Effect of chronic verapamil treatment on ventricular function and growth in chick embryos

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embryos. Am. J. Physiol. 261 (Heart Circ. Physiol. 30): H166-
H171, 1991.—Adjustment of myocardial mass to work load is a
fundamental characteristic of the heart. We studied the effect
of verapamil, a calcium channel blocker, on growth and function
of chick embryonic ventricle. We treated stage 18 chick
embryos with verapamil delivered to the extraembryonic vascular
bed by a miniosmotic pump and compared them with saline-treated control and untreated embryos. At stages 24, 27,
and 29, we measured ventricular pressure and dP/dt by a servo-
null system, dorsal aortic stroke volume and dV/dt by pulsed-
Doppler, and ventricular and embryo wet weights. Mean my-
ocyte profile area was measured by digital planimetry tech-
nique, and cell growth response by DNA and protein assay.
Verapamil treatment decreased ventricular pressure in experi-
mental (P < 0.05) compared with saline control and normal
embryos; at stage 27, 1.50 ± 0.21 vs. 2.17 ± 0.05 and 2.35 ±
0.08 (SEM) mmHg, respectively. Mean dorsal aortic blood flow
decreased in experimental (P < 0.05) vs. control and normal
embryos; at stage 27, 0.98 ± 0.07 vs. 1.54 ± 0.10 and 1.56 ±
0.07 mm/s, respectively. Stroke volume remained the same in
all experimental, normal, and control embryos except at stage
29. Ventricular weight decreased in experimental (P < 0.05) vs.
control and normal embryos; at stage 27, 1.09 ± 0.07 vs. 1.51 ±
0.08 and 1.54 ± 0.11 mg, respectively. Embryo weights, myocyte
size, and cytoplasmic fractional volume were similar in all
groups. Morphology of ventricles was normal. DNA was lower
in experimental (P < 0.05) compared with control and normal
embryos. Ratio of DNA to total protein was similar in each
group at each stage. Thus the smaller experimental heart
contained fewer cells of normal size. The capacity to decelerate
ventricular growth is present even at early stages of cardiovas-
cular development.

HEMODYNNIC FORCE is one determinant of myocardial
structure, composition, and function in the mature heart.
The adjustment of myocardial mass to work load is a
fundamental characteristic of the heart. The cellular
response to an increase in work load depends on the
maturity of the animal. In the mature heart, myocyte
hypertrophy is the response to increased functional load
(15). In juvenile heart, an increase in ventricular size by
cell hyperplasia and cellular hypertrophy is produced by
increased ventricular pressure (12, 13). The fetal heart
responds primarily by myocyte hyperplasia (14). The
response to a decrease in work load also varies with the
maturity of the animal. In the adult heart, myocardial
cell size decreases in response to mechanical unloading
(14). In fetal lamb, a prolonged decrease in preload
reduces left ventricular mass (6).

We hypothesized that hemodynamic force is also im-
portant in the morphogenesis and growth of the embryo-
onic heart. The chick embryo heart accelerates ventric-
ular growth by myocyte hyperplasia in response to a
sustained increase in ventricular pressure (4). We now
report the effects of chronic calcium channel blockade
on ventricular growth and development in the ventricle
of chick embryos. Verapamil suffused on the extraem-
byronic vascular bed decreased systemic and ventricular
blood pressure and decreased the rate of ventricular
growth without affecting morphogenesis or myocyte size.

MATERIALS AND METHODS

Fertile white Leghorn chicken eggs were incubated
blunt end up in a forced-draft 38.5°C incubator to Hamb-
erger-Hamilton stage 18 (3 days; see Ref. 7). The em-
byro was exposed by opening a small window in the egg
shell and removing the inner shell membrane adjacent
or the embryo. One end of PE-60 polyethylene tubing
was positioned on the surface of the extraembryonic
vascular bed, and the other end was attached to the flow
modulator of an Alzet miniosmotic pump (Alza, Palo
Alto, CA) (Fig. 1). The system was filled with 0.9% saline
solution, and the pump was immersed in normal saline.
The pump had a constant flow rate of 1 μl/h with a
reservoir capacity of 200 μl. We suffused the extraem-
byronic vascular bed of the experimental embryos with
verapamil at 1 ng/h and the sham controls with 0.9%
saline. Normal embryos had only the shell and shell
membrane removed. The window in the shell was covered
with parafilm, and the eggs were returned to the incu-
bator. Embryos were harvested at Hamburger-Hamilton
stages 24 (4 days), 27 (5 days), and 29 (6 days) (7). These
stages were selected because there is an approximate
twofold increase in embryo mass between stages (8).

Hemodynamic measurements. We measured the ven-
tricular pressure with a model 900 servo-null micropres-
Embryo and ventricular weight. An embryo was removed from the vitelline vascular bed and extraembryonic membranes, rinsed with normal saline, then gently blotted to remove excess water, and weighed on a Mettler electronic balance accurate to ±10 μg. The ventricle was weighed after the atria and great vessels were removed. At each stage, 6-10 groups of five pooled hearts were weighed.

Mean myocyte cytoplasmic area and proportion measurements. After the ventricles were harvested, the specimens were immediately fixed in 2.5% glutaraldehyde and 2.0% paraformaldehyde in 0.1 M cacodylate buffer (9). The samples were postfixed in osmium tetroxide, stained with uranyl acetate, dehydrated, and embedded in Spurr’s plastic. The orientation of myocytes in embryonic heart was random (10). Randomized thin sections were cut from each ventricle, stained with lead citrate, and photographed in a transmission electron microscope. Final magnification of the micrographs was ×6,500 and ×20,000.

Ten micrographs (×6,500) per embryo from the normal, saline control and experimental groups were ana-
FIG. 5. Dorsal aortic dV/dt was decreased in verapamil-treated embryos (▲) compared with normal (●) and saline control (■) across matched stages (*P < 0.05).

FIG. 6. Heart rate was decreased in verapamil-treated embryos (▲) compared with normal (●) and saline control (■) embryos across matched stages (*P < 0.05). There was also a decrease of 11% in heart rate when saline control was compared with normal embryo at stage 24.

FIG. 7. Stroke volume was similar in verapamil-treated embryo (▲) compared with normal (●) and saline control (■) embryos at stage 24 and 27 but was decreased at stage 29 (*P < 0.05).

FIG. 8. Ventricular wet weight was decreased in verapamil-treated embryos (▲) compared with normal (●) and saline control embryos (■) across matched stages (*P < 0.05) (A). There were no changes in embryo wet weight (*P > 0.05) (B).

Lyzed with the use of a Zeiss MOP-3 image digital analyzer to estimate mean myocyte cytoplasmic cross-sectional area. The myocyte cytoplasmic area was traced under a magnifying lens to define an accurate myocyte cell membrane.

Ten micrographs (~20,000) per embryo for each group were analyzed by point counting techniques to determine the fractional myocardial cytoplasmic volume of myofibrils and mitochondria (17, 18). Organelles other than those mentioned, including the ground substance but excluding nuclei and vacuoles, were categorized as cytoplasm. The point counting overlay grid consisted of 924 intercepts (points) per micrograph (21.0 × 29.7 cm). The number of intercepts falling on a given structure (Pi) and the total number of points (Pcytoplasm) are related to the relative volume fraction (Vr) of the structure per total cytoplasmic volume (Vcytoplasm) in the following way: Vr/Vcytoplasm = Pi/Pcytoplasm.

Intraobserver and interobserver errors were statistically insignificant by t test (P > 0.27 and P = 0.60, respectively) determined from repeated counts on 10 micrographs.

DNA and protein assay. The ventricle was digested in 0.5 M sodium hydroxide, neutralized to pH 7.4 with hydrochloric acid. The DNA concentration was quanti-
tated by the fluorescence of Hoechst dye 33258. Purified calf thymus DNA (Sigma Chemical, St. Louis, MO) was used as standard (2). The assay was linear from 10 ng to 1 μg of the calf thymus at a dye concentration of 0.1 μg/ml. The protein concentration of the ventricle was determined by a Bio-Rad protein microassay, using bovine serum albumin (Sigma) as the standard (1).

Statistical analysis. Data were expressed as means ± SE. We used a paired t test, analysis of variance, and Tukey’s honestly significant difference for statistical comparison (16). A P value of <5% (P < 0.05) was defined as significant.

RESULTS

Hemodynamics measurements. Each measure of hemodynamic function was decreased in the verapamil-treated embryos compared with sham treated and normal controls. Ventricular peak systolic, end-diastolic, and dP/dt pressures were decreased (Figs. 2 and 3). Dorsal aortic blood flow and dV/dt decreased from 25% to 50% (Figs. 4 and 5). A sustained decrease in heart rate was noted across stages (Fig. 6). Stroke volume was decreased only at stage 27 (Fig. 7).

Embryo and ventricular wet weight and cardiac morphology. Ventricular wet weights of the experimental embryos were decreased in all stages when compared with sham control and normal embryos (Fig. 8A). Embryo wet weights were similar at each stage (Fig. 8B). Heart morphology of verapamil-treated embryos was similar to normal and saline control embryos. The separation of the atrioventricular canal, formation of the atrial and ventricular septa, and the division of the conotruncus all proceeded at the same rate.

Myocyte characteristics. Mean myocyte cytoplasmic area was similar in experimental, normal, and control embryos (Fig. 9). The myocyte percent volume of mitochondria was 13.7 ± 0.5% in verapamil vs. 13.7 ± 0.5 and 11.0 ± 0.5% in control and normal embryos, respectively (Fig. 10). Although statistically significant, this 3% variation was likely within the biological norm. The proportion of myofibrils was similar in verapamil (24.2 ± 1.2%), control (20.9 ± 1.1%), and normal (23.6 ± 1.1%) embryos.

DNA and protein assay. DNA-to-protein ratios of the experimental ventricle were similar to normal and control embryos (Fig. 11). At stage 29, the ventricular total protein of the verapamil group was (in pg) 129.9 ± 7.8 vs. 163.2 ± 7.7 in control and 187.3 ± 17.3 in normal groups (Fig. 12A). A similar decrease also occurred in DNA at stage 29 with (in pg) 7.7 ± 1.6 in experimental, 13.4 ± 1.5 in control, and 17.7 ± 2.8 in normal embryos (Fig. 12B).

DISCUSSION

Ventricular work load correlates directly with myocardial mass in the developing and mature heart. In the mature animal, myocyte hypertrophy is the response to a sustained increase in functional load (14). In the immature postnatal animal, increased ventricular pressure results in a combination of hypertrophy and hyperplasia (15). In early postnatal mammals, the ventricle responds to both qualitative and quantitative changes (12).

Our research group is studying the interrelationship of form and function during embryonic development. We found that an increase in ventricular pressure from banding the outflow tract in stage 21 chick embryos results...
in myocyte hyperplasia independent of morphogenesis (4).

Conversely, decreased functional load also affects ventricular growth. In the mature cat, right ventricular myocardial structure returns to normal when a pressure overload is removed from banded pulmonary arteries (11).

Severing the chordae tendineae of a right ventricular papillary muscle in cats causes the muscle to atrophy; this is direct evidence that mechanical unloading affects myocardial cell size and structure (15). Atrophy is reversed by reattaching severed chordae tendineae, suggesting that papillary muscle atrophy is a response to a decrease in functional load (14). Similarly, in fetal lamb hearts, decreased preload reduces left ventricle/right ventricle mass ratio and chamber volume (6).

Our study reports the effects of chronic calcium channel blockade on ventricular pressure and ventricular growth. The chronically verapamil-treated embryos had decreased ventricular blood pressure and cardiac output and had smaller hearts by weight and size compared with normal controls. Mean myocyte cytoplasmic area and the proportion of myocyte organelles and DNA:protein ratio were similar in verapamil-treated, saline sham control, and normal hearts. The cellular response apparently was a decrease in myocyte number rather than a decrease in size, although absolute myocyte size, including the nucleus, was not determined.

Thus the embryonic ventricular growth can be regulated, slowed as well as accelerated. Ventricular growth is likely regulated by a mechanical-chemical link between ventricular function and myocyte growth (5). The mechanism(s) that regulates cell proliferation in these experiments may be either a direct effect on myocardial contractility or a change in calcium as a messenger for cell regulation. A change in myocyte stress or strain may be a mechanical signal that adjusts the rate of ventricular growth. Alternatively, a change in intracellular calcium may adjust a calcium-modulated messenger cascade that regulates the expression of growth factors or other modulators of growth.

These experiments also confirm that a fundamental characteristic of the heart, the capacity to adjust myocardial mass to work load, is present in the embryonic heart. Cardiac work load during embryonic and fetal development is an environmental influence that affects cardiovascular development. The embryonic heart is a rich model for defining the mechanisms that control physiological and pathological growth of the myocardium.

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