Biochem. J. (1992) 288, 79–85 (Printed in Great Britain)

Enhanced expression of inhibitory guanine nucleotide regulatory protein in spontaneously hypertensive rats

Relationship to adenylate cyclase inhibition

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We have previously shown that the stimulatory effects of guanine nucleotides, *N*-ethylcarboxamide-adenosine and other agonists on adenylate cyclase activity were diminished in aorta and heart sarcolemma of spontaneously hypertensive rats (SHR) [Anand-Srivastava (1988) Biochem. Pharmacol. **37**, 3017–3022]. In the present studies, we have examined whether the decreased response of these agonists is due to the defective GTP-binding proteins (G-proteins) which couple the receptors to adenylate cyclase, and have therefore measured the levels of G-proteins in aorta and heart from SHR and their respective Wistar–Kyoto (WKY) controls by using pertussis toxin (PT)- and cholera toxin (CT)-catalysed ADP-ribosylations and immunoblotting techniques using specific antibodies against G-proteins. The labelling with [^{32}P]NAD$^+$ and PT identified a 40/41 kDa protein in heart and aorta from WKY and SHR and was significantly increased in the hearts (~100%) and aorta (~30–40%), from SHR as compared with WKY. Immunoblotting revealed an increase in the levels of the G-protein α-subunits $G_i\alpha$-2 and $G_i\alpha$-3 in heart and $G_i\alpha$-2 in aorta, whereas no change in $G_o\alpha$ was observed in heart from SHR and WKY. On the other hand, no differences were observed in CT labelling or immunoblotting of stimulatory G-protein (G_s) in heart and aorta from WKY and SHR. In addition, CT stimulated the adenylate cyclase activity in heart sarcolemma from WKY and SHR to a similar extent. These results were correlated with adenylate cyclase inhibition and stimulation by various hormones. Angiotensin II (AII), atrial natriuretic factor (ANF) and oxotremorine-mediated inhibition was found to be greater in SHR as compared with WKY, whereas the stimulatory effects of adrenaline, isoprenaline, dopamine and forskolin were diminished in SHR aorta as compared to WKY. These results indicate that regulatory protein G_i is more expressed in SHR, which may be associated with the decreased responsiveness of stimulatory hormones and increased sensitivity of inhibitory hormones to stimulate/inhibit adenylate cyclase activity. It may thus be suggested that the enhanced G_i activity may be one of the mechanisms responsible for the diminished vascular tone and impaired myocardial functions in hypertension.

INTRODUCTION

The elevation of blood pressure in essential hypertension is due to a general increase in the resistance of peripheral vessels [1,2]. A part of this heightened peripheral resistance has been attributed to structural changes in the vessels [3], abnormalities in Ca^{2+} movements [4], and aberrations in cyclic nucleotide metabolism [5]. It has been suggested that the adenylate cyclase/cyclic AMP (cAMP) system is one of the biochemical mechanisms which participates in the regulation of arterial tone and reactivity [6]. Decreased cAMP levels in cardiovascular tissues have been implicated in the pathogenesis of hypertension [5,7], and various studies have reported increased [6], decreased [8] or unaltered [9] adenylate cyclase activity in spontaneously hypertensive rats (SHR).

The adenylate cyclase system is composed of three components: receptor, catalytic subunit and stimulatory (G_s) and inhibitory (G_i) guanine nucleotide regulatory (G-) proteins. The G-proteins act as transducers and, in the presence of guanine nucleotides, transmit the signal from the hormone-occupied receptor to the catalytic subunit. The stimulation and inhibition of adenylate cyclase by hormones are mediated through the G_s and G_i proteins of adenylate cyclase respectively [10]. The G-proteins are also targets of bacterial toxins that are useful probes for defining the interaction of the regulatory proteins with other components of the adenylate cyclase system. Bacterial toxins, such as cholera toxin (CT) and pertussis toxin (PT), have been shown to ADP-ribosylate, in the presence of [α-^{32}P]NAD$^+$, the α-subunits of G-proteins [G_s (45000 Da), G_i (40000–41000 Da) and G_o (39000 Da)] and thereby modify the characteristics of these proteins [11–19]. CT irreversibly activates G_s protein, mediating the stimulation of adenylate cyclase, whereas PT, in addition to G_o, acts on G_i protein, which regulates inhibition, and attenuates the GTP-dependent and receptor-mediated inhibition of adenylate cyclase [18]. The functions of G_o protein are not yet known, but it may interact with enzymes associated with Ca^{2+} mobilization and not with adenylate cyclase [20]. We have recently shown that the stimulatory effects of guanine nucleotides, *N*-ethylcarboxamide-adenosine and other agonists such as forskolin (FSK) and NaF on adenylate cyclase were diminished in aorta and heart sarcolemma of SHR [21]. The decreased response of adenylate cyclase activity to these agonists may be attributed to various factors, such as a decrease in the number of hormone receptors, defective G-proteins which couple hormone receptors to catalytic subunit of adenylate cyclase, or an impaired catalytic subunit, or the combination of all three. In the present studies, we have used bacterial toxins such as PT and CT and specific antibodies against G-proteins to examine if the G-proteins G_i and G_s are affected in hypertension and their relationship with adenylate cyclase stimulation and inhibition by various hormones.

Abbreviations used: cAMP, cyclic AMP; ANF, atrial natriuretic factor (99–126-peptide); AII, angiotensin II; PT, pertussis toxin; CT, cholera toxin; G_i, inhibitory G-protein; G_s, stimulatory G-protein; FSK, forskolin; WKY, Wistar–Kyoto rats; SHR, spontaneously hypertensive rats; GTP[S], guanosine 5′-[γ-thio]triphosphate.

EXPERIMENTAL

Rats

Female SHR and normotensive Wistar–Kyoto (WKY) rats (12 weeks old) were purchased from Charles Rivers Canada (St.-Constant, Quebec, Canada). Their blood pressure, measured by the tail-cuff method without anaesthesia, was 98.5 ± 3.3 and 146.5 ± 5.9 mmHg ($n = 15$) for the WKY and SHR groups respectively. Body weights were 172.5 ± 1.9 and 196.4 ± 1.9 g respectively.

Isolation of heart sarcolemma

After acclimatization for a few days, age-matched control WKY and SHR were decapitated, and their hearts were quickly removed and placed in ice-cold 10 mM-Tris/HCl buffer (pH 7.4). The heart sarcolemma was isolated essentially by the method described elsewhere [21,22]. The ventricles were washed thoroughly, cut into small pieces, and homogenized in a Virtis blender for 30 s in 10 vol. of 10 mM-Tris/HCl buffer (pH 7.4) containing 1 mM-EDTA. The homogenate was filtered through cheesecloth and centrifuged at 1000 *g* for 10 min. The sediment was suspended in 20–25 vol. of 10 mM-Tris/HCl buffer (pH 7.4), stirred in a cold-room for 30 min, and re-centrifuged at 1000 *g* for 10 min. This process was repeated twice more, first by suspending the sediment in 10 mM-Tris/HCl buffer at pH 8.0, and then in the same buffer but at pH 7.4. The sediment was again suspended in 20–25 vol. of 10 mM-Tris/HCl (pH 7.4), extracted with 0.4 M-LiBr for 45 min, and centrifuged at 1000 *g* for 10 min. It was then suspended in 10 mM-Tris/HCl (pH 7.4), stirred for 15–20 min, and centrifuged at 1000 *g* for 10 min. The sarcolemmal fraction thus obtained was suspended in a buffer containing 10 mM-Tris/HCl, 1 mM-dithiothreitol and 1 mM-EDTA (pH 7.4), and was used for determination of adenylate cyclase activity and ADP-ribosylation studies. As reported previously [22], the sarcolemmal fraction prepared by the hypo-osmotic-shock/LiBr treatment contained negligible amounts (2–4 %) of contamination by other subcellular organelles, such as mitochondria, sarcoplasmic reticulum and myofibrils. In addition, this preparation was also devoid of endothelial cells, since angiotensin-converting-enzyme activity (a marker for endothelial cells) was absent from this sarcolemmal fraction [22].

Preparation of aorta washed particles

Aorta washed particles were prepared as described previously [23,24]. The dissected aortae were quickly frozen in liquid N_2 and pulverized to a fine powder, in a percussion mortar cooled in liquid N_2. They were stored at -70 °C until assayed. After homogenization in a motor-driven Teflon/glass homogenizer in a buffer containing 10 mM-Tris/HCl and 1 mM-EDTA (pH 7.5), the homogenate was centrifuged at 16000 *g* for 10 min. The supernatant fraction was discarded, and the pellet was finally suspended in 10 mM-Tris/HCl/1 mM-EDTA and used for determination of adenylate cyclase activity and for ADP-ribosylation studies.

PT-catalysed ADP-ribosylation

ADP-ribosylation of various membranes or solubilized membrane preparations by PT was performed as described previously [25] by the method of Burns *et al.* [26]. The membranes from age-matched control WKY and SHR were incubated in 25 mM-glycylglycine buffer, pH 7.5, containing 15 μM-[α-^{32}P]NAD$^+$ (20 μCi/ml), 0.4 mM-ATP, 0.4 mM-GTP, 15 mM-thymidine, 10 mM-dithiothreitol and ovalbumin (0.1 mg/ml) with or without PT (5 μg/ml) for 30 min at 37 °C in a total volume of 100 μl. The reaction was terminated by addition of 20 μl of a 'stop' mixture containing 5 % SDS and 50 % β-mercaptoethanol. The contents were heated for 10 min in a boiling-water bath. The labelled proteins were analysed by subjecting the samples to SDS/PAGE by the method of Laemmli [27] with 12 %-polyacrylamide gels. After electrophoresis, the gels were fixed, stained, destained, dried and autoradiographed by exposure to Kodak XAR-5 film, as described previously [25]. They were calibrated by using molecular-mass standards (Pharmacia): phosphorylase *b* (94000 Da), albumin (67000 Da), ovalbumin (43000 Da), carbonic anhydrase (30000 Da), trypsin inhibitor (20000 Da) and β-lactalbumin (14400 Da).

CT-catalysed ADP-ribosylation

CT-catalysed ADP-ribosylation of the membranes from age-matched WKY and SHR was performed in a manner similar to that catalysed by PT, except that the CT (500 μg/ml) was preactivated for 20 min at 37 °C in a mixture containing 20 mM-dithiothreitol, 1 μg of BSA/ml and 25 mM-KH_2PO_4 (pH 8.0).

Immunoblotting

After SDS/PAGE, the separated proteins were electrophoretically transferred to nitrocellulose paper (Schleicher and Schuell) with a mini transfer apparatus (Bio-Rad) at 100 V for 1 hr or a semi-dry transblot apparatus (Bio-Rad) at 15 V for 45 min. After transfer, the membranes were washed twice in phosphate-buffered saline (PBS) and were incubated in PBS containing 3 % BSA at room temperature for 2 h. The blots were then incubated with antisera against G-proteins in PBS containing 1 % BSA and 0.1 % Tween-20 at room temperature for 2 h. The antigen–antibody complexes were detected by incubating the blots with goat anti-rabbit IgG (Bio-Rad) conjugated with horseradish peroxidase for 2 h at room temperature. The blots were washed three times with PBS before reaction with diaminobenzidine and H_2O_2 as described by Hsu & Soban [28] or with enhanced-chemiluminescence (ECL) Western-blotting detection reagents from Amersham.

Adenylate cyclase activity determination

Adenylate cyclase activity was determined by measuring [^{32}P]-cAMP formation from [α-^{32}P]ATP, as described previously [21,22]. The assay medium containing 50 mM-glycylglycine, pH 7.5, 0.5 mM-MgATP, [α-^{32}P]ATP [$(1–1.5) \times 10^6$ c.p.m.], 5 mM-$MgCl_2$ (in excess of the ATP concentration), 100 mM-NaCl, 0.5 mM-cAMP, 1 mM-3-isobutyl-l-methylxanthine, 0.1 mM-EGTA, 10 μM-guanosine 5′-[γ-thio]triphosphate (GTP[S]) (or otherwise as indicated), and an ATP-regenerating system consisting of 2 mM-phosphocreatine, 0.1 mg of creatine kinase/ml and 0.1 mg of myokinase/ml in a final volume of 200 μl. Incubations were initiated by addition of the membrane preparation (30–70 μg) to the reaction mixture, which had been thermally equilibrated for 2 min at 37 °C. The reactions, conducted in triplicate for 10 min at 37 °C, were terminated by addition of 0.6 ml of 120 mM-zinc acetate. cAMP was purified by co-precipitation of other nucleotides with $ZnCO_3$, by addition of 0.5 ml of 144 mM-Na_2CO_3 and subsequent chromatography by the double-column system, as described by Salomon *et al.* [29]. Under the assay conditions used, adenylate cyclase activity was linear with respect to protein concentration and time of incubation.

Protein was determined essentially as described by Lowry *et al.* [30], with crystalline BSA as standard.

MATERIALS

ATP, cAMP and isoprenaline (isoproterenol) were purchased from Sigma (St. Louis, MO, U.S.A.); creatine kinase (EC 2.7.3.2), myokinase (EC 2.7.4.3) and GTP[S] were from Boehringer

Mannheim (Montreal, Quebec, Canada). [α-^{32}P]ATP was from Amersham Corp. (Oakville, Ontario, Canada) and [α-^{32}P]NAD$^+$ from DuPont Canada (Mississauga, Ontario, Canada). The electrophoresis chemicals were obtained from Bio-Rad (Mississauga, Ontario, Canada). Atrial natriuretic factor (99–126-peptide) (ANF) was acquired from the Institut Armand Frappier (Montreal, Quebec, Canada). PT was from List Biochemicals (Campbell, CA, U.S.A.), and CT was from Sigma. AS/7, EC/2, GC/2 and RM/1 antibodies were from DuPont. Antibody A572 was kindly provided by Dr. Susan Mumby, University of Texas, Dallas [31].

RESULTS

We have recently shown that the stimulatory effects of guanine nucleotides such as GTP or guanosine 5′-[$\beta\gamma$-imido]triphosphate, which interact with G-proteins, on adenylate cyclase were diminished in the heart sarcolemma from SHR as compared with their respective age-matched WKY controls [21]. To investigate whether the GTP-stimulated enzyme activity is also impaired in aorta from SHR, we studied the effect of various concentrations of GTP[S[on adenylate cyclase activity in aorta from SHR and WKY, and the results are shown in Fig. 1. GTP[S] stimulated adenylate cyclase activity in a concentration-dependent manner in both WKY and SHR; however, the extent of stimulation was significantly lower in SHR. For example, 10 μM-GTP[S] augmented the adenylate cyclase activity by about 600%, whereas only about a 350% stimulation was observed in SHR. Similar results (not shown) were also obtained in cultured vascular smooth-muscle cells from aorta from WKY and SHR. These data indicate that the G-protein in the aorta from SHR is also impaired.

To investigate if the decreased stimulation of adenylate cyclase by GTP[S] in SHR is due to an increase in the inhibitory G-protein or to a decrease in the stimulatory G-protein, or both, we used PT and CT to ADP-ribosylate the G_i and G_s regulatory proteins respectively. Fig. 2(*a*) shows that PT in the presence of [α-^{32}P]NAD$^+$ catalysed the ADP-ribosylation of a protein band of 40/41 kDa, referred to as $G_i\alpha$, in heart sarcolemma solubilized by Lubrol PX at a final concentration of 0.3% as well as in aorta from WKY and SHR; however, the labelling of G_i was significantly enhanced (104$\pm$5%, $n = 5$) in heart sarcolemma from SHR as compared with WKY, whereas about a 35–40% (39.5$\pm$4%, $n = 5$) increase in $G_i\alpha$ labelling was observed in aorta from SHR, as judged by densitometric scanning.

Since PT catalyses the ADP-ribosylation of several G-proteins ($G_i\alpha$-1, $G_i\alpha$-2, $G_i\alpha$-3 and $G_o\alpha$) it was of interest to identify the G-proteins that are over-expressed in heart and aorta from SHR. To investigate this, the immunoblotting experiments were performed using specific antibodies against G_o and different isoforms of $G_i\alpha$. Fig. 2(*b*) shows that AS/7 antibodies, which have been reported to react with both $G_i\alpha$-1 and $G_i\alpha$-2 [32], recognized a single protein of approx. 40000 Da, referred to as $G_i\alpha$-2 ($G_i\alpha$-1 has been reported to be absent from heart; [33]), on immunoblots of heart sarcolemma and aorta from SHR and WKY rats. However, the relative amount of immunodetectable $G_i\alpha$-2 was greater (125–140%, 130$\pm$7, $n = 5$) in SHR as compared with WKY. In addition, the antibodies EC/2 against $G_i\alpha$3 and antibodies GC/2 against $G_o\alpha$ detected a single protein of 39/40 kDa in heart from WKY and SHR; however, no difference in the levels of $G_o\alpha$ was observed, whereas a slight increase in the levels of $G_i\alpha$-3 (20–25%, 24.2$\pm$2, $n = 5$) in SHR was noticed. The presence of $G_i\alpha$-3 and $G_o\alpha$ in heart has been demonstrated previously [33]. On the other hand, these antibodies could not

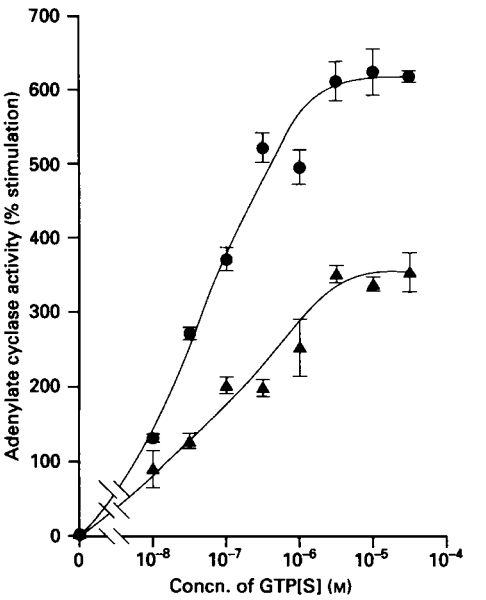

Fig. 1. Effect of GTP[S] on adenylate cyclase activity in aorta washed particles from 12-week-old WKY (●) and age-matched SHR (▲)

Adenylate cyclase activity was determined in the absence or presence of various concentrations of GTP[S] as described in the Experimental section. Values are means$\pm$S.E.M. of three separate experiments. Basal enzyme activity in WKY and SHR was respectively 40$\pm$6 and 36$\pm$5 pmol of cAMP/10 min per mg of protein. Six animals were utilized in each experiment.

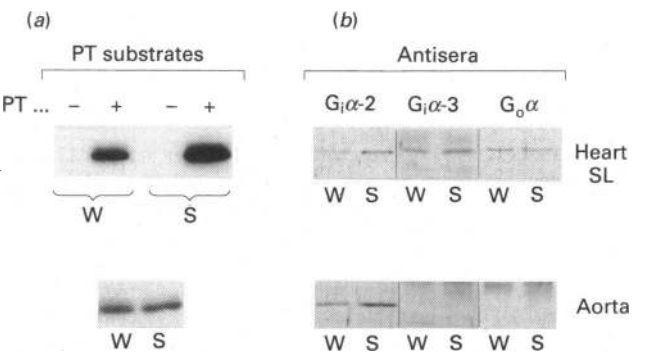

Fig. 2. Quantification of $G_o\alpha/G_i\alpha$ proteins in heart sarcolemma and aorta washed particles from 12-week-old WKY (W) and age-matched SHR (S) by PT-catalysed ADP-ribosylation and immunoblotting

Heart sarcolemmal membranes (4 mg/ml) were preincubated with Lubrol PX (0.3%) at 25 °C for 10 min and the membranes were centrifuged at 100000 *g* for 1 h. The supernatant was collected and used for ADP-ribosylation studies. The solubilized membranes were incubated with [α-^{32}P]NAD$^+$ in the absence (lanes 1, 3) or presence of 5 μg of PT/ml (lanes 2, 4), and aorta washed particles from WKY (W) and SHR (S) were incubated with [α-^{32}P]NAD$^+$ and 5 μg of PT/ml at 30 °C for 30 min. The ^{32}P-labelled proteins were analysed by SDS/PAGE followed by autoradiography as described in the Experimental section. The autoradiograms shown are representative of five separate experiments. (*b*) The membrane proteins from WKY and SHR were resolved by SDS/PAGE and transferred to nitrocellulose, which was then immunoblotted with antibody AS/7 for $G_i\alpha$-1, $G_i\alpha$-2, antibody EC/2 for $G_i\alpha$-3 and antibody GC/2 for $G_o\alpha$ as described in the Experimental section. $G_i\alpha$-1 and $G_i\alpha$-2 were detected by using the diaminobenzidine/H_2O_2 technique, and $G_i\alpha$-3 and $G_o\alpha$ were detected by ECL Western blotting technique as described in the Experimental section. The autoradiograms shown are representative of five separate experiments.

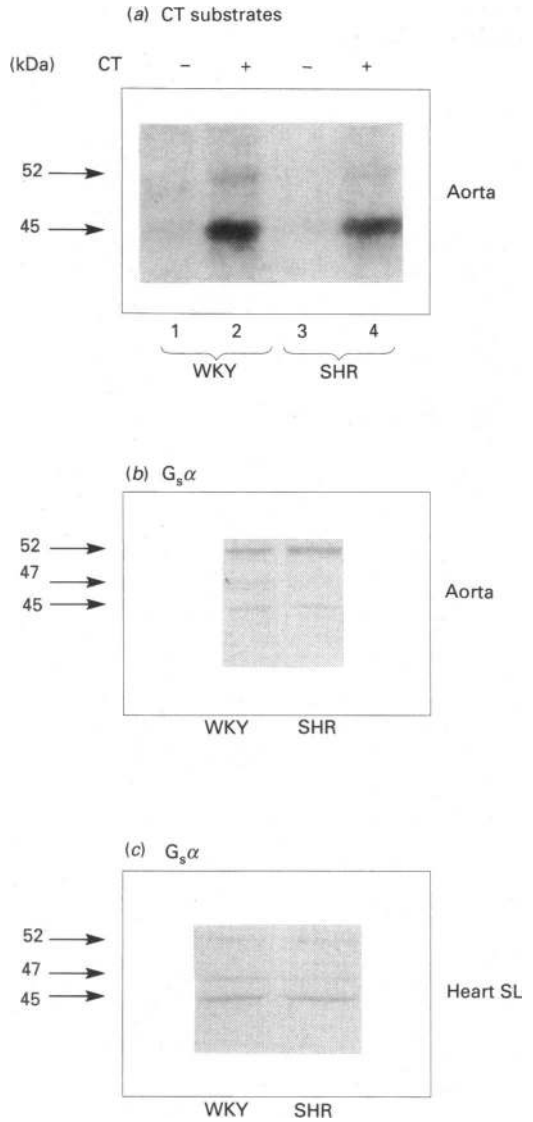

Fig. 3. Quantification of $G_s\alpha$ protein by CT-catalysed ADP-ribosylation (*a*) and immunoblotting (*b* and *c*) in aorta washed particles and heart sarcolemma from 12-week-old WKY and age-matched SHR

(*a*) Aorta washed particles from WKY (lanes 1, 2) and SHR (lanes 3, 4) were incubated with [α-^{32}P]NAD$^+$ in the absence (lanes 1, 3) and presence of 100 μg of CT/ml (lanes 2, 4) at 30 °C for 30 min. The ^{32}P-labelled proteins were analysed by SDS/PAGE as described in the Experimental section. The autoradiograms shown are representative of five separate experiments. (*b*) and (*c*) The membrane proteins from WKY and SHR were resolved by SDS/PAGE and transferred to nitrocellulose, which was then immunoblotted by using A572 antibody for heart and RM/1 for aorta as described in the Experimental section. $G_s\alpha$ was detected by using the diaminobenzidine/H_2O_2 technique as described in the Experimental section. The autoradiograms shown are representative of five separate experiments.

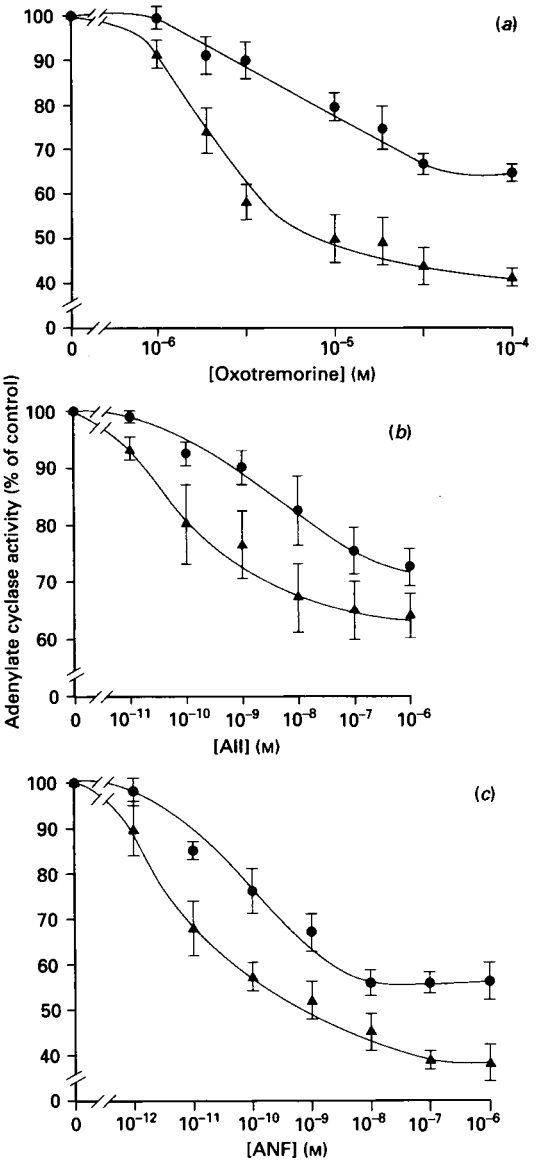

Fig. 4. Effect of various concentrations of (*a*) oxotremorine on adenylate cyclase activity in heart sarcolemma and (*b*) and (*c*) ANF on adenylate cyclase activity in aorta washed particles from 12-week-old WKY (●) and age-matched SHR (▲)

Adenylate cyclase activity was determined as described in the Experimental section. Values are means ± S.E.M. of three separate experiments. Six animals were utilized in each experiment. Basal enzyme activity in WKY and SHR in (*a*) was 553 ± 9 and 599 ± 43, in (*b*) 102 ± 2 and 118 ± 4, and in (*c*) 156 ± 4 and 201 ± 16 pmol of cAMP/10 min per mg of protein respectively.

detect any protein in aorta from WKY or SHR and indicate that $G_o\alpha$ and $G_i\alpha$-3 may not be present in aorta to the extent to be detected under the conditions used.

Fig. 3(*a*) shows the CT-dependent ADP-ribosylation of regulatory protein G_s in rat aorta from SHR and WKY rats. CT in the presence of [α-^{32}P]NAD$^+$ catalysed the ADP-ribosylation of two protein bands of 45000 and 52000 Da in both SHR and WKY rat aorta, and the labelling of a protein band of 45000 Da was not significantly different in SHR and WKY rats, as determined by densitometric scanning (WKY 38.9 ± 1.8, SHR 35.9 ± 1.1 arbitrary units; $n = 5$). On the other hand, the labelling of a protein of 52000 Da was very faint and could not be quantified by densitometry; however, it appears that, like the 45000 Da protein, this band in SHR was also of the same intensity as that of WKY.

To corroborate our results further, we used G_s-specific antibodies RM/1 (DuPont) and A-572 [31] to ascertain the levels of G_s in membranes from SHR and WKY rats. Figs. 3(*b*) and 3(*c*) show that the antibodies A-572 and RM/1 clearly detected 45 kDa, 47 kDa and 52 kDa forms of the stimulatory G_s in aorta (Fig. 3*b*) and heart sarcolemma (Fig. 3*c*); however, the levels of these proteins were similar in WKY and SHR. The presence of

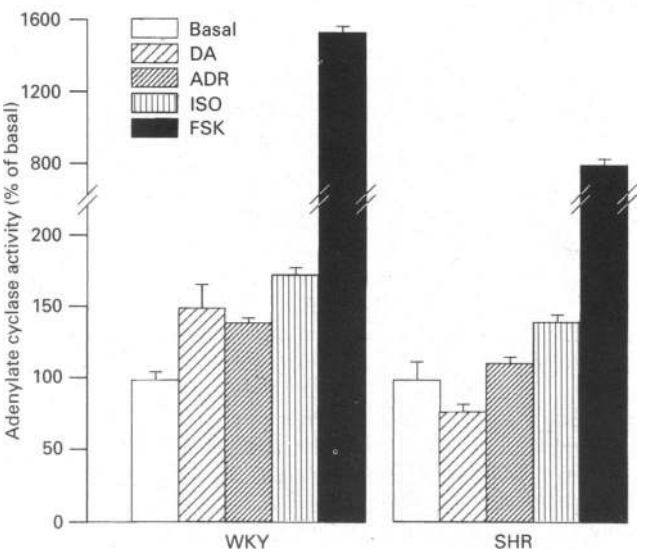

Fig. 5. Effect of stimulatory hormones on adenylate cyclase activity in aorta washed particles from 12-week-old WKY and age-matched SHR

Adenylate cyclase activity was determined in the absence of hormones (basal, □) or in the presence of 100 μM-dopamine (DA; ▨), 50 μM-adrenaline (ADR; ▩), 50 mM-isoprenaline (ISO; ▥) or 50 mM-FSK (■) as described in the Experimental section. Values are means ± S.E.M. of three separate experiments. Basal enzyme activity in the presence of 10 μM-GTP in WKY and SHR was 55 ± 4 and 42 ± 3 pmol of cAMP/10 min per mg of protein respectively. Six to eight animals were utilized in each experiment. Statistical analysis was performed with Student's t test for comparison between SHR and WKY: for dopamine and adrenaline, $P = 0.021$; for isoprenaline, $P = 0.013$; for FSK, $P < 0.01$.

three substrates of 45, 47 and 52 kDa for CT-catalysed ADP-ribosylation in heart membranes has been reported recently [34].

To investigate further if the unaltered expression of G_s in SHR could also be reflected in G_s functions, we studied the effect of CT on adenylate cyclase activity in heart sarcolemma from SHR and WKY rats. CT stimulated adenylate cyclase activity in heart sarcolemma from both WKY and SHR; however, the percentage stimulation was not significantly different in SHR as compared with WKY [WKY, basal + GTP (10 μM), 258 ± 34; CT, 690 ± 5; SHR, basal + GTP, 192 ± 25; CT, 554 ± 12 (pmol of cAMP)/10 min per mg of protein)]. These results indicate that G_s functions are also not altered in SHR.

Hormonal regulation of adenylate cyclase

Since G-proteins couple the hormone receptors to adenylate cyclase and mediate the stimulatory and inhibitory responses of hormones on adenylate cyclase, it was interesting to determine if the altered expression of $G_i\alpha$ is reflected in the hormonal regulation of adenylate cyclase. For this reason, we studied the effect of some hormones which stimulate or inhibit adenylate cyclase through G_s and G_i respectively on adenylate cyclase activity in heart sarcolemma and aorta from SHR and WKY rats.

Fig. 4(*a*) shows the effect of various concentrations of oxotremorine, which interacts with muscarinic receptors, on adenylate cyclase activity in heart sarcolemma from SHR and WKY rats. Oxotremorine inhibited the adenylate cyclase activity in a concentration-dependent manner in both SHR and WKY; however, the extent of inhibition was greater in SHR than in WKY. For example, at 5 μM, oxotremorine inhibited the adenylate cyclase activity by about 10% in WKY, which was increased to 40% in SHR. The enhanced inhibitory effect of oxotremorine on adenylate cyclase was associated with a decrease in the apparent K_i (3 μM as compared to 8.5 μM).

To investigate whether other hormones that inhibit adenylate cyclase through G_i regulatory protein can also result in the greater inhibition of adenylate cyclase in SHR as compared with WKY, we studied the effects of AII and ANF on adenylate cyclase in SHR- and WKY-rat aorta, a tissue which has been shown to have the receptors for both AII and ANF [23,24]. The results are demonstrated in Fig. 4(*b*) and 4(*c*). AII (Fig. 4*b*) and ANF (Fig. 4*c*) both inhibited the adenylate cyclase activity in a concentration-dependent manner in both SHR and WKY rats; however, the extent of inhibition was greater in SHR than in WKY and was associated with a decrease in K_i.

Since CT stimulated adenylate cyclase activity to a similar extent in both SHR and WKY and the levels of G_s were not different in membranes from SHR as compared with WKY, it was decided to examine if the enhanced expression of G_i can affect the G_s-mediated stimulatory responses of hormones in SHR. The results are shown in Fig. 5. Isoprenaline, dopamine, adrenaline and forskolin (FSK) all stimulated the enzyme activity to various degrees in aorta of SHR and WKY rats; however, the extent of stimulation was significantly decreased in SHR. For example, adrenaline, isoprenaline and dopamine stimulated the enzyme activity by about 42, 75 and 50% respectively in WKY rats, whereas in SHR the stimulations exerted by dopamine and adrenaline were completely abolished, and isoprenaline-stimulated enzyme activity was diminished by about 50%. Similarly FSK, which is believed to interact with the catalytic subunit of adenylate cyclase, stimulated the enzyme activity by about 1450% in WKY rats, which was decreased to 700% in SHR. Similar results (not shown) were also observed in the cultured vascular smooth-muscle cells from aorta from WKY and SHR.

DISCUSSION

The studies described in the present report elucidate some aspects of the mechanism responsible for eliciting the diminished sensitivity of some agents and hormones to stimulate adenylate cyclase activity in hypertension. It is well established that the effectiveness of various hormones such as glucagon, catecholamines, adenosine etc. to stimulate adenylate cyclase is depressed in myocardial membranes and aorta from SHR [35,36]. This decreased responsiveness to hormones has been shown to be associated with a decrease in the receptor number [36], a defective coupling and/or impaired catalytic subunit of adenylate cyclase system [21]. We have previously shown that all three components of adenylate cyclase system may be impaired in SHR [21]. Furthermore a great many studies have been performed on the receptors, especially β-adrenergic receptors and their regulation in hypertension [37], which suggest that down-regulation of the receptors may be one of the mechanisms responsible for the diminished sensitivity of adenylate cyclase in hypertension.

Since G-proteins play an important role in the regulation of adenylate cyclase, we mainly concentrated our studies on G-proteins and their activity in hypertension. In the present studies, we have demonstrated a significant decrease in the stimulatory effect of GTP[S] on adenylate cyclase in aorta from SHR as compared with WKY rats which indicate that G-protein in aorta may be impaired in SHR, as has been shown previously in heart sarcolemma [21]. However, our results on the CT-catalysed ADP-ribosylation and immunoblotting with antibodies against G_s did not demonstrate any alterations in the levels of $G_s\alpha$ in SHR as compared with WKY, and suggest that the G_s may not be responsible for the decreased ability of GTP[S] to stimulate

adenylate cyclase in SHR. In addition, the results demonstrating that CT treatment augmented the adenylate cyclase activity to a similar extent in heart sarcolemma and aorta (results not shown) from WKY and SHR further substantiate the notion that the function of G_s may not be altered in SHS. Similar results have also been demonstrated in failing human heart [38]; however, to our knowledge such studies have never been reported in hypertension. In contrast, Asano *et al.* [39] have reported a decreased function of G_s (vasorelaxation) in femoral arteries from SHR; however, the levels of G_s or G_i were not determined in those studies. The possible reason for this apparent discrepancy may also be the tissue (aorta/heart sarcolemma versus femoral arteries). A fundamental difference in biochemical and mechanical properties has been reported to exist between the vascular beds such as mesenteric arteries and cerebral arteries from SHR [40].

Our results on PT-catalysed ADP-ribosylation indicate an enhanced labelling of 40/41 kDa protein in aorta and heart from SHR as compared with WKY rats. In addition, by immunological studies we have demonstrated enhanced levels of $G_i\alpha$-2 and $G_i\alpha$-3 in heart and of $G_i\alpha$-2 in aorta from SHR as compared with WKY. Since $G_i\alpha$-2 and not $G_i\alpha$-3 has been implicated in the inhibition of adenylate cyclase [32,41,42], it may be possible that the enhanced levels of $G_i\alpha$-2 in SHR are responsible for the observed attenuation of adenylate cyclase activity stimulated by GTP[S]. The inhibition of GTP-stimulated adenylate cyclase activity by addition of G_i in the reconstitution system has also been demonstrated [43]. The mechanism by which $G_i\alpha$ inhibits adenylate cyclase activity has been a controversial subject. However, it has been suggested that free $\beta\gamma$ subunit, generated by the activation and dissociation of G_i, can inhibit adenylate cyclase activity, probably by binding to and thereby inactivating $G_s\alpha$–GTP [44], whereas other studies in G_s-deficient S49 cyc^- cells suggest a direct inhibitory action of $G_i\alpha$ on adenylate cyclase [45].

The enhanced inhibition of adenylate cyclase by AII, ANF and oxotremorine in SHR as demonstrated in the present studies can be attributed to the up-regulation of the receptors; however, no alterations in the AII receptors were observed in vascular membranes from SHR and WKY [46], and ANF-receptor binding sites were found to be decreased and not increased in SHR tissues [47]. Taken together, it may be suggested that the enhanced inhibition of adenylate cyclase by AII and ANF in SHR may not be at the receptor level and may involve the post-receptor events. Thus it may be suggested that the enhanced expression of $G_i\alpha$ in SHR may be one of the possible mechanisms which can explain the enhanced responsiveness of inhibitory hormones on adenylate cyclase in SHR. An enhanced expression of $G_i\alpha$ associated with enhanced inhibition of adenylate cyclase by adenosine and GTP in fat-cell membranes from hypothyroid rats has also been reported [48]. However, the present study is the first that demonstrates an enhanced expression of $G_i\alpha$-2 and its relationship with adenylate cyclase inhibition in hypertension. Similarly, the observed attenuated stimulation of adenylate cyclase by catecholamines in aorta from SHR as compared with WKY may be attributed to the decrease in the hormone-receptor binding sites [49,50] or to the defective coupling [51]. Since the G_s which couples the stimulatory hormone receptors to adenylate cyclase is not impaired in SHR, it appears that the increase in $G_i\alpha$-2 may partly be responsible for the attenuated responsiveness of catecholamines to adenylate cyclase stimulation. The modulation of G_s functions by G_i has also been reported by several investigators [38,43,48,52].

The decreased sensitivity of adenylate cyclase to stimulation by FSK in aorta from SHR as compared with WKY in the present studies may be due to the defective catalytic subunit itself [21], or to the over-expression of G_i, or both. The G_i-mediated inhibition of FSK-stimulated enzyme activity can be further substantiated by the results of various studies that have been performed on the effect of PT treatment on adenylate cyclase, and resulted in an augmentation of the FSK-stimulated enzyme activity [25]. On the other hand, the requirement for G_s and guanine nucleotides for the FSK activation of adenylate cyclase has also been reported [53]. Since the present studies do not demonstrate any alteration in G_s, the diminished responsiveness of adenylate cyclase to stimulation by FSK in hypertension cannot be attributed to the impaired G_s activity. Taken together, these data indicate that the enhanced expression of G_i in hypertension may partly be responsible for the diminished sensitivity of adenylate cyclase to FSK stimulation.

In conclusion, we have demonstrated that the expression of $G_i\alpha$ regulatory protein is enhanced in heart and aorta from SHR, whereas the levels of $G_s\alpha$ are not altered. The increased expression of $G_i\alpha$-2 appears to explain, in part, the increased responsiveness of adenylate cyclase to inhibitory hormones and decreased responsiveness to stimulatory hormones and agents which activate adenylate cyclase by a receptor-independent mechanism. It is suggested that the enhanced $G_i\alpha$-2 activity may be one of the mechanisms responsible for the diminished vascular tone and impaired myocardial functions in hypertension.

I am grateful to Dr. S. Mumby and Dr. A. G. Gilman for their kind gift of antibodies against G_s. I thank Christelle Thibault and Yuen Le Minh Quynh for performing immunoblotting experiments, Sylvie Picard and Line Pilon for their excellent technical assistance and Christiane Laurier for her valuable secretarial help. This work was supported by grants from the Quebec Heart Foundation and the Medical Research Council of Canada. I am the recipient of a Medical Research Council Scientist award from the Medical Research Council of Canada.

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Received 14 November 1991/6 May 1992; accepted 14 May 1992