BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **238,** 622–628 (1997)
ARTICLE NO. RC977350

Localization of Annexin V in the Adult and Neonatal Heart

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Received August 12, 1997

Annexins are a major family of intracellular Ca^{2+}-binding proteins which have been implicated in a variety of cellular functions. Several conflicting reports have been published on the location of annexin V in the heart. In this paper we have used confocal microscopy to demonstrate that annexin V is associated with the sarcolemma and intercalated discs of cardiac myocytes in sections of adult porcine and rat heart. In addition, we have used confocal microscopy of isolated rat myocytes to show that this association is stable even after cells were treated with the intracellular calcium chelator BAPTA-AM, to reduce cytosolic calcium levels to very low levels. This demonstrates that annexin V associates tightly with the sarcolemma and suggests that components in addition to phospholipid are involved in binding annexin V to the membrane. Furthermore, we show that, in sections of the neonatal rat left ventricle, annexin V has a different subcellular location than that observed in the terminally differentiated adult myocyte. In these differentiating neonatal cells, annexin V is also located in the nucleoplasm and at the periphery of the nucleus. These results demonstrate that the subcellular location of annexin V is differentially regulated and suggest that annexin V regulates calcium-dependent processes at both the sarcolemma and the nucleus.

Key Words: **annexin V; calcium; sarcolemma; heart; localization.**

Annexins are a family of at least thirteen proteins which contain repeats of a highly conserved 70 amino acid sequence [1,2]. Annexin V is the most ubiquitous annexin and is present in almost all mammalian cells including heart myocytes [3-5]. Although the exact physiological function of annexin V remains unclear it has been implicated in ion channel regulation [6-8], as an inhibitor of protein kinase C [9-13], as an inhibitor of blood coagulation [14,15] and as an inhibitor of phospholipase A_2 [16]. Annexin V has been shown to be located in both the cytosol and at the plasma membrane in a variety of cell types [2, 17-19]. Furthermore, recent evidence from platelets, fibroblasts and the osteosarcoma cell line MG-63, suggests that increases in cytosolic calcium levels induce a proportion of the annexin V to relocate from the cytosol to the plasma membrane [18-21]. In addition, a nuclear location for annexin V has also been reported [22-25], suggesting that annexin V may play a role at both the plasma membrane and in the nucleus.

As in other cell types, the role of annexin V in the heart is unknown. Several recent reports have produced conflicting findings as to the localization of annexin V. For example, several reports have shown that annexin V is located at the sarcolemma alone [26, 27] or at the sarcolemma and intercalated discs in myocytes [3-5]. In addition, Spreca and co-workers reported staining of the T-tubules are stained [4], while Doubell and co-workers also found immunoreactivity in the sarcoplasm and on the contractile elements [5]. Interestingly, in contrast to the above studies, Wang and co-workers have recently reported that annexin V is not located at the sarcolemma or intercalated discs, but that it has a cross-striated pattern within the myocytes with annexin V localized to the Z-lines [28]. Furthermore, the study of Van Bilsen and co-workers reported that annexin V is not present myocytes [29].

The aim of this study was therefore to resolve the controversy surrounding the location, and hence the function of annexin V in the heart. We therefore determined the location of annexin V in vibratome sections and in isolated cardiac myocytes. In addition, we provide evidence that endogenous annexin V associates with the sarcolemma in a novel manner and present evidence of a developmentally regulated change in the location of annexin V.

MATERIALS AND METHODS

Materials. Porcine heart left ventricle was used fresh from the abattoir. Hearts were removed from male adult or neonatal Wistar rats. A rabbit antiserum to human annexin V was a gift from Dr F. Seiler and Dr J. Römisch. Monoclonal antibodies against the cardiac ryanodine receptor were a gift from Dr J. Junker and antibodies against the SR specific Ca^{2+}-ATPase SERCA 2a were a gift from Dr

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0006-291X/97 $25.00

J. Colyer. All other chemicals and reagents were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.) or from BDH Chemicals (Poole, Dorset, U.K.) unless otherwise stated.

Purification of annexin V. Annexin V was purified from rat and pig heart using the method of Boustead et al [30]. The pure protein was then used to adsorb the antiserum for controls in immunocytochemical studies.

Preparation of tissue sections. Hearts were obtained immediately after death of the animals. Small pieces of pig and rat left ventricle (approx 5 × 2 × 2 mm) were placed into a large volume of 10% neutral buffered formalin (Sigma HT-50) which was changed 3 times, each after 30 mins. They were then left to fix at 4°C for 24-48 hours and were then mounted on a vibratome and submerged in PBS. Sections (≈50 μm thick) were cut in both longitudinal and transverse orientations and these were refixed for 5 mins in a small well of formalin and then transferred to PBS.

Preparation of isolated cells. Hearts were removed from freshly killed rats and perfused with collagenases and proteases to digest connective tissue. Single ventricular myocytes were isolated [31] and suspended in Hepes-Tyrodes containing 750 μM Ca^{2+}. Some cells were also suspended in Hepes-Tyrodes containing 5 mM EGTA plus 10 μM BAPTA-AM [32] for 90 mins. The myocytes were subsequently pelleted at 400 g_{av} in a bench top centrifuge and resuspended to 1ml in phosphate buffered saline (PBS). Cells were fixed by resuspension in 10% neutral buffered formalin (Sigma HT-50) for 5 mins. The cells were then centrifuged again, resuspended in distilled water, and were then spread onto ovalbumin-coated coverslips and air dried.

Fluorescent immunocytochemistry and confocal laser scanning microscopy. Cells and sections were permeabilized for 30 mins in PBS containing 2% (v/v) Triton X-100 and 50% (v/v) glycerol [33]. Subsequently, cells and sections were washed 3 times in PBS and treated with $NaBH_4$ (1 mg/ml; 3 × 5 mins) to reduce autofluorescence. After a further 3 washes in PBS, cells and sections were incubated with PBS containing 5% (v/v) goat serum for 3 hours at room temperature to block non-specific sites. Cells and sections were then incubated overnight in either undiluted tissue culture medium for monoclonal antibodies or polyclonal antiserum diluted 1:100 in PBS containing 5% goat serum plus 1 mM NaN_3. After further thorough washing they were then incubated with FITC-labelled affinity-purified second antibody for 3 hours. Following further washes with PBS, the cells and sections were mounted with Vectashield (Vector Laboratories). At the blocking and antibody steps, incubation solutions contained 2% (v/v) Triton X-100 and 50% (v/v) glycerol. Conventional epifluorescent light microscopy was performed using a Nikon Optiphot microscope and confocal microscopy was performed on the Leica laser scanning microscope.

The specificity of the antiserum to annexin V was established by Western blotting as described previously [18,20,21] and by adsorption of the antiserum with pure annexin V [18].

RESULTS AND DISCUSSION

Locations of Annexin V in Vibratome Sections

As mentioned above there is much discrepancy about the subcellular location, and hence the function, of annexin V in the heart. The subcellular localization of annexins V in both longitudinal and transverse sections of heart was therefore determined by immunofluorescence microscopy as described in the Materials and Methods section. When longitudinal sections were viewed it was apparent that annexin V was located predominantly at the sarcolemma and the intercalated discs, with some punctate staining seen within the myocytes (Fig. 1a). When transverse sections were viewed it was again apparent that the sarcolemma was the major site for annexin V within the myocytes with fainter staining of T-tubules (Fig. 1b). In addition, our results show that the coronary capillaries of the microcirculation are strongly stained for annexin V (Fig. 1), an observation that is consistent with the findings of other workers who have also reported that endothelial cells contain abundant amounts of annexin V [4, 5, 24].

Confirmation that annexin V in these vibratome sections was localised to the sarcolemma was obtained by comparison of annexin V with the staining pattern obtained with wheat germ agglutinin (WGA), a marker which has been previously shown to bind to glycoproteins in the plasma membrane [34-36]. On examination

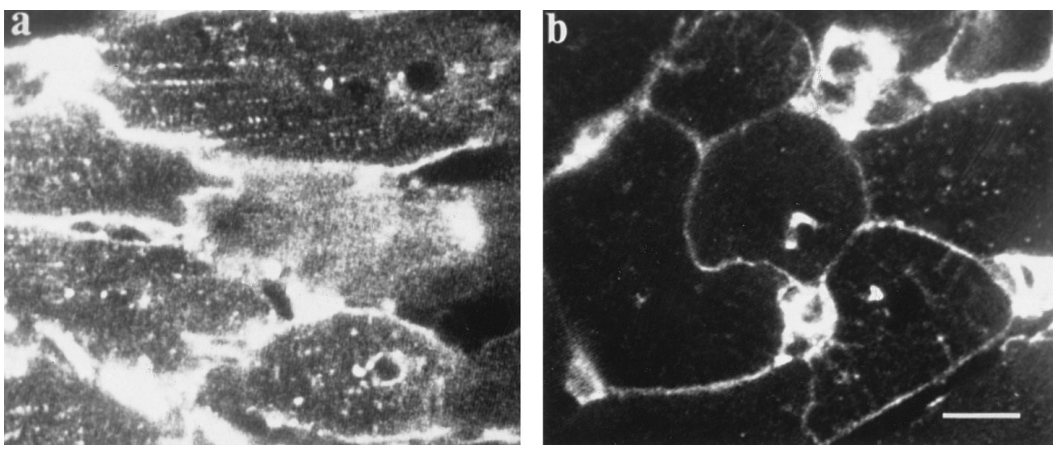

FIG. 1. Localization of annexin V in vibratome sections of the pig left ventricle. Longitudinal (a) and transverse (b) sections of formalin fixed pig heart were permeabilized with Triton X-100 and incubated with monospecific antiserum to annexin V. FITC-labelled second antibody was used to detect the primary antibody and the fluorescence in 1 μm thick optical sections was viewed with a confocal laser scanning microscope. Bar, 10 μm.

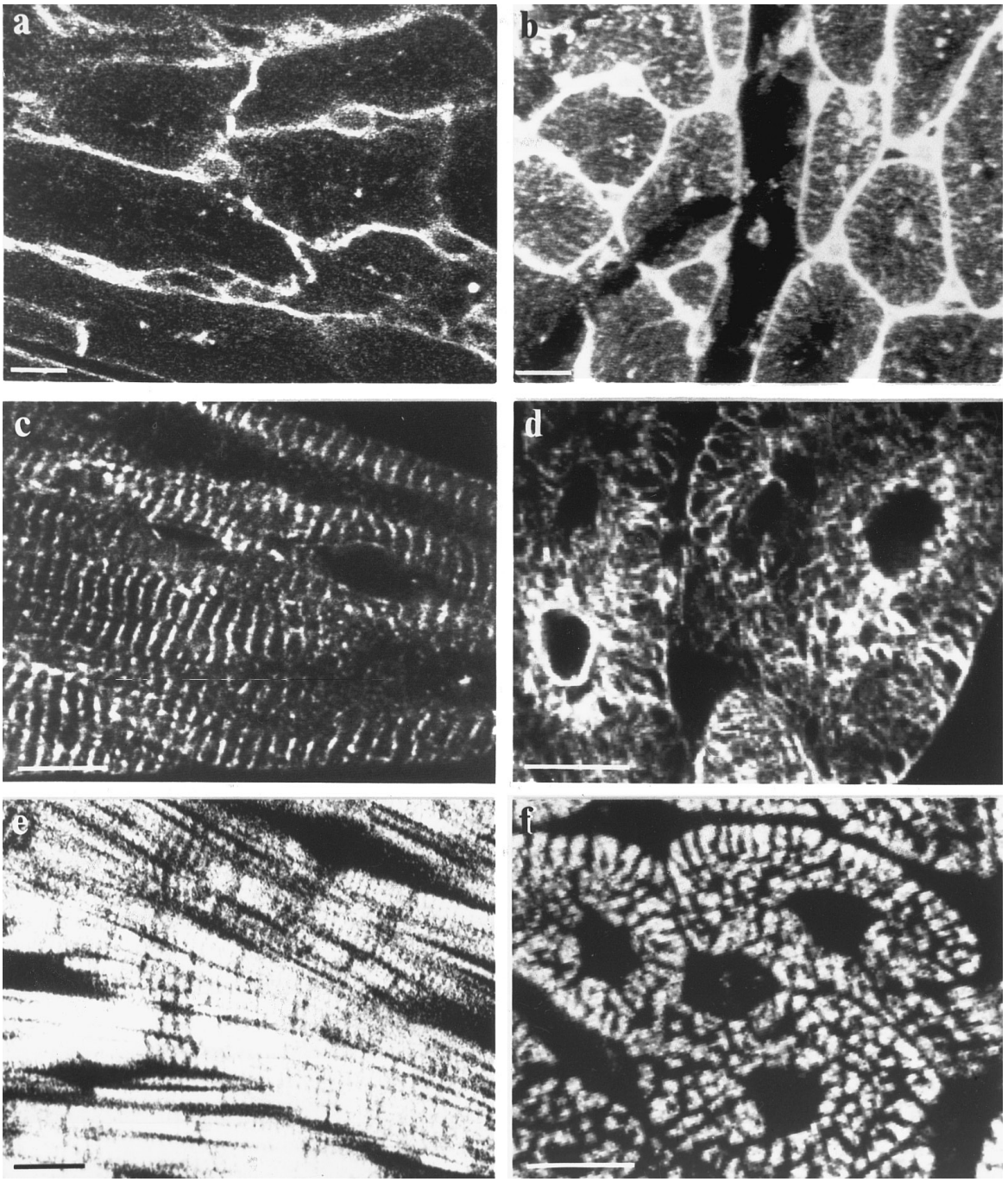

FIG. 2. Localization of the plasma membrane, the sarcoplasmic reticulum specific Ca^{2+}-ATPase and actin filaments in vibratome sections. Longitudinal (a, c and e) and transverse (b, d, and f) sections of formalin fixed pig heart were permeabilized with Triton X-100 and incubated with either the lectin WGA (a and b), a monospecific antibody raised against a synthetic peptide corresponding to the C-terminal 8 amino acids of the sarcoplasmic reticulum specific Ca^{2+}-ATPase (c and d) or rhodamine-labelled phalloidin (e and f). Fluorescent labelled proteins were viewed in 1 μm thick optical sections with a confocal laser scanning microscope Bars, 10 μm.

of the staining pattern it was apparent that it had a similar pattern in both longitudinal sections (Fig. 2a) and transverse sections (Fig. 2b). In contrast, the staining pattern for annexin V is completely different to that of the sarcoplasmic reticulum Ca^{2+} ATPase (Fig. 2 c and d) and of phalloidin which stains the myofibrils (Fig. 2 e and f). These results therefore demonstrate that, in myocytes, annexin V is predominantly found on the sarcolemma and intercalated discs, and is not associated with the SR or contractile apparatus.

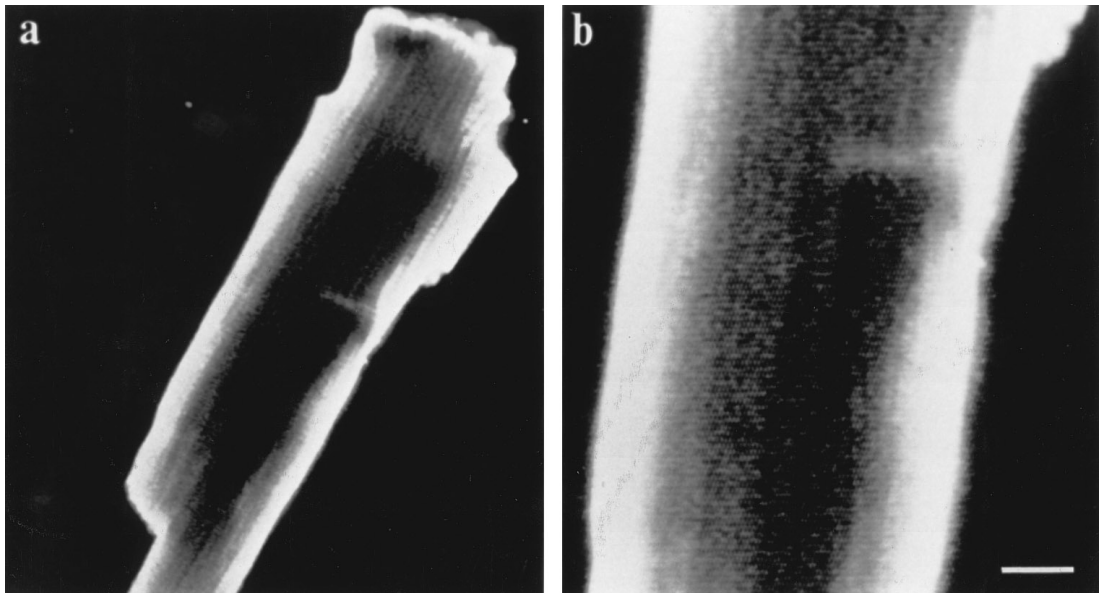

FIG. 3. Confocal images of the localization of annexins V in isolated myocytes. Formalin fixed rat ventricular myocytes were permeabilized with Triton X-100 and incubated with monospecific antiserum to annexin V. FITC-labelled second antibody was used to detect the primary antibody and the fluorescence was viewed with a confocal laser scanning microscope. Optical sections (1 μm thick) are shown through the middle of the cells. Bar, 10 μm (a), 5 μm (b).

Subcellular Localization of Annexin V in Isolated Ventricular Myocytes

The above results show that, in tissue sections annexin V is primarily found at the sarcolemma and intercalated discs. A possible criticism of studies on tissue sections is that during tissue fixation annexin V may have relocated from a cytosolic to a membrane location. We therefore investigated the location of annexin V in isolated cardiac myocytes. These isolated, living cells retain their morphology and physiological responses of cells *in situ*. On death they contract into unresponsive spherical structures. Living cells were therefore fixed immediately after isolation and then processed to locate annexin V by immunofluorescence microscopy. When sections through the centre of a myocyte were stained with antibodies to annexin V and the subcellular localization determined by confocal microscopy it was clear that annexin V is predominantly located at the sarcolemma and intercalated discs, with some faint sub-sarcolemmal staining inside the cells (Fig. 3). As annexin V has previously been reported to be associated with the sarcoplasm [5], the T-tubules [4], and the Z-lines [28] the staining pattern of annexin V in the isolated cells was subsequently compared with that of a variety of structural marker proteins. Fig. 4 reveals the intracellular staining of the SR specific Ca^{2+}-ATPase (Fig. 4a), the ryanodine receptor (Fig. 4b and 4c) and the myofibrils (Fig. 4d) on permeabilized cells. These results demonstrate that annexin V has a different staining pattern from the striated staining of the SR (compare Fig. 3 with 4a), the punctate staining of the terminal cisternae of the SR (compare Fig. 3 with Fig. 4b and 4c), and the myofibrils (compare Fig. 3 with Fig. 4d). In contrast, as with the vibratome sections discussed earlier, a similar staining pattern was observed when the subcellular location of annexin V was compared to that of the plasma membrane marker concanavalin A (compare Fig. 3 with Fig. 4e). These results therefore demonstrate that in isolated myocytes annexin V is primarily associated with the sarcolemma, and is not found in the sarcoplasm [5] or the contractile elements of these cells [28] as has previously been reported.

Is the Interaction of Annexin V with the Sarcolemma Dependent on Calcium?

Several groups including ours have reported that in biochemical subfraction experiments a proportion of the annexin V in a variety of tissues appears to bind to membranes in a manner that resists extraction with the calcium chelator EGTA [17-19, 37-39]. It was therefore interesting to investigate whether annexin V also associates with membranes of living cells in a manner that resists extraction with chelators of calcium. Isolated rat myocytes retain their normal morphology and ability to contract and relax. In addition, their intracellular Ca^{2+} concentration can conveniently be depleted by incubating the cells in the presence of extracellular EGTA and BAPTA-AM as described in the Materials and Methods section. Following depletion of cytosolic calcium it was apparent that the majority of annexin V remained associated with the sarcolemma (Fig. 5).

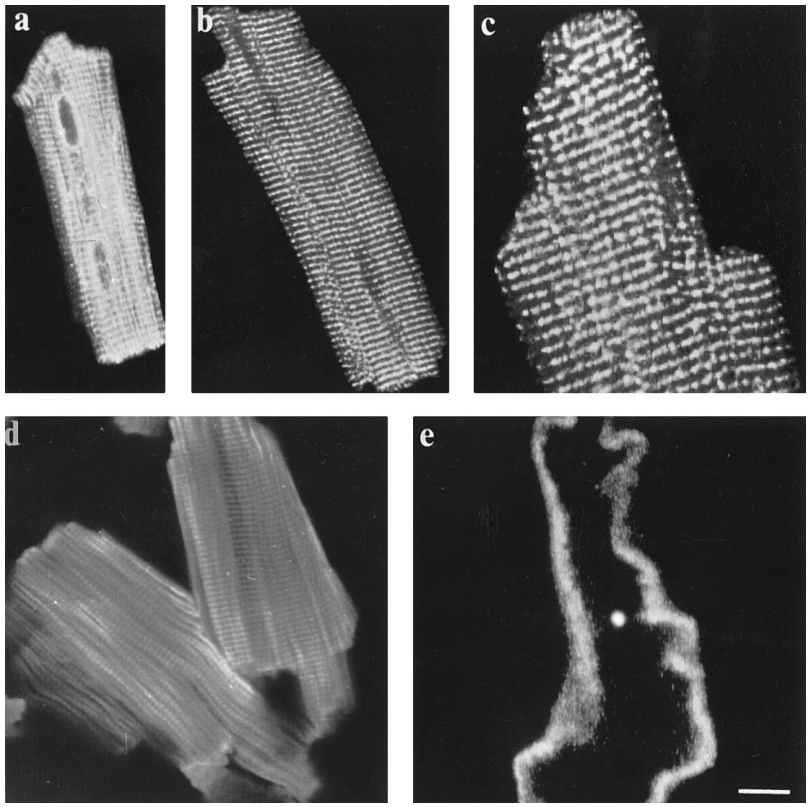

FIG. 4. Confocal images of the localization of structural markers in isolated myocytes. Formalin fixed myocytes were left unpermeabilized (e) or permeabilized with Triton X-100 (a to d) and incubated with monospecific polyclonal antibodies to the sarcoplasmic reticulum specific Ca^{2+}-ATPase (a) or monoclonal antibodies to the ryanodine receptor of the sarcoplasmic reticulum (b and c). FITC-labelled second antibody was used to detect the primary antibody and in (d) rhodamine-labelled phalloidin was used directly. FITC-labelled concanavalin A was used in (e) on the non-permeabilized cells. The fluorescence was viewed with a confocal laser scanning microscope. Optical sections (1 μm thick) are shown through the middle of the cells. Bar, 10 μm except 5 μm in (c).

However a small amount of annexin V did appear to relocate from the sarcolemma to a striated structure within the cell that most closely resembles the distribution of T-tubules within these cells. There was little evidence for the redistribution of annexin V into the sarcoplasm following chelation of intracellular calcium. Thus in the cardiac myocyte, annexin V is predominantly associated with the sarcolemma and intercalated discs, and can relocate only to a limited extent after depletion of cytosolic calcium levels. This is interesting in comparison with other cell types. In mammalian cells in culture [20, 21, 24] and in platelets [18, 19] very little annexin V is associated with the plasma membrane of unstimulated cells. Annexin V only relocates to the plasma membranes of these cells after elevation of cytosolic calcium levels. Our results therefore argue for a persistent requirement for annexin V on the plasma membrane of the cardiac myocyte that may correlate to the repeated rise in calcium in these cells with each heart beat. In other cells there is no frequent and repeated rise in calcium levels and thus possibly less requirement for annexin V at the plasma membrane.

Subcellular Localization of Annexin V in Vibratome Sections of Neonatal Rat Ventricle

Using calcium ionophores with fibroblasts [20] and with the osteosarcoma cell line MG-63 [21], we have previously shown that that elevation of cytosolic calcium levels alters the staining pattern of annexin V. In these cells, annexin V is seen to be associated with the nucleus and with the cytoplasm. However, following increases in intracellular calcium, annexin V relocates and binds to both the plasma and nuclear membranes. In addition, we have shown that when MG-63 cells are grown in the absence of growth factors, the nucleus becomes devoid of annexin V [24]. Importantly, if growth serum is subsequently added to these cells, the nucleus once again becomes positive for annexin V. These observations therefore suggest that annexin V may play two regulatory roles within these cells. First, one at the plasma membrane, and secondly one in the nucleus, possibly coupling cell activation by growth factors to cell growth. Adult myocytes which do not contain any nuclear annexin V are terminally differentiated cells, with limited growth and division. It was

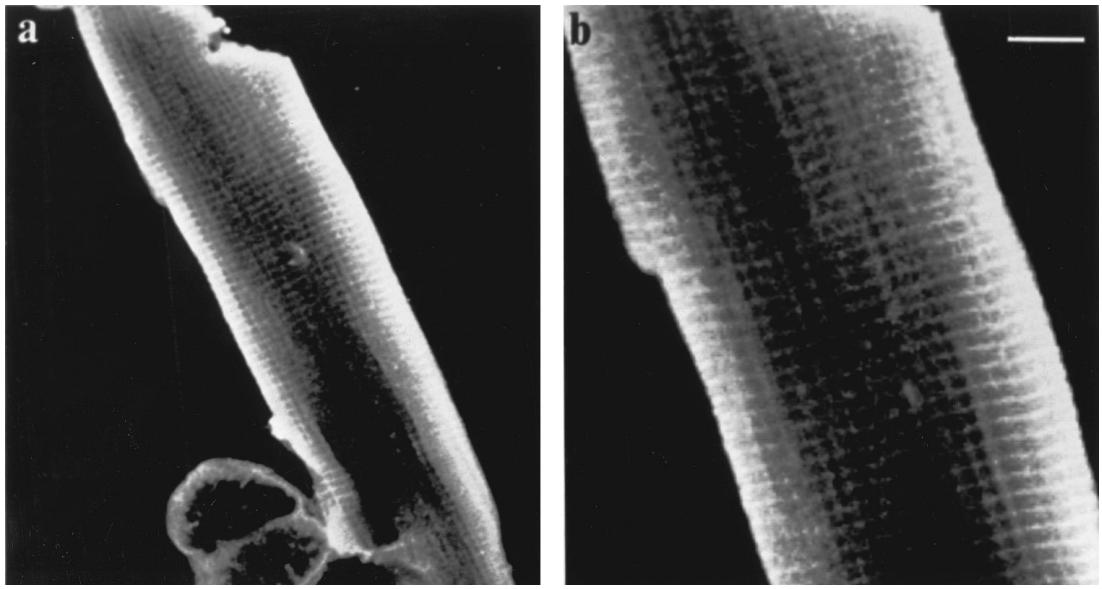

FIG. 5. Localization of annexin V in cells depleted of intracellular calcium. Isolated myocytes were fixed in the presence of EGTA and then permeabilized with Triton X-100. Subsequently they were incubated with monospecific antiserum to annexin V. FITC-labelled second antibody was used to detect the primary antibody and the fluorescence was viewed with a confocal laser scanning microscope. Optical sections (1 μm thick) are shown through the middle of the cells. Bar, 10 μm (a), 5 μm (b).

therefore interesting to investigate the subcellular location of annexin V in neonatal myocytes from hearts which were still actively growing. Vibratome sections of neonatal rat ventricle were therefore prepared as described in the Materials and Methods section. When longitudinal sections of neonatal rat ventricle were stained for annexin V it was apparent that, as for the adult tissue, annexin V was located on the sarcolemma and intercalated discs. (Fig. 6a small arrowhead) and in the endothelial cells of the coronary capillaries (Fig. 6a, thin arrow). However, in contrast to the staining previously observed in adult tissue, annexin V was also located within the nuclei (Fig. 6a, fat arrows) and in some cases as a ring at the nuclear membrane (Fig. 6a, b, large arrowheads). These observations implicate nuclear annexin V in cell growth and the development of the heart.

The work presented here demonstrates that annexin V is not playing a role at the contractile apparatus, as has previously been suggested, but is playing a role at the sarcolemma in adult myocytes, and at both the sarcolemma and nucleus in neonatal myocytes. Our re-

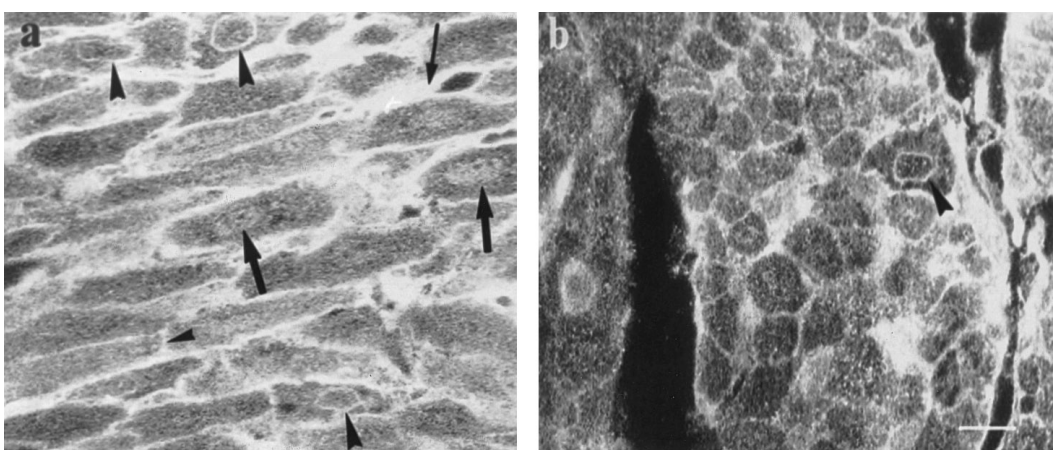

FIG. 6. Localization of annexin V in longitudinal and transverse vibratome sections of the left ventricle from a neonatal rat heart. Longitudinal and transverse sections of formalin fixed neonatal rat heart were permeabilized with Triton X-100 and incubated with monospecific antiserum to annexin V. FITC-labelled second antibody was used to detect the primary antibody and the fluorescence was viewed with a confocal laser scanning microscope. Optical sections (1 μm thick) are shown through the middle of the cells. Bar, 10 μm.

sults also demonstrate that, as with membrane associated annexin V in platelets [19], the annexin V which binds to the sarcolemma is also resistant to extraction with EGTA. In platelets, this membrane associated annexin V is released following solubilization of the membranes in the non-ionic detergent Triton X-100, and is mediated by non-lipid membrane components [19]. It is therefore possible that in cardiac myocytes annexin V binding proteins may mediate this tight association of annexin V with the membranes.

ACKNOWLEDGMENTS

This work was funded by a University of Leeds Mary and Alice Smith Memorial Scholarship, the British Heart Foundation (FS/95032). We also thank Dr. J. Junker (Campbell University, North Carolina) and Professor C. H. Orchard (Department of Physiology, Leeds) for the gift of antiserum against the cardiac ryanodine receptor, Dr. J. Colyer (Leeds) for the gift of antiserum against the SR specific Ca^{2+}-ATPase, and Dr. F. Seiler and Dr. J. Römisch (Behring, Marburg, Germany) for the gift of antiserum against annexin V. In addition we thank Juliet Fawcett and Munir Hussain of the Department of Physiology, Leeds for the supply of isolated myocytes.

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