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Expression and Localization of the Annexins II, V, and VI in Myocardium from Patients with End-Stage Heart Failure

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SUMMARY: Annexins II, V, and VI belong to a family of Ca^{2+} -dependent phospholipid-binding proteins that have been involved mainly in signal transduction, differentiation, membrane trafficking events, or binding to the extracellular matrix, or that might be effective as Ca^{2+} -channels. They are abundant in the mammalian myocardium and might play a role in ventricular remodeling and altered calcium handling during heart failure. To test this hypothesis, we compared the expression and distribution of these annexins in nonfailing ($n = 9$) and failing human hearts with idiopathic dilated cardiomyopathy ($n = 11$). Northern blot and slot blot analysis were used to determine the annexin mRNA levels and Western blots were used to quantify the amounts of annexin proteins. Distribution of annexins was studied by immunohistofluorescence labeling and compared with that of a sarcolemmal marker (Na^+/K^+ -ATPase) and of a myofibrillar protein (α -actinin). We showed that nonfailing hearts contained a higher amount of annexin VI than of annexin V or II (13.5 ± 1.8 , 3.7 ± 0.2 , and $2.5 \pm 0.5 \mu\text{g}/\text{mg}$ protein, respectively). In failing hearts, there was a parallel increase in both mRNA and protein levels of annexin II (146% and 132%, $p < 0.05$, respectively) and annexin V (152%, $p < 0.01$, 147%, $p < 0.005$, respectively); the protein level of annexin VI was also increased (117%, $p < 0.05$), whereas the increase of its mRNA level was statistically insignificant. We observed a predominant localization of annexin II in interstitium, and of annexins V and VI in cardiomyocytes at the level of the sarcolemma, T-tubules, and intercalated disks in nonfailing hearts, whereas in failing hearts enlarged interstitium contained all three annexins. Furthermore, annexin V staining at the level of cardiomyocytes almost disappeared. In conclusion, we showed that heart failure is accompanied by marked overexpression of annexins II and V, as well as translocation of annexin V from cardiomyocytes to interstitial tissue. The data suggest that annexins may contribute to ventricular remodeling and annexin V to impaired Ca^{2+} handling in failing heart. (*Lab Invest* 2000, 80:123–133).

The annexins constitute a family of proteins that have a distinctive property to bind phospholipids in a calcium-dependent way (for reviews see Raynal and Pollard, 1994; Waisman, 1995). Although the exact biological role of the annexins remains unknown, they have been involved in various intra- and extracellular processes, including mitogenic signal transduction, differentiation, membrane trafficking events, Ca^{2+} fluxes, and binding to the extracellular matrix. Myocardial annexins have been mostly studied

in animal species in which annexins II, V, and VI are particularly abundant. The annexin II has been found in rat (Jans et al, 1995), bovine (Zeng et al, 1993), and guinea pig (Trouvé et al, 1999) myocardium. In different tissues, annexin II has been reported to bind collagen, to promote contacts between vesicles, and to be involved in regulation of cell-extracellular matrix interactions (Waisman, 1995). Another important feature of annexin II is its inhibitory activity on G protein-regulated ip_3 production, suggesting a role in the development of hypertension (Schelling et al, 1996). Annexins V and VI are the major annexins in porcine (Pula et al, 1990), bovine (Iida et al, 1992; Wang et al, 1993; Zeng et al, 1993), rat (Doubell et al, 1993; Jans et al, 1995; Wang et al, 1995), and guinea pig (Trouvé et al, 1999) myocardium. Annexin V is able to form voltage-gated Ca^{2+} channels (Pollard et al, 1992) and to modulate activity of protein kinase C and phospholipase A_2 (Dubois et al, 1998; Mira et al, 1998; Van Bilsen et al, 1992). In myocardium, the localization of

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Figure 1.

mRNA analyses of cardiac annexins II, V, and VI. A, Representative Northern blot analysis of annexin II (*Anx II*), annexin V (*Anx V*), and annexin VI (*Anx VI*) mRNA from nonfailing (N) and failing human hearts with idiopathic dilated cardiomyopathy (IDCM) (F). Each lane contained equal amounts (25 μ g) of total RNA. B, Bar graph shows changes of mRNA levels of annexin II, V, and VI in failing hearts. mRNA levels were measured in nonfailing (light bars; $n = 9$) and failing (dark bars; $n = 11$) hearts by slot blot, and normalized to poly(A⁺) RNA. Values are mean $\pm$ SEM; * $p < 0.05$, ** $p < 0.01$, compared with nonfailing hearts.

annexin V is not clearly established: the presence of annexin V was first reported in endothelium and fibroblasts (Van Bilsen et al, 1992), then in myocytes in which it has been detected either in cytosol (Jans et al, 1995), in membranes (Luckcuck et al, 1997; Trouvé et al, 1999), or associated with Z-lines (Trouvé et al, 1999; Wang et al, 1995). Annexin VI has been localized in rat, bovine, and guinea pig hearts mainly in sarcolemma and intercalated discs of cardiomyocytes (Doubell et al, 1993; Iida et al, 1992; Luckcuck et al, 1997; Trouvé et al, 1999).

In heart pathophysiology, few reports deal with annexin expression or suggest a possible role in heart failure. Overexpression of annexin VI in the heart of transgenic mice was shown to result in dilated cardiomyopathy (Gunter-Hamblin et al, 1996) with impaired contractility and intracellular Ca^{2+} turnover (Song et al, 1995). Increases in the levels of annexins II, V, and VI were found in hypertensive guinea pigs at the onset of heart failure (Trouvé et al, 1999) and increases of annexins II and V, together with a decrease of annexin VI in failing human heart (Song et al, 1998). However, potential roles for annexins in heart failure cannot be considered without establishing their precise distribution in human heart and during heart failure.

The aim of this study was to perform parallel studies on expression and distribution of annexins II, V, and VI in failing (due to idiopathic dilated cardiomyopathy; IDCM) and nonfailing human hearts. Quantitative analysis showed coordinate upregulation of both mRNA and protein levels of annexins II and V in samples from failing hearts. In immunohistochemical studies, we showed that an increase in annexins II, V, and VI content in failing human heart was accompanied by

their accumulation in cardiac interstitium, whereas staining of cardiomyocyte membranes with antibodies to annexin V was almost lost.

Results

Annexins II, V, and VI mRNA in Nonfailing and Failing Human Myocardium

Northern blot analysis of mRNA extracted from human myocardium and hybridization with specific probes for annexins II, V, and VI demonstrated corresponding

Figure 2.

The effect of Ca^{2+} concentration on the annexins content of crude particulate preparation (CPP). Western blot analysis of annexin II (*Anx II*), annexin V (*Anx V*), and annexin VI (*Anx VI*) in CPP (10 μ g of protein) isolated as described in "Materials and Methods" (lane 1), in the presence of 1 mM Ca^{2+} (lane 2), or in the presence of 1 mM EGTA (lane 3).

bands (annexin II, 1.25 kb; annexin V, 1.35 kb; annexin VI, 2.3 kb) (Fig. 1A). The bands of annexin II and annexin V were larger in three different failing hearts compared with nonfailing hearts, whereas no obvious difference in annexin VI band was observed.

Quantification of annexin mRNA were further carried out by slot blot analysis (Fig. 1B). We found a significant increase in mRNA levels (normalized to poly(A⁺)RNA) for annexins II ($146 \pm 6\%$; $p < 0.02$; $n = 11$) and V ($152 \pm 15\%$; $p < 0.01$; $n = 11$) in failing comparing with nonfailing hearts ($n = 9$). A tendency toward an increase in annexin VI mRNA content ($120 \pm 13\%$; $n = 11$) was statistically insignificant.

Quantification of Annexins II, V, and VI Proteins in Human Myocardium

Western blot analysis was used to quantify the annexins in crude particulate preparations (CPP) of cardiac tissue (see "Methods") which contained all of the membrane-bound annexins. Because it was known

that annexins could be partially extracted by EGTA-containing solution (Trotter et al, 1995), we tested whether the presence or absence of Ca^{2+} during isolation of CPP might modify the yield of annexins (Fig. 2). Compared with CPP prepared without added Ca^{2+} (lane 1), there was no significant increase in the annexin content in the presence of 1 mM Ca^{2+} (lane 2) in homogenization medium, but under this condition annexin II appeared as two bands probably generated by activated proteolysis. In the presence of 1 mM EGTA (lane 3), some but not all of the annexins were extracted from CPP, and annexin VI seemed to be the most resistant to EGTA extraction. Thus, the amount of annexins in human heart sample was determined in CPP with neither EGTA nor Ca^{2+} added.

Figure 3 shows Western blot analysis of annexins II, V, and VI in CPP from nonfailing hearts. Nonspecific protein staining of the membrane with Coomassie blue (Fig. 3A) revealed multiple bands in lanes with CPP (lanes 1 to 3) and two bands for the mixture of pure

Figure 3.

Quantification of annexins II, V, and VI in CPP from nonfailing myocardium by Western blot analysis. A, Coomassie blue staining of the membrane. Lanes 1 to 3, CPP (10 μg); lane 4, a mixture of pure annexins V and VI (50 ng + 100 ng). Position of molecular weight markers (in kD) is shown by arrows. B, Immunostaining: the membrane was cut out and treated with antibodies to annexin II (lane 1), annexin V (lane 2), annexin VI (lane 3), and a mixture of antibodies to annexin V and annexin VI (lane 4). C, Quantification of annexins in human hearts. CPP samples from nonfailing hearts (10 μg protein, hollow circles) were run in parallel with different amounts of pure annexin (solid circles).

annexins V and VI (lane 4). After immunostaining of each lane, we observed one band after reaction with each of the anti-annexin antibodies and two bands in the last lane after reaction with both anti-annexin V and VI antibodies (Fig. 3B). The apparent electrophoretic mobility of annexin II, V, and VI was 36, 32, and 68 kDa, respectively. The corresponding apparent molecular weights were identical to those obtained for the pure human annexins.

For quantification of annexins II, V, and VI, specific bands in CPP were compared with known annexin concentrations on the same Western blot (Fig. 3C). Integrated density of the spots on ECL film had linear dependence on the amount of protein applied within the chosen concentration range. All of the measured samples were well within the calibration curve. It has been determined that protein content for annexins II, V, and VI was 3.7 ± 0.2 ($n = 4$), 2.5 ± 0.5 ($n = 6$), and 13.5 ± 1.8 $\mu\text{g}/\text{mg}$ ($n = 6$), respectively, in nonfailing human myocardium.

Comparison of Annexin Levels in Nonfailing and Failing Human Myocardium

Annexins and sarco-endoplasmic reticulum Ca^{2+} -ATPase (SERCA; a marker of heart failure [Hasenfuss et al, 1994]) contents of nonfailing and failing hearts were compared by Western blot analysis (Fig. 4A). One specific band of annexin II, V, and VI was detected in CPP from nonfailing as well as from failing hearts. The signals of annexin II and annexin V were stronger in failing compared with nonfailing hearts, whereas there was no obvious difference in the level of annexin VI. In addition, a decrease in the amount of SERCA was observed in all of the failing hearts.

The bar graph in Figure 4B shows that, compared with nonfailing hearts, the amounts of annexins II, V, and VI increased in failing myocardium to $132 \pm 9\%$ ($p < 0.05$), $147 \pm 7\%$ ($p < 0.01$), and $117 \pm 5\%$ ($p <$

0.05), respectively, whereas the amount of SERCA dropped substantially to $56 \pm 12\%$ ($p < 0.05$), as reported for similar human tissue preparations (Hasenfuss et al, 1994).

Immunolocalization of Annexins in Nonfailing and Failing Human Heart

The distribution of annexins II, V, and VI in cryosections of cardiac tissue was analyzed by immunofluorescence and was compared with that of α -actinin as a myofilament marker and of Na^+/K^+ -ATPase as a sarcolemma marker.

Annexin II was not detected in myocytes but was present in the endothelium (both vascular [Fig. 5A] and endocardial) and in the interstitium of normal myocardium (Fig. 5B). In failing hearts, an intense labeling of annexin II was observed in the interstitium (Fig. 5C). Double immunofluorescence study of transverse section at higher magnification showed that annexin II was mainly present (Fig. 5E) in places in which α -actinin was absent (Fig. 5F).

Annexin V (Figs. 6 and 7) in nonfailing hearts was localized mainly at the level of sarcolemma and in the endothelial cells (data not shown). Comparison of annexin V and Na^+/K^+ -ATPase labeling in nonfailing hearts is shown in Figure 6. A similar distribution of both proteins was observed in longitudinal tissue sections (Fig. 6, A and D) at the level of sarcolemma and intercalated discs. Furthermore, magnification of longitudinal sections (Fig. 6, B and E) showed, for both antigens, regular intracellular staining pattern of dotted transversely oriented narrow bands that coincided with the sarcomeric α -actinin labeling and that supported the assumption that annexin V was detected in regard to the Z-line, at the costamere level. Higher magnification of transverse sections clearly showed invaginations (arrows) containing Na^+/K^+ -ATPase (Fig. 6C) or annexin V (Fig. 6F) suggesting that annexin V was localized at or near T-tubules, as previously

Figure 4.

Comparison of the amount of annexins II, V, and VI in nonfailing and failing hearts by Western blot analysis. A, Western blot analysis of annexin II (Anx II), annexin V (Anx V), annexin VI (Anx VI), and sarco-endoplasmic reticulum Ca^{2+} -ATPase (SERCA) in CPP samples ($10 \mu\text{g}$ of protein) from nonfailing (N) and failing (F) hearts. B, Relative amounts of annexins II, V, and VI and SERCA in nonfailing (light bars) and failing hearts (dark bars). Values are mean $\pm$ SEM, $n = 9$; * $p < 0.05$, ** $p < 0.01$, compared with nonfailing hearts.

Figure 5.

Annexin II localization in nonfailing (N) and failing (F) hearts. Immunofluorescent labeling of annexin II in sections from nonfailing (A and B) and failing heart (C and D). Double immunolabeling of annexin II and α -actinin in transverse sections (E and F, respectively) of failing hearts. Arrow shows coronary vessels. Bar, 50 μ m.

described for Na^+/K^+ -ATPase (McDonough et al, 1996). At the same time, we cannot exclude annexin V association with adjacent sarcoplasmic reticulum because on some sections the pattern appears as faint bands parallel to the longitudinal axis of myofibers resembling that of cardiotin, a sarcoplasmic reticulum protein (Schaart et al, 1997).

Differences in labeling of annexin V in nonfailing and failing hearts is shown in Figure 7. Both transverse (Fig. 7, A and D) and longitudinal (Fig. 7, B and E)

sections showed that annexin V was mainly distributed at the level of sarcolemma in nonfailing hearts (as shown in Fig. 6) and was almost absent from T-tubules (Fig. 7D) and poorly labeled sarcolemma and intercalated discs (Fig. 7E) in failing hearts. Comparison of annexin V and α -actinin distribution showed that in failing hearts, annexin V was predominantly present in patches of fibrosis between the bundles of myocytes (Fig. 7E) where no α -actinin labeling (Fig. 7F) was detected.

Figure 6.

Na^+/K^+ -ATPase and annexin V localization in nonfailing hearts. Longitudinal sections of myocardium were labeled with antibodies to Na^+/K^+ -ATPase (A) and annexin V (B). Intracellular staining patterns at higher magnification are shown in B (Na^+/K^+ -ATPase) and E (annexin V). Transverse sections are shown in C (Na^+/K^+ -ATPase) and F (annexin V). Arrows point to T-tubules and arrow heads to intercalated discs. Bar, 25 μm (A and D); bar, 10 μm (B, C, E, and F).

Distribution of annexin VI in nonfailing and failing hearts is shown in Figure 8. Invaginations visible in transverse sections (Fig. 8A) and a striated pattern (Fig. 8B) coincident with α -actinin labeling at the Z-line of myocytes (Fig. 8C) suggested that annexin VI, like annexin V in nonfailing heart, was present at the level of sarcolemma, including T-tubules and intercalated discs. In failing hearts, invaginations of sarcolemma (Fig. 8D) and striated labeling of annexin VI (Fig. 8E) corresponding to that of α -actinin (Fig. 8F) were still observed within the myocytes. In addition, transverse and longitudinal sections of failing hearts showed that annexin VI was present in interstitium, between the bundles of myofilaments revealed by α -actinin staining (Fig. 8F).

Discussion

For the first time, the expression and the localization of annexins II, V, and VI were studied on the same samples of nonfailing and failing human hearts. We also demonstrated that in nonfailing human hearts annexins V and VI were present predominantly at the level of the myocyte membranes, whereas annexin II was mainly located in the interstitium. Myocardium from the patients with end-stage heart failure showed a significant increase in the expression of annexins II and V, accompanied by a marked staining of annexin II, V, and VI in the interstitium. At the same time, annexin V staining of myocytes became hardly visible.

In the present study, we determined the absolute amount of annexins II, V, and VI in membrane-enriched CPP from human myocardium and we showed that the amount of annexin VI was higher than

that of annexin II and annexin V. The preparation was chosen because it contained most of the tissue annexins (Fig. 2) and because we assumed that annexins that are tightly associated with membranes, such as those forming the Ca^{2+} channel, are of special interest for cardiac pathology. Using this preparation, we found a higher amount of annexin V than previously reported for human hearts (Reutelingsperger et al, 1994), but our findings agreed with those reported for normal rat hearts (Giambanco et al, 1993; Jans et al, 1995, 1998). In addition, the amount of annexin VI in human heart was higher than reported in rat heart (Giambanco et al, 1993). It is likely that the use of CPP leads to the recovery of the higher yields observed in this study, whereas EGTA extraction was employed in other studies.

In failing hearts, our results showed an increase in mRNA and protein levels of annexin II and annexin V, and clearly established that the accumulation of mRNA and protein occurred in parallel, likely due to transcriptional regulation. This is consistent with data reported by Song et al (1998) for failing hearts, obtained from a mixed group of patients with coronary artery disease and cardiomyopathy. However, we also found an increase in annexin VI that was not detected by Song et al, probably because a substantial part of annexin VI was lost in the EGTA extracts used in their study. A similar increase in the amount of annexins II and V, as well as of annexin VI, was demonstrated in left ventricles from hypertensive guinea pigs at the onset of heart failure (Trouvé et al, 1999), whereas in compensated hypertrophy, the amounts of annexin V were unchanged (Jans et al, 1998), suggesting that

Figure 7.

Annexin V localization in nonfailing (N) and failing (F) hearts. Immunohistofluorescence of transverse sections from nonfailing (A) and failing heart (D) labeled with antibodies to annexin V. Double immunolabeling of annexin V and α -actinin in longitudinal sections from nonfailing (B and C, respectively) and failing hearts (E and F, respectively). Arrows point to T-tubules and arrow heads to intercalated discs. Bar, 50 μ m.

expression of annexins varies with severity of heart pathology.

To clarify the consequences of this upregulation of annexins in the failing heart, we further investigated their distribution. We have shown that annexin II was mainly localized in the vascular endothelial cells and in the extracellular matrix of human heart (Fig. 5) as well

as of guinea pig heart (Trouvé et al, 1999). Parallel with the increase in annexin II mRNA and protein levels in failing hearts, there was an intense labeling of the interstitium between the cardiomyocytes (likely fibrotic tissue) and around the coronary arteries. Because annexin II is involved in membrane trafficking and extracellular matrix binding (Waisman, 1995), this

Figure 8.

Annexin VI localization in nonfailing (N) and failing (F) hearts. Immunohistochemistry of transverse sections from nonfailing (A) and failing heart (B) labeled with antibodies to annexin VI. Double immunolabeling of annexin VI and α -actinin in longitudinal sections from nonfailing (C and E, respectively) and failing hearts (D and F, respectively). Arrows show invaginations of sarcolemma and arrow heads indicate intercalated discs. Bar, 50 μ m.

would suggest that annexin II plays a role in the development of fibrosis and the organization of extracellular matrix in heart failure.

In this study, we described a high labeling of annexin V in the myocyte membranes of nonfailing human myocardium that was equivalent to that of Na^+/K^+ -ATPase (Fig. 6). Taken together, our results indicated that annexin V was present in sarcolemma, and intercalated discs, and in the T-tubules connected

to the terminal cisternae of sarcoplasmic reticulum—the complex in which the excitation-contraction coupling took place. Therefore, labeling of annexin V in human heart was similar to that of other species (Doubell et al, 1993; Jans et al, 1995; Luckcuck et al, 1997; Trouvé et al, 1999; Van Bilsen et al, 1992; Wang et al, 1995). Interestingly, immunofluorescence of annexin V in failing hearts (Fig. 7, D and E) was mainly observed in areas corresponding to fibrotic tissue. In

contrast, the labeling of annexin V in sarcolemma, T-tubules, and intercalated discs became faint. In some sections of failing human heart it was even difficult to distinguish annexin V labeling from that of annexin II, because myocyte labeling was almost absent, whereas the interstitium became extensively stained (Fig. 7D). This relocalization is a striking feature of the failing human heart and is likely attributable to a diversity in pathological states because it is often observed in end-stage heart failure, rarely in guinea pig at the onset of heart failure (Trouvé et al, 1999), and not detected in compensated hypertrophy (Jans et al, 1998), although annexin V localization in normal cardiac tissue is very similar in both species. In any case it suggests the involvement of annexin V in the development of heart failure.

Despite the absence of signal sequences in annexin molecules there is accumulating evidence of their ability to be translocated and secreted out of cells (Takehara et al, 1994). A recent suggestion to the use serum level of annexin V as a marker for myocyte damage during myocardial infarction (Kaneko et al, 1996) could also argue for a potential secretion of annexin V. Taking into account the ability of annexin V to bind collagen and to regulate cell-extracellular matrix interactions (King et al, 1997; Raynal and Pollard, 1994), we suggested that an early relocalization of annexin V could participate in the remodeling of ventricular myocardium. Conversely, the decrease in myocyte membrane-bound annexin V could induce local shifts of Ca^{2+} concentration leading to activation of protein kinase C (Dubois et al, 1998) and phospholipase A_2 (Mira et al, 1998), and/or activation of signaling pathways involved in cardiac remodeling (Sadoshima and Izumo, 1993).

In this study, we demonstrated that the content of annexin VI was higher than that of the annexins II and V. Annexin VI was mainly localized in myocytes at the level of the sarcolemma, T-tubules, and intercalated discs as in other mammalian species (Iida et al, 1992; Trouvé et al, 1999; Wang et al, 1993) and was similar to that of annexins V. In failing hearts, there was a slight increase in the amount of annexin VI, but unlike labeling with annexin V, both myocytes and interstitial tissue were still labeled. Therefore, these results did not argue in favor of a specific role for annexin VI in the altered Ca^{2+} handling and remodeling during heart failure.

In conclusion, our results showed that annexins II, V, and VI are abundant proteins of the human myocardium. Both quantification and localization of annexins in the failing human heart indicated an increase of the three annexins, and particularly of annexin V, in fibrotic areas. The ability of annexin II and V to bind proteins of the extracellular matrix suggested that they play a role in the remodeling of the ventricular myocardium during heart failure. Furthermore, relocalization of annexin V from myocytes into the interstitial space may have important functional consequences, including changes in Ca^{2+} homeostasis and in Ca^{2+} signaling pathways, which in turn may participate to

the impaired cardiac pump function in patients with end-stage IDCM.

Materials and Methods

Tissue Samples

Nine nonfailing hearts were obtained from adult patients who died from head trauma ($n = 8$) or smoke inhalation ($n = 1$). They were ultimately rejected for transplantation for reasons that were not related to their cardiac function.

Eleven failing hearts from patients with IDCM were obtained at the time of transplantation. All hearts exhibited morphological and histologic criteria of hypertrophy. The left ventricular ejection fraction was decreased to $16 \pm 2\%$ ($n = 11$). Transmural samples without visible signs of necrosis (0.5 to 2 g) were quickly excised from the anterior free wall of the left ventricle and immersed in liquid nitrogen. This study was approved by the Consultative Committee for the Protection of Human Subjects in Biomedical Research at the Pitié-Salpêtrière Hospital in Paris.

Northern and Slot Blot Analyses of Annexin mRNA

Total RNA from myocardium samples was prepared using an RNA plus kit (Bioprobe Systems, Montreuil sous Bois, France). The ratio of 18S to 28S bands and the yield of total RNA extracted were similar in both nonfailing and failing hearts.

cDNA probes of human annexin II (nucleotide 1 to 343) and human annexin V (nucleotide 1 to 159) were subcloned in pBluescript (SK+). The full-length annexin VI cDNA probe was inserted in pAT 153 plasmid and a 500 bp fragment was excised by *EcoR* 1-*PvuII*. Poly(A+) RNA was detected by a 25 to 30 mer poly(T) synthetic oligonucleotide (Pharmacia, Les Ulis, France). The cDNA probes were labeled by random priming (Megaprime DNA Labeling System; Amersham, Les Ulis, France) with (α - ^{32}P) dCTP (3000 Ci/mmol; NEN Life Science Products, Boston, Massachusetts) and the synthetic oligonucleotide was 5'-end-labeled with (γ - ^{32}P) dATP (6000 Ci/mmol, NEN).

Northern blots (25 μg RNA) and slot blots (2, 5, 10, and 20 μg) were performed according to the standard procedure described by Trouvé et al (1999). After washing, Northern and slot blots were exposed to a Phosphor screen for quantification with a BAS 1000 (FUJI Photo Film, Clichy, France) Bio-imaging Analyzer System. The results of densitometry were normalized to the total amount of poly(A+) RNA to correct for sample loading.

Characterization of Annexin II, V, and VI by Western Blot Analysis and Immunohistochemistry

CPP from myocardium samples (200 mg) were obtained according to Sainte Beuve et al (1997) in the presence of protease inhibitors. Protein concentrations were determined by the method of Lowry et al (1951) using bovine serum albumin as a standard.

CPP proteins were separated by electrophoresis in 10% polyacrylamide gels and then transferred to Hybond-P PVDF membrane (Amersham) (Towbin et al, 1979). Anti-annexin antibodies were obtained by immunization of a rabbit with recombinant human annexins (Trouvé et al, 1999). Monoclonal antibodies against dog cardiac SERCA have been described elsewhere (de la Bastie et al, 1990). The membranes were treated with antibodies diluted 1:5000 in blocking solution followed by the incubation with appropriate horseradish peroxidase-conjugated second antibodies diluted 1:5000 (Amersham). Peroxidase activity was detected by ECL using the ECLplus kit (Amersham). Coomassie blue staining of the total proteins was performed after stripping the membranes with 0.1 M/l sodium acetate (pH = 4) and 0.5 M/l sodium chloride. Quantification of both ECL spots on the film and Coomassie blue staining of proteins were performed by Gel Doc 1000 Molecular Analysis system (BioRad, Ivry/Seine, France). ECL spots were normalized by integrated density of the Coomassie blue staining of the lane.

For immunohistofluorescence studies, samples of left ventricular myocardium were placed in embedding medium Tissue-Tek (Miles-Diagnostics, Elkhart, Indiana) and frozen in isopentane at -155°C . Frozen tissue sections (7 to 8 μm) were fixed with acetone:methanol (1:1 for 20 minutes at -20°C), saturated with 5% bovine serum albumin in PBS (30 minutes at room temperature), incubated with mouse monoclonal anti- α -actinin antibodies (Sigma, Saint Quentin Fallavier, France) (1:50 in PBS for 30 minutes at 37°C), washed, then incubated with appropriate dilutions of anti-annexin II, V, or VI antiserum (1:40, 1:20, and 1:20, respectively, in PBS for 30 minutes at 37°C) or of anti- $\text{Na}^{+}/\text{K}^{+}$ -ATPase antiserum (Charlemagne et al, 1994). After three rinses in PBS, the sections were successively incubated for 30 minutes at room temperature with a 1:50 dilution of anti-mouse immunoglobulins conjugated with Texas red and a 1:50 dilution of anti-rabbit immunoglobulins conjugated with fluoresceine isothiocyanate fluorochrome. Secondary antibodies alone did not reveal any significant fluorescence signal after the incubation with the tissue slices. Fluorescence was visualized using a Leica DM RD microscope equipped with epifluorescence optics.

Statistical Analysis

Results were expressed as mean $\pm$ standard error of the mean (SEM). Differences between control and experimental groups were evaluated by a two-tailed unpaired Student's *t* test, and $p < 0.05$ was considered significant.

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