Annexin VI overexpression targeted to heart alters cardiomyocyte function in transgenic mice

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Gunteski-Hamblin, Ann-Marie, Guojie Song, Richard A. Walsh, Marie Frenzke, Gregory P. Boivin, Gerald W. Dorn II, Marcia A. Kaetzel, Nelson D. Horseman, and John R. Dedman. Annexin VI overexpression targeted to heart alters cardiomyocyte function in transgenic mice. *Am. J. Physiol.* 270 (*Heart Circ. Physiol.* 39): H1091–H1100, 1996.—Annexin VI is a member of a family of Ca^{2+}-dependent phospholipid-binding proteins that is expressed in many tissues, including the heart. It is a regulator of membrane-associated events, including the skeletal muscle ryanodine-sensitive Ca^{2+} release channel and the cardiac Na^+/Ca^{2+} exchanger. The potential roles of annexin VI in Ca^{2+} signaling in cardiac myocytes were evaluated by targeting its overexpression to the hearts of transgenic mice. Expression of full-length human annexin VI cDNA was targeted to the heart using the α-myosin heavy chain gene promoter (Subramaniam, A., W. K. Jones, J. Gulick, S. Wert, J. Neumann, and J. Robbins. *J. Biol. Chem.* 266: 24613–24620, 1991). Five transgenic lines exhibited at least 10-fold overexpression of annexin VI protein in both atria and ventricles. Pathological evaluation indicated mice overexpressing annexin VI had enlarged dilated hearts, acute diffuse myocarditis, lymphocytic infiltration, moderate to severe fibrosis throughout the heart, and mild fibrosis around the pulmonary veins of the lungs. Contractile mechanics of cardiomyocytes isolated from hearts of transgenic animals showed frequency-dependent reduced percent shortening and decreased rates of contraction and relaxation compared with control animals. Cardiomyocytes isolated from transgenic animals had lower basal levels of intracellular free Ca^{2+} and a reduced rise in free Ca^{2+} following depolarization. After stimulation, intracellular free Ca^{2+} returned to basal levels faster in transgenic cells than in cells from control animals. These data demonstrate that the overexpression of annexin VI in the heart disrupts normal Ca^{2+} homeostasis and suggests that this dysfunction may be due to annexin VI regulation of pumps and/or exchangers in the membranes of cardiomyocytes.

calcium; binding proteins; cardiomyopathy; annexins

TRANSIENT INCREASES in intracellular Ca^{2+} within cardiomyocytes serve as the initial signal for contraction in the heart. Movement of extracellular Ca^{2+} into cardiomyocytes occurs through voltage-dependent Ca^{2+} channels in the sarcolemma. Ca^{2+} entry via L-type channels triggers Ca^{2+}-induced Ca^{2+} release from the sarcoplasmic reticulum (SR) through the ryanodine-caffeine-sensitive Ca^{2+} release channel and activates contraction of myofilaments. During relaxation, Ca^{2+} is removed from the cytosol by uptake into the SR through the phospholamban-modulated SR Ca^{2+} pump and by the sarcolemmal Ca^{2+} pump and the Na^+/Ca^{2+} exchanger (see Langer, 19).

Various proteins within myocytes serve as mediators of the Ca^{2+} signal, thereby regulating the function of other cellular proteins through Ca^{2+}. These include calmodulin, troponin C, and the annexins. Annexins are a family of Ca^{2+}-dependent, phospholipid-binding proteins that do not contain the EF-hand motif typical of many other Ca^{2+}-binding proteins. Several annexins are expressed within the heart (22, 30). Annexin VI is a unique member of this family of proteins in that it contains eight conserved repeat domains instead of four (34). The primary mRNA transcript for annexin VI is alternatively spliced to produce two forms: one encodes an additional six amino acids at the beginning of the seventh repeat domain (28). This longer form is the predominant splice variant expressed in most tissues, including the heart (16). In vitro studies have suggested that annexin VI is a potent regulator of the skeletal muscle SR Ca^{2+} release channel (6, 12) and Na^+/Ca^{2+} exchanger (32) and is associated with atrial secretory granules (8). Immunolocalization studies have shown that annexin VI is one of the major cardiac annexins (9, 16, 22, 30).

The in vivo role of annexin VI is not known. One approach to elucidate its cellular role is to disrupt the gene in embryonic stem cells. Annexin VI, however, is expressed in a large number of tissues, suggesting that the protein is involved in many cellular processes. Therefore, overexpression of annexin VI targeted to the heart, both atria and ventricles, was accomplished so that the effect of aberrant annexin VI protein levels on normal heart function could be evaluated without affecting annexin VI expression in other tissues.

MATERIALS AND METHODS

Annexin VI transgene construct. An 0.85-kb *Xho* I-*Bam*H I fragment containing the SV40-intervening sequence and polyadenylation signal was isolated from vector pMAMneo and ligated into *Sma* I-*Bam*H I-digested vector pBluescript II (SK−), producing a 3.8-kb clone 25C. The human annexin VI cDNA encoding the longer splice variant (kindly provided by S. Moss, University College, London, UK) was digested with *Xho* I-*Apa* I to produce a 2.2-kb fragment containing the entire coding region (4, 27, 28). This fragment was inserted into *Xho* I-*Pst* I-digested clone 25C in which the *Pst* I site was blunt ended, producing 6.0-kb clone 3D. The 5.5-kb *BamH* I-*Sal* I fragment of the α-myosin heavy chain (α-MHC) gene promoter isolated from clone 20 (kindly provided by J. Robbins, Univ. of Cincinnati) was inserted into *Xho* I-digested clone 3D producing the 11.5-kb clone 2E-1 (see Fig. 1). Each of

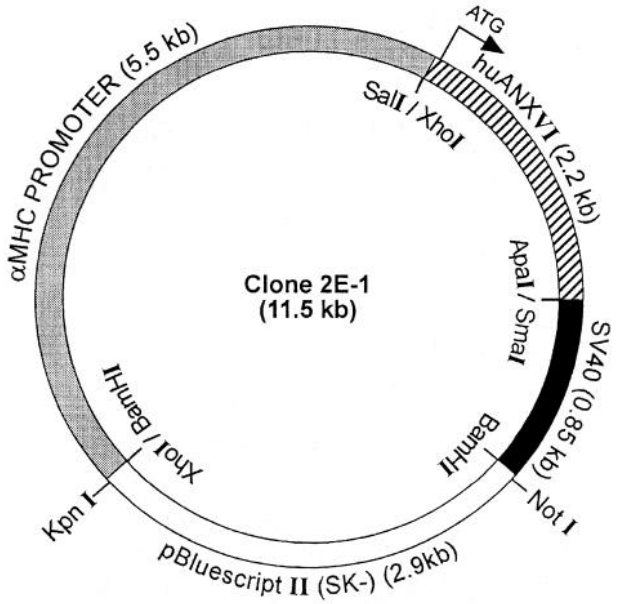

Fig. 1. Map of the annexin VI transgene construct. A 2.2-kb fragment containing the entire coding region of the human annexin VI cDNA (huANXVI) was ligated to the alpha myosin heavy chain gene promoter (α-MHC) and SV40 intervening sequence and polyadenylation signal (SV40). The restriction enzyme sites used in preparation of the construct in vector pBluescript II are shown. The *Not* I and *Kpn* I unique restriction enzyme sites used to release the transgene from the vector for injection into oocytes are also shown. Location of the translation initiation codon (ATG) and direction of translation are indicated.

the intermediate constructs and the complete clone, clone 2E-1, were confirmed by restriction enzyme mapping and sequencing.

Production of transgenic mice. The 8.5-kb DNA fragment was released from the pBluescript II (SK−) vector by *Not* I-*Kpn* I digestion and isolated on a 1% agarose gel. The desired fragment was cut out of the gel and purified using the Geneclean II system (Bio 101, La Jolla, CA) according to manufacturer's directions. Purified DNA was eluted from the Glassmilk using injection buffer [5 mM tris(hydroxymethyl) aminomethane (Tris) and 0.1 mM EDTA, pH 7.4]. Microinjection was performed in single–cell embryos derived from superovulated FVB/N females. Surviving microinjected embryos were implanted into pseudopregnant FVB/N foster mothers (13). Founder mice were identified using the polymerase chain reaction (PCR). Two PCR primers, TSHβ1 (5′-TCCTCAAAGATGCTCATTAG-3′) and TSHβ2 (5′-GTAACT-CACTCATGCAAAGT-3′), were used to amplify a 386-bp control fragment of the single copy thyroid-stimulating hormone β-gene in all of the PCR reactions. Primers to the 3′-untranslated sequence of the human annexin VI cDNA (huANXVI1: 5′-GGCTCTTATCTTCAGTGGAG-3′) and the 5′ end of the SV40-intervening sequence (SV401: 5′-GCAAG-CAAGAGTTCTATTAC-3′) were used to PCR amplify a 424-bp fragment from the transgene. Nontransgenic FVB/N mice were bred with the founder mice to produce stable transgenic lines.

Total protein isolation and Western blotting. Gel electrophoresis and immunoblot analyses were performed as previously described (15). Sheep anti-annexin VI antibody was prepared as described by Mathew et al. (24) and antigen affinity purified according to Kaetzel et al. (15).

Histochemistry. Tissues were removed from freshly killed animals, immediately fixed in 10% neutral buffered Formalin for 16–20 h at room temperature, and then transferred into phosphate-buffered saline (pH 7.4). Tissues were dehydrated through a gradient of alcohols, paraffin embedded, and sectioned at 4 μm thickness. Mounting of sections, preparation of hematoxylin-eosin and trichrome-stained slides, and analyses of pathology were done in the Division of Comparative Pathology (University of Cincinnati).

Immunolocalization. Preparation of tissue, sectioning, incubations, and photography were done as previously described (15) except that deparaffinized sections were incubated for 2 h with affinity-purified sheep anti-rat annexin VI antibody (OD_{280} = 0.123).

Cardiac myocyte preparation. Left ventricular myocytes were isolated from the hearts of mice overexpressing annexin VI and from nontransgenic littermate controls by a modified method (7). Briefly, animals were anesthetized with methoxyflurane, and the heart of each animal was rapidly excised and placed in a dish of oxygenated Ca^{2+}-free Joklik's modified buffer pH 7.2 (GIBCO-BRL, Gaithersburg, MD). The aorta was cannulated with a 23-gauge needle, flushed briefly with buffer, and mounted onto a perfusion apparatus. The right ventricular outflow tract was excised, and the coronary arteries were perfused at 2.2 ml/min first with Ca^{2+}-free Joklik's buffer for 4 min followed by Joklik's buffer containing 25 μM Ca^{2+}, 75 U/ml collagenase I, 75 U/ml collagenase II, 1% albumin, and 2% donor calf serum, pH 7.2. The perfusion temperature was maintained at 37°C, and all buffers were continuously bubbled with 95% O_2-5% CO_2. After 12–15 min of perfusion, the heart was removed from the perfusion apparatus and transferred to a watch glass containing low-Ca^{2+} Joklik's buffer (25 μM Ca^{2+} and 2% donor calf serum), and the left ventricle was isolated, minced, and gently pipetted into a 50-ml conical tube containing 10 ml of the buffer. The tissue was agitated to release loosened cells into the solution, which were then allowed to settle. Supernatant containing the isolated cells was immediately transferred to a new 50-ml conical tube. Isolation of the cells was repeated four times, and the cell supernatants were pooled. The pooled supernatant was centrifuged at 500 rpm for 1 min, and the cell pellet was resuspended in 20 ml low-Ca^{2+} Joklik's buffer. After the cells were allowed to settle for 15 min, they were resuspended in physiological buffer (in mM: 132 NaCl, 4.8 KCl, 1.2 $MgCl_2 \cdot 6H_2O$, 5 glucose, and 10 *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.2) supplemented with 1.4 mM Ca^{2+}.

Ca^{2+} measurements. Cytosolic free Ca^{2+} was measured by ratio imaging of fura 2 fluorescence as described by Borzak et al. (1) with minor modifications. Myocytes were loaded with fura 2 by incubation of a 1-ml suspension of isolated cardiac myocytes for 30 min at 37°C with 2.5 ml of 5 μM fura 2-acetoxymethyl ester (AM) in low-Ca^{2+} Joklik's buffer. Fura 2-AM-loaded myocytes were allowed to settle, and the resulting pellet was resuspended in physiological buffer as described above. The fura 2-AM-loaded cells were placed in a perfusion chamber on the stage of an inverted epifluorescence microscope (Olympus IMT-2) and constantly superfused with oxygenated physiological buffer at room temperature. Images of the cells were simultaneously acquired through a charge-coupled device (Panasonic model GP-CD60), viewed on a monitor (Sony PVM-122), and illuminated by a PTI Delta Scan-1 dual-beam spectrophotofluorometer (Photon Technology International, South Brunswick, NJ) at alternating excitation wavelengths of 340 nm (Ca^{2+} independent) and 380 nm (Ca^{2+} dependent). Emission was monitored at 510 nm. After background subtraction, images at 340 nm were divided by those at 380 nm to determine fluorescence ratios. Signals were analyzed by the OSCAR software (Photon Technology

International). Cells were stimulated to contract using a Grass S9 stimulator, and a platinum wire field electrode was connected to a bipolar switch box (model API-94, Innovative Lab Instruments, Cincinnati, OH).

Measurements of isolated cardiac myocyte mechanical properties. Measurements of morphological and contractile parameters were done in a perfusion chamber using the same isolated myocytes used for the fura 2-AM studies. Two platinum electrodes placed in the bathing fluid were connected to a Grass S9 stimulator, and the myocytes were field stimulated with pulses of 2 ms duration at frequencies of 0.25, 0.5, and 1.0 Hz (15, 30, and 60 beats/min). Percent shortening, rate of shortening, and rate of relengthening were measured as previously described (7). Myocyte dimensions measured from the videotaped images were compared with a calibration micrometer on the microscope stage. Measurements of the length and width of each selected myocyte were determined by recording the image of each selected cell on videotape before pacing. Cellular mechanics and intracellular Ca^{2+} were monitored in the following sequence: record baseline and signals of the paced cell at 0.25 Hz for 40 s, then continue to record and pace for 20 s at each of the stimulation rates of 0.5 and 1.0 Hz, then back to 0.25 Hz. Pacing was continuous through stimulation rate changes. Data average is reported as the means ± SE for the indicated number of animals. Four to eight myocytes were analyzed from each of five transgenic and five nontransgenic littermate control animals. The cells used for the mechanical studies were the same cells used for the Ca^{2+} dynamics. One-way and two-way analyses of variance (ANOVA) and Newman-Keuls multiple range test were used as appropriate for statistical analysis. Probability was considered significant at $P < 0.05$.

RESULTS

Analysis of transgenic lines. Sixteen transgenic founder animals were identified by PCR analysis of DNA isolated from tail clips to contain the human annexin VI transgene. The *line 13* founder was infertile. *Lines 1, 7, 9, 12*, and *15* did not produce transgenic offspring when bred for at least 50 pups and were not pursued further. The remaining 10 lines produced offspring that were verified as transgenic by PCR (data not shown). Transgenic offspring from *line 6* had lower average body weights compared with their nontransgenic littermates and lived only 29 days on average. *Line 6* transgenic mice exhibited difficulty in breathing, listlessness, and an unkempt appearance by *day 15* after birth. None of the *line 6* F1 generation mice lived long enough to breed for F2 generation animals. *Line 11* transgenic animals exhibited similar symptoms and moderate pathology, but their onset did not develop until the animals were at least 3 mo of age. Animals from *line 11* began to succumb to congestive heart failure at 5–6 mo of age. Transgenic animals from *lines 2* and *4* exhibited mild to moderate pathology. *Lines 2* and *4* animals were followed for more than 1 yr. A few transgenic animals from these lines died starting from 10 mo of age. To test for the effect of gene dosage, heterozygote mice (+/−) from *lines 2* and *4* were crossed. These offspring showed severe heart pathology and died before being weaned at *day 21*. Pathology done on aging animals from multiple lines indicated that, in general, heart function became more impaired as the animals aged. However, the degree of impairment varied from animal to animal even among littermates.

Determination of protein levels. Hearts from F1 and F2 generation offspring were removed, the atria and ventricle were separated, and the tissues were prepared for Western blot analysis to determine annexin VI protein levels. Total protein (20 μg) was loaded into each lane of a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel, electroblotted, and incubated with affinity-purified anti-annexin VI antibody. Results of total annexin VI protein levels in the ventricles of each transgenic line are shown in Fig. 2*A* (top line). *Lines 2, 4, 6, 11*, and *14* showed very high levels of overexpression (at least 10-fold) of annexin VI in the heart. Analysis of protein levels in atria showed similar results (data not shown). To determine whether overexpressing annexin VI in the heart affected endogenous protein levels of annexin V, which is also abundant in the heart, sheep anti-annexin V antibody was used in Western blot analysis of total ventricle protein from each transgenic line and a nontransgenic control animal. No consistent changes in annexin V protein levels were seen in ventricles from any of the annexin VI overexpressing lines (Fig. 2*A*, bottom line). Western blot analysis could not distinguish between endogenous murine annexin VI protein and human annexin VI protein derived from the transgene, since the two proteins are 95% homologous at the amino acid level (27), the proteins are the same size, and the antibody cross-reacts with both the human and murine species. Northern blot analysis verified that both murine and human annexin VI transcripts were present in each line in both heart and lung tissues. There was no significant difference in annexin VI protein levels in atria versus ventricles or in male versus female transgenic animals (Fig. 2*B*). Western blot analysis of total annexin VI protein levels from multiple tissues obtained from a nontransgenic control mouse, and mice from four of the lines overexpressing annexin VI, showed that annexin VI protein was found in all of the tissues examined, with overexpression being confined to the heart and lung (Fig. 2*C*).

Pathology of overexpressing lines. Most transgenic mice overexpressing annexin VI showed mild to severe lesions in their hearts. Features in common among the transgenic mice with lesions were mild (*lines 2* and *4*) to severe (*line 6*) multifocally extensive and diffuse fibrosis involving both the ventricles and atria. The mice frequently had myocyte necrosis, mild inflammation (lymphocytes and neutrophils), and occasionally mineralization associated with the fibrosis. In the ventricles, the areas most affected by the fibrosis were the myocardium and endocardium of the left ventricle. For an example of a severe case from a *line 6* transgenic animal, see Fig. 4*B*. Frequently, the entire wall of the atria was replaced by fibrous tissue. The lesion distribution and characteristics were suggestive of a loss of myocytes with replacement by fibrous tissue. Pathology was similar among all of the transgenic lines; lesions worsened as the animals aged.

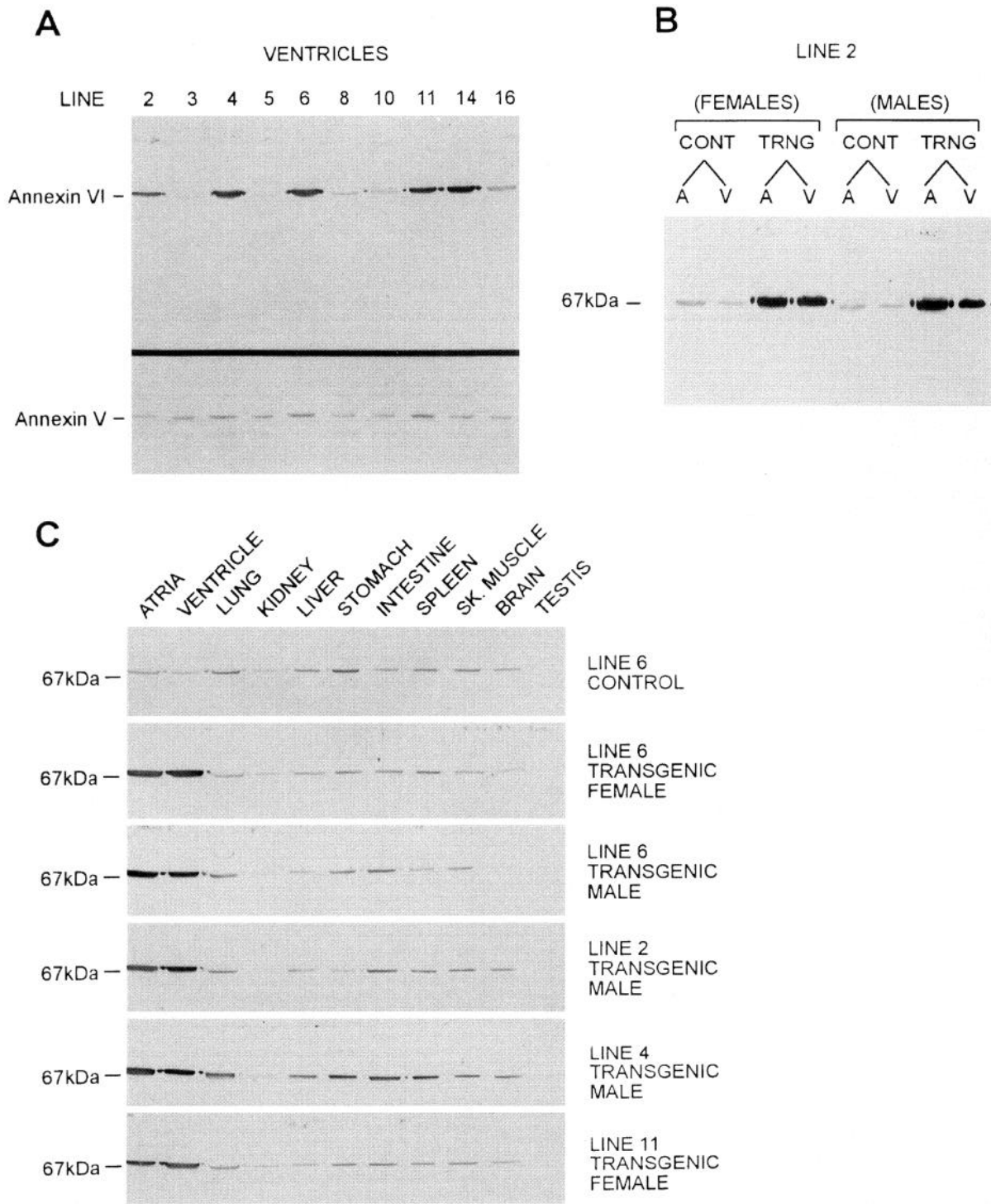

Fig. 2. Analysis of annexin VI protein levels. Relative levels of annexin VI protein (67 kDa) in samples from transgenic and nontransgenic control mice were compared. *A*: top line shows comparisons of annexin VI protein levels in ventricles from transgenic animals representing multiple lines. *Lines 2, 4, 6, 11*, and *14* show at least 10-fold overexpression of annexin VI in both ventricles and atria compared with levels in nontransgenic control animals; bottom line shows comparison of annexin V levels in same lines. Overexpression of annexin VI has no conclusive feedback regulatory effect on annexin V protein levels. *B*: annexin VI protein levels in atria (A) and ventricles (V) from transgenic (TRNG) and nontransgenic littermate controls (CONT) from *line 2*. Note that there is no difference in protein levels between male and female animals. *C*: total annexin VI protein in multiple mouse tissues from 4 transgenic lines that overexpress annexin VI. Mouse tissues analyzed are labeled across top of figure. Annexin VI is expressed in all tissues examined. Control is a nontransgenic littermate of the *line 6* male and female mice shown.

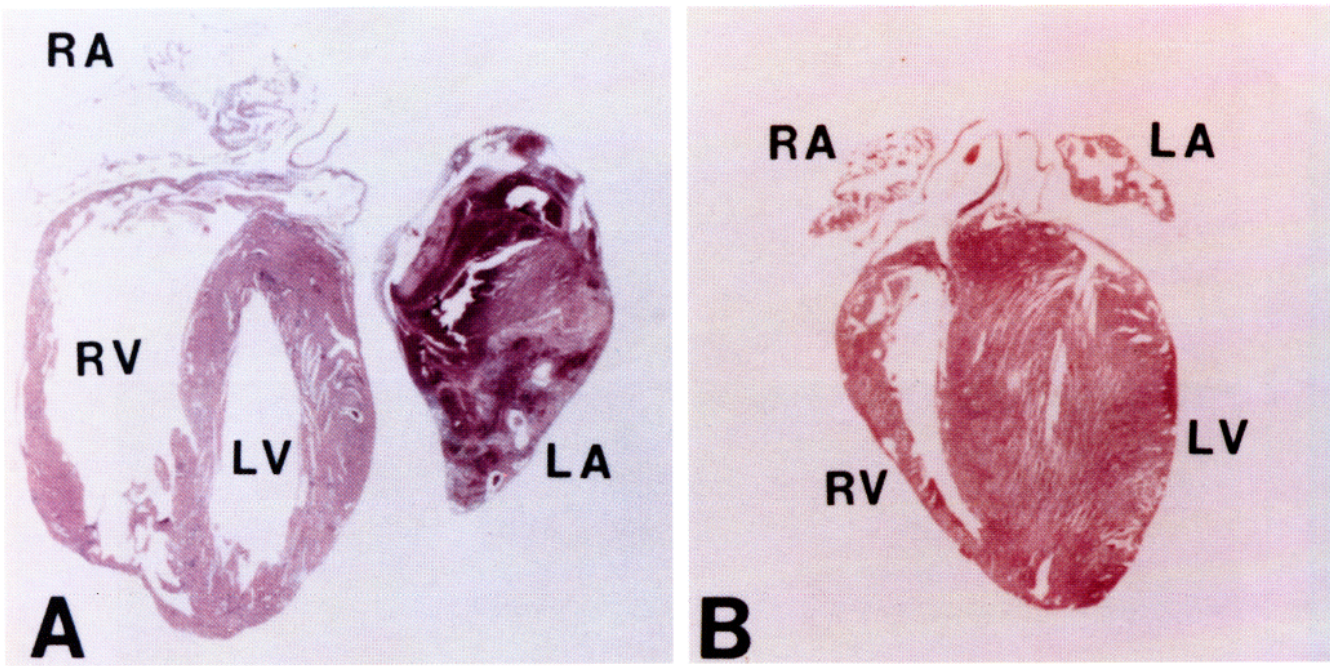

Fig. 3. Histology of whole heart sections from transgenic and nontransgenic age- and sex-matched mice. *A*: *line 6* transgenic heart [Hematoxylin and eosin (H & E) stain; ×2.5]. *B*: nontransgenic heart (H & E stain; ×2.5). RA, right atria; LA, left atria; RV, right ventricle; LV, left ventricle. Dilation of RV and RA and enlargement of transgenic heart is apparent. Note large LA thrombus partially occluding lumen in transgenic heart.

As cardiac function continued to be compromised, there was dilation of the right ventricle and compensatory cardiac hypertrophy (Fig. 3). Cardiac hypertrophy was determined by an increased heart weight-to-body weight ratio in the transgenic mice compared with control mice. The average heart weight-to-body weight ratios for transgenic mice were 0.93% and controls were 0.64%. Transgenic mice that had no histological or mild histological changes had heart weight-to-body weight ratios similar to the control mice. In most mice there was also severe dilation of the atria with large thrombi partially occluding the lumen (Fig. 3*A*). Abnormal blood flow, such as increased chamber turbulence or pooling, was believed to be the cause of the thrombi.

Because of the dilation, the atria typically appeared more severely affected than the ventricles. During embryogenesis, α-MHC gene expression varies. The predominant isoform in the atria throughout development is α-MHC, although this isoform is not predominant in the ventricles until 7 days after birth (3, 23). This results in increased expression of the transgene in the atrium at an earlier stage in development and may have led to more severe damage. Alternatively, because the atrial wall is thin and has less capacity to withstand myocyte damage than the ventricle, the lesions may be due to the decreased compliance of the atrium when damage was initiated.

The pulmonary vein muscular walls in the mouse develop from the same progenitor cells as cardiac myocytes and express the α-MHC gene (11). In the overexpressing transgenic mice, the muscular fibers of these vessels were frequently replaced by fibrous tissue. This suggests that the fibrosis is due to a loss of, or lack of, myocytes, since there did not appear to be displacement of the muscle but rather replacement.

There was also a reduction in the size and decreased cellularity of the thymus cortex in some of the animals with cardiac lesions. The cause of this change is unknown but is probably stress related. There were no significant lesions in any other organs.

Immunocytochemistry. Immunolocalization studies of heart and lung sections from transgenic and nontransgenic control animals were performed using a sheep anti-annexin VI antibody (15). A summary of the results is shown in Fig. 4. Sections from nontransgenic control animals showed localization of annexin VI to the sarcolemma (14) and intercalated disks (Fig. 4*C*). Mice overexpressing annexin VI showed very high levels of the protein throughout the heart, with individual cardiomyocytes showing variations in annexin VI levels (see Fig. 4*D*). Although sections from *line 6* are shown in Fig. 4, results were similar regardless of the line analyzed. These variations were consistent with overexpression targeted by the α-MHC promoter (5, 17), since individual mouse cardiomyocytes can express α/α- or β/β-homodimers or α/β-heterodimers of the myosin heavy chain genes (29). Cardiomyocytes overexpressing annexin VI showed protein diffusely throughout the cytosol but not within the nucleus.

Lung sections from transgenic animals had high levels of annexin VI protein confined to the musculature of the pulmonary veins but not the pulmonary arteries or bronchi (Fig. 4, *E* and *F*). This observation was consistent with overexpression of annexin VI targeted by the α-MHC gene promoter, in which expres-

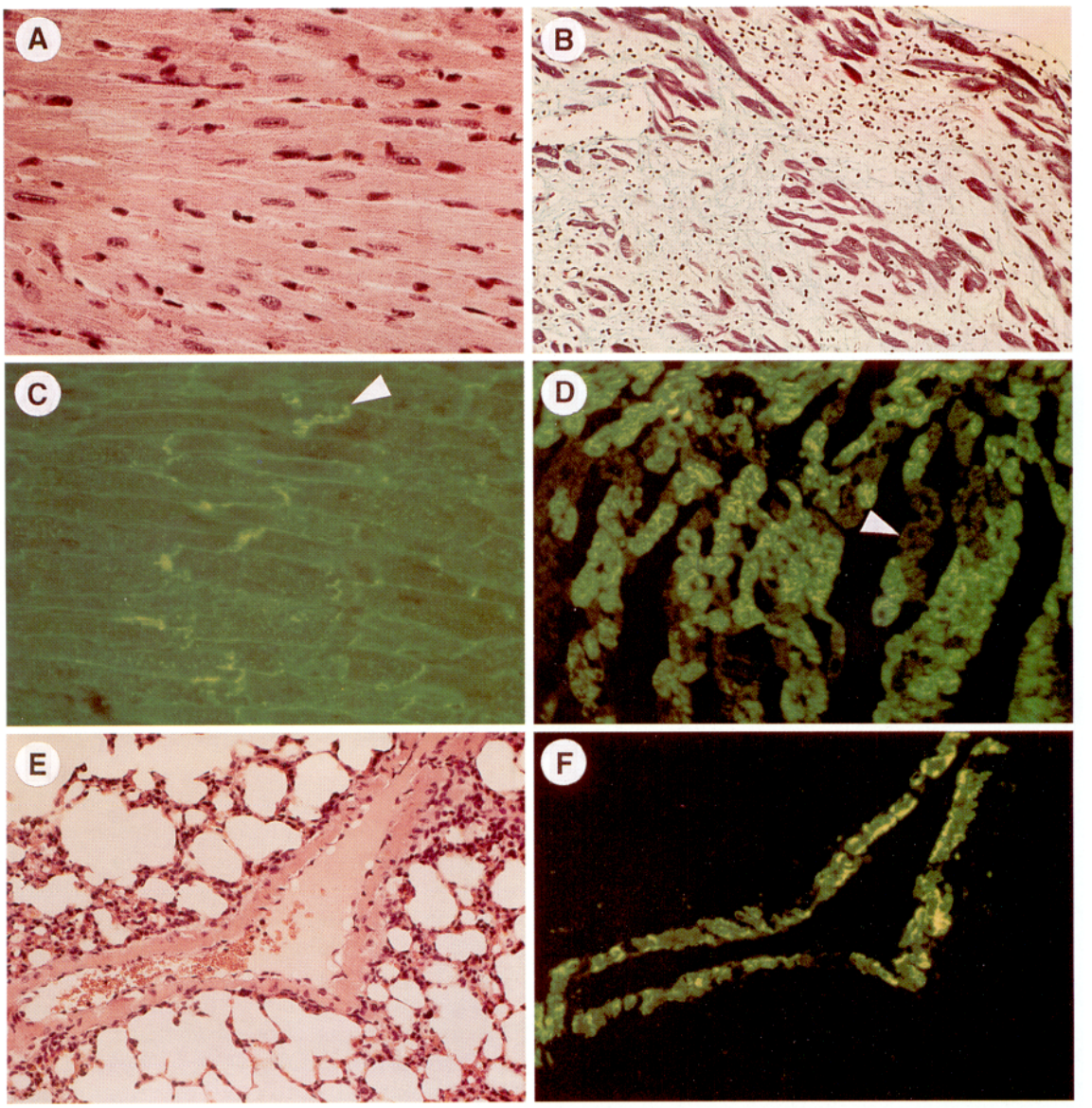

Fig. 4. Immunocytochemistry and histology of annexin VI in hearts and lungs of transgenic mice. H&E-stained sections, trichrome-stained sections, and sections incubated with affinity-purified sheep anti-rat annexin VI antibody from heart and lung are shown. *A*: nontransgenic *line 6* mouse heart H&E section, original magnification ×40; *B*: trichrome-stained transgenic *line 6* mouse section from left ventricle, ×20. Fibrosis separates muscular fibers. There are areas of myocyte necrosis and mild inflammation associated with fibrosis; *C*: immunolocalization of annexin VI in nontransgenic *line 6* mouse heart, ×40. Annexin VI is localized in normal cardiomyocytes to the sarcolemma and intercalated disks (arrowhead); *D*: immunolocalization of annexin VI in a transgenic *line 6* mouse heart, cross section, ×40. Individual cardiomyocytes varied in their level of annexin VI overexpression (arrowhead); *E*: H&E-stained section of transgenic *line 6* mouse lung pulmonary vein, ×40; *F*: same section as *E* showing localization of annexin VI overexpression, targeted by α-MHC promoter, to cells lining pulmonary vein, ×40. Exposure times were adjusted to give similar fluorescence intensity for publication, since tissue sections from transgenic animals had much greater fluorescence intensity corresponding to overexpression of annexin VI.

sion in the lung is specific to the cells lining the pulmonary veins (23).

Contractile properties and Ca^{2+} dynamics of cardiomyocytes. Cardiac myocytes isolated from transgenic animals were simultaneously analyzed for contractile properties and free Ca^{2+} levels upon stimulation using fura 2-AM. Results in Figs. 5 and 6 and Table 1 were obtained from *line 2* animals approximately 3 mo old. At 3 mo of age, *line 2* animals do not show any physical signs of illness such as difficulty breathing or listlessness. However, when pathology is conducted on hearts and lungs isolated from *line 2* animals at 3 mo of age, these animals show mild cardiomyocyte hypertrophy and fibrosis identical to the pathology seen in all of the lines analyzed. In all of the transgenic mice lines, pathology worsens as the animals age. *Line 2* animals were used for this study because pathology is milder at this age than in some of the other lines, making the hearts easier to manipulate. Transgenic and nontransgenic control littermates were directly compared. Car-

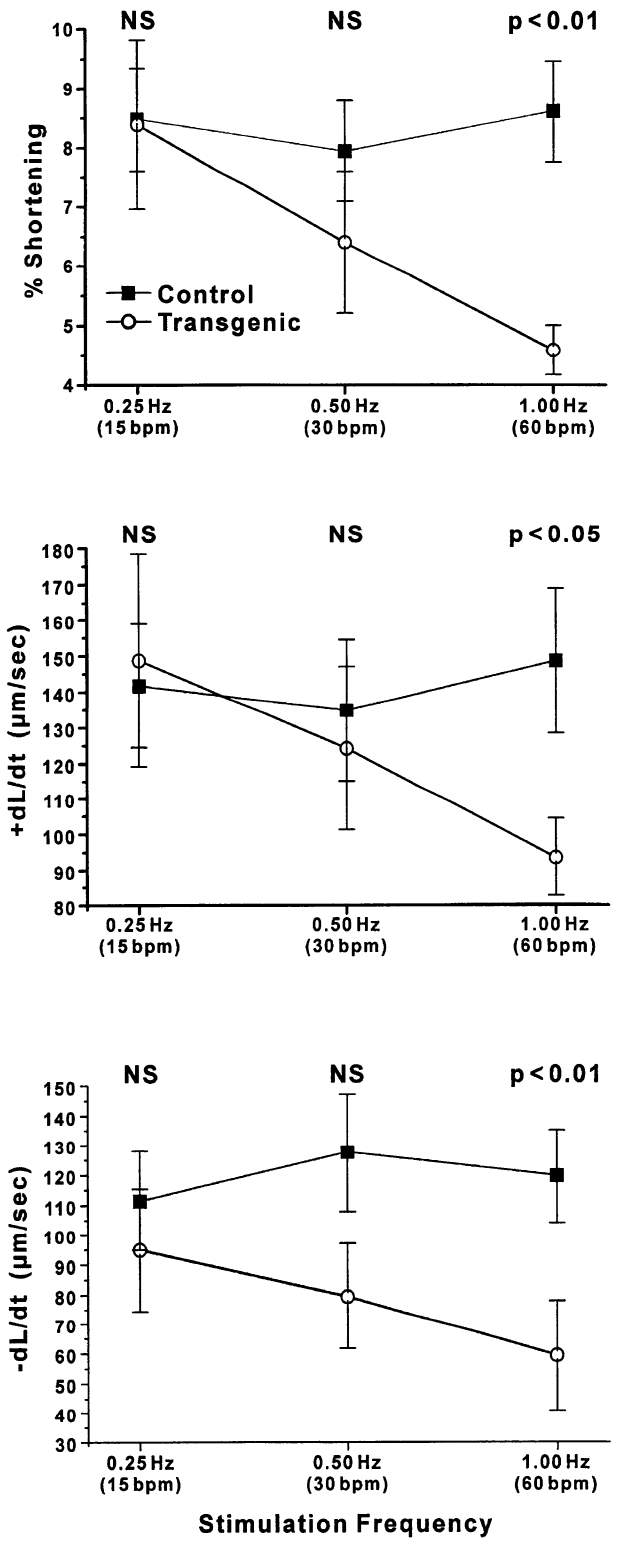

Fig. 5. Cardiac myocyte contractile properties in *line 2* transgenic mice. Relationships among percent shortening (% shortening), rate of shortening ($+dL/dt$), and rate of relengthening ($-dL/dt$) to stimulation rates are shown. Open circle, results from cardiomyocytes from transgenic animals; closed squares, nontransgenic littermate controls. Data shown are result of analysis of 4–8 cardiomyocytes from each of 5 transgenic and 5 nontransgenic animals from *line 2*. Differences in percent shortening, $+dL/dt$, and $-dL/dt$ are statistically significant at a stimulation frequency of 1.00 Hz (60 beats/min).

diomyocytes isolated from hearts of transgenic animals overexpressing annexin VI exhibited a significant frequency-dependent reduction in percent shortening, rate of contraction ($+dL/dt$), and rate of relaxation ($-dL/dt$) compared with age-matched nontransgenic control animals from the same line (see Fig. 5). Myocyte percent shortening in annexin VI transgenic mice was very close to that in control mice at 0.25 Hz (15 beats/min) stimulation frequency but began to decrease at 0.5 Hz (30 beats/min). When stimulation increased to 1 Hz (60 beats/min) significant ($P < 0.01$) depression appeared in the percent shortening of myocytes from the annexin VI transgenic mice. Changes in the $+dL/dt$ and the $-dL/dt$ were significant at 1 Hz and corresponded to the changes seen in percent shortening. Contractile properties and Ca^{2+} dynamics of *line 2* mice were verified by analysis of mice from *line 11*.

Fura 2-AM studies of cardiomyocytes from mice overexpressing annexin VI in the heart indicated that cells from transgenic animals had ~40% lower basal levels of free Ca^{2+} and that upon stimulation, peak Ca^{2+} levels were also significantly lower than in control mice (see Fig. 6*A*). The depression in the base level of intracellular Ca^{2+} did not change following increased stimulation frequency. The amplitude of the Ca^{2+} signal, an indicator of the peak systolic Ca^{2+} concentration, was significantly lower in annexin VI transgenic mice compared with controls at both 0.25 and 0.5 Hz stimulation but not at 1 Hz stimulation (Table 1). The time to 80% decline and 50% decline of Ca^{2+} signal (T_{80} and T_{50}), as well as peak time (the time from the beginning to the peak of the Ca^{2+} signal), did not exhibit any significant change (Table 1).

DISCUSSION

Overexpression of annexin VI was targeted to the heart of transgenic mice as an approach to analyze the role annexin VI plays in Ca^{2+} homeostasis and cardiac function. At least 10-fold overexpression of the protein was accomplished in multiple transgenic mouse lines in both atria and ventricles using the α-MHC promoter. Addition of the bacterial chloramphenicol acetyltransferase (CAT) reporter gene to the α-MHC promotor results in tissue-specific and developmental stage-specific expression of the CAT gene in FVB/N strain mice (11). This promoter has also recently been used to successfully overexpress the β_2-adrenergic receptor in the heart (25). Localization of annexin VI overexpression in the hearts of transgenic mice is consistent with α-MHC-directed CAT expression and also correlates with the expression pattern of this promoter when ligated to the β_2-adrenergic receptor. Mice overexpressing annexin VI showed altered cardiac myocyte Ca^{2+} dynamics and mechanical properties. Individual transgenic lines also showed differences in their ability to adjust to this overexpression. Because the transgene was under the regulation of the α-MHC promoter, it is possible that transgenic lines differentially control the activity of this promoter. The α-MHC promoter has been shown to be regulated by myocyte-specific enhancer-binding factor-2 (26) and thyroid hormone (21,

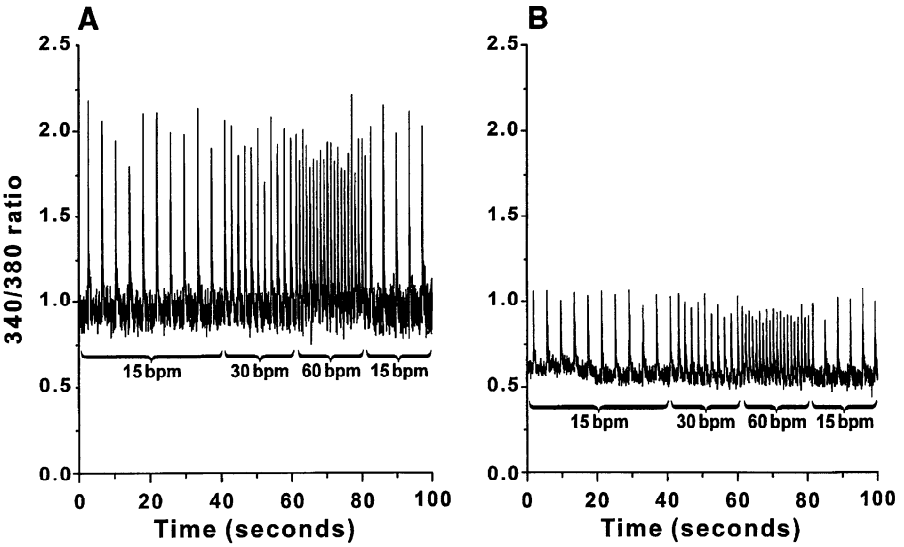

Fig. 6. Ca^{2+} dynamics in *line 2* transgenic mice. Typical Ca^{2+} signals from a single cardiomyocyte isolated from a nontransgenic (*A*) and transgenic (*B*) mouse from *line 2*. Cardiomyocytes from transgenic animals have lower basal levels of intracellular free Ca^{2+}. Upon stimulation, Ca^{2+} levels in myocytes from transgenic mice do not reach the same Ca^{2+} levels as cells from nontransgenic control mice. *Y*-axis: 340/380nm; *X*-axis: time (in s).

31). Thyroid hormone regulation has also been shown to vary among individual cardiomyocytes (20). Aging, hypertension (2), and cardiac hypertrophy (18) also trigger myosin isogene switching. Thus many factors may come into play in each transgenic line overexpressing annexin VI, which affect the ability of the individual line to survive the overexpression for a period of time.

Gross pathological changes were seen in the hearts of mice overexpressing annexin VI. Hearts, and to a lesser extent lungs, from transgenic animals had multifocal sites of fibrosis in areas of hypertrophy. This alteration could have been the result of ischemia due to abnormal blood flow. Overexpression of annexin VI in cardiomyocytes results in alteration of normal Ca^{2+} dynamics and contractility, which may lead to cell death. The level of involvement increased as the animals aged. Mice from *lines 2* and *4* survived over 1 yr. These mice, during the latter months, were very lethargic compared with control mice. We believe this was a compensatory change due to the heart lesions. Future examination of some of the clinical parameters of these mice will be performed to examine blood O_2 levels and response to exercise induction. All animals that died had severe cardiac lesions. Interpretation of the histological sections suggests that the fibrosis was a secondary response to the overexpression of annexin VI. The fibrous infiltrate replaced the muscular fibers of the heart and vascular wall of the lungs in areas of myocardial necrosis. Overexpressing annexin VI also led to cardiac hypertrophy and dilation. Animals with severe cardiac lesions had difficulty breathing and routinely showed signs of pulmonary congestion upon autopsy. The pulmonary congestion was not the result of CO_2 inhalation euthanasia.

Table 1. *Fura 2 ratios of isolated myocyte calcium transients from annexin VI transgenic mice*

	Pacing Rate, beats/min		
	15	30	60
Amplitude			
Control	0.974 ± 0.148	0.858 ± 0.141	0.783 ± 0.147
Transgenic	0.541 ± 0.082*	0.503 ± 0.077*	0.509 ± 0.093
Baseline			
Control	0.968 ± 0.055	0.982 ± 0.050	1.016 ± 0.051
Transgenic	0.526 ± 0.075†	0.566 ± 0.044†	0.564 ± 0.043†
Peak time			
Control	0.097 ± 0.005	0.092 ± 0.002	0.090 ± 0.004
Transgenic	0.108 ± 0.009	0.089 ± 0.003	0.096 ± 0.006
T_{80}			
Control	0.462 ± 0.036	0.415 ± 0.049	0.359 ± 0.027
Transgenic	0.510 ± 0.078	0.429 ± 0.043	0.341 ± 0.028
T_{50}			
Control	0.236 ± 0.018	0.217 ± 0.016	0.184 ± 0.011
Transgenic	0.262 ± 0.022	0.222 ± 0.011	0.200 ± 0.013

Values are means ± SE. Summary of results obtained from all cardiomyocytes analyzed from the same 5 transgenic ($n = 5$) and 5 nontransgenic littermate control animals ($n = 5$) from line 2 shown in Fig. 5. Amplitude, height of the Ca^{2+} peak upon stimulation; baseline, 340/380 ratio at rest; peak time, time from start to maximal Ca^{2+} signal; T_{80}, 80% decay of Ca^{2+} signal; T_{50}, 50% decay of Ca^{2+} signal. *$P < 0.05$; †$P < 0.01$; intergroup comparison.

Gruver et al. (10) have shown that overexpression of calmodulin, an EF-hand Ca^{2+}-binding protein, targeted to atria via the human atrial natriuretic factor promoter causes proliferation and hypertrophy of atrial cardiomyocytes during development. Although overexpressing annexin VI in the heart resulted in cardiac hypertrophy, no subjective cardiomyocyte hyperplasia was apparent. Hearts from transgenic mice overexpressing annexin VI also had severe fibrosis not apparent in mice overexpressing calmodulin in atria. These differences indicate that the two distinct Ca^{2+}-binding proteins have unique roles in heart regulation.

We have also produced mice containing a transgene in which only the first 129 amino acids of annexin VI are linked to the α-MHC promoter. Overexpression of this truncated annexin VI does not show the same phenotype or pathology described for the full-length construct and verifies the effects seen are due to overexpressing full length annexin VI and not to the transgene construct itself (C. L. Taylor, A. Gunteski-Hamblin, and J. R. Dedman, unpublished observations).

Annexin VI overexpression dramatically altered the contractile properties and Ca^{2+} dynamics of individual cardiac myocytes isolated from transgenic mice. Cardiomyocytes from transgenic animals had significantly reduced percent shortening, reduced $+dL/dt$, and reduced $-dL/dt$ compared with nontransgenic control littermates. Upon stimulation, cardiomyocytes from transgenic animals never reached the same intracellular free Ca^{2+} levels as nontransgenic control cells. A possible explanation for this phenomenon is that myofilament sensitivity to Ca^{2+} is increased as a compensatory mechanism to maintain basal contractility at the lower stimulation rate. At higher stimulation frequencies, Ca^{2+}-modulated contraction becomes more dependent on extracellular Ca^{2+} stores and intracellular Ca^{2+}-mediated Ca^{2+} release. Basal levels of intracellular free Ca^{2+} were also reduced by ~40% in cardiac myocytes from transgenic mice. This reduction could be due to the activation of the Na^+/Ca^{2+} exchanger or Ca^{2+} pump so that Ca^{2+} in the cytosol is continually cleared. The reduction in basal Ca^{2+} levels is maintained across stimulation frequencies (15–60 beats/min), but the reduction in the amplitude of the Ca^{2+} signal does not achieve statistical significance at the highest stimulation frequency (60 beats/min). Because the percent reduction is similar at all three stimulation frequencies, the differences would likely diminish as larger number of animals were analyzed. Further studies are being performed in which individual pumps, channels, and exchangers within these cardiomyocytes are modified with pharmacological agents to determine which Ca^{2+}-handling pathways are being affected by annexin VI. Additional studies will address the electrophysiological changes in these cardiomyocytes.

In conclusion, this study indicates that annexin VI serves as a regulator of intracellular Ca^{2+} homeostasis and ultimately heart performance. The overexpression of annexin VI alters cardiac myocyte contractile properties, Ca^{2+} dynamics, and heart pathology. These data suggest a possible role for annexin VI in heart performance.

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