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Calcium-dependent phospholipid binding proteins associated with the membranes of rabbit skeletal muscle

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By using extraction in the presence of Ca^{2+} and Triton X-100 and then in the presence of EGTA without detergent, a set of Ca^{2+}-dependent phospholipid binding proteins has been identified in the membranes of transverse tubules (T-tubules) and sarcoplasmic reticulum (SR), isolated from rabbit skeletal muscles. Longitudinal SR, junctional SR and T-tubule membranes yielded about 9, 14 and 3.3 μg of EGTA-soluble proteins per 1 mg of membrane protein, respectively. In the presence of 1 mM $CaCl_2$, 68 and 33 kDa proteins of T-tubules and junctional SR as well as 30 kDa protein of T-tubules were shown to bind to liposomes made of 1:1 w/w mixtures of (i) phosphatidylcholine and (ii) phosphatidylserine, phosphatidic acid, or phosphatidyl ethanolamine. In the presence of EGTA, the above-mentioned proteins were mostly found in the supernatants. Binding of the proteins with liposomes consisting of pure phosphatidylcholine was negligible.

Ca^{2+}-dependent phospholipid binding protein; Junctional sarcoplasmic reticulum; Transverse tubule; Liposome aggregation; (Rabbit skeletal muscle)

1. INTRODUCTION

The muscle fiber contains an intricate membraneous network that controls muscle contraction and relaxation by regulating the intracellular calcium concentration. The proteins associated with different portions of sarcoplasmic reticulum (SR) and transverse tubules (T-tubules) of sarcolemma, therefore, play an essential role in the process. However, the features of many proteins appearing in the skeletal muscle membranes, are still obscure (for review, see [1]). It concerns especially the so-called integral proteins with unknown function [2], which are included in this group solely on the basis that they are not extracted with high concentrations of salts and are not solubilized with low concentrations of various nonionic detergents. It seems that there are some proteins which do not span the membrane core and are located on the membrane surface due to hydrophobic interactions induced by Ca^{2+} binding to these proteins. Furthermore, the proteins were identified in various tissues, which were solubilized with Ca^{2+} chelators only after the prior treatment of the cells with low concentrations of nonionic detergent [3]. In vitro, the proteins can bind to membranes in a Ca^{2+} dependent manner and induce membrane aggregation, suggesting that they may play a role in Ca^{2+} dependent membrane processes [4,5]. Solubilization by Ca^{2+} chelators indicates that these proteins are either Ca^{2+} binding proteins or proteins associated with Ca^{2+} binding structures. Therefore, we have investigated whether the membranes of T-tubules and SR contain the proteins of that kind.

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2. EXPERIMENTAL

2.1. *Isolation and purification of membranes*

Total microsomal fraction was isolated from the fast white muscles of rabbit hindlimbs according to the procedure of Nakayama et al. [6]. Throughout the purification process, a mixture of protease inhibitors was present in each buffer to prevent protein degradation. The final concentrations of the inhibitors were: phenylmethanesulfonyl fluoride, 0.1 mM; iodoacetamide, 1 mM; polybrene, 25 mg/l; aprotinin, 2.8 mg/l; and trypsin inhibitor, 20 mg/l, and they will be simply omitted in the buffer descriptions unless otherwise noted. 2 mM $CaCl_2$ was added in each buffer and all manipulations were done at 4°C unless otherwise stated.

After the treatment with a French Press (American Instrument Co.), 5 ml of the microsomal suspension (10 mg of protein per 1 ml) was diluted with 20 ml of 34% (w/v) sucrose in 10 mM Mops/KOH, pH 7.4, and centrifuged at 100000 × g for 45 min (Beckman Ti-60 rotor). Slightly opalescent upper layer (crude T-tubules), middle layer (crude longitudinal SR, LSR) and heavy pellet (crude junctional SR, JSR) were collected, diluted 7-fold with 10 mM Mops/KOH, pH 7.4, and recentrifuged. The pellets were resuspended in a buffer (0.6 M KCl, 10% (w/v) sucrose, 10 mM Mops/KOH, pH 7.4) and layered on discontinuous sucrose density gradients constructed of 5 ml of 50% (w/v), 6 ml of 40% (w/v), 8 ml of 35% (w/v), 12 ml of 30% (w/v) and 5 ml of 23% (w/v) sucrose. The Beckman SW-27 rotor was run for 5 h at 22000 rpm. The 30% layer (for T-tubules), the layer on the top of 35% sucrose (for LSR) and the layer on the top of 40% sucrose (for JSR) were collected and stored at −20°C.

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2.2. *Isolation of EGTA-soluble proteins from membrane fractions*

The T-, LSR-, and JSR-fractions were diluted with 4 vols of 2 mM $CaCl_2$. After centrifugation at 100000 × *g* for 45 min, the pelleted material was resuspended to a protein concentration of 3 mg/ml in a buffer (75 mM NaCl, 1 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride, 1 mM NaN_3, 10 mM Hepes, pH 7.4) containing 1% Triton X-100 and 2 mM $CaCl_2$ [7]. Then all suspensions were stirred at 4°C for 15 min. The insoluble residues of LSR and JSR were collected by centrifugation at 25000 × *g* for 20 min (Beckman JA-20 rotor). The same procedure was used for the removal of heavy contaminations from the T-tubule preparations. The insoluble residue of T-tubules was sedimented at 100000 × *g* during 45 min. The pelleted materials were resuspended and twice washed in the detergent-free buffer containing 2 mM $CaCl_2$. Then the pellets were resuspended to a protein concentration of 3 mg/ml in the buffer containing 10 mM EGTA and dialyzed exhaustively against the same buffer for 18 h. The supernatants containing the EGTA-soluble proteins were collected by centrifugation at 25000 × *g* for 15 min and dialyzed against 20 mM Hepes, pH 7.4.

2.3. *Phospholipid binding*

Phospholipid (Serva, Heidelberg) vesicles were made in the presence of 240 mM sucrose from pure dioleoyl phosphatidylcholine (PC), or from 1:1 (w/w) mixtures of PC and dilauroyl phosphatidylethanolamine (PE), dipalmitoyl phosphatidylserine (PS) or phosphatidic acid (PA) as described in [8]. Liposomes prepared were used to identify Ca^{2+}-dependent phospholipid-binding proteins according to the procedure of [8].

2.4. *Gel electrophoresis*

Protein profiles of purified T-, LSR-, and TSR-membranes were analyzed by SDS-polyacrylamide 5–15% gradient gel electrophoresis as described in [9]. EGTA-soluble proteins and the proteins bound to liposomes were analyzed in homogeneous 10% gel using the same technique [9]. Molecular mass standards (Pharmacia, Uppsala) were phosphorylase (94 kDa), bovine albumin (66.3 kDa), egg albumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa). Gels were stained with Coomassie brilliant blue R-250 (Serva, Heidelberg).

2.5. *Protein assay*

Protein concentrations were measured as in [10] with bovine serum albumin as a standard.

3. RESULTS

To fully separate SR and T-tubule membranes, it was necessary to pass microsomal suspensions through a treatment with a French press cell and through a multistep sucrose gradient. The protein profiles of the fractions obtained can be seen in fig.1. LSR vesicles consist of predominantly Ca^{2+}-ATPase (105 kDa), 64 kDa protein and 51 kDa protein. Besides that, LSR contain a lot of minor components. The JSR fraction which consists of three distinct types of membranes, the junctional face membrane, Ca-pump membrane, and T-tubule membrane, displays a high content of calsequestrin. Additionally, a doublet of 350 and 325 kDa proteins known as a component of ryanodine receptor (for review, see [11]) is uniquely present in JSR. A glycoprotein band of about 160 kDa is present in SR but is reduced in the T-tubule fraction which is almost

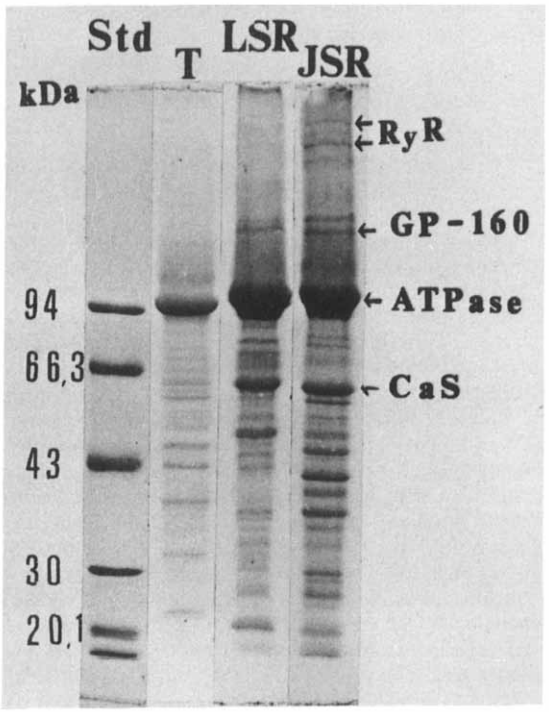

Fig.1. Electrophoretic analysis of membrane fractions prepared by the treatment of rabbit skeletal muscles with a French pressure cell and subsequent centrifugation in a multistep sucrose density gradient. Std, protein standards; T, transverse tubules; LSR, longitudinal SR; JSR, junctional SR; GP-160, integral 160 kDa glycoprotein; ATPase, Ca^{2+}-ATPase (105 kDa); CaS, calsequestrin (64 kDa).

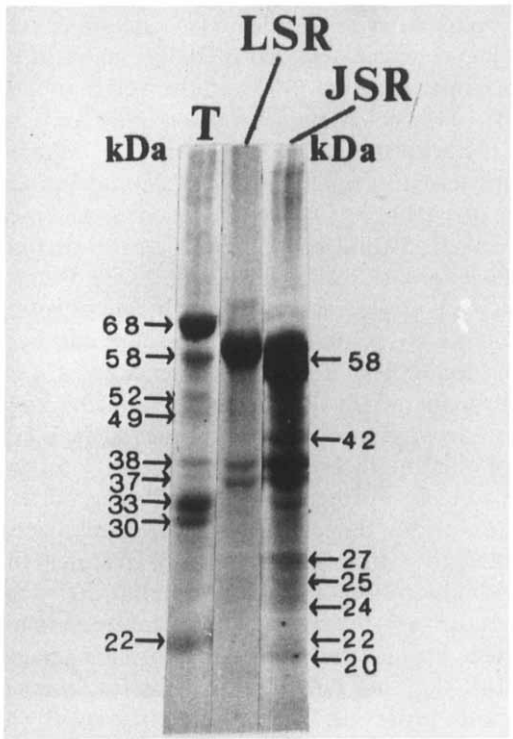

Fig.2. EGTA-soluble proteins extracted with 10 mM EGTA from a Triton X-100-insoluble residue of purified membrane fractions. Arrows indicate the position of certain polypeptides, numerals are the apparent molecular masses of the proteins. Other symbols as in fig.1.

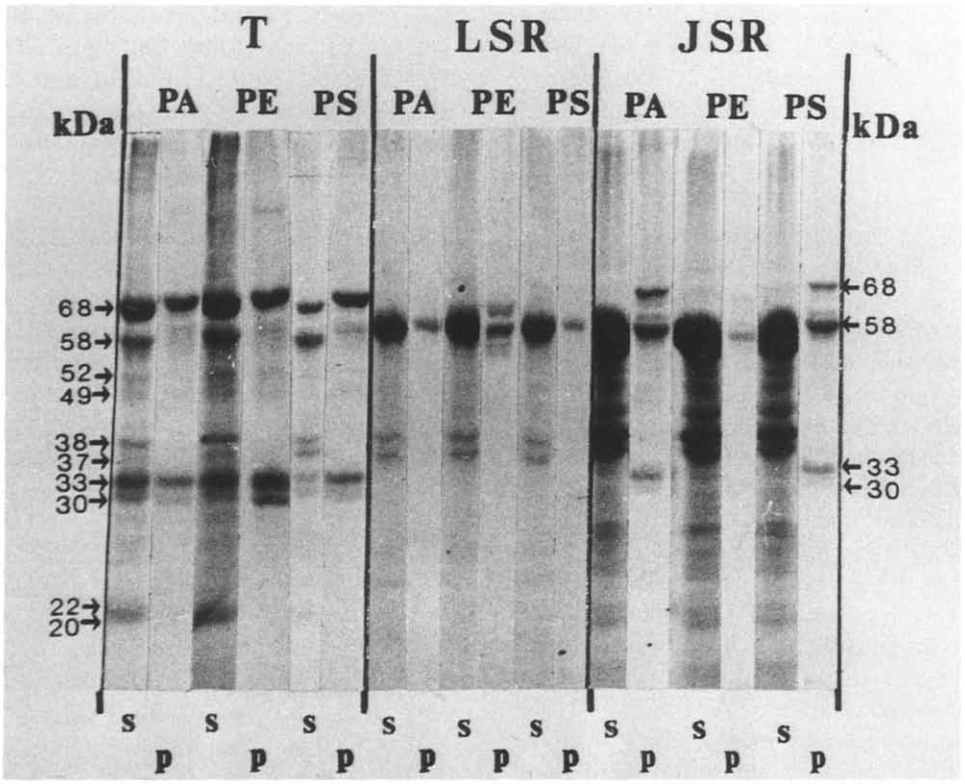

Fig.3. The Ca^{2+}-dependent association of EGTA-soluble proteins of the skeletal muscle membranes with phospholipid vesicles. PA, PE, and PS, liposomes made of 1:1 (w/w) mixtures of phosphatidylcholine and phosphatidic acid, phosphatidylethanolamine, or phosphatidylserine, respectively; s, supernatants; p, pellets. Other symbols as in fig.1.

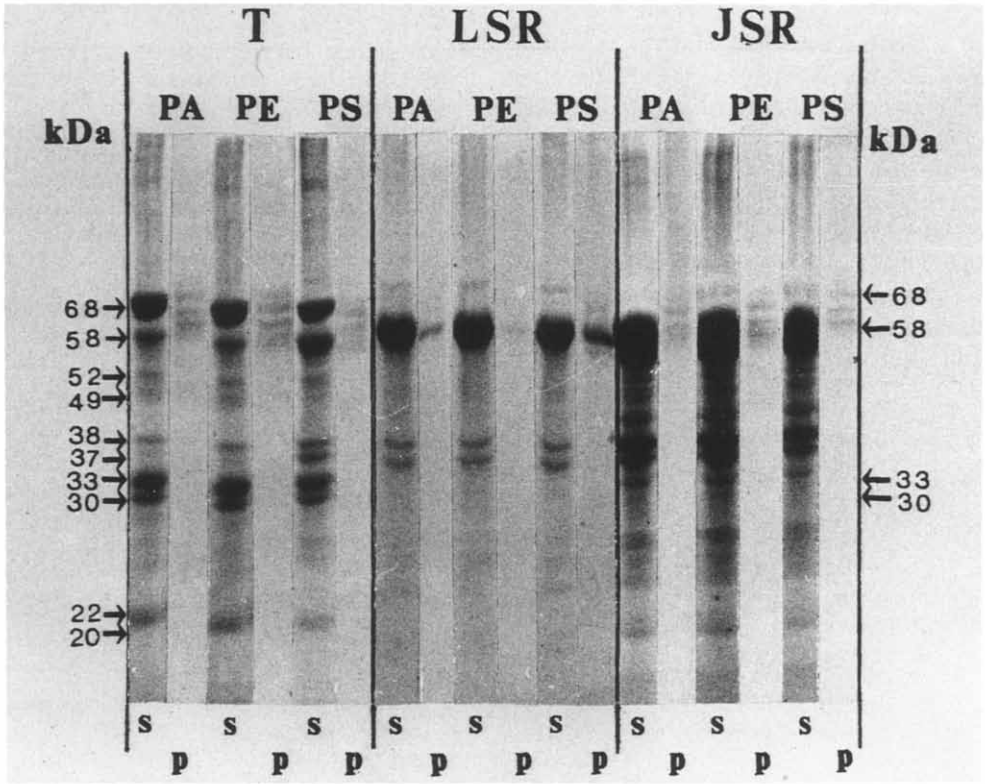

Fig.4. The effect of EGTA on the interaction of the EGTA-soluble proteins with phospholipid vesicles. All symbols as in fig.3.

devoid of high molecular mass polypeptides and enriched in 102 kDa polypeptide (i.e. Mg^{2+}-ATPase [12]) and in the set of minor low molecular mass components. So, the polypeptide patterns of T-, LSR- and JSR-membranes prepared according to the modified procedure of Nakayama et al. [6] are similar to those of standard preparations [13].

By using a different extraction first in the presence of Ca^{2+} and detergent and then in the presence of EGTA without detergent [7], some components were identified in the membranes of T-tubules and JSR, that are absent in the LSR membrane, as judged by SDS-polyacrylamide gel electrophoresis (fig.2). LSR, JSR and T-tubule membranes yielded about 9, 14 and 3.3 μg of EGTA-soluble proteins per 1 mg of membrane protein, respectively. To investigate their lipid-binding properties, the proteins were incubated with the sucrose-loaded liposomes of different composition.

Fig.3 shows that in the presence of 1 mM Ca^{2+} 68, 33 and 30 kDa polypeptides of T-tubules, 68 and 33 kDa polypeptides of JSR, and 58 kDa polypeptide of LSR and JSR are found in the liposome pellet. However, in the presence of EGTA, the 68, 33 and 30 kDa polypeptides associate very slightly with the liposomes (fig.4). In this case, the majority of these polypeptides remain in the supernatant. The same result was obtained when liposomes of pure PC were used (not shown).

4. DISCUSSION

Release of the proteins, observed under Ca^{2+}-free conditions in an EGTA-buffer, suggests a Ca^{2+}-dependent interaction with the membrane of T-tubules and JSR. In this context, the 68, 33 and 30 kDa polypeptides have some biochemical similarities to a class of Ca^{2+}-dependent membrane-binding proteins known generally as annexins [3] which fall into two distinct size classes of 67–71 kDa and 30–38 kDa. In vitro, these proteins are preferentially bound to the cytosolic face of plasma membrane preparations and can be re-extracted with EGTA or EDTA. Although the homology of proteins studied to those isolated from the total extracts of various muscles [14] and from other tissues [4,5] remains to be determined, their similar subcellular origin, similar Ca^{2+}-dependent association with the membranes and phospholipid vesicles and similar molecular masses suggest that they are closely related proteins. The availability of isolated components should make it possible to identify the proteins and to assess their function in skeletal muscle. At present, the physiological importance of the proteins remains obscure. However, Ca^{2+}-dependent reversible interaction of 68, 33 and 30 kDa polypeptides with the liposome membranes suggests that the biological action of the proteins may be affected significantly by changes in the intracellular Ca^{2+} levels of muscle cell.

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