Journal of Cellular Biochemistry 46:78–85 (1991)

Annexin VI Is Associated With Calcium-Sequestering Organelles

Parul Hazarika, Marcia A. Kaetzel, Adrian Sheldon, Norman J. Karin, Sidney Fleischer, T.E. Nelson, and John R. Dedman

Departments of Physiology and Cell Biology (P.H., M.A.K., A.S., N.J.K., J.R.D.) and Anesthesiology (T.E.N.), University of Texas Medical School, Houston, Texas 77225; Department of Molecular Biology, Vanderbilt University, Nashville, Tennessee 37235 (S.F.)

Abstract Annexin VI is a member of a Ca^{2+}-dependent, phospholipid-binding protein family. Although functions for this annexin have been proposed from in vitro studies, most remain controversial. Díaz-Muñoz et al. (*J Biol Chem* 265:15894, 1990) demonstrated that annexin VI modified, in a Ca^{2+}-dependent manner, the gating behavior of the sarcoplasmic reticulum Ca^{2+}-release channel, reconstituted into artificial bilayers, by increasing both the open probability and the mean open time. This effect was specific to the *trans* chamber, which represents the luminal side of the sarcoplasmic reticulum. In agreement with those findings, we show herein that annexin VI produced no effect on Ca^{2+}-uptake or -release by intact heavy sarcoplasmic reticulum vesicles (analogous to the *cis* chamber). We also used monospecific antibodies to evaluate the subcellular localization of annexin VI by immunofluorescent microscopy. Studies in rat skeletal muscle suggest that annexin VI is present surrounding individual myofibrils. Double immunolocalization studies with cultured muscle cells (chick myotubes) using anti-annexin VI and anti-SR Ca^{2+}-ATPase antibodies demonstrated superimposable staining patterns. In non-muscle tissue (normal rat kidney (NRK) cells), a punctate, perinuclear anti-annexin VI staining pattern was observed. Collectively, these data suggest that annexin VI may play a regulatory role in the Ca^{2+}-release/uptake cycle in the sarcoplasmic reticulum as well as in non-muscle organelles, a key process in stimulus-response systems.

Key words: sarcoplasmic reticulum, calcium regulation, calcium/phospholipid-binding protein, calcium-release/uptake, immunolocalization

Ca^{2+} acts as a second messenger in response to a variety of extracellular growth factors, hormones, cytokines, and other intercellular effectors. Transient elevation in intracellular free Ca^{2+} represents a signal involved in the regulation of a diverse number of cellular activities, including cell growth and division, motility, metabolism, adhesion, ion transport, and secretion [see 1]. Sub-micromolar levels of intracellular free Ca^{2+} are maintained against a large extracellular gradient by a Ca^{2+}-impermeable plasma membrane which contains Na^+/Ca^{2+}-exchangers and Ca^{2+}-pumps. Ca^{2+} entry occurs through a variety of Ca^{2+}-channels which have been defined by their sensitivity to pharmacological agents and by electrophysiological characteristics. Another mechanism for regulating intracellular Ca^{2+} levels is via activities which have been associated with the sarcoplasmic reticulum (SR) and smooth endoplasmic reticulum. In muscle, Ca^{2+}-release from the terminal cisternae of the SR occurs following sarcolemmal depolarization. Ca^{2+}-release from internal stores of smooth muscle and non-muscle cells has been shown to be regulated, in part, by inositol-(1,4,5)-triphosphate [2–6].

The intracellular Ca^{2+} signal is mediated via receptor or mediator proteins, such as calmodulin, troponin-C, and protein kinase C. More recently, several laboratories have independently identified another group of Ca^{2+}-binding proteins through their ability to interact with cytoskeletal elements, isolated membranes, secretory granules, or hydrophobic resins [see 7–9]. Sequence data indicate that there are ten unique gene products, each of which is composed of a repeat motif of 60–70 amino acids [8,10–14]. In vertebrates, these Ca^{2+}/phospholipid-binding proteins have been designated as annexins I–VIII

Received November 26, 1990; accepted December 21, 1990.

Address reprint requests to John R. Dedman, Department of Physiology and Biophysics, University of Cincinnati College of Medicine, Cincinnati, OH 45221-0016.

[15]. Two *Drosophila* annexin cDNAs have also been sequenced [16]. This gene superfamily is unrelated to calmodulin, troponin-C, and protein kinase C, with regard to potential structural mechanisms for binding Ca^{2+} or phospholipid. While there is a wealth of sequence information on these proteins, the cellular roles of the annexins are poorly defined [8,9].

We have previously shown by immunoblot analysis that annexin VI is expressed in all tissues examined, with high levels observed in muscle [17,18]. Since skeletal muscle is highly structured and its major functions have been well defined biochemically, muscle tissue was an appropriate system in which to explore annexin VI function. Based on preliminary muscle localization evidence [19], Díaz-Muñoz et al. [20] examined annexin VI effects on the ryanodine-sensitive sarcoplasmic reticulum Ca^{2+}-release channel which had been reconstituted into planar lipid bilayers. This annexin increased both the probability of opening and the mean open time. In order to further evaluate annexin VI function, it was examined in isolated intact SR vesicles and was localized in muscle and non-muscle cells.

MATERIALS AND METHODS

Measurement of Ca^{2+}-Uptake and -Release by Heavy SR Vesicles

To isolate heavy SR (HSR) vesicles, rabbit (back) white skeletal muscle was excised, minced on a tray of ice, and homogenized (1g tissue/4 ml buffer) in 20 mM histidine, 5 mM EGTA, pH 6.8 (4°C), in a Waring blender in 15 sec bursts. The pH was maintained with KOH at 6.8 during homogenization. The homogenate was centrifuged at 8000*g* for 20 min. The supernatant was filtered through glass wool, and actomyosin was then extracted by adjusting the filtrate to 600 mM KCl and centrifuging at 12,000*g* for 30 min. The pellet was resuspended in 20 mM histidine, 300 mM KCl, pH 6.8, and centrifuged at 16,000*g* for 30 min. The resulting HSR pellet was resuspended in 150 mM KCl, 300 mM sucrose, aliquoted, and stored at −80°C until used.

Ca^{2+}-uptake and Ca^{2+}-induced Ca^{2+}-release by HSR vesicles were measured essentially as described by Nelson et al. [21]. To measure Ca^{2+}-uptake, HSR vesicles (50 μg protein/ml final concentration) were added to 1 ml 20 mM histidine, 150 mM KCl, 5 mM NaN_3, 5 mM potassium oxalate, 16 μM arsenazo III (a metallochromic indicator dye), pH 6.8, along with 1 mM MgATP, 5 mM creatine phosphate, and 20 μg/ml creatine phosphokinase. Samples were stirred continuously in a cuvette which was maintained at 30°C by a water-jacketed cuvette holder. $CaCl_2$ (75 nmol) was added to the sample, and the uptake of Ca^{2+} from the medium by the HSR vesicles was monitored by measuring (A_{650}–A_{700}) using either a dual beam or diode array spectrophotometer.

Ca^{2+}-induced Ca^{2+}-release was measured with a similar experimental design, except that potassium oxalate was absent, the pH was 7.0, and 0.5–1.0 mg protein/ml (final concentration) HSR vesicles were used. $CaCl_2$ was added to the cuvette in increments of 10 nmol. At the end of each experiment, 200 nM ruthenium red, which completely blocks Ca^{2+}-channels, was added in order to calibrate the absorbance changes caused by changes in free Ca^{2+} levels. Additionally, A23187 ionophore was added to demonstrate the intravesicular Ca^{2+} content.

Heavy SR vesicles which were treated with annexin VI protein were incubated for approximately 15 minutes on ice with 10 μg annexin VI per 50 μg HSR for uptake studies or 10–30 μg annexin VI per 500 μg HSR for Ca^{2+}-release studies. Vesicles treated with antibodies were incubated for 1–2 hours on ice with 1 μg affinity-purified polyclonal antibodies per 500 μg HSR.

Production and Characterization of Anti-Annexin VI Antibodies

Sheep polyclonal antibodies were produced against rat liver annexin VI according to Mathew et al. [18]. The serum was antigen affinity-purified according to Kaetzel et al. [22]. The monoclonal antibodies used in these studies are described in Kaetzel et al. [23]; all of the antibodies produced by these hybridomas were IgGs, and were designated Mab 1–10.

Indirect Immunofluorescence of Cultured Cells and Tissue Sections

NRK cells were grown to 60–70% confluency on 11 × 22 mm coverslips in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Chick muscle cells were derived from 11 day chick embryos as described by O'Neill and Stockdale [24], plated in 35 mm dishes containing collagen-coated coverslips, and allowed to differentiate in vitro. Well-developed cultures (myotubes), as judged by the appearance of syncytia exhibiting phase-dense striations and spontaneous contractions, were fixed for 10 min in −20°C methanol and incubated successively with 5 μg/ml affinity-purified sheep anti-annexin VI [18] and 5 μg/ml monoclonal antibody CaF1,

which is specific for the avian fast isoform of the sarcoplasmic reticulum Ca^{2+}-ATPase [25]. Antibody binding was visualized with fluorescein-conjugated rabbit anti-sheep IgG, followed by rhodamine-coupled goat anti-mouse IgG (Organon Teknika), each at 1:80 dilutions.

Rat esophagus was fixed by immersion in 10% formalin in PBS, pH 7. The fixed tissue was washed with several changes of PBS, embedded in paraffin, and sectioned at 4 μm. Sections were deparaffinized with xylene and ethanol and hydrated, followed by incubation with a 1:10 dilution of normal goat serum (1 h at 37°C) to block non-specific binding. The sections were then incubated with a 1:2,000 dilution of monoclonal antibodies for 2 hours at 37°C, washed in PBS, and further incubated with fluorescein-conjugated goat anti-mouse IgG. Cells and tissue sections were viewed on a Nikon Optiphot epifluorescence microscope using the ×100 oil immersion objective. Photomicrographs were recorded on Kodak T-MAX (ASA 100) film.

RESULTS

Ca^{2+}-Uptake and Release Studies

To examine the effect of annexin VI on isolated rabbit heavy sarcoplasmic reticulum vesicles, two functions were evaluated, Ca^{2+}-uptake and Ca^{2+}-induced Ca^{2+}-release. Rates of ATP-dependent Ca^{2+}-uptake (by the SR Ca^{2+}-ATPase) were measured using intact heavy SR vesicles which had been 1) untreated, 2) treated with affinity-purified polyclonal anti-annexin VI, 3) treated with purified annexin VI, and/or 4) treated with 200 nM ruthenium red to inhibit the Ca^{2+}-release channel. The anti-annexin VI antibodies were shown to be monospecific for annexin VI [17]. Figure 1 shows representative tracings for Ca^{2+}-loading; the rates are summarized in Table I. While differences in Ca^{2+}-uptake rates as a result of different treatments were sometimes observed within specific individual experiments, there were no statistically significant differences in uptake properties when either annexin VI or its antibody were added to the vesicle preparation on the myoplasmic side.

Three parameters of Ca^{2+}-dependent Ca^{2+}-release from intact heavy SR vesicles were measured: amount of Ca^{2+} required to induce the first maximal release; amount of Ca^{2+} released during maximal releases; and duration of first maximal release. Heavy SR vesicles were either 1) untreated, 2) treated with purified annexin VI, or 3) treated with affinity-purified polyclonal

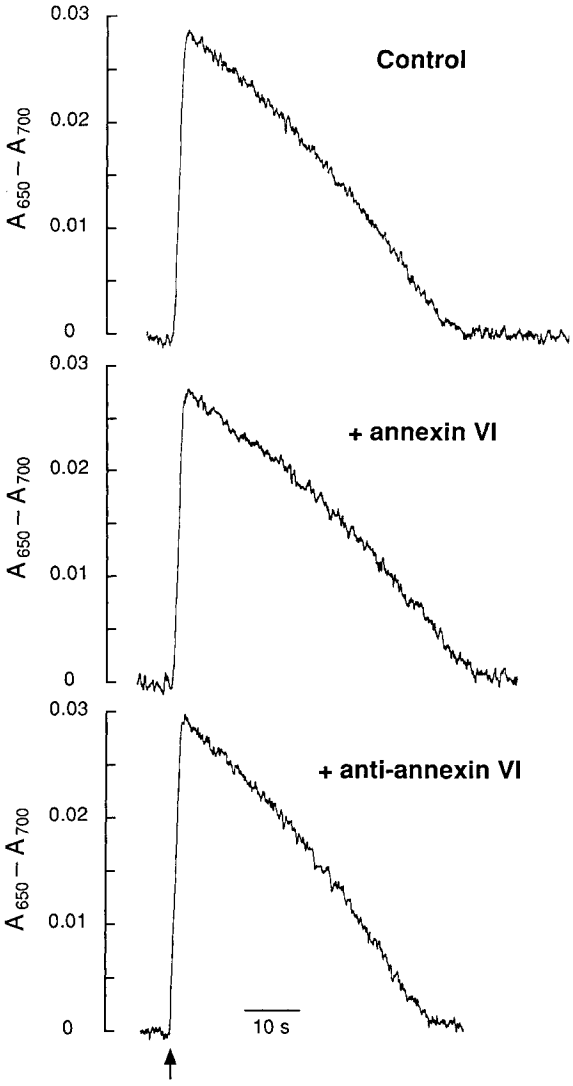

Fig. 1. Ca^{2+}-uptake by heavy sarcoplasmic reticulum (SR) vesicles treated with annexin VI or anti-annexin VI antibodies. Ca^{2+}-uptake (in the presence of oxalate) by untreated or treated rabbit heavy SR vesicles was measured as described in Methods. The arrow signifies the addition of 75 nmol Ca^{2+}. The net change in absorbance (A_{650}–A_{700}) of arsenazo III, a calcium indicator dye, is plotted against time. The addition of Ca^{2+} to the system produced an upward deflection, while the tracing returned to baseline during Ca^{2+}-uptake. Summation of these experimental results is shown in Table I.

anti-annexin VI (or an irrelevant antibody as a control for non-specific IgG effects). Figure 2 displays the tracings of typical experiments using the same preparations of heavy SR vesicles as in the uptake studies. The initial Ca^{2+} additions produce only transient increases in free Ca^{2+}, while subsequent additions of Ca^{2+} begin to elicit maximal Ca^{2+}-releases via the ryanodine-sensitive Ca^{2+}-channel [26]. Slight differences

TABLE I. Ca^{2+}-Uptake Rates of Heavy SR*

	Control	+annexin VI	+anti-annexin VI	+ruth. red	+ruth. red +annexin VI
Uptake rate	100%	109% ± 6%	94% ± 16%	236% ± 31%	180% ± 20%
(n)		(19)	(11)	(10)	(6)

*The Ca^{2+}-uptake rates are expressed as percent of control (i.e., no treatment; ± standard error with respect to control). The average control value is 32 nmol Ca^{2+}/mg HSR/sec. Ruth. red: ruthenium red, a Ca^{2+}-channel blocker.

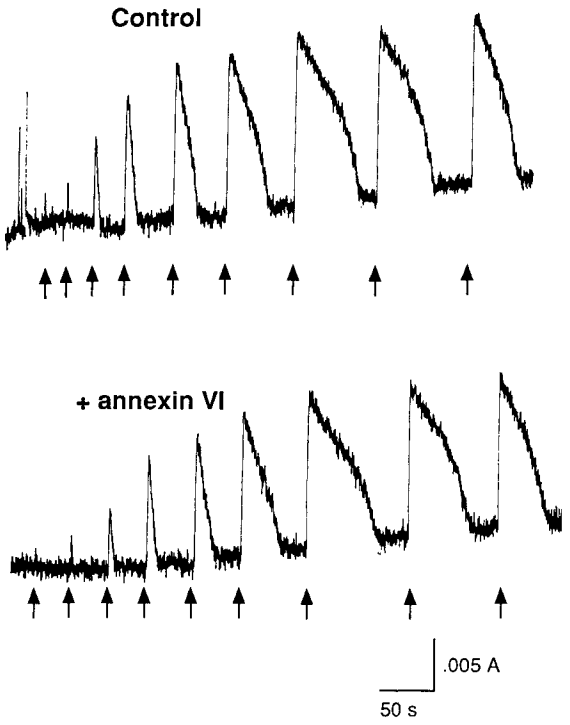

Fig. 2. Effect of annexin VI on Ca^{2+}-induced Ca^{2+}-release from skeletal muscle heavy SR vesicles. Heavy SR vesicles were treated with 10 μg annexin VI (or anti-annexin VI antibodies or irrelevant antiserum; plots were similar to those shown in this figure). Arrows signify successive additions of 10 nmol Ca^{2+}. The net change in arsenazo III absorbance is plotted against time. Initial transient increases in free Ca^{2+} (upward deflections, with corresponding return to baseline via Ca^{2+}-uptake) as a result of Ca^{2+} additions are followed by Ca^{2+}-induced Ca^{2+}-releases, which reach a maximal amplitude. Summation of these experimental results is shown in Table II.

could sometimes be observed between the various treatments; however, as summarized in Table II, these differences were not statistically significant. Preliminary studies indicate that while the anti-annexin VI antibodies immunoprecipitate annexin VI, they do not inhibit Ca^{2+}-dependent phospholipid binding. These data are consistent with the studies of Díaz-Muñoz et al. [20] in that annexin VI is ineffective on the myoplasmic side of the sarcoplasmic reticulum.

Localization of Annexin VI by Immunofluorescence

Production and characterization of monoclonal antibodies against annexin VI has been reported [23]. Mab-4 was shown to be monospecific for annexin VI when total extracts were analyzed by immunoblots [23]. Annexin VI was localized by immunofluorescence in rat esophagus. In striated muscle fiber cross-sections, anti-annexin VI antibody intensely stained the sarcoplasmic reticulum which surrounds the individual myofibrils within the muscle fiber (Figure 3). There was no staining of the sarcolemma which surrounds the individual fibers or of the contractile elements contained within the myofibril. Resolution of annexin VI localization was also obtained in cultured chick myotubes using double immunofluorescence. A monoclonal (mouse) antibody specific for chick Ca^{2+}-ATPase, a marker for SR, and an anti-annexin VI polyclonal antibody (sheep) were used (Figure 4). The 67 kDa annexin exhibited a striated pattern and appeared to co-localize with the Ca^{2+}-ATPase; fields 4A and 4B, and 4C and 4D, are superimposable. These data support the in situ association of annexin VI with the sarcoplasmic reticulum; this periodic pattern is consistent with the density variation which would be predicted for a sarcoplasmic reticulum-associated component [27].

Annexin VI is not unique to skeletal muscle [17,18]. It was, therefore, important to evaluate its localization in non-muscle cells since Ca^{2+} transients are critical events during stimulus-response coupling. Anti-annexin VI staining of cultured normal rat kidney (NRK) cells produced a dense, punctate pattern around the nucleus (Figure 5A). While interphase cells displayed polarized staining, mitotic cells demonstrated a bright granular fluorescence throughout the cytoplasm with the exclusion of the condensed chromosomes (Figure 5B). Resolution of annexin VI to specialized structures of the SR and in the cytoplasm of non-muscle cells will require electron microscopy.

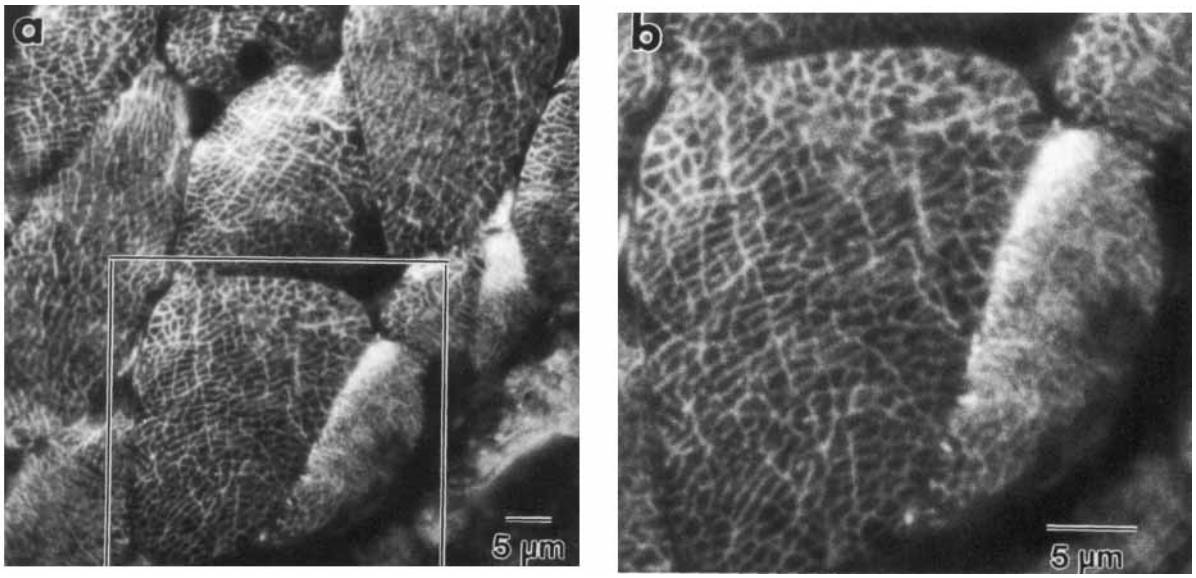

Fig. 3. Localization of annexin VI in rat skeletal muscle. Rat esophagus muscle was fixed in neutral buffered 10% formalin, embedded in paraffin, and sectioned at 4 μm. Sections were deparaffinized, hydrated, incubated with a 1:2,000 dilution of monospecific monoclonal antibody Mab-4, washed with PBS, and further incubated with *fluorescein-conjugated goat anti-mouse* IgG. The field was photographed on Kodak T-MAX (ASA 100) film with a Nikon Microflex UFX exposure attachment mounted on a Nikon Optiphot epifluorescence microscope. Area **b** is an enlargement of the inset in area **a**.

TABLE II. Ca^{2+}-Release Parameters of Heavy SR*

	Control	+annexin VI	+anti-annexin VI	+anti-irrelevant
nmol Ca^{2+} req. for first max. release	100%	100% ± 4%	102% ± 9%	98% ± 9%
(n)		(12)	(10)	(8)
nmol Ca^{2+} released per mg	100%	96% ± 2%	102% ± 5%	100% ± 9%
(n)		(42)	(33)	(21)
Duration (sec) of first max. release	100%	94% ± 11%	112% ± 17%	139% ± 26%
(n)		(9)	(10)	(7)

*The parameters are expressed as percent of control (i.e., no treatment; ± standard error with respect to control). The average control values for the +protein and +antibody treatments were, respectively, 62 or 42 nmol Ca^{2+} required for first maximal release; 48 or 47 nmol Ca^{2+} released per mg SR; and 77 or 138 second duration of first maximal release. The variation in control values is due to duration of vesicle storage at −80°C.

DISCUSSION

Annexin VI has been identified in various tissues by several laboratories [28–33]. The protein exists as a monomer and binds Ca^{2+} with micromolar affinity [18,32,34,35]. The protein sequence of annexin VI has been deduced from human [10,11] and mouse [36] cloned cDNA. Despite the growing body of information on the primary sequences and physicochemical properties of the annexins, the cellular functions for each protein remain controversial [8,37,38].

Díaz-Muñoz et al. [20] demonstrated that annexin VI modified the gating behavior of Ca^{2+}-channels of heavy sarcoplasmic reticulum (SR) membranes reconstituted in artificial planar lipid bilayers. The annexin increased the probability of opening and the mean open time. The Ca^{2+}-dependent effect of annexin VI was specific to the *trans* side of the bilayer, which corresponds to the luminal side of the SR. Studies described in this manuscript were undertaken to determine whether annexin VI or anti-annexin VI antibodies had an effect on either Ca^{2+}-uptake or Ca^{2+}-induced Ca^{2+}-release by intact isolated heavy SR vesicles (additions were to the myoplasmic, or *cis*, side). We observed that neither the protein nor its antibody affected those properties, presumably because the luminal (*trans*)

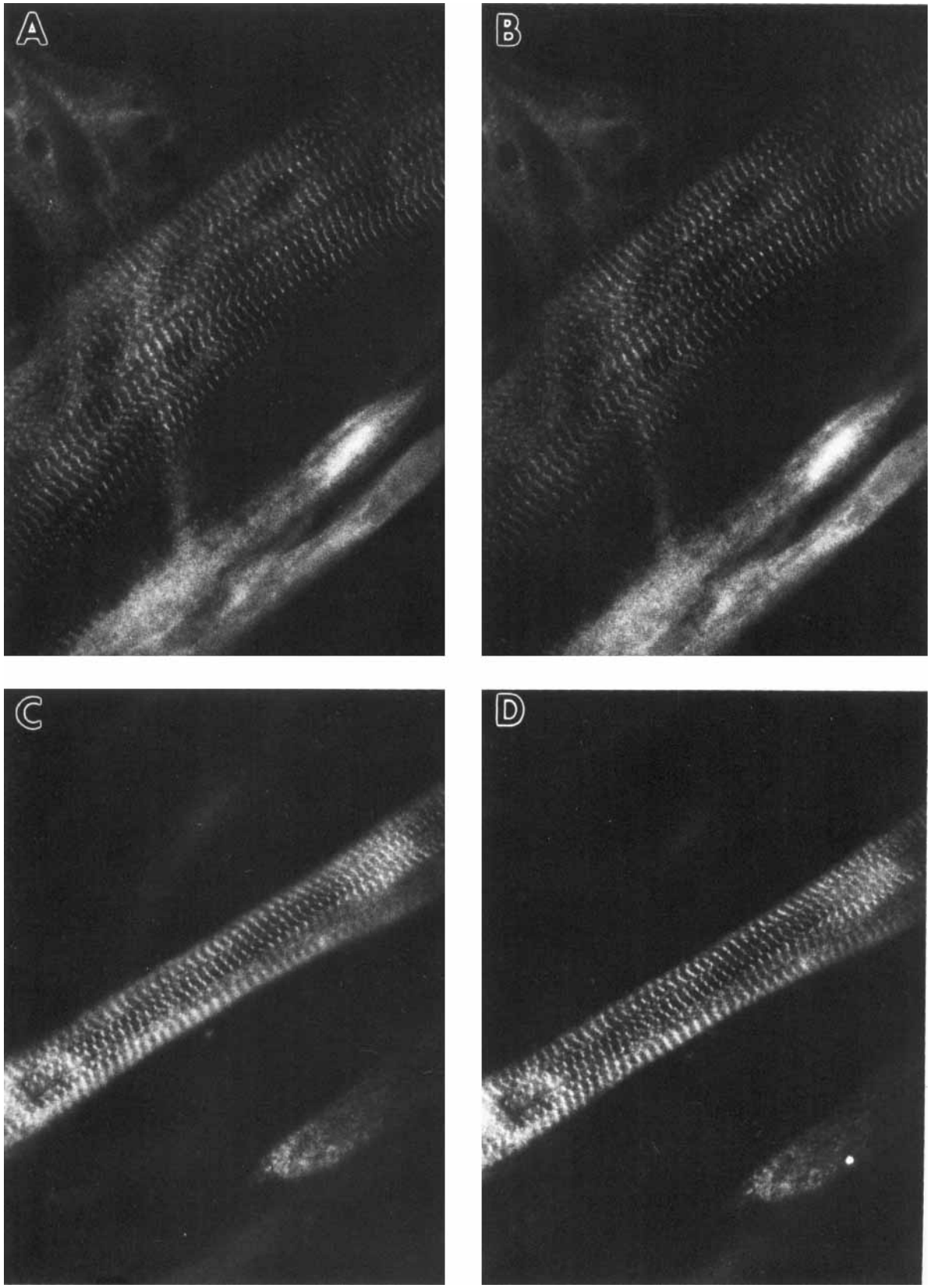

Figure 4. (Legend appears on page 84.

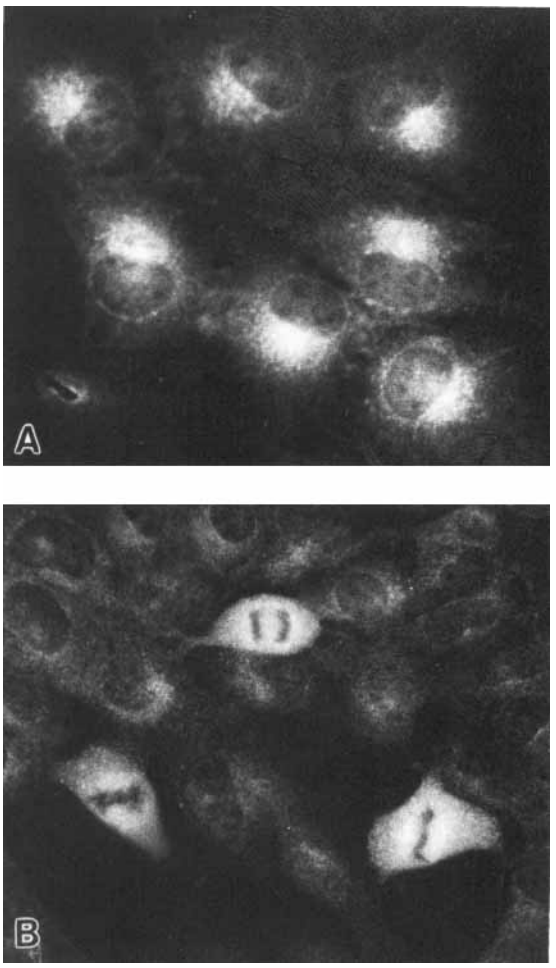

Fig. 5. Immunofluorescent localization of annexin VI in normal rat kidney (NRK) cells. NRK cells were grown to 60–70% confluency on coverslips, fixed with 3% formaldehyde, and permeabilized with acetone. The cells were stained with antibody Mab-4 and fluorescein-conjugated goat anti-mouse IgG, and photographed as described in Figure 4. **A:** Representative field of interphase cells. **B:** Representative field of cells in both interphase and mitosis. Magnification: ×1250.

side was inaccessible. These findings are consistent with those of Díaz-Muñoz et al. [20].

Another approach in establishing the physiological role of a protein is the resolution of its cellular localization. Annexin VI localization studies have not previously been reported for skeletal muscle. Several lines of evidence presented in this study suggest that annexin VI is associated with the sarcoplasmic reticulum and putative Ca^{2+}-sequestering vesicles in non-muscle cells. Annexin VI was localized as striations in cultured chick myotubes and as a sheath around individual myofibrils. The 67 kDa protein also co-localized with the SR Ca^{2+}-pump. Taken together, these observations indicate the association of annexin VI with the SR; this is consistent with the data presented concerning the regulation of the Ca^{2+}-channel. Further resolution of annexin VI localization can be made at the EM level.

Using cultured non-muscle cells, Owens and Crumpton [39] and Geisow et al. [40] independently reported annexin VI localization as a diffuse staining pattern throughout the cytoplasm of non-extracted cells and as a thick, reticulated pattern when cells were first extracted with 1% Triton X-100. More recent immunofluorescent localization studies of annexin VI in fibroblasts demonstrate a diffuse, punctate pattern excluded from the nucleus [41]. The interpretation of these images by Burgoyne and Geisow [41] is that annexin VI is localized on the inner surface of the plasma membrane. These authors suggest that the annexin VI localization is consistent with a cellular role for annexin VI in coupling plasma membrane proteins to the cytoskeleton and secretory vesicles.

Several investigators have identified protein components of the endoplasmic reticulum which demonstrate activities involved in Ca^{2+}-release/uptake cycles [42–48]. The localization of annexin VI in punctate vesicles surrounding the mitotic spindle is consistent with the Ca^{2+} sensitivity of the mitotic apparatus [49–53] and Ca^{2+} gradients during mitosis [54–56]. Furthermore, it has been demonstrated that the mitotic apparatus contains Ca^{2+}-sequestering vesicles [57–59]. The punctate staining of annexin VI in non-muscle cells may represent an association with organelles involved in Ca^{2+}-sequestration. Collectively, these data suggest that annexin VI

Fig. 4. Immunofluorescent localization of annexin VI and chick Ca^{2+}-ATPase in chick myotubes. Well-developed myotubes from 12-day-old chick embryo muscle cultured on coverslips were fixed in methanol, incubated successively with sheep anti-annexin VI antibody and monoclonal antibody against fast muscle Ca^{2+}-ATPase. The antibody binding was visualized with fluorescein-conjugated rabbit anti-sheep IgG (B, D), followed by rhodamine-conjugated goat anti-mouse IgG (A, C). Photographs **A** and **B**, and **C** and **D**, are paired images probed with anti-annexin VI or anti-Ca^{2+}-ATPase, as indicated above; fields A and B, and C and D, are superimposable. The fields were photographed on Kodak T-MAX (ASA 100) film mounted on a Nikon Optiphot epifluorescence microscope with a Nikon Microflex UFX exposure attachment, using the respective B2 and G (Nikon) filter cubes. Magnification: ×1380.

may be involved with Ca^{2+}-regulating organelles in both excitable and non-excitable tissues and may play a role in intracellular Ca^{2+} homeostasis. Reconstitution of isolated microsome activities is required in order to identify the role of annexin VI in non-skeletal muscle Ca^{2+} homeostasis.

ACKNOWLEDGMENTS

We would like to gratefully acknowledge Marina Lin for her helpful advice and suggestions, James Pastore, Danny Morse, and Linda Eshelman for their photographic expertise, Lawana Norris for her secretarial assistance, and Elsie Jackson and Barry Simpson for their valuable technical assistance.

This work was supported by grants from the National Institutes of Health (DK14632 [S.F.]; DK41740 [J.R.D.]) and the Texas Advanced Research Program (J.R.D.).

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