Guanine Nucleotide Regulatory Proteins in the Spontaneously Hypertensive Rat

Catherine J. Clark, Graeme Milligan, Alastair R. McLellan, and John M.C. Connell

We compared guanine nucleotide regulatory protein (G protein) levels and function in plasma membranes from resistance vessels (mesenteric arteries) isolated from spontaneously hypertensive (SHR) and normotensive Wistar rats. G protein function was deduced from studies of adenylate cyclase activity. Although the basal level of adenylate cyclase activity (±Mn$^{2+}$ ions) was significantly greater in SHR membranes, addition of agents that function via the stimulatory G protein — i.e., NaF (10$^{-2}$ M), (-)-isoproterenol (10$^{-4}$ M), and prostaglandin E$_2$ (10$^{-7}$ M) — resulted in a significantly lower stimulatory response in SHR membranes. Ligands that function via the inhibitory G protein — i.e., adrenalin (10$^{-5}$ M)/propranolol (10$^{-5}$ M) (this combination being equivalent to an $\alpha_2$-receptor agonist), carbachol (10$^{-3}$ M), and serotonin (10$^{-5}$ M) — were responsible for only slight inhibitory responses in both SHR and Wistar rat membranes, which were not significantly different. Western blotting identified the presence of $G_s$, $G_{i2}$, and $G_{i3}$ $\alpha$-subunits in rat vascular smooth muscle, but there were no differences in the levels of these G protein $\alpha$-subunits found in SHR and Wistar rat plasma membranes. The levels of the $\beta$-subunit in the two sets of membranes were also similar. In conclusion, there is a reduced response in adenylate cyclase activity to agents that function via the stimulatory G protein in SHR membranes. However, this is not a consequence of altered levels of the different G protein subunits.

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KEY WORDS • adenyl cyclase • guanine nucleotide regulatory protein • muscle, smooth, vascular
guanyline nucleotide regulatory proteins (G proteins) are key components in cellular signaling processes. G proteins are a family of cell membrane proteins that link cell surface receptors to intracellular second messenger systems; the effector systems, e.g., phospholipase C and adenylate cyclase, alter the intracellular concentration of second messengers. Generation of adenosine 3',5'-cyclic monophosphate (cAMP) by adenylate cyclase is under dual control from receptor-coupled G proteins, namely $G_s$, $G_{i2}$, and $G_{i3}$ $\alpha$-subunits in rat vascular smooth muscle, but there were no differences in the levels of these G protein $\alpha$-subunits found in SHR and Wistar rat plasma membranes. The levels of the $\beta$-subunit in the two sets of membranes were also similar. In conclusion, there is a reduced response in adenylate cyclase activity to agents that function via the stimulatory G protein in SHR membranes. However, this is not a consequence of altered levels of the different G protein subunits. (Hypertension 1993;21:204-209)

we examined whether altered G protein levels or function in VSM membranes from hypertensive animals might account for the abnormal regulation of adenylate cyclase.

Reduced function of $G_s$ proteins has been reported in platelets from the SHR. The significance of this and the relevance to vascular tissue remain unclear. However, it is of interest that abnormal expression and function of G proteins has recently been reported in human non-insulin-dependent diabetes mellitus and in animal models of diabetes. Essential hypertension and the SHR share a number of common pathophysiological features with diabetes mellitus, including resistance to the action of insulin. It was, therefore, also of interest to examine whether similar changes in $G_s$ protein function might be present in vascular tissue from the SHR.

Methods

Materials

All chemicals were supplied by Sigma Chemical Co. Ltd. (UK), with the exception of guanosine 5'-triphosphate (GTP), creatine kinase, and creatine phosphate, which were supplied by Boehringer Mannheim UK (Diagnostics & Biochemicals) Ltd. The radioisotopes ([$\alpha^32$P]adenosine 5'-triphosphate [ATP], [8$^3$H]cAMP, and anti-rabbit, whole antibody from donkey 125I) were supplied by Amersham International PLC. Horseradish peroxidase–labeled anti-rabbit immunoglobulin G (IgG) (donkey polyclonal) was supplied by the Scottish Antibody Production Unit, Law Hospital, Carluke.
Rats

SHRs and Wistar rats were supplied by Charles River UK Ltd. Mesenteric arteries were excised from 10- or 11-week-old male rats. Systolic blood pressures (tail-cuff method) and weights of 10 rats of each strain were measured before rats were killed by a blow to the head (SHR and Wistar, respectively: 208±2.7 and 144±2.5 mm Hg [p = 0.001], 258±1.9 and 286±2.7 g [p = 0.001], significance assessed by t test). Rats were fed rat and mouse maintenance diet, were allowed free access to water, and were kept under constant light conditions. For each plasma membrane preparation, 10 rats of each strain were killed.

Plasma Membrane Preparation

The mesenteries were defatted and then homogenized in ice-cold 0.25 M sucrose with a polytron (Northem Media, North Humberside, UK) at maximum setting for 2 x 12 seconds. The homogenate was centrifuged at 2,000g for 10 minutes at 4°C to obtain a crude plasma membrane fraction that was resuspended in 10 mM Tris-HCl, pH 7.5, aliquoted, and stored at -70°C. The protein concentration in the plasma membrane preparation was measured colorimetrically using Peterson’s modification of Lowry’s protocol based on bovine serum albumin as standard.

Adenylate Cyclase Assay

G protein function in plasma membranes was assessed by measuring ACA by the method of Salomon. In this assay, the formation of [32P]cAMP from [α-32P]ATP was measured in the presence of GTP and magnesium ions. The method consists essentially of two phases. After the described reaction phase, separation of the reaction product was achieved by sequential chromatography on Dowex 50 cation exchanger and on neutral alumina. The 32P counts were corrected for column recovery (70-80%) using [3H]cAMP.

The reaction mix (final volume, 50 μl) was essentially that described by Sharma et al and included an ATP regenerating system consisting of creatine phosphate (5 mM) and creatine kinase (50 units/ml), in addition to Tris-acetate, pH 7.6 (25 mM), magnesium acetate (5 mM), ATP (0.5 mM), cAMP (0.05 mM), dithiothreitol (1 mM), bovine serum albumin (0.1 mg/ml), GTP (0.02 mM), and [α-32P]ATP (2 x 106 cpm per assay). ACA was studied under basal conditions and in the presence of forskolin (10-4 M), MnCl2 (2 x 10-2 M), NaF (10-2 M), (-)-isoproterenol (10-4 M), prostaglandin E1 (10-5 M), adrenoline (10-5 M)/propranolol (10-3 M)/forskolin (10-4 M), carbachol (10-5 M)/forskolin (10-4 M), and serotonin (10-5 M)/forskolin (10-4 M). These concentrations of the various agents were saturating with respect to their effects (from previous dose-response curves, data not shown). The reaction was started by the addition of freshly thawed plasma membrane (5 μg per assay) and transfer of the assay tubes from an ice bath to a 37°C water bath for 15 minutes. This incubation period was chosen from the linear portion of the time course of the reaction. The reaction was terminated by the addition of a stopping solution, pH 7.5, consisting of sodium lauryl sulfate (2%), ATP (45 mM), and cAMP (1.3 mM).

Table 1. Specificities of Anti-G Protein Antisera

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Peptide used</th>
<th>Corresponding G protein sequence</th>
<th>Antiserum identifies</th>
</tr>
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<tr>
<td>CS1</td>
<td>RMHLROYEL</td>
<td>Gα4 385-394</td>
<td>Gα4</td>
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<tr>
<td>SG1</td>
<td>KENLKDGLF</td>
<td>Transducin α 341-350</td>
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<tr>
<td>1B</td>
<td>KNNLCEGLY</td>
<td>G3α 345-354</td>
<td>G3α</td>
</tr>
<tr>
<td>BN3</td>
<td>MSELDQLROE</td>
<td>β1 (1-10)</td>
<td>β</td>
</tr>
</tbody>
</table>

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis and Immunoblotting

The amount of plasma membrane protein to be loaded to achieve optimal blots was determined from plots of protein concentration against counts per minute obtained by excision and counting of labeled bands constructed for each antibody used. Protein molecular weight standards (molecular weight range, 14,300–200,000) were run in parallel with the samples under study. In addition, a “standard” human platelet plasma membrane preparation was included on all blots to facilitate comparison of data from different blots.

The plasma membrane protein was acid precipitated with trichloroacetic acid (24%), solubilized in Laemmli buffer, and then boiled for 3 minutes. The protein was resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on 10% acrylamide/0.27% N,N'-methylene-bis-acrylamide gels. Immunoblotting with specific antipeptide antisera was then performed as described in detail previously. Briefly, the resolved protein was electrophoretically transferred (blotted) to nitrocellulose paper, which was then blocked for 2 hours at 37°C in 5% dried skimmed milk (Marvel) in Tris-buffered saline. After thorough washing with distilled water, the blot was incubated at 37°C overnight with the appropriate antipeptide antisera. After appropriate washing, the blot was incubated at 37°C for 2 hours with horseradish peroxidase–labeled anti-rabbit IgG (donkey polyclonal). The horseradish peroxidase–labeled bands were visualized by development with a 0.025% solution of ortho-dianisidine. A photograph of the blot was taken at this stage. After blotting, the gel was retained, and residual protein, which had not been transferred electrophoretically to the nitrocellulose, was visualized by staining with a solution of Coomassie brilliant blue G. A photograph of the gel was also taken. The bands on the blot were quantified by incubation at 37°C for 2 hours with [35S]-labeled anti-rabbit IgG. The bands were subsequently cut out and counted using an NE 1612 gamma counter (NE Technology, Reading, UK).

Antisera

The antisera were generated in New Zealand White rabbits using a conjugate of the synthetic peptide and keyhole limpet hemocyanin as detailed by Goldsmith et al. The specificity of the antisera in recognizing Gα4 and Gαα protein species has been fully described elsewhere. The antiserum CS1 (Table 1) detected two bands of molecular mass 44 and 42 kd. These bands comigrated with Escherichia coli expressed recombinant forms of Gα proteins and, hence, represent authentic Gα proteins. G1α and G2α proteins have identical C-ter-
SHR — 43,000Da

**Statistical Analysis**

The adenylate cyclase assay results were analyzed by the Mann-Whitney U test for nonparametric data. Errors are SEM for eight experiments (adenylate cyclase assay) and four experiments (SDS-PAGE and immunoblotting with each antiserum) using different membrane preparations.

**Results**

Adenylate cyclase assays were performed using mesenteric artery plasma membranes isolated from SHR and Wistar rats. The basal level of ACA in SHR membranes was significantly higher than that measured in Wistar membranes (SHR versus Wistar, 690±73 versus 445±52 pmol cAMP per 15 min/mg; p=0.016). The basal level of ACA measured in the presence of Mn²⁺ ions was also higher in SHR membranes compared with Wistar membranes (SHR versus Wistar, 717±109 versus 502±48 pmol cAMP per 15 min/mg). Hence, the results of regulation of adenylate cyclase have been expressed as the percent change from basal to reflect the ability of ligands to alter ACA.

Figure 1 shows the percent change in ACA over basal in SHR and Wistar membranes after the addition of forskolin (10⁻⁴ M), NaF (10⁻² M), MnCl₂ (2×10⁻² M), (-)-isoproterenol (10⁻⁴ M), and prostaglandin E₁ (PGE₁) (10⁻⁵ M). There were significant differences in the extents of stimulation caused by the addition of NaF (SHR, 324±34%; Wistar, 781±87%; p=0.002), (-)-isoproterenol (SHR, 11±8%; Wistar, 48±11%; p=0.03), and prostaglandin E₁ (SHR, 19±10%; Wistar, 77±16%; p=0.004), with a significantly greater response to each agonist in Wistar membranes. In contrast, the response to forskolin was not significantly increased in Wistar membranes. The overall results were not altered by expressing data in relation to ACA measured in the presence of Mn²⁺ ions (data not shown), which uncouple adenylate cyclase from its regulatory G proteins (and which had similar effects in both rat strains).

Variable inhibition was seen in SHR and Wistar membranes in response to a range of ligands (epinephrine [10⁻⁵ M]/propranolol [10⁻⁵ M], carbachol [10⁻³ M], and serotonin [10⁻⁵ M]), but overall, no significant differences were seen (epinephrine/propranolol: SHR, -8.2±2.6%; Wistar, -0.4±9.7%; carbachol: SHR, -17.1±4.1%; Wistar, -4.3±13.6%; serotonin: SHR, -20.2±4.7%; Wistar, -3.6±9.6%). The extents of inhibition in both sets of membranes, in response to these ligands, were not increased in the presence of (-)-isoproterenol.

Preliminary immunoblotting experiments identified G_s (two forms: 44 and 42 kd), G₁₂, and G₃ α-subunits in VSM plasma membranes, whereas G_o and G₁ α-subunits were absent. The levels of these different G protein α-subunits and the β-subunit were compared in SHR and Wistar VSM membranes (Figures 2, 3, and 4; Table 2). There were no significant differences in the levels of the different G protein subunits present in SHR and Wistar membranes.

**Discussion**

Our results have shown a significant reduction in stimulation of ACA in VSM from the SHR when compared with Wistar controls. We chose to use the outbred Wistar strain, rather than Wistar-Kyoto rats, as the control group in this study in view of recent evidence that the Wistar-Kyoto rats obtainable from most commercial suppliers display marked genetic heterogene-
probed with transducin, G\textsubscript{j1}, G\textsubscript{j2}-specific antibody (SG1). Because vascular smooth muscle does not express detectable levels of G\textsubscript{j1} protein and transducin distribution is restricted to photoreceptor-containing tissues, the antisem SGI will only identify G\textsubscript{j2} protein in vascular smooth muscle. Sixty micrograms membrane protein was loaded in each lane. Lanes 1–3, Wistar membranes; lanes 4–6, spontaneously hypertensive rat membranes; lane 7, human platelet membranes.

**Figure 3.** Western blot shows nitrocellulose membrane probed with transducin, G\textsubscript{j1}, G\textsubscript{j2}-specific antibody (BN3). Fifty micrograms membrane protein was loaded in each lane. Lanes 1–3, Wistar membranes; lanes 4–6, spontaneously hypertensive rat membranes; lane 7, human platelet membranes.

**Figure 4.** Western blot shows nitrocellulose membrane probed with \(\beta\)-subunit-specific antibody (BN3). Fifty micrograms membrane protein was loaded in each lane. Lanes 1–3, spontaneously hypertensive rat membranes; lanes 4–6, Wistar membranes.

<table>
<thead>
<tr>
<th>G protein subunit</th>
<th>SHR (cpm)</th>
<th>Wistar (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G\textsubscript{j1} (44 kd)</td>
<td>3,221±126</td>
<td>3,180±232</td>
</tr>
<tr>
<td>G\textsubscript{j1} (42 kd)</td>
<td>1,819±95</td>
<td>1,928±58</td>
</tr>
<tr>
<td>Gj\textsubscript{2}</td>
<td>3,254±377</td>
<td>3,795±237</td>
</tr>
<tr>
<td>Gj\textsubscript{3}</td>
<td>717±44</td>
<td>662±44</td>
</tr>
<tr>
<td>(\beta)</td>
<td>1,517±75</td>
<td>1,439±91</td>
</tr>
</tbody>
</table>

SHR, spontaneously hypertensive rat; cpm, counts per minute. Values are mean±SEM for four experiments using different membrane preparations.

Table 2: Results of Quantifying Bands With Iodine-125-Labeled Secondary Antibody

This is a true decrease in responsiveness of adenylate cyclase in VSM membranes from the SHR, which is not accounted for by an intrinsic change in the basal activity of the enzyme.

These data are consistent with other studies that have examined ACA in vascular tissue from SHRs and control rats. Thus, it has been reported that ACA was reduced in myocardial tissue, mesenteric vascular tissue, and aortic tissue from the SHR in response to isoproterenol, without a major difference in basal values. However, these previous studies did not examine the underlying reason for the reduced adenylate cyclase response. Our own studies have examined whether this altered responsiveness could be explained by abnormality in the amount or function of either G\textsubscript{s} or G\textsubscript{j} proteins. We could find no evidence of reduced inhibition of ACA in response to a number of ligands that act via the G\textsubscript{s} subunit (including studies in which G\textsubscript{s} protein was activated in the presence of partly stimulated ACA with isoproterenol), suggesting that the function of this G\textsubscript{s} protein was normal. Similarly, G\textsubscript{2} and G\textsubscript{3} \(\alpha\)-subunit levels were not altered in SHR compared with Wistar membranes (the functional significance of G\textsubscript{j} subtypes has yet to be established, although mounting evidence suggests that at least G\textsubscript{j2} proteins can serve to inhibit adenylate cyclase). These data contrast with findings reported from studies on SHR platelet membranes reported by Coquil and Brunelle, who noted that G\textsubscript{i} function, as measured by activation with the GTP analogue S\textsuperscript{S}-guanylylimidodiphosphate, appeared to be slightly reduced. They suggested that this could account for the increased cAMP response noted in platelets from hypertensive rats in response to prostaglandin E\textsubscript{1}.

However, the relevance of platelet studies in hypertensive animals is uncertain, and no direct measurement was made of G\textsubscript{i} protein function in that study.

Our immunoblot studies do not show any alteration in the levels of the two G\textsubscript{i} \(\alpha\)-subunits within plasma membranes from the SHR, excluding a contribution from altered G\textsubscript{i} protein levels to the changes in ACA. We also demonstrated that \(\beta\)-subunit levels were not different when membranes from the two strains were compared. Although data suggest that \(\beta\)-y subunit complexes can contribute to regulation of ACA, our data
suggest that this does not account for the altered ACA in SHRs. Instead, our results may support an abnormality of G protein function. In the only other study to examine G protein function in the vascular tissue of the SHR, Asano and colleagues suggested that defects in relaxation of femoral artery strips in response to β-adrenergic receptor stimulation were due to altered function of a cholera toxin–sensitive G protein, i.e., G protein. This finding would, therefore, tend to support our own data.

The SHR is a model of hypertension that shows reduced sensitivity to insulin (insulin resistance), a finding that is also present in human essential hypertension. In a recent study of another animal model that displays insulin resistance (the diabetic [db/db] mouse), decreased adenylate cyclase response to isoproterenol was noted in adipose tissue. In that study, there was no change in the level of Gα proteins, although a marked increase in the level of the G1 α-subunit was demonstrated in adipocyte membranes from the db/db animals compared with their normal, lean littermates. G1 α-subunit is not, however, expressed in rat VSM, and it is not believed to have a role in the modulation of ACA; we have no data on adipose tissue adenylate cyclase regulation in the SHR. The apparent explanation for the decreased adenylate cyclase response in the db/db mouse appears to be a reduction in β-adrenergic receptor number. Cardiac membranes from the obese Zucker rat, which is not diabetic but is, nevertheless, profoundly insulin resistant and hypertensive, demonstrated reduced β-adrenergic stimulation of adenylate cyclase in comparison with lean controls. The heart tissue membranes from obese Zucker rats showed both reduced β-adrenergic receptor number and altered coupling between β-adrenergic receptors and G proteins. However, no alterations in either the level of G proteins or the functional interaction between G proteins and the catalytic moiety of adenylate cyclase was observed. Although β-adrenergic receptor number was not measured in our own study because of the large amount of membrane protein required for such measurements, other investigators have failed to agree on observed changes in β-adrenergic receptor number and affinity in SHR tissues. However, we did demonstrate a significantly decreased adenylate cyclase response to NaF, which activates G proteins independent of receptors, and suggest that this supports the notion that the decreased ACA observed is due to altered G protein function or coupling rather than altered receptor number. Further studies examining the activation of G proteins (e.g., with GTPyS) may clarify this point.

The reason for the apparent abnormality of G protein function is unclear, and the significance of reduced adenylate cyclase response to stimulatory agents in SHR vascular membranes also remains to be determined. Asano and colleagues suggest that this might account for some of the abnormality noted in relaxation of VSM in hypertensive rats, but our own data do not address this point. It is unclear whether the reduced responsiveness is a primary abnormality in the SHR or secondary to some other event. Studies in animals at an earlier stage in the development of hypertension and in other hypertensive models are appropriate to investigate this further.

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