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Annexin VI is Attached to Transverse-Tubule Membranes in Isolated Skeletal Muscle Triads

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Abstract. Annexin VI is a 68-kDa protein of the Annexin family, a group of Ca^{2+}-dependent phospholipid-binding proteins widely distributed in mammalian tissues including skeletal muscle. We investigated a) which membrane system contributes Annexin VI to skeletal muscle triads, and b) whether Annexin VI removal affects triad integrity or function. Annexin VI was present in isolated triads and transverse tubules but not in heavy sarcoplasmic reticulum vesicles, indicating that Annexin VI binds to either free or triad-attached transverse tubules. Extraction with EGTA of Annexin VI from triads did not alter their migration as a single band in sucrose density gradients or their ouabain binding-site density, indicating that triad integrity does not require Annexin VI. Caffeine-induced Ca^{2+} release kinetics and Ca^{2+} uptake rates were likewise not affected by Annexin VI removal from triads, suggesting that Annexin VI is not involved in these functions. Annexin VI purified from rabbit skeletal muscle displayed Ca^{2+}-dependent binding to liposomes containing phosphatidylinositol 4,5-bisphosphate and phosphatidylcholine. Binding saturated at 1/20 molar ratio phosphatidylinositol 4,5-bisphosphate/phosphatidylcholine and was optimal at free $[Ca^{2+}] \geq 20$ μM. Extraction of Annexin VI from triads did not affect the generation of phosphatidylinositol 4-phosphate, phosphatidylinositol 4,5-bisphosphate, or phosphatidic acid by endogenous lipid kinases, suggesting that despite its capacity to bind to negatively charged phospholipids, Annexin VI does not affect the kinase activities responsible for their generation.

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Introduction

Annexins are Ca^{2+}-dependent phospholipid binding proteins composed of two domains, a conserved core that is responsible for Ca^{2+}- and phospholipid-binding, and a variable N-terminal tail domain. All Annexins possess four repeats of the core domain, except Annexin VI, which has eight repeats. The tail domain presents marked differences in composition and size among the thirteen members of the Annexin family; it is considered a regulatory region because it contains the main sites for interaction with other proteins and for phosphorylation and proteolysis (Raynal & Pollard, 1994).

Annexins are widely distributed in mammalian tissues (Hayashi et al., 1989; Kaetzel et al., 1989) and the presence and spatial organization of Annexin molecules may influence overall membrane organization and signaling characteristics (Draeger, 2000). In the presence of Ca^{2+}, Annexins bind to negatively charged phospholipids, such as phosphatidylserine (PS), phosphatidic acid (PA) or phosphatidylinositol (PI) (Raynal & Pollard, 1994, Hayashi et al., 1989; Bandorowicz-Pikula et al., 1996). Scarce or negligible binding to amphiphilic phospholipids such as phosphatidylcholine (PC), phosphatidylethanolamine (PE) or sphingomyelin has been reported (Raynal & Pollard, 1994; Hayashi et al., 1989).

Annexins have been implicated in different cellular functions, including exocytosis and vesicle trafficking (Creutz et al., 1987; Lafont et al., 1998;

Turpin et al., 1998) ion channel modulation (Naciff et al., 1996), Ca^{2+} transport across membranes (Matsuda, Kaneko & Horikawa, 1997) and Ca^{2+} release from intracellular stores (Frey et al., 1999). Annexins modulate the activity of phospholipase A2 (Fauvel et al., 1987; Koumanov, Wolf & Béreziat, 1997) and protein kinase C (Russo-Marie, 1999) and seem to be involved in bone calcification (Genge et al., 1991) and thrombogenesis (Rand, 1998).

Annexin VI, the largest member of the family with a MW of 68 kDa, is widely distributed in mammalian tissues (Hayashi et al., 1989). Annexin VI binds to negatively charged phospholipids with a K_d in the nM range (Bandorowicz-Pikula et al., 1996). Annexin VI also binds ATP (Bandorowickz-Pikula & Awasthi, 1997) and ATP binding in the presence of Ca^{2+} decreases its α-helical structure (Bandorowickz-Pikula et al., 1999). Both ATP and membrane potential modulate the phospholipid binding activity of Annexin VI (Bandorowickz-Pikula & Awasthi, 1997; Hofmann et al., 1997).

As is the case for the other Annexins, the physiological functions of Annexin VI are not well established. In smooth muscle, Annexin VI participates in the formation of a membrane cytoskeleton complex (Babiychuk et al., 1999). Overexpression of Annexin VI in cardiac muscle alters cardiomyocyte function in transgenic mice (Gunteski-Hamblin et al., 1996). In cardiac muscle an accumulation of Annexins II, V and VI in interstitial tissue in left but not in right ventricles from hypertensive guinea-pigs has also been reported, suggesting a role for these proteins in fibrosis development and cardiac remodeling (Trouvé et al., 1999). However, Annexin VI null mutant mice do not present apparent defects in growth and development (Hawkins et al., 1999), indicating that the Annexin VI gene is not essential for mouse viability.

Annexin VI is abundant in skeletal muscle (Tagoe et al., 1994) where it binds to the α and β but not to the θ or ε isoforms of protein kinase C (Schmitz-Pfeiffer et al., 1998). It has been reported that Annexin VI is resistant to EGTA extraction from mammalian skeletal muscle sarcoplasmic reticulum (SR) vesicles, and that Annexin VI addition to the trans (luminal) but not to the cis (cytoplasmic) compartment activates SR Ca^{2+} release channels incorporated in lipid bilayers (Díaz-Muñoz et al., 1990). Based on these findings, Annexin VI was proposed as a luminal SR protein that modifies the function of Ca^{2+} release channels. However, a preliminary report situates Annexin VI in skeletal muscle triads as outside the SR and as forming part of the transverse tubule (T-tubule) membrane system (Brandt & Caswell, 1998).

In this work, we investigated a) which membrane system provides Annexin VI to skeletal muscle triads and b) the effects of Annexin VI removal on triad integrity and function. We found that Annexin VI was attached to T-Tubules and was absent from purified SR vesicles. Furthermore, Annexin VI removal did not affect triad integrity, SR Ca^{2+} transport or caffeine-induced Ca^{2+} release kinetics. We also found that in the presence of μM free [Ca^{2+}] Annexin VI has the capacity to bind to mixed liposomes composed of phosphatidylinositol 4,5-bisphosphate (PIP_2) and PC. Extraction of Annexin VI from triads did not affect the endogenous sequential phosphorylation of PI to phosphatidylinositol 4-phosphate (PI-4P) and PIP_2, or the generation of PA. These results suggest that, despite its capacity to bind to negatively charged phospholipids, Annexin VI does not affect the lipid kinases responsible for their generation.

Materials and Methods

Isolation of Membrane Fractions

All membrane fractions were obtained from rabbit fast skeletal muscle. Purified SR vesicles and T-Tubules were isolated as described elsewhere (Fernandez, Rosemblatt & Hidalgo, 1980; Hidalgo, González & Lagos, 1983). Triad membranes were obtained as detailed previously (Hidalgo et al., 1993); their density of ouabain binding sites was determined as described elsewhere (Hidalgo et al., 1986a). The protein concentration of membrane fractions was determined according to Hartree (1972) using bovine serum albumin as standard. All membrane fractions were stored at −80°C for up to one month.

Extraction of Annexin VI from Membranes

Isolated triads or T-tubules were diluted to 10 mg of protein per ml in a solution containing (in mM): 150 KCl, 20 MOPS/Tris, pH 7.4, 5 EGTA, plus a combination of protease inhibitors (1 μg/ml pepstatin, 1 μg/ml leupeptin, 0.4 mM benzamidine, 1 mM PMSF). However, some preparations required addition of up to 20 mM EGTA for complete Annexin VI extraction. After incubation for 20 min on ice, membranes were sedimented at 18,000 × g for one hour. The pellets were resuspended in 300 ml of the above solution minus EGTA and sedimented once again at 18,000 × g. The resulting pellets were resuspended in 0.3 M sucrose, 20 mM MOPS/Tris, pH 7.2, frozen in liquid nitrogen in small aliquots and stored for up to one month at −80°C.

Purification of Annexin VI from Skeletal Muscle

Triad membranes were diluted to 1 mg protein per ml in 30 ml of a solution containing (in mM): 150 KCl, 5 EGTA, 20 MOPS/Tris, pH 7.4. After incubation for 20 min on ice, vesicles were sedimented at 100,000 × g for 1 hr. The resulting supernatant was concentrated on 30-kDa Centricon filters (Millipore) to a final volume of 1 ml. After addition of $CaCl_2$ to obtain a final free concentration of 5 mM, the supernatant was fractionated on columns containing Phenyl Sepharose A equilibrated with a solution containing (in mM): 150 KCl, 1 $CaCl_2$, 20 MOPS/Tris, pH 7.4. After extensively washing the column with this solution, Annexin VI was eluted with a solution containing (in mM): 150 KCl, 5 EGTA, 20 MOPS/Tris, pH 7.4.

Alternatively, Annexin VI was purified from EGTA extracts by filtration on Bio-Gel P-100 columns. Fractions eluted from the columns were concentrated, extensively dialyzed in 150 mM KCl, 20 mM MOPS/Tris, pH 7.4 to remove EGTA, frozen in liquid nitrogen and stored at −80°C. The identity of purified Annexin VI was confirmed by reaction with specific polyclonal or monoclonal antibodies, after transferring the protein from SDS-gels to nitrocellulose blots. Western blots were revealed with commercial kits.

Calcium Release Kinetics

Triads were actively loaded with calcium at 25°C by incubating vesicles (0.1 mg/ml) in a solution containing (in mM): 0.05 $CaCl_2$, 150 KCl, 1.8 $MgCl_2$, 20 MOPS/Tris, pH 7.2, 1.5 ATP, 10 phosphocreatine plus 15 U/ml creatine kinase. After active loading for 5 min, extravesicular [Ca^{2+}] decreased to 0.1 μM. Calcium release from calcium-loaded triads was measured as described (Donoso et al., 2000), using an SX.18MV fluorescence stopped-flow spectrometer from Applied Photophysics (Leatherhead, U.K.). The increase in extravesicular [Ca^{2+}] was determined by measuring the fluorescence of Calcium Green-2 (Molecular Probes, Eugene, OR). Dye fluorescent emission was measured through a 515-nm cut-off long-pass filter, using an excitation wavelength of 488 nm. Calcium release was initiated by mixing 10 volumes of the solution containing calcium-loaded triads, with or without Annexin VI, with 1 volume of releasing solution containing (in mM): 44 caffeine, 150 KCl, 20 MOPS/Tris, pH 7.2 and 1 μM Calcium Green-2. After dilution the calculated values for free [Mg^{2+}] and free [ATP] were 0.5 mM and 0.19 mM, respectively (*see* below).

Binding of Annexin VI to Phospholipids

All binding assays were carried out in a solution containing (in mM): 150 KCl, 20 MOPS/Tris, pH 7.4, and variable concentrations of $CaCl_2$ and EGTA. Free [Ca^{2+}] was calculated with the WinMaxC program (www.stanford.edu/-cpatton/winmaxc2.html, file bers.ccm). The free [Ca^{2+}] of buffer solutions was checked with a calcium electrode (Orion, Beverly, MA) using a standard commercial kit to calibrate the electrode (WPI, Sarasota, FL). Annexin VI was incubated on ice for 20 min with 2.5 mg/ml of liposomes having different phospholipid compositions, as detailed in the text. Liposomes were prepared by extensive sonication of the corresponding lipid mixtures in a bath sonicator. After incubation of Annexin VI with liposomes, 0.15-ml fractions were sedimented at 180,000 × g in a Beckman Airfuge for 5 min. The resulting supernatants and pellets, resuspended to the original volume in sample buffer, were analyzed by electrophoresis in SDS-containing polyacrylamide gels.

Determination of ^{32}P-labeled PI-4P, PIP_2 and PA Generated by Triad Endogenous Lipid Kinases

All reactions were carried out at 20°C in a final volume of 0.1 ml of a solution containing 0.05 mg triad protein. The composition of the incubation solution was (in mM): 100 KCl, 5 $MgCl_2$, 2 dithiothreitol, 1.6 $CaCl_2$, 2 HEDTA, 1 ATP (containing [γ-^{32}P]-ATP at a specific activity of 22 mCi/mmol), 20 MOPS/Tris, pH 7.2. The resulting free Ca^{2+} concentration was 0.27 mM. The reaction was initiated by addition of ATP and was stopped at different times by addition of 1 ml 1 N HCl, followed by 2 ml chloroform/methanol (1/1). After vigorous shaking for 30 sec in a Vortex mixer, and incubation on ice for 10 min followed by brief sedimentation in a bench centrifuge, the organic and aqueous components formed two phases clearly separated. The resulting organic phase was collected by aspiration, evaporated to dryness under N_2, dissolved in a small volume of chloroform/methanol (1/1, v/v) and analyzed by thin-layer chromatography on HPTLC-HL plates using a solvent system composed of chloroform/methanol/4N ammonia (50/39/11, v/v). Phospholipid-containing spots were visualized with iodine vapors. Spots containing ^{32}P-labeled lipids were visualized by autoradiography, scrapped from the plates, transferred to vials and counted in a liquid scintillation counter.

Other Procedures

Densitometric scans of SDS-containing polyacrylamide gels, stained with Coomassie blue, were performed with the Universal software program from American Applied Biotechnology (Fullerton, CA).

Materials

All reagents used were of analytical grade. Protease inhibitors (Leupeptin, Pepstatin A, benzamidine and phenylmethylsulfonyl fluoride), alkaline phosphatase-coupled secondary antibodies, bovine serum albumin, Phenyl sepharose, phospholipids and ATP were obtained from Sigma (St. Louis, MO). Some experiments were carried out with PIP2 obtained from Calbiochem-Novabiochem Corp. (San Diego, CA). Bio-Gel P-100 was obtained from Bio-Rad Laboratories (Hercules, CA). The fluorescent calcium indicator Calcium Green-2 and the NBT/BCIP reagent kit to reveal Western blots generated with the monoclonal Annexin VI antibody, a kind gift of Dr. Neil Brandt, were from Molecular Probes, (Eugene, OR). Western blots generated with the polyclonal Annexin VI antibody from Santa Cruz Biotechnology (Santa Cruz, CA), were revealed with an ECL kit from Amersham Pharmacia Biotech, U.K. Uniplates HPTLC-HL were purchased from Analtech (Newark, DE), and [γ-^{32}P]-ATP) from New England Nuclear (Boston, MA).

Results

Extraction of Annexin VI from Skeletal Muscle Triads

Annexin VI purified from skeletal muscle had a MW of 68 kDa (Fig. 1*A*, lane 1). Isolated triads contained significant amounts of Annexin VI (Fig. 1*A*, lanes *2* to *4*) that was completely removed from the vesicles following incubation with 5 mM EGTA (Fig. 1*A*, lanes *5* to *7*). These observations were confirmed by Western blots with a commercial Annexin VI polyclonal antibody (Fig. 1*B*) that showed reaction with only one protein band that was absent from triads incubated with EGTA. As illustrated in Fig. 1*C*, washing triads with a solution containing 10 μM free [Ca^{2+}] extracted some minor protein bands in the first and second supernatants but did not extract Annexin VI (Fig. 1*C*, lanes *1* and *2*). A third wash of the vesicles with a solution containing 5 mM EGTA extracted to the supernatant all the Annexin VI present in the triads (Fig. 1*C*, lane *3*). Densitometric scans of triad-containing gels, such as the one shown in Fig. 1*A*, indicated that the Annexin VI content of

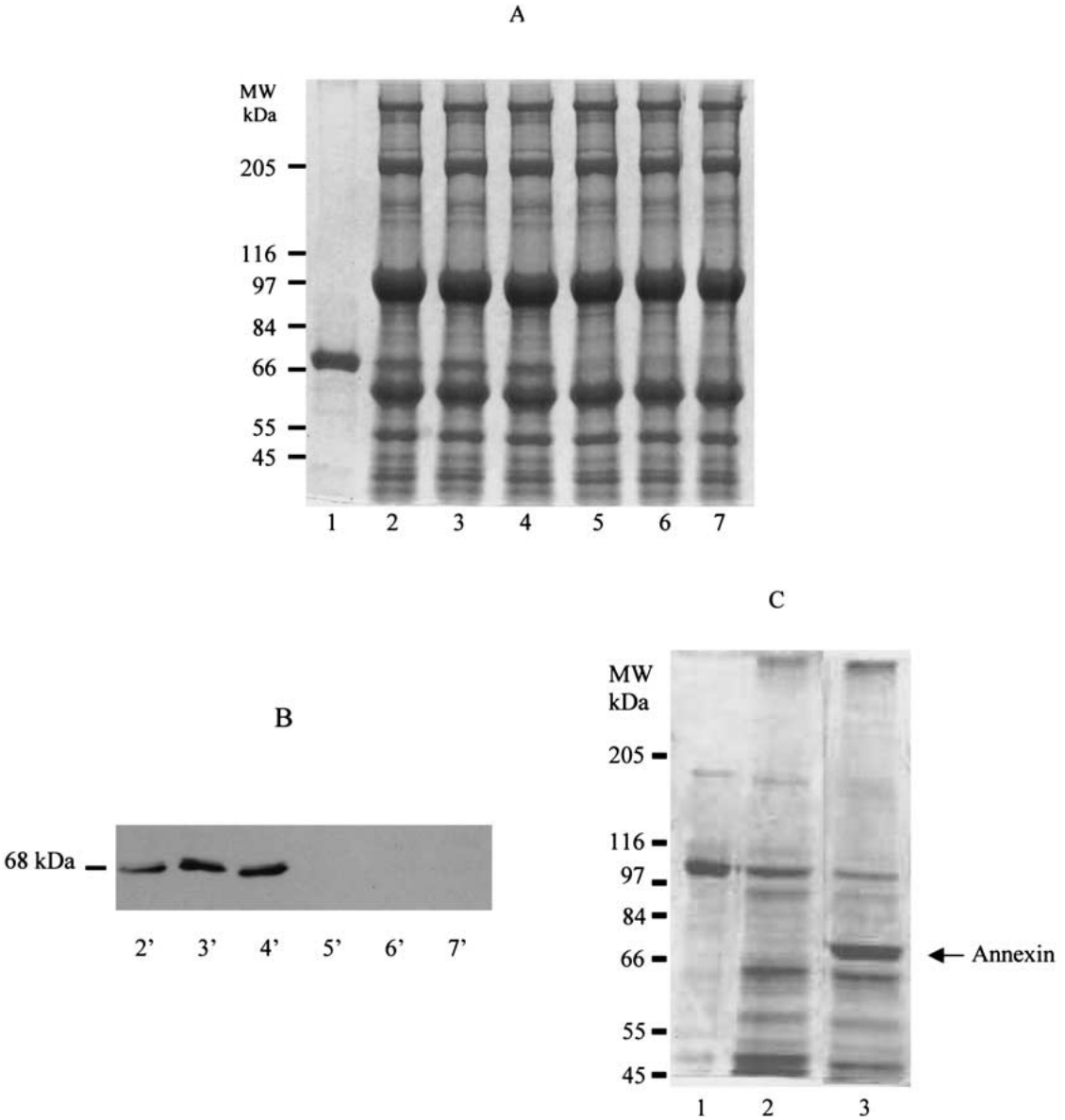

Fig. 1. Skeletal muscle triads contain Annexin VI. (*A*) SDS-7% polyacrylamide gels stained with Coomassie blue. Lane *1*, purified Annexin VI; lanes *2*, *3* and *4*: native triads; lanes *5*, *6* and *7*: triads after incubation with EGTA. (*B*) Immunoblot. Blots were incubated with a polyclonal antibody against Annexin VI. Lanes *2′*, *3′* and *4′* correspond to lanes *2*, *3* and *4* in Fig. 1*A*, and lanes *5′*, *6′* and *7′* to lanes *5*, *6* and *7* of Fig. 1*A*. (*C*) SDS-7% polyacrylamide gels stained with Coomassie blue. Lane *1*, supernatant of the first incubation of triads with a solution containing 10 μM $CaCl_2$; lane *2*, supernatant of the second incubation with a solution containing 10 μM $CaCl_2$, lane *3*, supernatant of the third incubation with a solution containing EGTA.

triads amounted to 4–6% of their total protein content.

Association of Annexin VI with Skeletal Muscle T-Tubules

Triad vesicles are composed of one T-tubule vesicle associated with two flanking SR terminal cisternae vesicles. To investigate which vesicular system contributes Annexin VI to triads, we analyzed the Annexin VI content of isolated T-tubules and SR vesicles. As illustrated in Fig. 2*A* (lane *1*), T-tubules contained a faint protein band migrating in the same position as Annexin VI. To extract this protein, T-tubules were first washed with a solution containing 10 μM free [Ca^{2+}]. After sedimentation, the resulting supernatant contained several protein bands but not Annexin VI (Fig. 2*A*, lane *2*). A second wash of purified T-tubules with a solution containing 5 mM EGTA extracted all the Annexin VI present in the vesicles, plus other unidentified proteins (Fig. 2*A*, lane *4*). The identity of Annexin VI in the

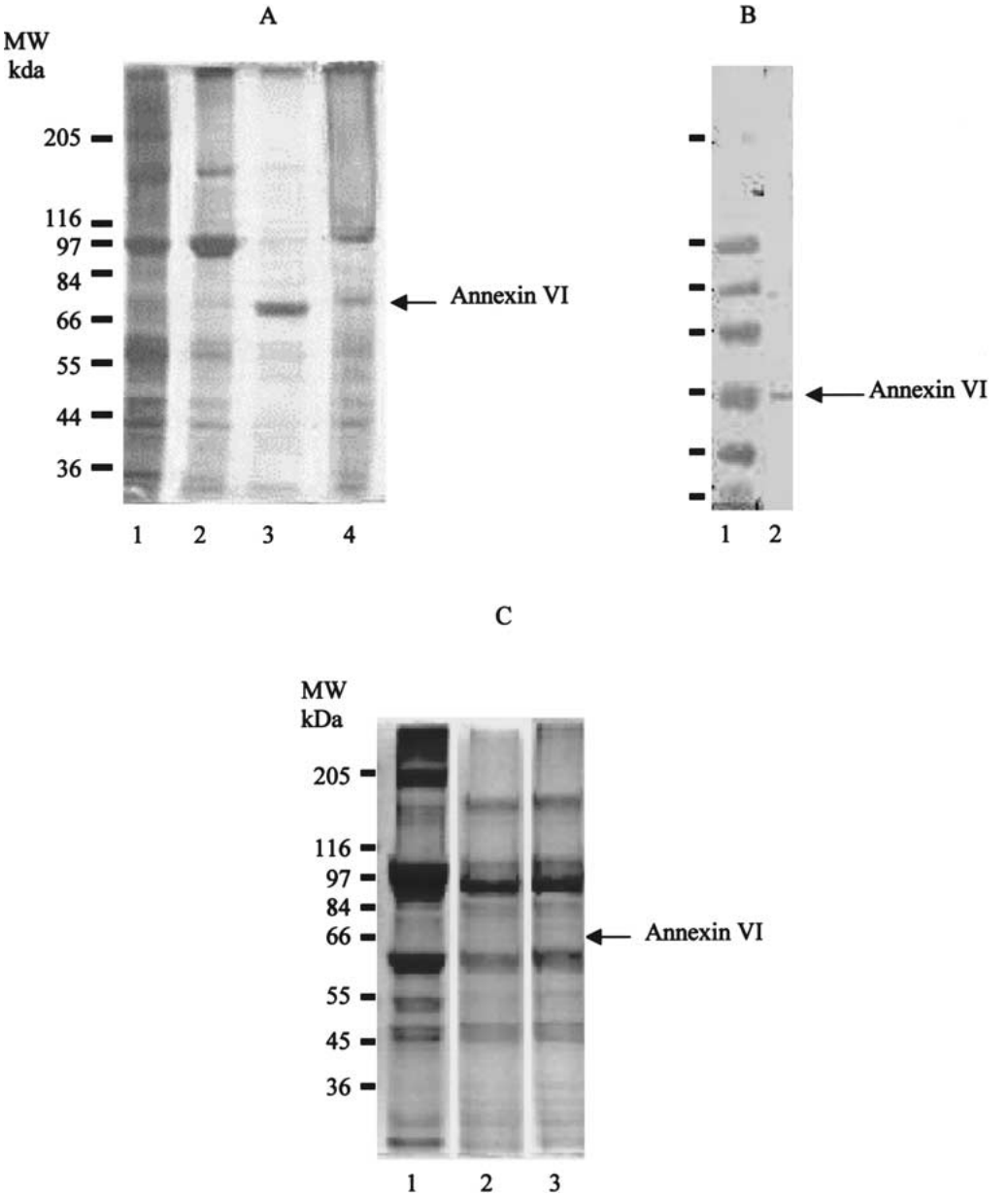

Fig. 2. T-tubules but not SR vesicles contain Annexin VI. (*A*) 7% polyacrylamide-SDS gel stained with Coomassie blue. Lane *1*, isolated T-tubules; lane *2*, supernatant from incubation of T-tubules with a solution containing 10 μM $CaCl_2$; lane *3*, purified Annexin VI; lane *4*, supernatant obtained after incubation of T-tubules with a solution containing EGTA. (*B*) Immunoblot. Lane *1*, MW standards; lane *2*: Annexin VI extracted from T-tubules revealed with a monoclonal antibody. (*C*) 7% polyacrylamide-SDS gel stained with Coomassie blue. Lane *1*, heavy SR membranes; lane *2*, supernatant obtained after incubation of heavy SR vesicles with a solution containing 10 μM $CaCl_2$; lane *3*, supernatant after incubation with a solution containing EGTA.

EGTA supernatant was confirmed by reaction with a monoclonal antibody specific for Annexin VI (Fig. 2*B*). In contrast, analysis of the protein composition of heavy SR vesicles devoid of T-tubules in an SDS-containing polyacrylamide gel heavily loaded with protein revealed that this fraction was highly enriched in calsequestrin, as expected from its terminal cisternae origin, but did not contain Annexin VI (Fig. 2*C*, lane *1*). Washing heavy SR vesicles first with a solution containing 10 μM free $[Ca^{2+}]$ followed by a second wash with a solution containing 5 mM EGTA failed to produce Annexin VI in the corresponding supernatants (Fig. 2*C*, lanes *2* and *3*).

Binding of Purified Annexin VI to Liposomes Containing PS or PIP_2

In the presence of 0.1 mM free $[Ca^{2+}]$, Annexin VI purified from skeletal muscle bound to liposomes

composed of PS/PC (1/4 molar ratio) but not to liposomes composed solely of PC. Annexin VI binding was assayed by determining the presence of Annexin VI in the pellets after sedimentation of liposomes (*see* Methods). As illustrated in Figure 3, after incubation with Annexin VI of liposomes containing only PS or PS/PC (1/4), all the protein was found in the sediments and none in the supernatants (Fig. 3*A* and *B*). In contrast, after incubation with liposomes containing solely PC, Annexin VI remained in the supernatant (Fig. 3*A*, lanes *5* and *6*). In the presence of 1 mM free [Ca^{2+}], purified Annexin VI also bound to liposomes composed of PIP_2/PC (1:12 molar ratio), as evidenced by the appearance of Annexin VI only in the pellet fraction (Fig. 3*B*, lanes *3* and *4*). The same binding behavior to liposomes composed of PS/PC (1/4) or PIP_2/PC (1/12) was observed after fractionation of the protein liposome complexes in sucrose density gradients, as illustrated in Fig. 3*B*, lanes *5* to *8*.

To test under lower free Ca^{2+} concentrations Annexin VI binding to liposomes composed of PIP_2/PC, we carried out binding experiments in the presence of 0.1 mM free [Ca^{2+}]. Molar ratios of PIP_2/PC ranging from 1/100 to 1/12 were used. In these conditions, Annexin VI bound to all liposomes containing PIP_2 but not to liposomes containing only PC (Fig. 3*C*). Maximal binding was obtained at 1/20 molar ratio PIP_2/PC, corresponding to 5% PIP_2 (Fig. 3*D*).

Binding of Annexin VI to liposomes composed of PIP_2/PC (1/12 molar ratio) was optimal in the pCa range <5**,** as illustrated in Figure 4. At free [Ca^{2+}] $\leq$ 10 μM, low amounts of Annexin VI were found in the pellets (Fig. 4*A*) and most of the protein remained in the supernatants (Fig. 4*B*), an indication of scant binding. At free [Ca^{2+}] $\geq$ 100 μM, Annexin VI was mostly present in the pellets and a very small fraction remained in the supernatants (Fig. 4*A* and *B*, last two lanes). To explore in finer detail the free [Ca^{2+}] required for Annexin VI binding to PIP_2/PC liposomes (1/12 molar ratio), we studied binding in the free [Ca^{2+}] range 10–80 μM. As illustrated in Fig. 4*C*, at 10 μM free [Ca^{2+}], Annexin VI was present mostly in the supernatant and not in the pellet (lanes *1* and *7*). However, at free [Ca^{2+}] $\geq$ 20 μM, Annexin VI was present mostly in the pellets (Fig. 4*C*, lanes 2 to 5, and lane 7).

Comparison of Triad Integrity between Control and Annexin VI-free Triads

Extraction of Annexin VI did not change the migration pattern of triad vesicles in sucrose gradients. Following Annexin VI removal, a single protein-containing fraction that equilibrated at the same density (40–43% w/v sucrose) as triads containing Annexin VI was obtained (*data not shown*). These results indicate that free T-tubules, which migrate as a lighter band in density gradients (25–27.5% w/v sucrose) were not released from triads following Annexin VI extraction. To confirm that triad integrity was no affected, we determined the density of ouabain binding sites, an indicator of T-tubule content, before and after Annexin VI removal from triads. Two independent determinations indicated that control triads had an [^{3}H]-ouabain binding site density of 16.4 $\pm$ 0.4 pmol/mg of protein, whereas Annexin-free triads had a density of 16.5 $\pm$ 1.4 pmol/mg protein.

Functional Comparison between Control and Annexin VI-free Triads: Ca^{2+} Uptake and Ca^{2+} Release

AnnexinVI removal did not affect Ca^{2+} transport rates (*data not shown*), and did not modify either the rate constants of caffeine-induced Ca^{2+} release, measured at pCa 7 in the presence of 0.5 mM free [Mg^{2+}] and 0.19 mM free [ATP]. Caffeine-induced Ca^{2+} release followed a time course best fitted by a double-exponential function, with a fast component that accounted for the largest fraction of Ca^{2+} released. As illustrated in Table 1, both exponential components of Ca^{2+} release were not significantly affected by Annexin VI removal.

Functional Comparison between Control and Annexin VI-free Triads: Activity of Endogenous Lipid Kinases

Isolated T-tubules and triads have endogenous lipid kinases that phosphorylate sequentially PI to PI-4P and PIP_2, and that generate PA presumably though the phosphorylation of endogenous diacylglycerol (Hidalgo et al., 1986b; Varsanyi et al., 1986). Previous results indicate that Annexin VI binds to the negatively charged phospholipids PS and PI (Raynal & Pollard, 1994; Bandorowicz-Pikula et al., 1996), and the present results show that Annexin VI also binds to PIP_2. Thus we investigated whether Annexin VI removal from triads affected the generation of the negatively charged phospholipids PA, PI-4P and PIP_2. As illustrated in Figure 5, control vesicles and vesicles devoid of Annexin VI showed no differences in the generation of PIP, PIP_2 and PA when measured after 120 sec of incubation with [γ-^{32}P]-ATP. The kinetics of formation of these compounds was also not affected by removal of Annexin VI from triads (*data not shown*).

Discussion

Annexin VI in Isolated Triads is Contributed by their T-Tubule Membranes

The present results suggest that Annexin VI is a transverse tubular protein, presumably bound to the cytoplasmic surface of T-tubules in a Ca^{2+}-depen-

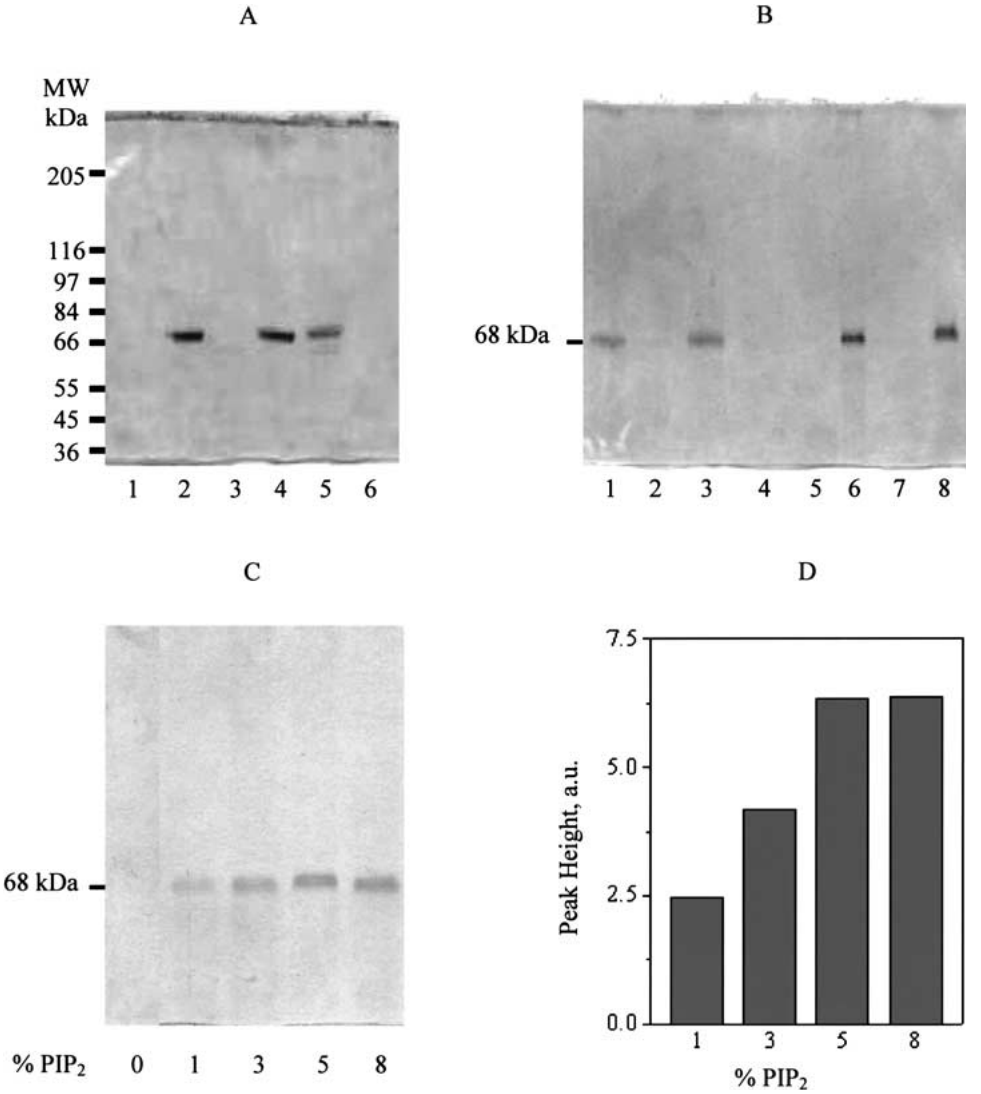

Fig. 3. Annexin VI binding to liposomes. (*A*) 7% polyacrylamide-SDS gel stained with Coomassie blue. Lanes *1* and *2* show supernatant and pellet, respectively, obtained after incubation of Annexin VI with liposomes containing only PS. Lanes *3* and *4*, supernatant and pellet, respectively, obtained after incubation of Annexin VI with liposomes that contained a mixture of PS/PC at 1/4 molar ratio. Lanes *5* and *6*, supernatant and pellet, respectively, obtained after incubation of Annexin VI with liposomes containing only PC. Experiments were carried out in the presence of 0.1 mM free $[Ca^{2+}]$, with 2 μM Annexin VI. (*B*) 7% polyacrylamide-SDS gel stained with Coomassie blue. Lanes *1* and *2*, pellet and supernatant, respectively, from incubation of Annexin VI with liposomes containing only PS. Lanes *3* and *4*, pellet and supernatant, respectively, from incubation of Annexin VI with liposomes that contained a mixture of PIP_2/PC at 1/12 molar ratio. Lanes *5* and *6*, light and heavy fractions, respectively, obtained from a sucrose density gradient loaded with liposomes containing a mixture of PS/PC (1/4 molar ratio). Lanes *7* and *8*, light and heavy fractions, respectively, obtained from a sucrose density gradient loaded with liposomes containing a mixture of PIP_2/PC at 1/12 molar ratio. All experiments were carried out with 3 μM Annexin VI in the presence of 1.0 mM free $[Ca^{2+}]$. (*C*) 7% polyacrylamide-SDS gel stained with Coomassie blue. All lanes show the pellets obtained after incubation of Annexin VI with liposomes containing variable % contents of PIP_2 relative to PC, as indicated in the figure. All experiments were carried out with 1.5 μM Annexin VI in the presence of 0.1 mM $[Ca^{2+}]$. (*D*) Binding of Annexin VI to liposomes containing variable contents of PIP_2 relative to PC. Peak heights were obtained from a densitometric gel scan of the lanes shown in Fig. 3*C*.

dent manner because it was extracted from either free or triad-attached T-tubules with EGTA. These results agree with a preliminary report describing Annexin VI as a transverse tubular membrane protein (Brandt & Caswell, 1998). The fact that isolated T-tubules contained less Annexin VI than triads indicates that some Annexin VI was lost from T-tubules during their lengthy isolation procedure.

We did not find Annexin VI in purified heavy SR vesicles devoid of T-tubules. This finding differs from a previous report indicating that Annexin VI is present in heavy SR vesicles and is resistant to extraction

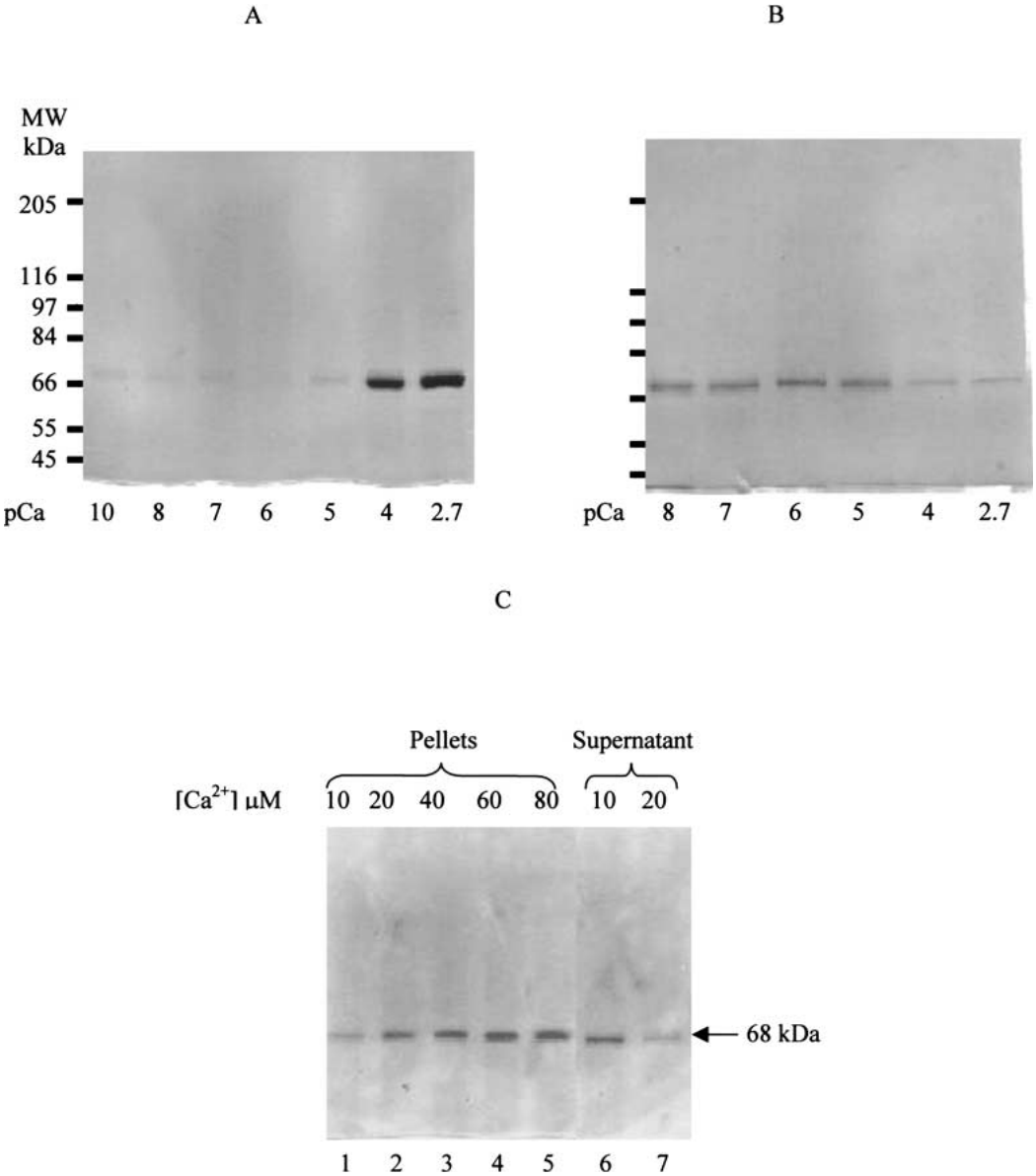

Fig. 4. Calcium dependence of Annexin VI-binding to liposomes containing PIP_2/PC. *A*, *B* and *C* illustrate 7% polyacrylamide-SDS gels stained with Coomassie blue. All experiments were carried out in the presence of 1.5 µM Annexin VI. (*A*) Pellets obtained after incubation of Annexin VI with liposomes containing PIP_2/PC at 1/12 molar ratio and the free $[Ca^{2+}]$, given as pCa, illustrated in the figure. (*B*) Supernatants obtained after incubation of Annexin VI with liposomes containing PIP_2/PC at 1/12 molar ratio and the free $[Ca^{2+}]$ given as pCa, illustrated in the figure. (*C*) Lanes *1* to *5*, pellets, lanes *6* and *7*, supernatants obtained after incubation of Annexin VI with liposomes containing PIP_2/PC at 1/12 molar ratio and the pCa values illustrated in the figure.

with 2 mM EGTA (Díaz-Muñoz et al., 1990). However, we used higher concentrations of EGTA, 5 to 20 mM, to extract Annexin VI from the membranes. Furthermore, it has been reported that skeletal muscle membranes isolated in the presence of Ba^{2+} or Ca^{2+} ions have tightly bound Annexin VI that cannot be extracted solely with EGTA; successful extraction requires EGTA plus detergent addition (Krasavchenko et al., 1999). However, it should be noted that our results indicate that Annexin VI was found attached to triads and T-tubules but not to SR vesicles after isolation of the different membrane fractions. Thus, we cannot assert that in vivo, or under different isolation procedures, Annexin VI does not bind to the SR membranes, especially since these membranes contain negatively charged phospholipids, although in lower proportions than T-tubules (Rosemblatt et al., 1982).

Binding of Annexin VI to PIP_2

We found in this work that Annexin VI bound not only to PS-containing liposomes but also to lipo-

Table 1. Caffeine-induced Ca^{2+} release kinetics

	Release rate constant, sec^{-1}	
	Control	Annexin VI-free triads
Experiment I		
*k*1	59.5 ± 0.6	60.9 ± 0.4
*k*2	6.0 ± 0.4	5.9 ± 0.1
Experiment II		
*k*1	58.4 ± 0.4	58.2 ± 0.4
*k*2	9.1 ± 0.2	7.6 ± 0.3

Caffeine-induced Ca^{2+} release followed a time course that could be fitted to a double-exponential function, with the corresponding rate constants *k*1 and *k*2. Values given for Experiments I and II correspond to data obtained with two different triad preparations. Values are given as mean ± SEM, and represent the average of at least 6 independent determinations carried out with each preparation. For further details, *see* text.

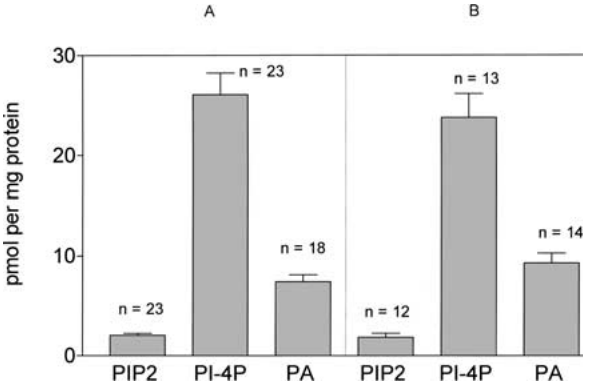

Fig. 5. Endogenous lipid kinase activities of triads with or without Annexin VI. Panel *A*: control triads. Panel *B*: triads without Annexin VI. For further experimental details, *see* text. Data, obtained from at least 3 independent determinations, are given as mean ± SEM.

somes containing PIP_2. Binding of Annexin VI to PIP_2-containing liposomes was strictly Ca^{2+}-dependent; binding was not observed at Ca^{2+} concentrations below 20 μM. The addition of PIP_2 to PC-containing liposomes, even at concentrations as low as 1%, induced Ca^{2+}-dependent binding of Annexin VI. This finding contrasts with previous reports showing lack of Annexin VI-binding to PIP_2 (Edwards & Crumpton, 1991). We do not know the reason for this discrepancy, but the highly negative nature of PIP_2 makes it a suitable candidate for Ca^{2+}-dependent Annexin VI binding. It has been reported that Annexin VI also binds to PI when assayed with two different techniques (Blackwood & Ernst, 1990; Edwards & Crumpton, 1991), making it likely that it will bind to PI-4P as well. We have shown previously that isolated SR membranes contain a lipid kinase activity that generates PI-4P, whereas T-tubules form both PI-4P and PIP_2 (Hidalgo et al., 1986b). It remains to be investigated if Annexin VI binds to these muscle membranes in vivo when local increases in phosphoinositide concentration occur concomitantly with a rise in cytoplasmic free $[Ca^{2+}]$.

Functional Effects of Annexin VI Removal from Triads

Previous findings indicate that Annexin VI regulates the function of the Ca^{2+} release channels incorporated in lipid bilayers when added to the trans (luminal) but not to the cis (cytoplasmic) compartment (Díaz-Muñoz et al., 1990). Yet we found that after muscle homogenization Annexin VI was present in T-tubules and triads, presumably bound to the cytoplasmic surface of transverse tubular membranes, but not in heavy SR vesicles. Furthermore, our results do not support a functional role for Annexin VI on Ca^{2+}-induced Ca^{2+} release from SR, because Annexin VI removal did not affect caffeine-induced Ca^{2+} release. It remains to be tested whether Annexin VI is involved in the physiological mechanism of depolarization-induced Ca^{2+} release. This process entails direct interactions between the voltage sensor proteins of T-tubules and the Ca^{2+} release channels of SR (Ríos & Pizarro, 1991) and it might be affected by removal of a triadic protein bound to the cytoplasmic leaflet of T-tubule membranes. However, the resulting changes in excitation contraction coupling, if any, are bound to be subtle because Annexin VI knockout mice do not present obvious defects in cardiac or skeletal muscle function (Hawkins et al., 1999).

Removal of Annexin VI Does not Affect the Activity of the Endogenous Lipid Kinases Present in Triads

Previous reports indicate that T-tubules and triads have endogenous lipid kinases that phosphorylate sequentially PI to PI-4P and PIP_2 (Hidalgo et al., 1986b; Varsanyi et al., 1986). In addition, triads from mammalian muscle also contain an endogenous kinase that generates PA, presumably by phosphorylation of endogenous diacylglycerol (Varsanyi et al., 1986). We found that Annexin VI had the capacity to bind to PIP_2 in the μM Ca^{2+} concentration range, and previous findings have reported Ca^{2+}-dependent Annexin VI binding to the negatively charged phospholipids PI and PA (Raynal & Pollard, 1994). Accordingly, we investigated if Annexin VI removal affects the activities of the endogenous lipid kinases that utilize PI, a negatively charged phospholipid, as substrate or that generate as products the negatively charged phospholipids PA, PI-4P and PIP_2. We found that removal of Annexin VI from triads does not affect these activities. These findings suggest that under the in vitro conditions used in this work, Annexin VI

does not modulate the function of the lipid kinases responsible for the production of PI-4P, PIP_2 or PA.

In conclusion, our findings indicate that T-tubules but not SR vesicles contribute Annexin VI to isolated triads. However, removal of Annexin VI from triads did not affect triad integrity, Ca^{2+} uptake, caffeine-induced Ca^{2+} release, or the activity of the endogenous lipid kinases responsible for the generation of PA and of the negatively charged phosphoinositides PI-4P or PIP_2. It is note-worthy in this regard that Annexin VI knockout mice are viable but do present apparent defects in growth and development (Hawkins et al., 1999). These findings indicate that the absence of Annexin VI does not impair mouse vital functions, including the normal functioning of their skeletal muscle system.

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