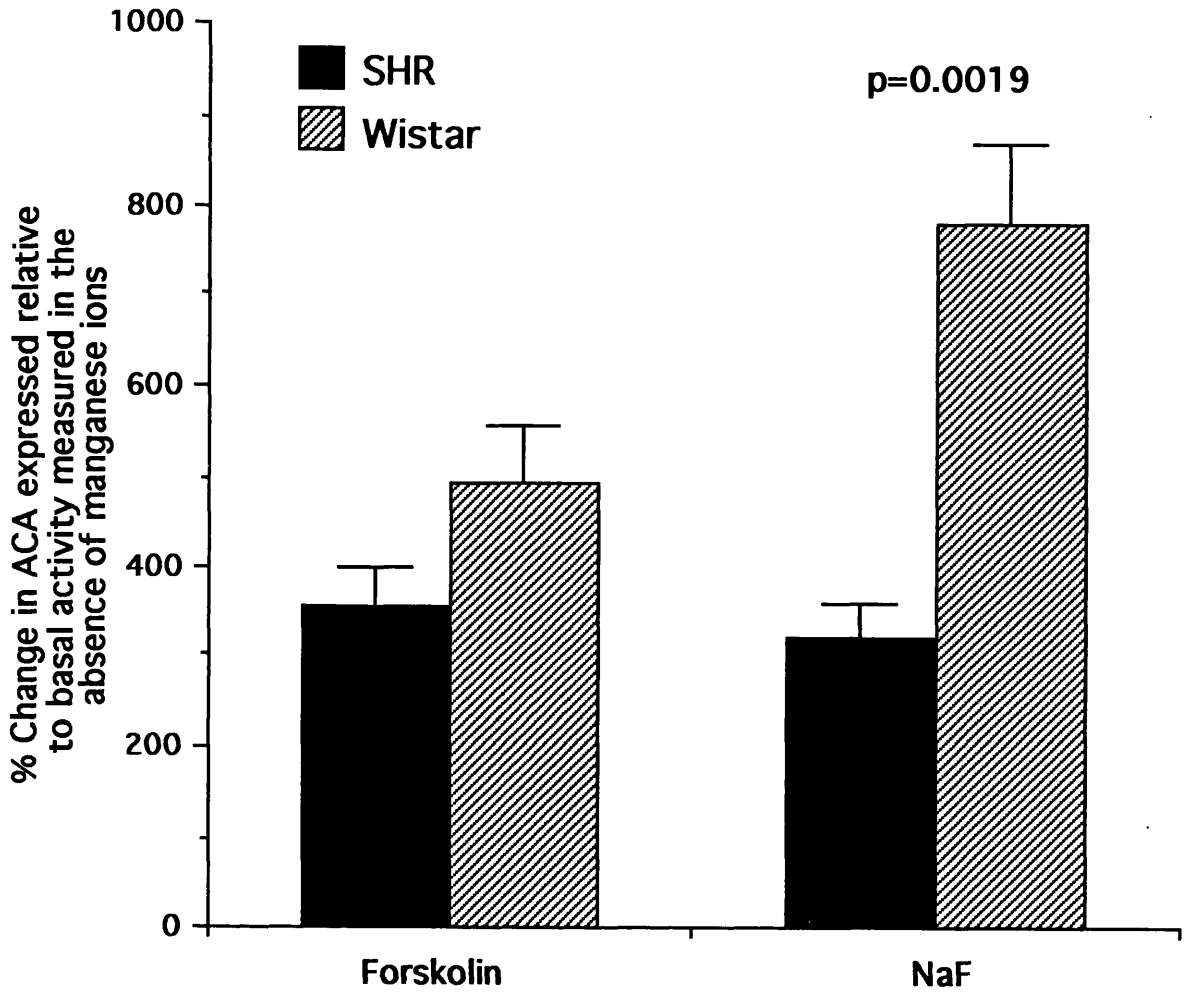

1000
800
600
400
200
0
SHR
Wistar
p=0.0019
% Change in ACA expressed relative to basal activity measured in the absence of manganese ions
Forskolin
NaF

Figure 3.2(a)

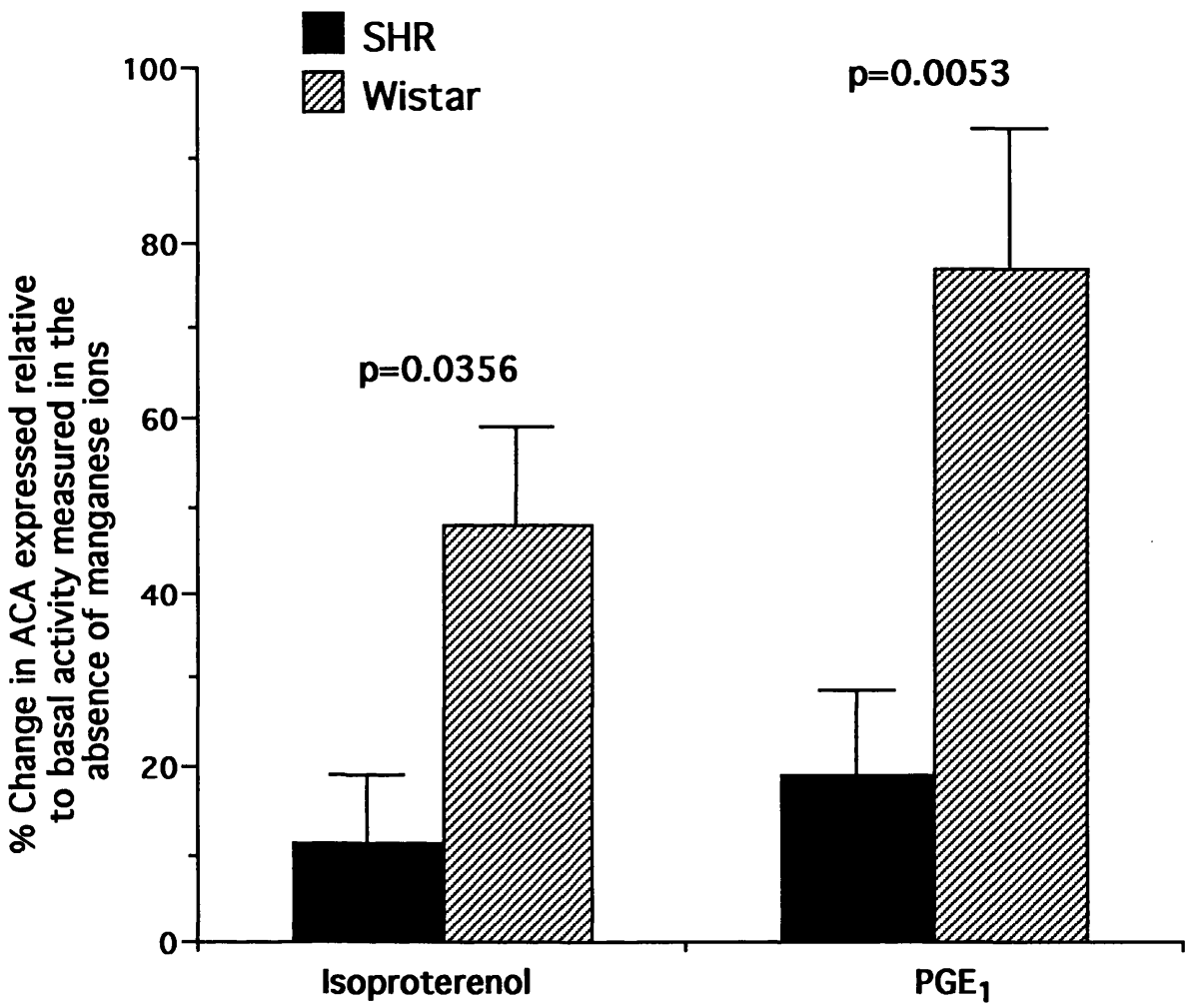

SHR
Wistar
p=0.0053
p=0.0356
100
80
60
40
20
0
% Change in ACA expressed relative to basal activity measured in the absence of manganese ions
Isoproterenol
PGE1

Figure 3.2(b)

compared to Wistar, but the difference was not statistically significant (SHR 358±41%; Wistar 493±63%).

The overall results were not altered by expressing data relative to basal activity measured in the presence of $MnCl_2$ ($2x10^{-2}M$). Figures 3.3(a) and 3.3(b) show the effects on ACA in response to forskolin ($10^{-4}M$), NaF ($10^{-2}M$) and isoproterenol ($10^{-4}M$), PGE_1 ($10^{-5}M$), respectively. There were significant differences in the extents of stimulation in the presence of NaF (SHR 335±53%; Wistar 662±64%, p=0.0039) and PGE_1 (SHR 16±6%; Wistar 52±8%, p=0.0086), with a decreased response in SHR membranes. The responses to forskolin and isoproterenol were reduced in SHR membranes compared to Wistar, but the differences were not statistically significant (forskolin: SHR 349±24%; Wistar 423±57% and isoproterenol: SHR 10±7%; Wistar 28±8%).

Variable inhibition was seen with forskolin-stimulated ACA in SHR and Wistar membranes in response to a range of ligands (adrenaline ($10^{-5}M$)/propranolol ($10^{-5}M$) (this combination being equivalent to an α_2-adrenoceptor agonist), carbachol ($10^{-3}M$) and 5-hydroxytryptamine ($10^{-5}M$)), but overall no significant differences were seen (adrenaline/propranolol: SHR -8±3%; Wistar -0.4±10%, carbachol: SHR -17±4%; Wistar -4±14% and 5-hydroxytryptamine: SHR -20±5%; Wistar -4±10%). The extents of inhibition in both sets of membranes, in response to these ligands, were not increased in the presence of isoproterenol (data not shown).

3.3.2 Regulation of adenylyl cyclase activity in VSMC membranes

Adenylyl cyclase activity assays were performed using VSMC membranes isolated from the mesenteric arteries of SHRs and Wistar rats. The basal level of ACA in SHR membranes was significantly higher than that measured in Wistar membranes: SHR 11965±1259 pmol cAMP/15min/mg; Wistar 2618±276, p=0.0304.

The results of regulation of adenylyl cyclase have been expressed as % change from basal activity measured in the absence of Mn^{2+} ions. Figure 3.4 shows the responses of SHR and Wistar VSMC membranes in the presence of forskolin ($10^{-4}M$), NaF ($10^{-2}M$) and isoproterenol ($10^{-4}M$). There were significant differences in the extents of stimulation caused by the addition of forskolin (SHR 689±52%; Wistar 1050±59%, p=0.0304), NaF (SHR 285±6%; Wistar

Figure 3.3 % Change in ACA expressed relative to basal activity measured in the presence of $MnCl_2$ ($2x10^{-2}M$) in SHR and Wistar mesenteric artery membranes in the presence of (a) forskolin ($10^{-4}M$) and NaF ($10^{-2}M$) and (b) isoproterenol ($10^{-4}M$) and PGE_1 ($10^{-5}M$). Data are mean±S.E. for eight experiments using different membrane preparations.

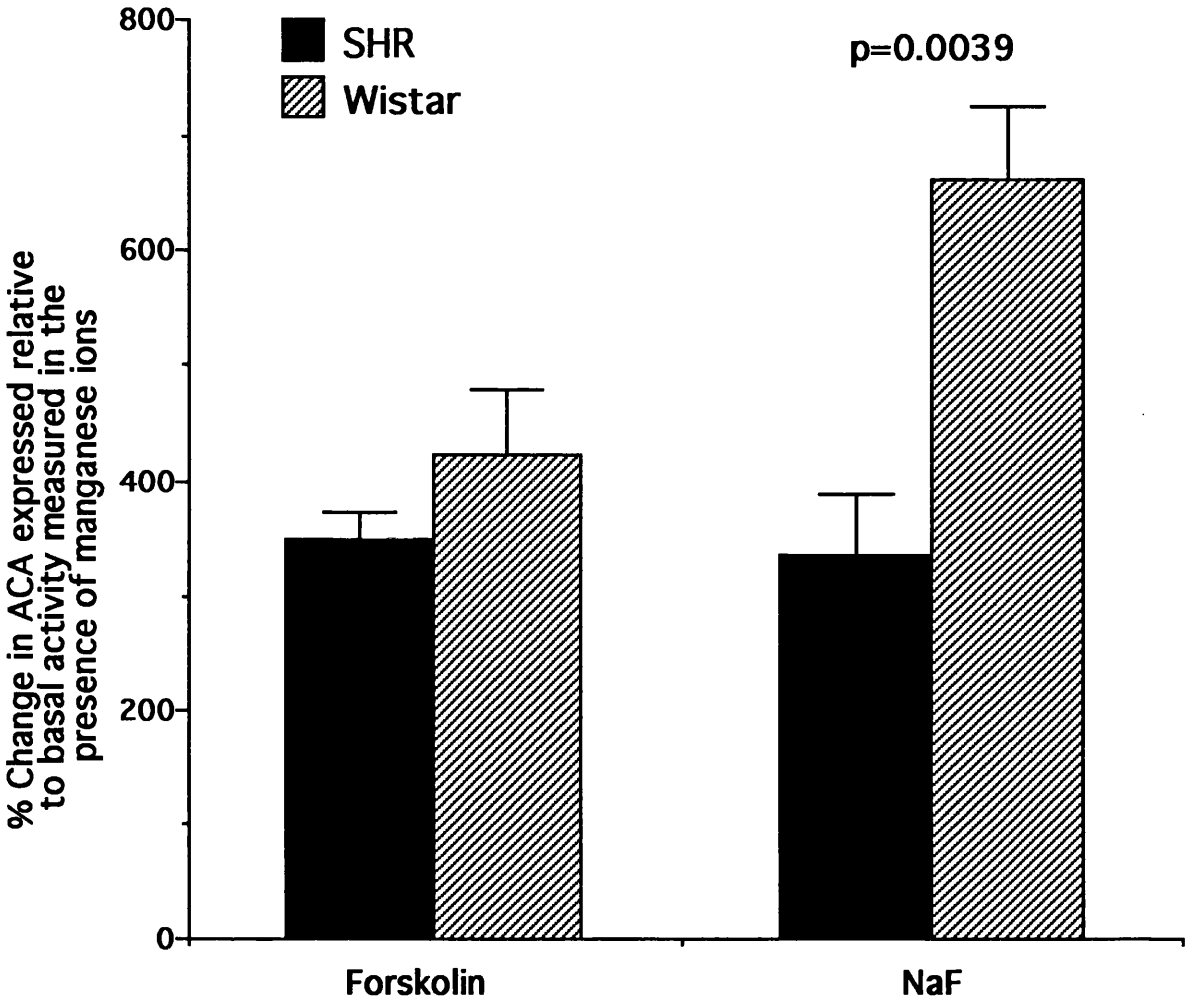

800
600
400
200
0
SHR
Wistar
p=0.0039
% Change in ACA expressed relative to basal activity measured in the presence of manganese ions
Forskolin
NaF

Figure 3.3(a)

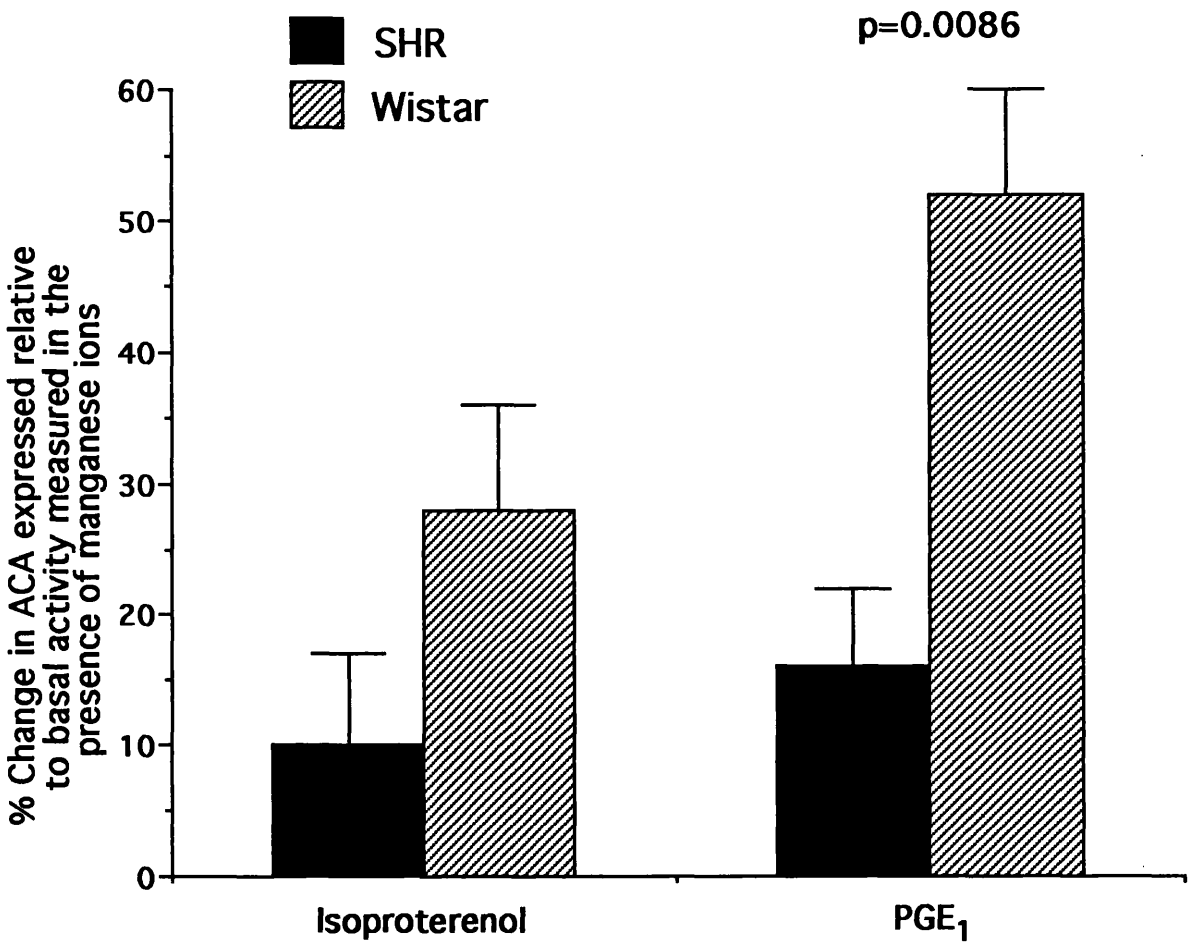
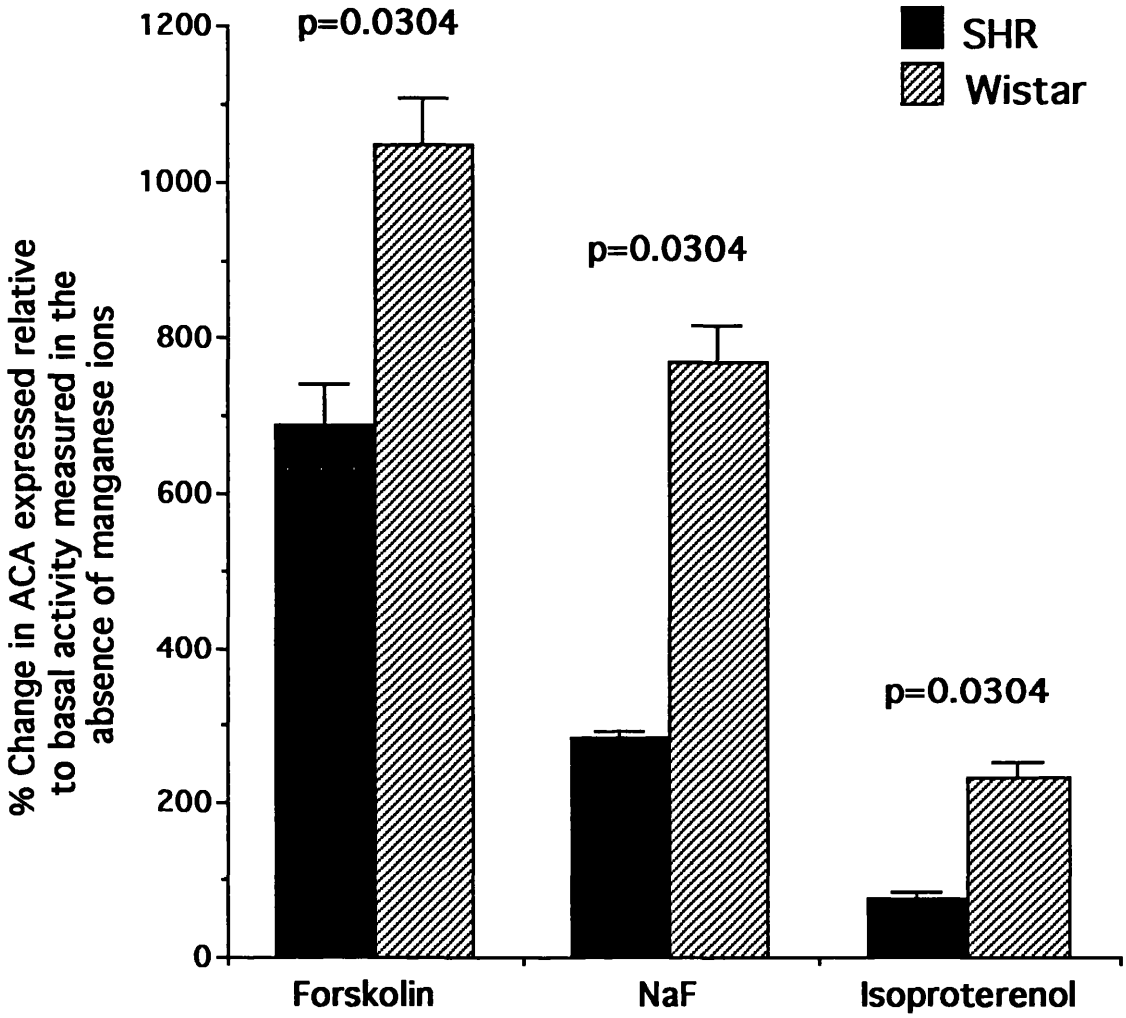

SHR
Wistar
p=0.0086
% Change in ACA expressed relative to basal activity measured in the presence of manganese ions
60
50
40
30
20
10
0
Isoproterenol
PGE1

Figure 3.3(b)

Figure 3.4 % Change in ACA expressed relative to basal activity measured in the absence of $MnCl_2$ in SHR and Wistar VSMC membranes in the presence of forskolin (10^{-4}M), NaF (10^{-2}M) and isoproterenol (10^{-4}M). Data are mean±S.E. for eight experiments using different membrane preparations.


p=0.0304
SHR
Wistar
1200
1000
800
600
400
200
0
% Change in ACA expressed relative to basal activity measured in the absence of manganese ions
p=0.0304
p=0.0304
Forskolin
NaF
Isoproterenol

769±49%, p=0.0304) and isoproterenol (SHR 76±7%; Wistar 230±23%, p=0.0304), with a decreased response in SHR membranes.

The inhibitions of forskolin-stimulated ACA in response to a combination of adrenaline (10^{-5}M) and propranolol (10^{-5}M) in SHR and Wistar membranes were measured. Similar extents of inhibition were observed in both sets of membranes: SHR -9±3%; Wistar -11±7%.

3.3.3 Levels of G-protein subunits in mesenteric artery membranes

Preliminary immunoblotting experiments identified $G_S\alpha$ (two forms; 44 and 42kDa), $G_i2\alpha$, $G_i3\alpha$ and the β-subunit in rat VSM plasma membranes, whereas $G_O\alpha$ and $G_i1\alpha$ were absent. The levels of these different G-protein α-subunits and the β-subunit were compared in SHR and Wistar mesenteric artery membranes using specific anti-peptide antisera, as described in Table 2.1 and section 2.13. Figures 3.5, 3.6, 3.7 and 3.8 are photographs of nitrocellulose sheets probed with CS1, SG2, I3C and BN3 antisera, respectively. The primary antibodies were detected by horseradish peroxidase-labelled anti-rabbit IgG and colour development with ortho-dianisidine as substrate. The bands on the nitrocellulose sheets were quantified with [^{125}I]-labelled anti-rabbit IgG (Table 3.1). There were no significant differences in the levels of the different G-protein subunits present in SHR and Wistar mesenteric artery membranes.

3.3.4 Levels of G-protein subunits in VSMC membranes

The levels of $G_S\alpha$ (both forms; 44 and 42kDa), $G_i2\alpha$, $G_i3\alpha$ and the β-subunit were quantified in SHR and Wistar VSMC membranes. As in mesenteric artery membranes, there were no significant differences in the levels of the different G-protein subunits present in SHR and Wistar VSMC membranes (results not shown).

3.4 Discussion

3.4.1 Reduced stimulation of adenylyl cyclase activity via G_S and receptors in SHRs

A significant reduction in the stimulation of ACA in VSM and VSMC from the SHR, compared with Wistar control, has been observed. The

Figure 3.5 Nitrocellulose sheet with SHR and Wistar mesenteric artery membranes probed with $G_{s}\alpha$-specific Ab (CS1). 50μg of membrane protein was loaded in each lane. Two forms of $G_{s}\alpha$ are apparent (44 and 42kDa), with the 44kDa form being predominant in VSM. 1-3=Wistar membranes, 4-6=SHR membranes and 7=human platelet membrane.

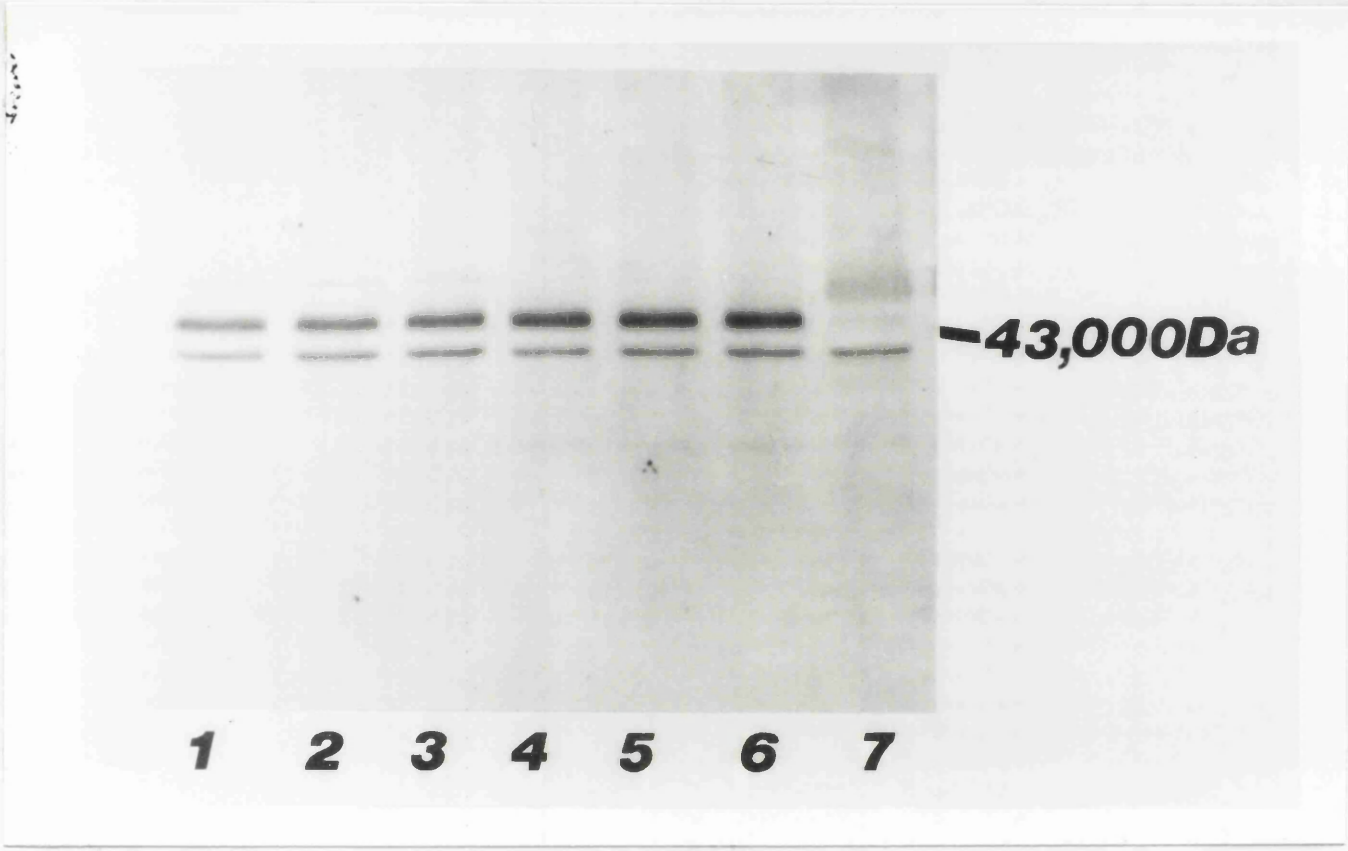

43,000Da
1
2
3
4
5
6
7

Figure 3.6 Nitrocellulose sheet with SHR and Wistar mesenteric artery membranes probed with transducin α, $G_i1\alpha$, $G_i2\alpha$-specific Ab (SG2). Since rat VSM does not express detectable levels of $G_i1\alpha$ and transducin distribution is restricted to photoreceptor-containing tissues, the antiserum SG2 will only identify $G_i2\alpha$ in rat VSM. 60μg of membrane protein was loaded in each lane. 1-3=Wistar membranes, 4-6=SHR membranes and 7=human platelet membrane.

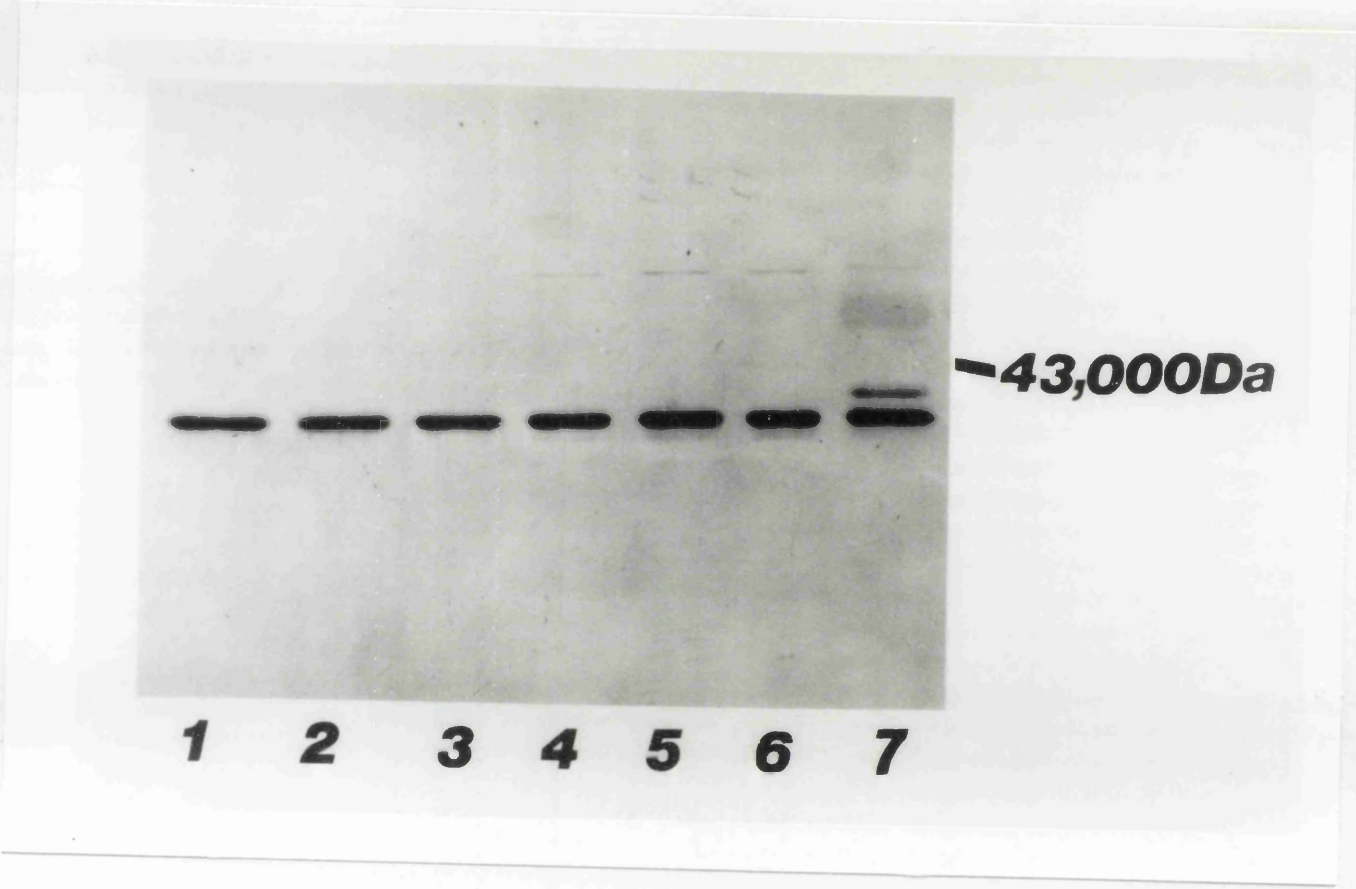
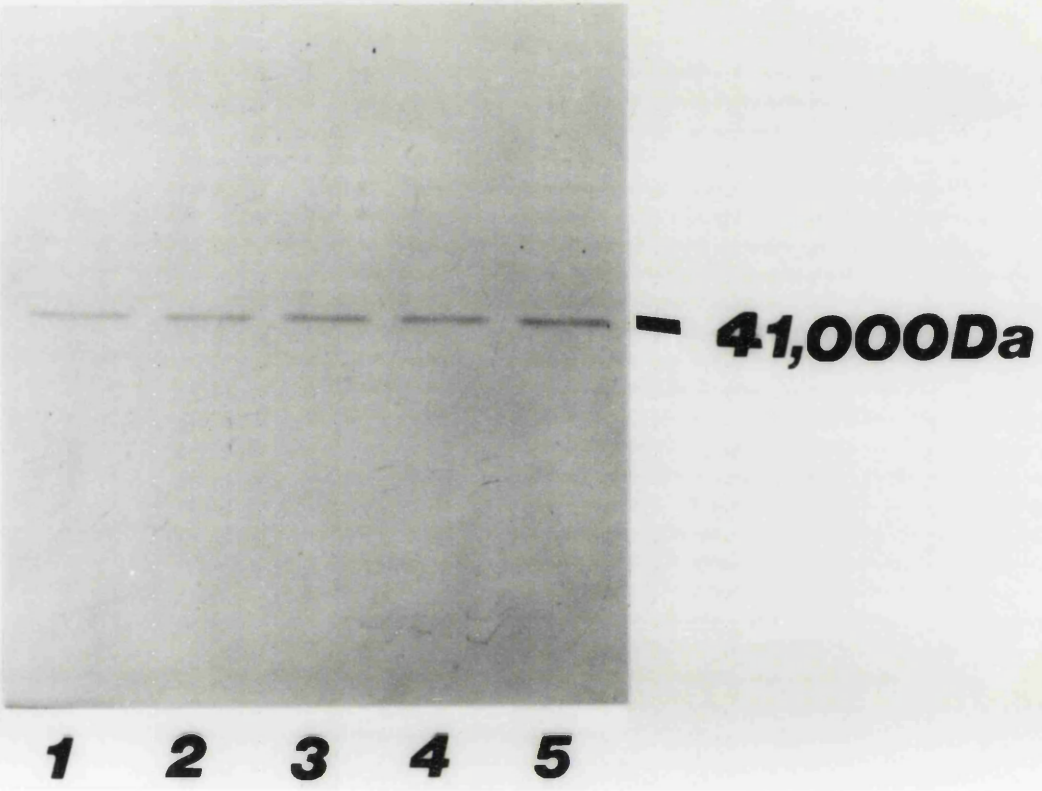
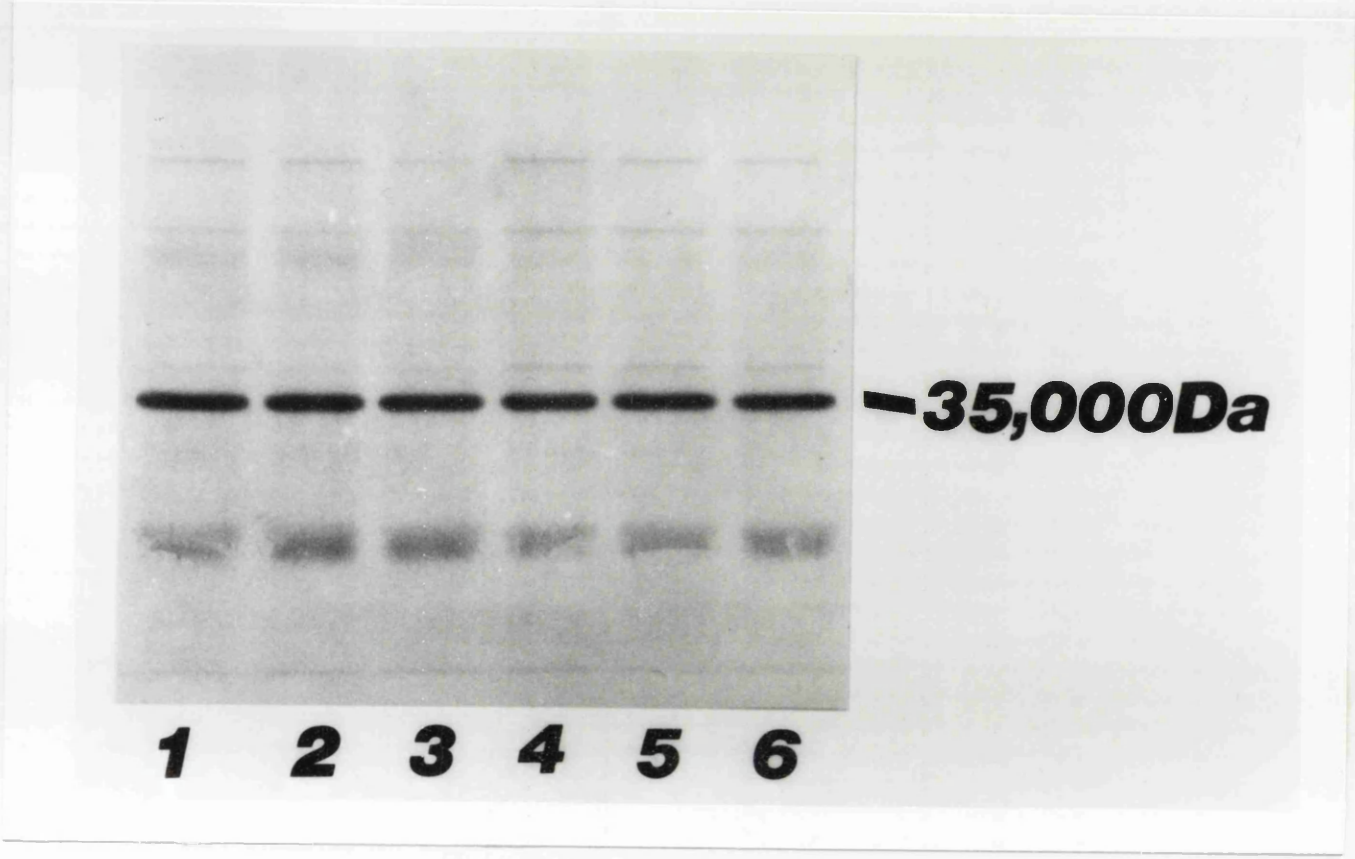

43,000Da
1
2
3
4
5
6
7

Figure 3.7 Nitrocellulose sheet with SHR and Wistar mesenteric artery membranes probed with $G_i3\alpha$-specific Ab (I3C). 50μg of membrane protein was loaded in each lane. 1-3=Wistar membranes and 4&5=SHR membranes.


41,000Da
1
2
3
4
5

Ficure 3.8 Nitrocellulose sheet with SHR and Wistar mesenteric artery membranes probed with β-subunit-specific Ab (BN3). 50μg of membrane protein was loaded in each lane. 1-3=SHR membranes and 4-5=Wistar membranes.


35,000Da
1
2
3
4
5
6

G-protein subunit	SHR (counts/minute)	Wistar (counts/minute)
$G_{s\alpha}$ (44kDa)	3221±126	3180±232
$G_{s\alpha}$ (42kDa)	1819±95	1928±58
$G_i2\alpha$	3254±377	3795±237
$G_i3\alpha$	717±44	662±44
β	1517±75	1439±91

Table 3.1 Quantification of bands on nitrocellulose sheets with SHR and Wistar mesenteric artery membranes using [^{125}I]-labelled Ab. Data represent mean±S.E. for four experiments using different membrane preparations. Statistical analysis of results did not disclose any significant differences.

overall results examining G_s and G_i levels and function were similar in membranes made directly from the mesenteric artery and in membranes prepared from VSMC isolated from mesenteric arteries. The increased basal ACA measured in SHR mesenteric artery membranes was also observed in membranes prepared from VSMC isolated from SHR mesenteric arteries. Thus, culturing vascular tissue did not perturb the regulation of ACA nor G-protein levels/function. The reduced adenylyl cyclase response to stimulation which has been demonstrated was seen with two different ligands, which activate the enzyme via receptors coupled to G_s, namely isoproterenol and PGE_1. Other investigators (Triner *et al.*, 1975) have reported difficulties in achieving hormonal stimulation of adenylyl cyclase in broken cell preparations of vascular smooth muscle, possibly reflecting physical disruption of the linkages among receptor/G-protein/adenylyl cyclase in such preparations. Indeed, relatively slight stimulatory responses to isoproterenol and PGE_1 were observed in this study. However, the responses were significantly reduced in SHR membranes and the abnormality was also noted with an agent, NaF, which activates adenylyl cyclase via receptor independent activation of G_s. The decreased adenylyl cyclase response in VSM does not appear to be a consequence of a direct change in the catalytic subunit itself: forskolin, which directly activates the catalytic subunit independently of receptors, produced responses in SHR and Wistar membranes which were not significantly different. Additionally, the decreased responsiveness in SHR membranes was also seen when the data were expressed as % change relative to basal activity measured in the presence of $MnCl_2$ (which uncouples adenylyl cyclase from its regulatory G-proteins, and may represent a more realistic basal measurement (Limbird *et al.*, 1979)). It seems, therefore, that there is a true decrease in responsiveness of adenylyl cyclase to stimulatory agents in VSM membranes from the SHR, which is not accounted for by an intrinsic change in the basal activity of the enzyme.

3.4.2 G_i levels and function

These data are consistent with other studies which have examined ACA in vascular tissues from SHRs and control rats (see section 3.1 for details). These previous studies did not, however, examine the underlying reason for the reduced adenylyl cyclase response in the

SHR. This study has examined whether this altered responsiveness could be explained by abnormalities in the amount or function of either G_S or G_i. No evidence of altered inhibition of ACA in response to a number of ligands, which act via G_i (including studies where G_i was activated in the presence of partly stimulated ACA (with isoproterenol)), was found, suggesting that the function of this G-protein was normal. Similarly, $G_i2\alpha$- and $G_i3\alpha$-subunit levels were not altered in SHR membranes compared with Wistar (rat VSM does not express detectable levels of $G_i1\alpha$). The functional significance of G_i sub-types has yet to be established, although there is mounting evidence which suggests that at least G_i2 can serve to inhibit adenylyl cyclase (Bushfield *et al.*, 1990b; McKenzie and Milligan, 1990; Simonds *et al.*, 1989a and Senogles *et al.*, 1990). There is evidence (Pobiner *et al.*, 1991) which suggests that G_i3 can elicit inhibition of ACA via angiotensin II receptors in rat hepatocytes. These data contrast with findings from studies on SHR platelet membranes reported by Coquil and Brunelle (1989), who noted that G_i function, as measured by activation with low concentrations of p(NH)ppG, was significantly reduced. They suggested that this could account for the increased cAMP response noted in platelets from hypertensive rats in response to PGE_1. However, the relevance of platelet studies in hypertensive animals is uncertain, and no direct measurement was made of G_S function in that study.

3.4.3 Evidence of increased G_i levels in SHRs compared with WKY rats

Anand-Srivastava *et al.* (1991) measured the levels of G-proteins in aorta and heart from SHRs and WKY rats. The level of G_i was significantly increased in the hearts and aortas from SHRs, while the level of G_S was similar in SHRs and controls. Stimulatory responses to epinephrine, isoproterenol, dopamine and forskolin were diminished in SHR hearts and aortas as compared to WKY (Anand-Srivastava, 1988). This group suggested that an increased level of G_i in SHRs may be associated with the decreased ACA in response to stimulatory hormones. It was suggested that the increased level of G_i may be one of the mechanisms responsible for the diminished vascular tone and impaired myocardial functions in hypertension. Anand-Srivastava *et al.* (1991) observed significantly higher mRNA levels of $G_i2\alpha$ and $G_i3\alpha$ in hearts and aortas from SHRs

as compared to WKY, however, the mRNA levels of G_S were not different in SHRs from WKY, indicating increased expression of genes of $G_i2\alpha$ and $G_i3\alpha$ and not of G_S in heart and aorta from SHRs. The level of $G_i\alpha$ was observed by Bohm *et al.* (1992) to be increased in membranes of hypertrophic hearts from SHRs without heart failure. In addition, myocardial β-adrenoceptors were reduced in SHRs. These studies used the WKY strain of rat as a normotensive control; however, genetic heterogeneity has been reported in this strain of rat (see 3.1 for details), highlighting concerns about the suitability of WKY rats as a normotensive control group in studies with the SHR.

3.4.4 G_S levels and function

Immunoblotting studies did not show any alterations in the levels of the two G_S α-subunits within plasma membranes from the SHR, excluding a contribution from altered G_S levels to the changes in ACA. Levels of the β-subunit were not different in membranes from SHRs and Wistar rats. Although there are data which suggest that $\beta\gamma$-subunit complexes can contribute to regulation of ACA (Federman *et al.*, 1992), this does not account for the altered ACA in SHRs. Instead, these data may support an abnormality of G_S function. Asano *et al.* (1988) suggested that defects in relaxation of femoral arterial strips, in response to β-adrenoceptor stimulation, were due to altered function of a cholera toxin-sensitive G-protein i.e. G_S. Work by Murakami and colleagues (1987) studying the β-adrenergic receptor-adenylyl cyclase system of cardiac membranes in SHRs suggested a reduction in the activity of G_S. Stimulation of ACA by either forskolin or purified G_S was higher in SHRs; however, stimulations of ACA by isoproterenol and GTP were similar in SHRs and controls. Although there was no difference in the amount of G_S in SHR membranes, the functional activity of G_S in cholate-extracted membranes from SHRs was significantly lower than that from control rats. There were no strain differences in the number and affinity of β-adrenergic receptors, the function and amount of G_i, and the amount of $\beta\gamma$-subunits. These findings would, therefore, tend to support the data presented here.

3.4.5 Role of β-adrenergic receptors in decreased stimulation of adenylyl cyclase activity

The SHR is a model of hypertension which shows reduced sensitivity to insulin (insulin resistance), a finding which is also present in human essential hypertension. In a study of another animal model which displays insulin resistance (the diabetic (db/db) mouse), a decreased adenylyl cyclase response to isoproterenol was noted in adipose tissue (Strassheim *et al.*, 1991). In that study, there was no change in the level of G_S, although a marked increase in the level of G_i1 α-subunit was demonstrated in adipocyte membranes from the db/db animals compared with their normal, lean littermates. However, a study by Begin-Heick (1992) observed a decreased level of $G_S\alpha$ (46kDa form) in adipocyte plasma membranes of db/db mice compared with homozygous controls, but there was no significant difference in the level of the 42kDa form of $G_S\alpha$. There were also reduced levels of $G_i1\alpha$ and $G_i2\alpha$ in the db mutant. G_i1 α-subunit is not, however, expressed in rat VSM, and it is not believed to have a role in the modulation of ACA. The apparent explanation for the decreased adenylyl cyclase response in the db/db mouse appears to be a reduction in β-adrenoceptor number. Cardiac membranes from the obese Zucker rat, which is not diabetic, but nevertheless profoundly insulin-resistant and hypertensive, demonstrated reduced β-adrenergic stimulation of adenylyl cyclase in comparison to lean controls (Strassheim *et al.*, 1992). The heart tissue membranes from obese Zucker rats showed both reduced β-adrenergic receptor number and altered coupling between β-adrenergic receptors and G_S. However, no alterations in either the level of G_S or the functional interaction between G_S and the catalytic moiety of adenylyl cyclase were observed. Although β-adrenoceptor number was not measured in this study, other investigators have failed to agree on observed changes in β-adrenoceptor number and affinity in SHR vascular tissues (Bhalla *et al.*, 1980 and Kumano *et al.*, 1983). However, these data demonstrate a significantly decreased adenylyl cyclase response to NaF, which activates G_S independently of receptors, and support the notion that the decreased ACA observed in the SHR is due to altered G_S function or coupling rather than altered receptor number.

3.4.6 Physiological relevance of reduced stimulation of adenylyl cyclase activity in SHRs

The reason for the apparent abnormality of G_S function is unclear, and the significance of reduced adenylyl cyclase response to stimulatory agents in SHR vascular membranes also remains to be determined. If the higher basal ACA found in SHR VSM membranes were part of a compensatory mechanism to reduce the elevated blood pressure, a higher response to isoproterenol would be expected in SHRs. However, since the response to isoproterenol was decreased in SHRs, the more likely interpretation is that the higher basal ACA in SHRs may be a compensatory mechanism for the deficient capability of the enzyme to respond to β-adrenergic stimulation. On the other hand, ACA, under physiological conditions, is unlikely to exist in the basal state, as it would be continually regulated (stimulated and inhibited) by circulating hormones. Therefore, measurement of basal ACA in this study is probably not representative of the *in vivo* situation. Indeed, the reduced responsiveness of adenylyl cyclase to β-adrenergic stimulation may be a more representative reflection of the true tissue adenylyl cyclase situation. It is unclear whether the reduced responsiveness to β-adrenergic stimulation is a primary abnormality in the SHR or secondary to some other event. Studies in other hypertensive models and in animals at an earlier stage in the development of hypertension are appropriate in order to investigate this further. Chapters 5 and 6 study G-protein function and levels in the Milan hypertensive strain compared with the Milan normotensive strain in adult and young rats, respectively.

Chapter 4 Comparison of vascular G-proteins in SHRs, Wistar rats, and WKY rats

4.1 Introduction

As has been discussed in section 3.1, altered regulation of ACA has been well documented in vascular tissue from the SHR (Amer *et al.*, 1974; Amer, 1973; Triner *et al.*, 1975; Bhalla and Sharma, 1982; Anand-Srivastava, 1988; Bhalla and Ashley, 1978; Bhalla *et al.*, 1980; Sharma *et al.*, 1982 and Kumano *et al.*, 1983), and may contribute to increased vascular reactivity. These studies used WKY rats as the normotensive control group. However, as mentioned previously, genetic heterogeneity has been reported in these rats from different commercial suppliers, and indeed, in rats from the same supplier (Kurtz and Morris, 1987 and Kurtz *et al.*, 1989). These reports highlight the difficulties in finding a suitable control group for studies with the SHR. Indeed, genetic heterogeneity has also been reported in SHRs from different sources (Nabika *et al.*, 1991). Hence, difficulties may arise when results from studies using rats from different suppliers are compared i.e. the differences in the genome of SHRs and WKY rats from different suppliers may vary, and these differences may interfere with the overall results of the study.

To address these points, G-protein levels and function were studied in vascular tissue from the SHR and also Wistar and WKY rats. Two normotensive control groups were included in these experiments to examine whether the differences between SHRs and WKY rats were also seen when a different normotensive control was used. Furthermore, the three strains of rat in this study were purchased from a different supplier to the one that supplied the rats for the study detailed in Chapter 3. Hence, the results from similar studies using rats from different sources could be compared.

Abnormalities of phospholipase C (PLC) activation have been reported in vascular and non-vascular tissues from the SHR (Millanvoye *et al.*, 1988; Kato and Takenawa, 1987 and Remmal *et al.*, 1987). The enzyme PLC alters the intracellular concentrations of the second messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DG), and is linked to cell surface receptors by a G-protein named G_p (Cockcroft and Gomperts, 1985). Recent evidence has indicated that this polypeptide is a member of the G_q/G_{11} subfamily of G-proteins (Gutowski *et al.*, 1991). Millanvoye

et al. (1988) have reported higher PLC activity in response to angiotensin II in SHR aortic smooth muscle cells compared with WKY. Increased activity of PLC has also been noted in erythrocyte ghosts from the SHR compared with WKY (Kato and Takenawa, 1987), while experiments involving thrombin stimulation of lipid metabolism by Remmal *et al.* (1987) suggested enhanced PLC activity in SHR platelets compared with WKY. It was, therefore, of interest to examine whether these reported increased PLC activities in the SHR reflected increased levels of G_q/G_{11}.

4.2 Materials and Methods

4.2.1 Rats

Spontaneously hypertensive, Wistar and Wistar-Kyoto rats were purchased from Harlan Olac Ltd., Oxon, United Kingdom. The rats were male and were sacrificed at 11 weeks of age. Prior to sacrifice, the rats had their systolic blood pressures and weights measured (see section 2.2 for details): SHR 202±9mmHg; Wistar 137±4; WKY 130±6 ($p=0.001$ for Wistar vs. SHR and WKY vs. SHR) and SHR 205±5g; Wistar 270±2; WKY 212±8 ($p=0.001$ for SHR vs. Wistar and WKY vs. Wistar), respectively. Measurements were made from ten rats of each strain and significance was assessed by *t*-test analysis.

4.2.2 Smooth muscle cell preparation and maintenance

VSMC were isolated by enzymic digestion from mesenteric arteries according to the procedure outlined in section 2.5.1. The cells were grown in culture through four passages by following the methods of sections 2.5.2 and 2.5.3.

4.2.3 Preparation of membranes

Production of plasma membranes from mesenteric arteries and VSMC is outlined in section 2.6.

4.2.4 Measurement of protein concentration

Protein concentrations in the membrane preparations were determined using the method outlined in section 2.7.

4.2.5 Adenylyl cyclase activity

The regulation of adenylyl cyclase activity was monitored by the method of Salomon (1979) outlined in sections 2.8.1 and 2.8.4.

4.2.6 Quantification of G-protein subunits

The levels of the G-protein subunits were quantified using a combination of SDS-PAGE (see section 2.9 for details) and immunoblotting with specific anti-peptide antisera (see section 2.10 for details).

4.3 Results

4.3.1 Regulation of adenylyl cyclase activity in VSMC membranes

Basal ACA (whether measured in absence/presence of Mn^{2+} ions) was similar in SHR and WKY membranes, but both were significantly lower than basal ACA measured in Wistar membranes (Figure 4.1). Table 4.1 shows that the addition of Mn^{2+} ions resulted in an inhibition of ACA in SHR, WKY and Wistar membranes. A high concentration of Mn^{2+} ions uncouples adenylyl cyclase from its regulatory G-proteins (Limbird *et al.*, 1979). In these VSMC, there appears to be a much greater measurable input from G_S than G_i, hence uncoupling these G-proteins would probably tend towards inhibition of ACA. A similar trend in activities was seen in the presence of forskolin (10^{-4}M), which activates the catalytic unit of adenylyl cyclase directly (Figure 4.1). In view of these inherent differences in enzyme activity, the responses to the various agents studied were expressed as % change over basal (measured in absence/presence of Mn^{2+} ions).

(i) Stimulatory effects expressed relative to basal activity measured in the absence of $MnCl_2$

Figure 4.2 shows the % change in ACA expressed relative to basal activity measured in the absence of Mn^{2+} ions in SHR, Wistar and WKY VSMC membranes after the addition of forskolin (10^{-4}M), NaF (10^{-2}M), isoproterenol (10^{-4}M) and PGE_1 (10^{-5}M). The response to forskolin was similar in Wistar and WKY membranes; however, a

Figure 4.1 Adenylyl cyclase activity in SHR, Wistar and WKY VSMC membranes measured in the absence and presence of $MnCl_2$ ($2x10^{-2}M$), and in the presence of forskolin ($10^{-4}M$). Data are mean±S.E. for seven (SHR and Wistar) and eight (WKY) experiments using different membrane preparations.

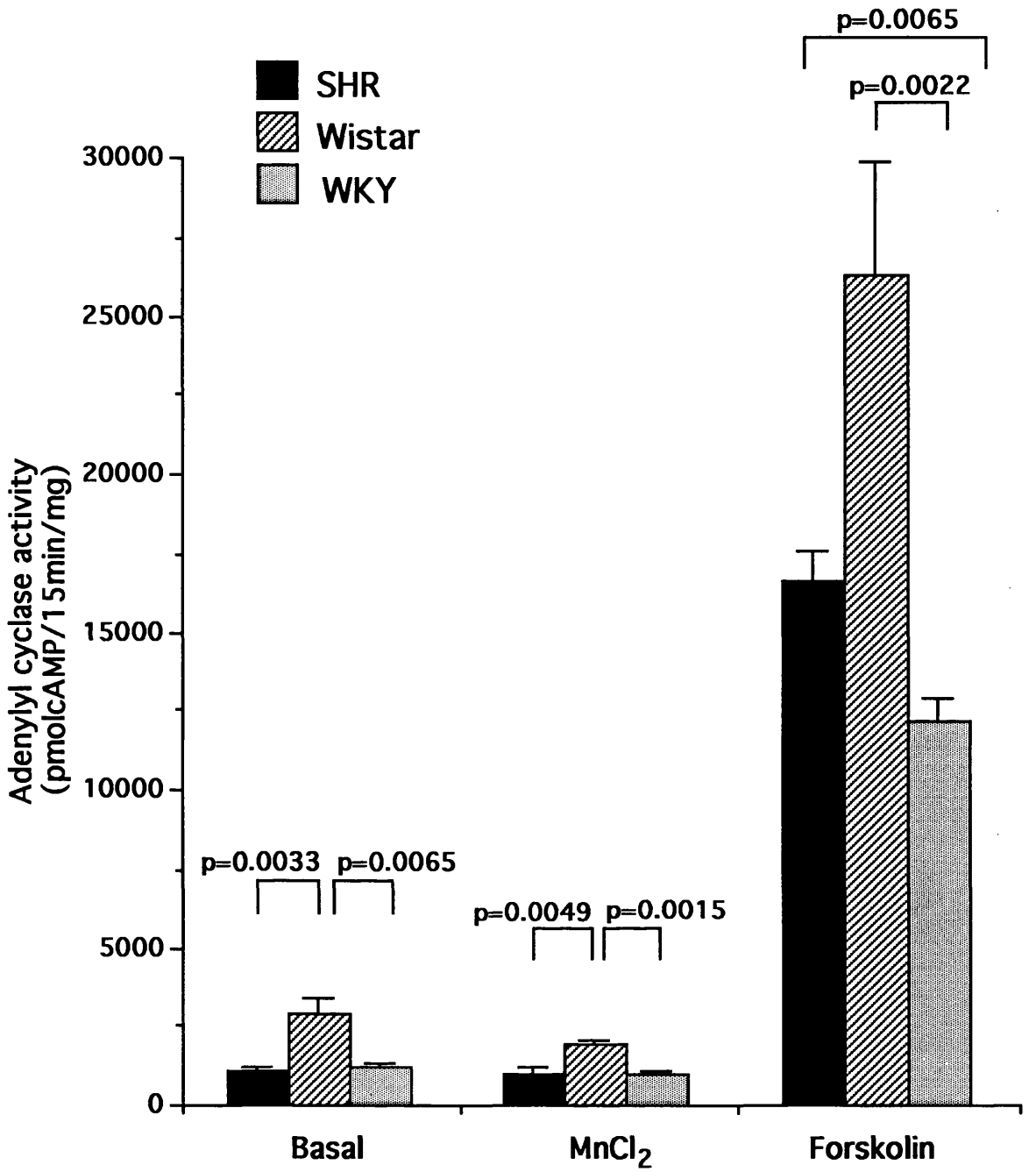
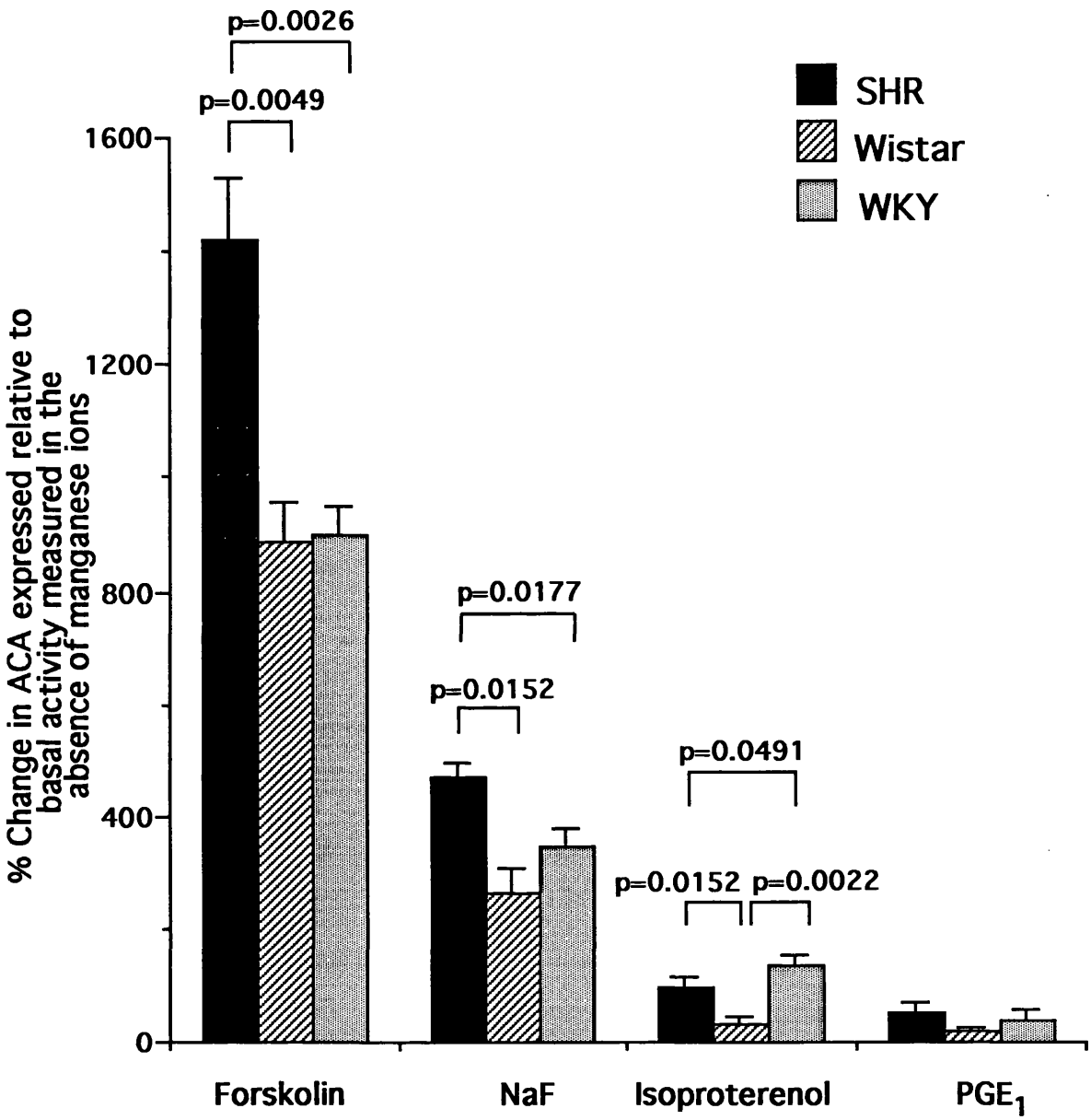

p=0.0065
p=0.0022
SHR
Wistar
WKY
30000
25000
20000
15000
10000
5000
0
Adenylyl cyclase activity
(pmolcAMP/15min/mg)
p=0.0033
p=0.0065
p=0.0049
p=0.0015
Basal
MnCl2
Forskolin

Figure 4.2 % Change in ACA expressed relative to basal activity measured in the absence of $MnCl_2$ in SHR, Wistar and WKY VSMC membranes in the presence of forskolin (10^{-4}M), NaF (10^{-2}M), isoproterenol (10^{-4}M) and PGE_1 (10^{-5}M). Data are mean±S.E. for seven (SHR and Wistar) and eight (WKY) experiments using different membrane preparations.


p=0.0026
p=0.0049
SHR
Wistar
WKY
1600
1200
800
400
0
% Change in ACA expressed relative to basal activity measured in the absence of manganese ions
p=0.0177
p=0.0152
p=0.0491
p=0.0152
p=0.0022
Forskolin
NaF
Isoproterenol
PGE1

	SHR	Wistar	WKY
Basal (no Mn^{2+} ions)	1107±48*	2837±522	1227±66*
Basal (+ Mn^{2+} ions)	1024±131*	1986±119	998±86*

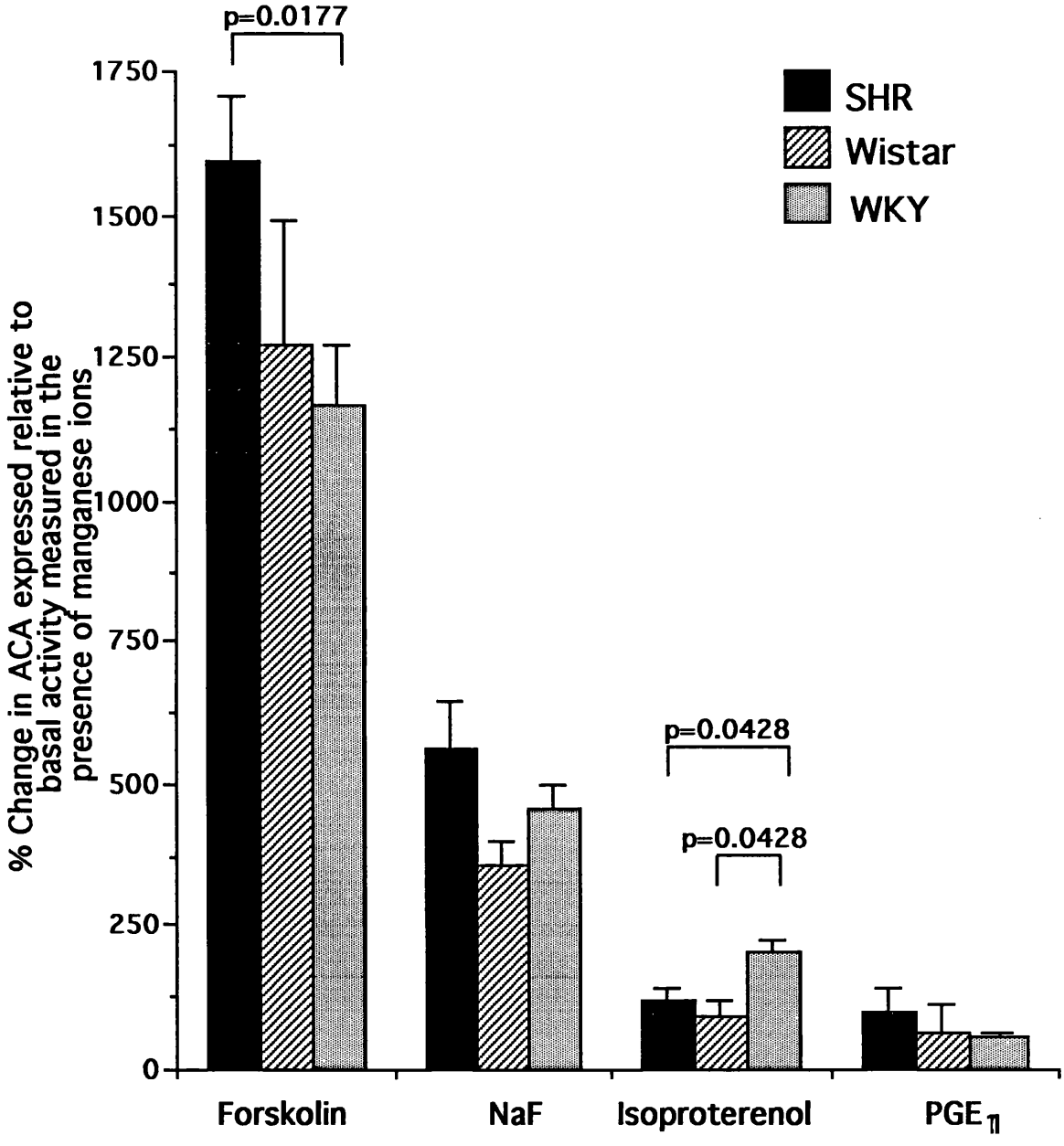
Table 4.1 Basal adenylyl cyclase activities in the absence and presence of $MnCl_2$ ($2x10^{-2}M$) in SHR, Wistar and WKY VSMC membranes. Adenylyl cyclase activity measurements represent pmol cAMP/15min/mg. Data represent mean±S.E. for seven (SHR and Wistar) and eight (WKY) experiments using different membrane preparations. *Statistically significant when compared with Wistar results (see Figure 4.1 for p values).

significantly increased stimulatory response was observed in SHR membranes compared with both normotensive controls (SHR 1418±111%; Wistar 886±69%; WKY 900±52%). Another receptor-independent stimulatory agent, NaF, also caused significantly greater stimulation in SHR membranes compared with both Wistar and WKY, while responses to NaF were similar in both control groups (SHR 472±24%; Wistar 260±49%; WKY 346±33%). Use of the β-adrenergic agonist, isoproterenol, and PGE_1 allowed measurement of receptor-dependent stimulation of ACA via two different types of receptors. The stimulatory effects in response to isoproterenol were significantly different in all three sets of membranes. The responses in SHR and Wistar membranes were significantly reduced compared to WKY, while the SHR response was significantly increased compared with Wistar (SHR 96±17%; Wistar 34±9%; WKY 138±15%). The stimulatory responses measured on the addition of PGE_1 were not significantly different in the three sets of membranes (SHR 52±18%; Wistar 16±10%; WKY 40±17%).

(ii) Stimulatory effects expressed relative to basal activity measured in the presence of $MnCl_2$

Figure 4.3 shows the % change in ACA expressed relative to basal activity measured in the presence of $MnCl_2$ ($2x10^{-2}M$) in SHR, Wistar and WKY VSMC membranes after the addition of forskolin ($10^{-4}M$), NaF ($10^{-2}M$), isoproterenol ($10^{-4}M$) and PGE_1 ($10^{-5}M$). The response to forskolin was similar in Wistar and WKY membranes; however, an increased stimulatory response was observed in SHR membranes compared with both normotensive controls (statistically significant in the case of WKY only) (SHR 1593±116%; Wistar 1271±219%; WKY 1169±104%). NaF was responsible for an increased stimulatory response in SHR membranes; however, this was not significantly different from the responses measured in the two control groups (SHR 565±80%; Wistar 356±45%; WKY 458±38%). Isoproterenol was responsible for similar stimulatory responses in SHR and Wistar membranes; however, both these responses were significantly reduced compared with WKY (SHR 121±22%; Wistar 89±32%; WKY 201±25%). The stimulatory responses measured on the addition of PGE_1 were not significantly different in the three sets of membranes (SHR 96±42%; Wistar 67±45%; WKY 59±5%).

Figure 4.3 % Change in ACA expressed relative to basal activity measured in the presence of $MnCl_2$ ($2x10^{-2}M$) in SHR, Wistar and WKY VSMC membranes in the presence of forskolin ($10^{-4}M$), NaF ($10^{-2}M$), isoproterenol ($10^{-4}M$) and PGE_1 ($10^{-5}M$). Data are mean±S.E. for seven (SHR and Wistar) and eight (WKY) experiments using different membrane preparations.


p=0.0177
1750
1500
1250
1000
750
500
250
0
% Change in ACA expressed relative to basal activity measured in the presence of manganese ions
SHR
Wistar
WKY
p=0.0428
p=0.0428
Forskolin
NaF
Isoproterenol
PGE1

(iii) Inhibition of isoproterenol-stimulated adenylyl cyclase activity

G_i function in SHR, Wistar and WKY membranes was demonstrated by the biphasic effects of GTP on isoproterenol-stimulated ACA (Figure 4.4). These GTP dose-effect experiments showed that activation of G_S and G_i occurred over similar concentration ranges of GTP in SHR, WKY and Wistar membranes. The extents of inhibition were measured from the differences in adenylyl cyclase activities at GTP concentrations of $5x10^{-5}M$ and $10^{-4}M$. G_i function was found to be similar in the three sets of membranes (SHR -5.6±1.5%; Wistar -9.2±2.3%; WKY -9.3±4.7%). However, although the GTP response in SHR and WKY membranes was similar, it was significantly reduced in comparison to the response measured in Wistar membranes. (The GTP response was significantly reduced in SHR membranes compared with Wistar at a GTP concentration as low as $10^{-7}M$, while the difference in GTP responses in WKY and Wistar membranes became significant at $5x10^{-6}M$ GTP.)

4.3.2 Levels of G-protein subunits in VSMC membranes

The levels of the different G-protein α-subunits (i.e. $G_S\alpha$ (44 and 42kDa forms), $G_i2\alpha$ and $G_i3\alpha$) and the level of the β-subunit were quantified in SHR, Wistar and WKY VSMC membranes using ^{125}I-labelled anti-rabbit IgG. There were no significant differences in the levels of the different G-protein α-subunits and the level of the β-subunit in SHR, Wistar and WKY VSMC membranes (Figures 4.5, 4.6, 4.7, 4.8 and Table 4.2).

The level of the α-subunit for G_q/G_{11} was also quantified in SHR, Wistar and WKY VSMC membranes, using an antiserum raised against a synthetic peptide which represents the C-terminal decapeptide of the α-subunit of these two G-proteins (Mitchell *et al.*, 1991). There were no significant differences in the levels of $G_q\alpha/G_{11}\alpha$ present in SHR, Wistar and WKY VSMC membranes (Figure 4.9 and Table 4.2).

4.3.3 Levels of G-protein subunits in mesenteric artery membranes

The levels of the various G-protein α-subunits and the level of the β-subunit were also quantified in SHR, Wistar and WKY mesenteric artery membranes. There were no significant differences in the levels of $G_S\alpha$ (both forms), $G_i2\alpha$, $G_i3\alpha$, the β-subunit and $G_q\alpha/G_{11}\alpha$ in

Figure 4.4 Dose-effect experiments for GTP on isoproterenol-stimulated ACA in SHR, Wistar and WKY VSMC membranes. ACA was monitored in the presence of isoproterenol (10^{-4}M). Data are mean±S.E. for five (SHR and Wistar) and four (WKY) experiments using different membrane preparations.

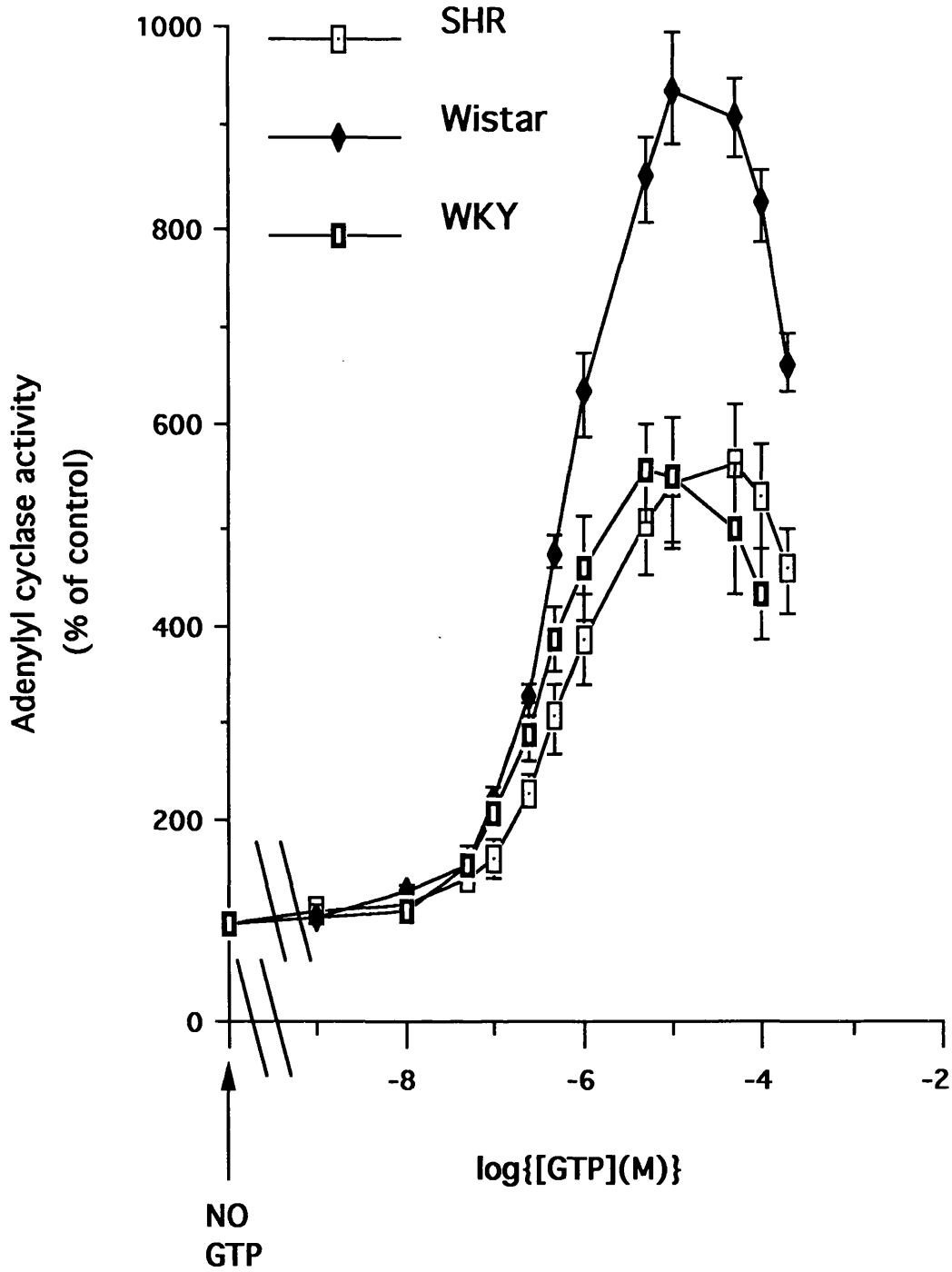

SHR
Wistar
WKY
1000
800
600
400
200
0
Adenylyl cyclase activity
(% of control)
-8
-6
-4
-2
log{[GTP](M)}
NO
GTP

Figure 4.5 Nitrocellulose sheet with SHR, Wistar and WKY VSMC membranes probed with $G_{s}\alpha$-specific Ab (CS1). 50μg of membrane protein was loaded in each lane. Two forms of $G_{s}\alpha$ are apparent (44 and 42kDa), with the 44kDa form being predominant in VSMC membranes. 1&2=Wistar membranes, 3&4=SHR membranes, 5&6=WKY membranes and 7=human platelet membrane.

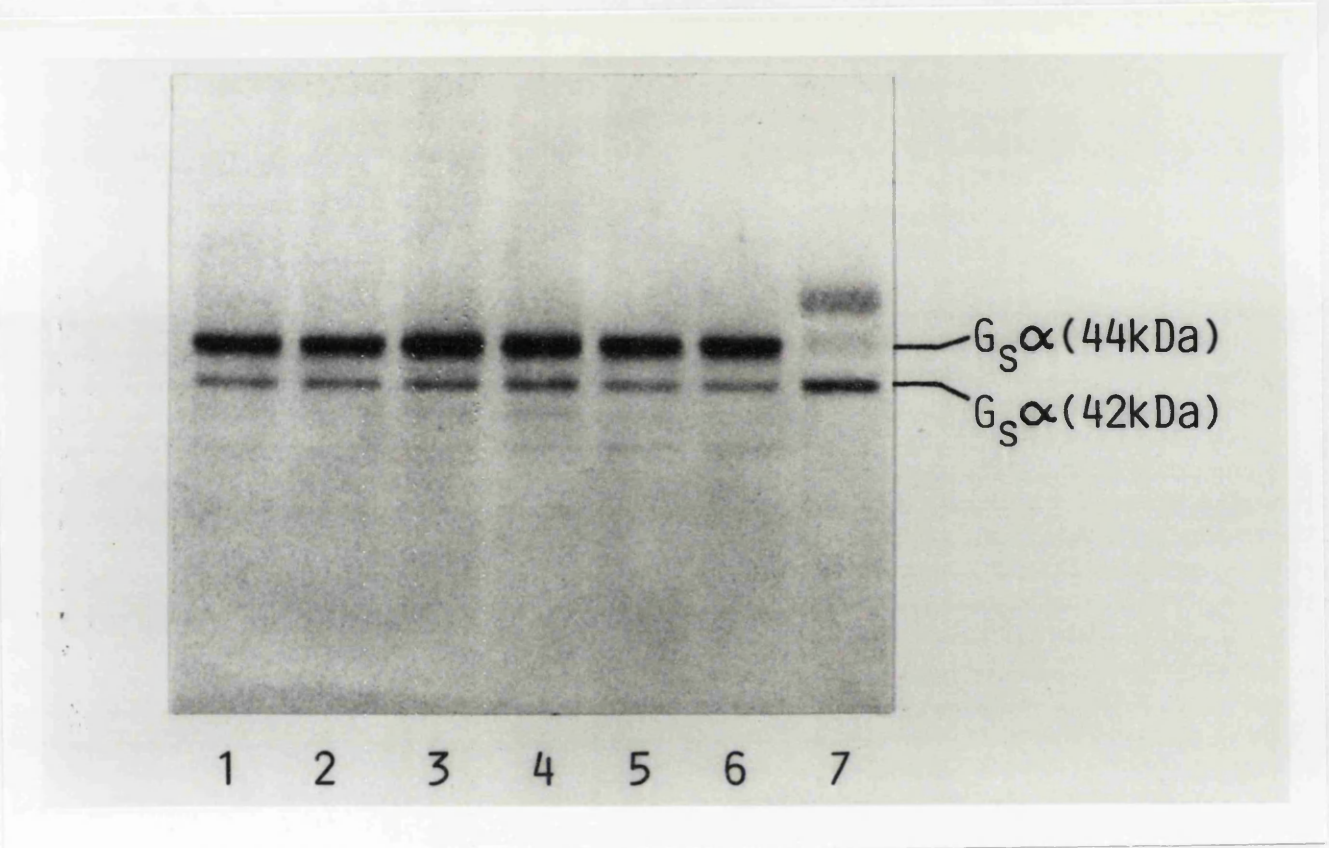

$G_s\alpha$(44kDa)
$G_s\alpha$(42kDa)
1 2 3 4 5 6 7

Figure 4.6 Nitrocellulose sheet with SHR, Wistar and WKY VSMC membranes probed with transducin α, $G_i1\alpha$, $G_i2\alpha$-specific Ab (SG2). Since rat VSMC membranes do not express detectable levels of G_i1 and transducin distribution is restricted to photoreceptor-containing tissues, the antiserum SG2 will only identify G_i2 in rat VSMC membranes. 60μg of membrane protein was loaded in each lane. 1&2=Wistar membranes, 3&4=SHR membranes, 5&6=WKY membranes and 7=human platelet membrane.

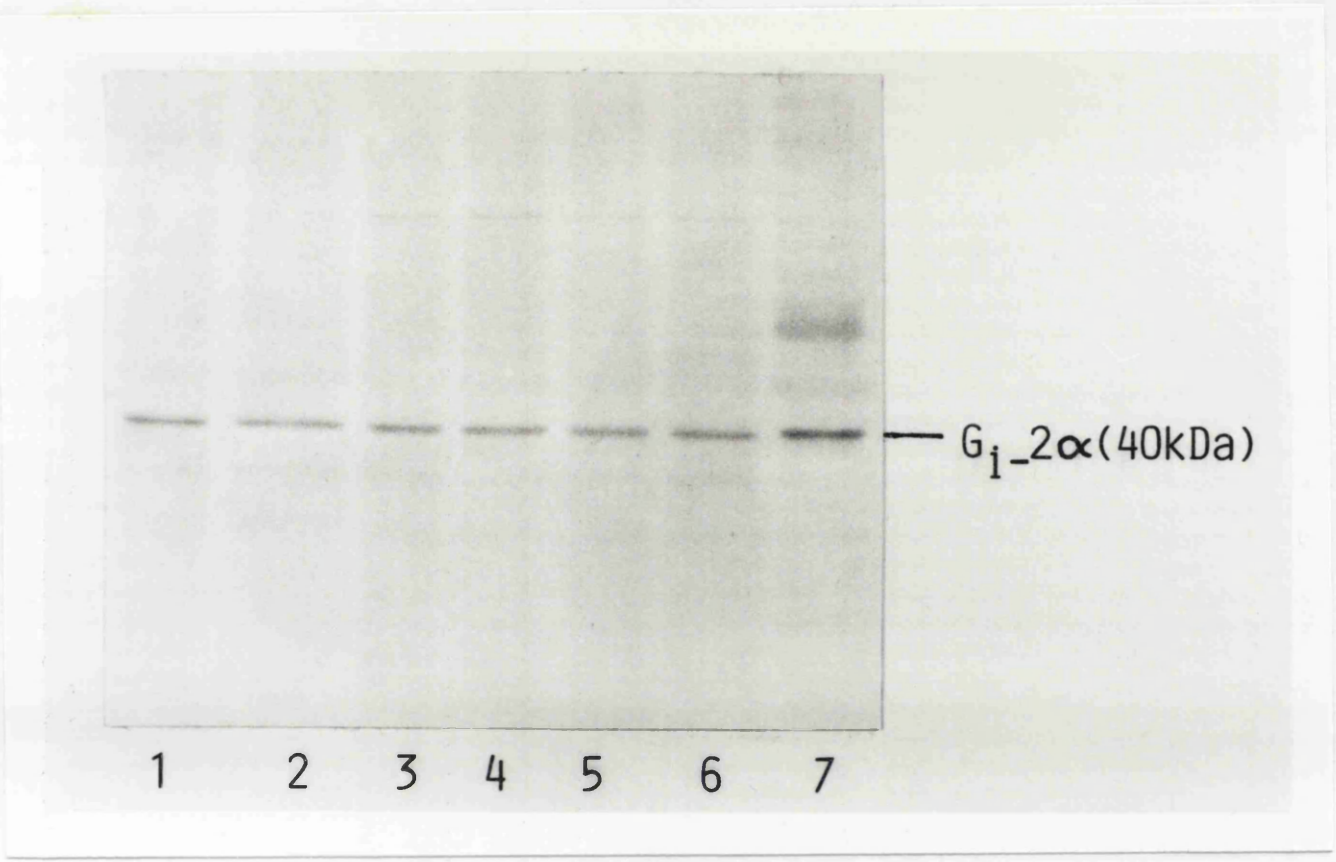

G_i_2α(40kDa)
1
2
3
4
5
6
7

Figure 4.7 Nitrocellulose sheet with SHR, Wistar and WKY VSMC membranes probed with $G_i3\alpha$-specific Ab (I3C). 50μg of membrane protein was loaded in each lane. 1&2=Wistar membranes, 3&4=SHR membranes and 5&6=WKY membranes.

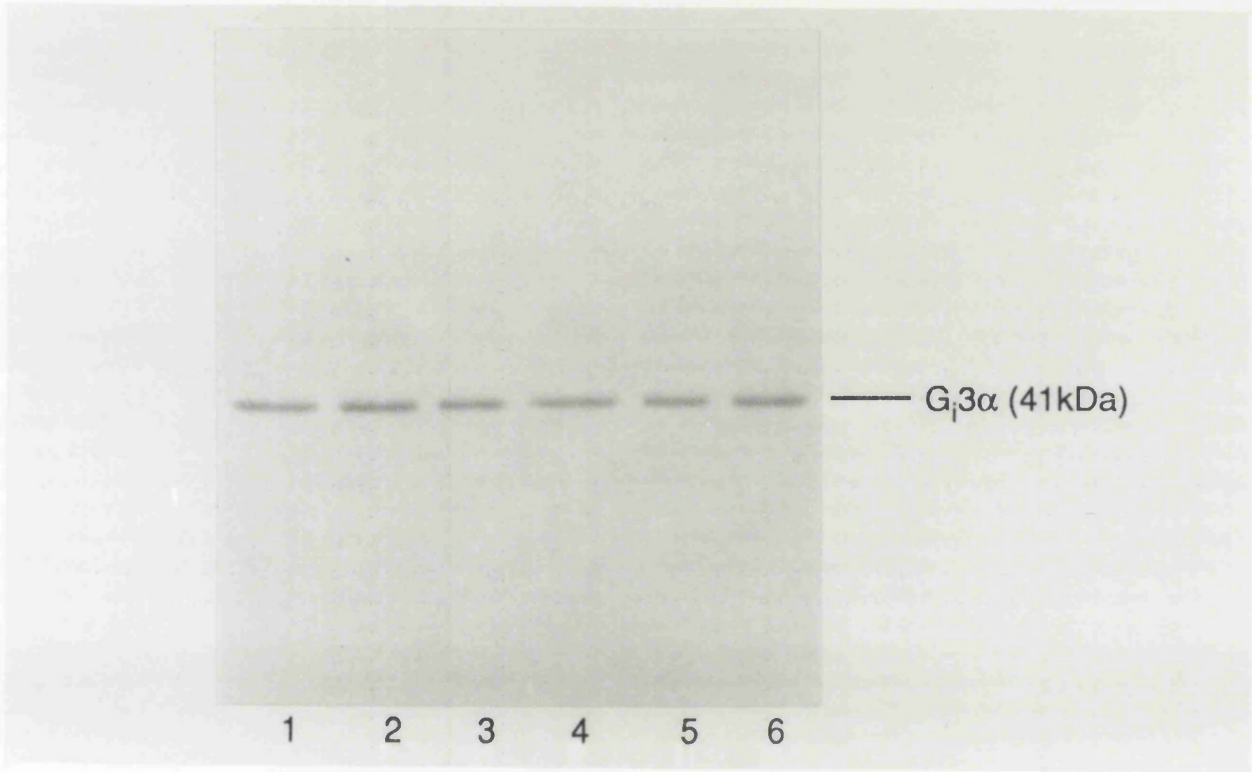

$G_i3\alpha$ (41kDa)
1
2
3
4
5
6

Figure 4.8 Nitrocellulose sheet with SHR, Wistar and WKY VSMC membranes probed with β-subunit-specific Ab (BN3). 50μg of membrane protein was loaded in each lane. 1&2=Wistar membranes, 3&4=SHR membranes, 5&6=WKY membranes and 7=human platelet membrane.

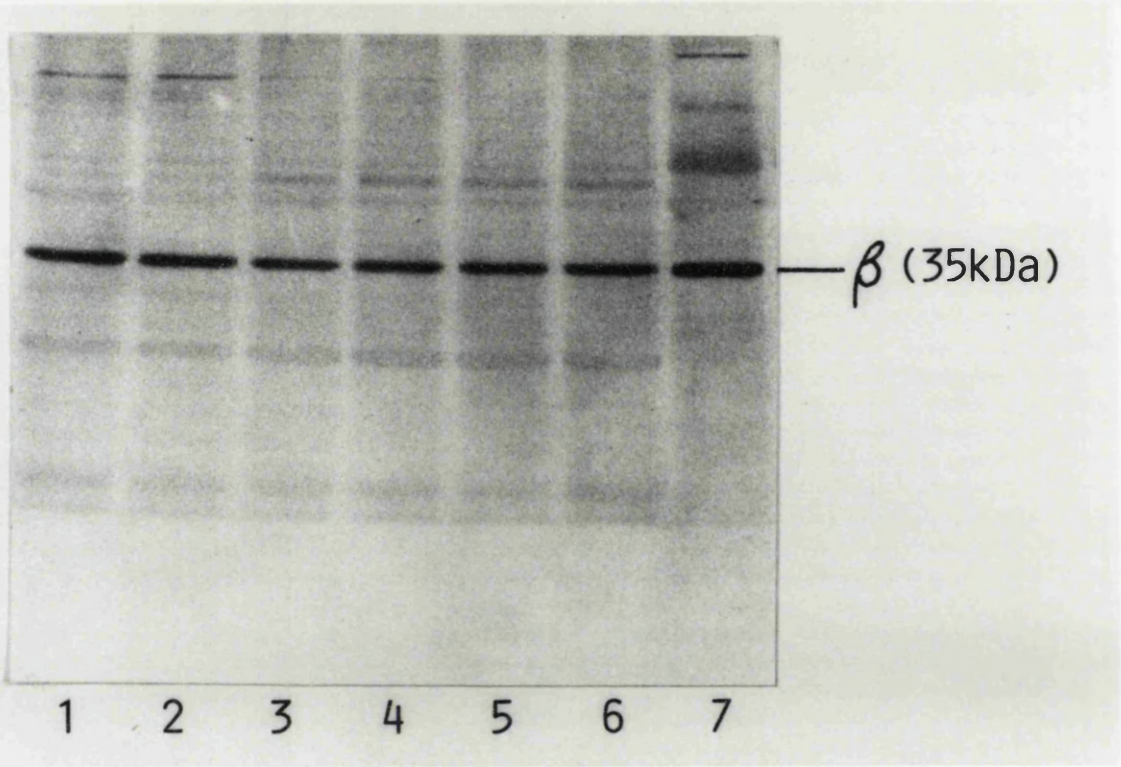
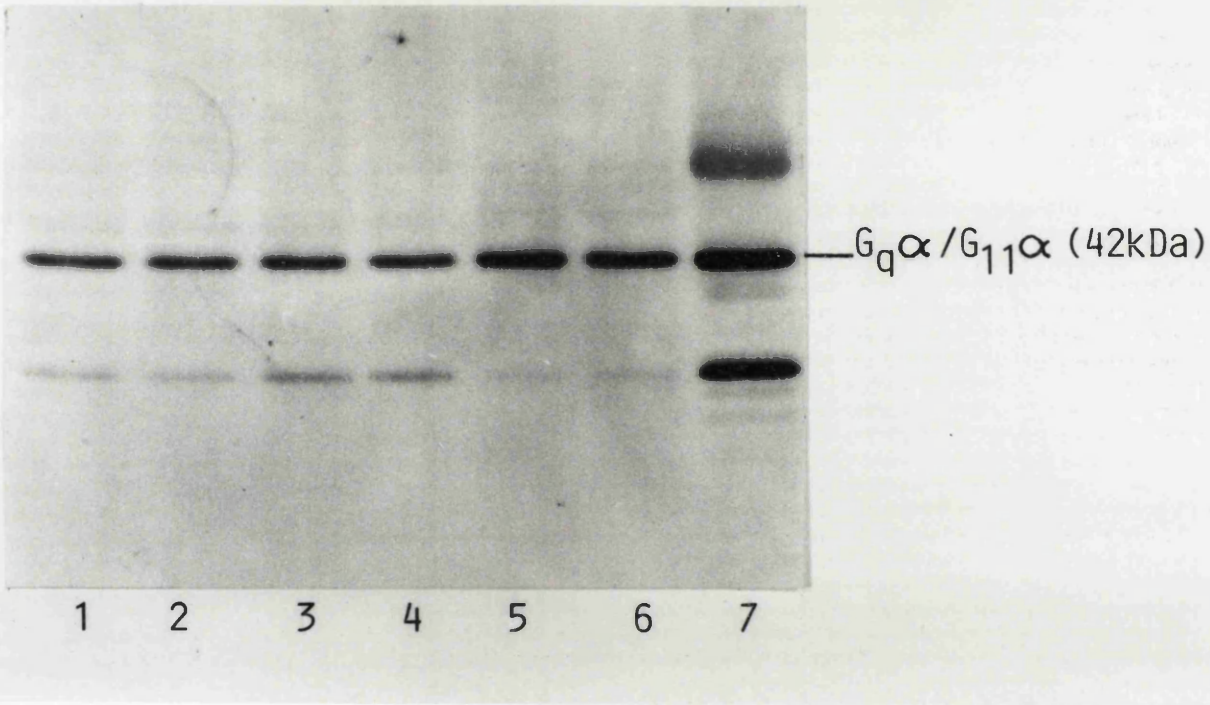

β (35kDa)
1 2 3 4 5 6 7

Figure 4.9 Nitrocellulose sheet with SHR, Wistar and WKY VSMC membranes probed with $G_{q}\alpha/G_{11}\alpha$-specific Ab (CQ2). 25μg of membrane protein was loaded in each lane. 1&2=Wistar membranes, 3&4=SHR membranes, 5&6=WKY membranes and 7=human platelet membrane.


$G_q\alpha$ / $G_{11}\alpha$ (42kDa)
1
2
3
4
5
6
7

G-protein subunit	SHR (cpm)	Wistar (cpm)	WKY (cpm)
$G_{s\alpha}$ (44kDa)	764±135	937±113	994±151
$G_{s\alpha}$ (42kDa)	137±39	95±26	139±23
$G_{i}2\alpha$	228±91	301±59	216±56
$G_{i}3\alpha$	131±25	165±31	142±16
β	1402±60	1608±212	1363±132
$G_{q\alpha}/G_{11\alpha}$	1572±78	1624±59	1701±115

Table 4.2 Quantification of bands on nitrocellulose sheets with SHR, Wistar and WKY VSMC membranes using [^{125}I]-labelled Ab. Data represent mean±S.E. for four experiments using different membrane preparations. Statistical analysis of results did not disclose any significant differences.

SHR, Wistar and WKY mesenteric artery membranes (Figures 4.10, 4.11, 4.12, 4.13, 4.14 and Table 4.3).

4.4 Discussion

4.4.1 Choice of rats

In this study, two normotensive control groups, namely the outbred Wistar strain and WKY strain, were included in experiments. The Wistar strain was chosen in addition to WKY rats as a normotensive group in view of evidence that the WKY rats obtainable from most commercial suppliers display marked genetic heterogeneity (Kurtz and Morris, 1987 and Kurtz *et al.*, 1989) (for details see section 3.1). Indeed, genetic heterogeneity has also been reported in SHRs supplied by commercial suppliers (Nabika *et al.*, 1991), thus, for this study, rats were purchased from a different supplier (Harlan Olac Ltd.) to the one that supplied the rats for the study detailed in Chapter 3 (Charles River Ltd.).

4.4.2 Adenylyl cyclase catalytic subunit

The basal activity of adenylyl cyclase in SHR VSMC membranes was similar to that measured in WKY membranes; however, the basal ACAs in both SHR and WKY membranes were significantly lower than that observed in Wistar membranes. On the addition of $MnCl_2$ ($2x10^{-2}M$), the basal ACAs in the three sets of membranes were reduced, resulting in a situation similar to basal measurements i.e. SHR and WKY ACAs were similar, but both were significantly reduced compared with Wistar. Hence, results were expressed as % change over basal measured in the absence/presence of Mn^{2+} ions.

Forskolin, which directly activates the catalytic subunit of adenylyl cyclase, produced similar stimulatory responses in both control groups; however, SHR membranes exhibited a significantly increased response compared with both controls (statistically significant compared with WKY only, when results expressed relative to basal activity measured in the presence of Mn^{2+} ions). These results suggest an alteration at the enzyme level, possibly an increased level/responsiveness of the catalytic subunit of adenylyl cyclase in SHRs compared with control(s). In Chapter 3, forskolin produced similar effects in SHR and Wistar VSM membranes (rats supplied by Charles River Ltd.), suggesting no alteration at the

Figure 4.10 Nitrocellulose sheet with SHR, Wistar and WKY mesenteric artery membranes probed with $G_S\alpha$-specific Ab (CS1). 50μg of membrane protein was loaded in each lane. Two forms of $G_S\alpha$ are apparent (44 and 42kDa), with the 44kDa form being predominant in VSM membranes. 1&2=human platelet membranes, 3&4=WKY membranes, 5&6=SHR membranes and 7&8=Wistar membranes.

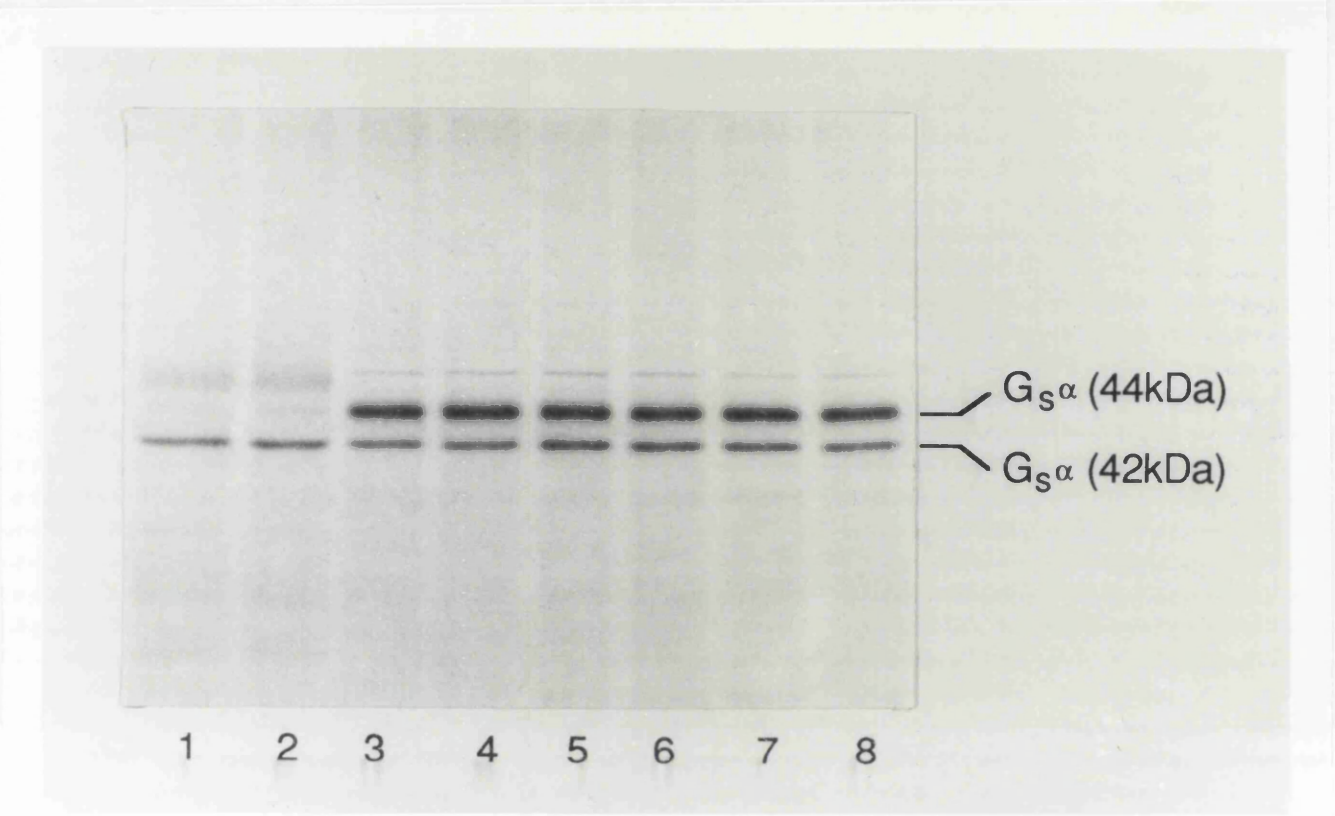

$G_s\alpha$ (44kDa)
$G_s\alpha$ (42kDa)
1
2
3
4
5
6
7
8

Figure 4.11 Nitrocellulose sheet with SHR, Wistar and WKY mesenteric artery membranes probed with transducin α, $G_i1\alpha$, $G_i2\alpha$-specific Ab (SG2). In rat VSM membranes, the antiserum SG2 will only identify $G_i2\alpha$. 60μg of membrane protein was loaded in each lane. 1&2=human platelet membranes, 3&4=WKY membranes, 5&6=SHR membranes and 7&8=Wistar membranes.

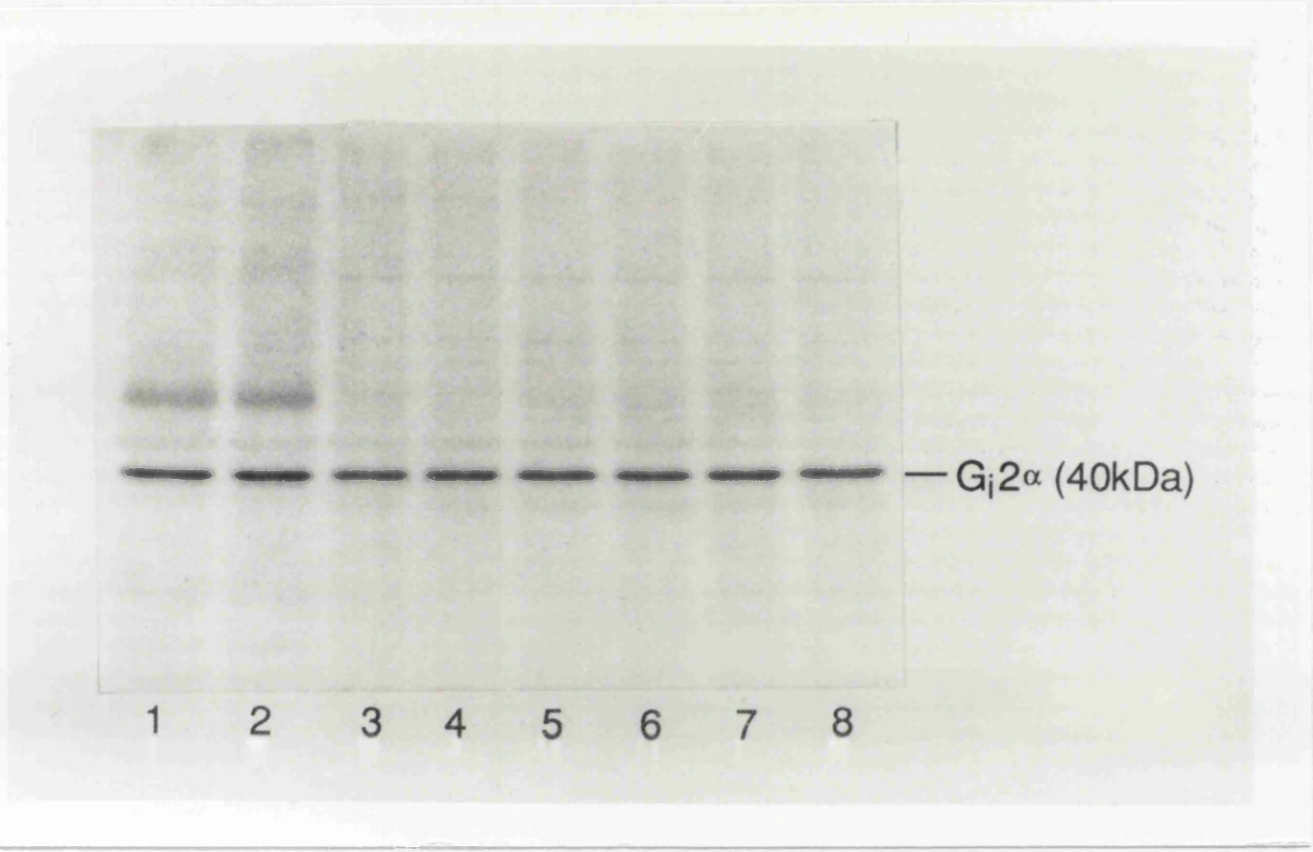

— $G_i2\alpha$ (40kDa)
1
2
3
4
5
6
7
8

Figure 4.12 Nitrocellulose sheet with SHR, Wistar and WKY mesenteric artery membranes probed with $G_i3\alpha$-specific Ab (I3C). 50μg of membrane protein was loaded in each lane. 1&2=Wistar membranes, 3&4=SHR membranes and 5&6=WKY membranes.

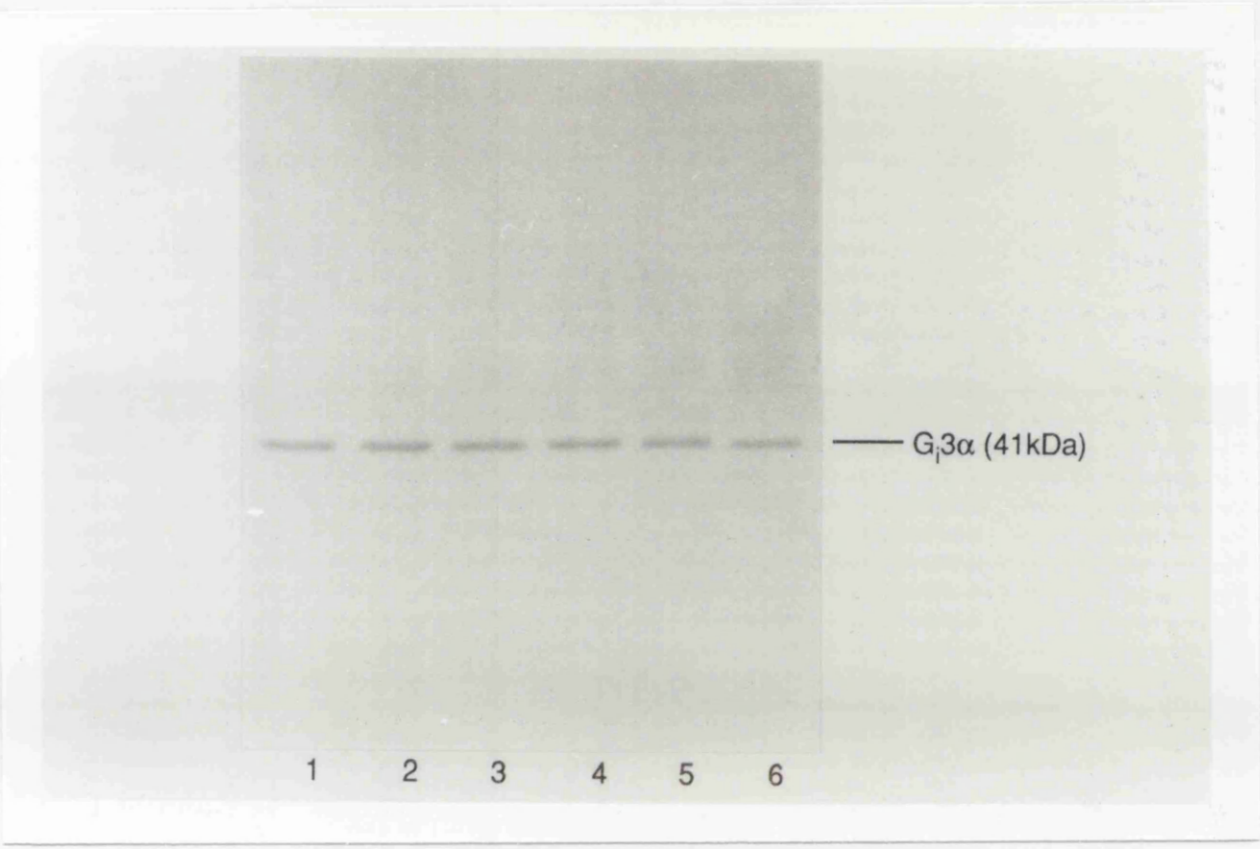

$G_i3\alpha$ (41kDa)
1
2
3
4
5
6

Figure 4.13 Nitrocellulose sheet with SHR, Wistar and WKY mesenteric artery membranes probed with β-subunit-specific Ab (BN3). 50μg of membrane protein was loaded in each lane. 1&2=Wistar membranes, 3&4=SHR membranes, 5&6=WKY membranes and 7&8=human platelet membranes.

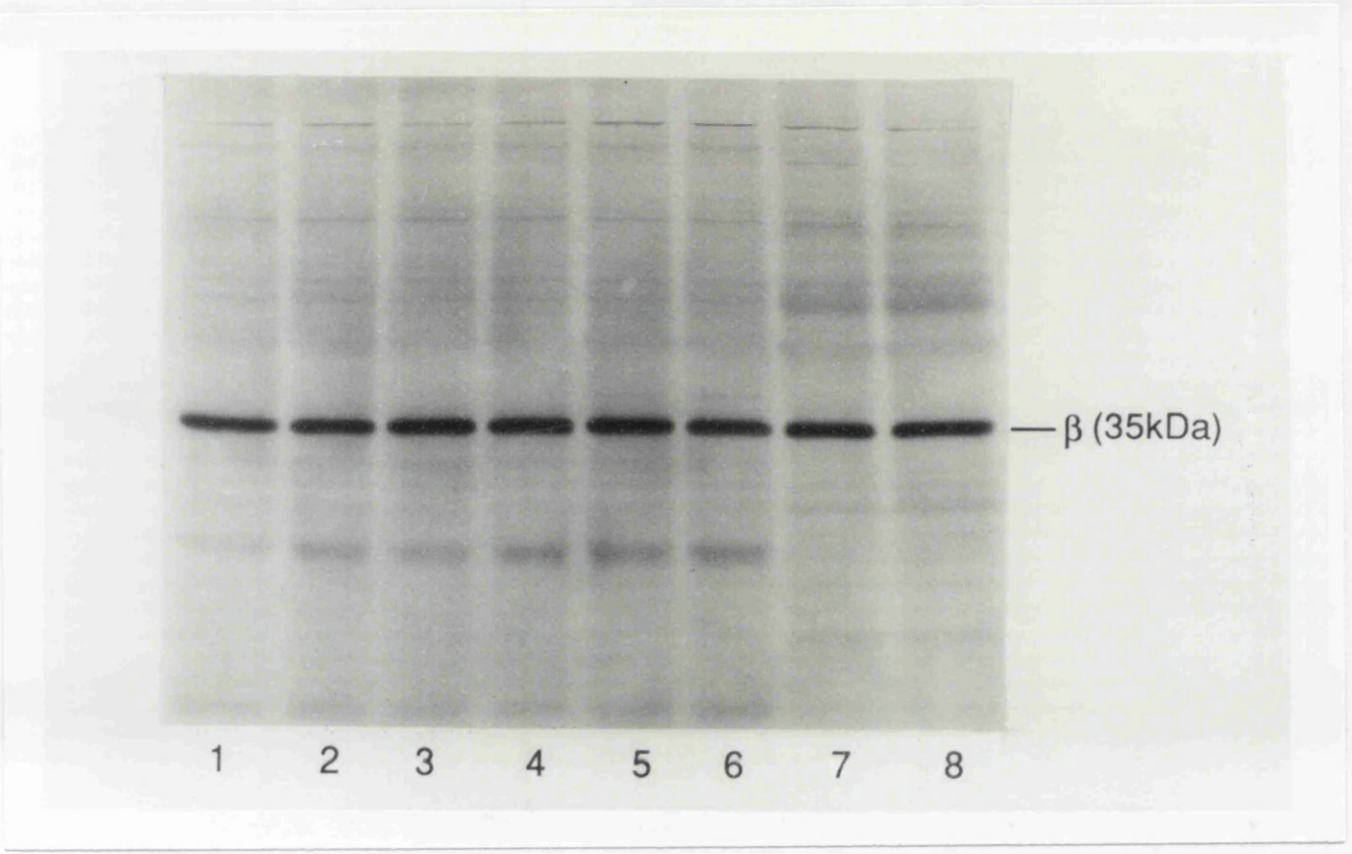
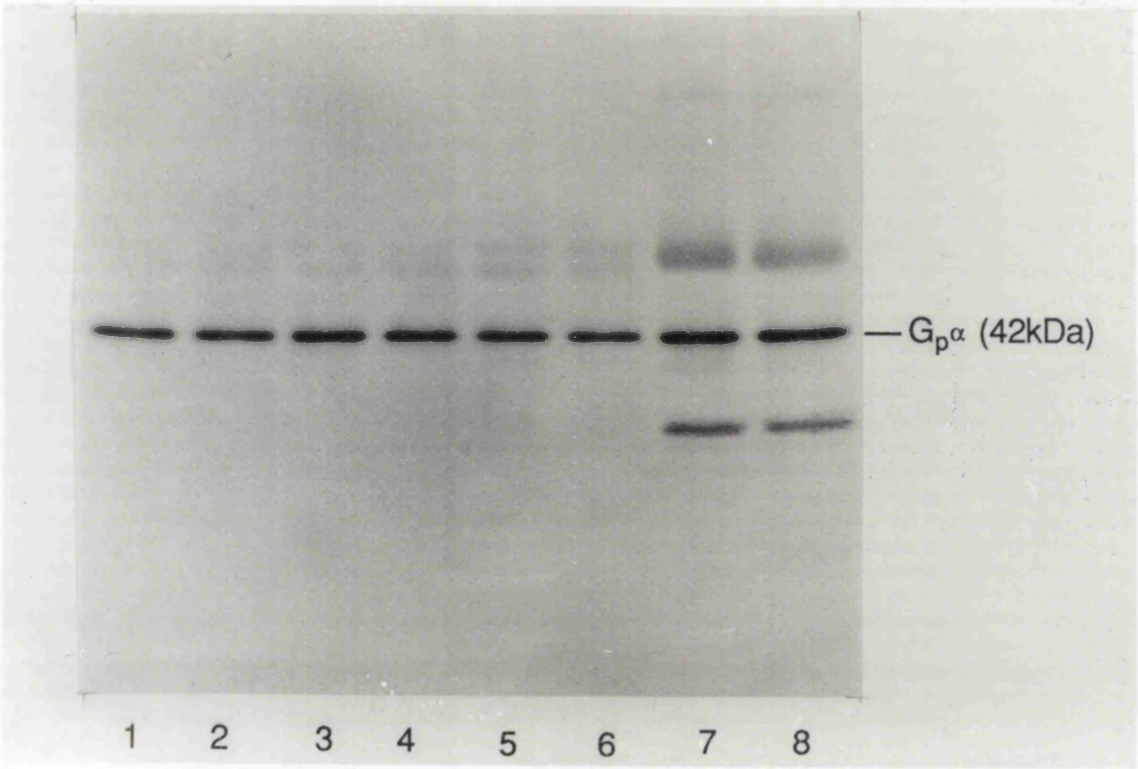

β (35kDa)
1
2
3
4
5
6
7
8

Figure 4.14 Nitrocellulose sheet with SHR, Wistar and WKY mesenteric artery membranes probed with $G_{q}\alpha/G_{11}\alpha$-specific Ab (CQ2). 25μg of membrane protein was loaded in each lane. 1&2=Wistar membranes, 3&4=SHR membranes, 5&6=WKY membranes and 7&8=human platelet membranes.


— $G_p\alpha$ (42kDa)
1
2
3
4
5
6
7
8

G-protein subunit	SHR (cpm)	Wistar (cpm)	WKY (cpm)
$G_{s\alpha}$ (44kDa)	197±20	247±21	193±17
$G_{s\alpha}$ (42kDa)	96±21	118±18	103±25
$G_{i}2\alpha$	71±9	102±17	85±21
$G_{i}3\alpha$	105±12	111±15	97±8
β	561±39	748±63	619±50
$G_{q\alpha}/G_{11\alpha}$	298±23	276±19	314±16

Table 4.3 Quantification of bands on nitrocellulose sheets with SHR, Wistar and WKY mesenteric artery membranes using [^{125}I]-labelled Ab. Data represent mean±S.E. for four experiments using different membrane preparations. Statistical analysis of results did not disclose any significant differences.

adenylyl cyclase catalytic subunit level in these SHRs. In a study of another animal model which displays insulin resistance, the streptozotocin-diabetic rat, Bushfield *et al.* (1990a) reported increased levels of the catalytic subunit of adenylyl cyclase in hepatocyte and liver plasma membranes compared with control, although basal ACA was actually decreased in hepatocyte plasma membranes from diabetic animals. Unfortunately, the level of the catalytic subunit of adenylyl cyclase was unable to be quantified in the three sets of membranes, as an appropriate antibody was not available.

4.4.3 G-protein subunit levels

The levels of the G-protein subunits responsible for the regulation of ACA were quantified in SHR, Wistar and WKY VSMC and mesenteric artery plasma membranes. No significant differences were found in the levels of $G_S\alpha$ (44 and 42kDa forms), $G_i2\alpha$, $G_i3\alpha$ and the β-subunit in SHR, Wistar and WKY membranes. This was also the case in VSM membranes from Charles River Ltd. SHRs and Wistar rats (see sections 3.3 and 3.4 for details). G-protein subunit levels have also been measured in aorta and heart from SHRs and WKY rats by Anand-Srivastava *et al.* (1991). The level of $G_i\alpha$ was significantly increased in the hearts and aortas from SHRs, while the level of $G_S\alpha$ was similar in SHRs and WKY rats. Indeed, Anand-Srivastava *et al.* (1991) observed significantly higher mRNA levels of $G_i2\alpha$ and $G_i3\alpha$ in heart and aorta from SHRs as compared to WKY rats, however, the mRNA levels of $G_S\alpha$ were not different in SHRs from WKY. However, these changes in SHR heart may not be primary abnormalities in hypertension, but may reflect changes secondary to increased blood pressure.

4.4.4 Function of G_S

G_S function was assessed in VSMC membranes by NaF, like forskolin, a receptor-independent stimulatory agent. When the results were expressed as % change relative to basal activity measured in the absence of Mn^{2+} ions, the situation was similar to that of forskolin i.e. the stimulatory responses to NaF were similar in Wistar and WKY membranes; however, SHR membranes produced a significantly increased response compared with both controls. These results suggest that the function of G_S does not appear to be altered in SHR

membranes compared with controls. When the results were expressed as % change relative to basal activity measured in the presence of Mn^{2+} ions, an increased response in SHR membranes was again noted, although it was not significantly different from the responses measured in the controls. Thus, while a significantly increased response to forskolin in SHR membranes compared to WKY was observed, this was reduced to a non-significant difference in the presence of NaF. This may suggest that an alteration in G_S function, i.e. decreased activity, exists in SHR membranes compared with WKY. In Chapter 3, a significantly decreased response to NaF was observed in SHR VSM membranes compared with Wistar using rats from a different supplier (see sections 3.3 and 3.4 for details), suggesting altered G_S function or coupling in these SHRs compared with Wistar controls. Indeed, Murakami and colleagues (1987) observed a reduction in the functional activity of G_S in SHR cardiac membranes. This group observed that the stimulation of ACA by either forskolin or purified G_S was higher in SHRs; however, stimulation of ACA by isoproterenol and GTP was similar in SHRs and WKY rats. Although there was no difference in the amount of G_S in SHR membranes, the functional activity of G_S in cholate-extracted membranes from SHRs was significantly lower than that from WKY rats. The group observed no strain differences in the number and affinity of β-adrenergic receptors, the function and amount of G_i, and the amount of βγ-subunits. The levels of both forms of $G_{S\alpha}$ and the β-subunit were not altered in the three sets of membranes investigated in this study. However, the reduction in ACA in SHR membranes in response to NaF compared with forskolin may reflect functional differences in G_S from SHR and WKY membranes.

4.4.5 Function of G_i

G_i function was demonstrated by showing biphasic effects of GTP on isoproterenol-stimulated ACA. In this instance, low concentrations of GTP promote activation of adenylyl cyclase by stimulating the coupling of the β-adrenoceptor to G_S, whereas high concentrations cause inhibition due to activation of G_i (Cooper, 1982 and Heyworth *et al.*, 1984). Biphasic effects due to GTP were observed in SHR, Wistar and WKY VSMC membranes showing that G_i function was not different among the strains. The extents of inhibition measured in the three sets of VSMC membranes were relatively low compared

with other systems (Coquil and Brunelle, 1989). Indeed, the ACA assay was not sensitive enough to measure the inhibition in response to low concentrations of p(NH)ppG and GTPγS, and also neuropeptide Y and lysophosphatidic acid in the three sets of membranes (data not shown). Chapter 3 reported that G_i function was also similar in SHR and Wistar VSM membranes (rats purchased from Charles River Ltd.). However, decreased G_i function has been reported in platelets from the SHR by Coquil and Brunelle (1989). However, the relevance of platelet studies in hypertensive animals is uncertain, and no direct measurement was made of G_S function in that study.

4.4.6 β-Adrenergic receptor number/affinity/coupling

Although the GTP response in SHR and WKY membranes was similar, it was significantly reduced in comparison to the response measured in Wistar membranes. This suggests an alteration in the coupling of β-adrenoceptors to G_S in SHR (and WKY) membranes compared to Wistar. Cardiac membranes from the obese Zucker rat, which is not diabetic, but nevertheless profoundly insulin-resistant and hypertensive, demonstrated reduced β-adrenergic stimulation of adenylyl cyclase in comparison to lean controls (Strassheim *et al.*, 1992). The heart tissue membranes from obese Zucker rats showed both reduced β-adrenergic receptor number and altered coupling between β-adrenergic receptors and G_S.

Reduced ACA in response to β-adrenergic stimulation in SHR vascular tissue has been well documented (Amer *et al.*, 1974; Amer, 1973; Triner *et al.*, 1975; Bhalla and Sharma, 1982; Anand-Srivastava, 1988; Bhalla and Ashley, 1978; Bhalla *et al.*, 1980; Sharma *et al.*, 1982 and Kumano *et al.*, 1983). Isoproterenol stimulation, in VSMC membranes, was significantly reduced in the SHR compared with WKY. Whether the results were expressed as % change relative to basal activity measured in the absence or presence of Mn^{2+} ions, did not alter the finding, consistent with decreased β-adrenergic receptor number/affinity in SHR (and Wistar) membranes compared with WKY. The reduced ACA in response to isoproterenol is consistent with the observed reduction in arterial relaxation in response to β-adrenoceptor stimulation in the SHR: studies by Asano and colleagues (1988) using isolated femoral artery material from the SHR suggested, on the basis of

indirect evidence, that this was a consequence of reduced G_S function.

Receptor-mediated stimulation of ACA was also examined using PGE_1. The responses PGE_1 produced were not significantly different in the three sets of membranes. However, when the increased response to NaF is compared with the decrease in ACA in response to PGE_1 in SHRs, the data suggest decreased PGE_1 receptor number/affinity in SHR membranes compared with Wistar and WKY. The extents of stimulation in response to isoproterenol and PGE_1 were measured in VSM membranes from Charles River Ltd. SHRs and Wistar rats (see sections 3.3 and 3.4 for details). In these rats, the stimulatory responses to both ligands were significantly reduced in SHR membranes compared with Wistar.

4.4.7 Activity of phospholipase C and levels of its regulatory G-proteins

The adenylyl cyclase enzyme is not the only effector system linked to cell surface receptors by G-proteins. The enzyme PLC, which alters the intracellular concentrations of the second messengers IP_3 and DG, is linked to cell surface receptors by a G-protein named G_p (Cockcroft and Gomperts, 1985). Recent evidence has indicated that this polypeptide is a member of the G_q/G_{11} subfamily of G-proteins (Gutowski *et al.*, 1991). Millanvoye *et al.* (1988) have reported higher PLC activity in response to angiotensin II in SHR aortic smooth muscle cells compared with WKY. Increased activity of PLC has also been noted in erythrocyte ghosts from the SHR compared with WKY (Kato and Takenawa, 1987), while experiments involving thrombin stimulation of lipid metabolism by Remmal *et al.* (1987) suggested enhanced PLC activity in SHR platelets compared with WKY. It was of interest to examine whether these reported increased PLC activities in the SHR were a consequence of increased levels of G_q/G_{11}. However, no significant differences were found in the levels of $G_{q\alpha}/G_{11\alpha}$ in SHR, Wistar and WKY VSM membranes.

4.4.8 Conclusions

Differences in the regulation of ACA have been identified among the three strains studied. There appeared to be an alteration at the adenylyl cyclase catalytic subunit level in SHR membranes compared with WKY. There may also be an alteration at this level in SHR

membranes compared with Wistar control, but this did not reach statistical significance. Depending on how the results were expressed, there may be decreased G_S activity in SHR membranes compared with WKY; however, the function of G_S in SHR and Wistar membranes appeared to be similar.

GTP dose-effect experiments on isoproterenol-stimulated ACA demonstrated that G_i function was not altered in SHR membranes as compared with Wistar and WKY; however, these experiments highlighted an alteration in the coupling of β-adrenoceptors to G_S in SHR membranes compared to Wistar. This alteration in β-adrenoceptor-G_S coupling was also present in WKY membranes compared to Wistar.

As with other studies (Amer *et al.*, 1974; Amer, 1973; Triner *et al.*, 1975; Bhalla and Sharma, 1982; Anand-Srivastava, 1988; Bhalla and Ashley, 1978; Bhalla *et al.*, 1980; Sharma *et al.*, 1982 and Kumano *et al.*, 1983), a reduced response of ACA to isoproterenol in SHR membranes compared to WKY was noted. This reduced β-adrenergic stimulation was also observed in Wistar membranes compared to WKY. It can be seen, therefore, that there are differences in the regulation of ACA among the three strains, and differences in the regulation of ACA between the two normotensive control strains are obvious. While these changes may reflect hypertension and its genetic antecedent, the possibility is raised, by inclusion of the Wistar control group, that the genetic heterogeneity reported in SHR and WKY strains (Nabika *et al.*, 1991; Kurtz and Morris, 1987 and Kurtz *et al.*, 1989) may contribute substantially to the differences detailed in this (and other) studies of ACA, and raise the possibility that these changes are not directly linked to the development of increased blood pressure.

Chapter 3 detailed a study examining G-protein levels and function in SHRs and Wistar rats supplied by Charles River Ltd. However, for this study, SHRs, Wistar and WKY rats were purchased from Harlan Olac Ltd., as genetic heterogeneity has been reported in both SHRs and WKY rats from different commercial suppliers (Nabika *et al.*, 1991; Kurtz and Morris, 1987 and Kurtz *et al.*, 1989). Differences in the regulation of ACA in SHR membranes compared with Wistar were observed in both studies; however, these differences were not similar and seemed to depend on where the rats were purchased. Hence, the remainder of this thesis involved work using VSMC (obtained from one source) isolated from another

rodent model of genetic hypertension, the Milan hypertensive strain and its isogenic control, the Milan normotensive strain.

Chapter 5 Comparison of vascular G-proteins in adult MHS and MNS rats

5.1 Introduction

The Milan hypertensive strain and the Milan normotensive strain descend from two mating pairs of Wistar origin rats from an outbred colony, which had mean blood pressures of 164mmHg and 168mmHg. The hypertensive strain was obtained by breeding for high blood pressure and the control strain was obtained by breeding for normal blood pressure (Bianchi *et al.*, 1984).

The MHS is a useful animal model for studying the mechanisms that may be responsible for human essential hypertension. For example, Bianchi *et al.* (1984) measured different factors in the prehypertensive phases of both rats and humans and compared the results with those obtained for appropriate controls of the same age. They chose the prehypertensive phase in order to detect changes that existed before the development of hypertension, and thus, cannot be secondary to the hypertension, *per se*. Moreover, during the time-interval between birth and the development of overt hypertension, the genetic factors responsible for hypertension may already have produced biochemical or physiological abnormalities. Due to the difficulty of defining a prehypertensive phase in human subjects, young normotensive subjects with two hypertensive parents were compared with subjects of the same age, but with two normotensive parents, on the assumption that, later in life, the former will develop hypertension more frequently (about 50%) than the latter (about 2%). This group observed that a number of features in the MHS are similar to those observed in human essential hypertension, with simliar changes in whole-kidney function (glomerular filtration rate and renal blood flow), morphology of the kidney and adrenal (by light or electron microscopy), 24 hour urinary electrolyte output, extracellular fluid volume, total and exchangeable body Na^+, plasma electrolytes, plasma and urine osmolarity, plasma aldosterone, and plasma catecholamines.

5.1.1 Renal abnormalities

The key role of the kidney in this animal model of genetic hypertension has been demonstrated. Studies of the effect of kidney cross-transplantation between adult MHS rats and adult MNS rats

showed that the MHS kidney produced hypertension in MNS. Transplantation has also been carried out between MHS and MNS rats at one month of age, when the difference in donor blood pressure was slight and of short duration, to determine whether this pressor effect of the MHS kidney was primary in nature or secondary to renal injury due to prolonged perfusion at high blood pressure of the MHS donor kidney. The blood pressure measured 120 days after kidney transplantation into one-month-old MHS rats was 152±2mmHg in recipients of MHS kidneys and 130±5mmHg in recipients of MNS kidneys ($p<0.01$). Therefore, MHS kidneys have a greater pressor effect even when taken from young donors with almost equal blood pressure, demonstrating that the pressor effect of the MHS kidney exists prior to hypertension (Fox and Bianchi, 1976). In addition, the relative sodium retention in MHS during the development of the blood pressure difference between the two strains is in keeping with a renal origin of hypertension in MHS. To evaluate further the role of the kidney, whole kidney clearance and micropuncture techniques were used to study whole kidney and single nephron filtration rate and proximal fluid reabsorption in both strains before, during and after the development of hypertension in MHS. Both single nephron and whole kidney glomerular filtration rates are significantly lower in young MHS before the development of hypertension, while in adult animals, whole kidney glomerular filtration rate is equal and superficial single nephron filtration rate is higher in MHS (discrepancy explained by lower number of glomeruli in MHS kidney) (Bianchi and Baer, 1976).

5.1.2 Ion flux changes

Na^+ transport across brush-border vesicles isolated from proximal tubular cells has been reported to be faster in MHS. When erythrocytes and proximal tubular cells of MHS rats were compared to those of MNS rats, the former had smaller volume and Na^+ content, whereas the Na^+ transport was faster and the Ca^{2+} ATPase at V_{max} was lower. This indicates that the cellular abnormality which may be responsible for the renal functional abnormality and hypertension is also present in erythrocytes. Moreover, MHS erythrocyte abnormalities are genetically determined within the stem cells and cosegregate with hypertension (Parenti *et al.*, 1986 and Bianchi *et al.*, 1986). In addition, the MHS is characterised in the prehypertensive phase by a significantly faster

bumetanide-sensitive cell membrane Na^+-K^+-Cl^- cotransport as compared to age-matched MNS (Salvati *et al.*, 1990). These observations are in keeping with data showing an increased sodium transport across renal tubules of the MHS, and support the hypothesis that the abnormality in sodium and water handling by kidneys from MHS can be related to an alteration in sodium transport across the luminal membrane of the proximal tubule cells (Parenti *et al.*, 1986). Indeed, the activity of the (Na^+-K^+)-ATPase from the kidney cortex of the MHS has been reported to be increased, resulting in an increased ATP-dependent ouabain-sensitive sodium transport in MHS rats, supporting the hypothesis that a higher tubular sodium reabsorption may be involved in the pathogenesis of hypertension in this rat strain (Parenti *et al.*, 1991).

As mentioned earlier, erythrocytes and proximal tubular cells of MHS rats possess similar cellular abnormalities, and because a correlation was also found in human hypertension between erythrocyte Na^+ transport abnormality and renal function, it was proposed that the erythrocyte may be used for studying the genetic molecular mechanisms of hypertension. In comparison to MNS, erythrocytes in MHS were observed to be smaller and had lower sodium concentration, and Na^+-K^+ cotransport and passive permeability were faster. These differences persisted after transplantation of bone marrow to irradiated F_1 (MHSxMNS) hybrids. Moreover, in normal segregating F_2 hybrid populations there was a positive correlation between blood pressure and Na^+-K^+ cotransport, suggesting a genetic and functional link in MHS between cell membrane cation transport abnormalities and hypertension (Ferrari *et al.*, 1987). The erythrocytes of MHS rats have a faster Na^+-K^+-Cl^- cotransport than erythrocytes from MNS rats. However, the difference in Na^+-K^+-Cl^- cotransport is no longer present in inside-out vesicles of erythrocyte membranes, and data support the hypothesis that an abnormality in the membrane skeletal proteins may play a role in the different Na^+-K^+-Cl^- cotransport modulation between MHS and MNS erythrocytes (Ferrari *et al.*, 1992).

Abnormalities at the cellular level have also been observed in MHS thoracic aortic vascular smooth muscle cells. It has been noted that smooth muscle cells from MHS rats exhibit increased growth in culture in comparison with the MNS, associated with higher Na^+-K^+-Cl^- cotransport activity (Vallega *et al.*, 1989). While the number and affinity of angiotensin II (Ang II) receptors were similar in VSMC from both strains, cultured smooth muscle cells from MHS

rats exhibited blunted phospholipase C, Na^+-H^+ exchange, and cytosolic Ca^{2+} responses to Ang II, suggesting that in this model of low renin hypertension, Ang II receptor response is blunted during increased growth rates (Socorro *et al.*, 1990).

5.1.3 α-Adrenergic receptor measurements

Since cerebral and renal α-adrenergic receptors play an important role in the control of blood pressure, the densities and affinities of α_1-adrenergic and α_2-adrenergic receptors have been examined in prehypertensive and hypertensive rats. In the cerebral cortex, no between-strain differences in α_1-adrenergic and α_2-adrenergic receptor density and affinity were observed in prehypertensive and hypertensive rats. In the renal cortex, the differences between MHS and MNS rats concerned α_2-adrenergic receptors only. MHS rats showed a lower affinity for the antagonist, [^{3}H]rauwolscine, in the prehypertensive period and absence of the normal age-related increase in receptor density. A lower density of [^{3}H]rauwolscine binding sites in the hypertensive period was also observed. In hypertensive rats, epinephrine bound to one class of sites in MHS rats and to two classes in MNS rats, while data suggest uncoupling of epinephrine receptors from its G-protein in MHS rats. The modifications of renal α_2-adrenergic receptors were more evident in the hypertensive period when the impaired tubular sodium reabsorption observed in the prehypertensive period had almost returned to normal. Taken together, these data suggest that the modification of renal α_2-adrenergic receptors in MHS rats could be a compensatory mechanism to counteract the increased renal sodium reabsorption, rather than the cause of the development of hypertension in MHS rats (Parini *et al.*, 1987).

5.1.4 Summary

In conclusion, although the MHS has not been as extensively studied as the SHR, the role of the kidney in this model of genetic hypertension has been demonstrated. Sodium transport in MHS erythrocytes and proximal tubular cells, in comparison to MNS, has been observed to be abnormal, and this has been implicated in the pathogenesis of hypertension in this rat strain. Although α-adrenergic receptors have been studied in the MHS and abnormalities noted regarding renal α_2-adrenergic receptors (Parini

et al., 1987), β-adrenergic receptors have not been examined. Since reduced stimulation of ACA through β-adrenergic receptors has been extensively reported in SHR vascular tissues, and may play a role in this strain of rat's increased vascular reactivity (Asano *et al.*, 1988), it was of interest to investigate the β-adrenergic receptor-adenylyl cyclase system in vasculature from the MHS. Little has been reported on membrane signal transduction in the MHS, hence, the regulation of the key intracellular enzyme adenylyl cyclase by stimulatory and inhibitory G-proteins was investigated in VSMC in this study. As the activity of PLC has been reported to be diminished in MHS VSMC in response to Ang II, the levels of its regulatory G-proteins (G_q/G_{11}) were measured to ascertain whether an abnormality in the levels of G_q/G_{11} contributed to the blunted PLC activity in response to Ang II. VSMC isolated from thoracic aortas from adult (hypertensive, 60-day-old) and young (prehypertensive, 30-day-old) rats were studied (Chapters 5 and 6, respectively).

5.2 Materials and Methods

5.2.1 Rats

Milan hypertensive strain and Milan normotensive strain rats were sacrificed at 60 days of age. Rats, the same age as those used for preparation of VSMC, had their systolic blood pressures measured: MHS 170±1.9mmHg; MNS 138±1.8mmHg, $p<0.001$. Measurements were made from 19 and 16 rats of MHS and MNS, respectively (significance was assessed by *t*-test analysis).

5.2.2 Smooth muscle cell preparation and maintenance

VSMC were isolated by enzymic digestion from thoracic aortas from four rats of each strain according to the procedure outlined in section 2.5.1. The cells were grown in culture through seven passages by following the methods of sections 2.5.2. and 2.5.3.

5.2.3 Preparation of membranes

Production of plasma membranes from VSMC is outlined in section 2.6.

5.2.4 Measurement of protein concentration

Protein concentrations in the membrane preparations were determined using the method outlined in section 2.7.

5.2.5 Adenylyl cyclase activity

The regulation of adenylyl cyclase activity was monitored by the method of Salomon (1979) outlined in sections 2.8.1 and 2.8.4.

5.2.6 Quantification of G-protein subunits

The levels of the G-protein subunits were quantified using a combination of SDS-PAGE (see section 2.9 for details) and immunoblotting with specific anti-peptide antisera (see section 2.10 for details).

5.2.7 β-Adrenergic receptor measurements

The number and affinity of β-adrenergic receptors in MHS and MNS VSMC membranes were measured according to the procedure outlined in section 2.14.

5.3 Results

5.3.1 Regulation of adenylyl cyclase activity

Basal ACA (whether measured in absence/presence of Mn^{2+} ions) was significantly increased in MHS membranes (Table 5.1). The addition of $MnCl_2$ ($2x10^{-2}M$) resulted in significantly different degrees of inhibition of ACA in MHS and MNS membranes (MHS -67.4±2.5%; MNS -41.3±3.0%, p=0.0002). (A high concentration of Mn^{2+} ions uncouples adenylyl cyclase from its regulatory G-proteins (Limbird *et al.*, 1979). In these VSMC, there appears to be a much greater measurable input from G_s than G_i, hence, uncoupling these G-proteins would result in a fall in ACA.)

A similar trend in activities was seen in the presence of forskolin ($10^{-4}M$), which directly activates the catalytic unit of adenylyl cyclase (MHS 15,709±532 pmol cAMP/15min/mg; MNS 6,597±442, p=0.0002). In view of these inherent differences in enzyme activity, the responses to the various agents studied were

	MHS	MNS	p value
Basal (no Mn^{2+} ions)	2477±232	858±45	p=0.0002
Basal (+ Mn^{2+} ions)	781±70	499±26	p=0.0058

Table 5.1 Basal adenylyl cyclase activities in the absence and presence of $MnCl_2$ ($2x10^{-2}M$) in adult MHS and MNS VSMC membranes. Adenylyl cyclase activity measurements represent pmol cAMP/15min/mg. Data represent mean±S.E. for ten experiments using different membrane preparations.

expressed as % change over basal (measured in the presence of Mn^{2+} ions, as this probably gives the closest approximation to the true basal activity). Figure 5.1 shows the % change in ACA over basal (in the presence of Mn^{2+} ions) in MHS and MNS membranes after the addition of (a) forskolin (10^{-4}M) and (b) NaF (10^{-2}M), isoproterenol (10^{-4}M) and PGE_1 (10^{-5}M). Use of the β-adrenergic agonist, isoproterenol, and PGE_1 allowed measurement of receptor-dependent stimulation of ACA through two different types of stimulatory receptor. Both agonists caused significantly greater stimulation in MHS membranes compared with MNS (isoproterenol: MHS 324±35%; MNS 130±15%, p=0.0002 and PGE_1: MHS 248±30%; MNS 107±14%, p=0.0008).

To examine whether altered regulation of ACA in MHS was only apparent at the receptor level of the adenylyl cyclase system, or whether abnormalities were present at other levels of the signalling pathway, the responses of ACA to forskolin and NaF were studied. There was a significantly greater stimulatory response to forskolin in MHS membranes compared with MNS (MHS 2034±172%; MNS 1254±114%, p=0.0046). However, the stimulations occurring in MHS and MNS membranes in response to another receptor-independent stimulatory agent, NaF, were not significantly different (MHS 490±38%; MNS 507±39%). When the data were expressed as % change from basal (no Mn^{2+} ions), NaF was the only stimulatory agent which produced significantly different stimulations in the two strains, with a reduced response observed in MHS membranes.

G_i function in MHS and MNS membranes was demonstrated by the biphasic effects of GTP on isoproterenol-stimulated ACA (Figure 5.2). These GTP dose-effect experiments showed that activation of G_S and G_i occurred over similar concentration ranges of GTP in MHS and MNS membranes, suggesting that there was no difference in the function of G_i in MHS membranes compared with MNS (MHS -24.1±2.0%; MNS -19.5±4.5%). However, the stimulatory GTP response in MHS membranes was increased in comparison to the response measured in MNS membranes. (G_i function was also measured using low concentrations of poorly hydrolysable analogues of GTP, namely p(NH)ppG and GTPγS. However, only very slight inhibitory responses were observed in these VSMC membranes (data not shown).)

In summary, these experiments examining the regulation of ACA, by expressing data relative to basal activity measured in the presence of Mn^{2+} ions, identified an increased ACA in MHS

Figure 5.1 % Change in ACA expressed relative to basal activity measured in the presence of $MnCl_2$ ($2x10^{-2}M$) in adult MHS and MNS VSMC membranes in the presence of (a) forskolin ($10^{-4}M$) and (b) NaF ($10^{-2}M$), isoproterenol ($10^{-4}M$) and PGE_1 ($10^{-5}M$). Data are mean±S.E. for ten experiments (nine for PGE_1) using different membrane preparations.

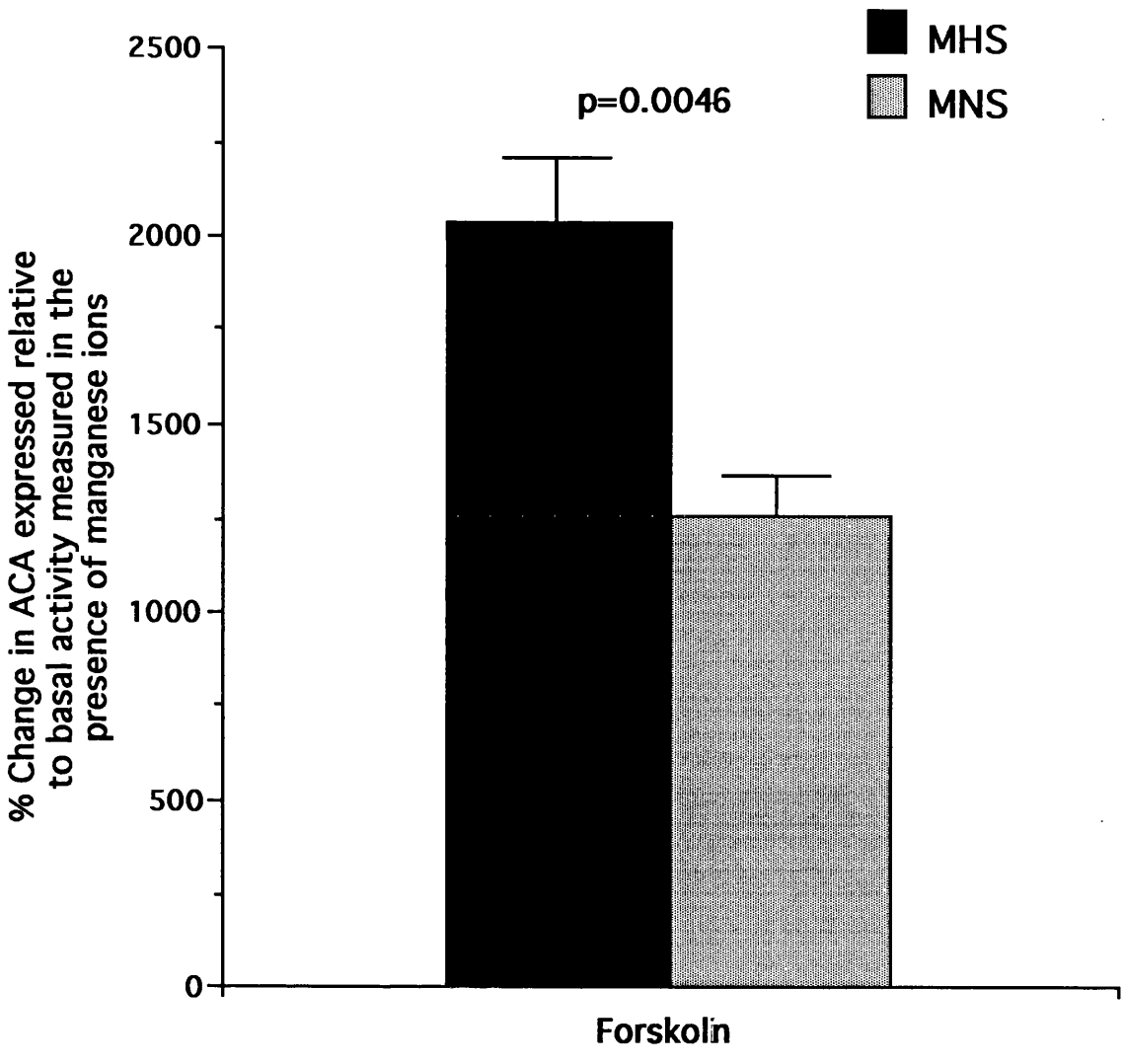

MHS
MNS
p=0.0046
2500
2000
1500
1000
500
0
% Change in ACA expressed relative to basal activity measured in the presence of manganese ions
Forskolin

Figure 5.1(a)

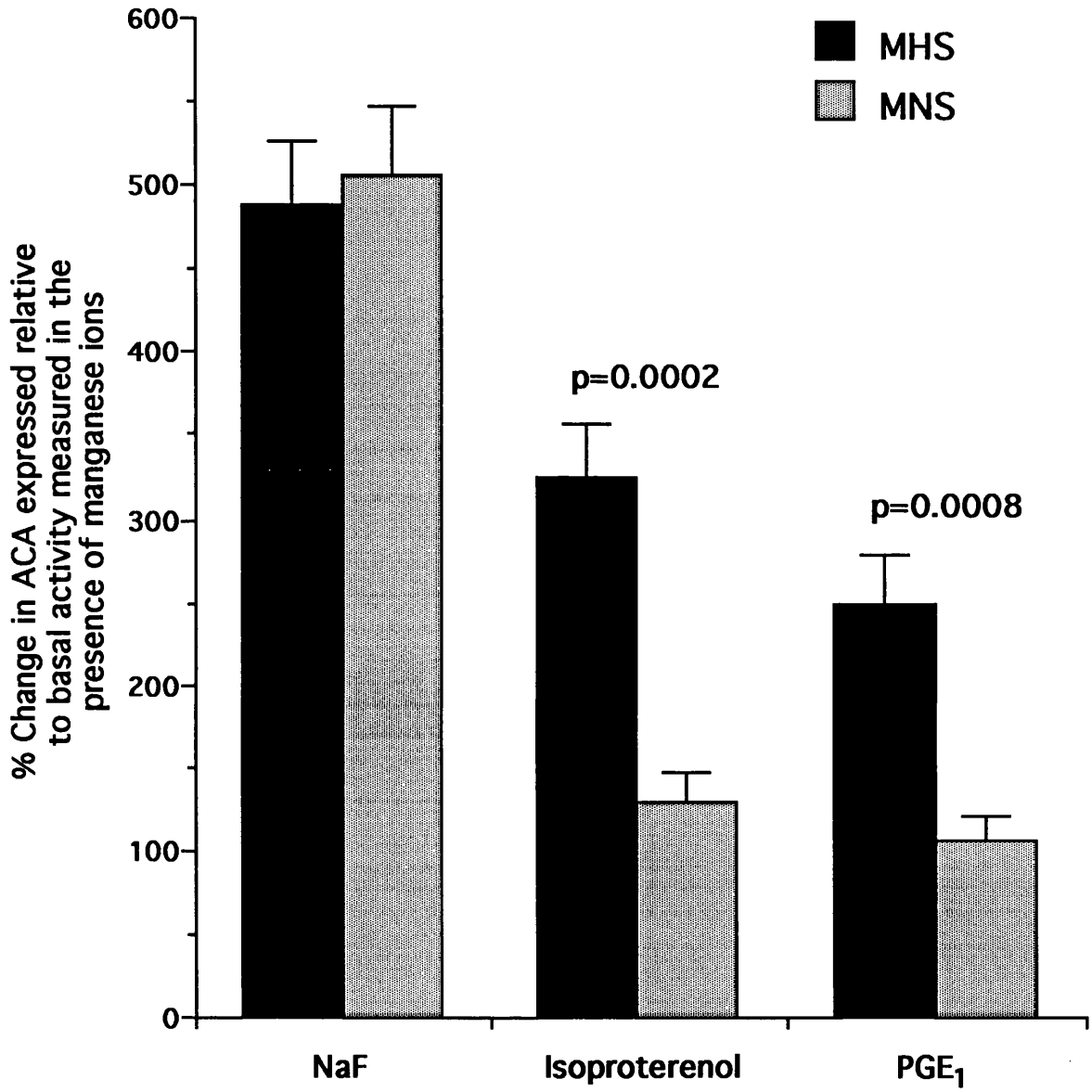
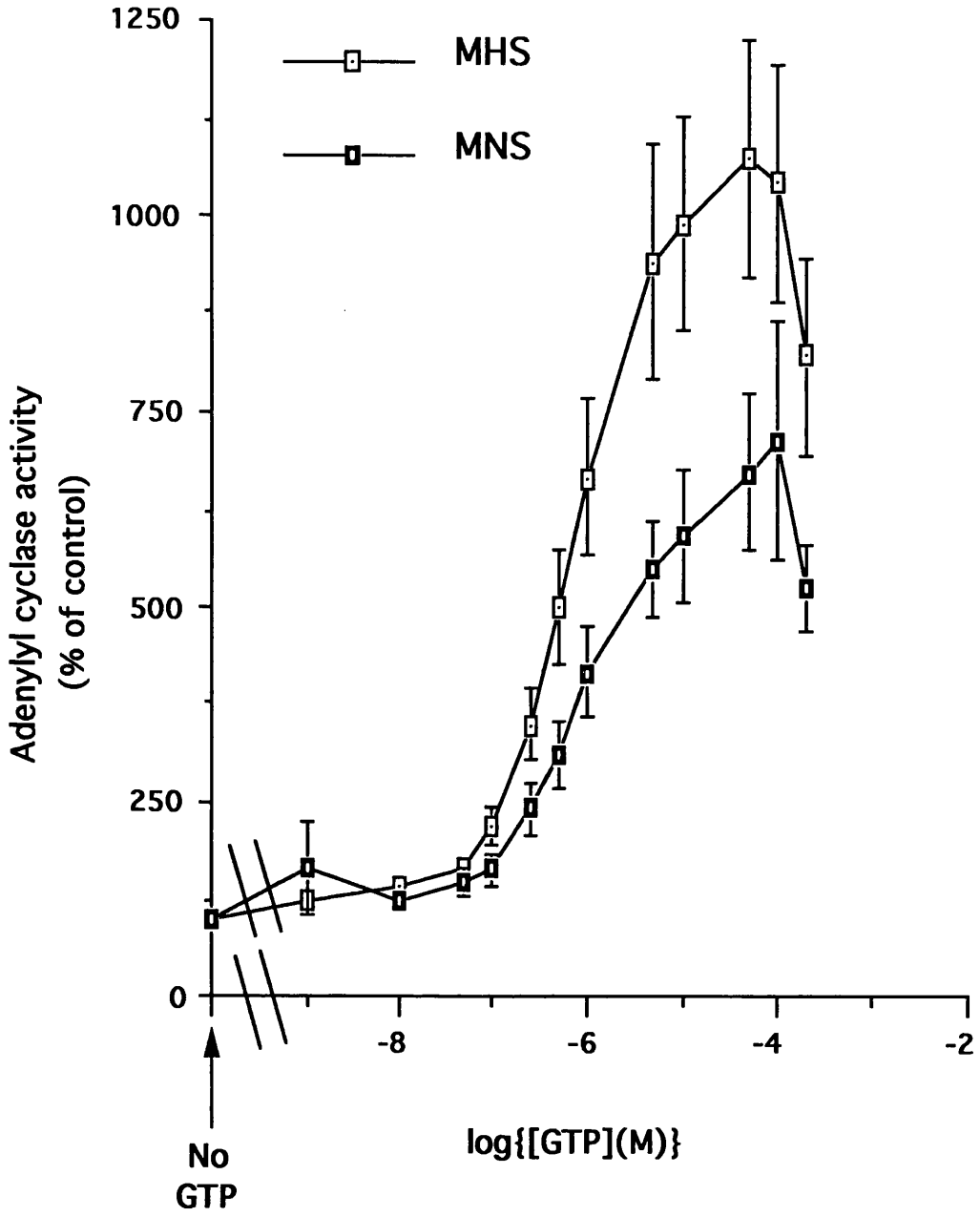

600
500
400
300
200
100
0
% Change in ACA expressed relative to basal activity measured in the presence of manganese ions
MHS
MNS
p=0.0002
p=0.0008
NaF
Isoproterenol
PGE1

Figure 5.1(b)

Figure 5.2 Dose-effect experiments for GTP on isoproterenol-stimulated ACA in adult MHS and MNS VSMC membranes. ACA was monitored in the presence of isoproterenol (10^{-4}M). Data are mean±S.E. for six experiments using different membrane preparations.


1250
1000
750
500
250
0
MHS
MNS
Adenylyl cyclase activity
(% of control)
-8
-6
-4
-2
No
GTP
log{[GTP](M)}

membranes in response to directly activating forskolin and two ligands which mediate stimulation via receptors. However, NaF, which stimulates ACA via direct activation of G_S, resulted in similar levels of activity of the enzyme in both strains. These results suggest an increased level/responsiveness of the adenylyl cyclase catalytic subunit, decreased level/activity of G_S and an increased number of β-adrenergic receptors in MHS VSMC membranes. When data were expressed relative to basal activity measured in the absence of Mn^{2+} ions, a decreased ACA in MHS membranes in response to NaF was identified, while similar stimulations were produced by forskolin, isoproterenol and PGE_1 in the two strains. These data suggest decreased level/activity of G_S and an increased number of β-adrenergic receptors in MHS VSMC membranes. Hence, although expressing the data in the afore-mentioned ways results in different trends regarding the degrees of stimulation, the interpretations of these results are similar with respect to changes at the G_S and β-adrenoceptor level.

5.3.2 G-protein subunit levels

Preliminary immunoblotting experiments identified $G_{S}\alpha$ (two forms; 44 and 42kDa), $G_{i}2\alpha$ and $G_{i}3\alpha$ in VSMC plasma membranes, whereas $G_{O}\alpha$ and $G_{i}1\alpha$ were absent (Figure 5.3). The levels of these different G-protein α-subunits and the level of the β-subunit were quantified in MHS and MNS VSMC membranes using [^{125}I]-labelled anti-rabbit IgG (Figures 5.4, 5.5, 5.6 & 5.7 and Table 5.2). The Figures 5.4, 5.5, 5.6 & 5.7 (also 5.8, see later) show representative nitrocellulose sheets for each G-protein subunit, however, experiments were repeated three times using different membrane preparations. There was a significantly reduced level (~20% reduction) of $G_{S}\alpha$ (both forms) in MHS membranes, while levels of $G_{i}2\alpha$ were similar in MHS and MNS membranes. The level of $G_{i}3\alpha$ was dramatically reduced in MHS membranes (~80% reduction) and a significant reduction in the level of the β-subunit (~20% reduction) was also observed in MHS membranes.

The level of the α-subunit for G_q/G_{11}, the G-proteins coupling receptor activation to generation of DG and IP_3 by PLC, was quantified in MHS and MNS membranes using an antiserum raised against a synthetic peptide which represents the C-terminal decapeptide of the α-subunit of these two G-proteins (Mitchell *et al.*, 1991) (Figure 5.8 and Table 5.2). There was no significant

Figure 5.3 Nitrocellulose sheet with adult MNS VSMC membrane probed with (a) $G_o\alpha$-specific Ab and (b) $G_{i1}\alpha$-specific Ab. 80μg of membrane protein was loaded in each lane. 1=MNS membrane and 2=rat brain membrane.

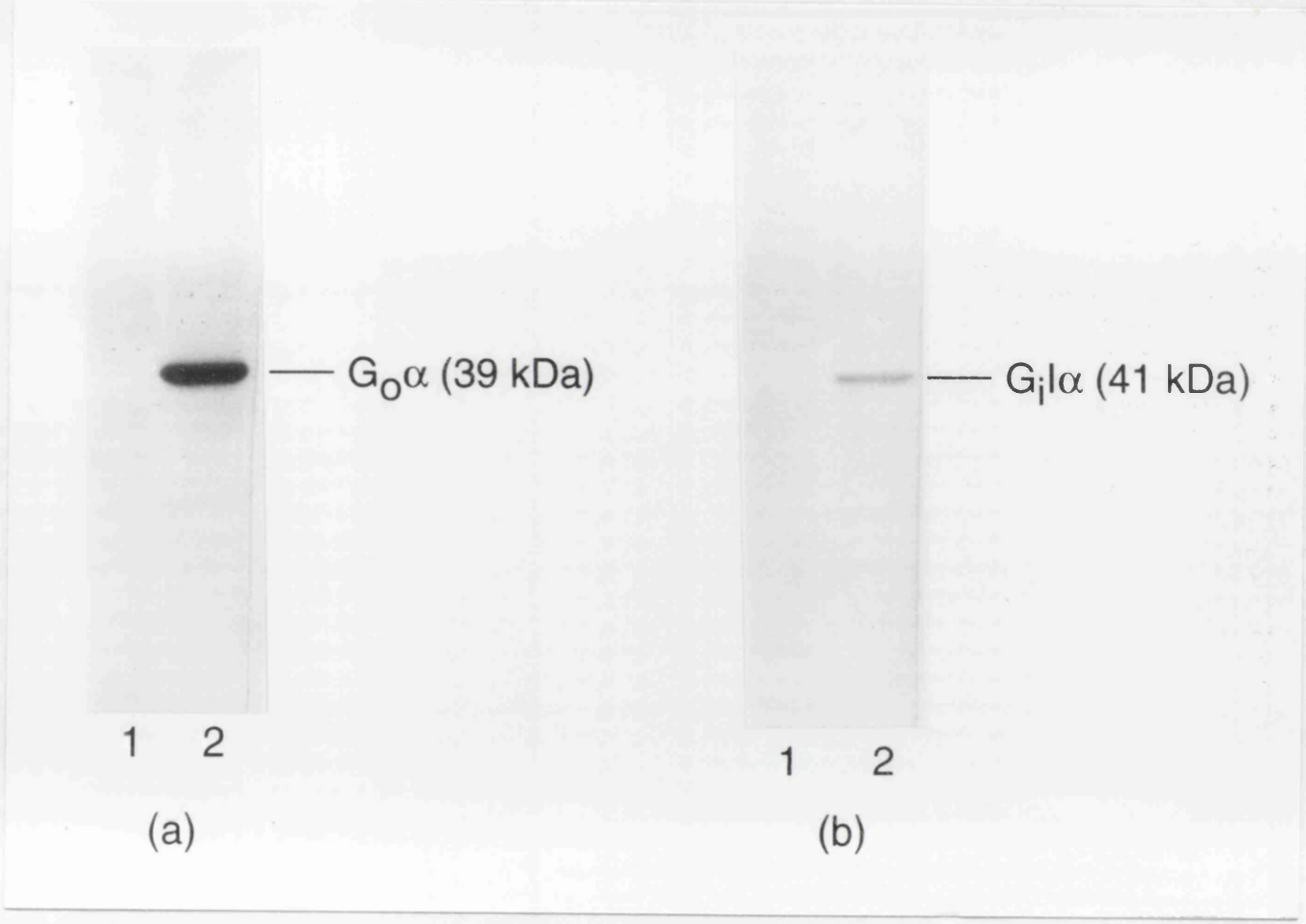

$G_o\alpha$ (39 kDa)
1 2
(a)
$G_i1\alpha$ (41 kDa)
1 2
(b)

Figure 5.4 Nitrocellulose sheet with adult MHS and MNS VSMC membranes probed with $G_S\alpha$-specific Ab (CS1). 50μg of membrane protein was loaded in each lane. Two forms of $G_S\alpha$ are apparent (44 and 42kDa), with the 44kDa form being predominant in VSMC membranes. 1-3=MHS membranes and 4-6=MNS membranes.

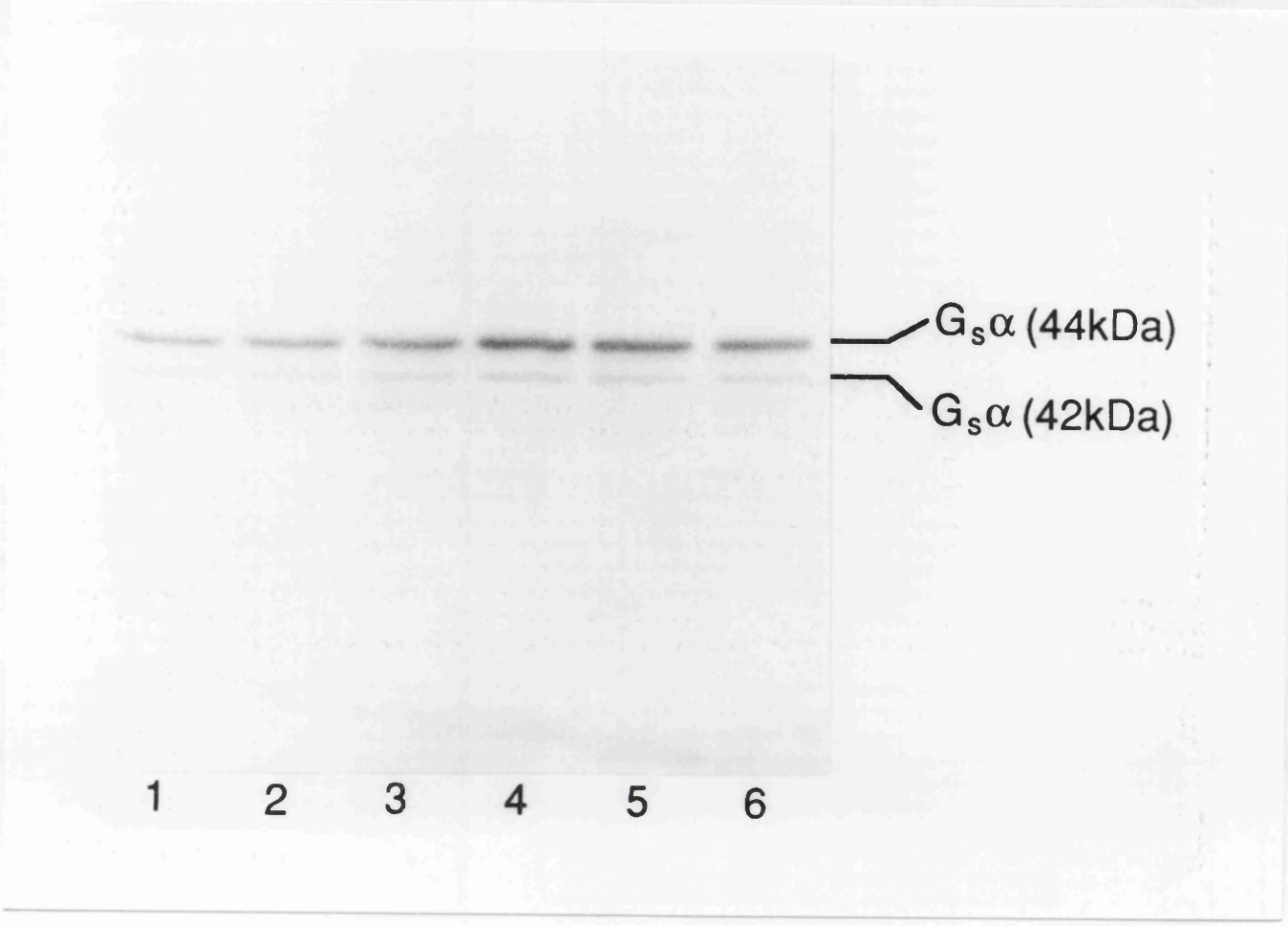

$G_s\alpha$ (44kDa)
$G_s\alpha$ (42kDa)
1
2
3
4
5
6

Figure 5.5 Nitrocellulose sheet with adult MHS and MNS VSMC membranes probed with transducin α, $G_{i}1\alpha$, $G_{i}2\alpha$-specific Ab (SG2). Since rat VSMC membranes do not express detectable levels of $G_{i}1$ and transducin distribution is restricted to photoreceptor-containing tissues, the antiserum SG2 will only identify $G_{i}2$ in rat VSMC membranes. 60μg of membrane protein was loaded in each lane. 1-3=MHS membranes and 4-6=MNS membranes.

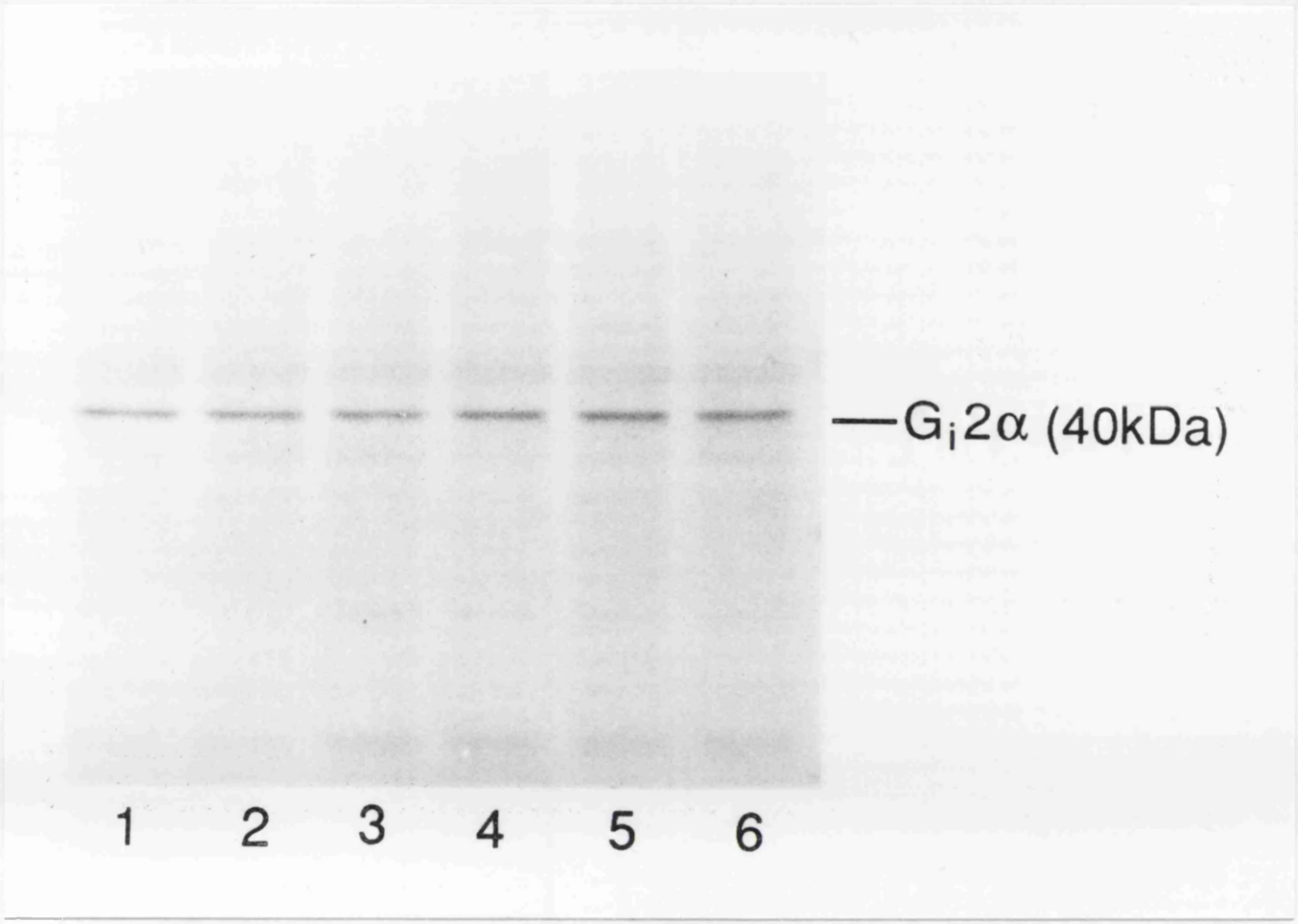

—$G_i2\alpha$ (40kDa)
1
2
3
4
5
6

Figure 5.6 Nitrocellulose sheet with adult MHS and MNS VSMC membranes probed with $G_i3\alpha$-specific Ab (I3C). 50μg of membrane protein was loaded in each lane. 1-3=MHS membranes and 4-6=MNS membranes.

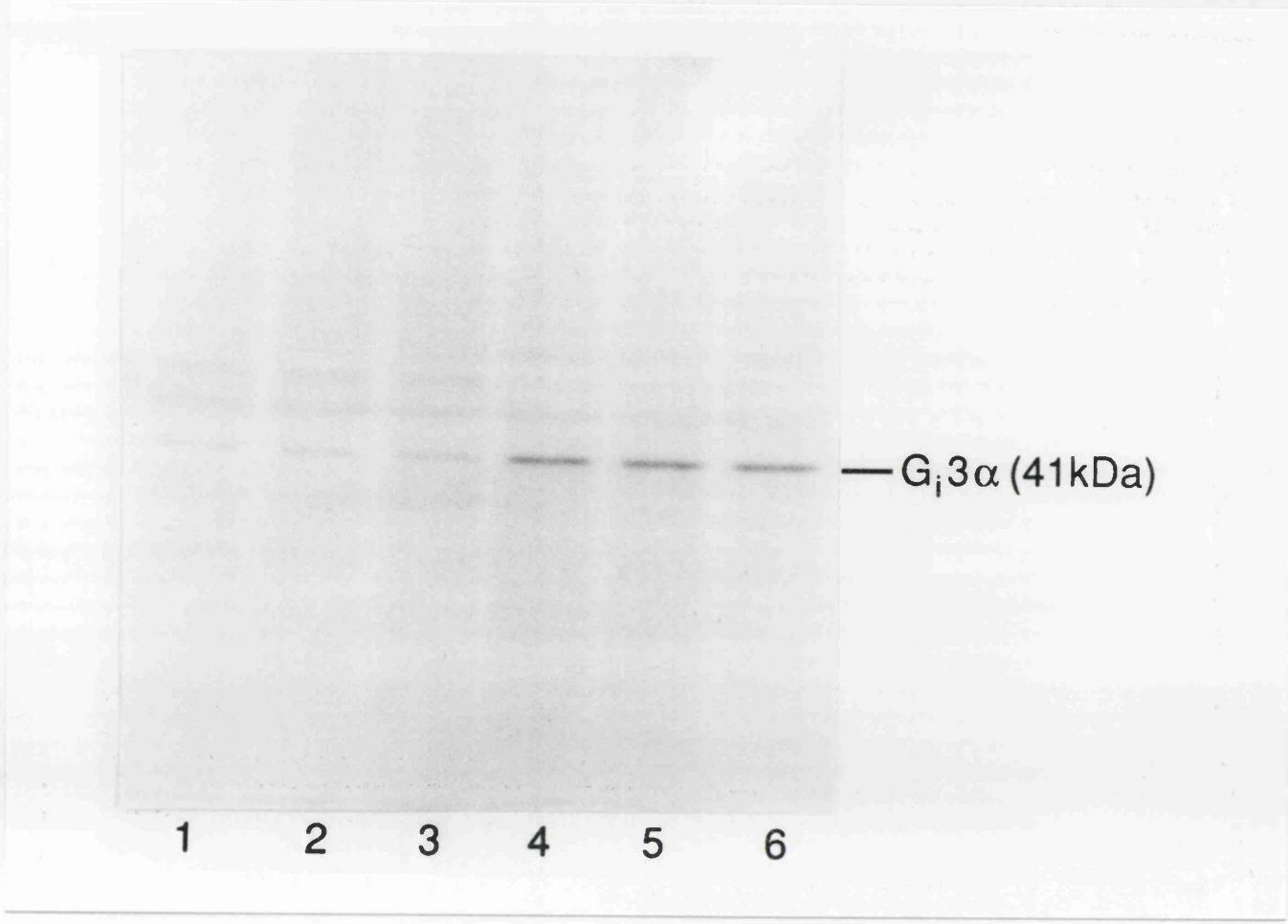

— $G_i3\alpha$ (41kDa)
1
2
3
4
5
6

Figure 5.7 Nitrocellulose sheet with adult MHS and MNS VSMC membranes probed with β-subunit-specific Ab (BN3). 50μg of membrane protein was loaded in each lane. 1-3=MHS membranes and 4-6=MNS membranes.

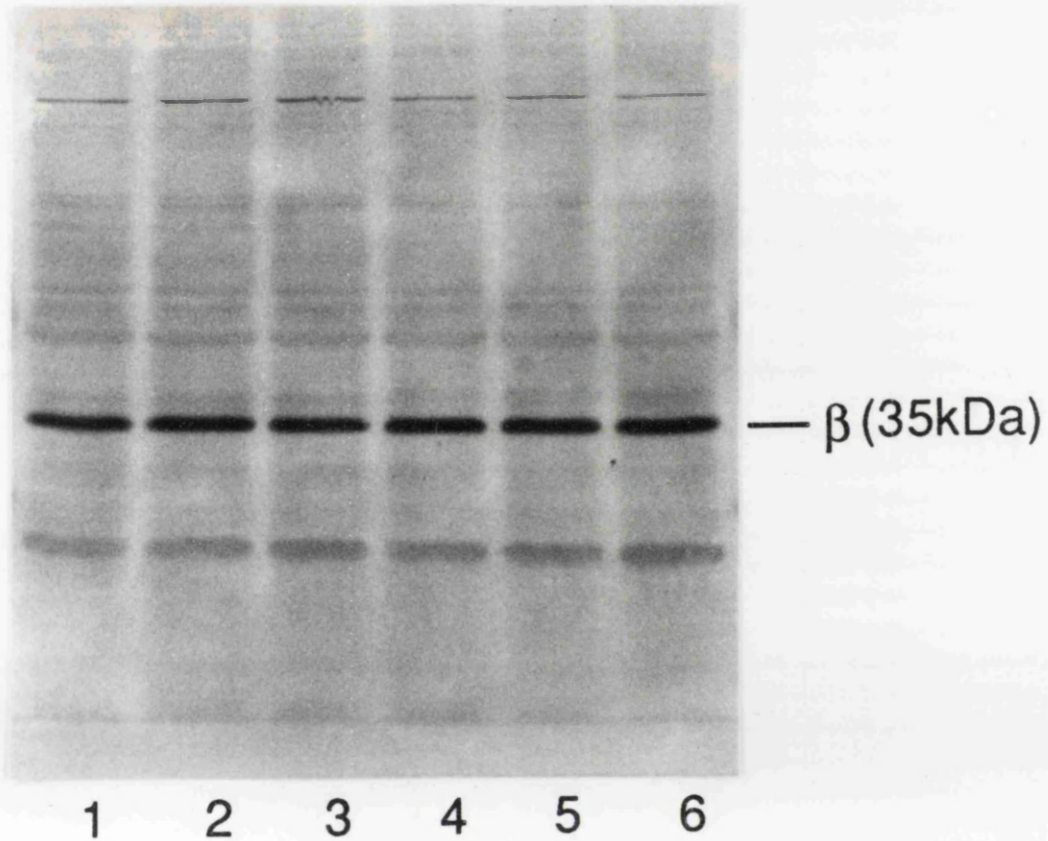
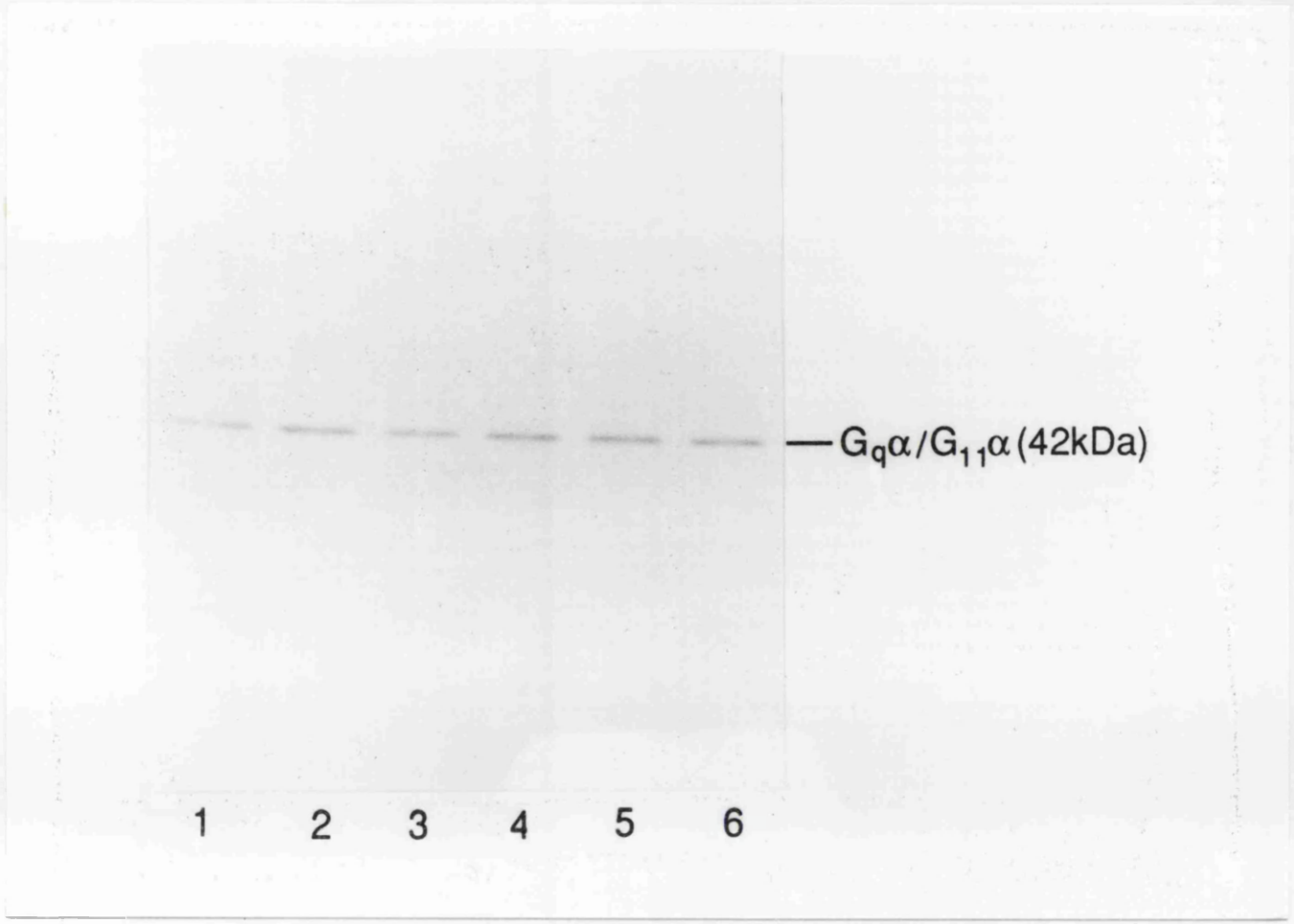

— β (35kDa)
1
2
3
4
5
6

Figure 5.8 Nitrocellulose sheet with adult MHS and MNS VSMC membranes probed with $G_{q}\alpha/G_{11}\alpha$-specific Ab (CQ2). 25μg of membrane protein was loaded in each lane. 1-3=MHS membranes and 4-6=MNS membranes.


— $G_q\alpha/G_{11}\alpha$ (42kDa)
1
2
3
4
5
6

G-protein subunit	MHS (cpm)	MNS (cpm)	
$G_{s\alpha}$ (44kDa)	2268±102	2997±143	*
$G_{s\alpha}$ (42kDa)	921±56	1203±72	*
$G_{i}2\alpha$	3235±165	3107±183	
$G_{i}3\alpha$	195±17	898±41	*
β	1311±62	1576±101	*
$G_{q\alpha}/G_{11\alpha}$	2286±121	2357±153	

Table 5.2 Quantification of bands on nitrocellulose sheets with adult MHS and MNS VSMC membranes using [^{125}I]-labelled Ab. Data represent mean±S.E. for four experiments using different membrane preparations. *Statistically significant difference.

difference in the level of $G_q\alpha/G_{11}\alpha$ present in MHS and MNS VSMC membranes.

5.3.3 β-Adrenergic receptor measurements

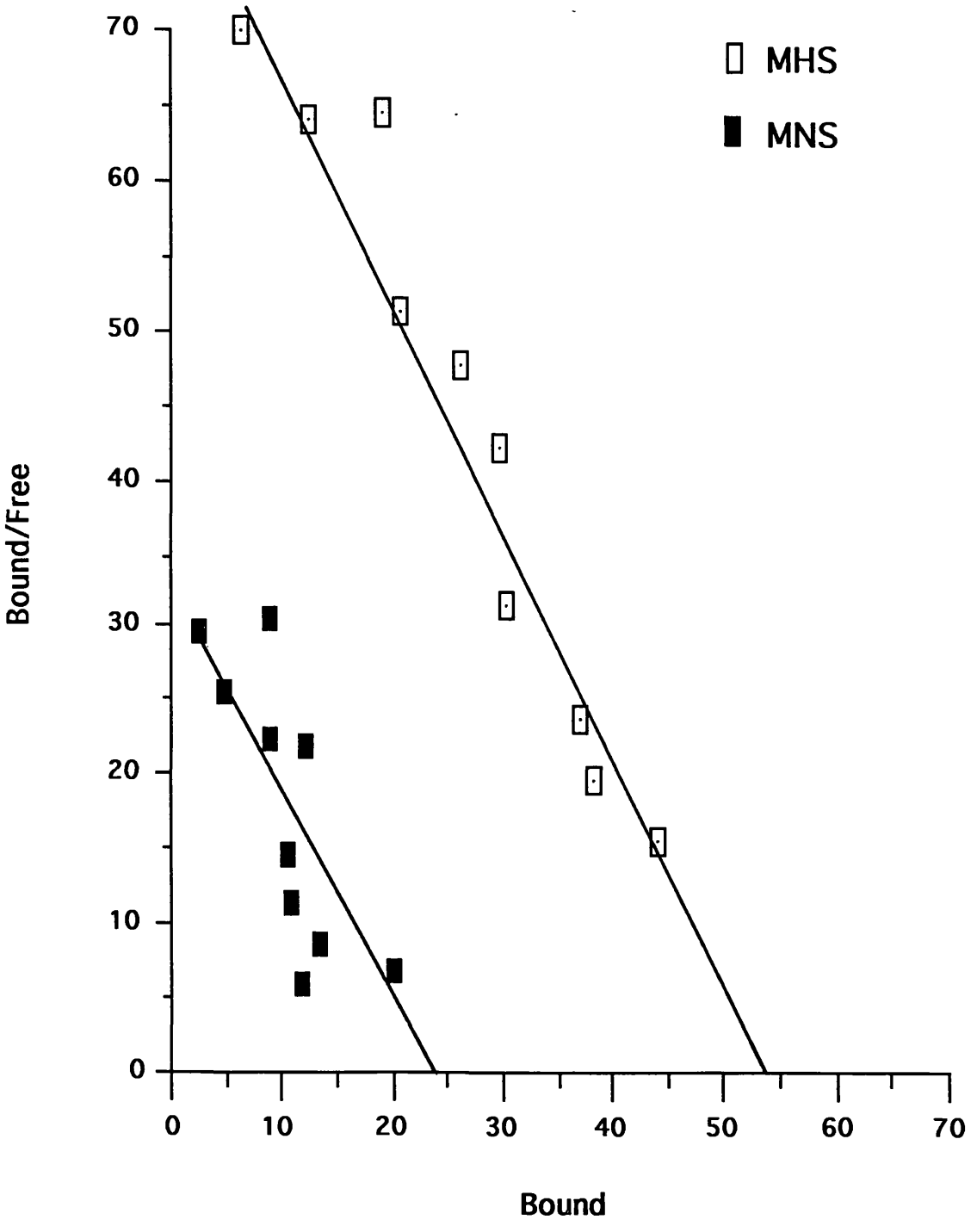
The number and affinity of β-adrenergic receptors in MHS and MNS VSMC membranes were measured from the binding of the antagonist, [^{125}I]CYP, in the presence and absence of propranolol. The affinities of the receptors for antagonist were similar for both strains (MHS K_d 78.6±20.8pM; MNS K_d 69.4±17.0pM), however, the number of receptors in MHS membranes was significantly increased compared with control (MHS B_{max} 54.40±3.44 fmoles/mg protein; MNS B_{max} 22.40±4.41 fmoles/mg protein, p=0.0119). (β-adrenergic receptor measurements represent mean±S.E. for five experiments using different membrane preparations.) Figure 5.9 shows the results from a representative binding assay using MHS and MNS VSMC membranes.

5.4 Discussion

The Milan hypertensive rat has been less extensively studied than other rodent models of genetic hypertension, and there is, therefore, limited information on the pathophysiological associations and causes of its high blood pressure. Angiotensin II receptor linked stimulation of PLC has been reported to be abnormal (Socorro *et al.*, 1990); using the antibody CQ2, which identifies the α-subunit of G_q/G_{11}, it was shown that the levels of these subunits were similar in VSMC membranes from MHS rats when compared with normotensive controls. The blunted PLC response to angiotensin II in MHS VSMC cannot, therefore, be explained by altered levels of its regulatory G-proteins, and alternative explanations must be sought.

Major differences in ACA in VSMC membranes were noted when MHS and MNS were compared. Thus, basal activity (measured in the absence and presence of Mn^{2+} ions) was significantly increased in MHS derived membranes, and corresponding increases in stimulatory responses to forskolin (which acts directly on the catalytic unit of adenylyl cyclase), isoproterenol and PGE_1 (which activate ACA via receptors) were seen when data were expressed relative to basal activity measured in the presence of Mn^{2+} ions. These data contrast with those which were reported earlier in vascular tissue and VSMC from the Okomoto SHR, where stimulated ACA responses were reduced when compared with control strains (Chapters 3 and 4).

Figure 5.9 Binding of [^{125}I]CYP to VSMC membranes from adult MHS and MNS rats. Radioligand bound (fmoles/mg protein) plotted as a function of the ratio of bound to free radioligand (bound/free x 10^{-5}). The intercepts with the abscissa are the maximal number of binding sites (B_{max}); the slope is the apparent affinity (K_d). The data show a typical experiment which was repeated four times with different membrane preparations.


MHS
MNS
Bound/Free
Bound
0
10
20
30
40
50
60
70

Several possible explanations for the increased ACA response in MHS VSMC must be considered, including changes in β-adrenoceptor number/affinity, alterations in stimulatory or inhibitory G-proteins (or coupling of these to receptors and/or catalytic unit of adenylyl cyclase) and changes in the amount or activity of the catalytic subunit of adenylyl cyclase.

5.4.1 β-Adrenergic receptor measurements

It is likely that part of the explanation lies in the increased β-adrenoceptor number demonstrated in MHS VSMC membranes; receptor affinity for antagonist was not altered. This change in β-adrenoceptor number has not previously been reported in Milan rats, and further studies are required to ascertain whether similar changes are present in other relevant tissues. In contrast, the reduced β-adrenoceptor number which has been reported in vascular tissues from SHRs has been implicated in the impaired vascular relaxation noted in arterial tissue in response to isoproterenol (Asano *et al.*, 1988). Vascular reactivity has not been extensively studied in the MHS, and the significance of the increased β-adrenoceptor number (and ACA) in this strain is unclear (discussed later).

5.4.2 G_S and G_i levels and activity

Alterations in the levels or activity of G_S or G_i seem unlikely to account for the increased ACA noted in MHS VSMC. Firstly, a minor decrease in the level of the α-subunit of G_S (both forms) was observed, by immunoblotting, in MHS VSMC membranes. The reason for this is uncertain, but reduced levels of the α-subunits for G_S would result in decreased ACA. Secondly, when ACA was stimulated by NaF, which directly activates G_S to stimulate ACA, no difference could be detected between MHS and MNS. In other words, bypassing β-adrenoceptors led to a loss of the increased response in ACA noted when receptors were activated by isoproterenol.

In theory, a reduced effect of the inhibitory action of G_i might explain an increased ACA response. For example, this explanation has been implicated in the increased ACA response noted in streptozotocin-diabetic animals, where significantly reduced levels and activity of G_i have been reported (Bushfield *et al.*, 1990a). However, in the current study, no evidence for altered G_i levels or

activity was shown to account for the increased ACA response. (To date, three sub-types of G_i have been identified, namely $G_{i}1$, $G_{i}2$ and $G_{i}3$. As discussed imminently, it is proposed that it is $G_{i}2$ that is the inhibitory G-protein involved in ACA regulation in these membranes. The role of $G_{i}3$ in the inhibition of adenylyl cyclase is discussed later.) The level of the $G_{i}2$ α-subunit, measured by immunoblotting, was not altered between the two strains. A minor reduction in the level of the β-subunit in MHS membranes was observed, which would not lead to an increased ACA. G_i function was studied by examining the biphasic effects of GTP on isoproterenol-stimulated ACA. In this instance, low concentrations of GTP promote activation of adenylyl cyclase by stimulating the coupling of β-adrenoceptors to G_S, whereas high concentrations cause inhibition due to activation of G_i (Cooper, 1982 and Heyworth *et al.*, 1984). In both MHS and MNS VSMC biphasic effects of GTP were observed with a similar degree of inhibitory effect at high concentrations, indicating that G_i function was not different between the two strains. These results also support the notion that in these membranes, $G_{i}2$ (as opposed to $G_{i}1$ or $G_{i}3$) is the inhibitory G-protein involved in ACA regulation. Thus, inhibition of ACA was observed despite the fact that the membranes do not express detectable levels of $G_{i}1$. Additionally, G_i function was similar in the two sets of membranes although the level of $G_{i}3$ in MHS was significantly reduced (discussed later).

In some tissues it is possible to observe receptor-mediated inhibition of ACA which is dependent on $G_{i}2$ function (McKenzie and Milligan, 1990). In this study, however, using a range of appropriate ligands (e.g. carbachol, 5-hydroxytryptamine and UK 14304) it has not been possible to demonstrate receptor-mediated inhibition of ACA for reasons which are unclear. Indeed, only slight receptor-mediated inhibitory responses were demonstrated in vascular tissue from another rodent model of essential hypertension, the SHR (Chapter 3).

The extent of stimulation of ACA (in the presence of isoproterenol) with a low dose of GTP was greater in MHS VSMC membranes when compared to MNS. This may suggest altered coupling of β-adrenoceptors to G_S in MHS membranes compared with control, and may contribute, in addition to the increased β-adrenoceptor number, to the increased ACA response to isoproterenol.

5.4.3 Adenylyl cyclase catalytic subunit level and activity

Lastly, differences in the amount and/or activity of adenylyl cyclase catalytic subunit may contribute to the observed differences in ACA. This possibility is supported by the effect of forskolin, which activates adenylyl cyclase directly. When expressed as absolute activity or as percentage stimulation from basal activity measured in the presence of Mn^{2+} ions, MHS was more responsive than MNS, suggesting that in the former strain there is a greater level/responsiveness of adenylyl cyclase catalytic subunit. The level of catalytic subunit was unable to be quantified directly in these studies, as an appropriate antibody was not available.

5.4.4 Summary

Taking all of these data together, therefore, the increased ACA response in MHS VSMC membranes appears to be a net change, reflecting possible increased adenylyl cyclase catalytic subunit activity/level, increased β-adrenoceptor coupling to G_S and an absolute increase in β-adrenoceptor number. These changes may be partially offset by a reduction in the level of G_S. The reason for these changes remains unclear, as is the pathophysiological significance. As discussed earlier, the increased ACA response to isoproterenol contrasts with findings in the SHR, where reduced responses have been noted. Cyclic AMP, generated in response to stimulation of ACA, normally causes vascular smooth muscle cells to relax and modulates intracellular effects induced by a rise in free calcium ions (Tomlinson *et al.*, 1985). It is possible that the changes noted in MHS VSMC membranes are secondary phenomena in response to another primary cause of increased vascular contractility. It should be pointed out, however, that these changes are present in cultured vascular smooth muscle cells which have been through several passages, and where secondary changes in response to a primary rise in blood pressure would be expected to be greatly attenuated. It appears more likely that the changes are genetically determined; how they relate to the development of high blood pressure remains to be studied.

5.4.5 The role of G_i3 in signal transduction

Finally, the unexpected and novel finding of a major reduction in the level of the α-subunit of G_i3 in MHS VSMC membranes when compared with MNS was observed. The functional role of G_i3 in membranes remains unclear, although there is increasing evidence that it may be involved in the regulation of voltage-gated transmembrane potassium flux (Codina *et al.*, 1988). It has recently been demonstrated that in anterior pituitary cells, the coupling of D_2 dopamine receptors to potassium currents is specifically via $G_i3\alpha$ (Lledo *et al.*, 1992). If so, a major reduction in G_i3 might be expected to have significant effects on transmembrane ion distribution. It may be relevant that altered ion transport distribution has been demonstrated in MHS cells (red blood cells, renal tubule cells and vascular smooth muscle cells), and it is possible that these changes may relate to the reduced $G_i3\alpha$ levels noted here.

The role of G_i3 in the coupling of inhibitory α_2-adrenergic receptors to adenylyl cyclase has also been investigated in different cell systems. CHO-K1 cells transfected with the human α_{2A}-adrenergic receptor were used to provide evidence of α_2-adrenergic receptor coupling to at least two sub-types of G_i, namely G_i2 and G_i3, and gave the first evidence of functional involvement of G_i3 in the inhibition of adenylyl cyclase (Gerhardt and Neubig, 1991). However, McClue *et al.* (1992) demonstrated that although G_i3 is activated by α_2-adrenergic agonists in membranes of clone 1C cells (produced by transfection of Rat 1 fibroblasts with genomic DNA encoding the α_2-C10 adrenergic receptor), it does not contribute to the transduction of receptor-mediated inhibition of adenylyl cyclase. The results presented here suggest that in membranes prepared from VSMC isolated from rat thoracic aortas, G_i3 is not involved in the inhibition of adenylyl cyclase, as G_i function (as measured by biphasic effects of GTP on isoproterenol-stimulated ACA) is unchanged in the presence of a dramatic reduction in the level of $G_i3\alpha$. However, work involving the cloned 5-HT_{1A} receptor may be of relevance to the diminished stimulation of PLC which has been reported in response to angiotensin II in MHS smooth muscle cells (Socorro *et al.*, 1990). The cloned 5-HT_{1A} receptor, stably expressed in HeLa cells, has been shown by Fargin *et al.* (1991) to mediate the effects of 5-HT to inhibit cAMP formation and to stimulate the hydrolysis of

phosphatidylinositol. Using specific antibodies to prevent receptor-G-protein coupling by binding to the regions of G-proteins putatively involved in interaction with receptors, this group demonstrated that G_i3 preferentially mediates the effects of 5-HT both to inhibit adenylyl cyclase and to stimulate PLC. The role of G_i3 in the coupling of Ang II receptors to PLC is not known, but if it is involved, then the substantial decrease in the level of G_i3 in MHS membranes may account for the diminished PLC activity in response to Ang II in MHS VSMC. Further studies examining the relationship of this change to the development of high blood pressure in the MHS are appropriate.

Chapter 6 Comparison of vascular G-proteins in young MHS and MNS rats

6.1 Introduction

A large proportion of the work that has been reported on the MHS rat has focussed on both the hypertensive and prehypertensive phases of development (see section 5.1 for details). Bianchi *et al.* (1984) chose the prehypertensive phase of the MHS to study, in order to detect changes that existed before the development of hypertension, and thus, cannot be secondary to the hypertension, *per se*. In addition, genetic factors responsible for hypertension may have produced biochemical or physiological abnormalities during the time-interval between birth and the development of hypertension, and are more strictly linked to the underlying mechanisms causing the hypertension. The study reported in Chapter 5 examined the adenylyl cyclase signalling pathway in adult, hypertensive MHS rats and identified abnormalities in comparison to age-matched control rats. This chapter examines the same system in young, prehypertensive MHS rats to observe whether similar abnormalities are present at an earlier stage in the development of hypertension in this animal model. VSMC were isolated from 30-day-old MHS and MNS rats, which had significantly different systolic blood pressures, but the MHS rats had not established hypertension.

6.2 Materials and Methods

6.2.1 Rats

Milan hypertensive strain and Milan normotensive strain rats were sacrificed at 30 days of age. Rats, the same age as those used for preparation of VSMC, had their systolic blood pressures measured: MHS 125±2.0mmHg; MNS 120±2.3mmHg, $p<0.05$. Measurements were made from 15 rats of each strain (significance was assessed by *t*-test analysis).

6.2.2 Smooth muscle cell preparation and maintenance

VSMC were isolated by enzymic digestion from thoracic aortas from four rats of each strain according to the procedure outlined in

section 2.5.1. The cells were grown in culture through seven passages by following the methods of sections 2.5.2 and 2.5.3.

6.2.3 Preparation of membranes

Production of plasma membranes from VSMC is outlined in section 2.6.

6.2.4 Measurement of protein concentration

Protein concentrations in the membrane preparations were determined using the method outlined in section 2.7.

6.2.5 Adenylyl cyclase activity

The regulation of adenylyl cyclase activity was monitored by the method of Salomon (1979) outlined in sections 2.8.1 and 2.8.4.

6.2.6 Quantification of G-protein subunits

The levels of the G-protein subunits were quantified using a combination of SDS-PAGE (see section 2.9 for details) and immunoblotting with specific anti-peptide antisera (see section 2.10 for details).

6.2.7 β-Adrenergic receptor measurements

The number and affinity of β-adrenergic receptors in MHS and MNS VSMC membranes were measured according to the procedure outlined in section 2.14.

6.3 Results

6.3.1 Regulation of adenylyl cyclase activity

Basal ACA (whether measured in the absence/presence of Mn^{2+} ions) was similar in MHS and MNS VSMC membranes (Table 6.1). The addition of $MnCl_2$ ($2x10^{-2}M$) resulted in similar inhibitions of ACA in MHS and MNS membranes (MHS -26.9±7.4%; MNS -15.6±4.9%). (A high concentration of Mn^{2+} ions uncouples adenylyl cyclase from its regulatory G-proteins (Limbird *et al.*, 1979). In these VSMC, there

	MHS	MNS
Basal (no Mn^{2+} ions)	867±113	799±47
Basal (+ Mn^{2+} ions)	572±30	664±36
Forskolin	9,273±626	8,893±496

Table 6.1 Adenylyl cyclase activities in the absence and presence of $MnCl_2$ ($2x10^{-2}M$), and in the presence of forskolin ($10^{-4}M$) in young MHS and MNS VSMC membranes. Adenylyl cyclase activity measurements represent pmol cAMP/15min/mg. Data represent mean±S.E. for ten experiments using different membrane preparations. Statistical analysis of results did not disclose any significant differences.

appears to be a much greater measurable input from G_s than G_i, hence uncoupling these G-proteins would result in a fall in ACA.) A similar trend in activities was seen in the presence of forskolin (10^{-4}M), which directly activates the catalytic unit of adenylyl cyclase (Table 6.1).

The responses to the various agents studied were expressed as % change over basal measured in the presence of Mn^{2+} ions, as this probably gives the closest approximation to the true basal activity. Figure 6.1 shows the % change in ACA over basal (in the presence of Mn^{2+} ions) in MHS and MNS VSMC membranes after the addition of (a) forskolin (10^{-4}M) and (b) NaF (10^{-2}M), isoproterenol (10^{-4}M) and PGE_1 (10^{-5}M). Use of the β-adrenergic agonist, isoproterenol, and PGE_1 allowed measurement of receptor-dependent stimulation of ACA through two different types of receptor. Isoproterenol caused a significantly reduced stimulation of ACA in MHS membranes compared with MNS, while PGE_1 produced similar stimulatory responses in both strains (isoproterenol: MHS 146±15%; MNS 208±17%, p=0.0155 and PGE_1: MHS 68±12%; MNS 80±16%).

To examine whether altered regulation of ACA in MHS VSMC membranes was only apparent at the β-adrenergic receptor level of the adenylyl cyclase system, or whether abnormalities were present at other levels of the signalling pathway, the responses of ACA to forskolin and NaF were studied. There was a significantly greater stimulatory response to forskolin in MHS membranes compared with MNS (MHS 1,519±68%; MNS 1,250±57%, p=0.0211). However, the stimulations occurring in MHS and MNS membranes in response to another receptor-independent stimulatory agent, NaF, were not significantly different (MHS 397±25%; MNS 426±33%). When the data were expressed as % change from basal (no Mn^{2+} ions), significantly reduced stimulations were observed in MHS VSMC membranes in comparison to MNS in response to both isoproterenol and PGE_1.

G_i function in MHS and MNS VSMC membranes was demonstrated by the biphasic effects of GTP on isoproterenol-stimulated ACA (Figure 6.2). These GTP dose-effect experiments showed that activation of G_s and G_i occurred over similar concentration ranges of GTP in MHS and MNS membranes, suggesting that there was no difference in the function of G_i in MHS membranes compared with MNS (MHS -21.8±2.2%; MNS -18.9±2.6%). The stimulatory GTP responses in MHS and MNS membranes were also similar (Figure 6.2). (G_i function was also measured using low concentrations of poorly hydrolysable analogues of GTP, namely

Figure 6.1 % Change in ACA expressed relative to basal activity measured in the presence of $MnCl_2$ ($2x10^{-2}M$) in young MHS and MNS VSMC membranes in the presence of (a) forskolin ($10^{-4}M$) and (b) NaF ($10^{-2}M$), isoproterenol ($10^{-4}M$) and PGE_1 ($10^{-5}M$). Data are mean±S.E. for ten experiments using different membrane preparations.

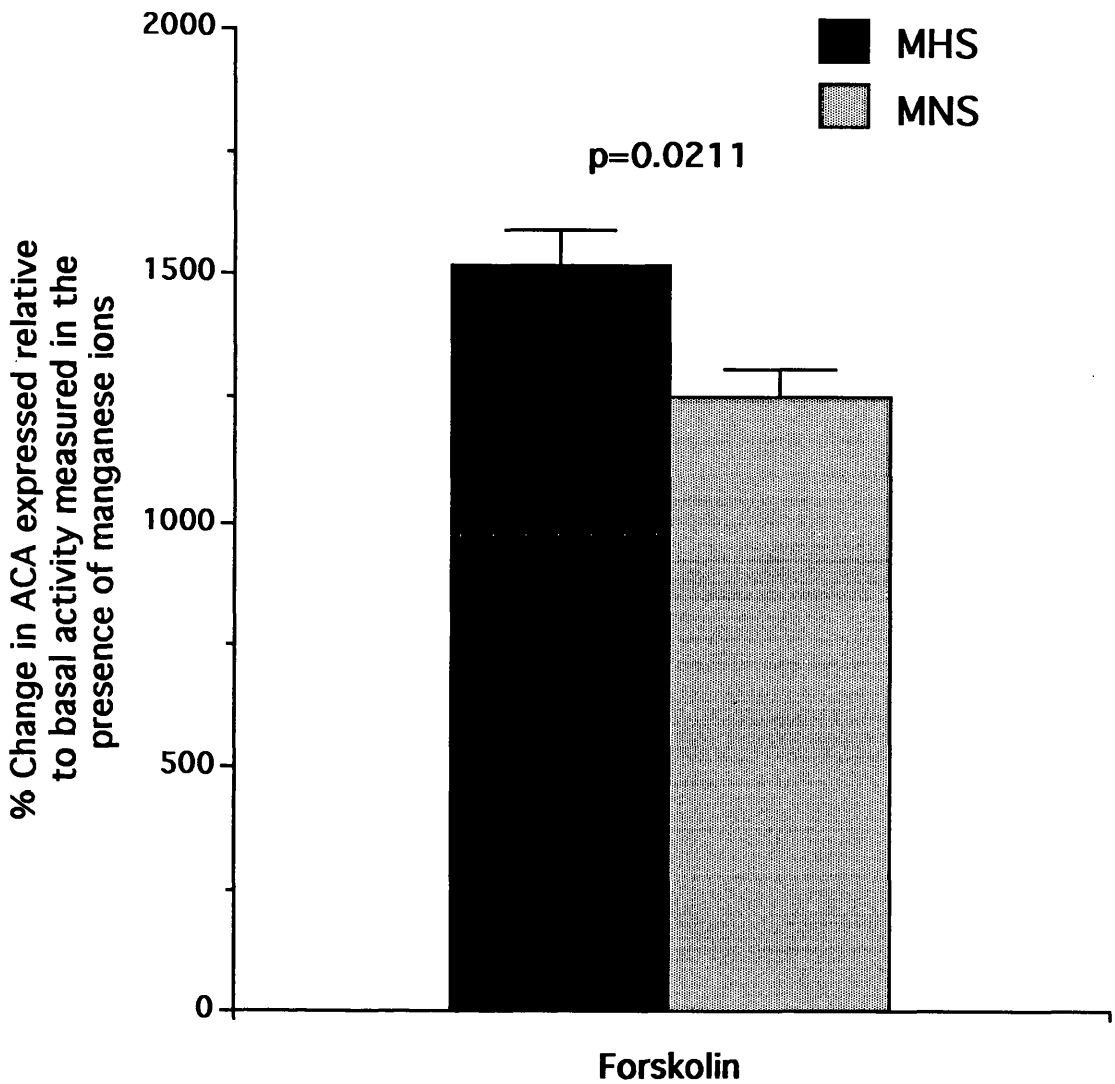

2000
1500
1000
500
0
MHS
MNS
p=0.0211
% Change in ACA expressed relative to basal activity measured in the presence of manganese ions
Forskolin

Figure 6.1(a)

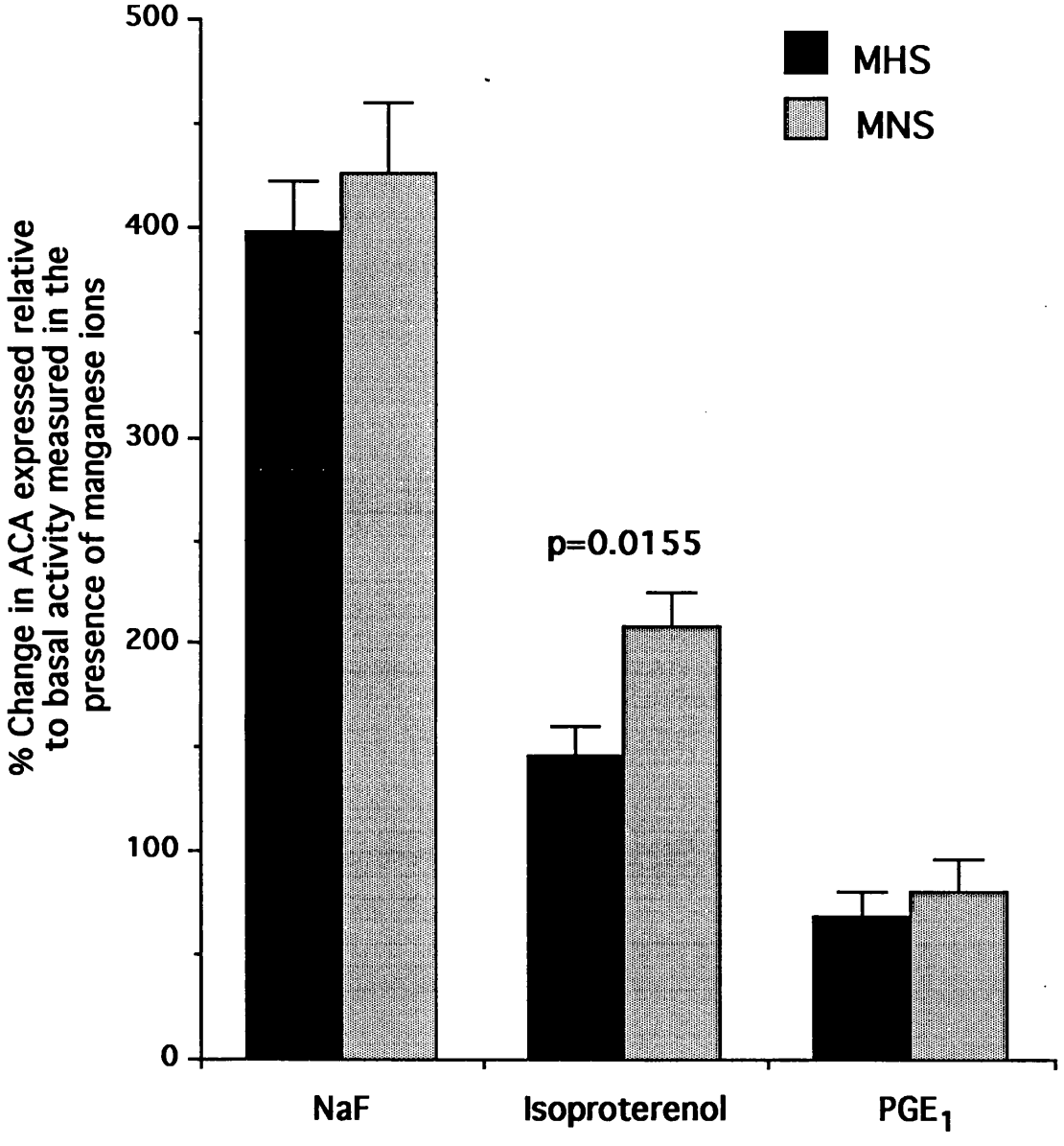

500
400
300
200
100
0
% Change in ACA expressed relative to basal activity measured in the presence of manganese ions
MHS
MNS
p=0.0155
NaF
Isoproterenol
PGE1

Figure 6.1(b)

p(NH)ppG and GTPγS. However, only very slight inhibitory responses were observed in these VSMC membranes.)

In summary, these experiments examining the regulation of ACA in relation to basal activity measured in the presence of Mn^{2+} ions in prehypertensive MHS VSMC membranes identified a decreased ACA in response to isoproterenol which mediates stimulation via β-adrenergic receptors. Directly activating forskolin produced an increased ACA in MHS membranes, but this difference was eliminated in the presence of NaF. These results suggest an increased level/responsiveness of the adenylyl cyclase catalytic subunit, decreased level/activity of G_S and a decreased number of β-adrenergic receptors in MHS VSMC membranes. When data were expressed relative to basal activity measured in the absence of Mn^{2+} ions, decreased enzyme activities in MHS membranes in response to both isoproterenol and PGE_1 were identified, while similar stimulations were produced by forskolin and NaF in the two strains. These data suggest a decreased number of β-adrenergic receptors in MHS VSMC membranes. Hence, whether the response of ACA to isoproterenol in MHS VSMC membranes is expressed relative to basal activity measured in the absence/presence of Mn^{2+} ions, there appears to be a reduction in enzyme response, suggesting decreased β-adrenoceptor number.

6.3.2 G-protein subunit levels

Preliminary immunoblotting experiments identified $G_S\alpha$ (two forms; 44 and 42kDa), $G_i2\alpha$ and $G_i3\alpha$ in rat VSMC plasma membranes, whereas $G_O\alpha$ and $G_i1\alpha$ were absent (data not shown). The levels of these different G-protein α-subunits and the level of the β-subunit were quantified in MHS and MNS VSMC membranes using [^{125}I]-labelled anti-rabbit IgG (Figures 6.3, 6.4, 6.5 & 6.6 and Table 6.2). The Figures 6.3, 6.4, 6.5 & 6.6 (also 6.7, see later) show representative nitrocellulose sheets for each G-protein subunit, however, experiments were repeated three times using different membrane preparations. There was a significantly reduced level (~20% reduction) of $G_S\alpha$ (both forms) in MHS membranes, while levels of $G_i2\alpha$ were similar in MHS and MNS membranes. The level of $G_i3\alpha$ was dramatically reduced in MHS membranes (~80% reduction) and a significant reduction in the level of the β-subunit (~20% reduction) was also observed in MHS membranes.

Figure 6.3 Nitrocellulose sheet with young MHS and MNS VSMC membranes probed with $G_S\alpha$-specific Ab (CS1). 50μg of membrane protein was loaded in each lane. Two forms of $G_S\alpha$ are apparent (44 and 42kDa), with the 44kDa form being predominant in VSMC membranes. 1-3=MHS membranes and 4-6=MNS membranes.

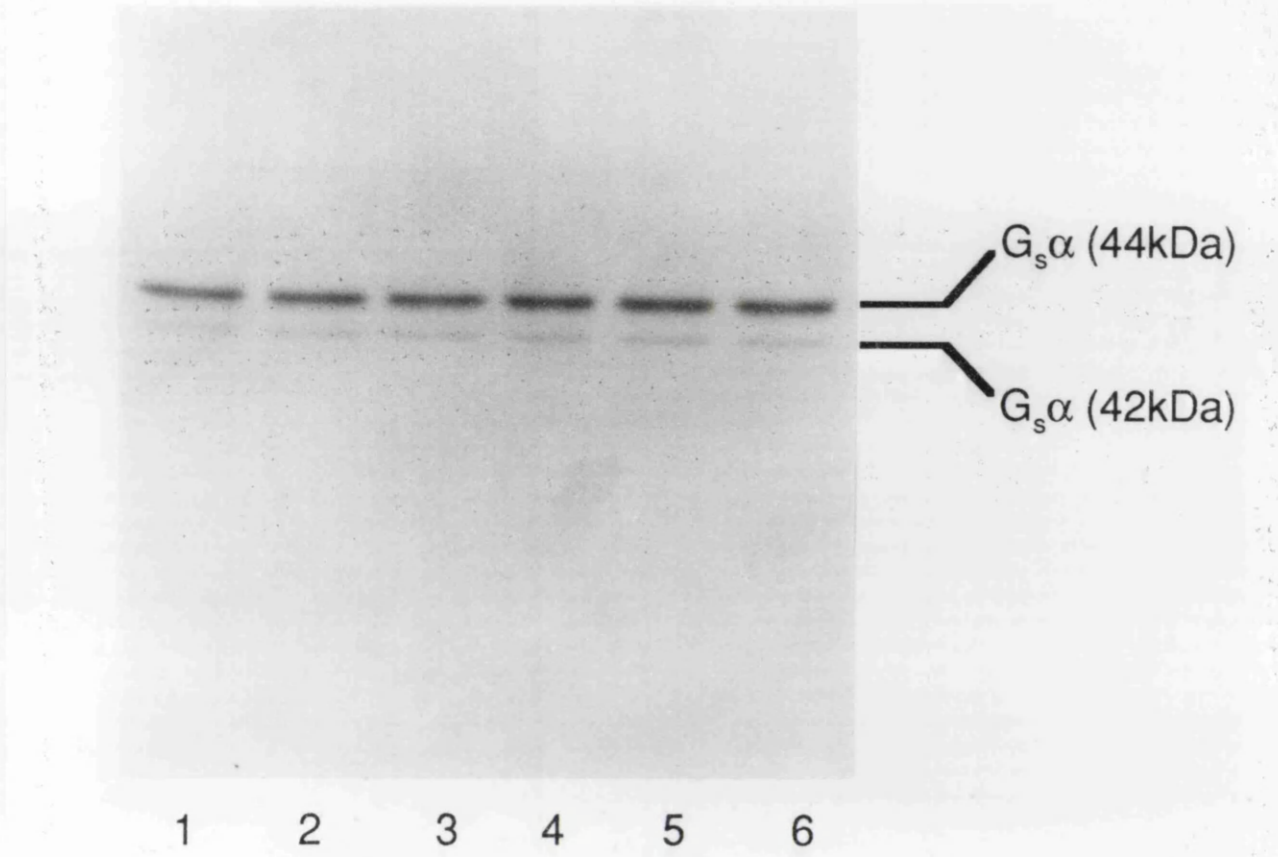

$G_s\alpha$ (44kDa)
$G_s\alpha$ (42kDa)
1
2
3
4
5
6

Figure 6.4 Nitrocellulose sheet with young MHS and MNS VSMC membranes probed with transducin α, $G_i1\alpha$, $G_i2\alpha$-specific Ab (SG2). Since rat VSMC membranes do not express detectable levels of G_i1 and transducin distribution is restricted to photoreceptor-containing tissues, the antiserum SG2 will only identify G_i2 in rat VSMC membranes. 60μg of membrane protein was loaded in each lane. 1-3=MHS membranes and 4-6=MNS membranes.

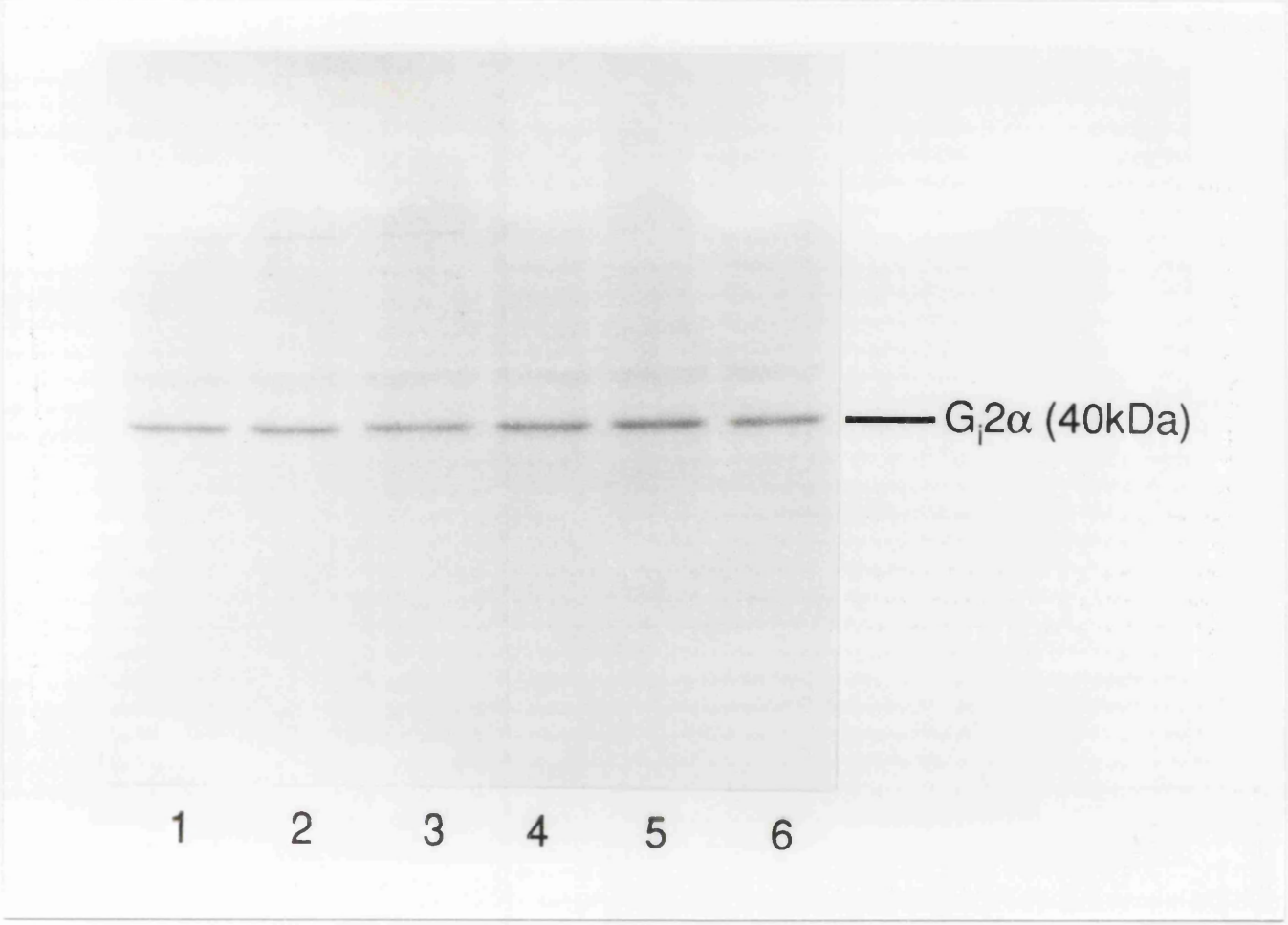

Gi2α (40kDa)
1
2
3
4
5
6

Figure 6.5 Nitrocellulose sheet with young MHS and MNS VSMC membranes probed with $G_i3\alpha$-specific Ab (I3C). 50μg of membrane protein was loaded in each lane. 1-3=MHS membranes and 4-6=MNS membranes.

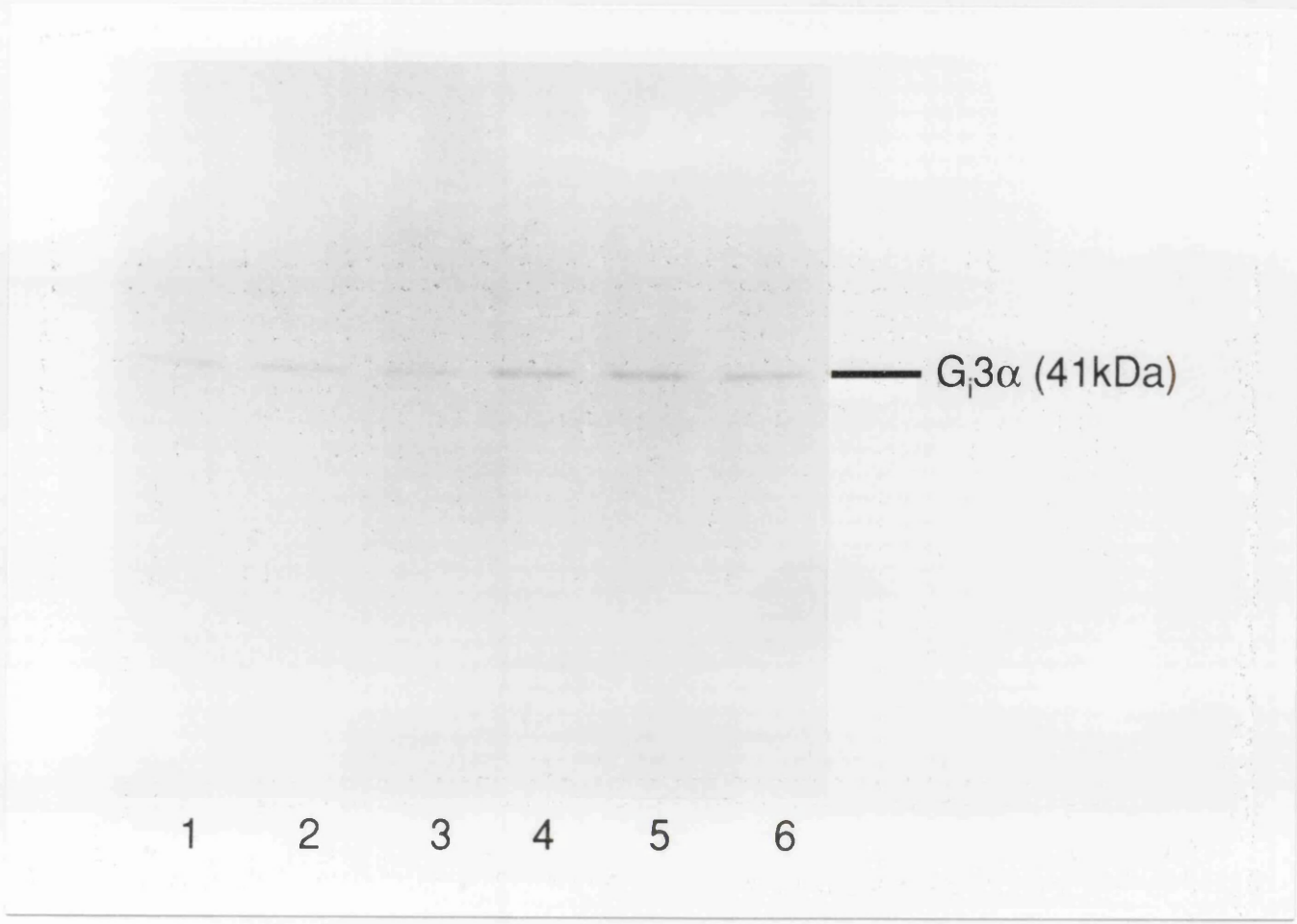

$G_i3\alpha$ (41kDa)
1
2
3
4
5
6

Figure 6.6 Nitrocellulose sheet with young MHS and MNS VSMC membranes probed with β-subunit-specific Ab (BN3). 50μg of membrane protein was loaded in each lane. 1-3=MHS membranes and 4-6=MNS membranes.

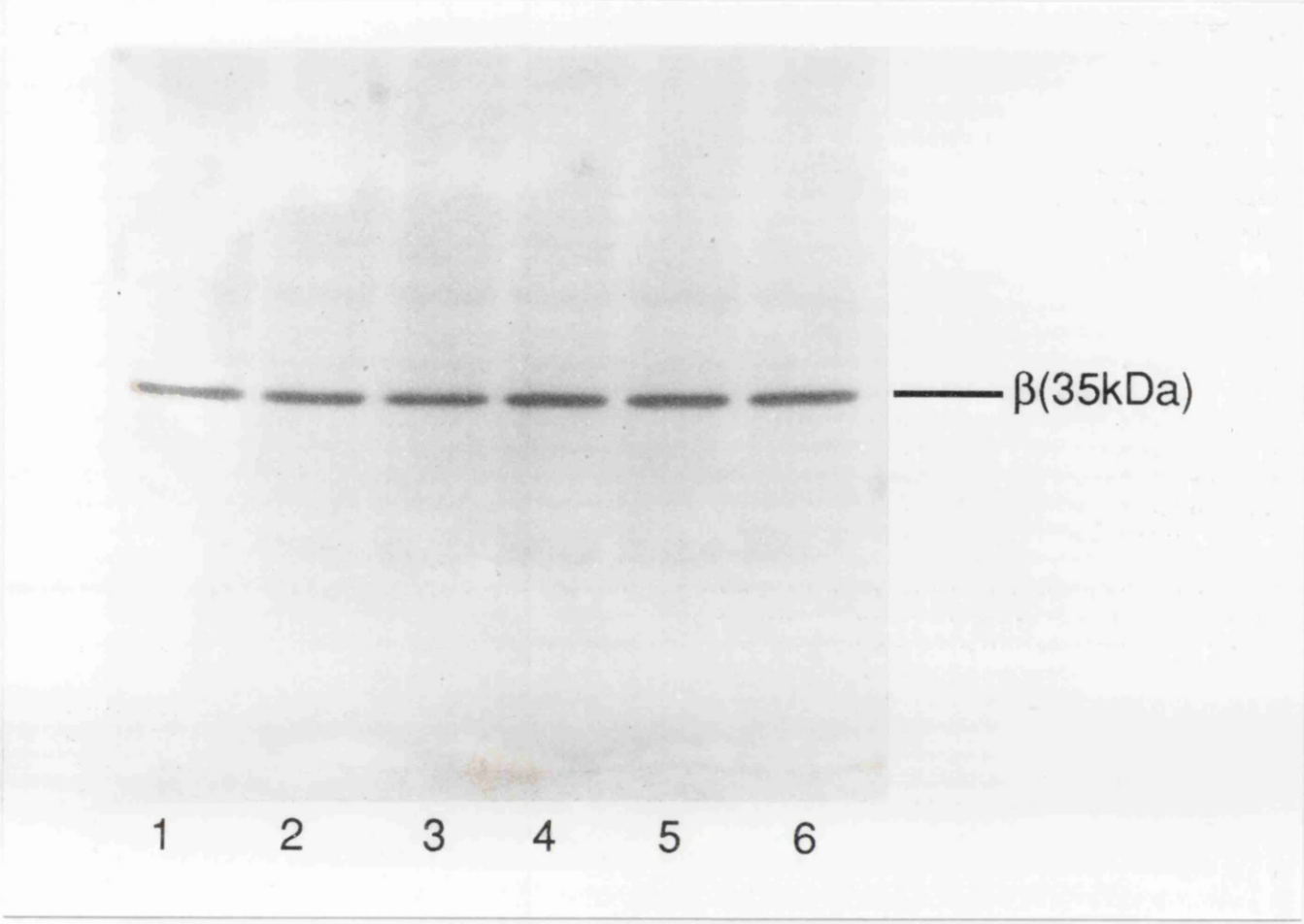
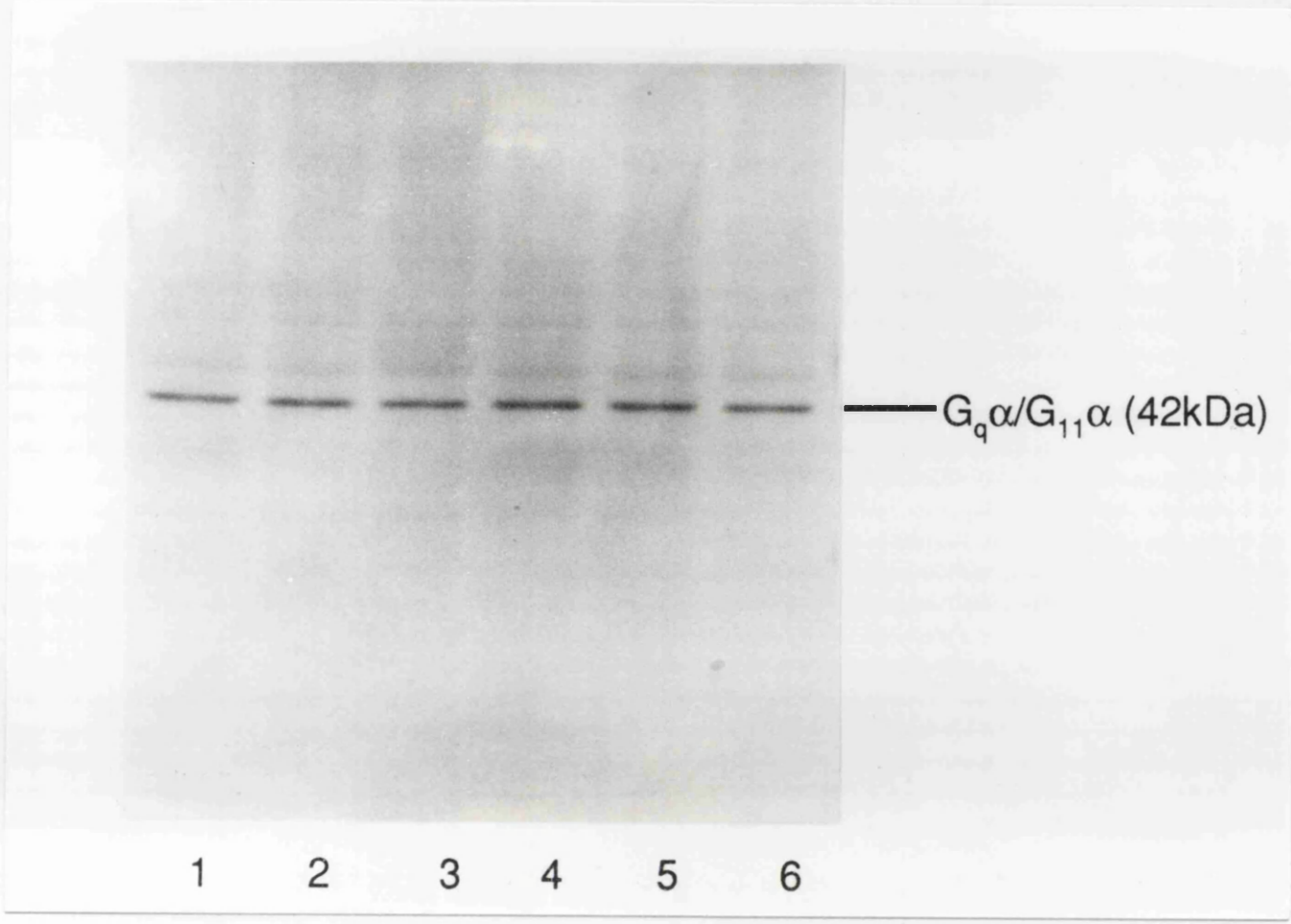

β(35kDa)
1
2
3
4
5
6

Figure 6.7 Nitrocellulose sheet with young MHS and MNS VSMC membranes probed with $G_{q}\alpha/G_{11}\alpha$-specific Ab (CQ2). 25μg of membrane protein was loaded in each lane. 1-3=MHS membranes and 4-6=MNS membranes.


$G_q\alpha/G_{11}\alpha$ (42kDa)
1
2
3
4
5
6

G-protein subunit	MHS (cpm)	MNS (cpm)	
$G_s\alpha$ (44kDa)	3107±256	3985±198	*
$G_s\alpha$ (42kDa)	1103±78	1445±93	*
$G_i2\alpha$	2853±104	2976±115	
$G_i3\alpha$	223±26	1125±89	*
β	1651±97	2059±135	*
$G_q\alpha/G_{11}\alpha$	1972±103	1899±54	

Table 6.2 Quantification of bands on nitrocellulose sheets with young MHS and MNS VSMC membranes using [^{125}I]-labelled Ab. Data represent mean±S.E. for four experiments using different membrane preparations. *Statistically significant difference.

The level of the α-subunit for G_q/G_{11}, the G-proteins coupling receptor activation to generation of DG and IP_3 by PLC, was quantified in MHS and MNS VSMC membranes (Figure 6.7 and Table 6.2). There was no significant difference in the level of $G_q\alpha/G_{11}\alpha$ present in MHS and MNS VSMC membranes.

6.3.3 β-Adrenergic receptor measurements

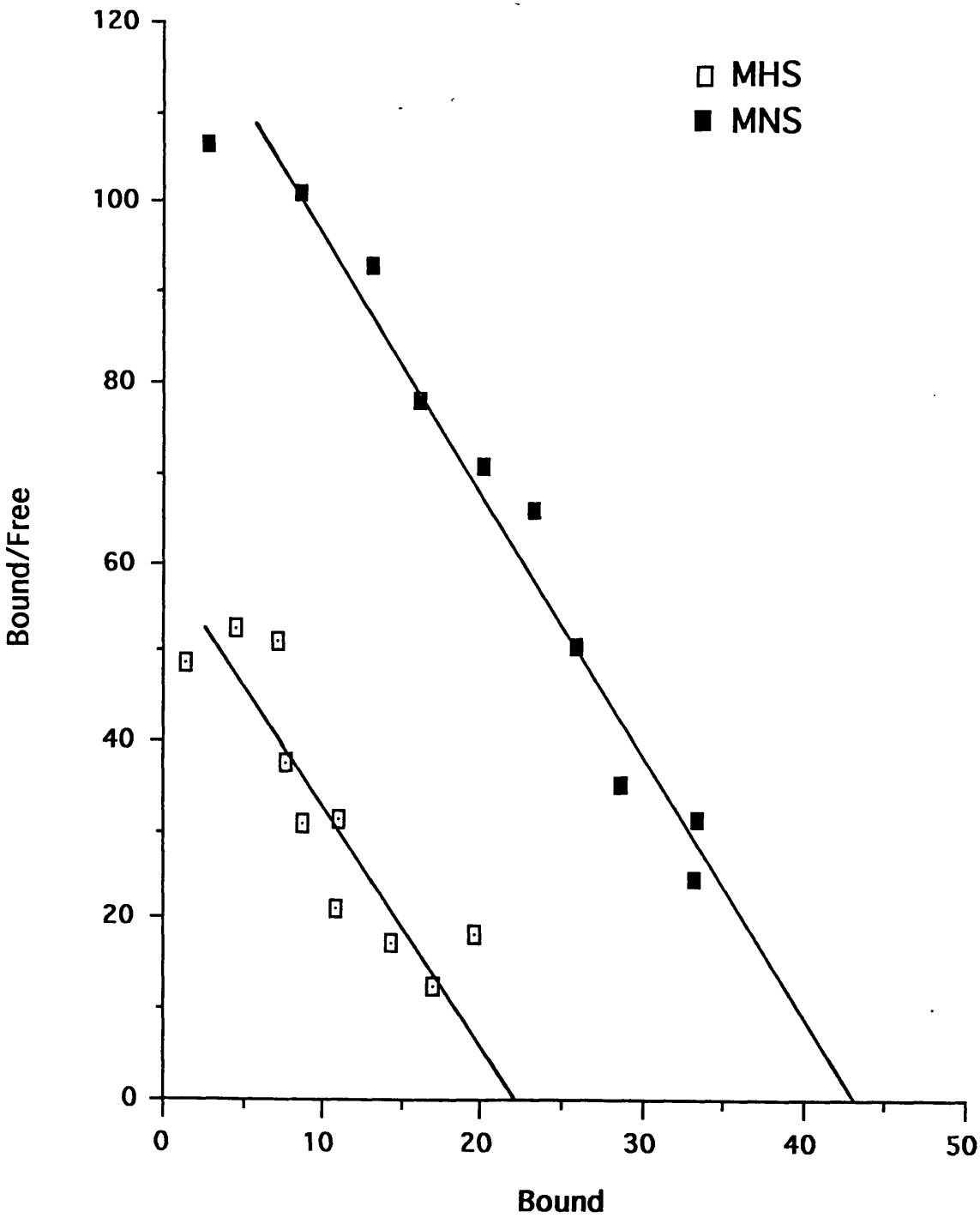
The number and affinity of β-adrenergic receptors in MHS and MNS VSMC membranes were measured from the binding of the antagonist, [^{125}I]CYP, in the presence and absence of propranolol. The affinities of the receptors for antagonist were similar for both strains (MHS K_d 33.3±5.6pM; MNS K_d 22.9±3.3pM), however, the number of receptors in MHS membranes was significantly decreased compared with control (MHS B_{max} 18.4±2.0 fmoles/mg protein; MNS B_{max} 31.7±2.9 fmoles/mg protein, p=0.0131). (β-adrenergic receptor measurements represent mean±S.E. for six experiments using different membrane preparations.) Figure 6.8 shows the results from a representative binding assay using MHS and MNS VSMC membranes.

6.4 Discussion

The Milan hypertensive rat has not been as extensively studied as other rodent models of genetic hypertension e.g. the SHR. Hence, there is limited information on the pathophysiological associations and causes of its high blood pressure. Stimulation of PLC in response to Ang II has been reported to be significantly diminished in MHS VSMC (Socorro *et al.*, 1990); however, as in adult MHS and MNS rats, the levels of the α-subunit of G_q/G_{11} were similar in young MHS and MNS VSMC membranes. Therefore, the blunted PLC response to Ang II in MHS VSMC cannot be explained by altered levels of these regulatory G-protein subunits in either the prehypertensive nor the hypertensive phase.

Significant differences in ACA in MHS VSMC membranes in response to stimulatory agents were noted. Basal activity of adenylyl cyclase (whether measured in the absence or presence of Mn^{2+} ions) was similar in membranes prepared from both strains. In the presence of forskolin (which acts directly on the catalytic unit of adenylyl cyclase), similar absolute levels of ACA were produced in MHS and MNS membranes. However, when results were expressed as % change over basal measured in the presence of Mn^{2+} ions (which

Figure 6.8 Binding of [^{125}I]CYP to VSMC membranes from young MHS and MNS rats. Radioligand bound (fmoles/mg protein) plotted as a function of the ratio of bound to free radioligand (bound/free x 10^{-5}). The intercepts with the abscissa are the maximal number of binding sites (B_{max}); the slope is the apparent affinity (K_d). The data show a typical experiment which was repeated five times with different membrane preparations.


MHS
MNS
Bound/Free
Bound
120
100
80
60
40
20
0
0
10
20
30
40
50

probably gives the closest approximation to the true basal activity), a significantly increased stimulation in response to forskolin was apparent in MHS membranes. This difference was eliminated in the presence of NaF, and actually reversed in response to isoproterenol. The reduced stimulation of ACA in response to isoproterenol in MHS membranes is similar to the situation previously reported in vascular tissue and VSMC from the Okomoto SHR (Chapters 3 and 4). Several possible explanations for the altered ACA response in MHS VSMC membranes must be considered, including changes in β-adrenoceptor number/affinity, alterations in stimulatory or inhibitory G-proteins (or coupling of these to receptors and/or catalytic unit of adenylyl cyclase) and changes in the amount or activity of the catalytic subunit of adenylyl cyclase.

6.4.1 β-Adrenergic receptor number

It is likely that part of the explanation lies in the decreased β-adrenoceptor number which was measured in MHS VSMC membranes; receptor affinity for antagonist was not altered. (As discussed later in section 6.4.4, comparing the results from this study in young MHS and MNS rats with those from a similar study using adult MHS and MNS rats (Chapter 5), reveals opposing regulation of ACA by isoproterenol in prehypertensive and hypertensive MHS rats, as a consequence of altered β-adrenergic receptor number.) The reduced β-adrenoceptor number which has been reported in vascular tissues from SHRs has been implicated in the impaired vascular relaxation noted in arterial tissue in response to isoproterenol (Asano *et al.*, 1988). Cyclic AMP, generated in response to stimulation of ACA, causes VSMC to relax and modulates intracellular effects induced by a rise in free calcium ions (Tomlinson *et al.*, 1985). Hence, a reduced response of ACA to isoproterenol in these animal models of essential hypertension may be of relevance to increased vascular reactivity associated with hypertension.

6.4.2 G_S and G_i levels and activity

Alterations in the levels or activity of G_S or G_i seem unlikely to account for the altered ACA noted in MHS VSMC membranes. The increased stimulation produced by forskolin in MHS membranes was eliminated by NaF, suggesting decreased G_S levels or activity. A

minor decrease in the levels of the α-subunits of G_S (44 and 42kDa) was shown, which may contribute to the NaF result; however, stimulation of ACA by isoproterenol resulted in a reversal of the trend in activities of the enzyme produced by forskolin i.e. decreased ACA was measured in MHS membranes. By stimulating ACA via β-adrenergic receptors, a reduced response was noted in MHS membranes, suggesting aberrations at the receptor level.

Data suggest that altered G_i levels or activity do not account for the altered ACA response. The level of the G_i2 α-subunit, measured by immunoblotting, was not altered between the two strains. G_i function was studied by examining the biphasic effects of GTP on isoproterenol-stimulated ACA. In this instance, low concentrations of GTP promote activation of adenylyl cyclase by stimulating the coupling of β-adrenoceptors to G_S, whereas high concentrations cause inhibition due to activation of G_i (Cooper, 1982 and Heyworth *et al.*, 1984). In both MHS and MNS VSMC biphasic effects of GTP were observed with a similar degree of inhibitory effect at high concentrations, indicating that G_i function was not different between the two strains. These results also support the notion that in these membranes G_i2 (as opposed to G_i1 or G_i3) is the inhibitory G-protein involved in ACA regulation. Thus, inhibition of ACA was shown despite the fact that the membranes do not express detectable levels of G_i1. Additionally, G_i function was similar in the two sets of membranes although the level of G_i3 in MHS was significantly reduced (discussed later in section 6.4.4).

A minor reduction in the level of the β-subunit was shown in MHS membranes, which would lead to a decreased ACA. It has been reported that certain sub-types of adenylyl cyclase are stimulated by βγ-subunits (Federman *et al.*, 1992). This effect of βγ-subunits requires co-stimulation by the GTP-bound form of the α-subunit of G_S. Hence, as minor decreases were noted in the levels of $G_S\alpha$ and the β-subunit, they may contribute to the decreased hormonal stimulation of ACA in MHS membranes.

The extent of stimulation of ACA (in the presence of isoproterenol) with a low dose of GTP was similar in MHS and MNS VSMC membranes. This suggests that the coupling of β-adrenoceptors to G_S is similar in both strains, and the reduced stimulation of ACA in response to isoproterenol observed in MHS membranes is probably a consequence solely of reduced β-adrenoceptor number.

6.4.3 Adenylyl cyclase catalytic subunit

Lastly, differences in the amount and/or activity of adenylyl cyclase catalytic subunit may contribute to the observed differences in ACA. Basal activities of adenylyl cyclase measured in the absence and presence of Mn^{2+} ions were not statistically different in the two strains. Indeed, in the presence of forskolin, similar absolute levels of ACA were observed in both sets of membranes. However, when this response was expressed as % change relative to basal activity measured in the presence of Mn^{2+} ions, a significantly increased stimulatory response was apparent in MHS membranes. This suggests an increased level/activity of the catalytic subunit of adenylyl cyclase in MHS membranes. However, the level of the enzyme's catalytic unit was unable to be quantified, as an appropriate antibody was not available.

6.4.4 Comparison of the adenylyl cyclase signalling system in the prehypertensive and hypertensive phases of MHS rats

As mentioned earlier, it is of interest and importance to compare these data with those from a similar study using older, hypertensive MHS and MNS control rats (Chapter 5). Similar changes in G-protein subunit levels were observed in both young and adult MHS rats. Thus, the levels of $G_{i}2\alpha$ and $G_{q}\alpha/G_{11}\alpha$ were similar in prehypertensive and hypertensive MHS rats compared with age-matched controls, while minor, but nevertheless significant, reductions in the levels of $G_{s}\alpha$ (both forms) and the β-subunit were observed in MHS VSMC membranes from both young and adult rats. The dramatic reduction in the level of $G_{i}3\alpha$ shown in adult MHS membranes was also observed to the same extent in young MHS rats. As discussed in Chapter 5 (section 5.4.5), the functional role of $G_{i}3$ in membranes remains unclear. However, if evidence can be found in the future linking $G_{i}3$ to the coupling of Ang II receptors to PLC, then the substantial decrease in the level of $G_{i}3$ in MHS membranes may account for the diminished PLC activity in response to Ang II in MHS VSMC. This work regarding PLC activity (Socorro *et al.*, 1990) was shown in VSMC from adult MHS rats. It would, therefore, be of interest to examine whether a similar diminished PLC activity is present in VSMC isolated from young MHS rats, as the reduced level of $G_{i}3\alpha$ was shown in both prehypertensive and hypertensive MHS rats. Also, as erythrocytes are genetically determined within the

stem cells, and any abnormalities in these cells would be genetically associated with hypertension, it would be interesting to investigate whether a reduced level of $G_i3\alpha$ is present in MHS erythrocytes.

The function of G_i was not altered in MHS rats in either of the age groups. Indeed, the extents of inhibition, as measured from the biphasic effects of GTP on isoproterenol-stimulated membranes, were ~20% in both strains, in both age-groups. However, there were notable differences in the stimulation of ACA in the two different age groups. An increased basal ACA (in the absence and presence of Mn^{2+} ions) was observed in MHS membranes from older rats, while in young rats, basal activities were similar in the two strains. In both adult and young rats, an increased stimulatory response to forskolin in the MHS was noted, which was eliminated in the presence of NaF. Interestingly, isoproterenol produced opposite effects in the differently aged MHS rats. As mentioned previously, isoproterenol stimulation of ACA was reduced in young MHS rats, although it was observed to be increased in adult MHS rats. Indeed, in young MHS rats a reduced number of β-adrenoceptors was measured, whereas in adult MHS rats an increased number of β-adrenoceptors and a greater stimulatory GTP response were observed. The number of β-adrenergic receptors in MNS membranes did not alter significantly with age; however, in MHS membranes the number of β-adrenergic receptors increased ~3-fold in hypertensive MHS VSMC membranes in comparison to the number of receptors measured in young MHS rats. The GTP responses observed in young and adult MHS membranes were similar, whereas, the GTP response in MNS membranes decreased with age. The reasons for these differences in the regulation of ACA in different age groups of rats are unclear, but since alterations in G-protein subunit levels are similar in both adult and young rats, despite the fact that isoproterenol produces opposite effects, it seems likely that the difference in isoproterenol response in MHS rats compared with MNS in both age-groups is a consequence of altered β-adrenoceptor number.

It is possible that the changes are secondary phenomena in response to another primary cause of increased vascular contractility. It should be pointed out, however, that these changes are present in cultured VSMC which have been through several passages, and where secondary changes in response to a primary rise in blood pressure would be expected to be greatly attenuated. It

appears more likely that the changes are genetically determined, illustrating the importance of measuring parameters at discrete stages in the development of hypertension. For example, a study examining α-adrenergic receptors in the cerebral and renal cortex of MHS rats has identified modifications between the prehypertensive and hypertensive phases (Parini *et al.*, 1987). Since cerebral and renal α-adrenergic receptors play an important role in the control of blood pressure, the densities and affinities of α_1-adrenergic and α_2-adrenergic receptors have been examined. In the cerebral cortex, no between-strain differences in α_1-adrenergic and α_2-adrenergic receptor density and affinity were observed in prehypertensive and hypertensive rats. In the renal cortex, the differences between MHS and MNS rats concerned α_2-adrenergic receptors only. MHS rats showed a lower affinity for the antagonist, [^{3}H]rauwolscine, in the prehypertensive period and absence of the normal age-related increase in receptor density. A lower density of [^{3}H]rauwolscine binding sites in the hypertensive period was also observed. In hypertensive rats, epinephrine bound to one class of sites in MHS rats and to two classes in MNS rats, while data suggest uncoupling of epinephrine receptors from its G-protein in MHS rats. The modifications of renal α_2-adrenergic receptors were more evident in the hypertensive period when the impaired tubular sodium reabsorption observed in the prehypertensive period had almost returned to normal. Taken together, these data suggest that the modification of renal α_2-adrenergic receptors in MHS rats could be a compensatory mechanism to counteract the increased renal sodium reabsorption rather than the cause of the development of hypertension in MHS rats. A similar compensatory mechanism could apply to the regulation of ACA by isoproterenol in young and adult MHS rats i.e. the increased β-adrenoceptor number in adult MHS VSMC membranes may be a compensatory mechanism to counteract the decreased β-adrenoceptor number in young MHS VSMC membranes. Also, basal ACA was significantly higher in adult MHS membranes compared with control (in the absence and presence of Mn^{2+} ions), while basal activities of the enzyme were similar in young MHS and MNS rats (in the absence and presence of Mn^{2+} ions). (It should be noted that the absolute level of basal ACA in MNS membranes is similar in both age-groups, while it is only the basal ACA in MHS membranes which changes with age.) The increased basal ACA and increased response of ACA to isoproterenol in adult MHS membranes may be a compensatory reaction to produce a higher

concentration of the vasodilator, cAMP, in response to an elevation in blood pressure.

It would be of interest to examine whether a similar pattern of alterations regarding basal ACA and β-adrenergic receptor number exists in other relevant tissues (e.g. myocardium) from prehypertensive and hypertensive MHS rats. Also, the relevance of the abnormalities regarding β-adrenergic receptor number and G_i3 α-subunit levels to the hypertensive phenotype could be investigated further by employing cosegregation studies. Every single one of the genetic differences present between disease and control strains represents a polymorphic marker that distinguishes with equal statistical power between strains, yet only a tiny fraction represent disease-related genes. Hence, differences observed on any level (physiological, biochemical, or molecular genetic) between the hypertensive and the normotensive strains are, in fact, much more likely unrelated than related to hypertension. Geneticists many years ago proposed a seminal test for a polymorphic marker's relevance to the hypertensive phenotype involving cosegregation of the marker with blood pressure in a freely segregating F_2 cohort bred from the two parental strains. This approach effectively filters out all polymorphic markers except the ones that are relevant to the phenotype of interest (Lindpaintner *et al.*, 1992). It would be interesting to examine whether the changes in β-adrenergic receptor number and/or G_i3 α-subunit levels cosegregated with blood pressure in F_2 MHS rats.

Chapter 7 Final Discussion and Conclusions

7.1 Final Discussion

7.1.1 Choice of genetically hypertensive rat

Genetically hypertensive rats are valuable models of human essential hypertension, as it is possible to have direct access to vascular tissues, and hypertension develops in these rats with little influence from the environment. There are several genetically hypertensive rat strains available internationally, and the work of this thesis has employed two such strains, namely the spontaneously hypertensive rat and the Milan hypertensive strain.

The SHR is the most widely studied rodent model of genetic hypertension, and closely resembles human hypertensives with respect to its pattern of development of hypertension, increased vascular smooth muscle contractility, and altered membrane cation transport. On the other hand, the MHS develops mild hypertension for the first two months after birth, but shows no further augmentation of hypertension during the process of aging. Hence, the developmental course of hypertension is different from essential hypertension, which tends to increase gradually with age. However, the key role of the kidney in the pathogenesis of hypertension in the MHS has been demonstrated (Fox and Bianchi, 1976), and a correlation has been found between erythrocyte Na^+ transport abnormality and renal function in both these rats and humans (Bianchi *et al.*, 1986).

Altered regulation of ACA has been demonstrated in SHR vascular tissues (see section 3.1 for details); however, the role of regulatory G-proteins in this abnormality has not been studied in detail. Investigators utilising the SHR in studies of ACA regulation used WKY rats as the normotensive control. The studies in this thesis have been aware of possible genetic heterogeneity in SHRs and WKY rats and have, therefore, used rats from different suppliers and included the Wistar rat as an additional normotensive control. The adenylyl cyclase system has not been examined in any tissue from the MHS, and so it was of interest to pursue these studies further. Two genetically hypertensive rat strains were studied to investigate whether similar abnormalities regarding the regulation of ACA existed in the two strains, despite possible differences in their pathogenetic mechanisms.

7.1.2 Expression of results

G-protein function was inferred from studies of adenylyl cyclase activity using the method of Salomon (1979). A range of agents was used in these studies to examine the abnormalities at specific levels of the adenylyl cyclase system. Stimulatory agonists (isoproterenol and PGE_1) were employed to investigate possible alterations at the receptor level, while NaF and forskolin revealed any alterations at G_S and the catalytic subunit of adenylyl cyclase, respectively. The responses to these agents were expressed relative to basal ACA, as there were generally differences in the basal activities of the enzyme in the strains being compared. In such instances, comparison of the absolute levels of ACA measured in response to a specific agent would not take this difference into account. Results were expressed relative to basal activity measured in the absence and presence of Mn^{2+} ions. The "Results" and "Discussion" sections of each of the four chapters encompassing these studies have considered the responses expressed relative to basal activity measured in the presence of Mn^{2+} ions in more depth. This is due to the fact that a high concentration of Mn^{2+} ions uncouples adenylyl cyclase from its regulatory G-proteins (Limbird *et al.*, 1979), and so probably gives a more realistic measurement of basal activity of the enzyme without any influence from G_S and G_i.

7.1.3 Comparison of similar studies employing SHRs from different sources

Chapters 3 and 4 give details of studies examining the contribution of G-proteins to the altered regulation of ACA in SHRs bought from different suppliers. Genetic heterogeneity has not only been reported in the WKY rat (Kurtz and Morris, 1987 and Kurtz *et al.*, 1989), but also in the SHR (Nabika *et al.*, 1991) from different sources. Chapter 3's study used the outbred Wistar strain of rat as an alternative normotensive control, while the study reported in Chapter 4 used both Wistar and WKY rats as controls to examine whether differences between SHRs and WKY rats were still seen when another normotensive control was included.

Chapter 4 reported differences in the regulation of ACA among SHRs, WKY and Wistar rats; however, differences in the regulation of ACA between the two normotensive control strains were obvious. While these changes may reflect hypertension and its genetic

antecedent, the possibility is raised, by inclusion of the outbred Wistar control group, that genetic heterogeneity in SHR and WKY strains may contribute substantially to the differences in the regulation of ACA, and raise the possibility that these changes are not directly linked to the development of increased blood pressure.

Differences in the regulation of ACA in SHR membranes compared with Wistar were observed in both Chapters 3 and 4. Although no significant differences in the levels of the various G-protein subunits in SHR and Wistar membranes were observed using rats from different suppliers, inconsistent differences in the regulation of ACA were obvious, and seemed to depend on where the rats were purchased. Hence, possible genetic heterogeneity between the two groups of SHRs from Charles River Ltd. and Harlan Olac Ltd. may have influenced the differing results of the similar studies.

7.1.4 Age-related changes in the MHS

Chapters 5 and 6 examined the regulation of ACA in VSMC prepared from the thoracic aortas of adult (hypertensive) and young (prehypertensive) MHS rats, respectively. The differences in the levels of the various G-protein subunits in MHS and MNS membranes were similar in the two age-groups. It was also observed that G_i function was unaltered in MHS membranes with respect to MNS in both age-groups. The abnormal regulation of ACA in the MHS was consistent with age regarding the responses to forskolin and NaF; however, the stimulations produced by isoproterenol were reversed when MHS and MNS rats were compared at the different ages. Young MHS rats exhibited a reduced ACA in response to isoproterenol in comparison to age-matched MNS controls, while an increased stimulation was produced by isoproterenol in adult MHS rats. This change in response to isoproterenol was paralleled by an appropriate alteration in β-adrenergic receptor number. It is unlikely that this age-dependent alteration in isoproterenol response (and β-adrenergic receptor number) is a primary cause of hypertension in the MHS, as the trend would be expected to be reversed if this were the case. Instead, it may represent some sort of compensatory mechanism in the MHS, to combat the increased VSM contractility which may result with elevated blood pressure.

7.1.5 Similar abnormalities in the SHR and MHS?

The work of this thesis has concentrated on two rodent models of human essential hypertension, which probably have different pathogenetic mechanisms. The role of G-proteins in the altered regulation of ACA was investigated in VSM from the two models and their controls. No significant differences were observed in the levels of the various G-protein subunits in SHR membranes compared with controls (Wistar and WKY). The function of G_i was unchanged in SHR, WKY and Wistar membranes; however, data suggest that the function of G_s may be decreased in the SHR compared with Wistar (Charles River Ltd. rats), and this may also be the situation in SHRs compared with WKY controls (Harlan Olac Ltd. rats). Significant reductions in the levels of $G_{s\alpha}$ (both forms), $G_{i}3\alpha$ and the β-subunit were shown in both young and adult MHS rats. The function of G_i was again unchanged in the two age-groups of MHS rats in comparison to age-matched controls. Hence, it was suggested that in these rat VSMC membranes, G_i3 does not contribute to the inhibition of adenylyl cyclase activity. G_s function appeared to be decreased in MHS membranes of both ages, and this decreased G_s activity may be explained, in part, by the reduced levels of $G_{s\alpha}$ noted in MHS VSMC membranes.

The responses of ACA to stimulation by isoproterenol in the two models were of notable difference. In the SHR (adult, hypertensive), a reduced response to isoproterenol was observed in comparison to Wistar (Charles River Ltd.) and WKY (Harlan Olac Ltd.) rats. A reduced response to isoproterenol was also noted in young MHS rats, whereas this was reversed with age, and an increased response to this β-adrenergic agonist was shown in adult MHS rats. The decreased isoproterenol stimulatory response in adult, hypertensive SHRs may contribute primarily to the hypertension in this model. Production of less cAMP via β-adrenergic receptors would, in effect, potentiate the existing increased VSM contractility in the SHR, and lead to increased severity of the hypertension. On the other hand, as mentioned in section 7.1.4, it is unlikely that the age-dependent alteration in isoproterenol response (increasing response with age) in the MHS is a primary contributor to its hypertension. A more plausible explanation is that the alteration in isoproterenol response (and indeed β-adrenergic receptor number) between young and adult MHS rats may represent some form of compensatory mechanism. As the MHS rat becomes older and its

blood pressure increases, production of more cAMP via an increased number of β-adrenergic receptors would tend to antagonise possible increases in VSM contractility associated with hypertension. Hence, as possible different pathogenetic mechanisms are responsible for the hypertension in the SHR and MHS, the stimulation of ACA by isoproterenol may play varying roles in their pathogenesis, with it being of primary importance in the case of the SHR, while it may serve as a compensatory relief to the effects of hypertension in the MHS.

7.2 Conclusions

Altered regulation of ACA has been identified in VSM from the SHR and MHS. Alterations in the levels of regulatory G-protein subunits were not observed in the SHR with respect to its controls; however, slight, but significant reductions in the levels of $G_S\alpha$ (both forms) and the β-subunit were observed in the MHS, and may contribute, albeit in a minor way, to its altered ACA. A substantial reduction in the level of $G_i3\alpha$ was shown in MHS VSMC membranes from both age-groups. If Ang II stimulates PLC via G_i3, then this could be the reason for the blunted PLC activity in response to Ang II in MHS VSMC (Socorro *et al.*, 1990). Also, as G_i3 has been implicated in the regulation of voltage-gated potassium channels (Yatani *et al.*, 1988), a reduced level of $G_i3\alpha$ may be linked to the altered transmembrane ion fluxes in MHS rats. It is possible that the activity of G_S is decreased in both the SHR and MHS, and data also suggest that changes may exist in the level/activity of the catalytic subunit of adenylyl cyclase. The stimulatory response of ACA to isoproterenol appears to be a consequence of β-adrenergic receptor number in the MHS, and this is probably also the case in the SHR. In adult rats, isoproterenol produced opposite responses in the SHR and MHS in comparison to their controls. This may reflect different pathogenetic mechanisms giving rise to hypertension in the two models.

It is worthy to note that similar studies using SHRs from different sources produced dissimilar results. Therefore, possible genetic heterogeneity with regard to SHRs (and WKY rats) from different suppliers must be borne in mind for future studies. Also, it is of interest to examine parameters at different stages in the development of hypertension, as age-related alterations may be

revealed, such as age-dependent β-adrenergic receptor number in MHS VSMC membranes.

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