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# Effect of Maturation and Aging on β-Adrenergic Signal Transduction in Rat Kidney and Liver

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**ABSTRACT.** The characteristics of the β-adrenergic signal transduction system were analyzed in kidney and liver membrane preparations from neonatal (2–3 days), mature (2 months), and old (2 years) rats. When comparing kidneys from adult to neonatal rats, we found a higher β-receptor density and a higher percentage of $\beta_1$-receptor subtype, lower immunoreactive $G_{s\alpha}$-protein, a lower ratio between the high and low molecular weight splice variant of $G_{s\alpha}$, lower immunoreactive $G_{i\alpha}$-protein, and lower basal adenylate cyclase activity. When comparing livers from adult to neonatal rats, we found lower β-receptor density and basal adenylate cyclase activity. Very few differences could be detected when comparing mature to old kidneys or livers. Stimulated adenosine 3′,5′-cyclic monophosphate (cAMP) synthesis was tissue- and age-dependent. In liver, G-protein- and β-receptor-stimulated cAMP synthesis mirrored basal adenylate cyclase activity and was highest in liver from neonatal animals. In contrast, cAMP synthesis was significantly more stimulated in kidneys from mature animals than from neonatal and senescent rats. We conclude that: (i) the stoichiometry of the components within the β-receptor/G-protein/adenylate cyclase complex is not fixed but is both tissue- and age-dependent; (ii) adenylate cyclase enzyme activity is possibly but not necessarily the rate-limiting step in the β-receptor-mediated synthesis of cAMP; and (iii) there is *in vivo* evidence for a preferential co-expression of the large splice variant of the $G_s$-protein and $\beta_2$-receptor subtype. It is speculated that this could have important physiological consequences for the development of the kidney. BIOCHEM PHARMACOL **60**;12:1787–1795, 2000.


**KEY WORDS.** G-proteins; aging; rat; β-adrenergic system; kidney and liver

It has been demonstrated previously that the coupling between β-receptors and G-proteins is decreased in several tissues of the aged rat compared to mature animals [1–6], this being compatible with the view that aging is accompanied by an attenuated β-receptor response [7]. We demonstrated that alterations in kinetics and thermodynamics of the binding [8] or alterations in membrane fluidity [9] could not explain the observations. We postulated that the age-related reduction in high-affinity binding between the β-receptor and $G_{s\alpha}$-protein** was caused by age-related alterations of the biochemical properties of the $G_s$-proteins.

$G_{s\alpha}$-proteins occur in two splice variants: $G_{s\alpha}$-large ($G_{s\alpha l}$; MW of 52 kD) and $G_{s\alpha}$-small ($G_{s\alpha s}$; MW of 45 kD), both of which have two isoforms that differ by the absence or presence of a single serine residue at the 5′-end of exon 4 [10]. Reconstitution experiments suggested that stimulation of adenylate cyclase is more effective via $G_{s\alpha l}$ than via $G_{s\alpha s}$ [11], but recombinant $G_{s\alpha l}$- and $G_{s\alpha s}$-proteins inserted in COS or S49 lymphoma cells stimulate adenylate cyclase equally well [12]. Recent evidence suggests that β-receptors couple preferentially with the $G_{s\alpha l}$-protein in liver from partially hepatectomized male rat [13], and the preferential coupling between β-receptors and $G_{s\alpha l}$-protein was recently confirmed in the insect Sf9 expression system [14, see 15 for a recent review]. $G_{s\alpha l}$ and $G_{s\alpha s}$ are simultaneously expressed in most tissues, but the ratio is variable [16]. Data in the literature concerning the effect of aging on the occurrence of the different G-proteins are at least confusing and show a high degree of variability depending on the detection method and species [17–21, see 15].

Taking into consideration our earlier results obtained in rat kidney [2] and liver [4] and the fact that we improved the method for quantitative immunological detection of tissue G-proteins by internal standardization [22], we studied the properties of the βAST system and in particular of G-proteins in these tissues. Two main items were addressed:

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** *Abbreviations:* βAST, β-adrenergic signal transduction system; $G_{s\alpha l}$, large splice variant of $G_{s\alpha}$; $G_{s\alpha s}$, small splice variant of $G_{s\alpha}$; cAMP, adenosine 3′,5′-cyclic monophosphate; KLH, keyhole limpet hemocyanin; ICYP, iodocyanopindolol; RT–PCR, reverse transcriptase–polymerase chain reaction; and ISO, isoproterenol.

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(i) the influence of maturation and aging both on the properties of the components of the βAST system in kidney and liver with special attention to $G_{s\alpha}$- and $G_{i\alpha}$-proteins and on the ratio between the two splice variants of the $G_{s\alpha}$-protein; (ii) changes in biochemical properties and the consequences for the activity of the βAST system.

Our studies reveal that maturation has profound effects on the levels of β-receptors and $G_{s\alpha}$- and $G_{i\alpha}$-proteins and, in the kidney, on the ratio between the splice variants of $G_{s\alpha}$-proteins; the effect of aging on these properties is limited. These changes induce major and unexpected alterations in cellular activity of the βAST system. The value of this *in vivo* model for the study of the expression of splice variants of $G_{s\alpha}$-proteins is discussed.

## MATERIALS AND METHODS

### *Materials*

Nitrocellulose blotting paper (Hybond-C extra), [$^{125}$I]cyanopindolol, and the commercially available cAMP assay kit were obtained from Amersham. Affinity-purified goat anti-rabbit immunoglobulin labeled with peroxidase ("secondary antibodies") were obtained from Sigma. All other reagents were from commercial sources.

### *Animals*

Male Wistar rats of 1–4 days, 2–4 months, and 24–26 months were used. Two-month-old rats were considered as sexually mature animals [23]. Mature and old rats were obtained from the Center for Experimental Animals of the University of Leuven (Belgium); neonatal rats and rats less than 2 months of age were bred in our animal facility from Wistar rats obtained from the same source. Rats were housed at a constant light–dark cycle of 12 hr and had free access to food and acidified water. White albino rabbits used for the generation of antibodies (see below) were obtained from a local supplier.

### *Preparation of Primary Antibodies*

Antisera were raised against synthetic peptides coupled to KLH through an extra COOH-terminal cysteine residue using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester as cross-linking agent. The selection of peptides (one-letter abbreviations) was based on data in the literature: TPEPGEDPRVTRAKY for $G_{s\alpha}$-protein and KQLQKDKQVYRATHR for $G_{i\alpha}$-protein [24]. 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 up to 12 months without apparent loss of activity. Reactivity and specificity of the antisera were tested using a conventional ELISA technique in which immunoplates were coated with the peptides, incubated with different dilutions of the antisera, postcoated with 1% BSA in PBS, and incubated with goat anti-rabbit labeled with alkaline phosphatase. Both antisera were shown to be selective for their respective peptides. Immunoblot experiments (see below) confirmed the selectivity of the antisera for the corresponding G-proteins. The antisera obtained against these peptides are shown to label all splice variants of the $G_{s\alpha}$- and $G_{i\alpha}$-proteins [12, *].

### *Sample Preparations*

Kidneys and livers were homogenized (dilution 1/20 w/v in gr/mL) in ice-cold buffer (Tris–HCl 50 mM pH 7.4 containing 2 mM EGTA and 1 mM phenylmethylsulfonyl fluoride) at 4° with an Ultra-Turrax (3 times 15 sec with 1-min interval) and then centrifuged at 1000 *g* for 10 min and at 20,000 *g* for 20 min. Sufficient kidneys from neonatal rats from one offspring were pooled to obtain at least 1 gram of tissue. The pellet was washed once in buffer by gentle homogenization with 4 to 5 strokes of a Potter–Elvehjem homogenizer followed by centrifugation. The pellet was dissolved (30 min at room temperature) in electrophoresis buffer (62 mM Tris containing 5% mercaptoethanol, 2% SDS, and 0.001% bromophenol blue) at approximately 2 mg protein/mL, boiled for 3 min, cooled to room temperature, and stored in aliquots at −20°.

Separate kidney cortex and medulla tissue were prepared from mature and senescent animals only; in neonatal animals, cortex and medulla could not be distinguished. From cortex, separate microtubules and glomeruli were prepared following the method of Sundaresan *et al.* [25]. Briefly, minced kidney cortical tissue (minimum 1 gram pooled tissue) was passed through stainless steel sieves of different mesh; tubules were collected at the 212-μm sieve while glomeruli were retained by the 63-μm sieve. Purity of the preparations was checked microscopically: for the tubule sample, 89 ± 4% (N = 12) of the macrocellular structures were identified as tubules, while the remainder were either smaller tubule fragments that were more difficult to identify or a minority (less than 3%) of glomeruli. The glomerular sample contained 83 ± 8% (N = 12) glomeruli, with the remainder again fragmented tissues. All these samples were prepared for electrophoresis as described for total kidney.

### *Protein Determination*

Proteins were determined by the method of Lowry *et al.* [26] modified according to Chang [27].

* Fraeyman N, Van de Velde E, Bazan A and Vandekerckhove J, unpublished results.

### *Electrophoresis, Blotting, and Immunostaining*

All samples were diluted to a protein concentration of 1 mg/mL and 80 μL was loaded in a sample slot. Electrophoresis and blotting were performed according to Laemmli [28] and Towbin *et al.* [29], respectively. Blots were quenched overnight at 4° in 0.4% bovine serum albumin in phosphate-buffered saline (PBS–BSA) and then incubated with primary antisera (1/2000 in PBS–BSA buffer) for 2 hr. Blots were washed in PBS containing 0.05% Tween 20 (PBS–Tween) and incubated with secondary antibody (1/3000 in PBS–BSA), followed by washing (3 times in PBS–Tween and once in 50 mM sodium acetate buffer, pH 4.5) and staining for 15–30 min for peroxidase activity, using aminoethyl carbazole in 50 mM sodium acetate buffer (pH 4.5) and $H_2O_2$ as substrate. The reaction was stopped by rinsing with tap water and the blots were stored in 0.05% $NaN_3$. Immunostained blots were scanned with reflectance absorbency (Joyce–Loebl Chromoscan 3), and the concentrations of $G_{s\alpha}$- and $G_{i\alpha}$-proteins were expressed in arbitrary units using the method of McFarlane-Anderson *et al.* [30] as modified [22]. The total amount of $G_{s\alpha}$-protein was calculated as the sum of the peak heights of the two subtypes. Control experiments were (results not shown): (i) various amounts of proteins, ranging from 20 to 100 μg, were subjected to the electrophoresis procedure; subsequent calculation of the cellular levels of $G_{s\alpha}$- and $G_{i\alpha}$-proteins yielded linear results; and (ii) the time dependence of the peroxidase-staining reaction was investigated for each tissue and for the $G_{s\alpha}$- and $G_{i\alpha}$-protein. Incubation times were 15 min for $G_{s\alpha}$ and 30 min for $G_{i\alpha}$, which yield a staining response within the linear part of the time–peak height curve. All samples used for statistical comparisons were run simultaneously, yielding acceptable errors as discussed in detail previously [22]. In each gel, one lane was used for an internal standard which was an aliquot of a kidney preparation stored at −70°. Occasionally, faint bands at MW 60,000 and 70,000 and at a MW below 30,000 were seen, which were due to binding of the second antibody alone.

### *Radioligand-Binding Experiments*

Saturation- and competition-binding experiments were performed as described before [2, 4] with 60–70 μg proteins from kidneys and 20–30 μg proteins from liver. Competition with ICYP binding (about 100 pM ICYP) was investigated using either the agonist isoproterenol ($10^{-9}$–$10^{-3}$ M) without GTP or in the presence of 0.1 mM GTP for the determination of the extent of high-affinity agonist binding or using the $\beta_1$-selective antagonist CGP 20,712A ((±)-2-hydroxy-5-[2-[[2-hydroxy-3-[4-[1-methyl-4-(trifluoromethyl)-1*H*-imidazol-2-yl]phenoxy]propyl]amino]ethoxy]-benzamide methanesulfonate, $10^{-11}$–$10^{-4}$ M) for the determination of the percentage of $\beta_1$-adrenoceptors.

### *Adenylate Cyclase Activity*

Adenylate cyclase activity was assayed on a crude membrane fraction as described before [2]. Approximately 100 μg of protein was used for both tissues. Adenylate cyclase activity was measured either without stimulation (basal activity) or after stimulation with different concentrations of GTP. From this concentration–response curve, the optimal GTP concentration for further experiments was found to be 33 μM. In addition, tissues were stimulated with 10 μM isoproterenol in the presence of 33 μM GTP. Adenylate cyclase activity was expressed either as pmol cAMP/mg protein.min or as the ratio of the net activity of the enzyme (stimulated activity:basal activity), expressed as percentage or as "fold increase".

### *RNA Purification and RT–PCR*

RNA was isolated from kidneys of animals two days and 2 months of age using RNA-Instapure™ (Eurogentec, Belgium) according to the manufacturer's instructions. The purity of RNA was examined on DMSO–glyoxal agarose gels. cDNA synthesis was performed with the Invitrogen cDNA cycle kit. The design of the PCR primers was based on the papers of Kozasa *et al.* [31] on the human genomic sequence and of Itoh *et al.* [32] on the rat cDNA sequence. According to the latter sequence, sense and antisense primers are located at 398–417 and 651–670, respectively. The cDNA for β-actin was amplified using commercial primers (Strategene) as an internal control. Optimalization of PCR was done using the Invitrogen optimizer kit.

### *Statistical Analysis*

Results are expressed as means ± SEM; comparison between age groups was carried out using one-way ANOVA. When significance was reached, comparison between two groups of data was performed using the two-sided Student's *t*-test with Bonferroni correction for multiple comparisons. Significance was accepted at the level of $P < 0.05$ or at the level of 0.01 after correction. All curve fittings were done using the GraphPad software.

## RESULTS

### β-*Adrenoceptor Analysis*

The data for neonatal and mature kidneys are shown in Fig. 1 and average values for the three age groups are summarized in Table 1. In kidney, receptor density (fmol/mg protein) was low in neonatal animals compared to the other age groups ($P < 0.05$). The percentage of $\beta_1$-adrenoceptors increased from 13% in neonatal rats to 66% in mature rats ($P < 0.05$). The percentage of agonist high-affinity binding site, the affinity for the radioactive ligand ICYP, and the affinity for the $\beta_1$-selective antagonist CGP 20,712A were indistinguishable among the three groups of animals. In liver, receptor density was 3- to 4-fold higher in

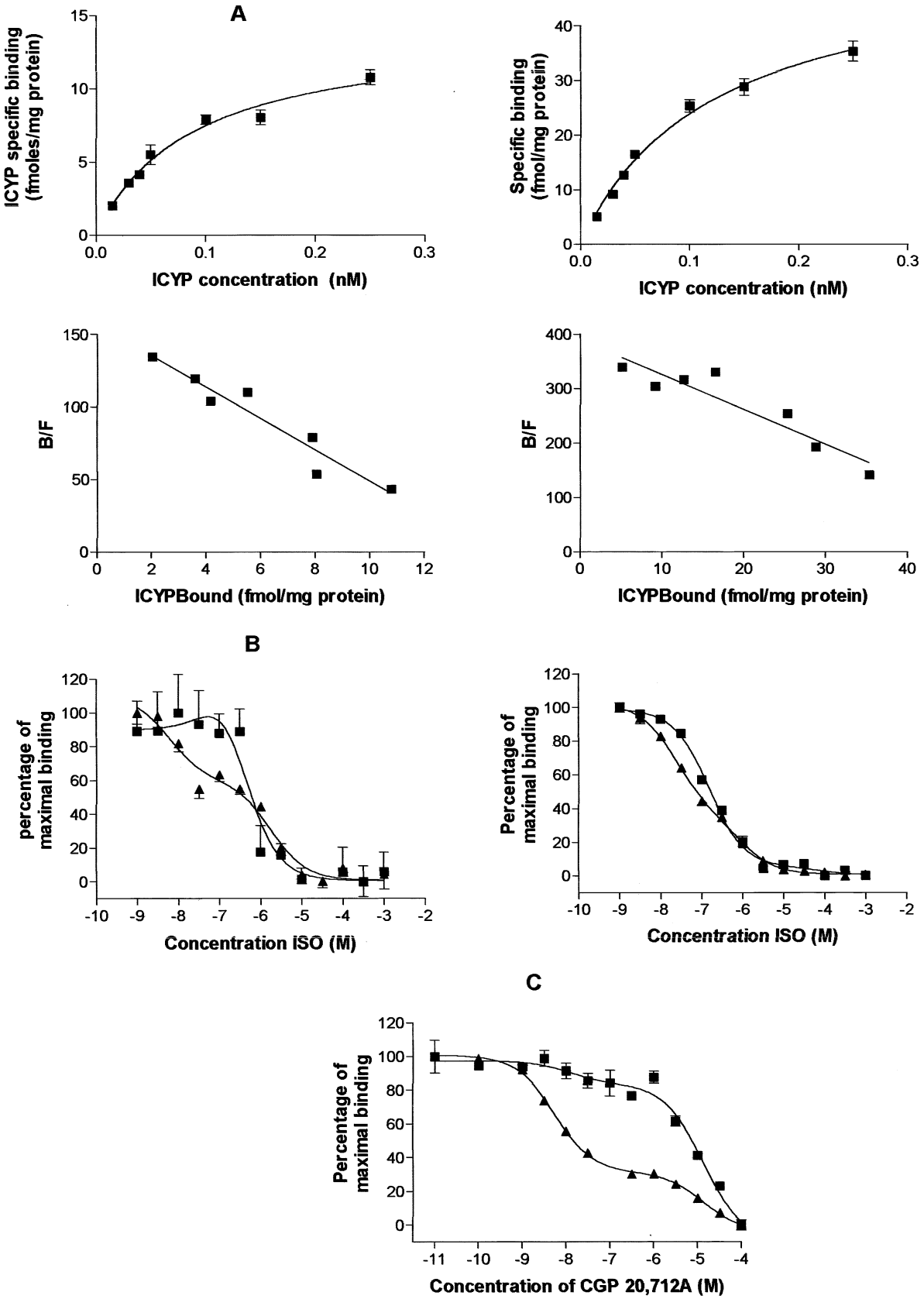


FIG. 1. Radioligand-binding characteristics of β-adrenoceptors in kidneys from neonatal and mature rats. (A) Representative examples of saturation-binding curve performed in triplicate and corresponding Scatchard plot for neonatal (left panel) and mature (right panel) rats. (B) Isoproterenol competition binding without (triangles) and in the presence (squares) of 10 μM GppNHp for neonatal (left panel) and mature (right panel) rats; the average of 3–5 curves in triplicate is shown. (C) CGP 20,712A competition binding for neonatal (full squares) and mature (full triangles) rats; the average of 3–5 curves in triplicate is shown.

**TABLE 1. Characteristics of the β-adrenoceptors in crude membrane preparations from kidneys and livers obtained from neonatal, mature, and senescent rats**

| | Neonatal (N = 6) | Mature (N = 6) | Senescent (N = 7) |
|---|---|---|---|
| Kidney | | | |
| $B_{max}$ | 29.8 ± 3.5* | 56.7 ± 3.6 | 45.1 ± 4.5 |
| $K_d$ | 10.0 ± 2.0 | 12.0 ± 2.0 | 28.8 ± 12.0 |
| $\beta_1$-adrenoceptor | 13 ± 4* | 66 ± 8 | 61 ± 3 |
| $Ki_{cgp}$ | 23 ± 4 | 15 ± 3 | 26 ± 7 |
| Agonist binding | 46 ± 7 | 63 ± 3 | 32 ± 4 |
| Ki (HA) | 6.1 ± 0.4 | 22.0 ± 2.1 | 14.3 ± 2.7 |
| Liver | | | |
| $B_{max}$ | 116.2 ± 8.5* | 28.4 ± 2.4 | 31.4 ± 4.3 |
| $K_d$ | 47.7 ± 5.3 | 33.1 ± 6.9 | 35.9 ± 3.4 |
| $\beta_1$-adrenoceptor | 18.3 ± 8.3 | 21.3 ± 3.1 | 25.1 ± 2.9 |
| $Ki_{cgp}$ | 1.2 ± 0.8 | 22.6 ± 9.8 | 7.9 ± 2.7 |
| Agonist binding | 42.5 ± 5.7 | 50.4 ± 3.7 | 41.6 ± 4.5 |
| $K_i$ (HA) | 10.5 ± 3.7 | 10.5 ± 4.4 | 15.7 ± 4.4 |

$B_{max}$: receptor density obtained from Scatchard plot analysis (fmol/mg protein); Kd: affinity of the ligand (pM); $\beta_1$-adrenoceptor: percentage of total β-adrenoceptor with high affinity for CGP 20,712A (selective $\beta_1$-antagonist); $Ki_{CGP}$: affinity of the high-affinity binding of CGP 20,712A obtained from competition-binding experiments; Agonist binding: percentage of total β-adrenoceptors with high affinity for isoproterenol obtained from competition-binding experiments; Ki(HA): affinity of the high-affinity binding site for isoproterenol obtained from competition-binding experiments.

* significantly different ($P < 0.05$) compared to the other age groups.

neonatal animals compared to mature and senescent rats; all other characteristics, including β-adrenoceptor subtype distribution and agonist high-affinity binding were similar for the three age groups.

### *G-protein Analysis*

The results of the G-protein analysis are shown in Fig. 2. In Fig. 2, A and B, representative Western immunoblottings using antisera against $G_{s\alpha}$ and $G_{i\alpha}$ peptides, respectively, are shown; in Fig. 2C, the average levels of both $G_{s\alpha}$-variants in kidney and liver are depicted. In kidney, a significant decrease upon maturation ($P < 0.01$) was found, while further aging was without effect. During maturation, the cellular level of $G_{s\alpha l}$-protein decreased, while the level of $G_{s\alpha s}$-protein increased significantly (ANOVA, $P < 0.05$); further aging was without effect. Consequently, the ratio between the high and low molecular weight forms ($G_{s\alpha l}/G_{s\alpha s}$) varied from 6.56 ± 0.91 (N = 10) in neonatal rats to 0.79 ± 0.04 (N = 8) and 0.81 ± 0.32 (N = 9) in mature and senescent rats, respectively (ANOVA, $P < 0.005$). In a separate series of experiments, the age dependence of the ratio $G_{s\alpha l}/G_{s\alpha s}$ in rat kidney was further investigated in animals from 2 days to 2 months old; this result is shown in Fig. 3. The ratio was again high in neonatal rats and decreased rapidly to approximately 1.5 in 1-month-old animals and to slightly less than 1 in 2-month-old animals. The ratio between high and low molecular weight proteins was comparable in total kidney, in kidney cortex and medulla, and in tubules prepared from mature and senescent animals (approx. 1). In glomeruli, a slightly higher value (between 1.5 and 1.7 for animals 2 months and 2 years of age, respectively) was found (results not shown). The results of the RT–PCR are shown in Fig. 4. In 2-day-old animals, most of the mRNA consisted of the unspliced form, which corresponds to the high molecular weight form of $G_{s\alpha}$. In the case of 2-month-old animals, however, an approximately equal amount of messenger for both splice variants was obtained. The level of the inhibitory $G_{i\alpha}$-protein in rat kidney was also age-dependent (ANOVA, $P < 0.05$): a significant decrease ($P < 0.05$) from neonatal to mature animals was followed by a slight increase till senescence, without reaching statistical significance in the latter case. In liver, maturation and aging had no significant effect either on the total level of $G_{s\alpha}$-protein or on the ratio $G_{s\alpha l}/G_{s\alpha s}$, which was slightly but significantly lower than one for each age group. The $G_{i\alpha}$-protein level decreased significantly upon maturation and remained constant upon further aging.

### *Adenylate Cyclase Activity*

In Fig. 5, the concentration–response curves for GTP-stimulated adenylate cyclase activity in kidneys of neonatal and mature rats are shown. Essentially similar curves were obtained for kidney of senescent rats and for liver of the three age groups (results not shown). $EC_{50}$ values varied between 1 and 5 μM GTP and were indistinguishable among the different age groups; maximal stimulation was reached at 30–40 μM GTP. For further experiments, a concentration of 33 μM GTP was used. Adenylate cyclase enzyme activity was measured under a number of conditions; these results are summarized in Table 2. For kidney, the basal enzyme activity was 4.3 and 4.7 times higher in neonatal animals as compared to mature and senescent rats, respectively ($P < 0.05$). In kidneys from mature and senescent rats, the GTP- and ISO + GTP-stimulated enzyme activity (expressed as fold increase over basal activity—see experimental section) was significantly higher than in kidneys from neonatal rats ($P < 0.05$). For liver, the basal activity was higher in neonatal rats compared to mature (2.2-fold) or senescent (1.3-fold) animals. The ratio between the basal activity and the response upon GTP stimulation declined significantly ($P < 0.05$) upon maturation and increased slightly upon aging, without reaching statistical significance in the latter case.

## DISCUSSION

In spite of numerous data, altered activity of the β-adrenergic signal transduction system upon maturation and aging remains enigmatic [33]. Based on our earlier reports [1–5, 8, 9], we postulated that a defect at the level of the G-proteins could be of importance. "Maturation" and "Aging" are indicative of the major steps in the life span of the animals (birth, sexual maturity, and senescence); systematic analysis of additional age groups has not been performed.

Analysis of the properties of the β-adrenoceptor essen-

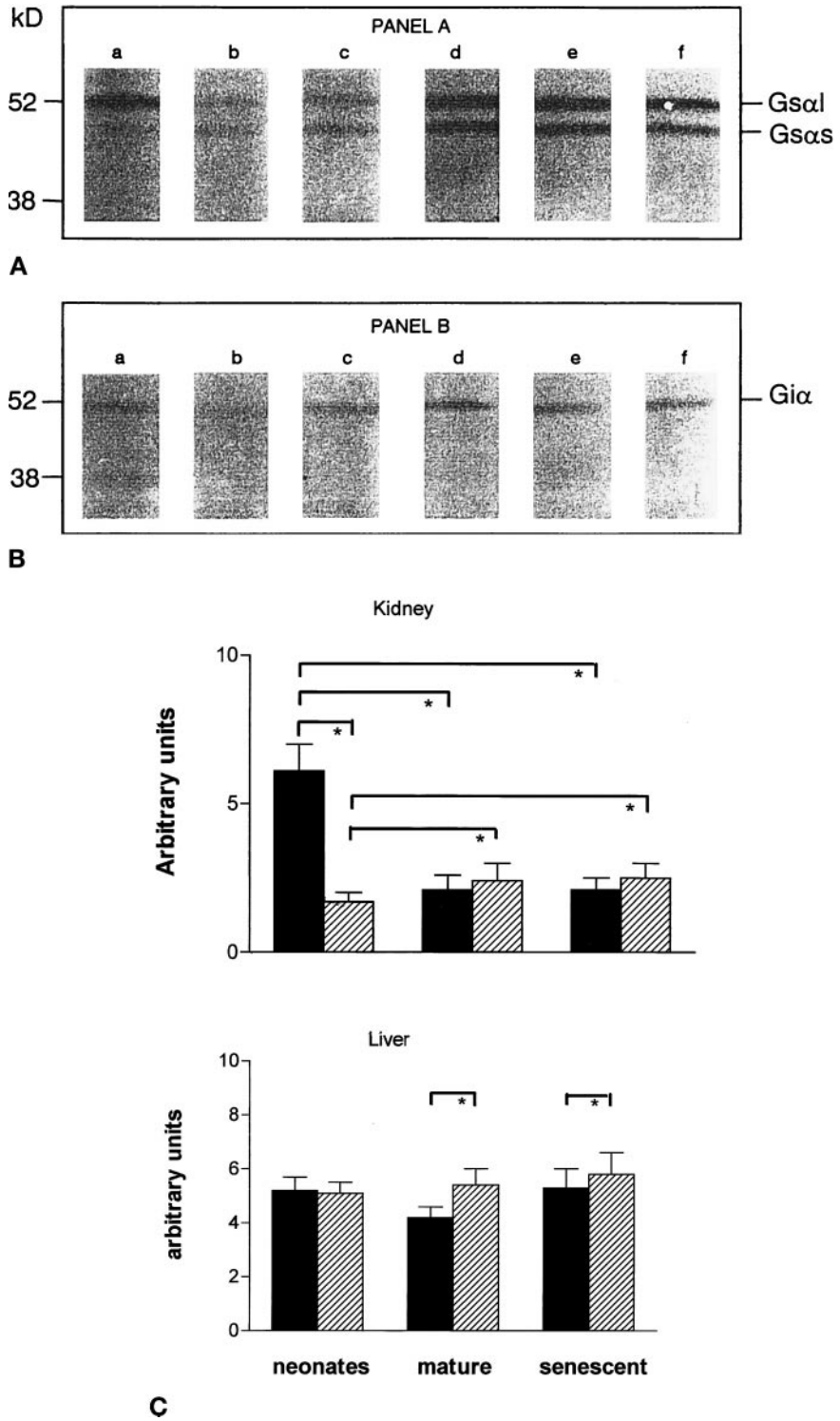


**FIG. 2. Immunoblots of $G_{s\alpha}$ and $G_{i\alpha}$ in rat kidney and liver. Representative example of immunoblots of G-proteins after SDS–electrophoresis for neonatal (lane 1), mature (lane 2), and senescent (lane 3) rat kidney (lanes a–c) and liver (lanes d–f). Detection is with anti-$G_{s\alpha}$-antibodies (panel A) and anti-$G_{i\alpha}$-antibodies (panel B). Details are given in the experimental section; molecular weight markers are indicated. Panel C: levels of $G_{s\alpha}$-subtypes in kidney and liver of neonatal, mature, and senescent rats (calculated from panel A). Units are arbitrary and calculated from peak height after densitometric scanning. Average value is shown with SEM and is derived from 8 to 20 experiments. Full bars: $G_{s\alpha l}$; hatched bars: $G_{s\alpha s}$. *: significantly different ($P < 0.05$ with Bonferoni correction).**

tially confirmed earlier data as far as kidney of 2-month-old and 2-year-old rats [2] and liver of the three age groups [4] is concerned. The ratio $\beta_2/\beta_1$-receptor subtype is 6.8 in kidney from neonatal and 1.7 in mature rats, and this is entirely due to a 10-fold increase in the $\beta_1$-receptor density. Age-dependent alterations in β-receptor subtype distribution have been found and discussed previously [2 and references therein], but the differences reported here are

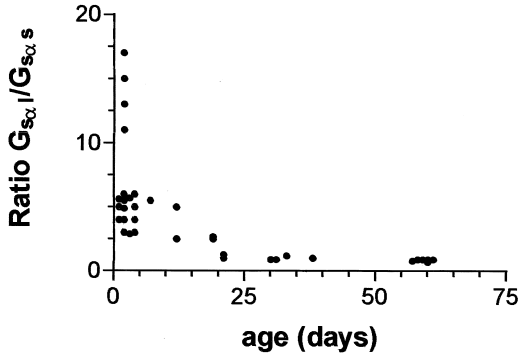


**FIG. 3. Effect of maturation on the $G_{s\alpha l}/G_{s\alpha s}$-ratio in rat kidney. Ratio between $G_{s\alpha}$-subtypes in kidneys of rats aged from 1 to 60 days. All individual ratios are shown. Calculations are as described in the experimental section.**

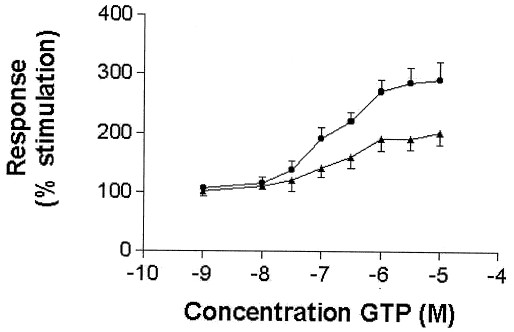


**FIG. 5. Concentration–response curves for the GTP-stimulated adenylate cyclase activity in kidneys of neonatal and mature rats. The basal enzyme activity is set as 100%; stimulated values are expressed as percentage increase above basal value. Average value of 5 to 7 separate determinations with SEM is shown. ●: neonatal rats; ▲: mature rats.**

much more pronounced than in earlier studies. Systematic comparison of the data in the literature on levels of G-proteins and splice variants and the effect of maturation and aging thereupon is not always possible because of methodological problems [34]. Using a modification of the immunoblotting analysis [22], we demonstrated in two separate series of experiments that, in rat kidneys, a significant decrease in the levels of $G_{s\alpha}$- and $G_{i\alpha}$-proteins and a major shift in the ratio between the two isoforms of $G_{s\alpha}$ (from 6.5 to 0.8) occur during maturation. Aging has limited effects on these parameters in kidney while in rat liver, virtually no effect on the levels of $G_{s\alpha}$ or on the ratio $G_{s\alpha l}/G_{s\alpha s}$ was seen during maturation or aging.

Based on our own data and those in the literature, some straightforward explanations for the results concerning the properties of β-receptors and $G_{s\alpha}$ in kidney could be excluded. We can assume that a technical bias of the results is unlikely, since the radioligand-binding assay for β-receptors can be applied to tissues independent of the age of the donor animal and the two splice variants of $G_{s\alpha}$ do not exhibit differences in immunological reactivity. One possible explanation would be that the changes in the kidney during maturation reflect the clonal growth of one or of a limited number of cell types expressing a particular β-receptor subtype ratio and $G_{s\alpha l}/G_{s\alpha s}$-ratio. This argument merits consideration in view of the age-dependent changes in the anatomy of the kidney: in neonatal kidneys, no specific tissue structure resembling medulla or cortex could be discerned, while in kidneys taken from mature and senescent rats, cortex and medulla can easily be dissected [35]. However, the electrophoresis pattern for the G-protein subtypes in different compartments of the kidney including cortex, medulla, and tubules and glomeruli using kidneys from 2-month-old and 2-year-old animals was identical. The slight increase in ratio to about 1.5 in


**FIG. 4. RT–PCR analysis of $G_{s\alpha}$- and β-actin messengers. Total kidney RNA was isolated from 2-day-old (lanes 1–3) and 2-month-old (lanes 5–7) animals. After cDNA synthesis, 1 μL (lanes 1 and 5) or 0.1 μL (lanes 2, 3, 6, and 7) was used for PCR amplification with primers for $G_{s\alpha}$ (lanes 1, 2, 5, and 6) and β-actin (lanes 3 and 7). One-fourth of the mixture was analyzed on a 3% agarose gel. Lane 4 contains a 100-bp-ladder (500 and 100 bp are present in higher amounts).**

**TABLE 2. Adenylate cyclase activity in rat kidney and liver from neonatal, mature and senescent rats**

| | Neonatal (N = 6) | Mature (N = 6) | Senescent (N = 7) |
|---|---|---|---|
| Kidney | | | |
| Basal activity | 75.5 ± 11.2* | 17.4 ± 1.4 | 16.1 ± 1.3 |
| GTP | 122.6 ± 11.6 | 40.1 ± 3.5 | 32.5 ± 1.5 |
| Fold increase (%) | 62 | 130 | 102 |
| GTP + ISO | 132.4 ± 3.7 | 42.7 ± 1.6 | 36.0 ± 0.7 |
| Fold increase (%) | 75 | 145 | 124 |
| Liver | | | |
| Basal activity | 29.4 ± 4.1* | 13.1 ± 0.9 | 9.8 ± 1.5 |
| GTP | 59.5 ± 5.5 | 19.3 ± 1.9 | 16.3 ± 2.6 |
| Fold increase (%) | 102 | 47 | 66 |
| GTP + ISO | 105.1 ± 17.0 | 22.4 ± 2.0 | 23.4 ± 3.1 |
| Fold increase (%) | 257 | 71 | 139 |

The enzyme activity is expressed in pmol cAMP formed in 10 min per mg of proteins. GTP: stimulation of adenylate cyclase activity with 33 μM GTP; GTP + ISO: stimulation of adenylate cyclase with 33 μM GTP and 10 μM isoproterenol; N: number of experiments.

* $P < 0.05$ compared to the other age groups.

glomeruli is not sufficient to explain the markedly increased ratio in total kidneys of premature rats. Although these results do not entirely exclude the possibility, they suggest that clonal growth is probably not the explanation for our observations. Finally, the results of the analysis of $G_{s\alpha}$-protein expression are confirmed by the RT–PCR results [36]. It is assumed that the translation process of the different G-proteins reflects the expression of the proteins, as suggested previously [30, 37, 38], although conflicting data have been reported [39]. Furthermore, it has been suggested that the decrease in expression of $G_{s\alpha}$-protein in kidney is due to the repression of the transcription process and not to increased instability of the protein [40], and our results confirm these findings. Alterations in the ratio between isoforms of different G-proteins is associated both with differentiation *in vitro* of cells such as neuroblastoma and 3T3-L1 fibroblast-like cells and with a number of pathological and non-pathological conditions such as pregnancy and labour [41]; these data have been reviewed recently [15]. Since it is known that glomerular filtration in rats is nearly absent at birth and that nephron formation continues to develop for two to three weeks [42], we can conclude that, similar to rat brain tissue [43], the relative abundance of $\beta_2$-receptors and $G_{s\alpha l}$-proteins is characteristic of undifferentiated kidney tissue.

The stoichiometry of the receptor–$G_s$-adenylate cyclase complex was studied beforehand in S49 lymphoma cells [44], and the authors concluded that adenylate cyclase was probably the limiting factor in the regulation of the agonist stimulation of the βAST system. Our results in rat liver are in keeping with this hypothesis. Indeed, although no direct measurement of the adenylate cyclase protein concentration was carried out, β-receptor-mediated synthesis of cAMP mirrors basal activity; hence, the most prominent influence of aging is likely to be on adenylate cyclase. The occurrence of multiple forms of the enzyme has been described [45], but hitherto no data on the effect of aging thereupon are available. In rat kidney, however, the situation was much more complex: in spite of a nearly 2-fold reduction in basal adenylate cyclase activity and the cellular content of $G_{s\alpha}$, the GTP- and ISO + GTP-stimulated cAMP synthesis was markedly higher in kidneys from 2-month-old animals as compared to neonates. These observations clearly argue against adenylate cyclase being the limiting factor in the regulation of βAST activity in the kidney of the rat. Our data indeed suggest that during kidney maturation, the most important influence on agonist-stimulated cAMP synthesis is the shift in the $\beta_2/\beta_1$-receptor subtype ratio and in the $G_{s\alpha l}/G_{s\alpha s}$-ratio (both from about 6.5 to about 0.7) rather than alteration in adenylate cyclase activity. The preference of $\beta_2$-receptors for the $G_{s\alpha l}$-isoform was demonstrated previously in CHO (Chinese hamster ovary) cells [46]. Our results not only strengthen this conclusion but demonstrate that this could also occur in kidney tissue *in vivo*.

Data on $G_{i\alpha}$-protein expression during maturation and aging are equally scarce. It is well known that in kidney, the three $G_{i\alpha}$-proteins are present, although the $G_{i\alpha 2}$-protein is most abundant [47], while for liver it has been shown that only the $G_{i\alpha 3}$-protein is present [48]. Since our results demonstrate that the age-related pattern in $G_{i\alpha}$-protein expression level is very similar for both tissues, the age effects on the individual members of the $G_{i\alpha}$-protein family were not investigated.

We conclude that the stoichiometry of the interaction of the components of the adenylate cyclase system is age- and tissue-specific. For rat kidney, the stoichiometry for the interaction of the components of the β-adrenergic signalling system is far more complex than that found in liver and that estimated previously from *in vitro* cell culture expression systems. It is clear that the developing rat kidney is an interesting *in vivo* model for the study of the regulation of the splicing mechanism of G-proteins and the coupling to β-receptors. It is tempting to speculate that these changes have important consequences for the development of the physiological functions in the kidney.

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