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β-Adrenergic and muscarinic receptor mRNA accumulation in the sinoatrial node area of adult and senescent rat hearts

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Abstract

The sinoatrial (SA) node is the cardiac pacemaker and changes in its adrenergic-muscarinic phenotype have been postulated as a determinant of age-associated modifications in heart rate variability. To address this question, right atria were microdissected, the SA node area was identified by acetylcholinesterase staining, and, using a RT-PCR method, the accumulation of mRNA molecules encoding β_1- and β_2-adrenergic (β_1- and β_2-AR) and muscarinic (M_2-R) receptor was quantified to define the proportion between β-AR and

Abbreviations: β-AR(s), β-adrenergic receptors; M-R(s), muscarinic receptors; β_1- and β_2-AR mRNA, mRNA coding for β_1- and β_2-adrenergic receptors; M_2-R mRNA, mRNA coding for muscarinic M_2 receptors; RT, reverse transcription; PCR, polymerase chain reaction; RA, right atria; LA, left atria; RV, right ventricle; LV, left ventricle.

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M_2-R mRNAs within the sinoatrial area of adult (3 months) and senescent (24 months) individual rat hearts. In adult hearts, the highest M_2-R/β-AR mRNA ratio was observed within the sinoatrial area compared with adjacent atrial myocardium, while in the senescent hearts, no difference was observed between sinoatrial and adjacent areas. This change was specific of the sinoatrial area since adult and senescent whole atrial or ventricular myocardium did not differ in their M_2-R/β-AR mRNA ratio, and was associated with a fragmentation of acetylcholinesterase staining of the senescent SA node. Quantitative changes in the expression of genes encoding proteins involved in heart rate regulation specifically affect the sinoatrial area of the senescent heart.

Keywords: β-Adrenergic and muscarinic receptor mRNA; Heart; Sino-atrial node; Senescence

1. Introduction

Heart rate is produced by the automatic repetitive depolarization of the cardiac pacemaker and is regulated by numerous interrelated neurohumoral and reflex mechanisms (review in Hainsworth (1995)). The SA node is innervated by both sympathetic and parasympathetic nerve endings, and these two branches of the autonomous nervous system directly modulate the heart rate. Autonomous nervous system acts via β-adrenergic (β-AR) in balance with muscarinic cholinergic receptors (MR) (Van Zwieten, 1991). Chronotropic, inotropic, and dromotropic activities of the heart are stimulated through both β_1- and β_2-adrenergic receptors by circulating and locally released catecholamines (Brodde, 1991). These two adrenergic receptor (AR) subtypes coexist in cardiac myocytes (Saito et al., 1988; Zhao and Muntz, 1993), are genetically distinct (Collins et al., 1989), and elicit qualitatively different physiological responses following independent stimulation by selective agonists (Xiao and Lakatta, 1993; Kuznetsow et al., 1995). A specific gene encodes the M_2 muscarinic receptor subtype (Bonner et al., 1987; Bonner, 1989), which is the dominant pharmacological and molecular form present in the mammalian heart (Hulme et al., 1990; Hoover et al., 1994). Postjunctional cholinergic muscarinic receptors can elicit negative chronotropic, inotropic or dromotropic responses when activated by muscarinic agonists or in response to cholinergic nerve stimulation (Fleming et al., 1987; Habecker et al., 1993).

Phenotypic changes in the β-adrenergic (βAR) and muscarinic receptors (MR) densities of the ventricles have repeatedly been reported in experimental models of cardiac overload as well as in cardiac insufficiency in humans and it has been postulated that the changes in HRV found in these conditions were not only determined by the numerous efferents of the ANS, but also depended upon the myocardial phenotype itself in terms of receptors content and transduction system (Carré et al., 1992, 1994a,b). The clinical situations in which HRV is altered usually associate disorders at each of these levels. For example, in cardiac failure, the central and baroreflexes are altered, together with a down-regulation of the

β1-adrenergic receptors (review in Brodde (1991)). The same is true in most of experimental models of cardiac hypertrophy. For example, aortic stenosis, a well-documented model of compensated cardiac hypertrophy in rats, influences the baroreflex and is also associated with a down-regulation of both the β1-adrenergic and muscarinic receptors (Mansier et al., 1993; Mondry et al., 1995). More recently, we were able, using transgenic technology, to target the overexpression of the β1-adrenergic receptors in mice atria (Bertin et al., 1993) and, by so doing, to attenuate HRV, showing that, in the absence of any modifications of reflex arcs, the atrial muscarinic/adrenergic receptor ratio is a determinant of HRV (Mansier et al., 1996). Nevertheless, a major criticism can be expressed concerning the above-experiments. The quantitation of the receptor content was indeed performed either from ventricular or atrial myocardium, and the above-interpretation assumes that the sinus node composition is comparable to that of the whole atria, or, even, to that of the ventricle, which, obviously, is far from being demonstrated.

To address these questions, we have selected a model of cardiac senescence, since ageing is associated with a global reduction of heart rate variability and progressive modifications in the vagal and sympathetic controls of heart rate regulation (Schwartz et al., 1991; Odemuyiwa, 1995), and have compared the β-adrenergic and muscarinic phenotypes of the sinoatrial area between adult and senescent rat hearts.

The specific objectives of our study were as follow: (i) To combine a molecular quantification of mRNA molecules encoding β-AR and M_2-R with a microdissection procedure and an histochemical localization of the SA node to define the equilibrium between β-adrenergic and muscarinic sequences within the SA node area of the adult heart. (ii) To study the consequences of cardiac senescence on this equilibrium by comparing the ratio between β-AR and M_2-R mRNA concentrations within the SA node areas dissected from adult and senescent hearts.

2. Materials and methods

2.1. Animals

Adult (3-month old) and senescent (24-month old) male Wistar rats were obtained from Iffa Credo (Lyon, France). Rats were housed in the animal facilities of INSERM U127 for 1 month prior to surgery with free access to water and standard laboratory food. Research was conducted at an INSERM laboratory that complies with the requirements of the french Ministry of Agriculture, and has been authorized to experiment with living animals according to executive order No. 87-848, October 19, 1987.

2.2. Dissection of the right atria

Rats were anesthetized [i.p. injection of sodium pentobarbital, 60 mg/kg body weight] before heparin (2000 IU/kg body weight) injection into the inferior vena cava (IVC). Animals were ventilated through an endotracheal tube using a Harvard

apparatus ventilator. After laparotomy, the infra-renal aorta was ligated above the mesenteric artery and cannulated close to the ligature with arterial tubing secured with a purse string in the aorta. The catheter was threaded through the aorta up to the origin of the left carotid artery and 2 ml of blood were withdrawn in order to avoid distension of the left heart when the cardioplegic solution is injected. The mediastinum was exposed through a bilateral thoracotomy. The two superior venae cavae and the IVC were ligated and, in a rapid sequential order, the IVC and supra-aortic vessels were tied off. A sterile saline solution (50 ml at $+4$°C) was progressively infused through the aortic catheter, under a pressure of 50 mmHg. This saline solution was vented through the pulmonary artery and the left atrium, while the heart was chilled in ice. After the cardiac arrest, the fat tissue and pretracheal ganglia were removed by microdissection from the posterior side of the heart, and the heart was excised and immediately transferred to an ice-cold saline solution. The right atrium was then excised, and a longitudinal anterior incision of this cardiac chamber was made along the superior and inferior vena cava axis (Fig. 1). The right atrium was dissected under magnifying glasses aided view, into four sections (RA/1, RA/2, RA/3, RA/4; Fig. 1). The left atrium (LA), and the left (L) and right (RV) ventricles were resected, and each myocardial sample was frozen in liquid nitrogen immediately after weighing and stored at -70°C until used for RNA extraction.

2.3. Histology

Histology was performed on separate animals. The right atria from adult or senescent animals were gently spread between the two steel plates of a specially designed forceps and frozen in liquid nitrogen. They were then flat mounted on a cryostat plate specimen using OCT compound (Tissue Tek, Miles Inc., Elkhart, IN) and serially cut into 10- or 25-μm thick sections collected on gelatin-coated slides. The sinus node was identified using staining for acetylcholinesterase activity (Karnovsky, 1964), with acetylthiocholine iodide as substrate. Sections were then lightly counterstained with 1% toluidine blue, dehydrated and coverslipped.

2.4. Cardiac RNA isolation

RNA was extracted from cardiac samples using a procedure modified from Chomczynski and Sacchi (1987). Frozen myocardial tissues were immediately homogenized in Trizol reagent (Gibco BRL Life Technology Inc.) with a polytron (Kinematica inc. Luzern, Switzerland). After extraction, total RNA was resuspended in DEPC-treated water, and the concentration of RNA was assessed spectrophotometrically. Integrity and concentration of total RNA preparations were checked by electrophoresis on 1.0% (w/v) agarose gels containing ethidium bromide.

2.5. Standard M_2-R template

The rat cardiac muscarinic receptor cDNA sequence cloned by Gocayne et al. (1987) was purchased from the American Type Culture Collection. This M_2-R cDNA includes the complete coding region, 50 bp of the 5′-nontranslated and 250 bp of the 3′-nontranslated regions of the rat cardiac sequence. The M_2-R recombined pGEM-3Z plasmid was digested by *Sty*I and *Spe*I to delete the 50-bp fragment located in the M_2-R cDNA between nucleotides 987 to 1040 of the coding region (Gocayne et al., 1987). The deleted DNA was blunt-ended and ligated, and DH5α competent bacteria (Life Technologies, Gibco BRL) were transformed with the ligation reaction products. Positive clones were analyzed by restriction enzyme mapping to select those including the deleted M_2-R cDNA.

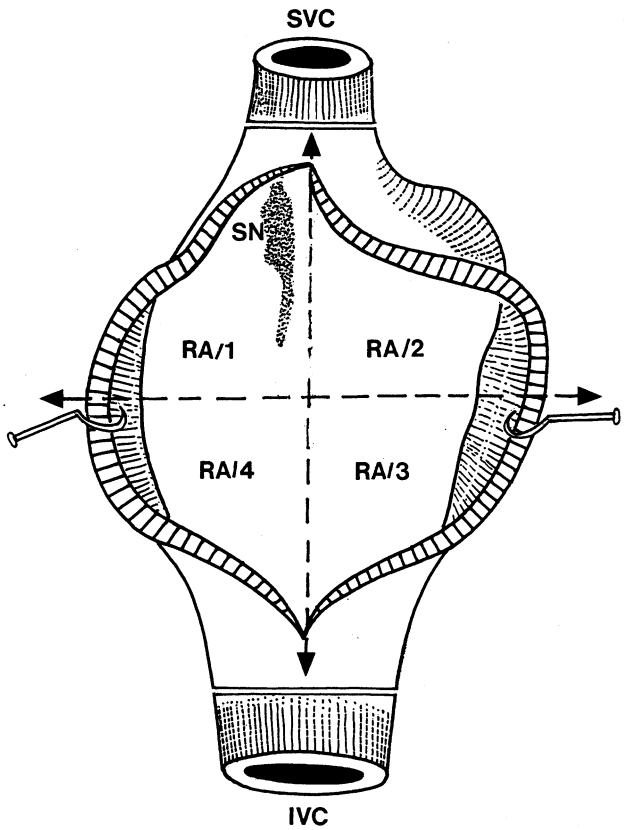

Fig. 1. Schematic representation of the procedure used to dissect the right atria into four sections. Schematic drawing of the right atria after incision along the superior (SVC) and inferior vena cava (IVC) axis. This axis and the perpendicular line (dotted lines with arrows) were used as an arbitrary 2D reference to dissect the right atria into the four sections RA/1, RA/2, RA/3 and RA/4. SN, SA node.

2.6. Transcription of template DNAs

2.6.1. Mutated β-AR templates

The mutant β_1 and β_2 DNA sequences were transcribed in vitro to produce sense mutated β_1- and β_2-adrenergic receptor RNA molecules (Elalouf et al., 1993). The transcription reactions were performed in the presence of $\alpha[^{32}P]$UTP, and the amounts of β_1 or β_2 transcripts were determined after measurement of the radioactivity incorporated in the RNA products. A total of 4–5 pmol of mutant β_1- and β_2-AR RNA were, respectively, obtained from 1 μg of template DNA. Mutated β_1- or β_2-adrenergic receptor RNA transcripts were used as internal standards to quantify the levels of β_1- and β_2-AR mRNA in cardiac RNA preparations.

2.6.2. Wild-type and deleted M_2-R templates

Full-length sense deleted or wild-type M_2-R RNAs were in vitro transcribed in the presence of $\alpha[^{32}P]$UTP from the corresponding cDNA templates using the method of Elalouf et al. (1993). The amounts of M_2-R transcripts were determined after measurement of the radioactivity incorporated in the RNA products. A total of 4–6 pmol of sense transcripts were obtained per reaction using wild-type or deleted M_2-R DNA template. Sense RNA molecules transcribed from the deleted M_2-R cDNA were used as an internal standard to quantify the cardiac products of M_2-R RT-PCR amplification. These sense RNA molecules were thus designed as standard M_2-R RNA.

2.7. Cardiac mRNA amplification

RT-PCR primers were synthesized by Bioprobe Systems, France. β-AR primers were those selected by Elalouf et al. (1993). The β_1 up (5′-CGCTCACCAACCTCTTCATCATGTCC-3′) and down (5′-CAGCACTTGGGGTCGTTGTAGCAGC-3′) primers corresponded, respectively, to bases 272–298 and 623–647, respectively, of the coding region of the β_1-AR sequence (Machida et al., 1990). The β_2 up (5′-TCTTCGAAAACCTATGGGAACGGC-3′) and down (5′-GGATGTGCCCCTTCTGCAAAATCT-3′) primers corresponded, respectively, to positions 1036–1059 and 1355–1378 in the coding and 3′ untranslated regions of the β_2 sequence (Gocayne et al., 1987). The M_2-R upstream (5′-GAACACAACAAGATCCAGAATGGCAAG-3′) and downstream (5′-CGGAGCATGGGCGCAATGATAG-3′) primers corresponded to bases 766–792 (coding region) and 1537–1559 (3′-nontranslated region), respectively, of the rat M_2-R cDNA sequence (Gocayne et al., 1987).

2.7.1. RT-PCR reactions

β-AR sequences were amplified according to Elalouf et al. (1993). The conditions used for M_2-R were the same as those used in β-AR reactions, except for the amount of internal standard molecules co-amplified with cardiac RNA and the cycling parameters. RT and PCR reactions were performed sequentially in a Techne

PHC-2 thermal cycling apparatus (OSI, France). RT was performed at 41°C for 45 min, using 200 U of Moloney Murine Leukemia Virus reverse transcriptase in a final volume of 50 μl. The amplification reaction was initiated in the presence of $\alpha[^{32}P]$dCTP [6000 Ci/mmol, NEN] and was carried out in a final volume of 100 μl with 1.25 U of a thermostable Taq DNA polymerase (AmpliTaq DNA polymerase, Perkin-Elmer Corp., USA). The reaction included 27 cycles (94°C, 1 min; 61°C, 1 min; 73°C, 1 min for β-AR PCR, and 94°C, 1 min; 62°C, 1 min; 73°C, 1.5 min for M_2-R PCR) and one additional cycle with a final elongation time of 10 min. Each reaction tube, except the reaction blank, included both cardiac total RNA (3 or 6 ng) and a constant amount (2000 molecules of mutated β_1- or β_2-adrenergic receptor RNA; 8000 molecules of standard M_2-R RNA) of internal standard RNA molecules. Each cardiac preparation was tested for DNA contamination in a parallel reaction (including 3 ng of cardiac RNA and either 2000 molecules of β_1 or β_2 standard RNA or 8000 molecules of standard M_2-R RNA) performed in the absence of reverse transcriptase. RT-PCR reactions included a reaction blank in which both reverse transcriptase and Taq polymerase activities were tested in the absence of RNA substrates.

2.7.2. Quantitative analysis

2.7.2.1. β-AR. To discriminate between cardiac and mutant RNAs, the RT-PCR products were digested (90 min at 37°C) with *Xho*I (10 U), and the products of *Xho*I digestion were separated on 3% agarose slab gels (Elalouf et al., 1993).

2.7.2.2. M_2-R. The reaction products were treated with *Spe*I in conditions that ensure a complete digestion of cardiac or wild-type DNA fragments (37°C for 90 min in 0.6 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT, 0.6 mM Tris–HCl, pH 7.5, using 10 U of *Spe*I per PCR assay). The DNA products were electrophoresed through a 3% agarose slab gel.

2.7.2.3. Quantitation of RT-PCR products. After gel fixation in 10% acetic acid and drying, two-dimensional image analysis was performed on a Fujix Bio-imaging Analyser System (BAS 1000). A minimum of two i.p. exposures were performed (1–3 h and overnight), the first to obtain nonsaturated images and the second to verify the absence of radioactive signals in samples corresponding to RT-PCR negative controls.

2.7.2.4. Calculations. The densitometric values obtained from the internal standards were used: $N = (P_c \times s)/(P_s \times c)$, where P_c is the densitometric value of the cardiac DNA band, P_s is the densitometric value of the internal standard DNA bands, c is the amount (3 or 6 ng) of cardiac RNA in the reaction, s is the amount of standard mutated β-AR (2000) or M_2-R deleted (8000) RNA molecules, and N is the mRNAs concentrations, expressed in number of molecules per ng of total RNA.

2.7.2.5. Exponential amplification of cardiac and control RNAs. Identical reaction efficiencies were observed in PCR reactions performed with cardiac or standard RNA molecules, as demonstrated by the parallel amplification curves obtained from these two categories of molecules (data not shown). Cardiac mRNA concentrations were determined at 28 cycles, that is within the exponential amplification phase of both cardiac and standard RNA sequences, as shown by the linear increase observed in the log of amplified target molecules when the number of PCR cycles was increased from 24 to 32 (data not shown).

2.8. Statistical analysis

Statistical analysis were assessed by one way analysis of variance (ANOVA). Statistical comparisons for individual variables were done by Fisher and Scheffé tests. Statistical significance was set at 5%. Values presented are mean $\pm$ S.E.M.

3. Results

3.1. Anatomical data

Mean body weight of adult ($n = 7$) rat group was significantly less than that of the senescent ($n = 8$) group (413 ± 15 versus 629 ± 24 g). The age-related cardiac enlargement was more pronounced in the left than in the right side of the heart ($+53 \pm 13\%$, $p < 0.002$ and $+57 \pm 6\%$, $p < 0.001$, respectively, for the left atria and ventricles, and $+30 \pm 8\%$, $p < 0.002$ and $+33 \pm 8\%$, $p < 0.002$ for the right atria and ventricles, respectively).

The comparison between adult and senescent homologous right atrial subsections did not reveal any significant difference in average mass. Adult and senescent values (in mg) averaged respectively: 19 ± 1 and 21 ± 1 for RA/1, 29 ± 3 and 35 ± 3 for RA/2, 25 ± 3 and 33 ± 3 for RA/3, 18 ± 1 and 24 ± 2 for RA/4.

3.2. Histological characterization

The SA node was localized by a strong acetylcholinesterase-positive area in the subsection RA/1 only (Fig. 2A, C). Morphometric investigations showed that in adult rat hearts, the maximum SA node area corresponded to 1/80 of the total area of the right atrium, and was around 200 μm thick, the overall atrial thickness being close to 500 μm. In senescent hearts, the SA node generally displayed heterogeneous acetylcholinesterase staining (Fig. 2C), being very strong in some cell clusters, while other areas of the SA node appeared unstained. In other atrial sections (RA/2 to RA/4), only thin acetylcholinesterase-positive tracts were observed, as shown in Fig. 2B and D.

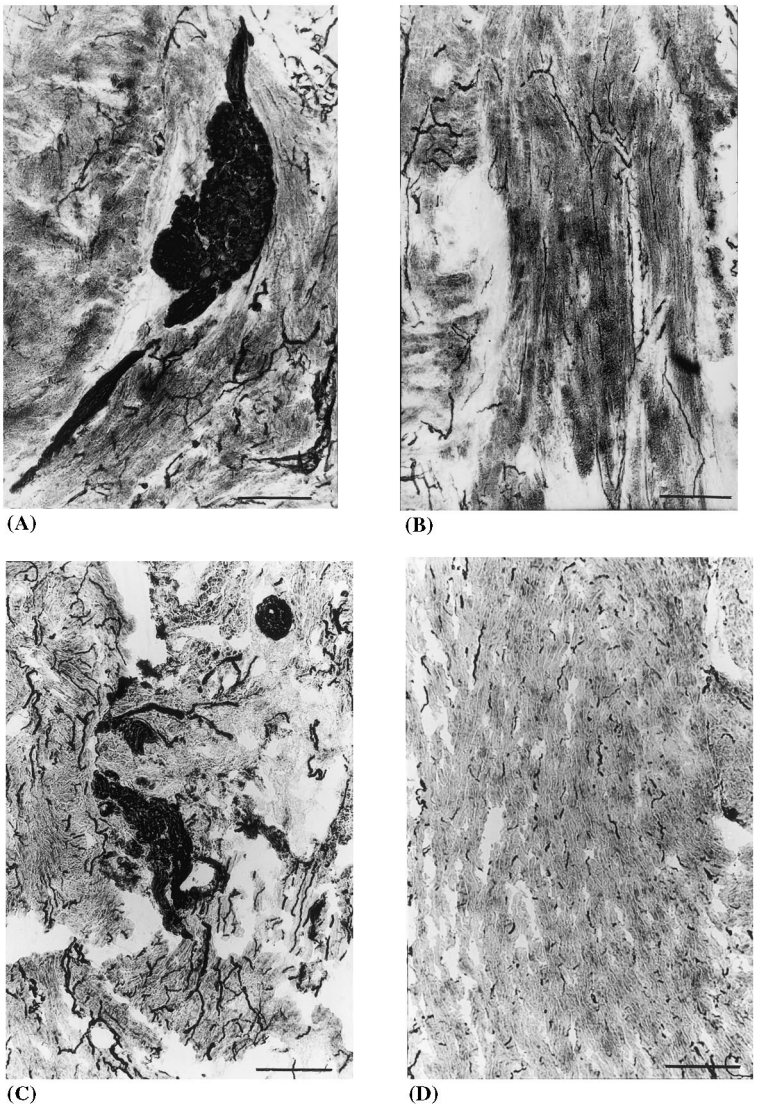

Fig. 2. Histochemical staining of acetylcholinesterase within RA/1 and RA/3 right atrial sections. Acetylcholinesterase staining of representative cryostat sections of flat-mounted right atria from adult (A, B) or senescent (C, D) rat hearts. Calibration bar = 200 μm. In adult rats, prominent staining of the SA node was observed in the RA/1 area (A). In senescent animals, the SA node staining was generally heterogeneous (C). Thin acetylcholinesterase-positive tracts were observed between the cardiomyocytes in the RA/3 section of adult (B) and senescent (D) rat hearts.

3.3. Specificity of the RT-PCR reactions

The specificity of β_1 and β_2-AR RT-PCR reactions has been previously checked (Elalouf et al., 1993). Mutated β-AR DNA differ from the cardiac β_1 or β_2 DNA

by one or two punctual mutations that generate a *Xho*I restriction site (Elalouf et al., 1993). The cardiac PCR products are thus identified as a 376 (β_1) or a 343 (β_2) bp fragment, while the mutated products are digested by *Xho*I into two distinct fragments [212 and 164 bp for mutated β_1 DNA, 261 bp and 82 bp for β_2 mutated DNA] (Fig. 3).

A unique DNA fragment was obtained from standard M_2-R (744-bp), wild-type M_2-R (794-bp) or cardiac (794-bp) RNA (Fig. 4). The deleted M_2-R RNA amplification product is identified as a unique *Spe*I-resistant 744 bp DNA band (Fig. 4). The reaction specificity was demonstrated by, (i) the absence of non-specific bands in the amplification products, by (ii) the correspondence of the PCR product size to that predicted from the cDNA sequence (Gocayne et al., 1987), by (iii) the identical restriction patterns obtained from M_2-R wild-type or cardiac RNA amplification products (data not shown), and by the absence of the specific DNA band in RT-PCR reactions performed with RNA extracted from liver, soleus muscle or brain tissue (data not shown).

3.4. Specific mRNA accumulation

In adult hearts, the mean concentration of β_1-AR mRNA was significantly greater in RA than in LV and RV (Table 1). No differences were observed among RA subsections in the adult and senescent hearts (Table 1).

For β_1-AR mRNA concentration, there were no significant differences between adult and senescent rats for any of the cardiac chambers or subsections of the right atria (Table 1).

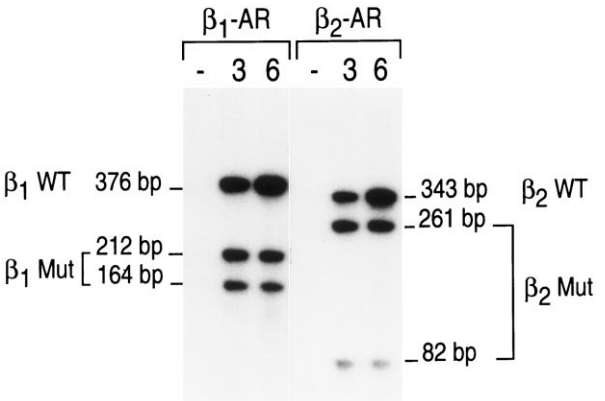

Fig. 3. RT-PCR amplification of β_1- and β_2-AR mRNAs in the adult rat heart. Cardiac total RNA was co-amplified with constant amounts (2000 molecules) of mutant β_1- or β_2-AR RNAs. Cardiac DNA fragments (bp): β_1, 376; β_2, 343. Mutants fragments (bp): β_1, $212+164$; β_2, $261+82$. Lane (−) corresponds to a co-amplification reaction including 3 ng of cardiac RNA and 2000 molecules of either β_1- or β_2-AR mutant RNA without reverse transcriptase. Lane 3 and 6, respectively, correspond to reactions with 3 and 6 ng of cardiac RNA.

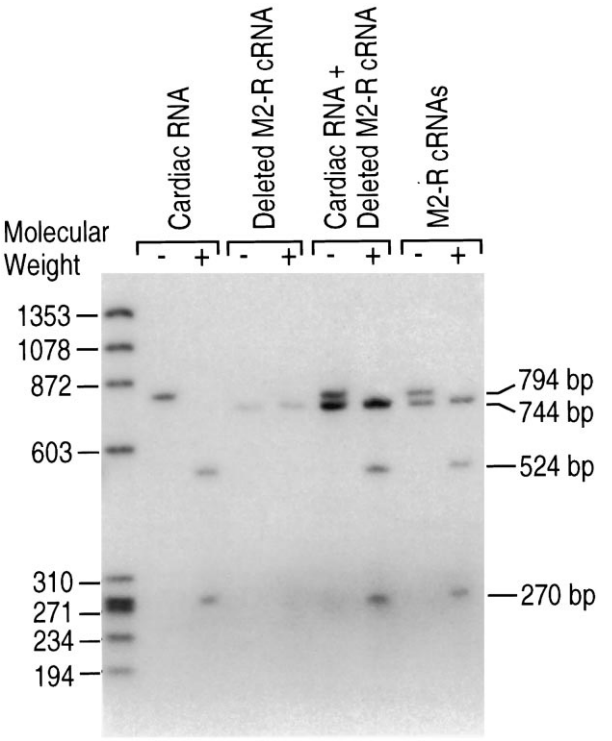

Fig. 4. RT-PCR amplification of M_2-R mRNA. Lanes 1–2: cardiac RNA amplification products. (−): undigested cardiac DNA is identified as a unique 794 bp.band. (+): *Spe*I-digested cardiac DNA is identified as two distinct DNA bands (524 and 270 bp). Lanes 3–4: deleted M_2-R RNA amplification product undigested (−) or digested (+) with *Spe*I is identified as a unique 744 bp DNA band. Lanes 5–6: co-amplification of cardiac RNA and deleted *Spe*I-resistant M_2-R RNA transcript used as a standard in the quantification of cardiac M_2-R mRNA accumulation. Undigested (−) DNA products are identified as a 794-bp cardiac and a 744-bp M_2-R standard DNA band. *Spe*I-digested (+) products were identified as a 744-bp standard DNA band and as two cardiac DNA products of 524 and 270 bp. Lanes 7–8: co-amplification of wild-type and deleted M_2-R RNAs. The DNA products are identified as two distinct bands (wild-type DNA: 794 bp, deleted DNA: 744 bp) in the absence of enzymatic digestion (−). *Spe*I digestion (+) discriminates between the deleted *Spe*I-resistant (744 bp) and wild-type (524 and 270 bp) DNA sequences. [^{32}P]dCTP-labelled ØX174 DNA was used as a molecular weight marker.

The mean value for β_2-AR mRNA concentration did not differ significantly among the adult or senescent sections (Table 1). For β_2-AR mRNAs, there were no significant differences between adult and senescent rats for any of the cardiac chambers or subsections of the right atria (Table 1).

The concentration of β_1-AR plus β_2-AR mRNA did not differ significantly among the sections (cardiac chambers or subsections of the right atria) or between the two age groups (Table 2).

M_2-R mRNA concentration was higher in atria than ventricles in both age groups (Table 1). Within adult RA, subsection RA/1 exhibited the highest M_2-R

mRNA concentration and was significantly different from RA/3 and RA/4 (Table 1). The senescent subsection RA/1 did not differ from any other senescent RA subsection, and was significantly different from adult RA/1 (Table 1).

3.5. mRNA ratios

3.5.1. β_1-AR mRNA/β_2-AR mRNA ratio

3.5.1.1. Adult group. Among cardiac chambers, the highest value was observed in RA (Table 2) which differed significantly from both left and right ventricles. Within RA, subsection RA/1 exhibited the highest ratio which was significantly greater than that of subsection RA/3 (Table 2).

3.5.1.2. Senescent group. The highest mean ratio was obtained in RA and was significantly greater than the ratio of RV and LV (Table 2). The senescent RA was significantly different from the adult RA, and no significant differences were observed among the senescent right atrial subsections (Table 2).

Table 1
mRNA concentrations in cardiac fractions

	β_1-AR mRNA		β_2-AR mRNA		M_2-R mRNA	
	Adult ($n=5$)	Senescent ($n=5$)	Adult ($n=5$)	Senescent ($n=5$)	Adult ($n=7$)	Senescent ($n=8$)
Right atria						
RA/1	918 ± 90	775 ± 39	382 ± 33	405 ± 37	9574 ± 454**	6264 ± 503***
RA/2	897 ± 44	837 ± 123	402 ± 33	467 ± 42	8161 ± 705	7079 ± 441
RA/3	790 ± 67	724 ± 48	442 ± 60	473 ± 72	6339 ± 551	6603 ± 935
RA/4	850 ± 101	774 ± 66	375 ± 30	467 ± 41	6784 ± 756	6861 ± 754
Cardiac chambers						
RA	955 ± 43*	810 ± 107	413 ± 32	508 ± 43	8686 ± 633[a]**	7652 ± 916[a]**
LA	762 ± 115	721 ± 130	387 ± 29	454 ± 46	9069 ± 661[a]**	7613 ± 1019[a]**
RV	663 ± 75	631 ± 77	449 ± 53	587 ± 41	3969 ± 436[a]	3753 ± 549[a]
LV	684 ± 43	578 ± 52	481 ± 47	540 ± 45	3695 ± 285[a]	4012 ± 840[a]

Receptor mRNA concentrations (in molecules of mRNA per ng of total RNA) in adult and senescent cardiac fractions. Total RNA from right atrial subsections (RA/1 to RA/4) and from left atria (LA), right (RV) or left (LV) ventricles, and the levels of β_1-, β_2-AR or M_2-R mRNA were measured after RT-PCR amplification of cardiac RNA in the presence of the corresponding internal standard as described in Section 2. Mean value for right atria (RA) were obtained by measuring mRNA levels from RA/1–4 pooled RNA preparations. Values are mean ± S.E.M.

[a] $n=6$ in these cases.

* $p<0.05$ as compared to LV.

** $p<0.01$ as compared to RA/3 or RA/4.

*** $p<0.05$ effect of senescence.

Table 2
β-AR mRNA concentration, and mRNA concentration ratios in adult and senescent hearts

	$\beta_{(1+2)}$-AR mRNA concentration		β_1-/β_2-AR mRNA ratio		M_2-R/$\beta_{(1+2)}$-AR mRNA ratio	
	Adult	Senescent	Adult	Senescent	Adult	Senescent
Right atria						
RA/1	1299 ± 86	1179 ± 58	2.49 ± 0.30**	1.98 ± 0.20	7.1 ± 0.5**	5.4 ± 0.5 #
RA/2	1299 ± 62	1304 ± 160	2.28 ± 0.16	1.77 ± 0.30	5.6 ± 0.5	5.3 ± 0.4
RA/3	1233 ± 105	1197 ± 113	1.89 ± 0.21	1.63 ± 0.19	5.5 ± 0.5	4.9 ± 0.9
RA/4	1225 ± 121	1241 ± 92	2.28 ± 0.20	1.69 ± 0.17	6.6 ± 0.8	5.7 ± 0.4
Cardiac chambers						
RA	1368 ± 49	1318 ± 136	2.37 ± 0.20*	1.61 ± 0.20* #	6.8 ± 0.4*	5.4 ± 0.6***
LA	1149 ± 128	1174 ± 155	1.98 ± 0.67*	1.60 ± 0.27*	8.4 ± 1.5*	6.4 ± 0.8***
RV	1113 ± 231	1218 ± 98	1.54 ± 0.20	1.09 ± 0.14	3.5 ± 0.3	3.0 ± 0.4
LV	1165 ± 87	1118 ± 83	1.45 ± 0.07	1.08 ± 0.09	3.4 ± 0.2	3.7 ± 0.9

Total amount of β-AR mRNA molecules (β_1-AR mRNA + β_2-AR mRNA) accumulated per ng of total RNA, β_1-AR/β_2-AR mRNA concentration ratios, and M_2-R/$\beta_{(1+2)}$-AR mRNA concentration ratios in adult ($n = 5$) and senescent ($n = 5$) cardiac chambers or right atrial subsections. RA/1 to RA/4: right atrial subsections 1–4. RA, right atria; LA, left atria; RV, right ventricles; LV, left ventricles. mRNA concentrations used for the determination of mean right atrial values (RA) were obtained by measuring mRNA levels from RA/1–4 pooled RNA preparations. Values are mean ± S.E.M.
* $p < 0.05$ as compared to LV.
** $p < 0.05$ as compared to RA/3.
*** $p < 0.05$ as compared to LV
# $p < 0.05$ effect of senescence.

3.5.2. M_2-R/$\beta_{(1+2)}$-AR mRNA ratio

The ratio between M_2-R and $\beta_{(1+2)}$-AR mRNA concentrations was significantly different between adult atria and ventricles (Table 2). The comparison between RA subsections showed a significant difference in the adult group between RA/1 and RA/3 (Table 2). In the senescent group, RA/1 which was significantly lower than its adult homolog, did not differ from any other senescent atrial subsection (Table 2).

4. Discussion

The main findings of the present study were the following: (i) Despite an heterogeneous distribution of β-AR subtype and M_2-R mRNA within the heart, the concentration of $\beta_{(1+2)}$ mRNA molecules remains constant whatever the age group. (ii) In the adult heart, the highest concentration of M_2-R mRNA and the highest M_2-R/$\beta_{(1+2)}$ mRNA ratio are observed in the SA node area. (iii) The M_2-R mRNA concentration and the M_2-R/$\beta_{(1+2)}$ mRNA ratio are reduced in the SA node area of the senescent heart.

4.1. The experimental approach

According to the usual yield of protein extraction from cardiac homogenates (about 100 μg per mg of tissue (Chevalier et al., 1991; Mansier et al., 1993) and to the amount of protein required to quantify receptor membrane densities using the classic method of radioligand binding, this method could not be applied to tissue fragments as small as those required for the present study. The same holds true when the alternative method of western blotting is considered, since, to be detected by western-blotting, an average-sized protein must comprise at least 1 part in 10^5 by weight of the total protein in the extract (Sambrook et al., 1989). Tissue section radioligand binding and autoradiography have also been used to study β-adrenergic receptor subtype distribution in rat heart myocardium (Zhao and Muntz, 1993). This method, which gives accurate information in terms of receptor relative densities, cannot be applied to the molecular characterization of small tissue fragments.

The present investigation at the level of mRNA molecules was justified by data from the literature which suggest a good correlation for β-AR and M_2-R between the abundance of receptor mRNA and of the level of the corresponding protein in a number of experimental models. Parallel changes in the abundance of β-AR mRNA and protein levels have been reported (references in Bristow et al. (1993)), and a similar relationship in the regulation of muscarinic mRNA and protein levels is suggested by available evidence (Hoover et al., 1994). This conclusion is furthermore supported by the results of our study which included a quantification of specific receptor mRNAs within left and right atria and ventricles in each age group and demonstrated a concordance between our results concerning the atrioventricular distribution of β-AR and M_2-R mRNAs in the adult rat heart and the corresponding receptor densities in rat atrial and ventricular tissues reported by other groups (see next paragraph).

The method of in situ hybridization using radioactive oligonucleotide probes and autoradiography has been used to study the atrioventricular distribution of M_2-R mRNA (Hoover et al., 1994). This method is quite adapted to the localization of specific mRNA sequences in a given tissue, but has a quantitative resolution that limits its application to situations where relatively large scale variations occur in the levels of specific mRNA accumulation.

Therefore, in the present study, we selected a RT-PCR method to quantify the amount of β-AR and M_2-R mRNA molecules in individual cardiac fractions of adult and senescent hearts.

4.2. Atrioventricular distribution of receptor mRNAs

β_1-/β_2-AR mRNA ratios of 1.98–2.37 and 1.45–1.54 were obtained in the present study for atrial and ventricular adult tissues, respectively. These results are consistent with the proportion reported by Zhao and Muntz (1993) of 2.22 and 1.77 between β_1- and β_2-AR subtypes in the atrial and ventricular myocytes of adult rat heart, respectively. Concerning M_2-R mRNA, our results show a larger difference

between atria and ventricles than observed in the β-AR mRNA distribution. In the adult heart, the atrial M_2-R mRNA concentration was on average about 2.3 times higher than the ventricular value. This is consistent with data of the literature indicating a 2.9 ratio between rat atrial and ventricular M_2-R densities (Hancock et al., 1987) and a proportion between rat atrial and ventricular M_2-R mRNA abundance of 3.2 (Hoover et al., 1994).

4.3. Cardiac β-adrenergic and muscarinic receptor densities in the senescent heart

The effects of age on β-adrenergic receptor density and affinity in ventricular tissues have been extensively studied (Abrass et al., 1982; Tumer et al., 1989; Chevalier et al., 1991; Mader et al., 1991). A general finding is a reduced responsiveness to β-adrenergic modulations of the senescent heart (Filburn and Lakatta, 1984). However, studies in rat models failed to reveal consistent age-related changes in cardiac β-adrenergic receptor density or agonist affinity (Kusiak and Pitha, 1983; Fan and Banarjee, 1985): an increase, decrease, or no change in β-adrenergic receptor density has been associated with the aging of the myocardium (Kusiak and Pitha, 1983; Fan and Banarjee, 1985; Narayanan and Derby, 1982; O'Connor et al., 1983; Chevalier et al., 1991). This discordance may result from experimental variations (as variations in cardiac membrane preparations) and from the age-related changes in cell size that may modify membrane receptor density without affecting their actual number (Cerbai et al., 1995). However, recent reports in which the densities of β_1- and β_2-AR were measured in human ventricles and in rat ventricular myocytes clearly evidenced that the age-related decrease in β-AR stimulation during ageing was associated with a selective reduction of β_1-AR density (White et al., 1994; Cerbai et al., 1995).

Discordant results have been reported about the consequences of ageing on the cardiac muscarinic system. Data obtained in human studies indicated that ageing was associated with a depression in the parasympathetic cardiac control (Dauchot and Gravenstein, 1975; Kelliher and Conahan, 1980), while studies performed on rat heart suggested an age-associated enhancement of muscarinic receptor responsiveness which may be partly attributed to an age-associated decline in cholinesterase activity (Kennedy and Seifen, 1990; Ferrari et al., 1991; Su and Narayanan, 1992). Discordant results have also been reported about the consequences of ageing on the number of cardiac muscarinic receptors. According to Narayanan and Derby (1983), no change was observed in the number of these receptors or their binding affinities in rat ventricular tissue, the density of these receptors being elevated in atria of senescent rat hearts. In a more recent report, Su and Narayanan (1992) found no age-related difference in receptor number and antagonist binding affinity in rat heart atria or ventricles. In contrast, a reduction of muscarinic receptor density in the left ventricle of senescent rat heart was reported to accompany a less pronounced reduction in β-AR density (Chevalier et al., 1991).

4.4. Localization of the SA node

In the present study, we have used the acetylcholinesterase activity staining as a marker of nodal tissue. The location of the SA node within RA/1 subsection is in agreement with data of the literature describing the SA node at the junction between the superior caval vein and right atrium (Oosthoek et al., 1993), and estimating its relative size using morphological studies (James and Spence, 1966; Opthof, 1988).

4.5. The SA node area in the adult heart

Immunolabelling and histochemical studies performed on the human heart have shown that the cardiac conduction system is more densely innervated than the myocardium in other parts of the heart, the SA node being the most densely innervated component of the conduction system and including a large proportion of acetylcholinesterase-positive nerves (Crick et al., 1994). Hancock et al. (1987) have evidenced higher choline acetyltransferase and acetylcholinesterase activities in right and left atria compared with ventricles, the highest activities of these two enzymes being located in the sinoatrial node of the adult rat heart. These authors have shown that the cardiac distribution of cholinergic markers overlaps the distribution of muscarinic receptors, indicating a localization of these receptors that was related to their signalling function in mediating the cardiac response to parasympathetic stimuli. The higher concentration of M_2-R mRNA and the higher proportion of β_1-AR mRNA in the SA node area are thus components of the phenotypic specificities of this atrial area that are related to its pacemaker function and its role as a target for neuronal regulations.

4.6. The senescent SA node

A number of studies have been focused on the determination of β-AR and muscarinic densities in the sinoatrial node of human and canine hearts, using quantitative light-microscopic autoradiography of radioligand binding sites (Beau et al., 1995; Rodefeld et al., 1996). These studies have evidenced in both species a greater density of muscarinic and β-adrenergic receptors, and a larger proportion of β_1-AR in the sinoatrial node than in adjacent atrial muscle. However, no data exist about the absolute molecular quantification of these receptor molecules within the SA node area and the consequences of cardiac senescence on the proportion of these two groups of receptor molecules within the pacemaker site of the mammalian heart.

As far as the concentration of M_2-R mRNA and the proportion between muscarinic and β-adrenergic sequences are concerned, the heart does not age as a unit: the age-related changes are focused on the SA node area. These changes are associated with morphological modifications as demonstrated by the fragmentation observed in acetylcholinesterase staining of the senescent SA node. The age-related changes observed in specific receptor mRNA expression within the SA node area

may thus reflect the cellular modifications that accompany the cardiac senescence and include a reduction in the number of muscle cells and an increase in the proportion of non-muscle cells (Anversa et al., 1986). The loss of phenotypic differences between the senescent right atrial subsections is consistent with an age-related augmentation of connective tissue in the SA node, as suggested by studies in elderly showing a substantial reduction of the number of pacemaker cells in the sinus node which is associated with an increasing connective tissue infiltration (reviewed in Bouman and Jongsma, 1986; Folkow and Svanborg, 1993).

The age-related changes that are observed in the SA node area are also part of a series of modifications that have been reported in the innervation of the senescent atrial tissue. Ultrastructural studies have demonstrated modifications in the density of post-glanglional noradrenergic and cholinergic axon distributions within the senescent rat atria which resulted from degenerative processes (Mc Lean et al., 1993). Age-related modifications have also been evidenced in the myocardial content and/or activity of specific catecholaminergic and cholinergic neurotransmitters (Lakatta, 1987; Ferrari et al., 1991; Su and Narayanan, 1992) which are involved in signalling functions and may also have a trophic action on their cardiac target cells.

Taken together, these data raise questions about the relationship between these groups of cellular and molecular events. Changes in β-adrenergic and muscarinic phenotype of the senescent SA node area may either participate in the mechanisms that induce age-related cellular remodelling in this atrial area or result from critical time-dependant cellular or tissular events. Numerous studies have established a major role of innervation in the control of gene expression in the skeletal muscle tissue (Michel et al., 1994), strongly suggesting that the atrial distribution of sympathetic and parasympathetic nerve endings and the concentration and/or activity of the corresponding mediators may be determinants of the SA node phenotype and that changes in these structural and functional parameters may at least in part account for the quantitative modifications associated with senescence in the regional distribution of muscarinic and β-adrenergic sequences within the SA node area.

4.7. Conclusion

Quantitative changes have been demonstrated in the present work in the SA node area of the senescent heart. These changes may be part of a larger group of age-related qualitative and quantitative modifications in gene expression that appear to participate in the adaptation of an ageing organism to environmental insults and their resulting dysregulations, as suggested by recent works reviewed in (Jazwinski, 1996).

In terms of M_2-R mRNA concentration and muscarinic/β-adrenergic mRNA proportion, the heart does not age as a unit: age-related changes are focused on the SA node area. A decreased concentration of M_2-R mRNA and a decreased proportion between β_1- and β_2-AR mRNAs are selectively observed in this specialized atrial area, where the total accumulation of β-AR mRNA molecules remains

unchanged. These quantitative changes are molecular markers of the senescence of the SA node area, and, as part of the biological modifications that differentiate adult and senescent SA node may contribute to the global reduction in heart rate variability observed in the senescent heart.

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