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Regional and Age-related Differences in mRNA Composition of Intracellular Ca^{2+}-release Channels of Rat Cardiac Myocytes

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L. Gorza, S. Vettore, A. Tessaro, V. Sorrentino and M. Vitadello. Regional and Age-related Differences in mRNA Composition of Intracellular Ca^{2+}-release Channels of Rat Cardiac Myocytes. *Journal of Molecular and Cellular Cardiology* (1997) 29, 1023–1036. We investigated the mRNA distribution of three different ryanodine receptors (RyR) and of the intracellular Ca^{2+}-release channel/inositol 1,4,5-trisphosphate receptor (IP_3R) type 1 in the rat heart during development and aging. *In situ* hybridization analysis shows that RyR1 mRNA is never expressed in the heart at any of the stages examined; RyR2 mRNA is detectable in cardiomyocytes in the early embryonic stages, whereas RyR3 mRNA accumulates in cardiomyocytes around birth. IP_3R mRNA appears at first in the primitive atrium at embryonic day 11 and in subsequent stages it is detectable also in a minor population of ventricular myocytes, which presumably correspond to conduction system precursors. In the adult heart, no apparent difference in hybridization signal intensity is observed between atrial and ventricular working myocytes either with RyR2, RyR3 or IP_3R cRNA probes, except for myocytes of the heart conduction system, which differ from working myocytes in the intensity of the hybridization signals for each probe. Additional differences are detected in the senescent heart with the IP_3R cRNA probe, which hybridizes with atrial myocytes stronger than with ventricular ones. RNase protection analysis confirms the temporal differences in RyR2 and RyR3 transcript accumulation observed during heart development and reveals a significant increase of IP_3R mRNA in the atrial myocardium during aging. Thus, the composition of intracellular Ca^{2+}-release channel mRNAs of the rat heart shows temporal and regional variations: such changes might reflect important differences in transcriptional regulation of these genes among myocytes.

Key Words: Ryanodine receptors; Inositol trisphosphate receptors; *In situ* hybridization; RNase protection; Heart development; Heart conduction system; Senescence.

Introduction

In cardiac myocytes, increase of intracellular Ca^{2+} concentration causes the activation of the contractile machinery and affects other functions, such as impulse generation and conduction (Billman, 1992). A major role in evoking the contractile response is played in the adult myocardium by the Ca^{2+} induced activation of the intracellular Ca^{2+}-release channel/ryanodine receptor (RyR) (Fabiato, 1983). The RyR gene family is composed of three members (reviewed by Nori *et al.*, 1996): RyR1, which is predominantly expressed in skeletal muscle; RyR2, which is predominantly expressed in the heart and the brain, and the recently identified RyR3, which is expressed in a broad variety of

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tissues. Northern blot and RNase protection analysis have given contrasting results concerning the expression of RyR3 mRNA in the ferret and the rabbit heart (Giannini *et al.*, 1992; Hakamata *et al.*, 1992). In the mouse heart, the RyR3 transcript was detected by RNase protection analysis (Giannini *et al.*, 1995) and *in situ* hybridization studies demonstrated a preferential accumulation in myocytes of the heart conduction system (Gorza *et al.*, 1995). Furthermore, the subtype 1 of the intracellular Ca^{2+}-release channels sensitive to inositol 1,4,5-trisphosphate (IP_3R) was detected in cardiac myocytes (Furuichi *et al.*, 1990; Gorza *et al.*, 1993b; Kijima *et al.*, 1993; Moschella and Marks, 1993). High concentrations of IP_3R were observed in a subset of cardiac myocytes, the Purkinje myocytes, which belong to the conduction system of the heart and are involved in impulse triggering and conduction (Gorza *et al.*, 1993b). The abundance of IP_3-sensitive Ca^{2+} channels in these myocytes might be mechanistically involved in the increase in automaticity observed in Purkinje myocytes after hormonal stimulation (reviewed in Gorza *et al.*, 1994).

During development, cardiac contractility appears to be mainly regulated by Ca^{2+} influx through the sarcolemma; nevertheless, ryanodine can reduce the inotropic response up to 50% in 1 day neonatal rat heart (Wibo *et al.*, 1991). In embryonic chicken hearts ryanodine depresses contractility as early as embryonic day (E) 5 and RyRs are immunologically detectable and able to bind ryanodine as early as E4 (Dutro *et al.*, 1993). In the mammalian heart, evidence on RyR2 mRNA accumulation and IP_3R expression has been provided for late stages of prenatal development (Arai *et al.*, 1992; Brillantes *et al.*, 1994, Gorza *et al.*, 1993b), whereas it is presently unknown whether transcripts specific for RyR and IP_3R are already present in the embryonic heart. Furthermore, it is not known whether changes in mRNA composition of intracellular Ca^{2+} channels occur in the heart during senescence, a condition characterized both in the human and the rat hearts by an high incidence of spontaneous arrhythmias (Carré *et al.*, 1992). Thus, the goal of the present study was to investigate the mRNA composition of intracellular Ca^{2+} channels of the rat heart, during development and aging, using nucleic acid probes specific for each intracellular Ca^{2+} channel type (Giannini *et al.*, 1995; Mignery *et al.*, 1990). Our results show that each transcript displays temporal and/or regional differences in accumulation, in either working or conduction myocytes, unraveling an unexpected heterogeneity in the expression of these transcripts among cardiac myocytes.

Materials and Methods

Tissue source

Wistar rats were used for this study. Male and female rats were mated overnight and noon of the subsequent day corresponded to day 0.5 of pregnancy. Several embryos from a same litter were collected at embryonic day (E)10, E11, E12, E13, E14, E15 and E18.

Some embryos from a litter were fixed with 4% paraformaldehyde and embedded in paraffin for *in situ* hybridization studies (Gorza *et al.*, 1993a). Hearts were excised from stage E18, 1-day-old, 1- and 3-month-old adult rats and 15- and 27-month-old senescent rats. The excised hearts were lightly fixed with 2% paraformaldehyde, cryoprotected with increasing concentrations of sucrose, as previously described (Gorza and Vitadello, 1989), and frozen in liquid nitrogen.

Hindlimb skeletal muscles, cerebrum and cerebellum, liver and testis were also excised from adult rats. For RNA preparation samples were excised and immediately frozen with liquid nitrogen.

Nucleic acid probes

Mouse RyR1, RyR2 and RyR3 cDNA subclones (Giannini *et al.*, 1995; Conti *et al.*, 1996) were used. Subclone pMB14 contained 1100 bp of the 3′ coding region of RyR1 cDNA; subclone pMB3 contained about 3000 bp of the 3′ untranslated region of RyR2 cDNA and subclone $pMT_{II}11$ contained about 1000 bp of the 3′ coding region of RyR3 cDNA. The regions chosen for probe generation correspond to regions of the nucleotide sequence with higher levels of divergence. In brief, sense and antisense RyR1 probes were obtained after linearization of clone pMB14 with *XhoI* and *Eco*RI, respectively, and were about 1100 nt in length. Sense and antisense RyR2 probes were obtained after linearization of clone pMB3 with *XhoI* and *Eco*RI and were 3000 and 150 nt in length, respectively. Sense and antisense RyR3 probe were obtained after linearization of clone $pMT_{II}11$ with *Bam*HI and *Bgl*II and were 750 and 430 nt in length, respectively.

A rat IP_3R cDNA (clone pI2a, Mignery *et al.*, 1990), containing a 2-kb *Eco*RI fragment of the 3′ coding and non-coding regions of the type 1 IP_3R mRNA, was linearized at the polylinker site and used as a template for sense and antisense cRNA

synthesis for *in situ* hybridization. A shorter antisense cRNA (about 280 nt), generated after digestion of the clone with *Sac I* and corresponding to the 3′ untranslated region was used in RNase protection assays.

Northern blotting

Total RNA was isolated from liver, heart and skeletal muscle of adult rats following the procedure described by Chomczynski and Sacchi (1987). Northern blot was performed as described in Vitadello *et al.* (1996). In brief, equal amounts (about 10 μg) of each sample, as determined by ethidium bromide staining of formaldehyde agarose gels, were transferred to nylon filters (Hybond N^+, Amersham) by capillary elution with 20 × SSPE. Blotted RNA was hybridized overnight at 42°C in the presence of 10×10^5 ct/min/ml of RyR1 probe, generated by random priming labelling with [^{32}P]-dCTP of pMB14 insert. High stringency washes were performed at 65°C with 0.1 × SSPE. Filter was exposed to X-ray film for 7 days.

RNase protection assay

Antisense cRNAs for RNase protection were transcribed *in vitro* in the presence of [α^{32}P]UTP (800 mCi/mmol) from pMB 3 and $pMT_{II}11$, using the same linearized template prepared for probe transcription for *in situ* hybridization, and from clone pI2a linearized with *Sac*I. Mouse β-actin cDNAs (Ambion) generated a transcript of 300 nt, which was used for normalization. Labelled probes were gel purified after 8 M urea–5% polyacrylamide electrophoresis.

Total RNA was extracted from tissues using the procedure described by Chomczynski and Sacchi (1987). RNA amount was determined by densitometry and by electrophoresis in formaldehyde agarose gels. RNase protection assay was performed as described in Rossetto *et al.* (1996). In brief, total RNA was hybridized overnight at 45°C in 80% formamide, 100 mmol/l Na citrate, 300 mmol/l Na acetate pH 6.4, 1 mmol/l EDTA with either 1×10^5 ct/min of RyR2 or IP_3R probes or with 2×10^5 ct/min of RyR3 probe. Total RNA 1 μg from each sample was hybridized with 6×10^4 ct/min of the β-actin probe. After digestion with RNase A and T1, samples were precipitated and separated by electrophoresis with 5% denaturing polyacrylamide gels. Molecular weight of protected fragments was determined using a 100-bp DNA ladder (Pharmacia) labelled with [^{32}P]dCTP with Klenow DNA polymerase. Autoradiographies were developed after 3-day exposure at −80°C for RyR 2; after 7-day exposure for β-actin probes and after 15-day exposure for RyR3 and IP_3R probes.

In situ hybridization

Sense and antisense cRNAs were transcribed *in vitro* and radiolabelled with ^{35}S as previously described (Gorza *et al.*, 1993b; Conti *et al.*, 1996). Probes were digested to 50–100 nt by mild alkaline hydrolysis. Paraffin sections and cryosections were used for *in situ* hybridization experiments. Prehybridization treatments consisted in 4% formaldehyde fixation, digestion with 20 μg/ml proteinase K and acetylation with 0.25% acetic anhydride in 0.1 mol/l triethanolamine. Hybridization was performed overnight at 52°C using for each section 10 μl of probe at a concentration of 1×10^5 ct/min/μl in 50% formamide, 10% dextran sulfate, 1 × Denhardt's, 10 mmol/l dithiothreitol (DTT) and 100 μg/ml yeast tRNA. Highest stringency wash was performed at 65°C in 50% formamide, 2 × Saline–Sodium Citrate SSC) and 0.1 mol/l DTT. Sections were dehydrated, dipped in KODAK NTB-2 autoradiographic emulsion diluted 1:1 with water, and exposed at 4°C. In each set of experiments, pairs of sections were hybridized with sense and antisense probe and exposed for 7 and 15 days, when hybridized with RyR1 and RyR2 probes, and for 15 and 21 days when hybridized with RyR3 and IP_3R probes. Slides were developed with KODAK D 19 for 3.5 min, fixed and examined with a Zeiss Axioplan microscope equipped with dark field optics.

Immunohistochemistry

Indirect immunoperoxidase was performed on cryosections of adult rat heart with α-smooth actin monoclonal antibody (Sigma) diluted 1:40 as previously described (Gorza *et al.*, 1993a).

Statistical analysis

Quantitative densitometry was performed on RNase protection experiments on samples of atrial and ventricular myocardium of 4-month-old rat hearts ($n = 3$) and 27-month-old rat hearts ($n = 4$). Autoradiographic bands were analyzed using a Shimadzu dual wavelength chromatoscanner CS-930

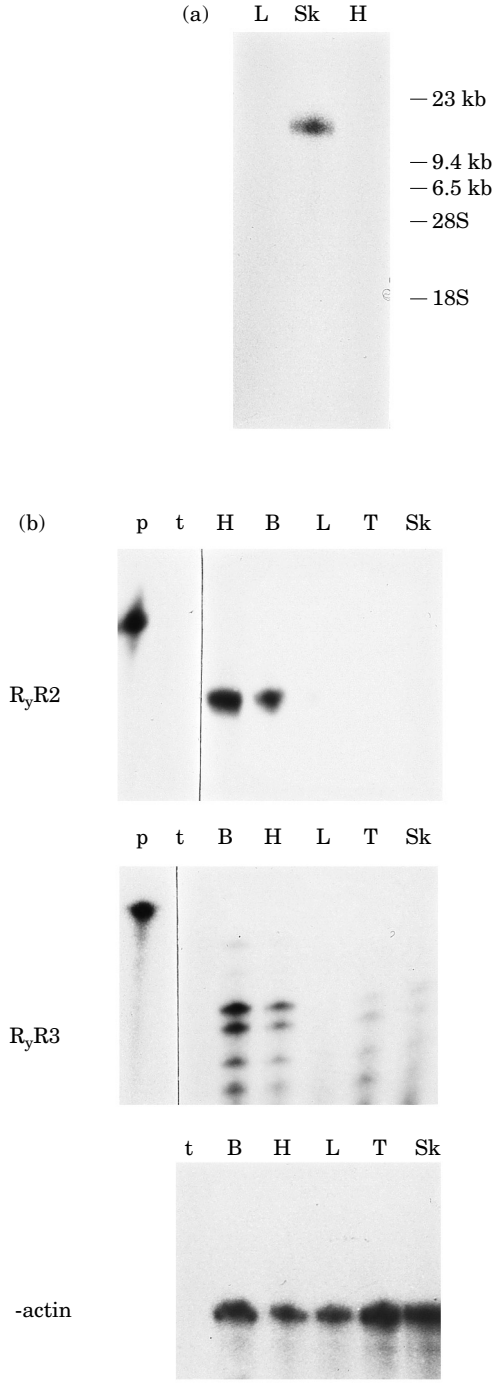


Figure 1 Specificity of mouse RyR probes for rat RyR mRNAs. (a) Northern blot of rat total RNA hybridized with RyR1 cDNA probe. Lanes were loaded with 10 μg of liver (L), skeletal muscle (Sk) and heart (H) total RNA. Migration of λ *Hind*III DNA markers (Pharmacia) and RNA 28S and 18S is indicated on the right. A single mRNA species corresponding to RyR1 mRNA is detectable in skeletal muscle. (b) RNase protection analysis of RyR2, RyR3 and β-actin cRNA probes. Hybridization was performed using 50 μg of total RNA from various tissues with both RyR2 and RyR3 probes, except for hybridization of heart total RNA with RyR2 probe, where only 10 μg were used. Total RNA 1 μg from each sample was used for hybridization with β-actin probe. p: probe; t: yeast tRNA; B: brain; T: testis. RyR2 probe protects a fragment of about 120 nt in heart and brain, whereas RyR3 probe protects two fragments of about 280 and 250 nt in heart, skeletal muscle, brain, testis but it does not protect fragments in liver. The finding of a doublet of protected fragments with RyR3 might be explained with the presence of slight differences in nucleotide sequence between hybrids, which are only partially affected by RNase treatment. β-actin probe showed a 240 nt protected fragment.

at a wavelength of 600 nm. Densitometric profiles were cut from paper and weighed. In order to compensate for difference in probe-specific activity between experiments, a RNA sample from a 3-month-old ventricular myocardium was used in each experiment as internal reference. Each sample value was normalized to the corresponding amount in β-actin mRNA. Statistical analysis was performed utilizing the unpaired Student's *t*-test.

Results

Specificity of RyR probes

The specificity of mouse RyR probes for rat RyR mRNAs is illustrated by Figure 1. Northern blot analysis performed with RyR1 1.1 kb DNA probe shows hybridization with a single mRNA species of about 14 kb expressed in rat skeletal muscle, whereas no reactivity is detectable with cardiac and liver total RNAs [Fig. 1(a)]. Due to the low amount of RyR3 transcript and in order to exclude possible cross-reactivities between RyR3 probe and RyR2 mRNA, RNase protection analysis was used to investigate the tissue specific expression of these two transcripts [Fig. 1(b)]. A protected fragment of 120 nt corresponding to RyR2 mRNA is detectable in heart and brain tissue and is apparently not expressed in skeletal muscle, testis and liver. The same profile of protected fragments corresponding to RyR2,3 mRNA, namely two fragments of 280 and 250 nt, is detected in heart and brain samples, and with much lower intensity in skeletal muscle and testis samples, whereas no protected fragment is detected after hybridization with liver total RNA.

RyR and IP_3R mRNA distribution in the developing rat heart

In situ hybridization analysis was performed on rat embryos. The earliest stage examined corresponded to E10 (about 13 somites) of rat development; only

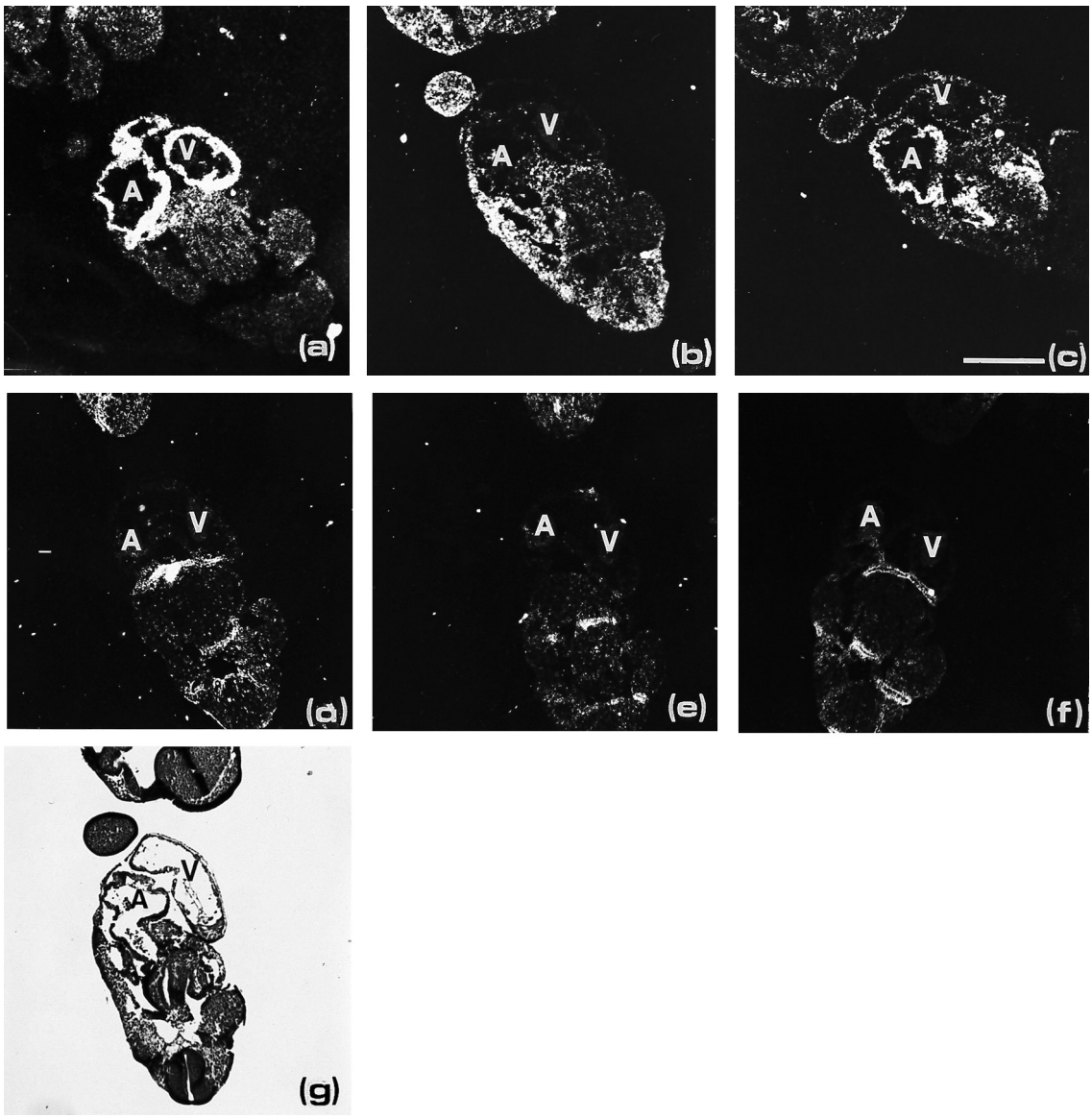


Figure 2 *In situ* hybridization of E11 rat embryo. Paraffin sections of whole embryo, obtained by cutting along a longitudinal plane, were hybridized with both antisense RyR2 (a), RyR3 (b), IP_3R (c) cRNA probes and sense RyR2 (d), RyR3 (e), IP_3R (f) cRNA probes. (g) Shows a serial section stained with hematoxylin. Antisense RyR2 probe hybridizes with cardiac myocytes of atrial (A) and ventricular (V) myocardium (a). Hybridization with antisense RyR3 probe is detectable in different regions of the embryo body, except the heart (b). Antisense IP_3R cRNA hybridizes with myocytes of the atrial myocardium, whereas myocytes of the ventricle do not display apparently any hybridization signal (c). No hybridization signal is detectable in the heart with sense probes; horizontal bright stripes correspond to section folds (d)–(f). Autoradiographic exposure was 15 days for all probes. Bar: 300 μm

very weak hybridization signals for RyR2 mRNA are detectable at this stage in myocytes of the heart tube (not shown). At E 11 of development, RyR2 mRNA accumulation appears to be exclusively localized in the heart, in myocytes of each heart chamber [Fig. 2(a)]. Conversely, at this same stage, RyR3 transcript is detectable in many embryonic tissues with the exception of heart cells, which apparently do not hybridize [Fig. 2(b)]. IP_3R mRNA accumulates in a few mesenchimal embryonic structures and in a population of heart cells, which correspond to atrial myocytes [Fig. 2(c)]. No hybridization signal is detected at this stage with the RyR1 probe (not shown) and with sense probes [Fig. 2(d)–(f)].

The hybridization pattern of RyR probes in the heart does not change at E12 and E15. No accumulation of RyR1 mRNA is observed in the heart, at variance with embryonic skeletal muscle cells which display positive hybridization signals [Fig.

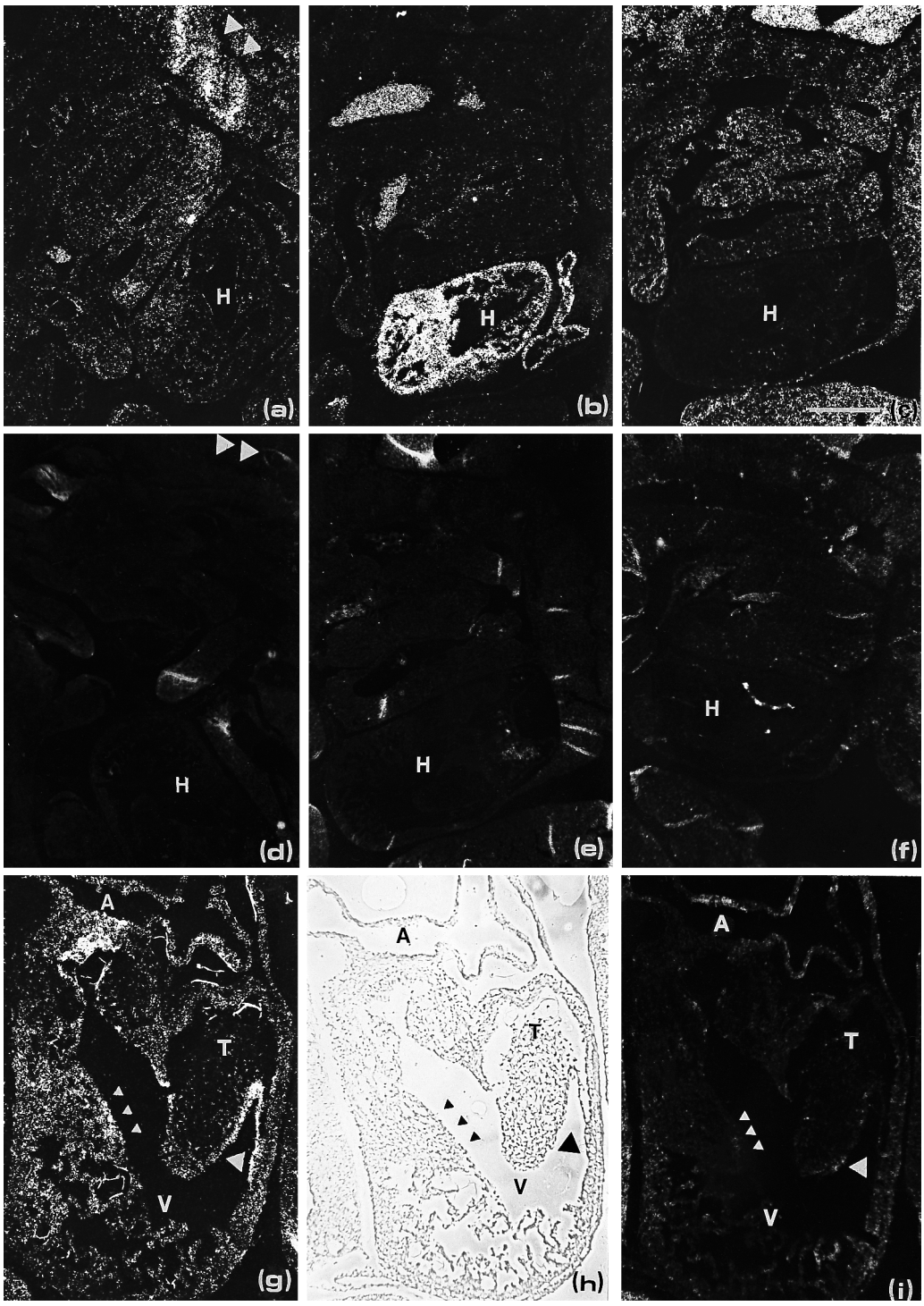


Figure 3 *In situ* hybridization of E13 rat embryo. Paraffin sections of whole embryo, obtained by cutting along a sagittal plane, were hybridized with antisense RyR1 (a), RyR2 (b), RyR3 (c) and IP_3R (g) cRNA probes. Serial sections were hybridized with sense RyR1 (d), RyR2 (e), RyR3 (f) and IP_3R (i) cRNA probes. Antisense RyR1 probe (a) hybridizes with muscle cells of the myotome (arrowheads), whereas it does not hybridize apparently with cardiac myocytes (H). Antisense RyR2 probe (b) hybridizes only with cardiac myocytes; antisense RyR3 probe, (c) shows hybridization signals in the neural tube and in the visceral mesoderm, whereas it does not apparently hybridize with heart cells. No positive signal is detected with sense probes (d)–(f), except for bright transverse stripes which correspond to folds. Accumulation of IP_3R mRNA in the heart is illustrated by a dark field micrograph taken at higher magnification (g): positive signals are detected in the atrium (A) and in a population of ventricular (V) myocytes localized on the top of the growing septum (small arrowheads). Large arrowhead points to strong hybridization signal displayed by myocytes localized in proximity of the truncus arteriosus (T). (h) shows the phase contrast of the same field illustrated in (g). No signal is detectable with the sense probe (i). Autoradiographic exposure was 7 days for sense and antisense RyR2 probes and 15 days for the other probes. Bar: (a)–(f) 300 μm; (g)–(i): 150 μm.

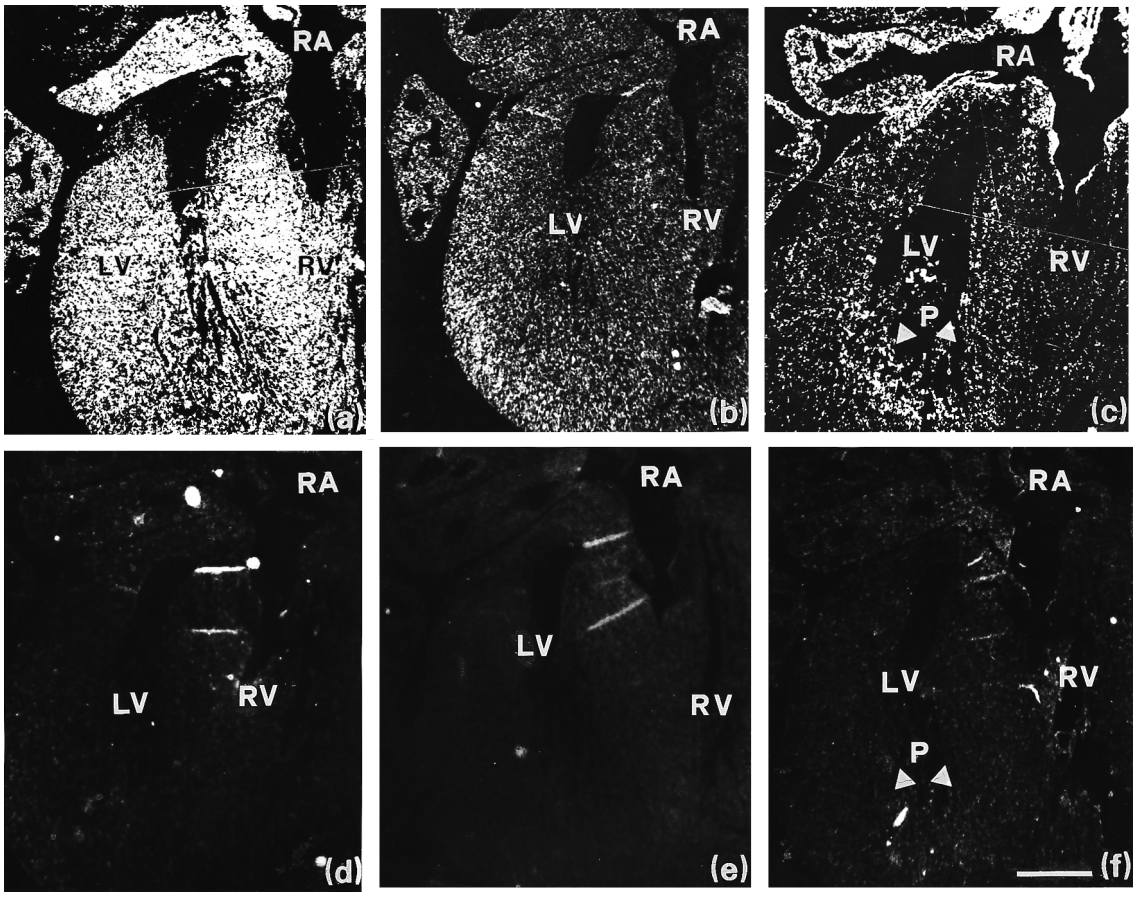
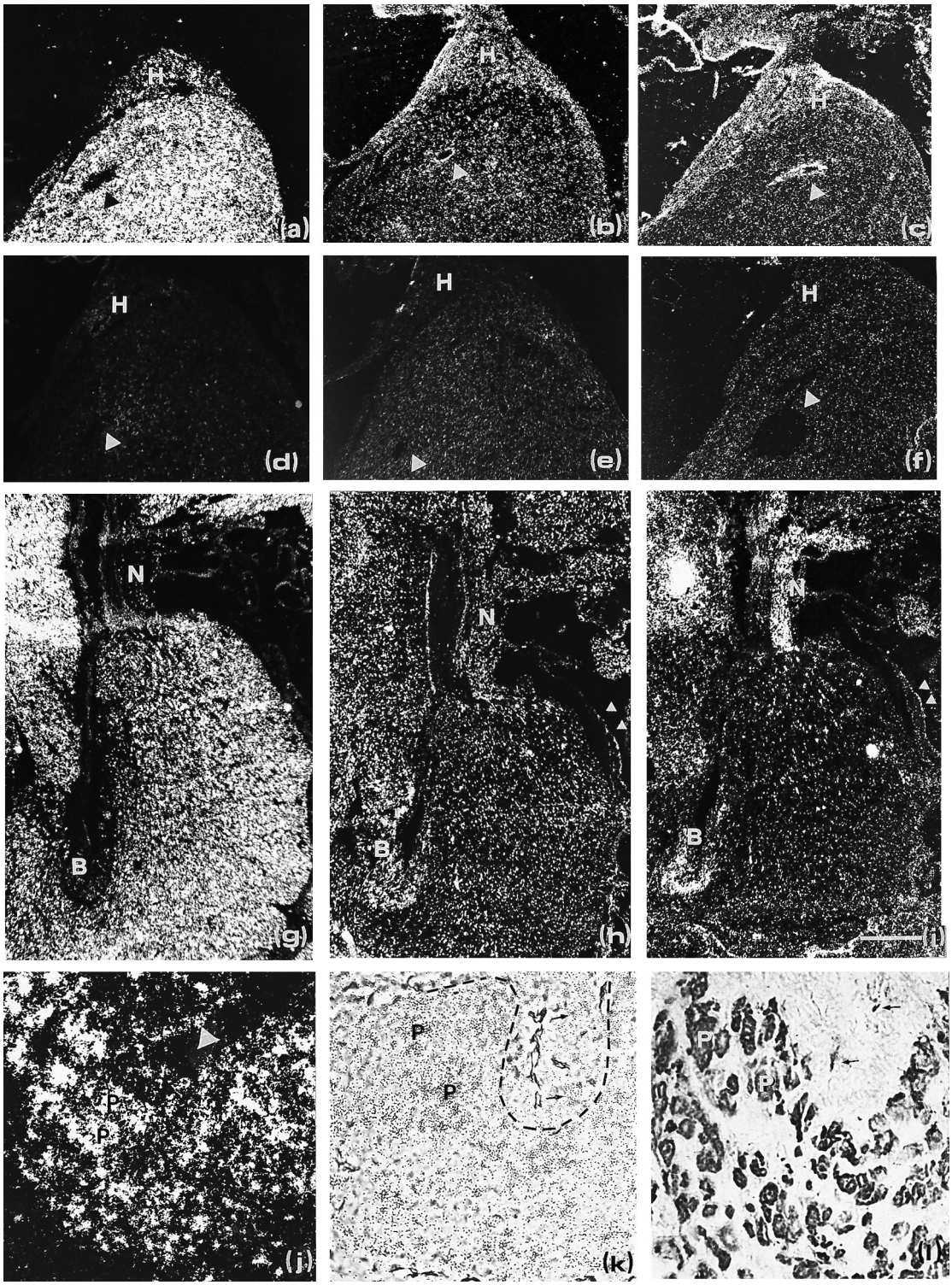


Figure 4 *In situ* hybridization of E18 rat heart. Paraffin sections of E18 heart were hybridized with antisense RyR2 (a), RyR3 (b) and IP_3R (c) probes and with sense RyR2 (d), RyR3 (e) and IP_3R (f) probes. Antisense RyR2 probe hybridizes strongly with fetal heart myocytes, whereas antisense RyR3 cRNA shows weak hybridization signals with both cardiac and mesenchymal cells. Antisense IP_3R probe hybridizes in the heart with atrial myocytes and with ventricular subendocardial myocytes (P, arrowheads), presumably corresponding to Purkinje myocytes. No hybridization signal was detected with sense probes (d)–(f); bright horizontal stripes correspond to section folds. RA: right atrium; RV: right ventricle; LV: left ventricle. Autoradiographic exposure was 15 days for all the probes. Bar: 300 μm

3(a)]. RyR2 transcript is still expressed exclusively in the heart, where it accumulates in every cardiac myocyte [Fig. 3(b)]; whereas RyR3 mRNA is detectable in the neural tube and in other visceral structures, but not in cardiac myocytes or other heart cell types [Fig. 3(c)]. No signal is detected at this stage when the sense probes are used [Fig. 3(d–f)]. On the other hand, IP_3R mRNA accumulation in E12 heart is changed: in addition to myocytes of the atrial myocardium, IP_3R mRNA becomes detectable in a minor population of ventricular myocytes, located on the top of the growing interventricular septum [Fig. 3(g), small arrowheads] and in proximity of the orifice of the truncus arteriosus [Fig. 3(g), large arrowhead], namely in regions where heart conduction system precursors have been identified (Gorza and Vitadello, 1989; Wessels *et al.*, 1992; Vitadello *et al.*, 1996). No hybridization signal is observed when the sense probe is used [Fig. 3(h)]. At E18, RyR 3 mRNA accumulation becomes detectable in both atrial and ventricular myocytes, which also express RyR2 transcript and still differ in IP_3R mRNA accumulation (Fig. 4). No hybridization signal is detected with the sense probes (Fig. 4). This same distribution for each probe is still present in 1-day newborn heart (not shown).

RyR and IP_3R mRNA distribution in the adult and senescent rat heart

Figure 5 illustrates the distribution of RyR2, RyR3 and IP_3R transcripts in working and conduction system myocytes of the adult rat. RyR2 mRNA is detectable in both atrial and ventricular working myocytes, which display strong hybridization signals, whereas heart conduction system myocytes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show apparently weaker hybridization signals [Fig. 5(a)]. RyR3 mRNA shows a complementary distribution: heart conduction system myocytes display stronger hybridization signals than working myocytes [Fig. 5(b)]. IP_3R mRNA accumulation in the adult heart differs from the pattern displayed until birth: no difference in the degree of the hybridization is detectable between atrial and ventricular myocytes (Figs 5 and 6), whereas the strongest hybridization signals are detected in myocytes of the heart conduction system [Fig. 5(c)]. No apparent signal is detectable with sense probes [Fig. 5(d)–(f)]. In addition to cardiac myocytes, RyR3 transcript is detectable in the subendocardial endothelium and in cells of the heart valves, where also positive signals for IP_3R transcript accumulation are occasionally observed (Fig. 5(h) and (i), arrowheads).

The difference in RyR3 transcript accumulation between working myocytes and heart conduction system myocytes appears to change with aging: in 15-month-old rat, the difference in signal intensity of RyR3 cRNA hybridization is no longer detectable in the proximal compartments, such as the atrioventricular node and bundle [Fig. 5(h)], whereas it persists in the distal compartments of the heart conduction system, namely in ventricular Purkinje bundles [Fig. 5 (j)–(k)]. Conversely, aging does not cancel the differences in intensity of hybridization observed with RyR2 and IP_3R probes between conduction system myocytes and ventricular working myocytes [Fig. 5(g) and (i)], which display the same pattern described for the young adult heart.

Atrial and ventricular myocytes of 27-month-old rats do not display any apparent difference in the degree of hybridization with RyR2 and RyR3 probes, which appear similar in intensity to 3-month-old samples (not shown). Conversely, in 27-month-old hearts, IP_3R probe hybridizes with atrial myocytes more intensely than with ventricular working myocytes (Fig. 6), whereas no apparent difference in signal intensity is detected between atrial and ventricular myocardium of young adult rats. The results of *in situ* hybridization analysis are summarized in Table 1.

RNase protection analysis

RNase protection analysis was performed in order to confirm the temporal differences in RyR2 and RyR3 mRNA accumulation observed during prenatal development by *in situ* hybridization analysis. Figure 7(a) shows that a faint band corresponding to protected RyR2 mRNA is detectable in E12 heart sample, whereas a clear band is evident at E16. On the other hand, no apparent protected fragment corresponding to RyR3 mRNA is detectable in E16 hearts, whereas protected bands can be visualized in the 1-day neonate heart [Fig. 7(a)]. Thus, consistent with *in situ* hybridization results, RyR3 mRNA accumulation in the heart appears delayed with respect to RyR2 mRNA. Furthermore, RNase protection analysis reveals an increased accumulation of IP_3R mRNA in senescent atrial myocardium, consistently with what observed using *in situ* hybridization [Fig. 7(b)]. After normalization of each sample to the corresponding β-actin mRNA content, the atrial myocardium of 27 month old rat shows a four fould increase in IP_3R mRNA ($P<0.01$) with respect to 3-month-old atrial myocardium and to both adult and senescent ventricular myocardium ($P<0.005$) (Fig. 8). RNase protection assays performed to demonstrate changes in RyR2 mRNA accumulation during senescence do not reveal any

Figure 5 *In situ* hybridization of adult rat heart. Cryostat sections from 1-month-old rat heart were collected at the level of the atrio-ventricular bundle (H) (a)–(f). Sections from 15-month-old rat heart were taken at the level of the atrio-ventricular node (N) and the left bundle branch (B) (g)–(l). (a) and (g) were hybridized with antisense RyR2 probe; (b),(h),(j)–(k) were hybridized with antisense RyR3 probe and (c) and (i) were hybridized with antisense IP_3R probe. (d), (e) and (f) correspond to hybridization with sense RyR2, RyR3 and IP_3R probe, respectively. Note weak hybridization signals with antisense RyR2 probe in atrioventricular bundle myocytes (H) and in atrioventricular nodal (N) and bundle branch (B) myocytes, whereas strong signals are detected with working myocytes (a and g). Conversely, RyR3 and IP_3R antisense probes show stronger hybridization signals with atrio-ventricular bundle (H) and bundle branch (B) myocytes than with working myocytes (b)–(c) and (h)–(i). Atrio-ventricular node myocytes (N) of 15-month-old rat heart do not differ in intensity of hybridization with antisense RyR3 probe with respect to working myocytes, whereas the same myocyte population hybridizes strongly with antisense IP_3R probe. Both RyR3 and IP_3R antisense probes also label the smooth muscle layer of coronary vessels [arrowhead in (b) and (c)], as well as scattered smooth muscle cells localized at the level of the heart valve leaflets [double arrowheads in (h) and (i)] and in the subendocardium [thin arrow in (k)]. (j) illustrates in more detail the hybridization with RyR3 antisense probe with the left bundle branch: silver grains are localized within Purkinje myocytes (P), as illustrated at higher magnification by bright field micrograph. (l) shows the same region in a serial section stained with α-smooth actin antibodies and indirect immunoperoxidase. Autoradiographic exposure was 15 days for sense and antisense RyR2 probes and 21 days for the other probes. Bar: (a)–(f): 270 μm; (g)–(i): 400 μm; (j): 70 μm; (k)–(l): 35 μm.

Table 1. Summary of the *in situ* hybridization signals observed with antisense cRNA probes for intracellular Ca^{2+}-release channel mRNAs in different regions of the developing, adult and senescent rat hearts.

	Probes			
Stage	*RyR1*	*RyR2*	*RyR3*	*IP_3R*
E10	n.d.	present	n.d.	n.d.
E11	n.d.	present	n.d.	stronger with A myocytes
E12–15	n.d.	present	n.d.	stronger with A and with few V myocytes
E18–1Day	n.d.	present	present	same as E12–15 stage
1–3 Month	n.d.	weaker with CS	stronger with CS	no difference between A and V, stronger with CS
15 Month	n.d.	weaker with CS	stronger with P	no difference between A and V, stronger with CS
27 Month	n.d.	weaker with CS	stronger with P	stronger with A than with V, stronger with CS

n.d., not detectable; A, atrial myocardium; V, ventricular myocardium; CS, conduction system myocytes; P, Purkinje myocytes.

difference in the amount of this transcript with respect to the adult heart (not shown).

Discussion

The present study investigates the composition of intracellular Ca^{2+}-release channels of working and specialized myocardium during rat development and aging. Our results show that: (1) skeletal RyR1 mRNA is never expressed in the heart; (2) working and conduction myocytes show a distinct composition in intracellular Ca^{2+}-release channel mRNAs which appears to be temporally and regionally regulated; (3) atrial myocytes of the senescent heart show an increased accumulation of IP_3R mRNA.

The minor functional role played by intracellular Ca^{2+}-release channels in excitation–contraction coupling during heart development has been explained with both the different time of appearance of sarcoplasmic reticulum channels, pumps and regulatory proteins and the late development of T-tubules (Wibo *et al.*, 1991; Arai *et al.*, 1992; Brillantes *et al.*, 1994; Anger *et al.*, 1994; Mahony and Jones, 1986). In the mammalian heart, sensitivity of cardiac myocytes to ryanodine (Tanaka and Shigenobu, 1989), together with the accumulation of RyR 2 mRNA (Arai *et al.*, 1992; Brillantes *et al.*, 1994), was demonstrated at late stages of fetal development. Our study shows that RyR2 mRNA accumulates in mammalian heart from the early stages of development, suggesting an important role for this gene also during heart development. Interestingly, it has been demonstrated that ryanodine reduces heart contractility in the chicken embryo already at day 5 *in ovo* (Dutro *et al.*, 1992), which corresponds to E13 in the rat (Sissman, 1970).

Furthermore, only RyR2 mRNA, among RyR transcripts, is apparently detectable in the embryonic heart. Contrary to other skeletal muscle genes, such as skeletal α-actin, slow skeletal troponin I and fast myosin alkali light chain, which are transiently upregulated in embryonic cardiac myocytes (Carrier *et al.*, 1992; Gorza *et al.*, 1993a, Kelly *et al.*, 1995), RyR1 mRNA is never detected in the embryonic heart, despite of its accumulation in skeletal muscle cells early in development. Besides, *in situ* hybridization and RNase protection analyses indicate that RyR3 mRNA accumulation in the heart occurs only at late stages of fetal development, suggesting that the expression of this intracellular Ca^{2+}-release channel type might be required as sarcoplasmic reticulum maturation proceeds.

Our results point to an additional important finding, namely the early and regionally restricted accumulation of IP_3R mRNA. At E11 of development it appears to be restricted to the atrial myocardium, whereas at E13 also becomes detectable in a subset of ventricular myocytes, which presumably correspond to conduction system myocytes on the basis of their distribution (Gorza and Vitadello, 1989; Wessels *et al.*, 1992). The differential accumulation of type 1 IP_3R mRNA between atrial and ventricular working myocardium observed during prenatal development is no longer detectable in the adult heart, whereas it reappears in the 27-month-old rat heart. RNase protection confirms that the accumulation of IP_3R mRNA in the senescent atrial myocardium is about four times more abundant than in the ventricle. Differences in mRNA composition between atrium and ventricle have been reported for other cardiac sarcoplasmic reticulum transcripts, such as Ca^{2+}-ATPase (SERCA2) and phospholamban (Koss *et al.*, 1995; Moorman *et al.*, 1995): during embryonic development and in adult rat and mouse hearts, SERCA2 mRNA is more

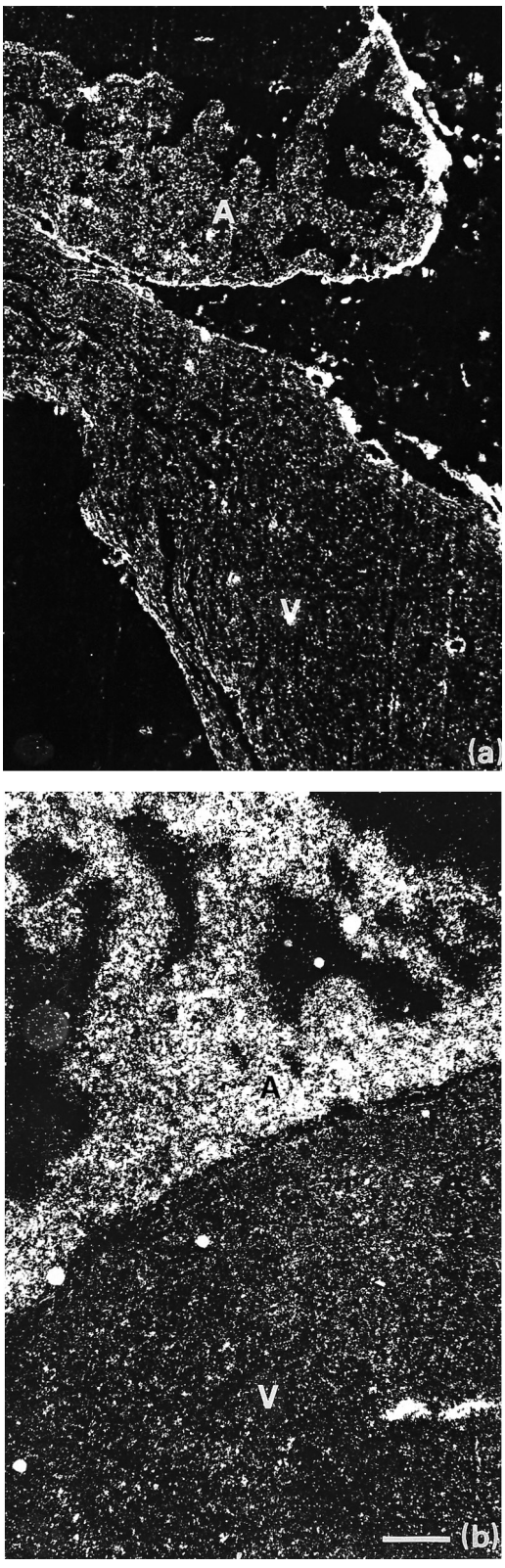

Figure 6 *In situ* hybridization of senescent rat heart. Cryosections from adult (a) and 27-month-old rat heart (b) were hybridized with antisense IP_3R cRNA. Note higher hybridization signals in the auricular appendix of the atrial myocardium (A) of the senescent rat. Autoradiographic exposure was 15 days. Bar: 200 μm.

abundant in the atrium than in the ventricular myocardium, whereas phospholamban mRNA is two times more concentrated in the ventricle.

During senescence, the SERCA2 gene is downregulated further in ventricular working myocytes, mimicking what occurs in the presence of cardiac hypertrophy (Lompré *et al.*, 1991). Conversely, IP_3R mRNA accumulation is not apparently changed in the presence of mild compensatory hypertrophy (L. Gorza, unpublished observations), although a twofold increase in the amount of IP_3R mRNA has been described in the ventricular myocardium in the presence of heart failure (Go *et al.*, 1995). In the aged myocardium, increased accumulation of IP_3R mRNA is detectable only in atrial working myocytes, which display a similar distribution during prenatal development. Such evidence allows us to speculate that the expression of this type of intracellular Ca^{2+}-release channel in cardiac myocytes might play a pivotal role in the regulation of chronotropism. Indeed, bursts of beating activity have been registered in young avian embryos, following exposure to ryanodine and thus after cessation of heart beating (Dutro *et al.*, 1993) or following carbachol exposure and inositol phosphate metabolism activation (Muwagba *et al.*, 1992). Besides, Holter registration performed in aged rats demonstrated almost no sinus rhythm and the presence of spontaneous supraventricular arrhythmias as most frequent abnormalities (Carré *et al.*, 1992).

The possible recruitment of IP_3R in myocytes of the heart conduction system, which contain high concentrations of this intracellular Ca^{2+}-release channel, has been proposed as a mechanism involved in changes in automaticity occurring after hormonal stimulation (Gorza *et al.*, 1993b). The present work indeed shows that IP_3R mRNA accumulates in ventricular conduction system myocytes from early embryonic development and also persists in the senescent hearts. Besides, conduction system myocytes display an intriguing composition in RyR transcripts: they apparently do not contain RyR1 mRNA and show a complementary pattern of hybridization with both RyR2 and RyR3 probes. Very low levels of hybridization for RyR2 mRNA are detected in conduction system myocytes with respect to working myocytes; whereas higher hybridization signals than working myocytes are observed in these myocytes with RyR3 probes, suggesting a composition in intracellular Ca^{2+}-release channel mRNAs distinct from working myocytes. Indeed, the presence of RyR mRNAs in conduction myocytes is consistent with the immunological demonstration of the channel(s)

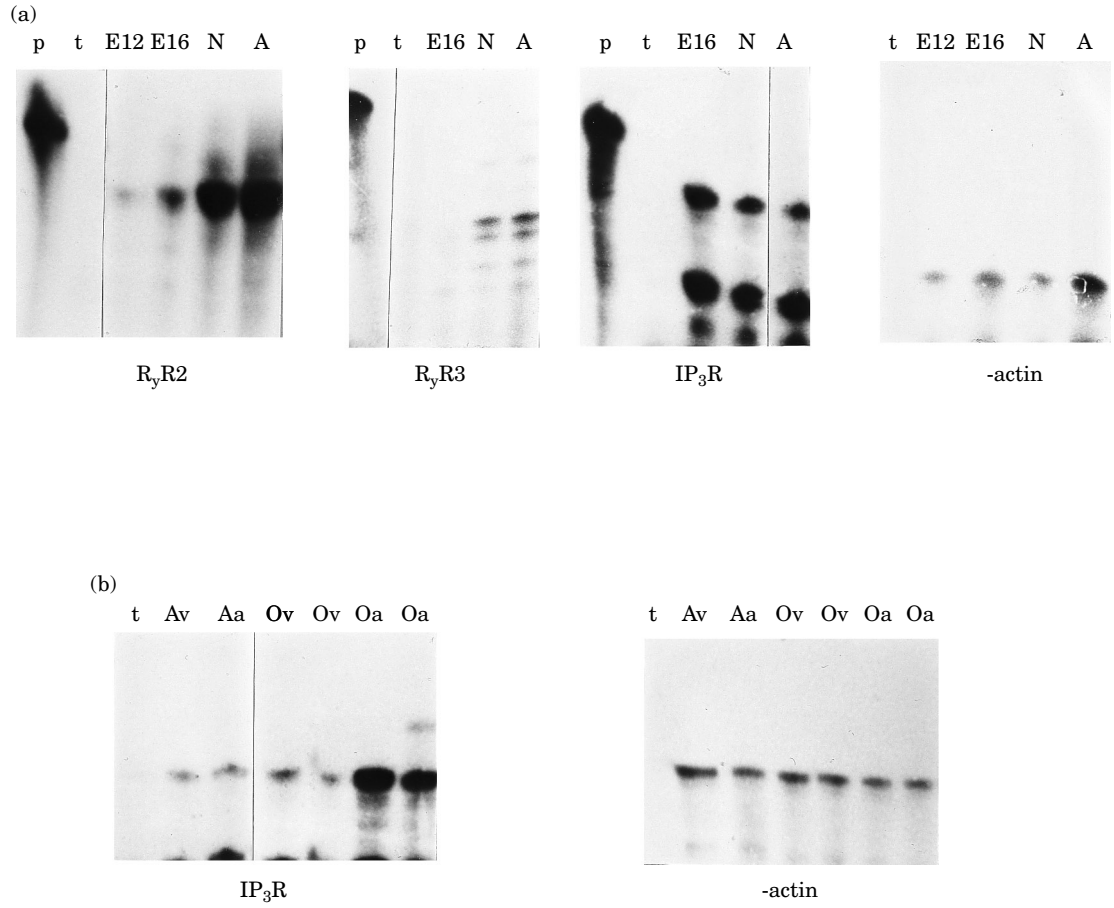

Figure 7 RNase protection assays. (a) shows the temporal difference of RyR2, RyR3 and IP_3R mRNAs accumulation during development. RyR2 probe was hybridized with 25 μg of total RNA from yeast tRNA (t), E12 whole rat heart (E12), E16 whole rat heart (E16), 1-day whole neonate heart (N), and 10 μg of adult ventricular myocardium (H). RyR3 probe was hybridized with 50 μg of total RNA from the same samples; IP_3R probe and β-actin probe were hybridized with 20 and 1 μg of total RNA from each sample, respectively. p: probe. A 120-nt protected fragment is detected with RyR2 probe in E16 heart, whereas a faint signal is observed with E12 heart. RyR3 probe shows two protected fragments of about 280 and 250 nt only in neonatal and adult hearts. (b) illustrates differences in IP_3R mRNA accumulation in 27-month-old atrial myocardium. Av and Ov correspond to adult and old ventricular myocardium, respectively; Aa and Oa correspond to adult and old atrial myocardium, respectively. IP_3R probe and β-actin probe were hybridized with 20 and 1 μg of total RNA from each sample, respectively.

(Jorgensen *et al.*, 1993). However, the functional role of RyR3 in conduction system myocytes remains to be elucidated. It has been shown that RyR3 is not activated by caffeine, but requires Ca^{2+} released after subliminal activation of IP_3 sensitive Ca^{2+} stores (Giannini *et al.*, 1992). Studies performed on skinned preparations of dog Purkinje myocytes have shown that an higher free Ca^{2+} concentrationis required to induce Ca^{2+} release from the sarcoplasmic reticulum of these myocytes (Fabiato, 1982). Thus, it is possible that the abundancy of IP_3R and RyR3 in conduction system myocytes might be functionally related.

In summary, intracellular Ca^{2+}-release channel genes vary in transcript accumulation in cardiac myocytes among heart regions, during development and aging and between working and conduction system myocytes; such an heterogeneity might indeed contribute to the establishment of important differences in the control of Ca^{2+}homeostasis among cardiac myocytes.

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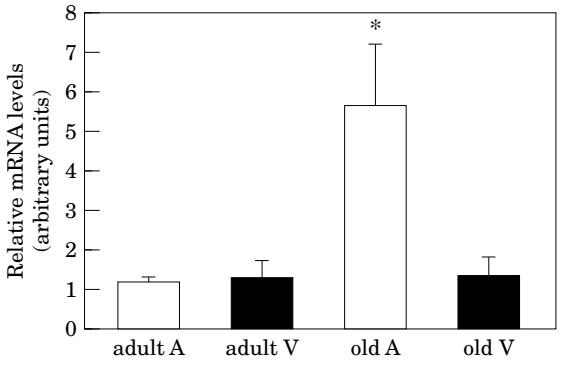

Figure 8 Relative normalized IP_3R mRNA levels in adult ($n=3$) and 27-month-old atrial and ventricular myocardium ($n=4$). Densitometry was performed on RNase protected fragments as described in Materials and Methods and values were normalized to β-actin and expressed in arbitrary units. * indicates significant increase of IP_3R mRNA levels in the old atrial myocardium compared with the adult atrial myocardium ($P<0.01$), and the adult and senescent ventricles ($P<0.005$).

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