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Presence of Functional Sarcoplasmic Reticulum in the Developing Heart and Its Confinement to Chamber Myocardium

Antoon F. M. Moorman,*,[1] Cees A. Schumacher,* Piet A. J. de Boer,* Jaco Hagoort,* Karel Bezstarosti,† Maurice J. B. van den Hoff,* Gerry T. M. Wagenaar,* Jos M. J. Lamers,† Frank Wuytack,‡ Vincent M. Christoffels,* and Jan W. T. Fiolet*

**Experimental & Molecular Cardiology Group, Cardiovascular Research Institute Amsterdam, Academic Medical Center, 1105 AZ Amsterdam, The Netherlands; †Department of Biochemistry, Cardiovascular Research Institute, COEUR, Erasmus University Rotterdam, Rotterdam, The Netherlands; and ‡Department of Physiology, University of Leuven, Louvain, Belgium*

During development fast-contracting atrial and ventricular chambers develop from a peristaltic-contracting heart tube. This study addresses the question of whether chamber formation is paralleled by a matching expression of the sarcoplasmic reticulum (SR) Ca^{2+} pump. We studied indo-1 Ca^{2+} transients elicited by field stimulation of linear heart tube stages and of explants from atria and outflow tracts of the prototypical preseptational E13 rat heart. Ca^{2+} transients of H/H 11+ chicken hearts, which constitute the prototypic linear heart tube stage, were sensitive to verapamil only, indicating a minor contribution of Ca^{2+}-triggered SR Ca^{2+} release. Outflow tract transients displayed sensitivity to the inhibitors similar to that of the linear heart tube stages. Atrial Ca^{2+} transients disappeared upon addition of ryanodine, tetracaine, or verapamil, indicating the presence of Ca^{2+}-triggered SR Ca^{2+} release. Quantitative radioactive *in situ* hybridization on sections of E13 rat hearts showed ~10-fold higher SERCA2a mRNA levels in the atria compared to nonmyocardial tissue and ~5-fold higher expression in compact ventricular myocardium. The myocardium of atrioventricular canal, outflow tract, inner curvature, and ventricular trabecules displayed weak expression. Immunohistochemistry on sections of rat and human embryos showed a similar pattern. The significance of these findings is threefold. (i) A functional SR is present long before birth. (ii) SR development is concomitant with cardiac chamber development, explaining regional differences in cardiac function. (iii) The pattern of SERCA2a expression underscores a manner of chamber development by differentiation at the outer curvature, rather than by segmentation of the linear heart tube.

Key Words: **cardiac development; sarco(endo)plasmic reticulum Ca^{2+} pump; chicken; rat; human.**

INTRODUCTION

The development of the heartbeat and the ensuing development of excitation–contraction coupling are fundamental to the function of the early primary heart tube. With subsequent chamber formation the low-capacity, peristaltic-contracting primary heart tube is transformed into a high-capacity, fast-contracting chamber pump. Zones of slow conduction persist at the atrioventricular canal and outflow tract. They are essential for embryonic cardiac function. Owing to their prolonged contraction they display a sphincter-like function, allowing them to substitute the adult type of one-way valves in a heart in which atrioventricular and semilunar valves have not yet developed (Boucek *et al.*, 1959; Paff *et al.*, 1962; Lieberman *et al.*, 1965; Arguello *et al.*, 1986; de Jong *et al.*, 1992; Moorman *et al.*, 1998).

The transformation of the linear primary heart tube into a four-chambered heart has remained an esoteric topic. Part

[1] To whom correspondence should be addressed at the Department of Anatomy & Embryology, AMC, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands. Fax: 31 20 6976177. E-mail: a.f.moorman@amc.uva.nl.

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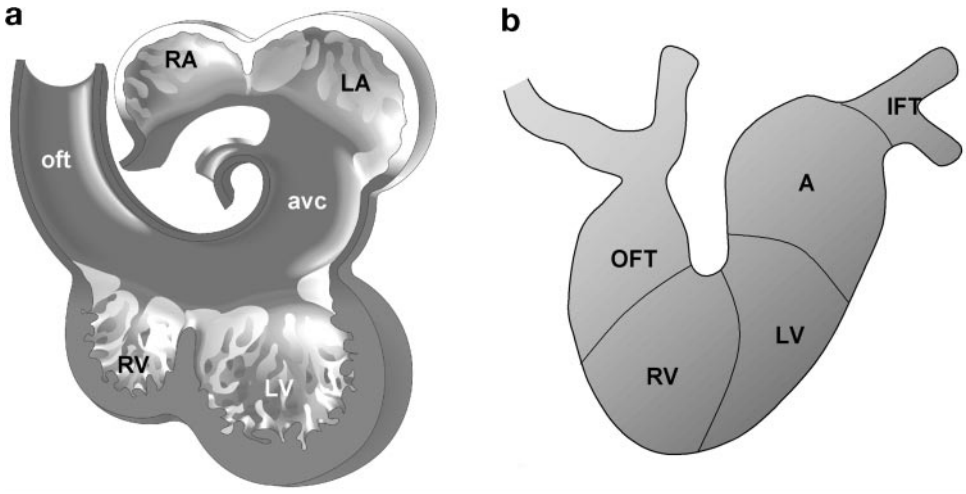


FIG. 1. Concepts of cardiac chamber development. (A) Ballooning model (de Jong *et al.*, 1997; Moorman *et al.*, 1998; O'Rahilly *et al.*, 1987); (B) segmental model. In the ballooning model it is supposed that the chambers develop by differentiation and ballooning at the outer curvature from the primary heart tube, which remains recognizable as the smooth-walled part of the myocardium at the inner curvature. It is based on the morphology of the developing heart. The cartoon should be considered a template, designed to facilitate the understanding of the transition of a tubular heart into a four-chambered heart. It should not be considered a reconstruction of an existing stage. In the conventional segmental model, the developing compartments are considered to be part of the whole tube and thus extend at the inner curvature. L/R A, left/right atrium; avc, atrioventricular canal; L/R V, left/right ventricle; oft, outflow tract.

of the confusion may originate from the implicit assumption that the linear heart tube consists of a linear array of segments, which are difficult to arrange in the parallel configuration that is characteristic of the formed heart (Fig. 1). Morphological evidence is, however, in favor of chamber formation by a process of local differentiation and proliferation (ballooning) (Fig. 1) of trabeculated working myocardium at the outer curvature of the looped heart, leaving smooth-walled myocardium at the inner curvature, atrioventricular canal, and outflow tract (Davis, 1927; van Mierop, 1969; de Jong *et al.*, 1997). The adult configuration is in essence being achieved by a relative rightward expansion of the primary myocardium of the atrioventricular canal, to directly connect the right atrium and the right ventricle (Wessels *et al.*, 1992, 1996).

Crucial to the development of fast-contracting chambers from the peristaltic-contracting heart tube is the proper control of the concentration of free intracellular Ca^{2+} ions, which requires a regulated movement of Ca^{2+} ions across both the sarcolemmal and the sarcoplasmic membranes (Bers, 1991). Cardiac relaxation is achieved by the clearance of Ca^{2+} from the cytosol, basically by the action of the sarcolemmal Na^{+}–Ca^{2+} exchanger (NCX) and the sarco(endo)plasmic Ca^{2+}-ATPase (SERCA2) (Bers, 1991), the Ca^{2+} affinity of which is lowered by the nonphosphorylated form of phospholamban (James *et al.*, 1989; Koss *et al.*, 1997). In the heart, primarily the NCX1 (Nicoll *et al.*, 1990) and the SERCA2a isoforms (Brandl *et al.*, 1987; Lompré *et al.*, 1989; Komuro *et al.*, 1989; Anger *et al.*, 1994) are expressed. Phospholamban is encoded by a single gene and is expressed in cardiac, slow-twitch, and smooth muscle cells (Fujii *et al.*, 1988; Eggermont *et al.*, 1990). The relative contribution of the sarcolemmal and sarcoplasmic system is species-specific and depends on the developmental stage and the physiological/pathological state of the heart (Bers, 1991; Koban *et al.*, 1998). As yet no differences in NCX expression have been observed between the distinct developing cardiac components at either the mRNA or the protein level (Koban *et al.*, 1998). These observations do not support a role of the sarcolemmal NCX in the establishment of functionally distinct cardiac compartments. On the other hand the sarcoplasmic SERCA2a mRNA displays anteroposterior polarity in the developing heart and subsequently compartmentalized expression, suggesting a correlation of the encoded Ca^{2+} pump with the development of functionally distinct compartments (Moorman *et al.*, 1995). The complementary pattern of expression of the mRNA encoding phospholamban (Moorman *et al.*, 1995) is in line with this concept.

Although we have reported previously regionalized expression of SERCA2a mRNA (Moorman *et al.*, 1995), lack of quantification and of three-dimensional analyses has prevented the display of potential relationships with the way in which cardiac chambers develop. The segmental model predicts a segmental SERCA2a expression at both the inner and the outer curvature. The ballooning model, i.e., chamber development at the outer curvature (Fig. 1), predicts a "primary" transcriptional program in the myocardium at the inner curvature, atrioventricular canal, and outflow tract, which is interrupted at the outer curvature by the

transcriptional program of the working myocardium of the developing chambers. We observe this latter pattern for both SERCA2a mRNA and protein expression. In agreement, a strong resemblance is observed in the drug sensitivity pattern of Ca^{2+} transients of early primary heart tubes and of outflow tracts, whereas the development of sarcoplasmic reticulum (SR) function is concomitant with cardiac chamber development. These observations complement the elegant observations, made by Boucek and colleagues about half a century ago, concerning the manner of contraction of the different compartments of the embryonic heart (Boucek *et al.,* 1959; Paff *et al.,* 1962). They observed sluggish contractions in the outflow tract and rapid contractions in the atria and hence considered the outflow tract the least differentiated part of the embryonic heart.

MATERIALS AND METHODS

Animals, Cardiac Tissue Sources, and Isolation

Rat embryos. Adult Wistar rats, obtained from the Broekman Institute (Someren, The Netherlands), were kept under a controlled dark–light cycle (light 12 h). Animal welfare was in accordance with institutional guidelines of the University of Amsterdam. Embryonic age was calculated from dated matings and postnatal age from time of birth. Embryos were harvested by cesarean section of pregnant rats after CO_2/O_2 anesthesia and processed for immunohistochemistry and *in situ* hybridization. For the Ca^{2+} uptake measurements embryonic day (E) 13 rat embryos were kept in ice-cold 0.9% NaCl solution during isolation of the hearts. Ventricles were isolated using a dissection microscope and collected in a tube at −40°C immediately after isolation. The ventricles of the hearts of the mothers served as adult myocardial controls and comprised the left ventricular free wall and ventricular septum. After isolation all tissues were frozen in liquid nitrogen and stored at −80°C until use.

Human embryos. Human embryos were obtained after termination of pregnancy at the Academic Medical Center of Amsterdam and at the Postgraduate Medical School in Budapest. The respective local medical-ethical committees approved the studies.

Chicken embryos. Fertilized White Leghorn eggs were obtained from a local hatchery (Fa Drost BV, Loosdrecht, The Netherlands) and incubated for appropriate periods in a moist atmosphere at 37°C. Embryos were staged according to Hamburger and Hamilton (1951).

Cytoplasmic Ca^{2+} Transients

Electrically stimulated cytoplasmic Ca^{2+} transients were measured in freshly isolated indo-1-loaded atrial and outflow tract preparations of E13 rat (n = 23) and H/H 25+ (n = 12) chicken hearts as well as in the prototypical linear heart tube stage of H/H 11+ chickens (n = 9).

Microscopically dissected multicellular preparations were loaded with 5 μmol/L indo-1/AM at 37°C for 30 min in a continuously aerated, Hepes-buffered (pH 7.4) and neonatal calf serum supplemented (30% v/v; Gibco Life Technologies) Tyrode's solution. This solution had the following ionic composition (in mmol/L): [Na^+] 155, [K^+] 4.7, [Ca^{2+}] 2.6, [Mg^{2+}] 2.0, [Cl^-] 149, [phosphate] 1.4, [HCO_3^-] 4.3, and [Hepes] 17. After loading, the preparations were washed twice with fresh Tyrode's solution without calf serum to remove residual extracellular indo-1/AM and kept for another 10 min to allow for complete deesterification of intracellular indo-1/AM.

The indo-1-loaded atrium or outflow tract preparations were attached to poly-L-lysine-treated coverslips. Subsequently they were placed on an inverted fluorescence microscope with quartz optics between two platinum field stimulation electrodes and positioned parallel at a distance of 8 mm (40 V/cm) in a temperature-controlled (37°C) perfusion chamber (volume 30 μl). Indo-1 fluorescence was excited at 340 nm and measured in dual emission mode at 410 and 516 nm with a 40× objective (Fiolet *et al.,* 1995). Fluorescence signals were sampled at 1 kHz. Data are expressed as ratio values (410 nm/516 nm) in arbitrary units after correction of signals for background fluorescence (less than 10% of overall signals) measured in unlabeled preparations.

Each atrium and outflow tract preparation was conditioned by 5 min of stimulation at a cycle length of 500 and 1000 ms, respectively, before recording of control Ca^{2+} transients. Subsequently, either sarcolemmal L-type Ca^{2+} channels were inhibited by 25 μmol/L verapamil or SR Ca^{2+}-release channels were inhibited by 100 μmol/L tetracaine or 50 μmol/L ryanodine, or 100 μmol/L thapsigargin was applied to inhibit SR Ca^{2+} uptake. In some embryonic chicken preparations 100 μmol/L cyclopiazonic acid (CPA) was applied as an alternative to thapsigargin or 5 mmol/L Ni^{2+} to inhibit the Na^+/Ca^{2+} exchanger. Fresh preparations were used for each individual drug application. The preparations with the numbers in parentheses were as follows. E13 rat (n = 23): atria (n = 12) verapamil (2), tetracaine (3), ryanodine (3), thapsigargin (4); outflow tracts (n = 11) verapamil (2), tetracaine (4), ryanodine (3), thapsigargin (2). H/H 25+ chicken (n = 20): atria (n = 13) verapamil (1), tetracaine (4), thapsigargin (3), CPA (3), Ni^{2+} (2); outflow tracts (n = 7) tetracaine (4), CPA (1), Ni^{2+} (2); ventricles (n = 5) verapamil (1), ryanodine (1), thapsigargin (3). H/H 11+ chicken primary heart tube (n = 11): verapamil (3), tetracaine (3), ryanodine (3), Ni^{2+} (2).

Sarcoplasmic Reticulum Ca^{2+} Uptake

Tissue samples of adult rat ventricles and embryonic rat ventricles from the same litter were pooled to obtain sufficient material and stored at −80°C before use. Oxalate-supported Ca^{2+} uptake was measured at 37°C after homogenization in an ice-cold buffer containing (mmol/L) imidazole 10, sucrose 300, $MgSO_4$ 5, and $Na_2S_2O_5$ 10 (pH 7.0). Reaction mixtures (pH 7.0) contained (mmol/L) KCl 100, imidazole 20, $MgCl_2$ 10, ATP 5, $CaCl_2$ 0.2 (= 118 μm/L), 0.2 mCi $^{45}Ca^{2+}$/ml, and 25–100 mg homogenate protein/ml (Lamers *et al.,* 1993; Feher *et al.,* 1990). In addition, NaN_3 10, ruthenium red 0.01, and potassium oxalate 10 were included, to inhibit mitochondrial (Solaro *et al.,* 1974) and sarcolemmal Ca^{2+} transport, to inhibit the SR calcium-release channel, and to prevent accumulation of free Ca^{2+} in the SR, respectively. Blank reactions were performed in the absence of ATP. SR specificity of Ca^{2+} uptake was tested by addition of 2 μmol/L thapsigargin. Reaction samples were filtered through 0.45-μm filters (Millipore, Bedford, MA) 1, 2, and 4 min after initiation of the reactions. The $^{45}Ca^{2+}$ content of the SR vesicles remaining on the filters was estimated by liquid scintillation counting. The rate of Ca^{2+} uptake (nmol/mg protein/min) was linear up to about 5 min. Differences between means were analyzed with Student's t test. P values less than 0.05 were considered significant.

Tissue Processing for in Situ Hybridization and Immunohistochemistry

Embryonic and fetal tissues were fixed for 4–5 h or overnight in a cold, freshly prepared solution of 4% formaldehyde in PBS for *in situ* hybridization or in a cold mixture of methanol, acetone, acetic acid, and water (35:35:5:25) for immunohistochemistry. E10 embryos were studied *in utero*. After fixation, specimens were dehydrated in a graded alcohol series at room temperature and were left overnight in 1-butanol or chloroform. Fixation and dehydration took place on a shaking device. Subsequently the specimens were embedded in paraplast, sectioned (7 μm thick), and mounted onto 3-aminopropyltriethoxysilane-coated glasses for *in situ* hybridization or onto poly-L-lysine-coated glass slides for immunohistochemistry.

Quantitative in Situ Hybridization

Pretreatment, hybridization, processing for autoradiography of sections, and preparation of probes were performed as described elsewhere (Jonker *et al.*, 1997; Moorman *et al.*, 1999). ^{35}S-labeled cRNA probes to SERCA2a mRNA were made by *in vitro* transcription, using linearized pBluescript containing the 2200-bp *Eco*RI 3′ end fragment of the rat cardiac SERCA2a cDNA generated from clone pRH39 (Lompré *et al.*, 1989; Moorman *et al.*, 1995). Hybridization with sense probe and RNase treatment of the sections did not yield significant signal. In this procedure the autoradiographic signal (optical density) is linearly related to the radioactivity present, for a fixed time of exposure, and time of development of the photographic emulsion, as assessed in calibrated microscopic samples (Jonker *et al.*, 1997; Moorman *et al.*, 1999).

Image measurements, processing, and alignment was done using NIH Image (version 1.61, NIH, http://rsb.info.hih.gov/nih-image). After manual alignment of the sections, unwanted background outside the volume of interest was removed. To get a smooth and clear volume, SCIL_Image (version 1.3, TNO-TPD, http://www.tpd.tno.nl) was used for 3D image processing. The original signal values were superimposed in the smoothed volume, from which volume-rendered 3D reconstructions were made using T3D (version 1.1.3, Fortner Research, http://www.fortner.com).

Immunohistochemistry

After deparaffination sections were incubated for 30 min in H_2O_2 diluted to 3% (v/v) in PBS (pH 7) to block endogenous peroxidase activity. Subsequently, they were incubated in a buffer containing 10 mmol/L Tris–HCl, 5 mmol/L EDTA, 150 mmol/L NaCl, 0.25% (w/v) gelatin, and 0.05% (v/v) Tween 20 (final pH 8.0) to reduce background staining. Next, sections were incubated with a polyclonal antiserum against porcine SERCA2a (Eggermont *et al.*, 1990) or with a monoclonal antibody against canine phospholamban (Phosphoprotein Research, Bardsea, UK) which recognizes phospholamban irrespective of its phosphorylation state (Drago *et al.*, 1994). Antigen–antibody binding was visualized by the indirect unconjugated peroxidase–antiperoxidase technique and diaminobenzidine as substrate or by alkaline phosphatase-coupled secondary antibodies and NBT/BCIP (Boehringer Mannheim; No. 1681451) as substrate.

RESULTS

Ca^{2+} Transients

For the prototypical linear heart tube, chicken H/H 11+ embryonic hearts were used, because a comparable rat stage is hard to isolate. In rat, cardiac looping and chamber formation start while the primary cardiac tube is still being made. As the prototype for the preseptational embryonic heart, E13 rat and chicken H/H 25+ (data not shown) hearts were used to measure cytoplasmic Ca^{2+} transients. We decided to compare Ca^{2+} transients in explants of rat atrial (working) myocardium with those of outflow tract (primary) myocardium, as these compartments can be unambiguously isolated free from contaminating ventricular or atrioventricular canal myocardium.

Figure 2 shows representative examples of the effect of blockade of either sarcolemmal or SR calcium transport systems on Ca^{2+} transients. Ca^{2+} transients were measured in multicellular preparations of the atrium (left side) and the outflow tract (middle) of E13 rat hearts and of the primary heart tube in stage H/H 11+ chicken hearts (right). Verapamil was used to block the sarcolemmal L-type Ca^{2+} channel. Tetracaine and ryanodine were used to inhibit the SR Ca^{2+}-release channel. Atrial preparations could be stimulated up to 3 Hz. The Ca^{2+} transients were characterized by rapid upstroke and relaxation rates. The outflow tract and primary heart tube preparations failed to follow high stimulation rates and in most instances the maximal rate was about 1 Hz. The Ca^{2+} transients in the outflow tract and primary heart tube showed some delay with respect to the stimulus, were substantially slower than the atrial transients, and sometimes were kinetically distorted due to the slowly propagating calcium wave in the microscopic field (see for instance the middle bottom of Fig. 2). Cytoplasmic Ca^{2+} transients were completely blocked by verapamil (upper traces) in the atrium, the outflow tract, and primary heart tube. Specific inhibition of SR calcium release channels by either tetracaine (middle) or ryanodine (bottom) abolished the Ca^{2+} transients in the atrium, but failed to do so in the outflow tract and primary heart tube. This differential drug sensitivity was consistently observed in all atrium and outflow tract preparations of E13 rat (and H/H 25+ chicken, not shown) and in all primary heart tube preparations of H/H 11+ chicken. Thus, a strong resemblance is observed in the drug sensitivity pattern of Ca^{2+} transients of outflow tract and of the primary heart tube.

Application of the cardiac SR Ca^{2+} pump specific inhibitor thapsigargin (100 μmol/L) had no effect on Ca^{2+} transients measured in outflow tract preparations. In atrial preparations Ca^{2+} transients were only partly and variably reduced by thapsigargin to about 60% on the average; 100% inhibition was never observed (data not shown). Similar results were obtained using the alternative SR Ca^{2+} pump inhibitor CPA (100 μmol/L). Limited uptake, described for both drugs in intact cardiac preparations (Baudet *et al.*, 1993), might provide an explanation for this variability and

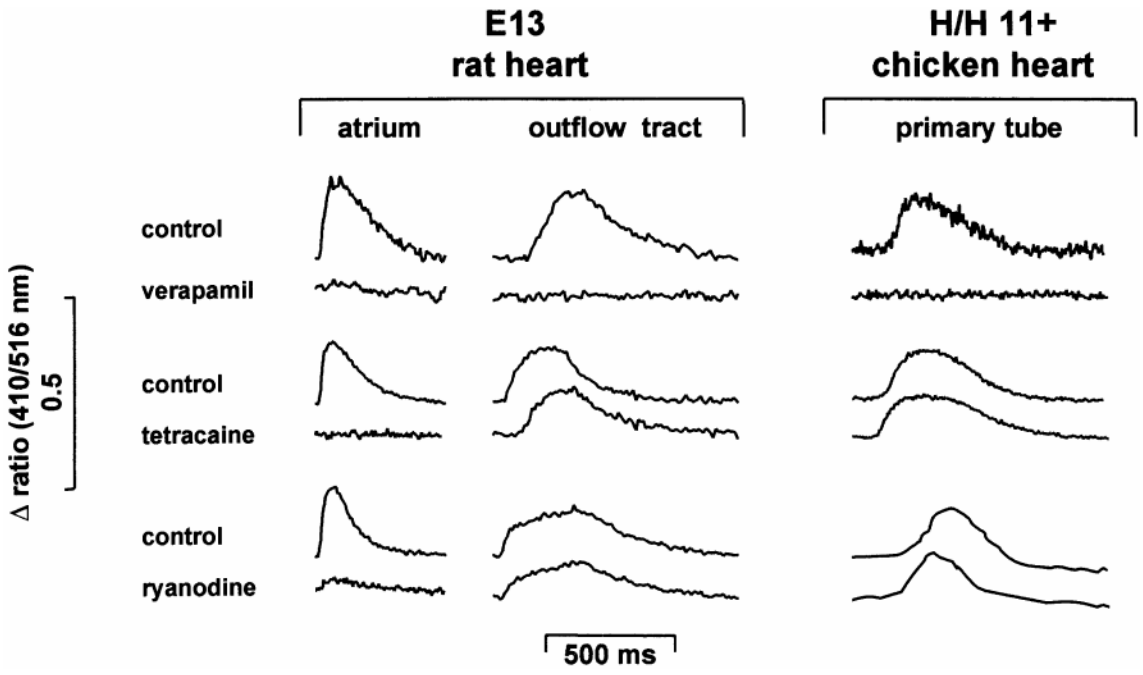


FIG. 2. Calcium transients in multicellular preparations of atria (left) and outflow tracts (middle) of E13 rat embryo hearts and primary heart tubes (right) of H/H 11+ chicken hearts. Preparations were field-stimulated (40 V/cm) at a cycle lengths of 500, 1000, and 1000 ms, respectively. A fresh preparation was used for each individual drug tested. Control transients were recorded after 5 min of conditioning stimulation. Subsequently, transients were measured again 1 min after application of either 25 μmol/L verapamil (upper tracings), 100 μmol/L tetracaine (middle tracings), or 50 μmol/L ryanodine (lower tracings).

incompleteness of inhibition. In support of this explanation is our observation that Ca^{2+} uptake into SR vesicles obtained from homogenates of adult and E13 ventricles were much more sensitive to thapsigargin (see below).

In some H/H 25+ atrial and outflow tract preparations, and in H/H 11+ primary heart tubes, 5 mmol/L Ni^{2+} was applied to inhibit Na^+/Ca^{2+} exchange (data not shown). In the presence of Ni^{2+} calcium transients were unaffected in atria. In outflow tract and primary heart tube preparations Ni^{2+} (like verapamil) entirely suppressed calcium transients. Complete inhibition was achieved within 2 min in outflow tracts but instantaneously in the primary heart tube. This suggests a minor contribution of the Na^+/Ca^{2+} exchanger in atria to the generation of calcium transients, but crucial participation (in concert with the L-type Ca^{2+}-channel) in outflow tract and primary heart tube. However, some caution on conclusions is justified, because it is well known that Ni^{2+} is not entirely specific for the Na^+/Ca^{2+} exchanger, but also affects the L-type Ca^{2+} channel current to some extent.

Oxalate-Supported Ca^{2+} Uptake

To further support the presence of a functional SR in embryonic hearts we compared, as a measure of SR function, the rate of ATP-dependent oxalate-supported Ca^{2+} uptake at saturating Ca^{2+} concentrations in homogenates prepared from frozen E13 ventricles and in similarly prepared homogenates from adult ventricles. Limitation of the embryonic material forced us to use ventricular tissue only, as the ventricles comprise about 90% of the cardiac mass. We measured in adult ventricular homogenates a Ca^{2+} uptake rate of 11.5 nmol $\cdot$ mg^{-1} protein $\cdot$ min^{-1} (n = 10; *SD* 2.4), which can be ~70% blocked by thapsigargin, a specific inhibitor of the cardiac SR Ca^{2+} pump. In the embryonic ventricular homogenates Ca^{2+} uptake rates amount to 5.2 nmol $\cdot$ mg^{-1} protein $\cdot$ min^{-1} (n = 5; *SD* 0.5), which is about half of the adult activity on a protein tissue base. This activity could be blocked effectively to ~80% by thapsigargin. In line with the above-mentioned data, these results demonstrate that embryonic ventricular cells have a functional sarcoplasmic reticulum.

Quantitative Analysis of SERCA2a mRNA Expression in E13 Rat Hearts

As the size of the embryonic heart does not permit unambiguous separation of the individual component parts for RNA isolation and Northern analysis, we have developed a quantitative radioactive *in situ* hybridization protocol (Jonker *et al.*, 1997; Moorman *et al.*, 1999). Reliable

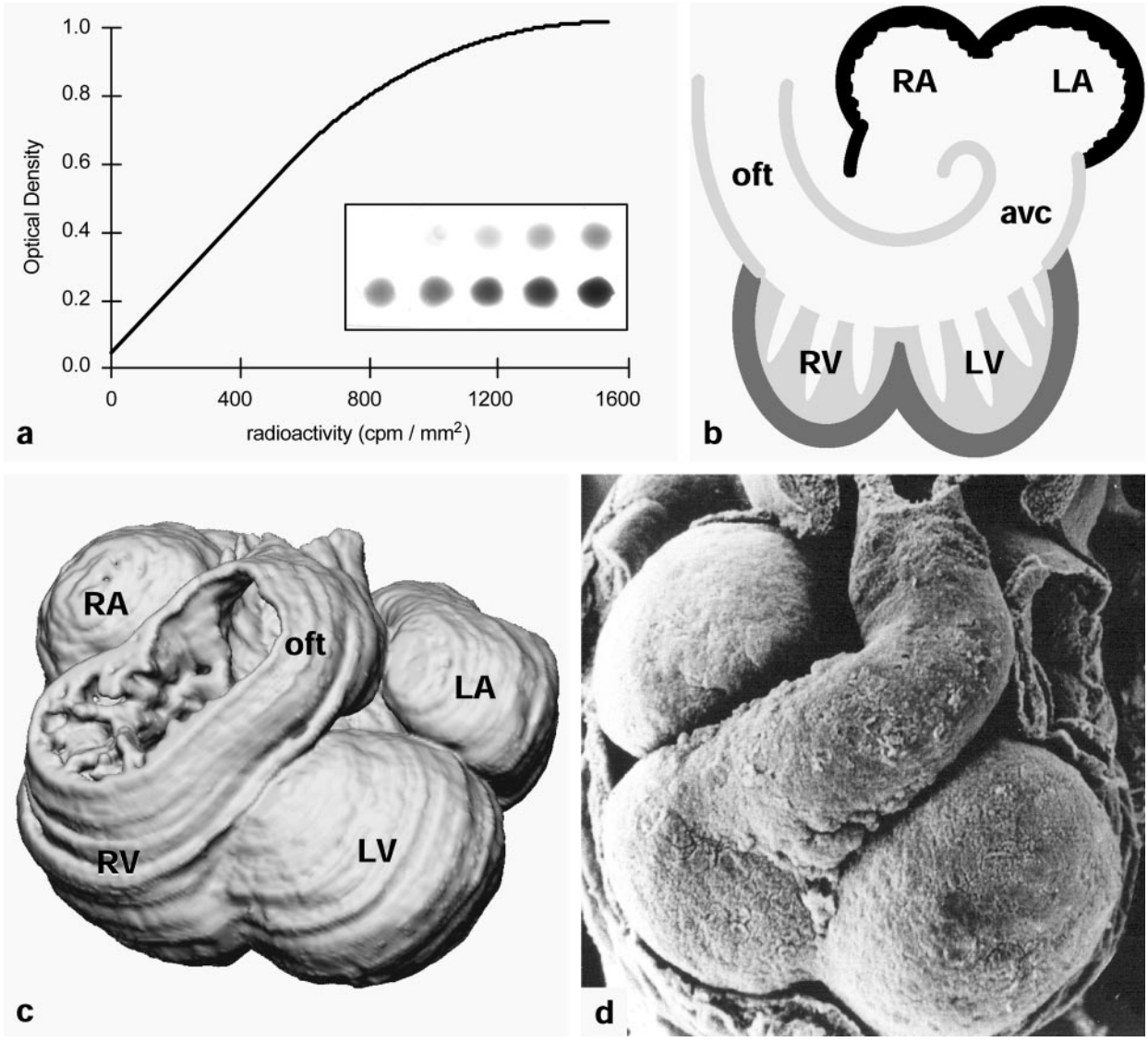


FIG. 3. Quantitative *in situ* hybridization and 3D reconstruction. (a) Calibration curve; (b) summary of SERCA2a mRNA expression, high expression is indicated in black, intermediate expression in gray, and low expression in light gray; (c) reconstruction of all sections; (d) scanning EM picture of a comparable staged heart.

measurements of autoradiographic signal (OD) requires that OD values be unambiguously related to the radioactivity distribution in the section, which in turn reflects the amount of probe locally present in the section. To this end a calibration curve has been made using gelatin calibration spots, containing known amounts of radioactivity. These spots were exposed and developed similar to, and simultaneous with, the tissue sections to be examined. Figure 3a shows that the optical density is proportionate to the amount of radioactivity present in the spots. This calibration curve can be used to convert OD values (up to OD 0.8) into amounts of radioactivity locally present in the sections. Specific signal due to hybridization of the ^{35}S-labeled riboprobe to SERCA2a mRNA in myocardial tissue has been expressed relative to nonspecific signal in nonmyocardial tissue. The ratio specific signal/nonspecific signal has been color coded to facilitate the interpretation of the spatial distribution of the mRNA.

Serial sections of complete E13 embryonic rat hearts have been hybridized with an ^{35}S-labeled riboprobe to SERCA2a mRNA and have been 3D reconstructed. A summary of the patterns of expression is given in Fig. 3b and the entire reconstruction in comparison with a scanning EM picture in Figs. 3c and 3d. A selection of transverse and sagittal

sections is shown in the Figs. 4 and 5. Photographs of the entire series can be obtained from the corresponding author.

Highest expression is seen in the working myocardium of atria, ~10-fold nonmyocardial control tissue, and ventricles (compact myocardium of the ventricular free wall and septum), ~5-fold control tissue. Primary myocardium of atrioventricular canal and outflow tract and the trabecular ventricular component display low expression, ~3-fold control tissue. A salient feature of all sections is that the primary myocardium of atrioventricular canal, inner curvature, and outflow tract displays a distinctly lower SERCA2a mRNA expression compared to the developing atrial and ventricular chambers as clearly seen in Fig. 4c′. Note also that the caudal, lower wall of the primary heart tube (sinus venosus/atrioventricular canal) is directly attached to the body wall. The upper, cranial wall of the atrioventricular canal (primary heart tube) is not directly attached to the body wall because in-between the working myocardium of the atrial chambers has developed.

Distribution Patterns of SERCA2a and PLB Protein

SERCA2a protein can be detected from E10 onward in the developing rat heart (Fig. 6a), similar to the encoding mRNA (Moorman *et al.*, 1995). Expression is restricted to the myocardium, highest in the inflow myocardium and lowest in the outflow tract. No antibody binding is apparent in the cardiac cushions and the rest of the embryonic tissue, demonstrating that specific immunostaining has been achieved. At E13 the pattern of SERCA2a protein is comparable to the mRNA pattern, that is, highest in atrial myocardium and lowest in outflow tract myocardium (Fig. 6b). The clear compartment-differentiating pattern of the mRNA is equally well represented in the protein pattern, indicating that control is primarily exerted at the pretranslational level. From E13 onward the SERCA2a protein pattern gradually acquires the adult pattern: high in the atrium, distinctly lower in the ventricles, and in a transmural gradient with lower expression at the luminal side (Fig. 6c).

Phospholamban protein displays a pattern of expression similar to that of its encoding mRNA (Moorman *et al.*, 1995). It cannot be detected in E10 hearts, it is present in E13 hearts in an anteroposterior gradient, being highest in the outflow tract (Fig. 6d), and it acquires its adult pattern around E16, that is, highest expression in the ventricles and a distinctly lower expression in the atria (Fig. 6e).

In a 7-week human heart, Carnegie stage 19, which is comparable with E15 rat, SERCA2a protein expression is essentially similar to the expression in the rat embryonic heart (Fig. 6f).

DISCUSSION

It is generally accepted that calcium-induced calcium release from the SR, and energy-dependent reuptake, underlies cardiac contraction in the adult mammalian heart (Fabiato *et al.*, 1979; Bers, 1991). Calcium release from the SR is triggered by depolarization-induced inward L-type calcium channel current carrying a small amount of calcium, which after each cycle is extruded via the sarcolemmal NCX. This study unequivocally demonstrates the presence of functional SR in the mammalian embryonic heart long before birth as a prominent characteristic of the developing chambers at the outer curvature.

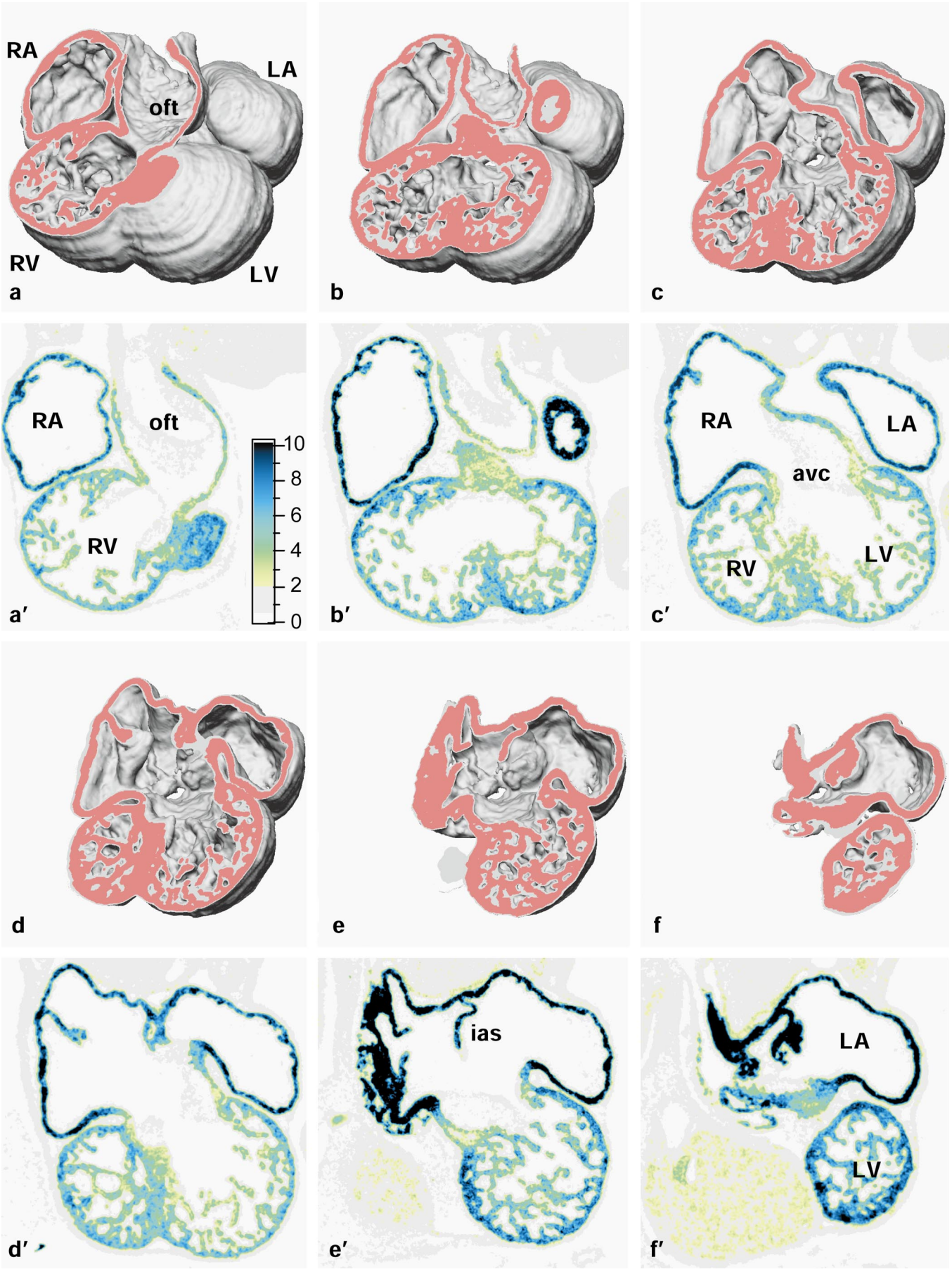
SR Function before Birth

The significance of prenatal SR function in the mammalian heart is generally underappreciated, although in early cardiac development presence of SERCA2a, phospholamban, and oxalate-supported Ca^{2+} uptake has been demonstrated (Will *et al.*, 1983; Jorgensen *et al.*, 1984). This notion emerged from studies reporting a poorly morphologically developed SR (not necessarily implying a poorly functionally developed SR) before birth (Nakanishi *et al.*, 1987) and increase of SERCA2a mRNA, protein, and activity (Lompré *et al.*, 1991; Arai *et al.*, 1992; Dumas *et al.*, 1999; Vetter *et al.*, 1995) with a concomitant decrease in NCX mRNA, protein, and activity (Artman, 1992; Boerth *et al.*, 1994; Vetter *et al.*, 1995) after birth. The presumption of poorly developed SR before birth has, in turn, invoked the proposal that sarcolemmal L- and/or T-type Ca^{2+} channels working in concert with reversed mode operating NCX play a predominant role in calcium entry to compensate for a deficient SR.

A confusing element in many developmental studies is the changing tissue base of total RNA and total protein relative to one another with development (van den Hoff *et al.*, 1999). Prenatally, the heart changes from a hyperplastic synthetic organ to a hypertrophic force-producing organ, reflected by a fourfold developmental *de*crease in total RNA and a threefold *in*crease in total protein per cardiac wet weight (van den Hoff *et al.*, 1997). Perinatal NCX mRNA levels and protein and activity have been reported to be four- to eight- and two- to fourfold adult levels, respectively (Boerth *et al.*, 1994; Artman, 1992; Vetter *et al.*, 1995; Koban *et al.*, 1998). Correction for changes in tissue base results in an even more pronounced developmental decrease of NCX mRNA, whereas NCX protein levels do not change significantly. The latter conclusion matches the observation that there is no significant developmental change in the surface-to-volume ratio expressed as sarcolemmal membrane plus T-tubular area per unit cell volume (Page *et al.*, 1974), implying that the NCX membrane density may not change significantly with development.

SR Function Is Present in the Developing Chambers

Functional differences are to be expected between rat atrial and outflow tract compartments based upon the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LV
f′

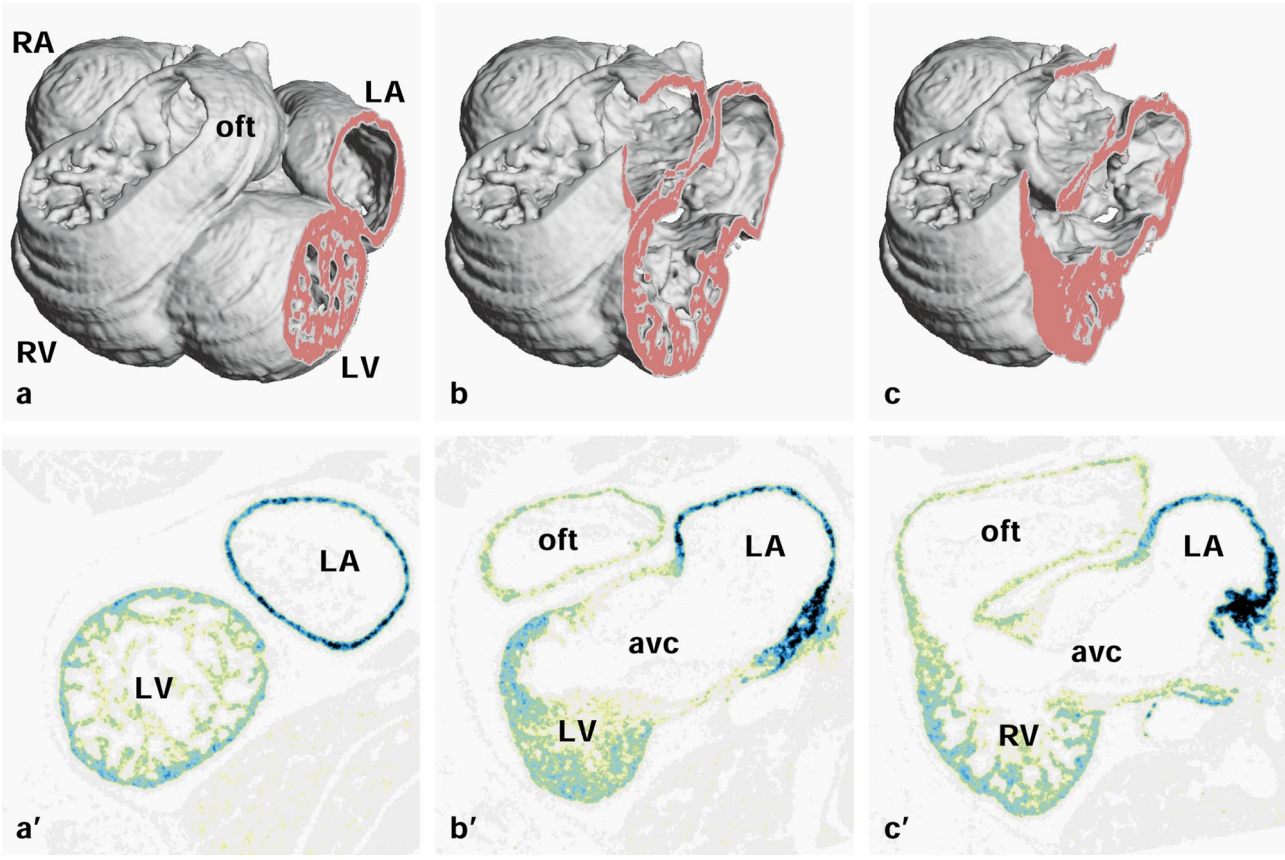

FIG. 5. Quantitative *in situ* hybridization on sagittal sections of a serially sectioned E13 embryo rat heart, hybridized with a probe to SERCA2a mRNA. Note the contiguous low expression at the inner curvature and the segmental expression at the outer curvature. See legend to Fig. 4 for abbreviations.

mRNA and protein patterns of expression. Indeed, atrial Ca^{2+} transients were entirely abolished by blockade of SR Ca^{2+} release channels both in the open and in the closed state with ryanodine or tetracaine, respectively, in contrast to the outflow tract and primary heart tube. In all tissue compartments inhibition of sarcolemmal L-type Ca^{2+} current with verapamil entirely abolished Ca^{2+} transients. These findings validate the following conclusions. First, activation of sarcolemmal calcium current is essential for the generation of cytoplasmic Ca^{2+} transients, whether or not a functional SR is operative. Second, functional SR is absent in the embryonic outflow tract, but present in atria (and ventricles, as assessed by Ca^{2+} uptake measurements in isolated SR vesicles). The sensitivity of outflow tract preparations to Ni^{2+} (in contrast to the atrial preparations) suggests a concerted action of L-type Ca^{2+} channel and Na^{+}/Ca^{2+} exchanger in the generation of calcium transients. Such a mechanism of interaction deserves further investigation, which, however, is beyond the scope of this study.

Mutant mice lacking the ryanodine receptor type 2 display embryonic lethality (Takeshima *et al.*, 1998). The total abolishment of Ca^{2+} transients in the atria by SR-specific inhibition, indicating that neither sarcolemmal L-type Ca^{2+} channel-dependent Ca^{2+} entry nor reversal of NCX significantly contributes to the Ca^{2+} transients, might well explain the cause of this lethal mutation.

FIG. 4. Quantitative *in situ* hybridization on transverse sections of a serially sectioned E13 embryo rat heart, hybridized with a probe to SERCA2a mRNA. Reconstructions (a–f); sections (a′–f′). The calibration bar shows in gray the signal present in nonmyocardial tissue and in a gradient from yellow to blue/black the strength of hybridization relative to the signal in nonmyocardial tissue. oft, outflow tract; avc, atrioventricular canal; L/R A, left/right atrium; L/R V, left/right ventricle; ias, interatrial septum.

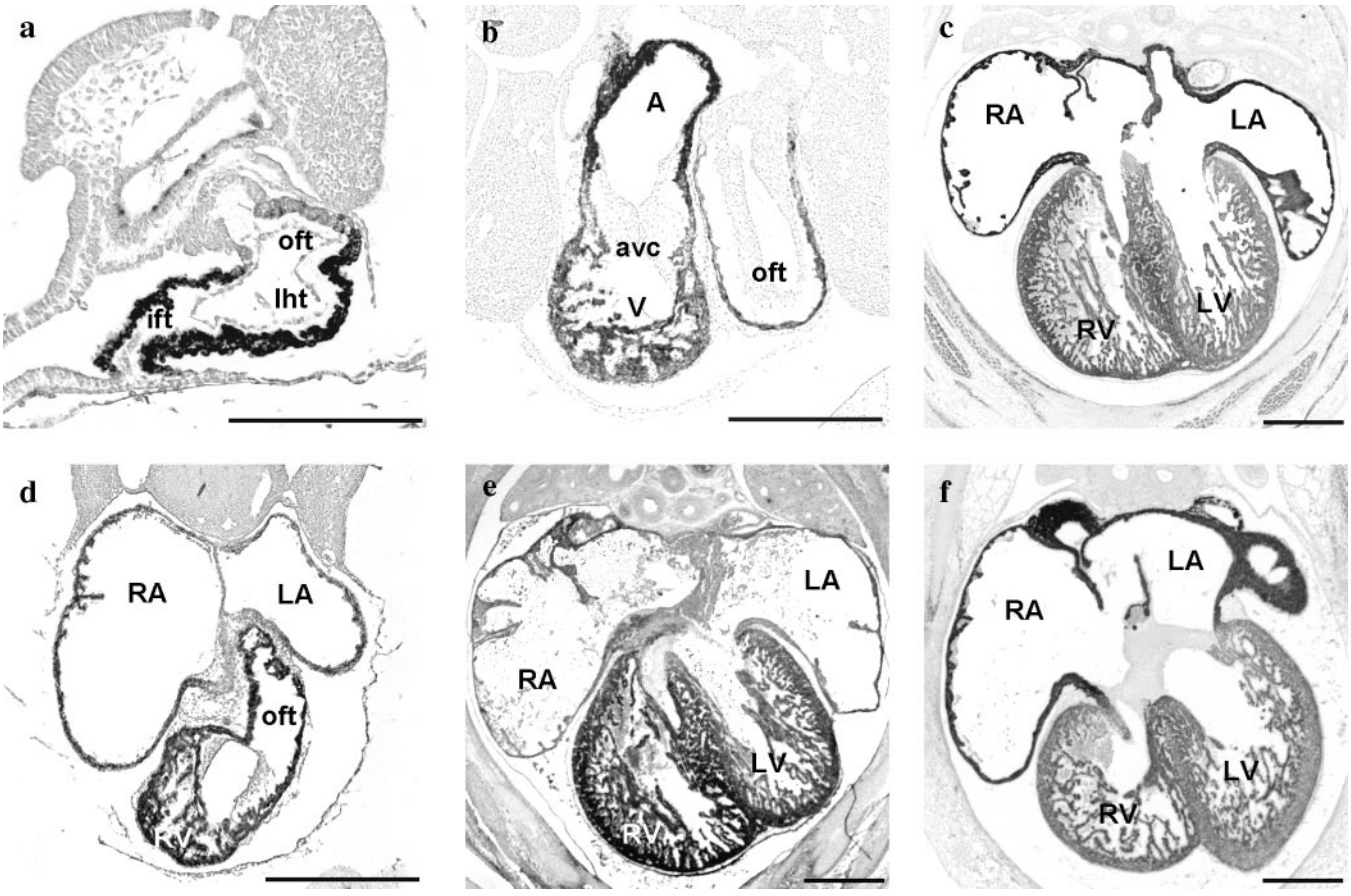

FIG. 6. Immunohistochemical detection of SERCA2a protein (a, b, c, f) and phospholamban (d, e) in E10 (a), E13 (b, d), and E16 (c, e) rat embryo hearts and in a 7-week human heart. See legend to Fig. 4 for abbreviations; lht, looping heart tube. SERCA2a protein expression is clearly higher in the upstream part of the heart than in the downstream part. Moreover expression is distinctly lower in the atrioventricular canal and outflow tract.

The Linear Heart Tube and the Embryonic Outflow Tract and Atrioventricular Canal Share Absence of SR Function

In chicken H/H 11+ linear heart tubes Ca^{2+} transients were not sensitive to inhibitors of Ca^{2+}-triggered SR Ca^{2+} release, but sensitive to L-type calcium channel and NCX inhibition. With chamber formation, Ca^{2+} transients in developing working myocardium, in contrast to the flanking outflow tract, become sensitive to inhibitors of Ca^{2+}-triggered SR Ca^{2+} release. Sensitivity to inhibition of L-type calcium channel and NCX remains in both compartments. The differential sensitivity is well represented in the mRNA and protein distribution pattern. SERCA2a mRNA and protein are low in the myocardium of outflow tract, atrioventricular canal, and early linear heart tube. In the linear heart tube stage SR structures have not yet been laid down (Komazaki *et al.*, 1997), which of course explains absence of SR activity. However, in outflow tract myocardium of E13 rat hearts SR tubules are well represented (Knaapen *et al.*, 1997). Yet no Ca^{2+}-triggered SR Ca^{2+} release could be detected. This may be attributed to the high levels of phospholamban in the outflow tract, which keep the SR Ca^{2+} pump in the low-Ca^{2+}-affinity state (Koss *et al.*, 1997).

We have not been able to measure activities in the myocardium of the atrioventricular canal, owing to its small size. Based on the matching mRNA, protein, and activity levels in the atrial, ventricular, and outflow compartments, respectively, it seems reasonable to suppose that the atrioventricular canal will have comparable properties as the outflow myocardium.

Chamber Development: Functional and Morphological Implications

The combined mRNA, protein, and activity data from chicken and rat embryonic hearts suggest that regional differences in SR distribution and/or function are responsible for the separate functions of the embryonic cardiac compartments. Atrial and ventricular chambers remain

flanked by primary myocardium at the atrioventricular canal, at the outflow tract, and at the inner curvature. The contiguous domain of low SERCA2a expression in atrioventricular canal, outflow tract, and inner curvature and the alternating high and low expression at the outer curvature support a model of chamber development by differentiation from the primary heart tube at the outer curvature (de Jong *et al.*, 1997), rather than by entire segmentation of the tube (Figs. 1A and 1B). In accordance with this notion, the patterns of expression of many other genes allow the distinction of a so-called "primary" transcriptional program in atrioventricular canal, inner curvature, and outflow tract versus a "chamber" transcriptional program (V. M. Christoffels *et al.*, 2000).

The flanking segments of atrioventricular canal and outflow tract are characterized by slow conduction velocity (de Jong *et al.*, 1992; Moorman *et al.*, 1998), a matching low level of gap junctions and of connexin expression (Gros *et al.*, 1978, 1994; van Kempen *et al.*, 1991; Moorman *et al.*, 1998). Previously, we concluded that the flanking segments acquired a peristaltic contraction owing to this slow conduction, enabling them to substitute the adult type of one-way valves (de Jong *et al.*, 1992). This study complements these observations and provides the molecular basis for the assignment of the observed long contraction duration in these areas to low SR activity, matching the slow conduction velocities.

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