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Original article

Association of annexin A5 with Na^+/Ca^{2+} exchanger and caveolin-3 in non-failing and failing human heart

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Abstract

Annexin A5 is a Ca^{2+} dependent phosphatidylserine binding protein mainly located in the T-tubules and sarcolemma of cardiomyocytes. Our objectives were to determine whether annexin A5 was associated with various protein(s) and whether such an association was modified in failing (F) hearts. The association between annexin A5 and the cardiac Na^+/Ca^{2+} exchanger (NCX) was demonstrated by immunohistofluorescence, annexin A5-biotin overlay and co-immunoprecipitations (IPs) performed with microsomal preparations (MPs) from non-failing (NF) (n = 8) and F (dilated cardiomyopathy, n = 7) human hearts. We moreover found caveolin-3 in the immunoprecipitates, indicating the presence of multimolecular subsarcolemmal complexes. Surface plasmon resonance assays in NF MPs allowed us to demonstrate direct interaction between the NCX and caveolin-3 and immobilized annexin A5. Interaction was Ca^{2+}-dependent and inhibited by the specific antibody. In addition, dissociation by zwittergent 3-14 (ZW 3-14) of the complexes from MPs increased specific interactions. In F hearts, specific interactions were blunted in native MPs but were fully recovered after treatment with ZW 3-14. In conclusion, we demonstrated that a direct interaction between annexin A5 and the cardiac NCX occurs in complexes including caveolin-3. In F hearts, despite the increase in the exchanger level, almost all of the NCX was involved in complexes. These interactions probably occurred in the intracytoplasmic regulatory loop of the exchanger, suggesting a different regulation of the exchanger in heart failure, consistent with a role in altered Ca^{2+} handling.

Keywords: Annexin A5; Na^+/Ca^{2+} exchanger; Caveolin-3; Non-failing and failing human heart; Surface plasmon resonance

1. Introduction

Annexins are a family of calcium-dependent phospholipid-binding proteins [1,2]. They share a conserved core domain with repeating structures of 70–80 amino acid residues that contain Ca^{2+} binding sites. Annexins are involved as mediators in Ca^{2+} signalization, signal transduction, membrane trafficking and organization [3–5]. Recently, the role of annexins as organizers of membrane macromolecular domains and their association with caveolins have been evidenced [6,7]. In addition, in cardiac muscle annexins A6 and A7 have been suggested as modulators of Ca^{2+} homeostasis. Annexin A6 overexpression in transgenic mice induced dilated cardiomyopathy associated with decreases in contractility and intracellular Ca^{2+} levels [8] whereas in annexin A6-null-mutant mice an increase in myocyte contractility and faster Ca^{2+} removal has been reported [9]. Like annexin A6, annexin A7, known mainly as a skeletal muscle annexin, has been suggested as a regulator of Ca^{2+} handling proteins in the heart because of a decrease in the force-frequency relationship in annexin A7 null mutant mice [10].

Among annexins, annexin A5 is well known for its high affinity for phosphatidylserine (PS) and formation of complexes with cytoskeletal and extracellular matrix components. Depending on Ca^{2+} concentration it was reported to be translocated to nuclear or plasma membrane and to inhibit

Abbreviations: EDTA, ethylenediaminetetraacetic acid; F, failing; HBS-P, Hepes buffer salin-surfactant P20; IP, immunoprecipitation; MP, microsomal preparation; NCX, Na^+/Ca^{2+} exchanger; NF, non-failing; PS, phosphatidylserine; RU, resonance unit; SERCA2a, sarco/endoplasmic reticulum Ca^{2+}-ATPase isoform 2a; SPR, surface plasmon resonance; ZW 3-14, zwittergent 3-14.

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protein kinase C and PLA2 activities [3,4]. In the myocardium, annexin A5 is an abundant protein localized mainly in T-tubules and sarcolemma of myocytes [11–14]. Based on similarities with the properties of the annexin family, we first hypothesized that annexin A5 may be able to interact with Ca^{2+} handling proteins involved in Ca^{2+} cycling and therefore play a role in Ca^{2+} homeostasis. Because annexin A5 ar $Ca^{2+}$is translocated to sarcolemma and interstitial tissue under pathophysiological conditions where the intracellul level is altered, such as in failing (F) human hearts [14,15], and is externalized during myocyte apoptosis [16], we then hypothesized that interaction of annexin A5 with other protein(s) could be modified and play an important role during heart failure. Therefore, our aim was first to identify the annexin A5-binding protein(s) then to investigate whether this complex might be modified during heart failure. By using overlay experiments, immunoprecipitation (IP) and surface plasmon resonance (SPR) we identified a trimolecular complex consisting of the Na^+/Ca^{2+} exchanger (NCX) as the Ca^{2+} handling protein associated with annexin A5 and of caveolin-3, which has been previously reported by Bossuyt et al. [17] to be associated with NCX in heart extracts. Taken together, our results suggest that annexin A5, like caveolin-3 [17], binds to the regulatory intracytoplasmic loop of NCX. Furthermore, our data showed an increased association of NCX with annexin A5 and/or caveolin-3 in F human hearts.

2. Materials and methods

2.1. Patients

Studies were performed with left ventricular myocardium (LV) from end-stage F hearts from patients with idiopathic dilated cardiomyopathies (n = 7). They had been presenting increases in LV mass (460 ± 70 g) and had a decreased LV ejection fraction (14 ± 5%). Non-failing (NF) LV was obtained from brain-dead organ donors that could not be used for transplantation for technical reasons (n = 7). This study was approved by the Consultative Committee for the Protection of Human Subjects in Biomedical Research at the Pitié-Salpétrière Hospital (Paris, France).

2.2. Microsomal preparations (MPs) and solubilization by CHAPS and zwittergent 3-14 (ZW 3-14)

MPs were obtained from 0.3 g tissue according to Mansier et al. [18]. MP were solubilized with 3% CHAPS in 1 mol/l NaCl, 2 mmol/l $CaCl_2$, 1 mmol/l DTT, 20 mmol/l Tris–HCl pH 7.4 for 1 hour at 4 °C or with 2% ZW 3-14 in 100 mmol/l NaCl, 30 mmol/l Imidazole pH 6.8, 8% sucrose for 15 min at RT. Detergent-solubilized proteins were recovered after centrifugation for 5 min (airfuge centrifuge, Beckman).

2.3. NCX antibodies

In this study, two monoclonal NCX antibodies were used: 1) C2C12 raised against the intracellular cytoplasmic loop (a.a 371–525) of dog cardiac NCX (Alexis Biochemicals) and 2) 300–127 raised against the N-terminal end of rabbit cardiac NCX (Abcam). First, we found that both antibodies can be used for Western blots at 1/1000 dilutions. Second, for IP, Abcam antibody was preferred to C2C12 antibody because NCX intracellular loop is a preferential site of interaction with other proteins [17,19]. Third, in SPR assays, to prevent interaction between NCX and immobilized annexin A5, C2C12 antibody was preferred to Abcam antibody because interaction might only involve intracellular NCX sites. Interestingly and in agreement with such an assumption, we found that Abcam antibody did not inhibit interaction between MP and immobilized annexin A5.

2.4. Western blot analysis

Proteins were subjected to SDS-PAGE and blotted to PVDF membrane (Amersham). Annexin A5 was revealed as previously described in [14]. Monoclonal anti-caveolin-3 (Becton Dickinson France) was used at 1/1000 dilution. Immunoreactive bands were visualized by enhanced chemoluminescence (ECL+, Amersham), quantified by Biorad gel-Doc 1000 scanning and normalized to Coomassie blue stained proteins.

2.5. Annexin A5 overlay

MP were subjected to 7.5% SDS-PAGE and blotted to PVDF membrane. They were incubated in presence of 1.8 mmol/l $CaCl_2$ or 1 mmol/l ethylenediaminetetraacetic acid (EDTA) with 1 μg/ml biotinylated annexin A5 (Boehringer–Mannheim) in 10 mmol/l Tris buffer pH 7.5, 150 mmol/l NaCl and 0.1% Tween 20 containing 5% milk for 12 hours at 4 °C then with Streptavidin-peroxydase. Immunoreactive bands were visualized and quantified as described above.

2.6. Immunohistofluorescence

Cryostat sections (7–8 μm) were treated as previously described in [14] with polyclonal anti-annexin A5 antibodies and with monoclonal C2C12 anti-NCX antibody. Fluorescence was visualized using a Leitz DM RD microscope equipped with epifluorescence optics.

2.7. IP

Anti-annexin A5, anti-NCX and anti-caveolin-3 IPs were performed with CHAPS- or ZW 3-14 solubilized MP (400 μg) according to Zhang et al. [20] either with polyclonal anti-annexin A5 antibodies (1/5000) and 50 μl of protein A Sepharose beads, or with anti-NCX (300-127) or anti-caveolin-3 monoclonal antibodies and 50 μl of anti-mouse IgG/agarose beads (Sigma, Ref: A-6531). Specificity of the assays was tested in absence of primary antibodies.

2.8. Monitoring of binding interactions by SPR

A BIAcore X SPR-based biosensor system (BIACORE AB, Uppsala, Sweden) was used to determine the interac-