0031-3998/95/3806-0892$03.00/0
PEDIATRIC RESEARCH

Vol. 38, No. 6, 1995
Printed in U.S.A.

Inotropic Effect of Increasing Concentration of Ca^{2+} in the Fetal Rat Heart with Retinoic Acid-Induced Malformations

IVANA OŠŤÁDALOVÁ, TOMAS PEXIEDER, BOHUSLAV OŠŤÁDAL, AND FRANTIŠEK KOLÁŘ

Institute of Physiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic [I.O., B.O., F.K], and Institute of Histology and Embryology, Faculty of Medicine, University of Lausanne, Switzerland [T.P.]

ABSTRACT

Cardiac malformations (pulmonary trunk stenosis, ventricular septal defect, and double outlet right ventricle) were induced by the administration of two doses of retinoic acid (RA) to Wistar rats on d 13 of pregnancy. Contractile performance of the isolated perfused rat heart and its inotropic response to Ca^{2+} (0.6–10.0 mmol · L^{-1}) was studied in 20-d-old fetuses. The body weight of RA-exposed fetuses was significantly lower compared with controls. RA negatively influenced the contractile parameters of the fetal rat heart. The most pronounced effect was, except at a Ca^{2+} concentration of 2.5 mmol · L^{-1}, observed at developed force at all other concentrations. Simultaneously, the sensitivity to Ca^{2+}, expressed as the Ca^{2+} concentration at which 30% of maximum was attained, was significantly lower in RA-exposed hearts. This implies that the malformed heart is more dependent on the extracellular sources of Ca^{2+}. (***Pediatr Res* 38: 892–895, 1995**)

Abbreviations

RA, retinoic acid
ed, embryonic day
DF, developed force
$(+dF/dt)_{max}$, rate of maximal contraction
$(-dF/dt)_{max}$, rate of maximal relaxation
CHD, congenital heart disease
TGA, transposition of great arteries
DORV, double-outlet right ventricle
VSD, ventricular septal defect

CHD is the most frequent major malformation in human population (1). It has been estimated that about 8% of CHD are due to chromosomal anomalies, 2% to teratogenic agents in the environment, but 90% have a multifactorial origin (2–4). Most of the embryogenetic theories are, however, purely speculative (5); one reason is the absence of an adequate animal model for experimental analysis of the pathogenesis of CHD.

As early as in 1972, Shenefelt (6) described RA-induced cardiac malformations in the hamster. Davis (7), Davis and Sadler (8), Irie *et al.* (9), and Pexieder *et al.* (10) described RA-induced cardiovascular malformations in mice and, finally, Pexieder *et al.* (10) developed a similar model in rats. It has been shown that the administration of all-*trans*-RA to pregnant dams induced cardiac malformations such as TGA and double outlet right ventricle. RA obviously interferes with cardiac development. It passes across the cell membrane to be bound by cellular RA-binding protein, which facilitates its transfer across the nuclear envelope. Inside, the nuclear RA receptors are involved in its interactions with DNA and DNA transcription. This mechanism is responsible for the various biologic effects known to influence almost every tissue in an organism. Pharmacokinetic studies on RA in the pregnant mouse indicate that its plasmatic half-life is 6–10 h (11).

Many of the cardiovascular malformations are amenable to surgical intervention (12, 13) designed to correct abnormal morphology, such as erroneous connections between the ventricles and the great arteries. However, there is almost no evidence of whether the macroscopical abnormality is associated with altered structural and functional properties of the embryonic heart. The knowledge of these parameters may be important for indications for cardiac surgery (14) and for outcome prediction.

We have previously observed that the contractile parameters and inotropic responsiveness of the rat heart changes significantly during the early phases of postnatal ontogeny. The time course of this development is closely related to the maturation of the systems involved in calcium handling (15). Nakanishi *et al.* (15a) described perinatal development of the cardiac con-

Received September 15, 1994; accepted June 6, 1995.
Correspondence: Dr. Ivana Ošťádalová, Institute of Physiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, 140 00 Prague 4, Czech Republic.
Supported in part by research a grant from Schweizerischer Nationalfonds zur Forderung der wissenschaftlichen Forschung (Verkstaerkte Zusammenarbeit mit Ost-Europaeischen Staaten, No. 70 TP-032909) and by a grant of the Czech Ministery of Health (Z129).

tractile system in the rabbit. At this stage of ontogenesis the rabbit heart is, however, structurally and functionally different in comparison with rat heart. Information on the prenatal development of inotropic responsiveness in the rat heart is still lacking. The aim of the present study was to compare the inotropic effect of increasing concentration of Ca^{2+} in the fetal rat heart with and without RA-induced malformations.

METHODS

Assessment of heart function. A total of three pregnant Wistar rats (day of sperm in vaginal smear is ed 0) were given 125 mg · kg^{-1} of all-*trans*-RA (Sigma), dissolved in olive oil, by gastric tubing on ed 13 and 13.5. The control pregnant rats (n = 3) were treated at the same stage and in the same way. On ed 20 all pregnant females were anesthetized by thiopental, and their fetuses were removed, weighed, and killed by cervical dislocation. The experimental (n = 7) and control (n = 8) hearts served for measurement of contractility. The hearts were quickly dissected, and a stainless steel cannula (with an external diameter of 0.45 mm) was inserted into the aorta. The hearts were rapidly perfused in the Langendorff mode under constant pressure (13 cm H_2O), proportional to the mean arterial blood pressure for the perinatal stage of rat development (16). The hearts were perfused with the Tyrode solution containing (mmol · L^{-1}): NaCl 145.0; KCl 5.9; $CaCl_2$ 1.25; $MgCl_2$ 1.2; glucose 11.0; mannitol 1.1, and HEPES 5.0. The pH was adjusted to 7.4 by Tris, and the solution was saturated with 100% O_2. Mannitol was used as a scavenger of oxygen radicals. The temperature was maintained at 37°C. The hearts were electrically stimulated at a rate of 2 Hz using silver electrodes attached to the base of the heart. The stimulation was performed with pulses of alternating polarity, 1-ms duration, and voltage set at 50% above the threshold level. The resting force was gradually increased by means of a micromanipulator to the level at which the developed force was approximately 80% of the maximum force reached at optimum preload. The contractile function of the isolated heart was measured using an isometric force transducer (17) connected by means of a glass fiber, a two-arm titanium lever, and silk suture (0.7 metric, Ethicon) to the apex of the heart. The contractile force (DF, g) and its first derivative [$(+dF/dt)_{max}$, $g.s^{-1}$ and $(-dF/dT)_{max}$, $g.s^{-1}$] were evaluated automatically from the force signal using an on-line computer (18). After measurement and drying on filter paper the control hearts were weighed and the experimental hearts were prepared for morphological evaluation (see below).

Estimation of inotropic response to Ca^{2+}. The hearts were initially perfused with 1.25 mmol · L^{-1} Ca^{2+} for 40 min. The Ca^{2+} concentration was then decreased to 0.625 mmol · L^{-1} and thereafter gradually increased (1.25, 2.5, 5.0, 7.5, and 10.0 mmol · L^{-1}). The maximal effect of each concentration was recorded and expressed as *1*) absolute values, *2*) a percentage of the value reached at the lowest Ca^{2+} concentration, and *3*) a percentage of the maximum response.

Morphological examination. After assessment of heart function, the ventricles from RA-treated hearts were perfused with a fixing solution (a mixture of 2% glutaraldehyde and 1% formaldehyde in 0.1 mol · L^{-1} cacodylate buffer; the osmolarity of cacodylate buffer was adjusted to 330 mOsm · L^{-1} by NaCl). After fixation the type of cardiac defect was determined using microdissection and scanning electromicroscopic determination (19) of the phenotype.

Data analysis. All data were presented as means±SEM. The statistical significance of differences ($p<0.05$) between experimental and control group for each concentration was evaluated by the Mann-Whitney nonparametric test (see Figs. 1 and 2) or by two-way analysis of variance with logarithmic transformation to stabilize variances (see Table 1).

RESULTS

Morphologic characteristic of heart defects. The body weight of fetuses exposed to RA decreased significantly compared with controls (2.52 ± 0.07 g *versus* 3.30 ± 0.14 g).

Among the seven RA-exposed hearts analyzed morphologically, after the functional investigations, five fetal hearts were diagnosed as having a DORV, one heart had a VSD and one heart pulmonary trunk stenosis.

Inotropic response to Ca^{2+}. The effect of increasing Ca^{2+} concentration on the contractile parameters [DF, $(+dF/dt)_{max}$, $(-dF/dt)_{max}$] of malformed and control hearts is shown in Figure 1, *A–C*. In malformed hearts the absolute values of DF were, excepting a Ca^{2+} concentration of 2.5 mmol · L^{-1}, significantly lower ($p<0.05$) over the whole range of Ca^{2+} concentrations. This trend was obvious also for curves of $(+dF/dt)_{max}$ and $(-dF/dt)_{max}$. The absolute values of $(+dF/dt)_{max}$ and $(-dF/dt)_{max}$ in malformed hearts were significantly decreased at Ca^{2+} concentrations of 0.625, 1.25, and 5.0 mmol · L^{-1}.

The magnitude of the inotropic response to Ca^{2+} (DF), expressed as the percentage of the lowest value, increased with increasing concentrations in both groups. This value in the malformed hearts was, however, starting from a Ca^{2+} concentration of 2.5 mmol · L^{-1}, significantly higher than in controls (Fig. 2*A*). The maximum rate of force development $[(+dF/dt)_{max}]$ was higher starting from the Ca^{2+} concentration of 2.5 mmol · L^{-1}; on the other hand, the values of the maximum rate of relaxation did not differ (Table 1).

The sensitivity to extracellular Ca^{2+}, expressed as the concentration at which half-maximum DF was attained, did not differ in control and malformed hearts (2.69±0.19 and 2.73±0.20 mmol · L^{-1}, respectively). However, the concentration at which 30% of the maximum DF was attained was significantly higher in malformed than in control hearts (1.56±0.07 and 1.15±0.08 mmol · L^{-1}, $p<0.01$), suggesting a decreased sensitivity at the low Ca^{2+} concentration (Fig. 2*B*).

DISCUSSION

It has been established that RA is a potent cardiovascular teratogen in rodents (for review, see Ref. 20). Our results have, however, clearly shown that the administration of RA significantly influenced the growth of rat fetuses, supporting the view that its effect is more complex and the heart is not the only target tissue (21). Furthermore, Ruberte *et al.* (22, 23) and Dolle *et al.* (24) have shown that cellular RA-binding protein

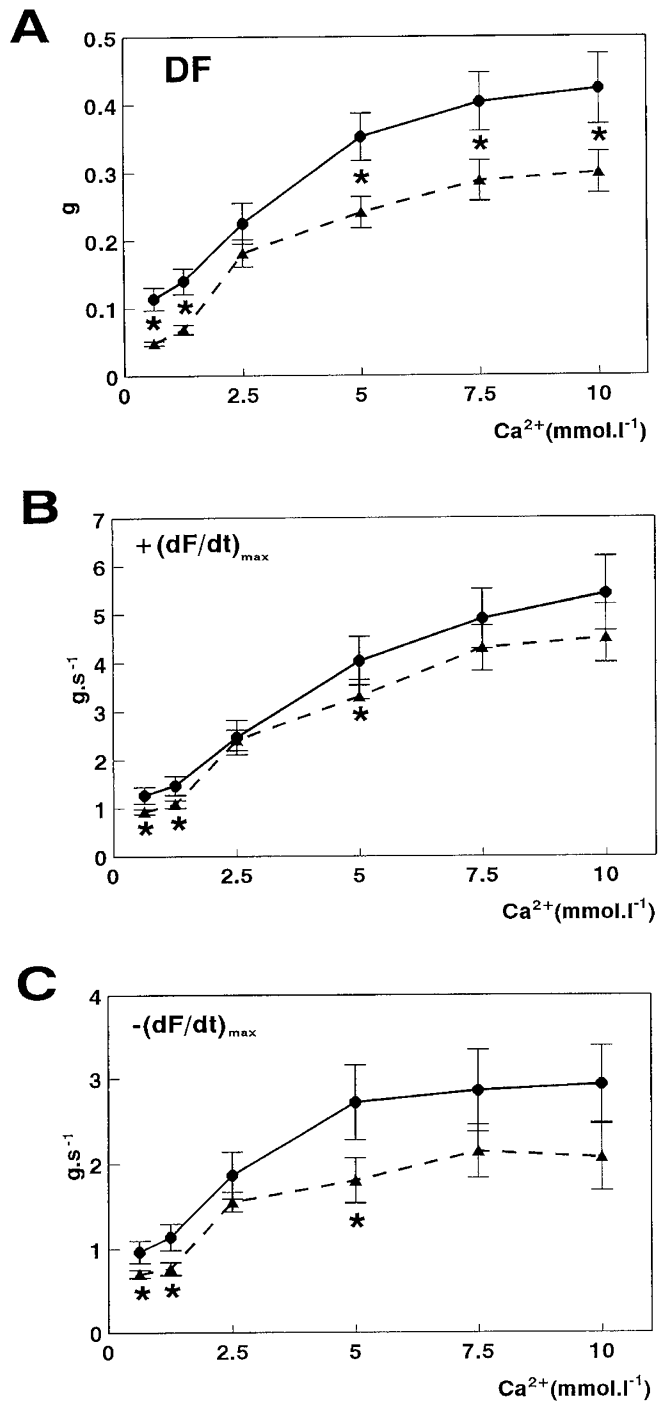

Figure 1. Inotropic response to increasing Ca^{2+} concentration expressed as absolute values DF (*A*), $(+dF/dt)_{max}$ (*B*) and $(-dF/dt)_{max}$ (*C*) of the control (*circles*, eight hearts) and malformed hearts (*triangles*, seven hearts). Data are means±SEM; *significantly different ($p < 0.05$).

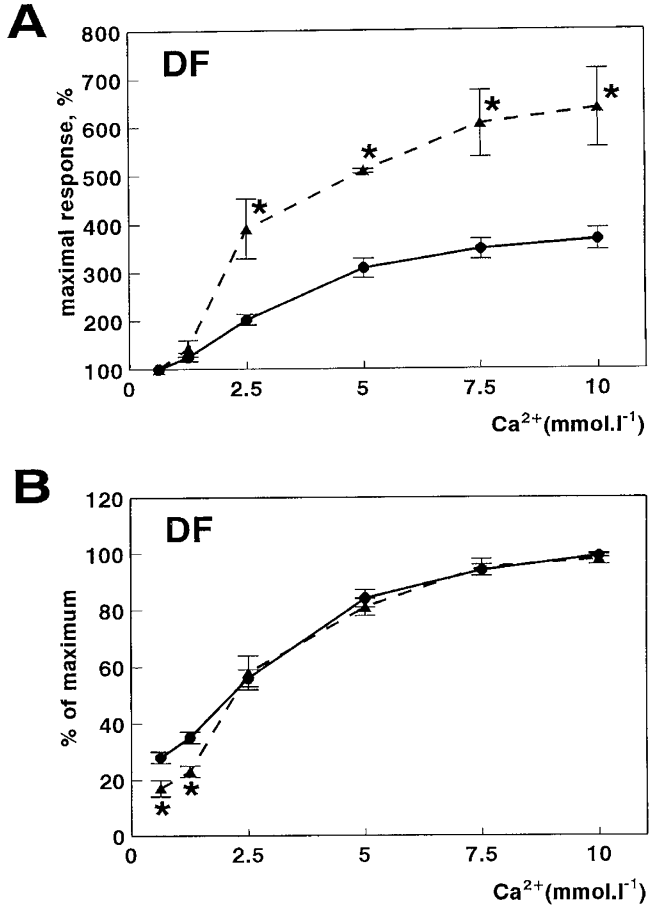

Figure 2. Inotropic response to increasing Ca^{2+} concentration expressed as a percentage of the lowest value of DF (*A*) and as a percentage of the maximum value of DF (*B*) of the control (*circles*, eight hearts) and malformed hearts (*triangles*, seven hearts). Data are means±SEM; *significantly different ($p < 0.05$).

Table 1. *Inotropic response to increasing Ca^{2+} concentration (maximum response)*

Ca^{2+} concentration (mmol · L^{-1})	$(+dF/dt)_{max}$ (%)	$(-dF/dt)_{max}$ (%)
Controls		
0.62	100	100
1.25	119 ± 6	121 ± 6
2.50	201 ± 15	200 ± 16
5.00	318 ± 18	283 ± 27
7.50	417 ± 44	281 ± 32
10.00	410 ± 25	287 ± 29
Malformations		
0.62	100	100
1.25	117 ± 10	107 ± 10
2.50	274 ± 24*	231 ± 23
5.00	370 ± 41*	287 ± 41
7.50	461 ± 45*	318 ± 49
10.00	471 ± 57*	299 ± 82

Controls ($n = 8$); RA-induced malformations ($n = 7$). Values are means ± SEM.

* $p < 0.05$ *vs* controls.

and RA receptors have a specific pattern of localization in different embryonic tissues, such as the cardiogenic plate, the adjacent mesenchyme, and neural crest cells.

RA given in a proper dosage and at proper time of gestation induces extracardiac and cardiac malformations whose phenotype is species- and strain-dependent. In the rat (11) as well as in NMRI mice the most frequent lesions are DORV (43% of the offspring), VSD (16% of the offspring), and aortic arch anomalies (4.2%). In the ICR strain of mice, the most frequent were right and left TGA followed by DORV and VSD. Not yet published data on RA-exposed mice (Pexieder, T, personal communication) indicate an abnormal architecture of ventric-

ular myocardium such as decreased thickness of the compact layer, disorganized spongy layer, and aberrant papillary muscle. When the RA treatment was combined with short-time fasting, the incidence of TGA rose up to 65% of the offspring (25).

Our results demonstrate that RA negatively influenced the contractile parameters of the fetal rat hearts. The most pronounced was this effect on DF at most Ca^{2+} concentrations. Simultaneously, the sensitivity to Ca^{2+}, expressed as the concentration at which 30% of maximum was attained, was significantly lower in malformed hearts. This implies that the malformed heart is significantly more dependent on the extracellular sources of Ca^{2+}, suggesting that the mechanisms responsible for intracellular Ca^{2+} transients are influenced (26). It was found that the amount of contractile proteins in RA-treated mouse fetal hearts was significantly less than in controls (25). It has been shown previously that some functional sarcoplasmic reticulum already exists during the late fetal life (27) as well as in newborn rats (28). During further development, the ability of sarcoplasmic reticulum to accumulate Ca^{2+} increases, and there is a progressive maturation of Ca^{2+} release from the sarcoplasmic reticulum (15, 29). It may be suggested that the RA-induced cardiac malformations might be connected with the significant changes of structures involved in Ca^{2+} in the fetal heart. To our knowledge, this is the first time that altered inotropic responsiveness could be demonstrated in structurally abnormal (malformed) fetal hearts.

On the basis of our data, however, it is difficult to distinguish whether the described changes are due to an RA effect on Ca^{2+} handling or whether they are the consequences of abnormal cardiac morphology. Furthermore, the number of hearts observed in the study is inadequate for a subgroup of cardiac lesions.

In this connection it is necessary to mention that the teratogenic effect of RA on the rat heart is very heterogeneous, both qualitatively and quantitatively. Similar heterogeneity may exist also in the pathogenetic mechanism responsible for the functional consequence of structural changes. Detailed analysis of myocardial ultrastructure in RA exposed hearts, with particular attention to the systems involved in the Ca^{2+} handling, may be very helpfull in this respect. Moreover, no data are as yet available on the hemodynamic alterations in different types of RA-induced malformations. Recent results (25) that show the impact of RA on cardiac protein synthesis in mice might be the expression of the fetal heart adaptation, trying to cope with the abnormal structure.

Acknowledgment. The authors thank Dr. J. Vorlíček for statistic analysis.

REFERENCES

1. Eurocat Report No 4 1991 Surveillance of congenital anomalies, years 1980–1988. Dept of Epidemiology, Catholic University of Louvain, Bruxelles
2. Pexieder T, Bloch D, Beuret A 1989 Eurocat collaborative study of congenital heart disease. Eur J Epidemiol 5:254–262
3. Ferencz C, Corea-Villasenor A, Rubin JD, Boughman JA, Loffredo C 1991 The epidemiology of cardiovascular malformations (CVM): ten years later. Teratology 43:450–468
4. Patterson DF, Pexieder T, Schnarr WR, Navratil T, Alaili R 1993 A single major-gene defect underlying cardiac conotruncal malformations interferes with myocardial growth during embryonic development: studies in the CTD line of Keeshond dogs. Am J Hum Genet 52:388–397
5. Pohanka I, Vitek B 1972 Survey of embryology of the heart and the most important pathogenetic theories of great vessel transposition. Cesk Patol 17:8–16
6. Shenefelt RE 1972 Morphogenesis of malformations in hamsters caused by retinoic acid: Relation to dose and stage of treatment. Teratology 5:103–118
7. Davis LA 1981 The role of retinoic acid in the production of endocardial cushion defects. Anat Rec 199:64–72
8. Davis LA, Sadler TW 1981 Effects of vitamin A on endocardial cushion development in the mouse heart. Teratology 24:138–148
9. Irie K, Ando M, Takao A 1990 All-trans retinoic acid induced cardio-vascular malformations. In: Bockman DE, Kirby ML (eds) Embryonic Origins of Defective Heart Development. Academy of Science, New York, pp 387–388
10. Pexieder T, Pfinzenmaier Rousseil M, Prados-Frutos JC 1990 Prenatal pathogenesis of the transposition of great arteries. Teratology 41:584–596
11. Pexieder T, Pfinzenmaier RM, Prados-Frutos JC 1992 Prenatal pathogenesis of the transposition of great arteries. In Transposition of the Great Arteries 25 Years after Rashkind Balloon Septosomy. Steinkopff Verlag, Darmstadt, pp 11–27
12. Ebert PA 1990 Atlas of Congenital Cardiac Surgery. Churchill Livingstone, Edingburgh
13. Puga FJ 1990 Congenital heart surgery: present and future. In: Long WY (ed) Fetal and Neonatal Cardiology. WB Saunders, Philadelphia, pp 714–717
14. Šamánek M, Bass A, Ošťádal B, Hučín B, Stejskalová M 1989 Effect of hypoxaemia on enzymes supplying myocardial energy in children with congenital heart disease. Int J Cardiol 25:265–270
15. Ošťádalová I, Kolář F, Ošťádal B, Rohlíček V, Rohlíček J, Procházka J 1993 Early postnatal development of contractile performance and responsiveness to Ca^{2+}, verapamil and ryanodine in the isolated rat heart. J Mol Cell Cardiol 25:733–740

15a. Nakanishi T, Seguchi M, Takao A 1988 Development of the myocardial contractile system. Experientia 44:936–944

16. Litchfield JB 1958 Blood pressure in infant rats. Physiol Zool 31:1–6
17. Rohlíček V 1983 Photoelectric transducer. Physiol Bohemoslov 22:89
18. Rohlíček V 1989 A microcomputer based analyser for evaluation of contractile properties of a cardiac muscle. Physiol Res 38:389–401
19. Pexieder T 1986 Standardized method for the study of normal and abnormal cardiac development in chick, rat, mouse, dog and human embryos. Teratology 33:91C–92C
20. Pexieder T, Pfinzenmaier Rousseil M 1992 Comments on "Target tissue specificity of retinoic acid-induced stress proteins and malformations in mice." Teratology 45:121–122
21. Anson JF, Laborde JB, Pipkin JL, Hinson WG, Hansen DK, Sheehan DM, Young JF 1991 Target tissue specificity of retinoic acid-induced stress proteins and malformations in mice. Teratology 44:19–28
22. Ruberte E, Dollé P, Chambon P, Morriss-Kay G 1990 Retinoic acid receptors and cellular retinoid binding proteins. II. Their differential pattern of transcription during early morphogenesis in mouse embryos. Development 111:45–60
23. Ruberte E, Dollé P, Krust A, Zelent A, Morriss-Kay G, Chambon P 1990 Specific spatial and temporal distribution of retinoic acid receptor gamma transcripts during mouse embryogenesis. Development 108:213–222
24. Dollé P, Ruperte E, Leroy P, Morriss-Kay G, Chambon P 1990 Retinoic acid receptors and cellular retinoic binding proteins. I. A systematic study of their differential pattern of transcription during mouse organogenesis. Development 110:1133–1151
25. Pexieder T, Blanc O, Pelouch V, Ošťádalová I, Milerová M, Ošťádal B 1994 Late fetal development of retinoic acid induced transposition of great arteries—morphology, physiology and histochemistry. In: Clark EF, Markwald RR, Takao A (eds) Mechanisms of Abnormal Cardiovascular Development. Futura Press, Mount Kisco, NY, pp 297–307
26. Fabiato A, Fabiato F 1978 Calcium-induced release of calcium from the sarcoplasmic reticulum of skinned cells from adult human, dog, cat, rabbit rat and frog hearts and from fetal and newborn rat ventricles. Ann NY Acad Sci 307:491–522
27. Ošťádalová I, Kolář F, Ošťádal B 1995 Inotropic effect of low extracellular sodium on perfused perinatal rat heart. Can J Physiol Pharmacol 73:50–54
28. Wibo M, Bravo G, Godfraind T 1991 Postnatal maturation of excitation-contraction coupling in rat ventricle in relation to the subcellular localization and surface density of 1,4-dihydropyridine and ryanodine receptors. Circ Res 68:662–673
29. Chin TK, Friedman WF, Klitzner TS 1990 Developmental changes in cardiac myocyte calcium regulation. Circ Res 67:574–575