AGE-RELATED ALTERATIONS IN ADENYLYL CYCLASE SYSTEM OF RAT HEARTS

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Cardiac membranes from 26-, 52- and 104-week-old Wistar rats were used to investigate the age-related alterations in the β -adrenergic receptor-adenylyl cyclase system. The densities and affinities of β -adrenoceptors did not change with aging. There were no significant changes in the total amount of stimulatory G-protein (G_s), and in G_s activity measured in a reconstitution assay using human platelet membranes. The major isoform of $G_s\alpha$, however, changed from a 45,000 to 52,000 dalton peptide with aging. The total amount of pertussis toxin substrates (G_{i2} and G_o) decreased significantly with aging. This finding was supported by the fact that pertussis toxin-induced potentiation of adenylyl cyclase activity was markedly reduced in the aged group. The activity of catalytic protein assessed by forskolin-stimulated adenylyl cyclase activity was decreased at 104 weeks. On the other hand, GTP analogue-stimulated adenylyl cyclase activity was significantly potentiated in the same group. These results suggest that the decreased sensitivity to catecholamines observed in aged hearts is mainly due to a dysfunction of catalytic protein, and that decreased G_i activity partially compensates for this catalytic dysfunction.

THE responsiveness of the cardiovascular system to catecholamines declines with aging¹⁻³ in contrast to the coincident increase of circulating catecholamines⁴⁻⁶. Dysfunction of the adenylyl cyclase system (AC system) is thought to be a mechanism for such alteration in response. To date, alterations in the β -adrenergic receptor and catalytic protein have been reported.^{7,8} However alterations of the stimulatory G-protein (G_s) and inhibitory G-protein (G_i) have been poorly characterized. The purpose of this investigation was to elucidate age-related changes in post-receptor components of AC system.

Key words:

Adenylyl cyclase system
GTP-binding protein
Aging

MATERIALS AND METHODS

The purified porcine brain islet-activating protein (IAP) substrates were kindly donated by Dr.T. Katada (Tokyo Institute of Technology, Yokohama, Japan) and Dr.M. Ui (Tokyo University, Tokyo, Japan). [³²P]-nicotinamide adenine dinucleotide (NAD) was obtained from NEN (USA) and [¹²⁵I]-cyanopindolol (ICYP) was obtained from Amersham International (USA). Radioimmunoassay kits for cAMP were from Yamasa Shoyu (Japan). Wistar rats were purchased from Shizuoka Laboratory Animal Center (Japan). Cholera toxin was from Sigma Chemical Corporation (USA). Other materials and chemicals were obtained from commercial sources.

(Received April 18, 1990; accepted October 26, 1990)

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TABLE I BODY WEIGHT AND HEART WEIGHT. MAXIMUM BINDING (Bmax) AND DISSOCIATION CONSTANT (Kd) OF ICYP IN HEART MEMBRANES

Group	Body weight	Heart weight	Heart/Body weight	ICYP binding Bmax	Kd
weeks (n)	g	mg	mg/g	fmol/mg	pM
26 (6)	330.8±29.3	723.8±54.4	2.19±0.07	57.6± 8.8	66.3±17.3
52 (6)	433.9±43.7 ^{*1}	930.9±68.5 ^{*2}	2.15±0.12	55.3± 5.0	51.6± 6.6
104 (6)	365.9±18.0	867.2±92.9	2.46±0.34	59.8±12.3	54.8±11.5

Crude membranes of 26-, 52- and 104-week-old Wistar rats were incubated with various concentrations of ICYP for 30 min at 30°C. Bound ligand was separated from free ligand by rapid filtration through glass-fiber filter, and filters were counted using a gamma-counter (Aloka, Japan). Non-specific binding was determined in the presence of 10 μ M propranolol. The results from six animals in each group were analyzed in Scatchard plots, and are shown as the mean $\pm$ SD. ^{*1}; significantly different from 26-week-old rats ($p < 0.005$) and 104-week-old rats ($p < 0.05$). ^{*2}; significantly different from 26-week-old rats ($p < 0.005$).

Fig.1. Scatchard analysis of ICYP binding to rat heart membranes.

Crude membranes from 26-, 52- and 104-week-old Wistar rats were incubated with various concentrations of ICYP for 30 min at 30°C. This figure shows the means of duplicate determinations from a single representative animal in each group. The mean values from multiple observations are listed in Table I.

Crude membrane preparation

Crude membranes were prepared from the hearts of 26-, 52- and 104-week-old male Wistar rats. Hearts were excised just after decapitation, and washed in 10 mM phosphate buffer (pH7.5) (PB) containing 5 mM EDTA, 1 mM EGTA, 5 mM MgCl₂ and 50 kallikrein inhibitory unit (KIU)/ml aprotinin at 4°C. All the following procedures were carried out at 0–4°C. After the atria and

great vessels were removed, the ventricles were homogenized with a Polytron-type homogenizer in PB. The homogenate was centrifuged at 1,250×g for 30 min. The resultant supernatant was centrifuged again at 100,000×g for 60 min, and the pellet was washed twice in the same buffer. The final pellet was suspended in PB and stored at –80°C until use. The final protein concentrations of the crude membranes were 1–2 mg/ml. The protein yields were about 0.5–1.5%, and essentially the same in all groups.

β -adrenergic receptor binding

Heart membranes were incubated with various concentrations of ICYP (25–1600 pM) for 30 min at 30°C. After the incubation, bound ligand was separated from free ligand by rapid filtration on a Whatman GF/C glass fiber filter. Non-specific binding was obtained in the presence of 10 μ M propranolol. The specific ICYP bound to β -receptors was determined by subtracting the non-specific bound from the total bound. The detailed procedures were the same as those described by Okajima et al⁹

Assay of adenylyl cyclase activity

Adenylyl cyclase activity was measured by radioimmunoassay in the presence of various agents such as 100 μ M isoproterenol, 100 μ M guanosine 5'-(3-o-thiotriphosphate) (GTP γ S), 100 μ M guanosine 5'-(β , γ -imido) triphosphate (GppNHp) and 100 μ M forskolin. The detailed procedures were the

TABLE II ADENYLYL CYCLASE ACTIVITY IN HEART MEMBRANES

Condition	26-week-old	52-week-old	104-week-old
<i>Adenylyl cyclase activity (pmol cAMP/mg/min)</i>			
<i>basal</i>	18.9 ± 9.8	15.1 ± 6.2	13.7 ± 3.7
<i>ISO</i>	20.8 ± 3.9	17.1 ± 4.5	15.4 ± 3.7
<i>GTPγS</i>	30.1 ± 2.4	26.3 ± 3.1	66.4 ± 20.1 ^{*1}
<i>GppNHp</i>	23.1 ± 4.4	22.0 ± 2.5	42.1 ± 12.1 ^{*1}
<i>forskolin</i>	410.4 ± 53.9	415.7 ± 40.0	248.1 ± 25.3 ^{*2}

Crude membranes of 26-, 52- and 104-week-old Wistar rats were incubated for 10 min at 30°C with various additions. The concentration of isoproterenol (ISO), GTP, GTPγS, GppNHp, and forskolin were 100 μM. Cyclase activity was assayed by the radiomunoassay kit for cAMP (Yamasa shoyu, Japan) in eight rats in each group. ^{*1}; significantly different from 26- and 52-week-old rats ($p < 0.001$). ^{*2}; significantly different from 26- and 52-week-old rats ($p < 0.01$).

Fig.2. Polyacrylamide gel analysis of radiolabeled products resulting from cholera toxin-catalyzed ADP-ribosylation of the cholate extracts.

Cholate extracts of 26-, 52- and 104-week-old Wistar rats were incubated with [³²P]-NAD for 1 hr in the presence of 75 μg/ml cholera toxin at 30°C. The ADP-ribosylated extracts were subjected to SDS-PAGE (10%T) and subsequent autoradiography. The amounts of protein applied to SDS-PAGE were 0.67 μg (Lane 1), 0.72 μg (Lane 2), and 0.64 μg (Lane 3) for 26-, 52 and 104-week-old rats, respectively. The total amounts of cholera toxin substrates were determined by filtration methods, and the mean values from multiple observations are summarized in Fig. 3. (A; self-ADP-ribosylated A-protoxin of cholera toxin, D; dye front, Kda; kilo dalton)

same as those described by Honma et al!⁰

Extraction of membrane proteins using sodium cholate

Crude membrane fractions were centrifuged at 100,000×g for 60 min at 4°C. The pellet was suspended in 20 mM Hepes-Na (pH8) containing 1 mM dithiothreitol (DTT), 1 mM EDTA and 1% sodium cholate. After incubation for 60 min on ice, the mixture was centrifuged at 180,000×g for 60 min at 4°C. The resultant supernatant was stored at -80°C as "cholate extract". The final concentration of the cholate extracts was 1-2 mg/ml. The protein yield of this procedure was essentially the same in all groups.

Bacterial toxin-catalyzed ADP-ribosylation

The procedures used for ADP-ribosylation were essentially the same as those described previously!¹¹ For ADP-ribosylation by cholera toxin, 50 mM KH₂PO₄ (pH7.6) was used instead of Tris-HCl (pH8). The reaction was stopped by the addition of an equal volume of Laemmli sample buffer!¹² and the sodium dodecyl sulfacte (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography were performed as described previously!¹³ To determine the amount of radiolabeled G-proteins, the reaction was terminated by the addition of 5 volume of 2% SDS solution containing 0.1% bovine serum albumin (BSA) and 5 volume of 30% trichloroacetic acied. The precipitated protein was then filtered on a nitrocellulose membrane, and counted with li- quid scintillator.

Fig.3. Amounts of cholera toxin substrates in the cholate extracts.

Cholate extracts of 26-, 52- and 104-week-old Wistar rats were incubated with cholera toxin and [32 P]-NAD as described in the legend to Fig. 2. After incubation, the reaction was terminated by the addition of 2% SDS solution containing 0.1 mg/ml BSA, and 30% TCA. Bound radioactivity was separated using nitrocellulose filters, and counted with a liquid scintillator, as described under "MATERIALS AND METHODS". Results represent mean $\pm$ SD for three different experiments.

Assay of G_i activity

The procedure for ADP-ribosylation was the same as that described in the previous section except that 2.5 μ M NAD was used instead of [32 P]-NAD. Crude membranes of 26- and 104-week-old Wistar rats (50 μ g) were incubated with the preactivated IAP (20 μ g/ml) for 1 hr at 30 $^{\circ}$ C. After incubation, the mixture was centrifuged at 100,000 $\times$ g for 1 hr at 4 $^{\circ}$ C, and the pellet was suspended in 20 mM Hepes-Na (pH8). Then, forskolin-stimulated adenylyl cyclase activity was assayed as described under "Assay of Adenylyl Cyclase Activity". Results were normalized by the forskolin-stimulated adenylyl cyclase activity obtained in the non-treated membranes.

Assay of G_s activity

The cholate extract was activated by 1 hr incubation at 30 $^{\circ}$ C with 20 mM Tris-HCl (pH8) containing 1 mM EDTA, 1 mM DTT,

Fig.4. Polyacrylamide gel analysis of radiolabeled products resulting from pertussis toxin-catalyzed ADP-ribosylation of the cholate extracts.

Cholate extracts were incubated with [32 P]-NAD for 1 hr in the presence of 20 μ g/ml pertussis toxin at 30 $^{\circ}$ C. The ADP-ribosylated extracts were subjected to SDS-PAGE (10%T) and subsequent autoradiography. A; Cholate extracts of 26-week-old Wistar rat (30 μ g, Lane 1), purified G_o (80 ng, Lane 2) and purified G_{i1} (40 ng, Lane 3) were ADP-ribosylated by pertussis toxin. B; Cholate extracts from 26-, 52 and 104-week-old Wistar rats were ADP-ribosylated by pertussis toxin. The amounts of applied protein were 0.67 μ g (Lane 1), 0.72 μ g (Lane 2), and 0.64 μ g (Lane 3) for 26-, 52- and 104-week-old rats, respectively. The total amounts of pertussis toxin substrates were determined by filtration methods, and the mean values from multiple observation are summarized in Fig. 5. (Kda; kilo dalton)

100 mM NaCl, 0.1% Lubrol-PX, 10 mM $MgCl_2$ and 3.3 μ M GTP γ S. The reaction was terminated by the addition of ice-cold 10 mM $MgCl_2$ solution containing 333 μ M GTP. Then G_s activity in the preactivated cholate extract was determined by a reconstitution assay with human platelet membranes as described previously¹⁴

Protein assay

Protein concentration was assayed by the amido-black staining method¹⁵ BSA was used as protein standard.

Statistical analyses

All experiments were performed in duplicate. Multiple observations were reported as the mean $\pm$ SD. Results were analyzed by Cochran-Cox t-test with $p < 0.05$ considered

Fig. 5. Amounts of pertussis toxin substrates in the cholate extracts

Cholate extracts of 26-, 52- and 104-week-old Wistar rats were incubated with 20 μ g/ml pertussis toxin and 5 μ M [32 P]-NAD as described in the legend to Fig. 4 and "MATERIALS AND METHODS". Results represent mean $\pm$ SD for three different experiments.

to be statistically significant.

RESULTS

Body weight and heart weight

The mean body weights and heart weights of 52-week-old rats were significantly higher than those of other groups. However, the ratio of heart weight to body weight was essentially the same in all groups, indicating that cardiac hypertrophy did not develop during the aging process (Table I).

β -adrenergic receptor

There were no significant changes in the maximum binding (B_{max}) and dissociation constant (K_d) for ICYP with aging (Table I, Fig. 1). In the membranes from the three groups, the competitive curves of isoproterenol displacing ICYP were made steeper and shifted to the right by the addition of 100 μ M GppNHp (data not shown). The patterns of isoproterenol competition for ICYP binding in the presence and absence of

Fig. 6. Effects of GTP γ S-activated G_s on adenylyl cyclase activity of human platelet membranes.

Cholate extracts from 26-, 52- and 104-week-old Wistar rats were activated by 1h incubation with 3.3 μ M GTP γ S at 30 $^{\circ}$ C, then diluted with ice-cold 10 mM $MgCl_2$ solution containing 333 μ M GTP to stop the reactions. An aliquot of the mixture was incubated with human platelet membranes for 15 min at 30 $^{\circ}$ C (14), then adenylyl cyclase activity was assayed. Results represent mean $\pm$ SD for six different experiments. The detailed procedure was described under "MATERIALS AND METHODS".

GppNHp were similar among these three groups. This may be because there was no difference in the coupling of G_s to β -adrenergic receptors in the cardiac membranes of these rats¹⁶

Adenylyl cyclase activity

The basal activity and the isoproterenol-stimulated adenylyl cyclase activity decreased during aging, though these decreases were not statistically significant. Adenylyl cyclase activity stimulated by non-hydrolyzable GTP analogues, such as GTP γ S and GppNHp, were significantly potentiated in the 104-week-old group. On the other hand, the forskolin-stimulated adenylyl cyclase activity was significantly decreased in the 104-week-old group (Table II).

Fig. 7 Pertussis toxin-induced potentiation of adenylyl cyclase activity.

Crude membranes from 26-, 52- and 104-week-old Wistar rats were incubated with the preactivated pertussis toxin. After the incubation, membranes were centrifuged, and pellets were suspended in 20 mM Hepes-Na (pH8), then forskolin-stimulated adenylyl cyclase activity was assayed. The results were normalized by the forskolin-stimulated adenylyl cyclase activity obtained in non-treated membranes. Results represent mean $\pm$ SD for four different experiments. The detailed procedure was as described under "MATERIALS AND METHODS".

Cholera toxin-catalyzed ADP-ribosylation of G_s

The cholate extracts were ADP-ribosylated by cholera toxin in the presence of [32 P]-NAD, and subjected to SDS-PAGE, and subsequent autoradiography. As shown in Fig. 2, three different isoforms of $G_s\alpha$ -subunit were radiolabeled in rat heart membranes, and their molecular weights were estimated to be 45,000, 47,000, 52,000 dalton as reported previously!⁷ The major isoform changed from 45,000 to 52,000 dalton with aging. Although hearts from the 104-week-old group contained relatively higher amount of G_s than did those from the other groups, there were no significant changes (Fig. 3).

Pertussis toxin-catalyzed ADP-ribosylation of G_i

Two distinct polypeptides were radiolabeled by pertussis toxin in the presence of [32 P]-NAD, and their molecular

weights were estimated to be 40,000 and 39,000 dalton when the purified pertussis toxin-labeled substrates were used as molecular weight standards (Fig. 4A). The total amounts of the pertussis toxin labeled substrates significantly decreased in the 104-week-old group (Fig. 5).

G_s activity

The G_s activities of cholate extracts were measured in a reconstitution experiment using human platelet membranes and did not change significantly with aging (Fig. 6).

G_i activity

Pertussis toxin-induced potentiation of cyclase activity was diminished in 104-week-old rats, indicating that G_i function was disturbed in the aged group (Fig. 7). Forskolin-stimulated adenylyl cyclase activities of pertussis toxin-treated membranes from 26- and 104-week-old rats were 223.7 ± 23.1 and 141.0 ± 19.1 (pmol cAMP/mg/min), respectively. Those of pertussis toxin non-treated membranes were 168.5 ± 14.7 and 125.6 ± 107 (pmol cAMP/mg/min), respectively.

DISCUSSION

A reduction in agonist-stimulated adenylyl cyclase activity with aging has been documented for several tissues, including rabbit brain¹⁸ and rat adipocytes!⁹ In these tissues, alterations in agonist-stimulated adenylyl cyclase activity were accompanied by a parallel change in receptor density. However, in the case of cardiovascular tissue, conflicting results have been proposed by several laboratories. Abrass et al reported that there were no changes in the density or in the affinity of myocardial β -adrenergic receptors in Fisher 344 rats! Kusiak and Pitha showed that the density of β -adrenergic receptors was slightly increased in old Wistar rats!⁸ Fan and Banerjee reported that β -adrenergic receptors declined by 20–25% as Fisher 344 rats matured from adolescence to adulthood, but that no further decrease occurred with senescence!²⁰ The reason for different results from different investigators is not clear. In this study, the density and affinity of β -adrenergic receptors did not change during the aging process (Table I and Fig. 1).

Age-related dysfunction of the catalytic protein of adenylyl cyclase has also been reported in several tissues, such as rat heart^{8,20,21} rat lung^{22,23} and human lymphocytes.⁷ We used forskolin-stimulated cyclase activity to evaluate the function of catalytic protein, and confirmed that the catalytic activity declined with aging (Table II). In this study, we did not detect significant reduction in basal and isoproterenol-stimulated adenylyl cyclase activity with aging. These results might indicate that the cell-free system was not suitable for the evaluation of signal transducing efficiency throughout the stimulatory pathway of adenylyl cyclase system. It is well known that isoproterenol-stimulated adenylyl cyclase activity is less than non-hydrolyzable GTP analogue-stimulated adenylyl cyclase activity in the crude membranes of heart. Inaccessibility of isoproterenol might be the major cause of this phenomenon. Membranes are fragmented during the homogenization procedure, and finally compose small vesicles. These vesicles can be divided into three categories: right-side-out vesicles, inside-out vesicles and leaky vesicles. In the case of inside-out vesicles, ligand-binding sites of β -adrenergic receptors are presumably protruding to the internal space of the vesicles, so as a hydrophilic compound, isoproterenol has decreased accessibility for these receptors. On the other hand, isoproterenol can interact β -adrenergic receptors in right-side-out vesicles, since their ligand-binding sites would be located outside the vesicles. In these vesicles, however, cyclase activity may not be detected by the assay system we use, because the catalytic proteins are facing inside vesicles and hydrophilic ATP, the substrate for the catalytic protein, cannot pass through the lipid bilayer. Thus, we preferentially measure the isoproterenol-stimulated adenylyl cyclase activity in the leaky vesicles which are very few in normal membrane preparations. In our membrane preparation, the forskolin-stimulated adenylyl cyclase activity, which was measured in the presence of alamethicin (a channel forming antibiotic) was twice as high as activity without alamethicin, indicating that about 50% of the membrane fractions were inside-out and the remaining 50% membranes were right-side-out (data not shown).

Compared with studies on β -adrenergic receptors and catalytic protein, investigations dealing with age-related changes in G-proteins have been few and have yielded inconclusive results. In this study, three different isoforms of $G_s\alpha$ were ADP-ribosylated by cholera toxin (Fig. 2) as we reported previously.¹⁷ The major isoform changed from 45,000 to 52,000 dalton with aging, though the total amount of these isoforms did not change (Fig. 3). This kind of isoform switching has also been observed during the maturation of rat reticulocytes.²⁴ Several laboratories have isolated and characterized cDNA clones which encode $G_s\alpha$.²⁵⁻³⁰ Recently, Kozawa et al revealed that the $G_s\alpha$ gene isolated from human genomic libraries was composed of 13 exons and 12 introns, and suggested that four types of $G_s\alpha$ mRNA may be generated from a single gene by alternate use of certain exons with two different splice junctions.²⁷ Although the functional significance of these isoforms is not known, they could have different roles in the interaction between receptors and effectors.

Two peptides were ADP-ribosylated by pertussis toxin in this study, and their molecular weights were estimated to be 40,000 dalton (major isoform) and 39,000 dalton (minor isoform) (Fig. 4A). Because the 39,000 dalton peptide was detected by an antiserum prepared against G_o , it was thought to be $G_o\alpha$ (data not shown). Recently, Jones et al reported four types of recombinant clones encoding four distinct pertussis toxin substrates.³¹ Suki et al proposed the terms, G_{i1} , G_{i2} , G_{i3} and G_o , as the nomenclature for these pertussis toxin substrates.³² Among the three α -subunits which compose the G_i -family, $G_{i1}\alpha$ and $G_{i2}\alpha$ may correspond to the 41,000 dalton peptide and 40,000 dalton peptide, respectively.³³ The major pertussis toxin substrate detected in this study seemed to be $G_{i2}\alpha$, because it migrated between purified $G_{i1}\alpha$ and G_o on SDS-PAGE (Fig. 4A). This finding was supported by the fact that $G_{i2}\alpha$ mRNA is expressed more intensely than $G_{i1}\alpha$ and $G_{i3}\alpha$ mRNA in cardiac cells.³¹ In this study, the total amount of pertussis toxin substrates decreased in the aged group (Fig. 5). Moreover, pertussis toxin-induced potentiation of adenylyl cyclase activ-

ity was significantly reduced in the 104-week-old rats (Fig. 7). These results indicate that the hormone-stimulated inhibition of adenylyl cyclase activity is diminished in the aged animal probably because of a decrease in pertussis toxin substrates, in particular G_{i2} .

Adenylyl cyclase activity stimulated by GTP γ S or GppNHp was significantly higher in the 104-week-old group (Table II). Because the catalytic activity was diminished in the 104-week-old group, the nonhydrolyzable GTP analogue-induced potentiation of activity suggests the existence of compensatory mechanism at the level of G-proteins: increased G_s activity and/or decreased G_i activity. If the alteration of G_s was to bring about the potentiation, one possibly would be that activated 52,000 dalton $G_{s\alpha}$ stimulates catalytic proteins more effectively than does activated 45,000 and 47,000 dalton $G_{s\alpha}$, because the total amount of $G_{s\alpha}$ did not change with aging. To clarify the relation between the isoform switching of $G_{s\alpha}$ and the GTP analogue-induced potentiation of adenylyl cyclase activity, we assessed G_s activity in reconstitution assay using human platelet membranes. Contrary to our expectation, G_s activities of the three groups were essentially the same (Fig. 6). This result suggests that the non-hydrolyzable GTP analogue-induced potentiation of cyclase activity was mainly derived from the decrease of G_i .

We conclude that the age-related reduced sensitivity to catecholamines is mainly due to a dysfunction of catalytic protein, and that decreased signal transduction via G_i seems, to some extent, to compensate for the dysfunction of catalytic protein. Further research will be required to clarify the physiological roles of the prominent switching of $G_{s\alpha}$ isoforms, and the decrease of G_i during the aging process.

Acknowledgments

We gratefully acknowledge Dr. Paul A. Insel (Department of Pharmacology, University of California San Diego) for his helpful discussion and critical review of this manuscript.

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