Am J Physiol Heart Circ Physiol 298: H1571–H1576, 2010.
First published March 12, 2010; doi:10.1152/ajpheart.00721.2009.

Effects of mechanical loading on early conduction system differentiation in the chick

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Submitted 4 August 2009; accepted in final form 12 March 2010

Sankova B, Machalek J, Sedmera D. Effects of mechanical loading on early conduction system differentiation in the chick. *Am J Physiol Heart Circ Physiol* 298: H1571–H1576, 2010. First published March 12, 2010; doi:10.1152/ajpheart.00721.2009.—The primary ring, a horseshoe-shaped structure situated between the left and right ventricle and connected superiorly to the atrioventricular canal, is the first specialized fast ventricular conduction pathway in the embryonic heart. It has been first defined immunohistochemically and is characterized as a region of slow myocyte proliferation. Recent studies have shown that it participates in spreading the ventricular electrical activation during stages preceding ventricular septation in the mouse, chick, and rat. Here we demonstrate its presence using optical mapping in chicks between *embryonic days* (ED) *3–5*. We then tested the effects of hemodynamic unloading in the organ culture system upon its functionality. In ED3 hearts cultured without hemodynamic loading for 24 h, we observed a significant decrease in the percentage activated through the primary ring conduction pathway. A morphological examination revealed arrested growth, collapse, and elongation of the outflow tract and disorganized trabeculation. A similar reversal toward more primitive activation patterns was observed with culture between ED4 and ED5. This phenotype was completely rescued with the artificial loading of the ventricles with a droplet of silicone oil. We conclude that an appropriate loading is required during the early phases of the conduction system formation and maturation.

chick embryo; optical mapping; cardiac conduction system; ventricular activation sequence

CARDIAC CONDUCTION SYSTEM (CCS) is of myogenic origin and differentiates gradually from a pool of working cardiac myocytes (1, 7). This process is subject to some plasticity, since ectopic differentiation is possible under appropriate stimuli in vivo (24) as well as in vitro (8).

A functional deployment of various CCS components follows progress in cardiac morphogenesis. The first component to appear is the pacemaker at the inflow at the tubular stage (13); conduction delay in the atrioventricular canal appears at the time of chamber formation, and ventricular CCS function matures concomitant with ventricular septation (2, 15). This functionality is heralded by epicardial activation patterns proceeding from ventricular apex to the base and, in millimeter size embryonic hearts, is most conveniently studied using optical mapping approach (16, 19, 22).

Before maturation of the His bundle and the bundle branches system, there exists another preferential activation pathway along the future interventricular septum—the so-called primary ring. It is a ring-shaped structure situated between the developing left and right ventricle, superiorly in continuity with the atrioventricular canal. It has been first defined immunohistochemically in the embryonic human heart (27) and is characterized as a region of slow myocyte proliferation in the chick (25). Functional studies have shown that this structure participates in the spreading of ventricular electrical activation during the stages preceding ventricular septation in the mouse (17), chick (22), and rat (23). In the chick and rat embryo, we have originally called these patterns the anterior and posterior conduction pathway, since we did not recognize them at that time as a part of the primary ring tissues. Although there is yet no direct proof originating from the fate mapping studies of the cells of the primary ring and because the details of connection between the slow-conducting atrioventricular ring and the fast-conducting primary ring are not elucidated, we hypothesize, together with the Dutch group, that the primary ring region would normally contribute to the future specialized ventricular conduction tissue, specifically the His bundle and bundle branches. This hypothesis is corroborated by it functional characteristic, timing of appearance, and unpublished recent data from transgenic mice expressing the LacZ transgene under the control of different parts of connexin-40 promotor region (Lucile Miquerol, personal communication). Detailed studies of these questions will be difficult since there are no 100%-specific early conduction system markers, and even connexin-40, despite its clear functional significance and later specificity for the His-Purkinje system, shows a rather ubiquitous expression at embryonic stages in mice and is detected only later in development in the chick (9).

We have previously demonstrated that the maturation of the developing ventricular conduction system is sensitive to mechanical loading. Increased pressure loading in ovo leads to an accelerated maturation of the His-Purkinje system (15), accompanied by an upregulation of mRNA of its functional marker, connexin-40 (9). An increase was also noted in the amount of mRNA for endothelin-converting enzyme, a key member of biosynthetic pathway of endothelin-1, which was shown to induce the conversion of working myocytes to the conduction phenotype in vitro (8). Conversely, the decreased loading of the developing left ventricle induced in ovo by left atrial ligation leads to the delay of CCS maturation, with the left bundle branch being most severely affected (15) and accompanied by the downregulation of connexin-40 and endothelin-converting enzyme (22).

It is difficult to separate in vivo the direct effects of myocyte conditioning via altered stress, which itself might be sufficient (at particular stages) for CCS phenotype induction (25) from the effects of an altered shear stress on the ventricular endocardium resulting from perturbed hemodynamics (6). In an

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attempt to separate these two influences, we studied the development of ventricular CCS in cultured isolated hearts either completely unloaded or with ventricles artificially loaded with a droplet of viscous silicon oil (14, 18). We show that complete unloading in vitro leads to the reversal of normal development of the ventricular activation sequence (base to apex→primary ring→apex to base) toward more primitive patterns, whereas artificial loading is sufficient to induce a progression similar to the situation in vivo. Thus mechanical stretching of myocytes is required and sufficient epigenetic factor during the early stages of CCS differentiation.

MATERIALS AND METHODS

Egg incubation and in vitro heart culture. Fertilized White Leghorn chicken eggs were obtained from the Institute of Molecular Genetics, Academy of Sciences of the Czech Republic (Kolec, Czech Republic) and stored at 16°C until the beginning of incubation. The eggs were incubated blunt end up in a humidified incubator at 37.5–38.0°C until embryonic days (ED) 3, 4, and 5. At ED3 and 4, the embryos were removed from the eggs under sterile conditions, and the hearts were excised with part of the adjacent dorsal body wall structures and cultured in 24-well plates in 1 ml of sterile DMEM media with 2% fetal chick serum and 1:1,000 insulin-transferrin-selenium medium supplement (Sigma, Germany) for 24 h in a cell culture incubator (37°C, 5% CO_2-95% room air). The artificial loading of ventricles with silicon oil was performed in a subset of E4 hearts as described previously (14, 18). Briefly, silicon oil of suitable viscosity was injected with a pulled glass capillary needle through the sinus venosus and atrium to the ventricle (Fig. 1). The size of the droplet corresponded to the maximum end-diastolic volume (if there was an excess, it usually leaked out through the overdilated atrioventricular or outflow tract cushions). It was previously shown that such loading increases ventricular contractility by the Frank-Starling law and increases oxygen consumption and glycolysis compared with isolated, unloaded hearts (14, 18).

Functional recordings. Freshly isolated hearts from ED3, -4, and -5 chick embryos together with unloaded and loaded cultured hearts were stained with voltage-sensitive dye 4-{β-[2(di-*n*-butylamino)-6-napthyl]vinyl}pyridinium (Invitrogen) and subjected to optical map-

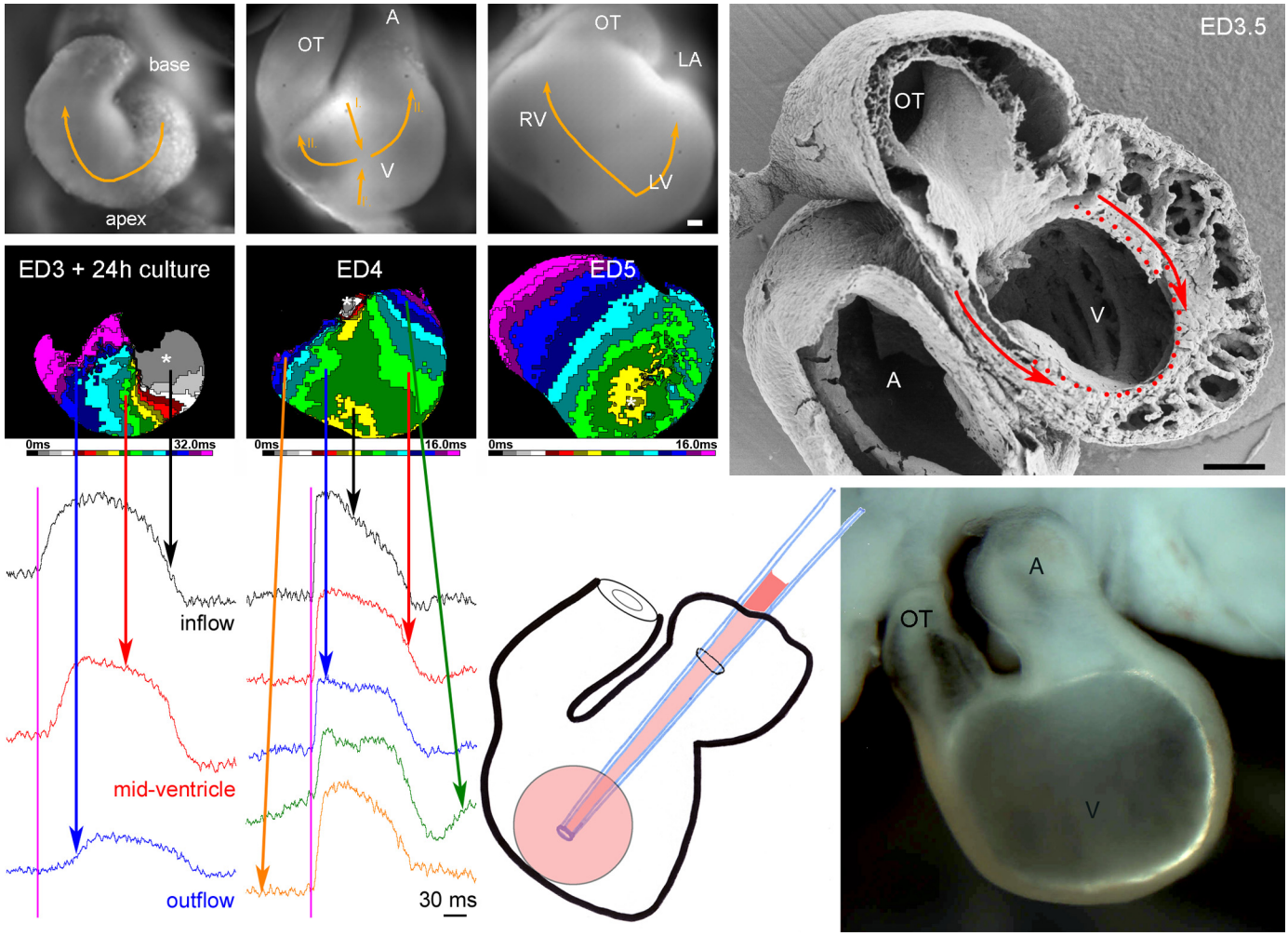

Fig. 1. Classification of ventricular activation patterns in chick embryonic hearts. *Top*, *left* to *right*: 3 patterns (base to apex, primary ring, and apex to base) most prevalent at these developmental stages. Direction of spread of activation is indicated by orange arrows. *Top*, *far right*: propagation of activation through the primary ring in a sagittal view [previously unpublished scanning electron micrograph obtained in conjunction with our previous study (21)]. *Bottom*, *first two panels*: optically recorded action potentials; note decreased upstroke velocity in the cultured heart. *Bottom*, *last two panels*: the technique of artificial loading and show silicone-loaded heart after 24 h in culture. A, atrium; LA, left atrium; LV, left ventricle; OT, outflow tract; RV, right ventricle; V, ventricle; ED, embryonic day. Scale bars = 100 μm. Temporal scale is 2 ms per color isochrone in the top first panel and 1 ms per isochrone in the top second and third panels. *Earliest activated region.

ping of epicardial action potential propagation as recently described in detail (12). The spatial resolution with ×10 water immersion objective resulted in 16-μm pixel size, and the temporal resolution was 1 kHz. Spatiotemporal activation maps were constructed using BV_Analyzer software bundled with the Ultima L high speed camera (SciMedia) using the same algorithms (maximal upstroke velocity for activation time detection) and digital filtering as described previously (15).

Morphological evaluation. After the recordings, the hearts were fixed with either 4% paraformaldehyde for whole mount confocal analysis (11) or with Dent's fixative (80% methanol-20% dimethylsulfoxide) for standard paraffin histology. The images were acquired on either a Leica SPE confocal microscope (Leica) or an Olympus BX51 compound microscope fitted with DP71 CCD camera (Olympus) and processed in Adobe Photoshop (Adobe Systems). For additional morphological features of the ventricles, we have also referred to the scanning electron micrograph series (e.g., Fig. 1) from our previous work (21).

Statistical analysis. The activation patterns present at these stages of development could be grouped into three categories (Fig. 1): base-to-apex loop (most primitive, predominating at ED3), primary ring (most frequent at ED4), or most mature apex to base (indicative of functionality of right or left bundle branches). Each group had a minimum of 10 hearts. Differences in the frequency of these patterns between groups were analyzed using Pearson's χ^2-test (15), and values of $P < 0.05$ were considered statistically significant.

RESULTS

Three patterns of ventricular activation encountered between ED3–5 are illustrated in Fig. 1. During normal in ovo development, a gradual transition from the most primitive base-to-apex activation, following blood flow in the looped tubular heart to the mature apex-to-base pattern, revealing the initiation of function of the bundle branches was observed (Figs. 2 and 3). A schematic representation of the activation pathway along the primary ring structure within the forming interventricular septum is shown in Fig. 1.

The effect of complete hemodynamic unloading in culture on the activation patterns was dramatic. Hearts explanted at ED3 and cultured for 24 h all exhibited the most primitive base-to-apex pattern of activation (Fig. 2). This was highly significantly different (χ^2, $P = 8 \times 10^{-7}$) from the normal spectrum at ED4, where the activation through the primary ring predominated. Hearts explanted at ED4 showed a similar reversal of the activation sequence, but about one quarter

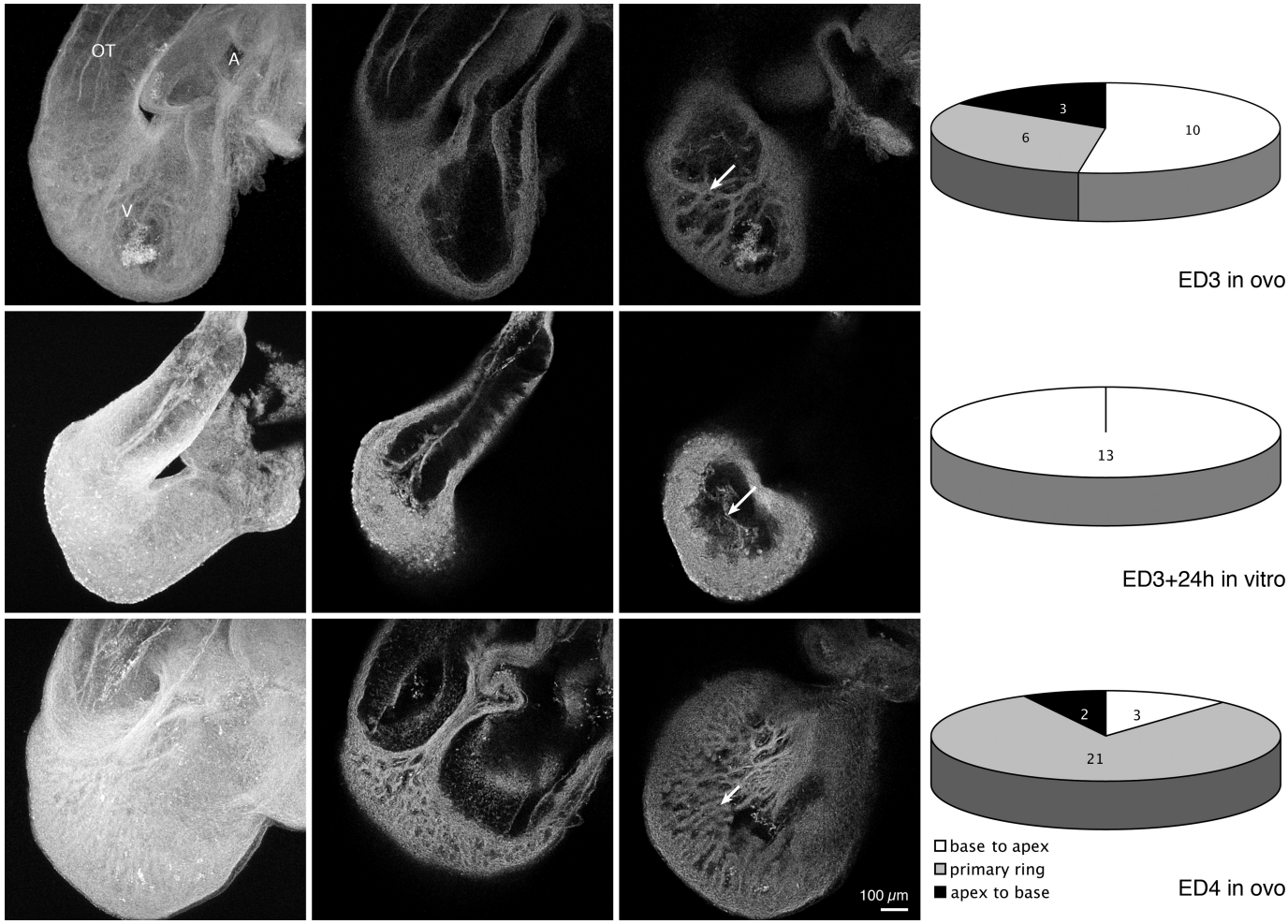

Fig. 2. Morphology of hearts and ventricular activation patterns between ED3 and ED4. First column shows maximum intensity projection of confocal series (*middle two panels*) through the hearts. The last column shows the increase in proportion of hearts activated through the primary ring between ED3 and ED4 and complete abolition of this pattern by in vitro culture without mechanical loading. Arrows point to ventricular trabeculae, which show abnormal morphology in the cultured hearts; there is also elongation of the outflow tract and collapse of its lumen.

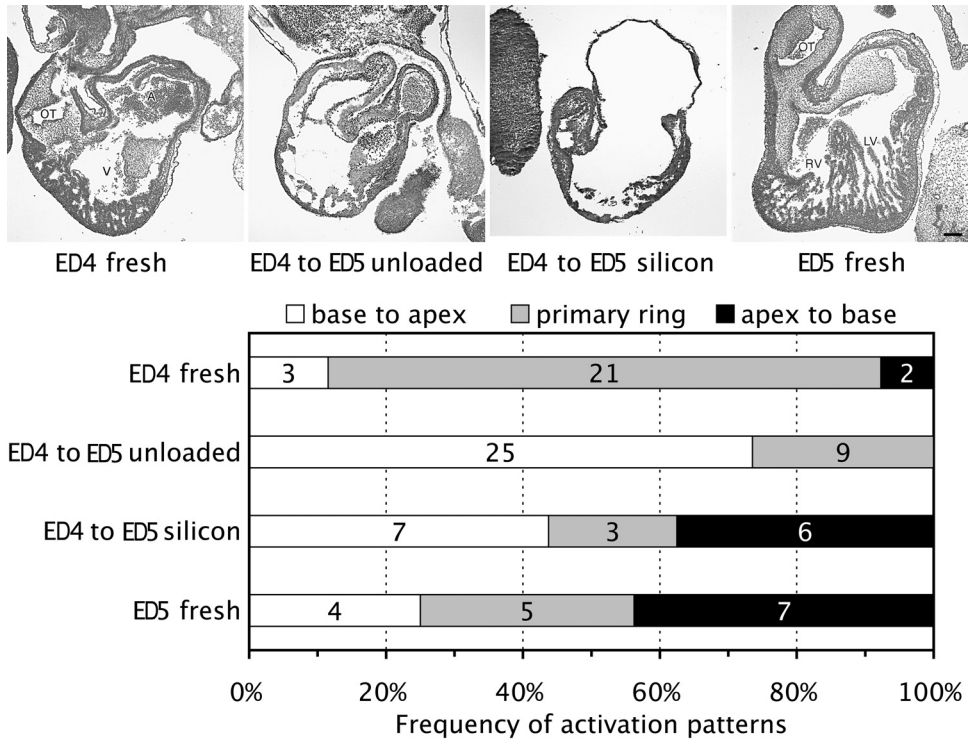

Fig. 3. Heart structure and activation patterns between ED4 and ED5. Histological sections in the frontal plane show increase in trabecular abundance that is inhibited in culture. Activation pattern maturation progresses to increasing proportion of mature apex-to-base pattern that is not observed in culture. Rescue experiment with artificial loading of ventricle with silicon oil shows normalization of activation pattern spectrum. Scale bar = 100 μm.

showed a more advanced activation through the primary ring (Fig. 3). This was still highly significantly different ($P = 6 \times 10^{-5}$) from patterns normally encountered at ED5, where the apex-to-base activation was the most frequent but the other possibilities were also fairly common.

Morphologically, the preseptation stages between ED3–5 were characterized by an increasing complexity of ventricular trabeculation (Figs. 2 and 3), which contributed to the formation of the interventricular septum from ED4 and starting the septation of the atria and outflow tract. The effect of culture on cardiac morphology was manifested by the arrested growth, elongation, and collapse of the outflow tract and the perturbation of the trabecular architecture, more severe with explantation at ED3 (Fig. 2).

The artificial loading of the ventricle with a droplet of silicon oil at ED4 (Fig. 3) normalized the functional maturation of the ventricular conduction system. The distribution of activation patterns was similar to the one normally found at ED5 in ovo ($P = 0.49$) and significantly differed from that of their cultured unloaded counterparts ($P = 0.0007$).

To separate the effect of active myocyte contraction induced by loading from that of passive stretch itself, we performed the same silicon-loading experiment in the presence of excitation-contraction uncoupler blebbistatin (Sigma, 25 μmol), the same substance that we used during optical mapping. The activation patterns of silicon-loaded ED4 hearts cultured for 24 h in the presence of blebbistatin were similar to those without this motion inhibitor, pointing to a crucial role of passive stretching in the early conduction system differentiation.

DISCUSSION

The patterns of ventricular activation described in this study are similar to those previously reported in the chick (3, 15) and confirm the gradual transition from the base-to-apex peristaltoid-like activation toward the mature apex-to-base pattern, implicating the functional His-Purkinje system. In fact, the present work is more similar to the data of Chuck and colleagues (2, 3) than to our own older data (15), since we show the presence of apex-to-base activation patterns from the earliest stages examined. We attribute this apparent difference to the remarkable sensitivity of the CCS to temperature since our first description extending up to ED10 was performed at 35°C rather than the customary 37°C to prevent hypoxia-related arrhythmias (atrioventricular conduction block) at later stages of development.

In comparison to our previous studies in chicks (22, 25) and rats (23), we change the interpretation of the observed ventricular activation patterns. Having studied carefully the mouse data by Rentschler and colleagues (17) as well as the morphological studies by Wessels and associates (10, 26), we now recognize both the anterior and posterior preferential conduction pathways coursing from the atrioventricular canal along the forming interventricular septum as being part of the primary ring (Fig. 1). While only the epicardial mapping data are presented in the present study, it was previously shown that there is a good correlation between epicardial and endocardial maps in the chick embryo, both at the later stages where the activation courses down the interventricular septum and then spreads through the trabecular network (15) as well as at the earlier stages (ED3-ED6) where separate epicardial and endocardial surfaces of bisected hearts showed an independence of the anterior and posterior activation pathway through the primary ring (22). Our data thus confirm the functional importance of this structure as the first fast ventricular activation pathway predating the His-Purkinje system.

Two hypotheses governing the induction of the CCS were proposed: *1*) direct myocyte conditioning by mechanical factors (25) and *2*) paracrine induction via soluble factors such as endothelin or neuregulin acting at short distance (5, 17). Both these hypotheses agree that the conversion from primitive working myocytes to conduction myocytes can only happen during a narrow time window. There is solid evidence that endothelin can induce in vivo the ectopic formation of Purkinje fibers in the chick during stages where such a conversion normally develops (24). In agreement with our recent study on endothelin receptor inhibition, which was able to delay CCS function maturation at ED8 and 9 but not at ED2 to 4 (20), we show here that at the early stages of CCS formation (ED3–5), the necessary and sufficient stimulus is simply adequate myocyte stretching. In the absence of mechanical loading, the CCS function does not manifest and trabecular development is abnormal.

There are few reported pharmacological interventions with the primary ring function. In our experiments with endothelin receptor blockade using dual antagonist bosentan (20), we found that while there was clearly an inhibitory effect at the later stage of the His-Purkinje system formation, the primary ring function was unperturbed by treatment between ED2 and ED4. In contrast, experiments with neuregulin treatment in whole mouse heart organ culture (17) could be interpreted as a positive effect on the primary ring-dependent activation pathway, which is indeed also present in the early mammalian heart (23, and unpublished observations). This would be consistent with the upregulation of the CCS markers reported by Rentschler and colleagues (17).

The mechanisms for the transmission of mechanical loading into changes in gene expression that lead to the conduction phenotype are still largely unknown. There are Gd-sensitive stretch channels, the blockade of which was shown to lead to the inhibition of conduction system differentiation in the chick embryo (9). In later stages of CCS formation, the sensor might be the endocardium or arterial endothelium, since its perturbation by the removal of glycocalyx leads to an abnormal oscillatory stress response and failure of endothelin production (1, 28). Our unpublished results from chick gene chip array comparing sham-operated and banded hearts did not uncover any likely candidate genes; however, the proteomics approach did bring up some candidate proteins, which are currently the subject of further identification (Sedmera and Krug, unpublished observations).

In conclusion, this study extends our previous studies where cardiac and CCS development was manipulated using hemodynamic interventions (9, 15) with resulting accelerated CCS maturation by an increased pressure loading of the ventricles. It is conceivable that CCS originally developed under mechanical influences with a later reinforcement by paracrine signals from the endocardium.

ACKNOWLEDGMENTS

We thank Eva Kluzakova, Marie Jindrakova, and Michal Tuma for excellent technical assistance.

GRANTS

This work was supported by Ministry of Education Grants VZ 0021620806, AV0Z50450515, and AV0Z50110509 and Grant Agency of the Czech Republic Grant 304/08/0615. D. Sedmera is also a recipient of the Purkinje Fellowship from the Academy of Sciences of the Czech Republic.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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