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Annexin A6 at the cardiac myocyte sarcolemma – Evidence for self-association and binding to actin

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Abstract

The plasma membrane of the heart muscle cell and its underlying cytoskeleton are vitally important to the function of the heart. Annexin A6 is a major cellular calcium and phospholipid binding protein. Here we show that annexin A6 copurifies with sarcolemma isolated from pig heart. Two pools of annexin A6 are present in the sarcolemma fraction, one dependent on calcium and one that resists extraction by the calcium chelator EGTA. Potential annexin A6 binding proteins in the sarcolemma fraction were identified using Far Western blotting. Two major annexin A6 binding proteins were identified as actin and annexin A6 itself. Annexin A6 bound to itself both in the presence and in the absence of calcium ions. Sites for self association were mapped by performing Western blots on proteolytic fragments of recombinant annexin A6. Annexin A6 bound preferentially not only to the N terminal fragment (domains I–IV, residues 1–352) but also to C-terminal fragments corresponding to domains V + VI and domains VII + VIII. Actin binding to annexin A6 was calcium-dependent and exclusively to the N-terminal fragment of annexin A6. A calcium-dependent complex of annexin A6 and actin may stabilize the cardiomyocyte sarcolemma during cell stimulation.

Keywords: Annexin A6 (VI); Heart; Sarcolemma; Actin

1. Introduction

The plasma membranes of heart muscle cells must resist the mechanical stresses of a life time of constant activity. The cardiomyocyte sarcolemma is invested with a cytoskeletal network that structures the membrane (Towbin and Bowles, 2002, 2006). The membrane cytoskeleton is likely to play a vital role in preventing damage to the cardiomyocyte. There is good evidence for the association of actin with the cardiomyocyte sarcolemma (Calaghan et al., 2004). In addition to actin, the sarcolemmal cytoskeleton includes various actin-binding proteins including spectrin (Burridge et al., 1982; Glenney and Glenney, 1983), vinculin (Pardo et al., 1983) and, of especial importance, dystrophin (Byers et al., 1991). In X-linked dilated cardiomyopathy, the importance of the sarcolemma cytoskeleton is emphasised by the finding that dystrophin that lacks actin-binding activity leads to congestive heart failure and premature death (Towbin and Bowles, 2002; Vatta et al., 2002).

Annexin A6 (previously annexin VI) is a member of the annexin family of calcium and phospholipid binding proteins (Gerke et al., 2005). Early studies identified annexin VI as a major calcium-dependent component of lymphocyte plasma membranes (Davies et al., 1984; Owens and Crumpton, 1984; Owens et al., 1984) and intestinal epithelial cell cytoskeletons (Shadle et al., 1985). Annexins are strongly implicated in various processes including interactions between membranes and the actin-based cytoskeleton (Hayes et al., 2004; Gerke et al., 2005).

Heart tissue is a rich source of annexin A6 (Doubell et al., 1993; Luckcuck et al., 1998) and several studies have located annexin A6 in heart myocytes predominantly at the sarcolemma and intercalated discs (Doubell et al., 1993; Luckcuck

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et al., 1998; Iida et al., 1992; Wang et al., 1993; Gunteski-Hamblin et al., 1996). There is evidence from knock-out and overexpression studies that annexin A6 plays an important role in cardiomyocyte physiology. Thus, overexpression of annexin A6 targeted to the heart results in several pathological changes including enlarged dilated hearts and acute diffuse myocarditis (Gunteski-Hamblin et al., 1996). Additionally, annexin A6 null-mutant mice show altered mechanical properties of their cardiomyocytes (Song et al., 2002).

Studies have shown that a pool of annexin A6 is associated with membranes in a manner that is resistant to extraction by calcium chelators (Bianchi et al., 1992; Pula et al., 1990). This suggests that the binding of annexin A6 to membranes can involve more than simple calcium-dependent binding to acidic phospholipids. One explanation for this could be association with proteins and there is good evidence for binding of annexin A6 to various proteins. In particular, annexin A6 has been shown to bind to spectrin (Watanabe et al., 1994), actin (Hayashi et al., 1989; Hosoya et al., 1992) and to plasma membrane proteins that have C2 domains including PKC alpha (Schmitz-Peiffer et al., 1998; Orito et al., 2001) and p120GAP (Davis et al., 1996; Orito et al., 2001) for which the binding site on annexin A6 has been mapped (Chow and Gawler, 1999).

Here we have shown that annexin A6 copurifies with the cardiomyocyte sarcolemma. Far Western analysis indicated that annexin A6 binds to several sarcolemmal proteins. We have identified one of these as actin. We have also found that annexin A6 binds to itself. Annexin A6 is likely to regulate interactions of actin with the sarcolemma in response to intracellular variation in calcium levels; this may account for the observed physiological effects of annexin A6 depletion and overexpression in heart cells.

2. Materials and methods

2.1. Materials

Affinity-purified goat antibodies against annexin A6 were purchased from Santa Cruz Biotechnologies (Heidelberg, Germany). Mouse monoclonal antibodies against Na^+K^+ ATPase and the Na^+Ca^{2+}-exchanger were purchased from Affinity BioReagents (Cambridge, UK). A monoclonal antibody which recognises all actin isoforms (C4 clone) was purchased from Chemicon (Hampshire, U.K.). HRP-conjugated rabbit anti-goat IgG and HRP-conjugated goat anti-mouse IgG were from Pierce (Cheshire, UK). GST-annexin A6 was prepared using a plasmid which was a gift from Professor Stephen Moss (University College London). All other chemicals were purchased from Sigma–Aldrich Ltd. (Poole, Dorset, U.K.) or BDH Ltd. (Poole, Dorset, U.K.).

2.2. Isolation of sarcolemmal membranes and Triton X-100 insoluble cytoskeleton fractions

Porcine cardiac sarcolemmal membranes were isolated in buffers containing 1 mM calcium as previously described (Jones, 1988). All the buffers used contained 1 μg/ml each of the protease inhibitors leupeptin, aprotinin, and pepstatin and also included 0.2 mM PMSF. Protein concentrations were determined using bicinchoninic acid (BCA) reagent (Smith et al., 1985) and bovine serum albumin as a standard.

2.3. Electrophoresis and Western blotting

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970). Proteins were transferred from SDS-PAGE gels to nitrocellulose membrane, according to the method of Towbin et al. (1979). After staining with Ponceau S (0.1% (w/v) Ponceau S, 3% (v/v) acetic acid) to detect the quality of transfer, the nitrocellulose was incubated in TBS-Tween (10 mM Tris-HCl pH 7.4, 0.15 M NaCl, 0.1% (v/v) Tween-20) containing 5% (w/v) powdered milk for 1 h to prevent non-specific binding. Primary antibodies were diluted in TBS-Tween containing 1% (w/v) milk powder. Nitrocellulose blots were probed for 16 h with either a 1:500 dilution of goat anti-annexin VI, or 1:2000 dilutions of monoclonal antibodies to Na^+K^+ ATPase or GST in PBS containing 0.1% (v/v) Triton X-100. Bound IgG was detected with the appropriate HRP-conjugated second antibodies. Peroxidase activity was detected using the ECL detection system (Amersham, U.K.).

2.4. Quantitative analysis of antigen levels

To quantify levels of antigens from Western blots, dilutions of samples were analysed to ensure that responses were not saturated. In the case of annexin A6, purified recombinant annexin VI was used to construct standard curves to determine the amount of annexin A6 in various samples. Annexin A6 immunoreactivity was quantified by densitometry using NIH-Image 1.52.

2.5. Immunofluorescence microscopy

Immunofluorescence microscopy on rat heart cardiomyocytes was performed as described previously (Luckcuck et al., 1998; Messerli et al., 1993) using affinity-purified goat anti-annexin A6 (Santa Cruz) or a monoclonal antibody against vinculin followed by the appropriate FITC-labelled second antibodies. Pre-incubation of the anti-annexin A6 antibodies with the blocking peptide removed all immunoreactivity (not shown). Samples were viewed using a Leica laser confocal scanning microscope.

2.6. Far-Western assay for annexin VI binding proteins

Recombinant GST-annexin A6 and annexin A6 were prepared as described previously (Davis et al., 1996). Proteins in samples of heart homogenate and purified sarcolemma were separated by SDS-PAGE were transferred onto nitrocellulose and blocked in 5% (w/v) PBS milk for 1 h. Blots were incubated with 10 μg/μl annexin A6, GST or GST-annexin A6 in PBS (140 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.8 mM KH_2PO_4) containing 0.5% skimmed milk for 1 h at 25 °C.