Characterization of mammalian heart annexins with special reference to CaBP33 (annexin V)

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Porcine heart was observed to express annexins V (CaBP33) and VI in large amounts, and annexins III and IV in much smaller amounts. Annexin V (CaBP33) in porcine heart was examined in detail by immunochemistry. Homogenization and further processing of heart in the presence of EGTA resulted in the recovery of annexin V (CaBP33) in the cytosolic fraction and in an EGTA-resistant, Triton X-100-soluble fraction from cardiac membranes. Including Ca^{2+} in the homogenization medium resulted in a significant decrease in the annexin V (CaBP33) content of the cytosolic fraction with concomitant increase in the content of this protein in myofibrils, mitochrondria, the sarcoplasmic reticulum and the sarcolemma. The amount of annexin V (CaBP33) in each of these subfractions depended on the free Ca^{2+} concentration in the homogenizing medium. At the lowest free Ca^{2+} concentration tested, 0.8 μM, only the sarcolemma appeared to contain bound annexin V (CaBP33). Membrane-bound annexins V (CaBP33) and VI partitioned in two fractions, one EGTA-resistant and Triton X-100-extractable, and one Triton X-100-resistant and EGTA-extractable. Altogether, these data suggest that annexins V and VI are involved in the regulation of membrane-related processes.

Annexin; Calcium; Membrane; Binding; Heart

1. INTRODUCTION

Annexins, also known as lipocortins, constitute a novel family of intracellular Ca^{2+}-binding proteins sharing the ability to bind to acidic phospholipids and biological membranes in a Ca^{2+}-dependent way (for reviews see [1-3]). Two acidic Ca^{2+}-binding proteins, CaBP33 and CaBP37, originally purified from bovine brain by Ca^{2+}-dependent affinity chromatography [4] were shown to be annexins on the basis of their ability to bind to acidic phospholipids and brain membranes in the presence of Ca^{2+} [5]. A substantial fraction of the CaBP33/CaBP37 mixture bound to membranes was resistant to Triton X-100, in the presence of Ca^{2+}, and extractable with EGTA from the Triton X-100-resistant residue [5], suggesting an association of these two proteins with the cytoskeleton in the brain. Actually, the CaBP33/CaBP37 mixture was shown to bind saturably, reversibly and with a relatively high affinity to a cytoskeleton preparation obtained from brain membranes, particularly to a set of low M_r polypeptides present in the cytoskeleton preparation [6]. Rat brain turned out to express only CaBP33 [5]. While two proteins comigrating with CaBP33 and CaBP37, respectively, in SDS gels were purified from porcine heart by Ca^{2+}-dependent affinity chromatography, only the lower M_r species (CaBP33) was recognized in Western blots by a polyclonal antiserum raised against the unfractionated bovine brain CaBP33/CaBP37 mixture [5]. Also, the yield of the putative porcine heart CaBP37 was extremely low.

Concomitantly with our observation that CaBP33 and CaBP37 were brain annexins, Woolgar et al. [7] demonstrated that three annexins could be isolated from porcine brain, a 68 kDa annexin homologous to the 68 kDa annexin (annexin VI) found in liver, adrenal medulla, and lymphocytes, endonexin I (annexin IV), and a 32 kDa annexin homologous to human placental endonexin II (annexin V). These homologies were established by an immunochemical approach using antisera raised against liver 68 kDa annexin, endonexin I from adrenal medulla, and placental endonexin II [7]. Amino acid sequence analysis of CaBP33 (manuscript in preparation) demonstrated that this protein was highly homologous to endonexin II (annexin V) [8], suggesting that CaBP33 could be the brain (iso)form of endonexin II (annexin V). The identity of CaBP37 remains to be elucidated.

We sought to have more detailed information on cardiac annexins, with special reference to annexin V (CaBP33). Our results indicate that the mammalian heart expresses several annexins, among which the most prominent species are CaBP33 (annexin V), and a 70 kDa protein (annexin VI). Subcellular fractionation studies indicate that myofibrils, mitochondria, the sarcoplasmic reticulum (SR) and the sarcolemma (SL) all have annexin V (CaBP33) bound, provided Ca^{2+} has been included in the homogenization buffer, and that

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the free Ca^{2+} concentration during tissue homogenization and subsequent steps dictates the amounts of annexin V (CaBP33) recovered in any of the above subfractions. Evidence will also be presented indicating that, once bound to cardiac membranes in the presence of Ca^{2+}, annexin V (CaBP33) and annexin VI partition in two parts, one resistant to EGTA and extractable with Triton X-100, and one resistant to Triton X-100 and extractable with EGTA.

2. MATERIALS AND METHODS

2.1. *Isolation of cardiac annexins*

To identify as many annexins as possible in porcine heart, the procedure described by Boustead et al. [9] was used with minor changes. All operations were done at 4°C. The EGTA-solubilized proteins [9] were loaded onto a column (1 × 10 cm) of DEAE Sephacel equilibrated with 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5 mM 2-mercaptoethanol (buffer A). The column was washed with 100 ml of buffer A and eluted with increasing concentrations of NaCl in buffer A (50 ml/step). Individual protein peaks as well as the unfractionated EGTA extract and proteins not retained by the resin were analyzed by SDS-PAGE [10] and immunoblotting [11] using a rabbit antiserum raised against the unfractionated bovine brain CaBP33/CaBP37 mixture [5]. The protein peak from the DEAE Sephacel column (protein peak II) containing polypeptides of 33 and 70 kDa in large amounts (see Results) was concentrated by pressure dialysis and gel filtered through Sephacryl S-200 (2 × 90 cm) in buffer A to achieve final purification of the above polypeptides. Protein peak III (see results) was similarly treated to purify a 70 kDa doublet therein. The other protein peaks from the DEAE Sephacel column were concentrated likewise and not further fractionated. All protein samples were dialysed against deionized, double-distilled water and lyophilized.

2.2. *Binding of cardiac annexins to phospholipids*

Individual pure proteins and protein fractions from the DEAE Sephacel column containing heterogeneous populations of proteins were assayed for their ability to bind to liposomes of different phospholipid composition as described [9]. Briefly, proteins were incubated at room temperature in 0.5 ml of 20 mM Tris-HCl, pH 7.5, 0.1 M KCl, 2 mM $MgCl_2$, 1 mM EGTA containing liposomes of either phosphatidylcholine (PC) or cardiolipin (CL) (150 μg each) in the absence and presence of 100 μM free Ca^{2+}. After 20 min, the suspensions were centrifuged at 12 000 × *g* for 10 min, and the supernatants were saved. Individual pellets were resuspended in 0.5 ml of the appropriate incubation buffer and centrifuged as above. This operation was repeated once more. Individual final pellets were resuspended in 0.5 ml of 20 mM Tris-HCl, pH 7.5, 5 mM EGTA. Equal volumes of supernatants and pellets were subjected to SDS-PAGE. Liposomes were obtained as previously described [12].

2.3. *Distribution of annexins V (CaBP33) and VI in cardiac subfractions*

Aliquots (10 g) of porcine heart tissue were homogenized in 50 ml of 20 mM Tris-HCl, pH 7.5, 0.1 M KCl, 1 mM $MgCl_2$, 0.25 mM PMSF, containing 5 mM EGTA (buffer E), no other additions (buffer B), or 1 mM $CaCl_2$ (buffer C). Homogenates were filtered through cheese-cloth and centrifuged at 750 × *g* for 8 min. The supernatants (S_1) were saved. The upper layers of individual pellets containing myofibrils [13] were washed 5 more times in the respective buffers by cycles of resuspension and centrifugation. Individual final pellets were resuspended in buffer E and centrifuged at 150 000 × *g* for 30 min to obtain three EGTA-extracts from myofibrils. The S_1 supernatants were centrifuged at 60 000 × *g* for 20 min to obtain P_2 pellets that were resuspended in the respective buffers and centrifuged again. The resultant supernatants were centrifuged at 150 000 × *g* for 60 min to yield the cytosolic fractions (S_2). The P_2 pellets containing mitochondria plus cardiac microsomes [14] were resuspended in the respective buffers minus KCl and centrifuged. This operation was repeated two more times before extraction of individual P_2 pellets with 1% (w/v) Triton X-100 in buffers E, B, and C minus KCl, respectively. After 15 min, the suspensions were centrifuged at 150 000 × *g* for 90 min to obtain three Triton X-100-extracts. The resultant pellets containing the Triton X-100-unsoluble materials were washed 3 times in buffers E, B, and C minus KCl, respectively, by cycles of resuspension and centrifugation. Individual final pellets were resuspended in 20 mM Tris-HCl, pH 7.5, 5 mM EGTA, and centrifuged to obtain three EGTA-extracts. Individual S_2 supernatants and Triton X-100-extracts were brought to 60% saturation with solid $(NH_4)_2SO_4$ and centrifuged. The supernatants were brought to 80% saturation with $(NH_4)_2SO_4$ and centrifuged. Individual pellets were resuspended in buffer A and dialyzed against the same buffer. All operations were done at 4°C. All soluble and solubilized fractions were subjected to SDS-PAGE and immunoblotting as mentioned in the preceding section.

Fractionation of the post-myofibril supernatant from porcine heart to obtain the SL and SR fractions was done following the methods of Suko and Hasselbach [14] and Tuana et al. [15] with minor changes. The mitochondrial and SR_1 pellets [14], and the SL and SR_2 pellets [15] were washed 3 times in the separation buffer minus sucrose (20 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 0.25 mM PMSF, plus or minus $CaCl_2$ as required) by cycles of resuspension and centrifugation before extraction with 20 mM Tris-HCl, pH 7.5, 5 mM EGTA. All operations were done at 4°C. The extracts were subjected to SDS-PAGE and immunoblotting as above.

2.4. *Purification of the CaBP33/CaBP37 mixture, annexin V (CaBP33) and annexin VI from bovine brain*

The CaBP33/CaBP37 mixture was purified as in [4]. The two proteins were separated from one another as in [5]. Annexin VI was purified as in [7].

3. RESULTS AND DISCUSSION

Subjecting the Ca^{2+}-precipitated, EGTA-extractable proteins obtained from heart tissue to DEAE chromatography resulted in the separation of four protein peaks (Fig. 1A). Peaks I and IV contained heterogeneous populations of polypeptides (Fig. 1B) and were not further fractionated. Peak II contained polypeptides of 70 and 33 kDa in large amounts plus minor contaminants (Fig. 1B). Close inspection of gels revealed that the 70 kDa polypeptide was a doublet (also see Fig. 2B). Protein peak II was subjected to gel filtration to obtain pure 70 and 33 kDa proteins (Fig. 2). The 70 kDa doublet comigrated in SDS gels with bovine brain annexin VI (Fig. 1B). The 33 kDa protein comigrated in SDS gels with bovine brain annexin V (CaBP33) (Fig. 1B) and was recognized by the antiserum raised against the bovine brain CaBP33/CaBP37 mixture in Western blots (Figs 1B and 2A), suggesting that the 33 kDa protein was authentic annexin V (CaBP33). The possibility that the 33 kDa protein in DEAE protein peak II might be annexins III or IV was excluded since: (i) the antiserum against the CaBP33/CaBP37 mixture as well as the anti-brain annexin V (CaBP33) antiserum recognized by immunoblotting a 33 kDa polypeptide in EGTA-extracts from skeletal muscles that has been reported not to con-

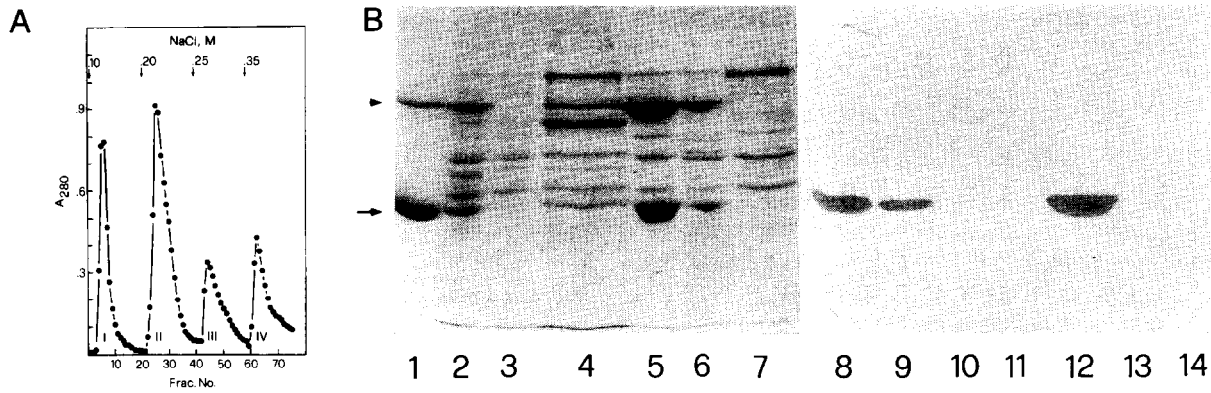

Fig. 1. Isolation of porcine heart annexins. (A) DEAE Sephacel chromatography of Ca^{2+}-precipitated, EGTA-extractable proteins. These were loaded onto a column of DEAE Sephacel and eluted with increasing concentrations of NaCl as indicated. Fractions of 2.5 ml were collected and monitored for protein content. (A_{280}). (B) SDS-PAGE (lanes 1–7) and immunoblotting (lanes 8–14) of (1,8) annexin V (CaBP33) plus annexin VI from bovine brain, (2,9) the material loaded onto the column, (3,10) the material unretained by the resin, (4,11) protein peak I, (5,12) protein peak II, (6,13) protein peak III, and (7,14) protein peak IV. Immunoblotting was done using the antiserum against the bovine brain CaBP33/CaBP37 mixture [5]. Identical results were obtained with the anti-bovine brain annexin V (CaBP33) antiserum. The arrow and arrowhead point to the positions of annexins V (CaBP33) and VI, respectively. 10% acrylamide gels were used in these as well as in experiments illustrated in Figs 2–7. Gels were stained with Coomassie blue.

tain annexin IV [16]; and (ii) annexins III and IV bound to DEAE resins are eluted with 0.01 M NaCl [16], whereas annexin V and bovine brain annexin V require 0.15–0.20 M NaCl for elution from similar resins ([5,18] and Fig. 1A). Moreover, PAGE under non-denaturing conditions showed that porcine heart annexin V (CaBP33) comigrated with the slow migrating band of the bovine brain CaBP33/CaBP37 mixture, with no additional bands (not shown). As to the DEAE protein peak III (Fig. 1A), this also was processed by Sephacryl chromatography (not shown) to obtain a 70 kDa polypeptide in pure form (cf. Fig. 3C).

Porcine heart annexin V (CaBP33) bound in the presence, but not in the absence, of 100 μM free Ca^{2+} to the negatively charged phospholipid, cardiolipin (CL) (Fig. 3A). This binding was completely reversed by EGTA, as judged by SDS-PAGE (Fig. 3A). No binding of annexin V (CaBP33) to liposomes of phosphatitylcholine (PC) was observed, irrespective of the presence or absence of Ca^{2+} (Fig. 3A). As to the 70 kDa doublet obtained from DEAE protein peak II, only the higher M_r species appeared to behave as an annexin, in that it bound to CL, but not to PC, vesicles in the presence of Ca^{2+}, although a certain amount of this protein co-sedimented with CL and PC liposomes even in the absence of Ca^{2+} (Fig. 3B). Most of the polypeptides in DEAE protein peaks I and IV sedimented with CL vesicles (Fig. 3D) and with PC vesicles (not shown) independently of Ca^{2+}, with the exception of a 33 kDa polypeptide in protein peak I which sedimented with CL, but not PC, vesicles Ca^{2+}-dependently (not shown). This 33 kDa polypeptide probably was a mixture of annexins III and IV [16] since it was detached from the DEAE column at 0.1 M NaCl (Fig. 1A). The 70 kDa doublet purified from DEAE protein peak III also was tested in the liposome binding assay (Fig. 3C). Of the two polypeptides, only the lower M_r species, that was the most abundant, sedimented with CL vesicles in the presence, but not in the absence, of Ca^{2+}. No binding of this polypeptide to PC vesicles was observed (not shown). At present we do not know whether the higher M_r species in the 70 kDa doublet from DEAE peak II and the lower M_r species in the 70 kDa doublet in DEAE peak III are identical or related proteins.

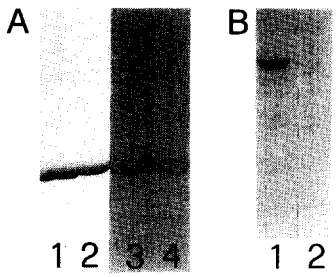

Fig. 2. Purification of porcine heart annexin V (CaBP33) and annexin VI (the 70 kDa doublet in DEAE protein peak II). (A) SDS-PAGE (lanes 1,2) and immunoblotting (lanes 3,4) of (1,3) porcine heart annexin V (CaBP33) and (2,4) bovine brain annexin V (CaBP33). (B) SDS-PAGE (lane 1) and immunoblotting (lane 2) of porcine heart annexin VI. Immunoblotting was done as described in Fig. 1B. Note that the antisera do not cross-react with annexin VI.

It is generally accepted that membrane-associated annexins resist extraction with Triton X-100, albeit in the presence of Ca^{2+}, and bind to cytoskeleton elements in a Ca^{2+}-dependent way [5,6,17–21]. However, annexins I and II, and the CaBP33/CaBP37 mixture from bovine brain have been shown to exist in a membrane-bound form that is resistant to EGTA and is solubilized with Triton X-100 [5,22–24]. To monitor the distribution of annexins V (CaBP33) and VI in porcine heart, in a first series of experiments three subfractions, i.e. the cytosol, myofibrils, and a post-myofibril membrane

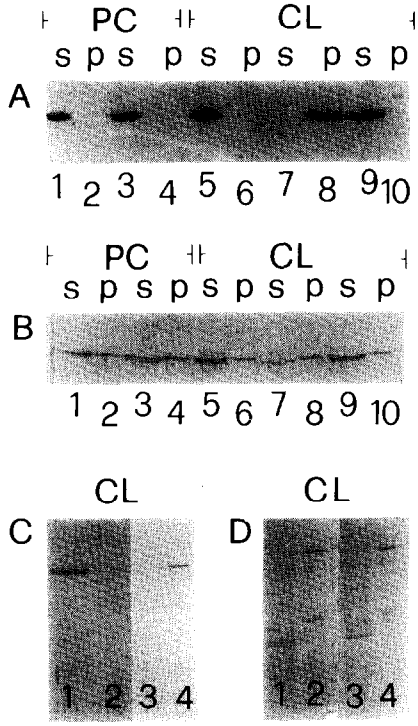

Fig. 3. Binding of cardiac annexins to phospholipids. (A) Binding of porcine heart annexin V (CaBP33) to liposomes of PC (lanes 1–4) and CL (lanes 5–10). Identical samples of supernatants (s) and pellets (p) obtained after centrifugation of suspensions of liposomes previously incubated with 30 μg of annexin V (CaBP33)/ml in the absence (lanes 1,2,5,6) and presence (lanes 3,4,7,8) of 100 μM free Ca^{2+} were subjected to SDS-PAGE. No binding of annexin V (CaBP33) to PC liposomes occurs irrespective of the absence or presence of Ca^{2+}, whereas Ca^{2+}-dependent binding of the protein to CL liposomes is observed. Lanes 9, 10 refer to an experiment in which the protein was incubated with CL vesicles in the presence of 100 μM free Ca^{2+} for 20 min before addition of EGTA to 1 mM EGTA completely reverses the Ca^{2+}-dependent binding of the protein to CL vesicles. (B) Binding of porcine heart annexin VI (purified from DEAE protein peak II) to liposomes of PC (lanes 1–4) and CL (lanes 5–10). Conditions were as described in (A). Only the higher M_r species of the doublet behaves in part as an annexin. (C) Binding of the 70 kDa doublet purified from DEAE protein peak III to liposomes of CL. Conditions were as described in A in the absence (lanes 1,2) and presence (lanes 3,4) of 100 μM fre Ca^{2+}. Only the lower M_r species of the doublet behaves as an annexin. (D) Binding of proteins from DEAE protein peak IV to CL vesicles. Conditions were as described in (A) in the absence (lanes 1,2) and presence (lanes 3,4) of 100 μM free Ca^{2+}, except that 120 μg of the protein mixture/ml were used. None of the polypeptides in this protein mixture behaves as an annexin.

fraction containing mitochondria, the SR and the SL, were analyzed. As the total $CaCl_2$ concentration in the homogenizing medium increased from 0 to 1 mM, the amount of annexin V (CaBP33) in the cytosolic fraction decreased, judging from immunoblot analyses (not shown), whereas it concomitantly increased in myofibrils (not shown) and in the Triton X-100-resistant residue from membranes (Fig. 4). No appreciable changes in the annexin V (CaBP33) content were observed in the Triton X-100-soluble fractions from membranes (not shown). Thus the increase in the

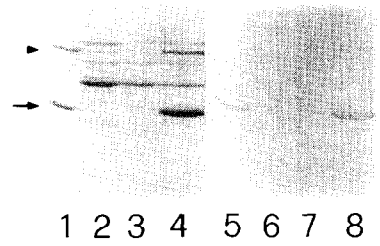

Fig. 4. Annexins V (CaBP33) and VI in the EGTA-extracts from post-myofibril membranes previously solubilized with Triton X-100. (Lanes 1–4) SDS-PAGE of (1) annexins V (CaBP33) and VI from porcine heart, (2–4) the EGTA-extracts from membranes prepared in the presence of EGTA, with no additions, or in the presence of Ca^{2+}, respectively. (Lanes 5–8) The same materials as in lanes 1–4 were subjected to immunoblotting as described in Fig. 1B. Note that large amounts of annexins V (CaBP33) and VI are found exclusively in the EGTA-extract from membranes prepared in the presence of Ca^{2+}. The arrow and arrowhead point to the position of annexins V (CaBP33) and VI, respectively.

annexin V (CaBP33) content of myofibrils and the Triton X-100-resistant residue had occurred at the expense of the cytosolic annexin V (CaBP33). Since a substantial amount of the protein was recovered in the Triton X-100-extract from membranes obtained in the presence of EGTA, we concluded that this fraction of annexin V (CaBP33) was tightly bound to membranes. We also performed experiments in which membranes prepared in the presence of either 5 mM EGTA or 1 mM $CaCl_2$ were first extracted with EGTA, then washed 4 times in EGTA, and finally extracted with Triton X-100. Virtually no annexin V (CaBP33) was recovered in the EGTA-extract from membranes prepared in EGTA, whereas a large amount of it was recovered in the EGTA-extract from membranes prepared in $CaCl_2$,

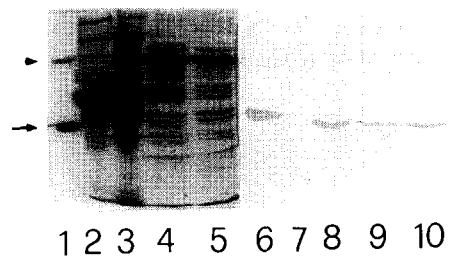

Fig. 5. Annexin V (CaBP33) and EGTA- and Triton X-100-extracts from cardiac membranes. These were prepared in the presence of EGTA (lanes 2,4,7,9) and in the presence of Ca^{2+} (lanes 3,5,8,10) and first extracted with EGTA (lanes 2,3,7,8) and then with Triton X-100 (lanes 4,5,9,10) as described in the text. Lanes 1,5 contain annexins V (CaBP33) and VI purified from bovine brain. SDS-PAGE and immunoblotting were done as described in Fig. 1B (lanes 1–5 and lanes 6–10, respectively). Note that annexin V (CaBP33) is found in the EGTA-extract from membranes obtained in the presence, but not in the absence, of Ca^{2+}, and that both Triton X-100-extracts contain appreciable amounts of the protein. The arrow and arrowhead point to the positions of bovine brain annexins V (CaBP33) and VI, respectively.

as expected (Fig. 5). On the other hand, the amounts of annexin V (CaBP33) in the two Triton X-100-extracts were simular or the amount of Triton X-100-soluble annexin V (CaBP33) in the Triton X-100-extract from membranes prepared in $CaCl_2$ was larger than that from membranes prepared in EGTA (Fig. 5). These data suggested that: (i) annexin V (CaBP33) was induced to bind to cardiac membranes by Ca^{2+}; (ii) membrane-bound annexin V (CaBP33) partitioned in two fractions, one dissociable by EGTA and tentatively considered bound to the polar surface of the membrane lipid bilayer and/or to cytoskeleton elements, and one undissociable by EGTA and extractable with Triton X-100; and (iii) Ca^{2+} caused annexin V (CaBP33) to bind tightly to the lipid bilayer and/or to one or more membrane intrinsic proteins, but once bound to these targets the protein no longer required Ca^{2+} for it to remain bound to membranes. If these conclusions are correct, then the Triton X-100-soluble annexin V (CaBP33) obtained from membranes prepared in EGTA resulted from Ca^{2+}-dependent binding of the protein to membranes in vivo. The 70 kDa polypeptide comigrating with bovine brain annexin VI in SDS gels appeared to behave similarly to annexin V (CaBP33) (Figs 4 and 5).

Next we examined the distribution of the Ca^{2+}-dependently bound annexin V (CaBP33) in cardiac membrane subfractions. To this end, mitochondria, the SR and the SL were purified in the presence of 1 mM Ca^{2+}. Individual EGTA-extracts from these membranes were subjected to SDS-PAGE and immunoblotting using the anti-CaBP33/CaBP37 antiserum. Annexin V (CaBP33) was recovered in all EGTA-extracts (Fig. 6). Again, the 70 kDa polypeptide (annexin VI) was likewise detected (Fig. 6). When the above membrane subfractions were prepared in the presence of increasing free Ca^{2+} concentrations (Fig. 7), annexin V (CaBP33) was detected in the SL starting from 0.8 μM free Ca^{2+}, in the SR starting from 100 μM free Ca^{2+}, and in mitochondria starting from 2 μM free Ca^{2+}. These data suggested that the SL and, possibly, mitochondria, but not the SR, could be targets of annexin V (CaBP33) at physiological free Ca^{2+} concentrations.

Previous reports have shown that the mammalian heart expresses several annexins including annexins III, IV, V, and VI [5,16,25,26]. In all these cases, identification of annexins was done by an immunochemical approach using antisera raised against antigens from other tissues. In the present report, we looked for cardiac annexins by applying to porcine heart the so-called Ca^{2+}-precipitation method [9]. Large amounts of annexin V (CaBP33), appreciable amounts of annexin VI, and relatively low amounts of annexins III and IV could be purified from this organ. Annexins I and II have been reported to be present in extremely low amounts in the heart [1-3]. Since at concentrations as low as 0.8-2.0 μM free Ca^{2+} annexin V (CaBP33) was detected in the EGTA-extracts from the SL and mitochondria, whereas only at 100 μM free Ca^{2+} could the protein be seen in the EGTA-extract from the SR, recovery of annexin V (CaBP33) in cardiac subcellular fractions can be reasonably attributed to Ca^{2+}-dependent binding of annexin V (CaBP33) to these membranes, rather than to Ca^{2+}-induced precipitation. On the other hand, artifactual redistribu-

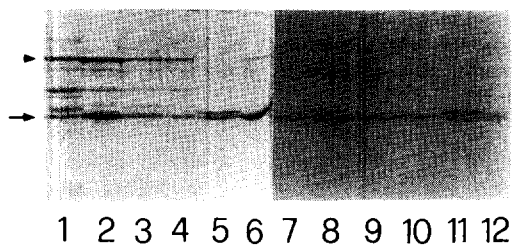

Fig. 6. Detection of annexins V (CaBP33) and VI in cardiac membrane subfractions obtained in the presence of Ca^{2+}. Mitochondria, SL, SR_1, and SR_2 (see section 2.3) were obtained in the presence of 1 mM $CaCl_2$ and extracted with EGTA. (Lanes 1-6) SDS-PAGE of (1-4) EGTA-extracts from mitochondria, SL, SR_1, and SR_2, respectively, (5) the bovine brain CaBP33/CaBP37 mixture, and (6) annexins V (CaBP33) and VI from bovine brain. (Lanes 7-12) The same protein fractions as in lanes 1-6 were subjected to immunoblotting as described in Fig. 1B. The arrow and arrowhead point to the positions of annexins V (CaBP33) and VI, respectively.

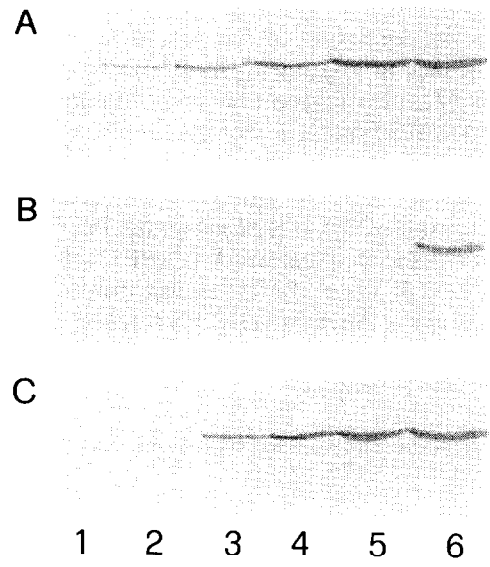

Fig. 7. Distribution of annexin V (CaBP33) in the sarcolemma (A), the sarcoplasmic reticulum (B), and mitochondria (C) as a function of free Ca^{2+} concentration. The three membrane subfractions were purified in the presence of 0 (lane 1), 0.8 (lane 2), 2 (lane 3), 11 (lane 4), 50 (lane 5), and 100 (lane 6) μM free Ca^{2+}. After separation, individual subfractions were extracted with EGTA and centrifuged to obtain the EGTA-extracts. These were subjected to SDS-PAGE (not shown) and immunoblotting as described in Fig. 1B. Note that no annexin V (CaBP33) is found in the absence of Ca^{2+}, that at concentrations as low as 0.8 μM free Ca^{2+} the protein is found in the sarcolemma, and that the protein is found in the sarcoplasmic reticulum only at ≥ 100 μM free Ca^{2+}

tion of this protein during tissue homogenization because of the presence of Ca^{2+} should be considered. Immunocytochemical analyses of the protein in cardiac tissue and in cultured cardiomyocytes will help elucidate this point.

The finding that several annexins, including annexins V (CaBP33) and VI are recovered in EGTA-resistant, Triton X-100 soluble fractions from membranes contrasts with the observation that annexins that have bound to phospholipids in the presence of Ca^{2+} come again in solution on addition of EGTA. Clearly, liposomes are not directly comparable with natural membranes. Membrane proteins could have an important role in defining the binding properties of annexins and in conditioning the structural features of membrane-bound annexins. Tight binding of annexins to natural membranes could imply partial penetration of these proteins into the lipid bilayer. In a recent report [27], this possibility has been excluded for a number of annexins as far as artificial membranes are concerned. However, data have been presented showing that human placental microvillar annexins, that had bound to CL or phosphatidylinositol vesicles in the presence of Ca^{2+}, necessitate Triton X-100 for their complete solubilization [28]. Studies on natural membranes targeted with appropriate probes would help elucidate this issue. The functional meaning of membrane-bound annexins V (CaBP33) and VI remains to be determined, but it appears to be in line with the proposed role of annexins in the regulation of the structural organization of membranes in relation to both exocytosis and endocytosis [3], also taking into consideration that the CaBP33/CaBP37 mixture from bovine brain interacts with a number of polypeptides in cytoskeletons from brain membranes [6]. As annexin V has been reported to be secreted (see [3]), it is possible that the fraction of membrane-associated annexin V (CaBP33) that is resistant to EGTA might be in transit toward the extracellular space. Alternatively, this fraction of the protein might be linked to membrane intrinsic proteins to regulate their functions.

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