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EFFECT OF AGE ON β-RECEPTORS, $G_{s\alpha}$- AND $G_{i\alpha}$-PROTEINS IN RAT HEART

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Abstract—β-adrenoceptors, $G_{s\alpha}$- and $G_{i\alpha}$-proteins were investigated in a crude plasma membrane preparation from ventricles of young (2–4 months) and senescent (22–24 months) Wistar rats. Receptor density, ligand affinity and β_1/β_2-receptor ratio were independent of the age of the rats. The percentage of β-receptors coupled to G-proteins increased with age. An age-related increase in the level of $G_{s\alpha}$ (124%) was paralleled by an increase in the ratio between the high and low molecular weight form of $G_{s\alpha}$. The level of $G_{i\alpha}$-protein almost doubled (170%) upon aging. We conclude that the age-related differences are small at the level of the β-adrenoceptor molecule, but that the increase in $G_{i\alpha}$-proteins could be responsible for the age-related reduction in myocardial inotropic and chronotropic responses. Moreover, we suggest that the changes in degree of high affinity coupling between β-receptor and G_s-protein are possibly linked to alterations in the ratio between the G_s-molecular weight subtypes.

Key words: age; β-receptors; G-proteins; heart

In rat myocardial tissue, stimulation with β-adrenergic agonists results in inotropic and chronotropic responses and it is generally accepted that these effects are diminished with aging [1–4]. Moreover, Sakai *et al.* [4] found that the contractile response of cardiac myocytes to increases in bathing $[Ca^{2+}]$ did not vary with age and concluded that the age-associated contractile deficit during β-adrenergic stimulation is specific to the β-adrenergic signal transduction (β-AST)† pathway. Since the β-AST-system consists of at least three components (receptor, G_s-protein and adenylate cyclase—EC 4.6.1.1.), each of them could be affected by age. There is, however, no consensus on the age-related modifications at the level of this transduction system. In the majority very few changes in the β-adrenoceptor density are found [2, 5–7] although occasionally a decreased [8–10] receptor density was reported. β-receptor stimulation leads to enhanced synthesis of cAMP and this process is thought to be mediated by high affinity, GTP-sensitive binding of agonists to β-receptors. Scarpace [11] found that in rat heart, aging was accompanied by a decrease in coupling between β-receptor and G-protein, which corresponds to what we found in other rat tissues [12–14]. Moreover, guanine nucleotide-stimulated adenylate cyclase activities were reduced in aged rat hearts [15, 16], suggesting age-related alterations at the level of the G-protein. Additionally, it was reported that in myocardial failure, the persistent sympathetic drive produces a downregulation of myocardial β-adrenoceptors [17–19] and an increased expression of $G_{i\alpha}$ [20, 21]. Since plasma norepinephrine levels are also increased in aging rats [22], it is tempting to speculate that similar alterations occur in myocardial tissue of the senescent rat.

These observations prompted us to reinvestigate the effect of aging on the properties of the β-adrenergic signal transduction system in rat heart ventricles with special emphasis on both $G_{s\alpha}$- and $G_{i\alpha}$-proteins, which respectively stimulate and inhibit the effector enzyme adenylate cyclase. While receptor characteristics remained constant, the cellular content of both $G_{s\alpha}$- and $G_{i\alpha}$-proteins was affected by age and the possibility that this is responsible for the diminished inotropic and chronotropic responses in aged rats is discussed.

MATERIALS AND METHODS

Animals. Male Wistar rats either 2–4 months or 22–24 months old were obtained from the Proefdierencentrum of the University of Leuven (Belgium). They were housed individually and had free access to food and acidified water until 24 hr before sacrifice.

Materials. ICYP was obtained from Amersham (Amersham, U.K.); the specific activity was 2000 Ci/mmol. (−)-Isoproterenol (a β-nonselective agonist) and goat-anti-rabbit affinity purified IgGs labelled with peroxidase (EC 1.11.1.7; secondary antibody) were obtained from Sigma (Poole, U.K). GppNHp was purchased from Boehringer-Mannheim (Brussels, Belgium). The β_1-subtype selective antagonist CGP 20,712A {(±) - (2 - (3 - carbamoyl - 4-hydroxyphenoxy)-ethylamino)-3-[4-(1-methyl-4-trifluoromethyl-2-imidazolyl)-phenoxy]-2-propanol-

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† Abbreviations: β-AST, β-adrenergic signal transduction; IgGs, immunoglobulin Gs; BSA, bovine serum albumin; SDS, sodium dodecylsulphate; KLH, Keyhole Limpet Hemocyanin; PBS, phosphate buffered saline; ICYP, [^{125}Iodo] (−) cyanopindolol; GppNHp, guanylyl-imidobisphosphate; SEM, standard error of the mean.

methane sulphonate} was a gift from Ciba Geigy (Groot-Bijgaarden, Belgium). The β_2-subtype selective antagonist ICI 118,551 [erythro-(±)-1-(7-methylindan-4-yloxy) -3- isopropylaminobutan-2-ol] and the β_1-subtype selective antagonist ICI 89,406 (1-(2-cyanofenoxy)-3-β-(3-fenylureido)-ethylamino-2-propanol) were gifts from ICI (Destelbergen, Belgium). All other chemicals were of the highest purity grade commercially available.

Tissue preparation. Animals were killed by decapitation and the heart ventricles were homogenized in buffer [NaCl/Tris/ethylene glycol-bis(β-aminoethyle ether)*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 154/10/2 mM, pH 7.4], using an Ultra Turrax (3 times for 15 sec with 1 min interval at a dilution of 1/20, wet weight/volume). The homogenate was centrifuged for 10 min at 3000 *g* (Sorvall, SS34-rotor). The supernatant was then centrifuged for 20 min at 27,000 *g* and the pellet was washed twice in homogenization buffer (Potter Elvehjem, 4 strokes). The final pellet was resuspended in Tris/$MgCl_2$/EGTA, 50/20/2 mM, pH 7.4 at 1–2 mg protein/mL for ligand binding experiments. This suspension was used either directly (isoproterenol competition binding) or stored at −70°, for at most 2 weeks. The protein content was determined with the dye-binding method of Macart and Gerbaut [23] using BSA as standard. For immunoblotting experiments, the final pellet was resuspended in Laemmli [24]-electrophoresis buffer (62 mM Tris containing 5% mercaptoethanol, 2% SDS and 0.001% bromophenol blue) at approximately 2 mg protein/mL. Protein concentration was determined by the method of Lowry [25] as modified for the determination of proteins in detergent-containing buffers [26].

Ligand binding experiments. Saturation and competition binding experiments were performed as described previously [12]. Saturation binding was done with a concentration range of 1 to 300 pM ICYP (9 points in duplicate); nonspecific binding was calculated from a parallel incubation (9 points in duplicate) including 100 μM isoproterenol and was, at the value of the dissociation constant, 20.1 ± 4.1% (N = 6) for ventricles of young and 24.6 ± 2.7% (N = 6) for ventricles of senescent rat; all incubations were performed at 37°. In competition binding experiments, approximately 100 pM ICYP was incubated with 12 concentrations of either the agonist isoproterenol (from 10^{-3} M to 10^{-10} M) with or without GppNHp (250 μM) at a temperature of 30° or with the antagonists CGP 20, 712A, ICI 118, 551 or ICI 89,406 (all from 10^{-4} M to 10^{-11} M) at 37°. In both types of experiments approximately 40 μg protein was used for each point; the samples were incubated for 60 min which is sufficient for reaching complete equilibrium [27]. Bound ligand was separated from free ligand by filtration through Whatman GF/C glass fiber filters (Skatron Cell Harvester). Radioactivity was counted in a Packard gamma counter with 80% efficiency.

Saturation binding curves were analysed either by Scatchard analysis [28] or by the iterative curve-fitting program LIGAND [29]; displacement curves were fitted using the iterative program GraphPAD [30]. Curves were always fit according to a one and a two site model and the best fit was chosen based on a partial *F*-test [31]. K_i-values were calculated using the formula of Cheng and Prusoff [32]. Statistical significance was calculated using the unpaired two tailed *t*-test. Significance was accepted at the 0.05 level.

Production of antisera. Antibodies against $G_{s\alpha}$- and $G_{i\alpha}$-proteins (primary antibodies) were raised in white albino rabbits against a synthetic peptide coupled to KLH through an extra added COOH-terminal cysteine residue using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester as cross-linking agent [33]. The selected amino acid sequence was "TPEPGEDPRVTRAKY" for $G_{s\alpha}$ and "KQLQKDKQVYRATHR" for $G_{i\alpha}$ [33]. Rabbits were injected intradermally with the KLH-peptide solubilized (1 mg/mL) in a 1/1 mixture of PBS and either complete Freund's adjuvant (for the first injection) or incomplete Freund's adjuvant (for the booster injections). Each rabbit received between 0.8 and 1 mg of antigen at the first injection and between 0.4 and 0.8 mg at each of the three booster injections which were given every 21 days. Serum was stored at −70° for months without apparent loss of activity. Reactivity of the antisera was tested using an ELISA in which immunoplates were coated with the synthetic peptides and was found to be high and specific for the corresponding antigens (results not shown). Moreover, the antisera obtained against these peptides were previously shown to label all splice variants of the $G_{s\alpha}$- and $G_{i\alpha}$-protein respectively [33, 34] and this result was confirmed with our antisera.

Electrophoresis and immunoblotting. Protein samples were boiled for 3 min and 40 μL of protein was loaded. The procedure of Laemmli [24] was further followed in all details with a 9% separation gel and 4% stacking gel. After electrophoresis, gels were equilibrated in blotting buffer for 20 min. Blotting was done according to Towbin [35] on nitrocellulose for 3 hr at 300 mA (Transblot Biorad); the temperature was kept below 20°. The blots were quenched overnight in 0.4% bovine serum albumin in phosphate-buffered saline (PBS–BSA) and then incubated with primary antibody, diluted 1/2000 in PBS–BSA, for 2 hr. Blots were washed in PBS containing 0.05% Tween-20 (polyoxyethylene sorbitanmonolaureate; PBS–Tween) and incubated during 1.5 hr with secondary antibody, diluted 1/3000 in PBS–BSA followed by washing in PBS–Tween. The blots were subsequently stained for peroxidase activity using aminoethylcarbazole in 50 mM natriumacetate (pH = 4.5) and H_2O_2 as substrate. Quantitation of the blots was done according to McFarlan-Anderson *et al.* [36]. Stained blots were scanned with reflectance densitometry (Joyce-Loebl Chromoscan 3) and peak heights were recorded. Net peak height was corrected for the exact protein level of the applied sample. These arbitrary units were used as estimates for the level of G_α-proteins in the membrane fraction. The total $G_{s\alpha}$-content was calculated as the sum of the two subtypes. In order to avoid interblot variability, all samples were determined in the same run under the same conditions of time and temperature. The blots were stained for 15 (α_s-assay) or 5 (α_i-assay) min,

Table 1. Rat characteristics: total body weight in g, wet ventricle weight in g and the ratio ventricle weight/body weight

	Young (N = 7)	Senescent (N = 7)
Body weight	316.7 ± 39.1	474.0 ± 29.5*
Ventricle weight	0.78 ± 0.09	1.04 ± 0.09
Weight ratio (10^{-3})	2.47 ± 0.20	2.19 ± 0.10

Means ± SEM, N: number of rats.
* Significantly different from young rats ($P < 0.05$).

Table 2. Quantitative data of the saturation and competition binding experiments with ICYP as ligand

	Young	Senescent
Saturation binding	(N = 14)	(N = 4)
B_{max}	42.2 ± 3.9	41.6 ± 3.9
K_d	36.5 ± 4.2	35.9 ± 7.2
Competition binding		
CGP20,712A	(N = 6)	(N = 5)
% HA	54.5 ± 4.1	53.8 ± 1.7
K_i,HA	2.3 ± 0.6	4.2 ± 0.2
K_i,LA	26.4 ± 10.0	28.6 ± 5.9
ICI 89,406	(N = 6)	(N = 6)
% HA	50.4 ± 2.6	50.1 ± 1.6
K_i,HA	1.2 ± 0.5	5.8 ± 2.9
K_i,LA	2.2 ± 0.7	6.6 ± 3.3
ICI 118,551	(N = 7)	(N = 6)
% HA	36.5 ± 2.4	33.3 ± 2.0
K_i,HA	2.5 ± 0.5	1.2 ± 0.3
K_i,LA	7.3 ± 2.8	3.4 ± 1.0

Receptor density (B_{max} in fmol/mg protein) and equilibrium dissociation constant (K_d in pM); percentage of high affinity binding sites (% HA), equilibrium inhibition constants of the high affinity binding sites (K_i,HA in 10^{-9} M) and equilibrium inhibition constants of the low affinity binding sites (K_i,LA in 10^{-7} M). Means ± SEM; N: number of experiments.

which is within the linear kinetics of the enzyme reaction, as was assured by preliminary experiments. Finally, various amounts of protein sample, ranging from 20 to 100 μg, were subjected to the electrophoresis procedure and subsequent calculation of the cellular levels of $G_{s\alpha}$- and $G_{i\alpha}$-protein yielded linear results (results not shown).

RESULTS

The characteristics of the rats are summarized in Table 1. There is a weight gain of approximately 150 g in the senescent rats, while the ventricle weight increases slightly from 0.78 to 1.04 g. This corresponds with a nonsignificant tendency for an age-dependent decrease in the ratio ventricle weight/body weight.

The data on the β-receptor density and the results of the determination of the β-receptor subtypes are

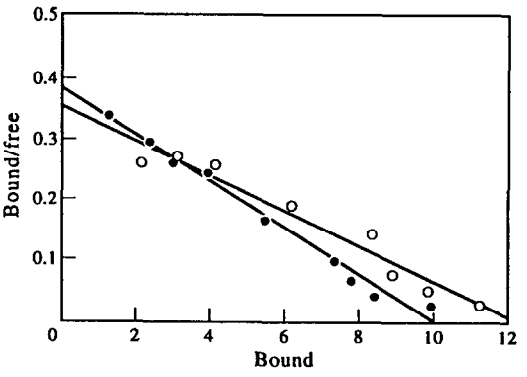

Fig. 1. Representative examples of saturation binding curves with ICYP as ligand in ventricle plasma membranes of young (closed symbols) and senescent (open symbols) rats. The amount of bound ligand is expressed in pM.

summarized in Table 2. In Fig. 1 representative examples of saturation experiments with ventricle crude membrane preparations are shown. Receptor density (B_{max} in fmol/mg protein) and affinity of the receptor for the ligand (K_d in pM) were unaffected by the age of the animal. Representative examples of the antagonist competition curves are depicted in Fig. 2. Using the β_1-selective antagonists CGP 20,712A and ICI 89,406, the percentage of receptors with high affinity is comparable in rats of both ages (ca. 54 and 50%, respectively). Using the β_2-subtype selective antagonist ICI 118,551 the percentage of receptors with high affinity for the ligand also remains constant with aging (approximately 36.5% in young and 33.3% in senescent animals).

The results of the agonist high affinity binding experiments are summarized in Table 3; representative examples of the agonist competition binding are depicted in Fig. 3. The percentage of receptors with high affinity for isoproterenol increases significantly with aging; the affinity of the high and low affinity binding site for the agonist is independent of the age of the animal. The affinity of the binding site in the presence of GppNHp is comparable for both age groups (approximately 0.5 μM) and slightly lower than the low affinity binding site in the absence of GppNHp.

A representative example of an immunoblot experiment with heart ventricles of young and senescent rats is depicted in Fig. 4; in Table 4 average values for all determinations are given. When the antibody against $G_{s\alpha}$ is used, two clearly discernible bands with molecular weights of 52 kDa ($G_{s\alpha large}$, $G_{s\alpha l}$) and 45 kDa ($G_{s\alpha small}$, $G_{s\alpha s}$) are seen. The ratio between the two subtypes ($G_{s\alpha l}/G_{s\alpha s}$) increases significantly upon aging. The total $G_{s\alpha}$ content increases slightly but significantly upon aging. $G_{i\alpha}$-protein is recognized upon immunoblots as one single band in SDS–PAGE at a molecular weight of 44 kDa. The intensity of the band increases significantly 1.7-fold upon aging.

DISCUSSION

The purpose of the present study was to investigate

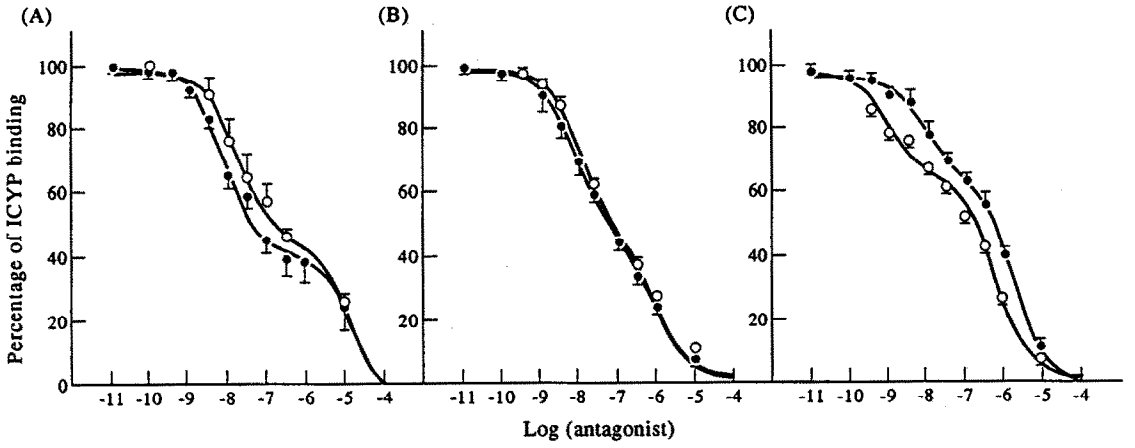

Fig. 2. Mean competition binding curves with ICYP as ligand and the β_1-selective antagonists CGP 20,712A (A) or ICI 89,406 (B) or the β_2-selective antagonist ICI 118,551 (C) as displacer in ventricle plasma membranes of young (closed symbols) and senescent (open symbols) rats. The binding at the lowest and highest concentration of competitor is set at 100 and 0%, respectively. Curves are the mean of at least four different experiments in duplicate.

Table 3. Quantitative data of the competition binding experiments with ICYP as ligand and the nonselective β-agonist (−)isoproterenol as displacer

	Young (N = 12)	Senescent (N = 6)
% HA	42.2 ± 4.3	56.4 ± 1.7*
K_i,HA	23.0 ± 4.9	23.9 ± 12.6
K_i,LA	16.6 ± 5.0	8.3 ± 2.9
K_i,Gp	5.2 ± 0.7	3.8 ± 0.8

* Significantly different from young rats ($P < 0.05$).

Percentage of high affinity binding sites in the absence of GppNHp (% HA), equilibrium inhibition constant of the high affinity binding site (K_i,HA in 10^{-9} M), equilibrium inhibition constant of the low affinity binding site (K_i,LA in 10^{-7} M) and equilibrium inhibition constant of the agonist binding site in the presence of 250 μM GppNHp (K_i,Gp in 10^{-7} M). Means ± SEM; N: number of experiments.

the age-related alterations in rat myocardial tissue involved in the reduced inotropic and chronotropic responses to β-adrenergic agonists, with special emphasis on both $G_{s\alpha}$- and $G_{i\alpha}$-proteins, which respectively stimulate and inhibit adenylate cyclase.

Receptor density in our preparations was about 40 fmol/mg protein, which is comparable with some data from the literature [8, 37–40] and two to three times higher than what is found by others [2, 5–7, 41, 42]. It is, however, striking that this discrepancy parallels the use of different rat strains: Wistar [8, 37–40] or other (Fischer-344 [2, 5, 6, 41, 42] and Sprague–Dawley [7]) rats. Hence, we believe that the genetic differences between those rats are responsible for these variations. Our results show furthermore that in Wistar rats receptor density is hardly affected by age, which is compatible with data in the literature [2, 5–7] but in contrast with

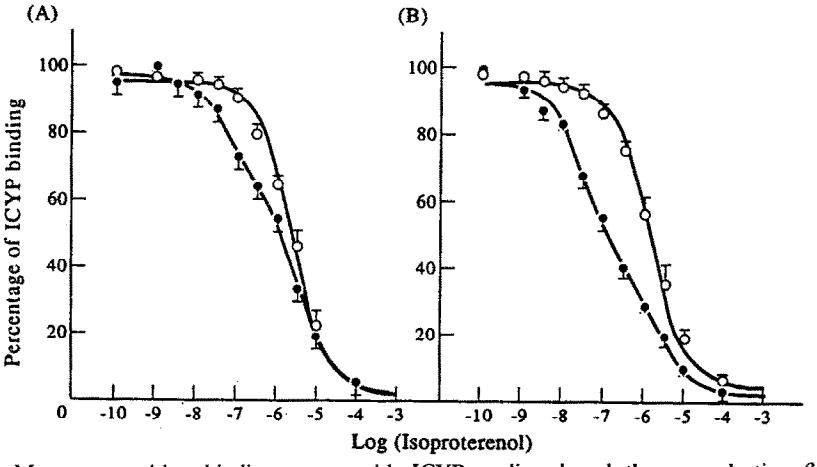

Fig. 3. Mean competition binding curves with ICYP as ligand and the non-selective β-agonist (−)isoproterenol as displacer in ventricle plasma membranes of young (A) and senescent (B) rats. The binding at the lowest and highest concentration of competitor is set as 100 and 0%, respectively. Closed symbols indicate competition binding without GppNHp and open symbols competition binding with 250 μM GppNHp. Curves are the mean of at least four different experiments in duplicate.

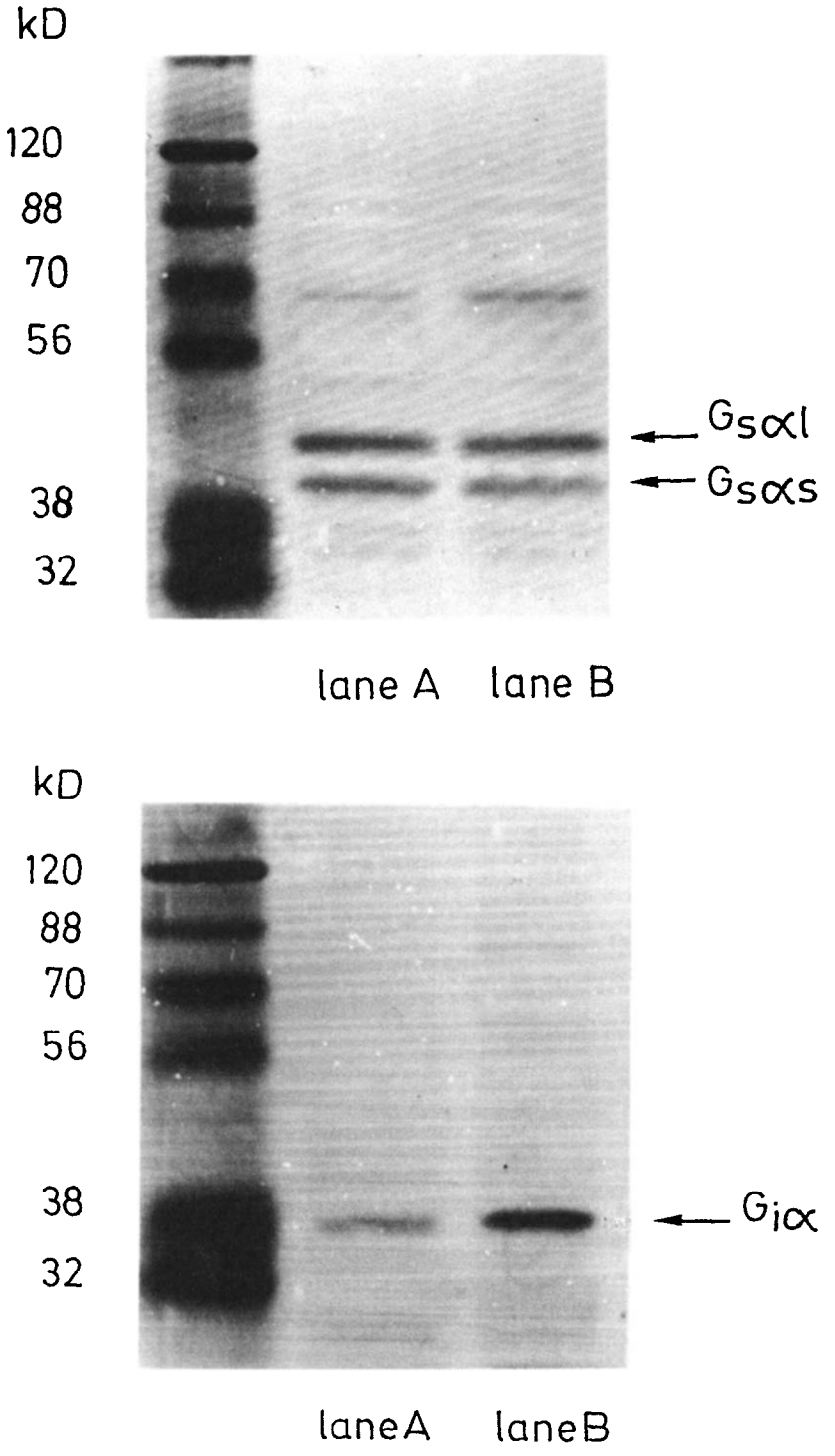

Fig. 4. A representative example of an immunoblot experiment with heart ventricles of young (lane A) and senescent rats (lane B), using anti-$G_{s\alpha}$-antibodies (upper part) and anti-$G_{i\alpha}$-antibodies (lower part) as primary antibodies. Molecular weight markers are as indicated.

those of Gudmundsdottir *et al.* [8]. Part of this discrepancy is possibly due to differences in diets fed to the animals. The latter authors show indeed that the diet can influence receptor density markedly.

The results with the antagonists are straightforward. The ratio between the percentage β_1- and β_2-receptors was independent of the age of the animal, confirming previous studies on this item

Table 4. Average values for $G_{s\alpha}$- and $G_{i\alpha}$-protein content in cardiac ventricle membranes of young and senescent rats

	Young (N = 4)	Senescent (N = 4)
Total $G_{s\alpha}$-protein content	58.2 ± 2.3	72.3 ± 1.2*
Ratio $G_{s\alpha l}/G_{s\alpha s}$	0.56 ± 0.02	0.77 ± 0.02*
Total $G_{i\alpha}$-protein content	19.6 ± 0.5	33.6 ± 2.0*

* Significantly different from young rats ($P < 0.05$).
Values are given in arbitrary units. Means ± SEM; N: number of experiments.

[41, 43]. It varied between 1.4 and 1.6, which is slightly lower than what was found by Tumer *et al.* [41, 43] in ventricular tissue of Fischer-344 rats. This difference could be caused by the difference in radioligand concentration used in his (approximately 1/2 of the K_d) versus our experiments (approximately 3 times the K_d). This high concentration was chosen for our experiments, since it has been shown before that the concentration of radioligand should be nearly saturating for correct estimation of the receptor subtype distribution [44].

To our surprise, we found that the degree of high affinity coupling between receptor and agonist, which indicates the percentage of G-protein coupled receptors, was significantly increased upon aging. This increase is in contrast with what is found in female Fischer-344 rats [11]. The influence of strain and gender and of differences in experimental conditions, including differences in concentrations of ICYP used in competition binding experiments [44], could cause part of the discrepancy. This result is also in contrast with our previous observations in other rat tissues, including kidney [13] and liver [14]. This observation suggests that age might affect G-proteins and this was clearly illustrated in our subsequent experiments.

In ventricular tissue, indeed, aging is also accompanied by a significant shift in the ratio between the high and low molecular weight form of $G_{s\alpha}$-protein in favour of the former. Again, this is in contrast with the results in kidney and liver* where the ratio between both isoforms was of a similar magnitude as in heart tissue of young animals, but displayed a slight tendency to decrease with age. Considering these parallel changes between the percentage of high affinity-coupled receptors and the ratio between the high and low molecular weight form of $G_{s\alpha}$ in heart as well as in kidney and liver tissue, our results suggest that the high molecular weight form could have a higher degree of receptor coupling than the low molecular weight form, i.e. that, at each time, a larger portion of the high molecular weight form of $G_{s\alpha}$ should be involved in the coupling to the β-receptors as compared to the low molecular weight form. Different physiological functions have indeed been attributed to the different isoforms: they were found to display differences in the rate of GDP-release which, in turn, could have important consequences for the phosphorylation and inactivation of the nucleotide-free G-protein [45, 46]. It is clear that our interpretation of the results is in keeping with this hypothesis, although one should notice that all forms of $G_{s\alpha}$ activate adenylate cyclase and Ca^{2+}-channels to an equal extent [47, 48] and that the isoforms interact with β_1- and β_2-receptors with similar affinities [47]. Experiments with purified proteins in reconstitution systems might confirm this hypothesis.

The total $G_{s\alpha}$-protein content in the membrane fraction of rat ventricular tissue increased approximately 24% with aging. This is in contrast with the results of Böhm *et al.* [7], who reported no difference in the amount of $G_{s\alpha}$ between old and young rats. These authors however detected only one single α_s-band after one-dimensional gel electrophoresis, while there is overwhelming evidence that $G_{s\alpha}$-exists in two molecular weight forms in rat heart. Hence, comparison between both results is not feasible.

Finally, it was found that the $G_{i\alpha}$-protein content in the membrane fraction of rat ventricular tissue almost doubled with aging. This result is in keeping with the work of Böhm *et al.* [7], who also found a 170% increase. Moreover, Reithman *et al.* [49] and Mende *et al.* [50] also reported an increase (of ca. 22 and 70%, respectively) in the level of $G_{i\alpha}$ upon prolonged treatment of rat heart muscle with norepinephrine, a situation that is, to a certain extent, comparable with the aged condition, since plasma norepinephrine levels are also increased in senescent rats. They suggest that this increase is responsible for the observed desensitization of adenylate cyclase stimulation. Finally, as mentioned earlier, it was reported that in myocardial failure, the persistent sympathetic drive also produces an increased expression of $G_{i\alpha}$ [20, 21] and this might be related to the depressed cAMP response in the failing heart [51]. Recent articles provide clues as to how this blunting of the adenylate cyclase response could occur. Indeed, Dobson *et al.* [52, 53] indicate that there is an elevated interstitial level of adenosine in heart tissue of aged rats and that this adenosine exerts a greater anti-adrenergic effect in the aged rat heart. Hence, we believe that the stimulation of inhibitory $G_{i\alpha}$-coupled A_1-adenosine receptors could contribute to the reduced adenylate cyclase activity in aged rats.

In summary, our results suggest that, notwithstanding the slight increase of total G_s-protein content and of high affinity G-protein receptor coupling, the reported increase in $G_{i\alpha}$ might be at least one as yet unknown membrane alteration leading to the observed age-related reduced chronotropic and inotropic responses.

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