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Immunological Development and Cardiovascular Function Are Normal in Annexin VI Null Mutant Mice

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Annexins are calcium-binding proteins of unknown function but which are implicated in important cellular processes, including anticoagulation, ion flux regulation, calcium homeostasis, and endocytosis. To gain insight into the function of annexin VI, we performed targeted disruption of its gene in mice. Matings between heterozygous mice produced offspring with a normal Mendelian pattern of inheritance, indicating that the loss of annexin VI did not interfere with viability in utero. Mice lacking annexin VI reached sexual maturity at the same age as their normal littermates, and both males and females were fertile. Because of interest in the role of annexin VI in cardiovascular function, we examined heart rate and blood pressure in knockout and wild-type mice and found these to be identical in the two groups. Similarly, the cardiovascular responses of both sets of mice to septic shock were indistinguishable. We also examined components of the immune system and found no differences in thymic, splenic, or bone marrow lymphocyte levels between knockout and wild-type mice. This is the first study of annexin knockout mice, and the lack of a clear phenotype has broad implications for current views of annexin function.

The annexins are a family of calcium-binding proteins encoded by at least 12 different genes in mammals and by numerous other genes in invertebrates and plants. Annexins have been intensively studied because of their possible involvement in a wide range of important biological processes, including phospholipase A_2 and protein kinase C inhibition, apoptosis, blood coagulation, vesicle trafficking, cell transformation, and calcium homeostasis (1, 7). All annexins bind to negatively charged phospholipids in the presence of Ca^{2+} (such as those enriched in the inner leaflet of the plasma membrane), and the idea that soluble cytosolic annexins become associated with intracellular membrane lipids in activated cells is a likely paradigm for annexin function. Calcium binding by annexins is mediated by a complex binding site that has more structural similarity to the Ca^{2+}-binding site in phospholipase A_2 than to other Ca^{2+}-binding proteins, such as calmodulin (10). Most annexins have four tandem repeats of the conserved domain that defines the protein family, and it is the α-helical bundles created by these repeats that form the Ca^{2+}-binding sites.

Annexin VI is unique within the family in that it contains eight conserved repeats. In the crystal structure, these are arranged as two four-repeat lobes oriented perpendicularly to one another and separated by a linker between repeats four and five (2). As with other annexins, the function of annexin VI is far from clear, but there are reports of its association with endocytic vesicles (11, 16), and it has been linked with budding of clathrin-coated pits (12, 15). However, the observation that endocytosis occurs normally in A431 cells that lack annexin VI supports the view that this process is not an essential function of the protein (23). Annexin VI is also strongly expressed in the heart, and studies with transgenic mice showed that annexin VI overexpression targeted to cardiomyocytes led to cardiomyopathy and heart failure (8). Analysis of cardiomyocytes isolated from these mice revealed that basal Ca^{2+} levels and the amplitude of Ca^{2+} spikes were both reduced, with concomitant changes in contractile properties. Loss of annexin VI has also been linked to cell transformation. Thus, annexin VI was strongly down-regulated in melanocytes during their progression from a normal to a highly metastatic malignant phenotype (6). Consistent with this, stable expression of annexin VI in A431 cells restrains both their growth in culture and their ability to form tumors in vivo (24, 25).

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MATERIALS AND METHODS

Preparation of an annexin VI targeting construct. The mouse annexin VI targeting construct was generated by a novel long-range genomic fusion PCR technique with the Expand Long Template or the Expand High Fidelity PCR kit (Boehringer Mannheim). The template was genomic DNA prepared from the embryonic stem (ES) cells, of the 129 cell line, to be targeted. Two amplification products were generated, one with primers MAVI-1 (5′-TTGGTTTCTGCTGC AGAACCTGAGACCAT-3′) and MAVI-2 (5′-GATTTGCGGTTTAAACTCT GGGAAGTCGTGGACAGAGC-3′), which yielded a 7.5-kb genomic fragment extending from exon 2 to exon 3, and a second with primers MAVI-3 (5′-ACT TCCCAGAGTTTAAACGACGCAAATCAGGATGCTGAGG-3′) and MAVI-4 (5′-TTCCTGGCTCTGCTTGTTGCTGCGGGAGGT-3′), which yielded a 2.2-kb product extending from exon 3 to exon 4. Primers MAVI-2 and MAVI-3 have a central region of complementarity into which we introduced a *Pme*I site. A 9.7-kb fusion PCR product was generated with primers MAVI-1 and MAVI-4, with a mixture of the two amplimers as a template. A 5.8-kb *Bam*HI/*Bcl*I fragment of the fusion product was then cloned into pBluescript SK. Finally, a neomycin resistance cassette was cloned into the engineered *Pme*I site in exon 3 of the cloned fusion product, and the plasmid was linearized for transfection.

Generation of annexin VI null mutant mice. The ES cell line E14-1, of 129/ola origin (19), was cultured on feeder layers of Neo^r STO cells (kindly provided by Mike Owen, Imperial Cancer Research Fund, London, United Kingdom) and transfected with the annexin VI targeting construct. Neomycin-resistant ES cell colonies were screened by PCR for integration of the construct at the correct locus. ES cells containing one disrupted annexin VI allele were injected into 3.5-day-old blastocysts from C57BL/6 donors and returned to pseudopregnant F_1 hybrid foster females. Chimeric offspring were checked for germ line transmission of the mutation by crossing them with C57BL/6 mice, and mice heterozygous for the mutation in annexin VI were used to establish a breeding colony.

Analysis of cardiovascular function. Measurements of heart rate and mean arterial blood pressure (MAP) in the conscious mouse under resting conditions and during and after toxic shock (caused by intravenous administration of *Escherichia coli* lipopolysaccharide [LPS] at 4 mg/kg of body weight) were performed as described elsewhere (18).

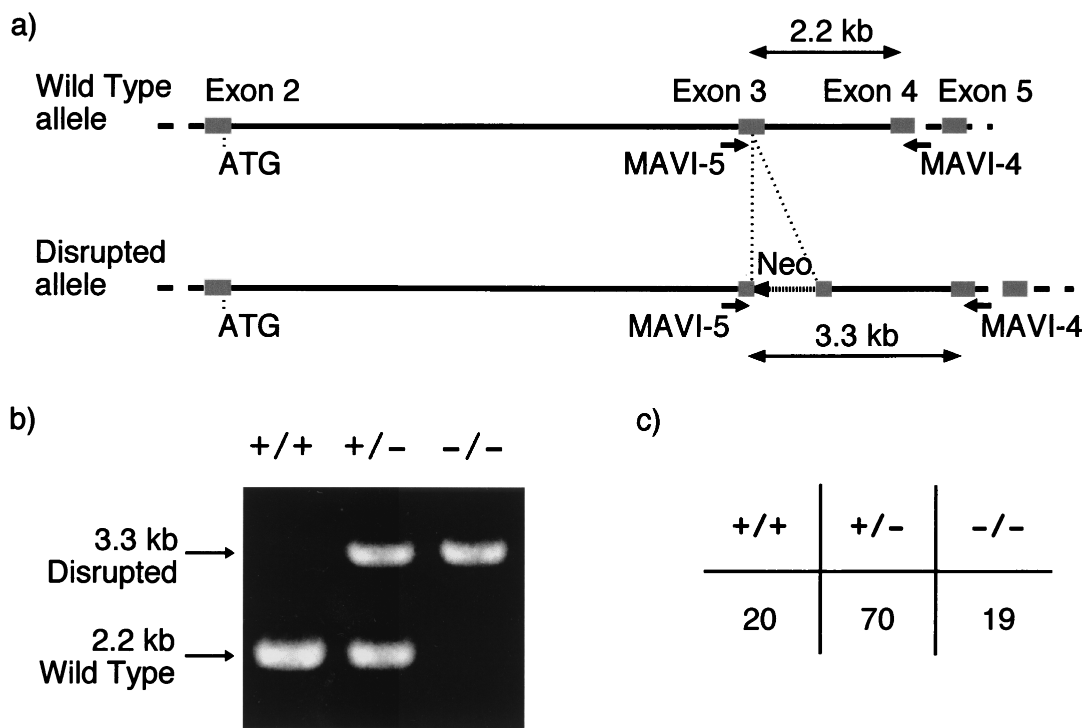

FIG. 1. Disruption of the mouse annexin VI gene locus. Genotyping was done by genomic PCR of DNA extracted from tail snips. Primers MAVI-4 (which lies beyond the end of the targeting construct) and MAVI-5 were used as shown in panel a. Disrupted alleles of annexin VI have a 1.1-kb insert in exon 3 which increases the size of this PCR product from 2.2 to 3.3 kb. The results of PCRs with these primers and template DNA from wild-type (+/+), heterozygous (+/−), and homozygous null (−/−) mice, as shown in panel b, were resolved on a 1% agarose gel and visualized by ethidium bromide staining. The ratios of +/+, +/−, and −/− pups from +/− × +/− matings are shown in panel c.

Flow cytometry. Analyses were performed as described previously (19) with splenocytes, thymocytes, and bone marrow cells. All antibodies were from PharMingen, and triple-staining fluorescence-activated cell sorting (FACS) was done on a Becton Dickinson FACSCalibur and analyzed with CellQuest software.

SDS-PAGE and Western blotting. Tissue samples (~1 mm^3) were homogenized on ice in 1 ml of lysis buffer (10 mM Tris-HCl [pH 7.4], 50 mM NaCl, 10 mM EGTA, 1 mM dithiothreitol, 1% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, and 1 mM each of the small peptide inhibitors chymostatin, leupeptin, and pepstatin). Particulate matter was pelleted by centrifugation, and supernatant protein concentrations were determined with the Bio-Rad detergent-compatible protein assay kit. Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide gels and transferred to Immobilon P (Millipore) for Western blotting. Membranes were probed as described previously (23) with antisera against annexins I (rabbit polyclonal), II (mouse monoclonal, kindly provided by V. Gerke, Muenster, Germany), V (rabbit polyclonal), and VI (rabbit polyclonal). Protein bands were visualized by using chemiluminescence or Western blue (Promega).

RESULTS

As a first step towards generating annexin VI null mutant mice, we used a novel PCR-based technique to prepare a targeting construct (8a). The targeting construct contained the gene for neomycin resistance inserted into exon 3. Intron-exon boundaries are highly conserved between human and mouse annexins, so although the mouse annexin VI gene had not been characterized, predictions based on the structure of the human annexin VI gene proved to be accurate (21, 22). The wild-type and disrupted alleles were readily distinguished by PCR with primers MAVI-4 and MAVI-5 (5′-ACCGAGGCTCTGTCCA CGACTTCCC-3′), located in exons 4 and 3, respectively, on either side of the Neo^r gene (Fig. 1a). Note that although MAVI-4 was used to amplify the original fragment of genomic DNA, it was excluded from the targeting construct. This technique yielded a single product of 2.2 kb in control (+/+) mice,

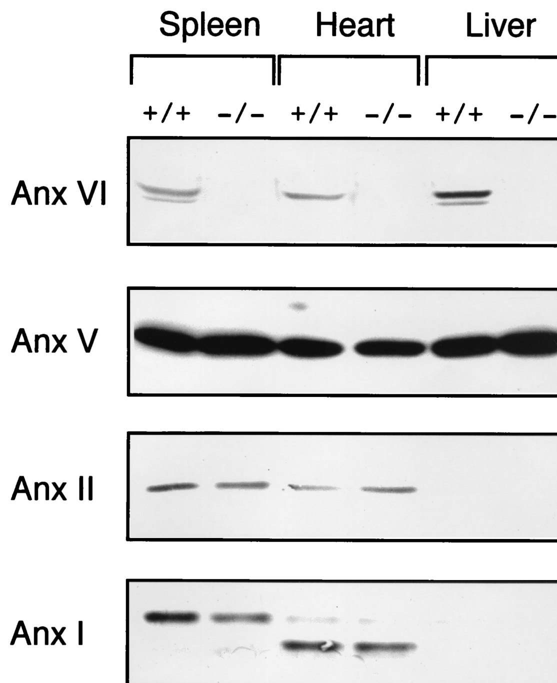

FIG. 2. Expression of annexins (Anx) in annexin VI knockout mice. Protein samples (40 μg of total protein per lane) from splenocytes, whole heart tissue, and liver tissue were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes for Western blotting. The annexin V blot was developed by chemiluminescence, while the others were developed with Western blue substrate.

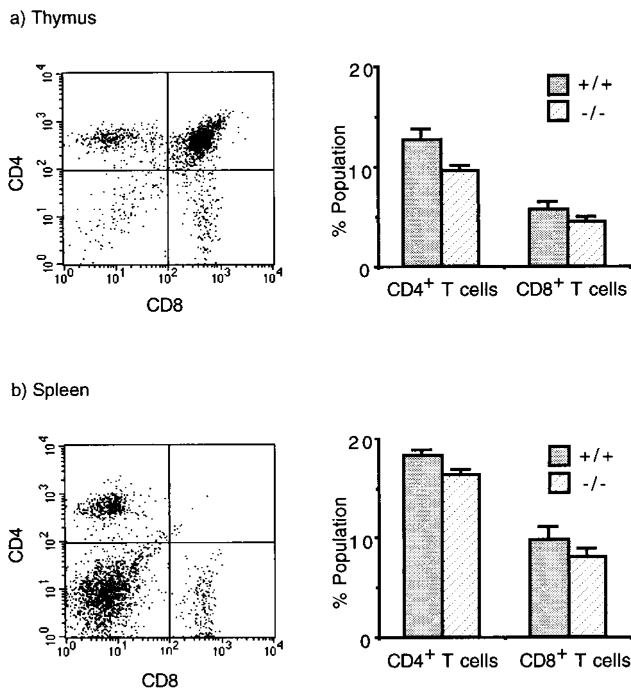

FIG. 3. Developmental profiles of thymic and splenic T cells. Thymocytes and splenocytes were prepared in duplicate from wild-type and annexin VI −/− mice, triple stained with phycoerythrin-conjugated anti-CD4, fluorescein isothiocyanate-conjugated anti-CD8, and BIO-conjugated anti-CD3, and analyzed by FACS. Representative staining profiles are shown in each case. The average percentages of helper ($CD4^+$ $CD8^-$) and cytotoxic ($CD4^-$ $CD8^+$) T cells in each population are given in the bar charts, with error bars showing average differences from the means.

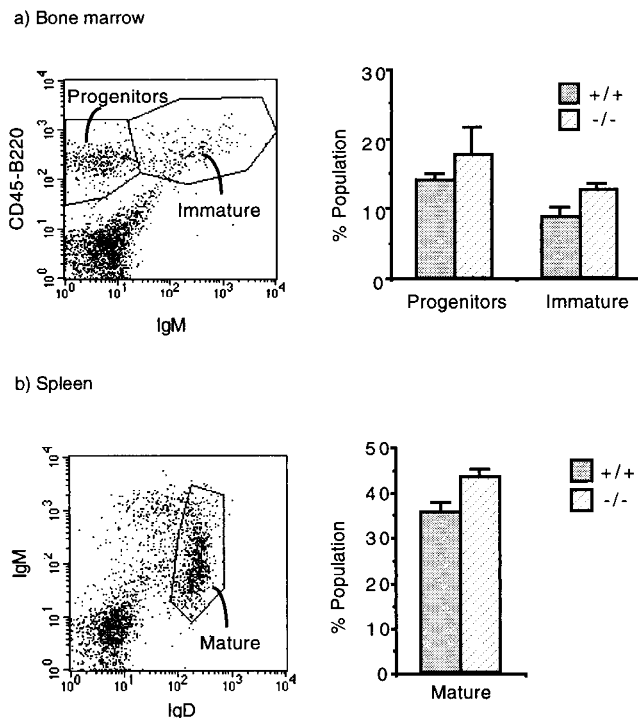

FIG. 4. Developmental profiles of bone marrow and splenic B cells. Bone marrow cells and splenocytes were prepared in duplicate from wild-type and annexin VI −/− mice, triple stained with phycoerythrin-conjugated anti-IgM, fluorescein isothiocyanate-conjugated anti-IgG, and anti-CD45-B220, and analyzed by FACS. Representative staining profiles are shown in each case. The average percentages in each population of those classes of B cells labeled on the FACS plot are given in the bar charts, with error bars showing the average differences from the means.

two products of 2.2 and 3.3 kb in heterozygous (+/−) mice, and a single 3.3-kb product in homozygous null (−/−) mutant mice (Fig. 1b). Mice that carried one copy of the disrupted gene were interbred to generate pups that were +/+, +/−, and −/− for annexin VI as determined by genomic PCR of DNA extracted from tail snips. Pups from 13 litters from heterozygous parents were genotyped in this way, and the ratios of +/+, +/−, and −/− mice (Fig. 1c) did not differ significantly from the predicted Mendelian ratios of 1:2:1 for nonlethal alleles. Thus, under laboratory conditions annexin VI knockout pups were no less viable than their wild-type littermates.

At a gross phenotypic level, loss of annexin VI had no discernible impact. The morphology of muscle and the major organs, including the heart, lung, brain, kidney, liver, thymus, spleen, testes, and ovaries, was examined histologically, and no obvious changes were observed in annexin VI −/− mice. This implies that annexin VI is not required during the development of gross body structures in the mouse. The knockout mice have also to date shown no loss of viability up to 1 year of age compared to wild-type mice. Furthermore, +/+ × +/+, +/− × +/−, and −/− × −/− matings revealed no differences in fertility with respect to average litter sizes, indicating that loss of annexin VI also has no significant effect under these conditions on the ability to mate or on the ability to carry, give birth to, or suckle young as determined by pup viability (results not shown).

Given the absence of an overt phenotype under laboratory conditions, we investigated the possibility that another member of the annexin gene family might compensate for the loss of annexin VI. This might be particularly true for annexin V, which according to cladistic analysis is the closest relative of annexin VI in the gene family (22). Since such functional compensation might be reflected in altered gene expression, we examined the levels of expression of annexins I, II, V, and VI by Western blotting of whole-tissue protein extracts from splenocytes, heart, and liver of +/+ and −/− mice (Fig. 2). Although we surveyed only a sample of the mammalian annexin gene family, those that we investigated are, like annexin VI, the most widely expressed and therefore the most prone to changes in expression linked to functional compensation. However, these blots clearly revealed no major differences in expression between +/+ and −/− mice for any of these annexins, except for the disrupted annexin VI.

We next examined components of the immune system, given that annexin VI is strongly up-regulated during development of both B and T lymphocytes (3). The immature and mature T-cell populations of the thymus and spleen were classified by their surface expression of CD3, CD4, and CD8. For each tissue, a typical wild-type FACS distribution plot for CD4 and CD8 is shown (Fig. 3). The numbers of cells in each population (determined from the quadrants of the FACS plot), averaged for two wild-type and two knockout mice, are shown in each case in the corresponding bar chart. These results show that annexin VI −/− mice are not significantly different from wild-type mice in terms of the proportions of $CD4^+$ and $CD8^+$ T cells in the thymus or spleen. Furthermore, there were no significant differences in the proportions of immature ($CD3^-$) and mature ($CD3^+$) T cells in the thymus (data not shown).

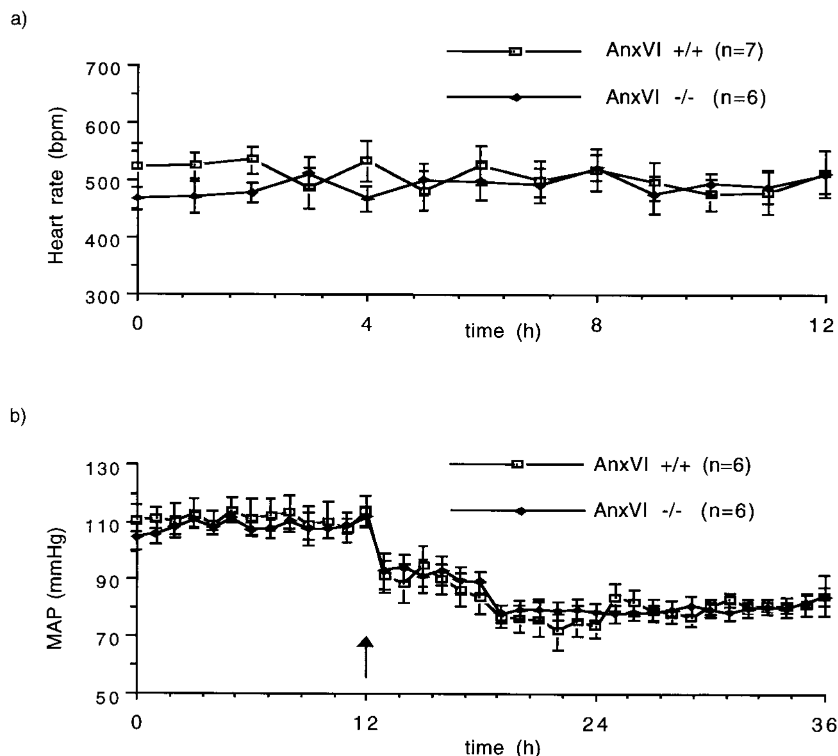

FIG. 5. Blood pressure, heart rate, and endotoxic shock in annexin VI (AnxVI) knockout mice. MAPs and heart rates were measured in freely moving conscious mice. Resting heart rates (a) are shown for wild-type and annexin VI −/− mice over a 12-h period. Resting MAPs (b) are also shown over a 12-h period, after which (arrow) 4 mg of *E. coli* LPS per kg was administered via the femoral vein, and the resulting MAP changes were monitored for a further 24 h. Error bars indicate standard errors of the means.

Triple staining of surface antigens was also used to characterize subpopulations of B cells. While all B cells express the CD45/B220 antigen, surface expression of immunoglobulin M (IgM) and IgD changes with maturation. The results for the IgM/B220 and the IgM/IgD staining patterns, displayed as FACS plots and bar charts (as for Fig. 3), again show that the proportions of developing B-cell precursors ($B220^+$ IgM^-) and immature B cells (IgM^+ $B220^+$) in the bone marrow and of intermediate and late B cells in the spleen are not significantly different between wild-type and annexin VI −/− mice (Fig. 4). Similarly, analysis of myeloid, granulocytic, and monocytic cells in the bone marrow failed to reveal differences between the control and annexin VI null mutant mice (data not shown).

To investigate possible effects of loss of annexin VI on the heart and the circulatory system, the resting heart rate and MAP in conscious mice were measured. This is a relatively new technique that allows long-term analysis of these parameters over a period of days in conscious mice. Although the surgical procedure involves the implantation of a cannula in the femoral artery, the mice regain consciousness and are able to move freely and behave normally. This approach also allows the administration of substances known to affect the circulatory system, such as *E. coli* endotoxin (LPS), which induces conditions that mimic septic shock in humans. The average resting heart rate and MAP in +/+ and −/− mice were recorded over a 12-h period (Fig. 5) and were not significantly different. Administration of LPS at 12 h led to a dramatic reduction of MAP with a prolonged recovery phase, 24 h of which is shown and all of which was also unaffected by loss of annexin VI.

DISCUSSION

The annexin VI gene is thus not a gene essential for mouse viability. Indeed, there are no obvious effects of loss of annexin VI on the adult structure and, most probably, on the development of any tissues. So although annexin VI expression is developmentally regulated during, among other processes, limb bud formation (17), its expression is not required to form these structures. At a cellular level, the predictions of experiments suggesting that annexin VI is required for budding of clathrin-coated pits (15) or for caveolar endocytosis (20) are thus also confounded; both such processes are likely to be essential for the viability of cells and thus the organism. However, annexin VI −/− mice are viable. Our results do not preclude annexin VI having some minor modulatory role in vesicle trafficking, and confirmation of normal endocytic and caveolar endocytosis in annexin VI −/− mice will require analysis at a cellular level.

While the lack of an overt phenotype could reflect a subtle cellular role for annexin VI, it is also possible that another member of the family of 10 known annexins in the mouse could compensate for the function of annexin VI. However, in the heart, liver, and spleen, none of annexins I, II, and V are obviously up-regulated (or down-regulated) at the level of protein expression as determined by Western blotting. While annexin VI has been shown to be expressed at high levels in the spleen and lymph nodes, in particular in mature lymphocytes (3), flow cytometric analysis of the relative myeloid (not shown) and B- and T-lymphocyte populations revealed no significant alterations in mice lacking annexin VI. Annexin VI is

thus not required in lymphocyte ontogeny as had been suggested, although it may modulate lymphocyte function or be involved in immune or proliferative responses.

As with lymphocyte development and function, a broad-range approach that would allow the detection of a wide range of changes in mice lacking annexin VI was used to investigate heart and circulatory function. The fact that annexins V and VI are the major cardiac annexins suggests that these proteins have important roles in heart function (5). Furthermore, experiments suggesting a role for annexin VI in the regulation of the ryanodine receptor (4, 9) and studies of annexin VI overexpression in the heart (8) all supported this hypothesis. While the fall in resting blood pressure for mice lacking inducible nitric oxide synthase was reduced under conditions employed in this study (18), indicating that genetic manipulation can affect this variable, no such changes were observed in annexin VI knockout mice. Thus, LPS-induced circulatory collapse was normal in annexin VI $-/-$ mice, indicating that the pathways leading to cytokine production and the loss of vascular tone are normal, while the response of the heart to and its recovery from the loss of vascular tone were also unaffected.

While these data show that annexin VI is not crucial to heart function, it is possible, given the familial relationship between annexins V and VI, that disruption of both genes would reveal a phenotype in the heart. Alternatively, annexin VI may have a role in the heart under conditions not yet tested, such as in reperfusion injury or during or after an infarction. In this context, it is interesting that K201, a new benzothiazepine derivative which protects against cardiac reperfusion injury, inhibits the calcium channel activity of annexin V and binds to the exit pore of the putative ion conductance pathway (13, 14). The experimental system used in this study will also be useful in examining the possible effects of caffeine on cardiovascular function, given that caffeine increases blood pressure and excites the heart and that it also directly affects the ryanodine receptor, a possible site of action of annexin VI. Direct analysis of calcium mobilization and its link to muscle contraction in isolated cardiomyoctes will also be of interest in order to allow comparison with the results obtained with the isolated cardiomyoctes of transgenic mice overexpressing annexin VI (8).

The results presented here provide the first clear information regarding the role of any annexin in development and organ function. The fact that loss of annexin VI does not grossly affect any of these processes raises questions about several proposed roles for annexin VI in endocytosis, lymphocyte development, and heart function, and it suggests that annexin VI may have a rather subtle role. However, while the annexin VI gene is not essential, the generation of annexin VI $-/-$ mice will allow a wide range of further experiments at the cellular level and should ultimately lead to the generation of mice lacking multiple annexins.

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