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Research Article

Isoforms of protein 4.1 are differentially distributed in heart muscle cells: Relation of 4.1R and 4.1G to components of the Ca^{2+} homeostasis system

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ABSTRACT

The 4.1 proteins are cytoskeletal adaptor proteins that are linked to the control of mechanical stability of certain membranes and to the cellular accumulation and cell surface display of diverse transmembrane proteins. One of the four mammalian 4.1 proteins, 4.1R (80 kDa/120 kDa isoforms), has recently been shown to be required for the normal operation of several ion transporters in the heart (Stagg MA et al. Circ Res, 2008; 103: 855–863). The other three (4.1G, 4.1N and 4.1B) are largely uncharacterised in the heart. Here, we use specific antibodies to characterise their expression, distribution and novel activities in the left ventricle. We detected 4.1R, 4.1G and 4.1N by immunofluorescence and immunoblotting, but not 4.1B. Only one splice variant of 4.1N and 4.1G was seen whereas there are several forms of 4.1R. 4.1N, like 4.1R, was present in intercalated discs, but unlike 4.1R, it was not localised at the lateral plasma membrane. Both 4.1R and 4.1N were in internal structures that, at the level of resolution of the light microscope, were close to the Z-disc (possibly T-tubules). 4.1G was also in intracellular structures, some of which were coincident with sarcoplasmic reticulum. 4.1G existed in an immunoprecipitable complex with spectrin and SERCA2. 80 kDa 4.1R was present in subcellular fractions enriched in intercalated discs, in a complex resistant to solubilization under non-denaturing conditions. At the intercalated disc 4.1R does not colocalise with the adherens junction protein, β-catenin, but does overlap with the other plasma membrane signalling proteins, the Na/K-ATPase and the Na/Ca exchanger NCX1. We conclude that isoforms of 4.1 proteins are differentially compartmentalised in the heart, and that they form specific complexes with proteins central to cardiomyocyte Ca^{2+} metabolism.

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Introduction

Interactions between the cytoskeleton and the plasma membrane play central roles in determining the resilience of membrane structures to mechanical stress and also in organising signalling systems. A key role has emerged in recent years for adaptor proteins that join cross-linked cortical actin filaments to transmembrane proteins. Among the F-actin cross-linkers on the cytoplasmic face of the membrane is spectrin, an elongated tetrameric protein [1,2]. A general model for spectrin–actin complexes is that they are joined to the plasma membrane either by direct interaction with the lipid bilayer [3–5], or through two key adaptor proteins, ankyrin and protein 4.1 [1,2]. Ankyrin has attracted particular interest because the products of two ankyrin genes, ankyrinB and ankyrinG, are associated with human heart disease and they control the distributions and activities of signalling proteins central to regulation of heart beating: the Na^+ channel NaV1.5, the Na^{2+}/Ca^{2+} exchanger NCX1, Na^+/K^+-ATPase and inositol trisphosphate receptor [6,7].

Protein 4.1, by contrast, is less well characterised in the heart. Vertebrates have four genes encoding protein 4.1; the corresponding proteins are 4.1R, 4.1G, 4.1B and 4.1NN [2]. They have a common domain structure: an N-terminal FERM domain, a central spectrin-actin binding domain (SAB) and a C-terminal domain (CTD). The FERM domain binds various membrane proteins (certain ion channels, cell adhesion molecules and proteins carrying blood group antigens), calmodulin and PIP_2 [8–12]. The SAB domain binds spectrin and actin [13–16]. In the brain the CTD binds a number of G-protein coupled receptors and ligand gated ion channels [e.g. 12,17–19]. At the N-terminal of the protein is a variably spliced "headpiece" (U1 domain) that binds calmodulin and regulates the activity of the FERM domain [20]. Between the SAB and CTD is a U3 region that is unconserved between each of the four proteins. All of the 4.1 proteins are subject to extensive differential mRNA splicing [21].

In previous work, we detected differentially spliced mRNAs for all four in mouse heart, but only 4.1R, 4.1G and 4.1N mRNA in human heart [22]. Of the four proteins, only 4.1R has been localised [22,23]. It is present on the cytoplasmic face of the entire cardiomyocyte plasma membrane, including intercalated discs. It is also in a striated internal array that probably corresponds to T-tubules.

Recently, we investigated the phenotype of 4.1R transgenic mice [23]. In these mice, the first two initiation codons AUG1 and AUG2 [24] had been knocked out [25]. A third initiation codon (AUG3) exists within the FERM domain that is used if AUG1 and AUG2 are removed by mRNA splicing [26,27]. In the transgenic mice (here called 4.1R(AUG1 + 2) null) AUG3 was still present, and a corresponding 50 kDa 4.1R protein was expressed in the heart [23]. The hearts displayed phenotypic alterations, namely bradycardia and an extended ECG Q–T period. In electrophysiology, numerous ion channel activities were altered, including those associated with NaV1.5 and NCX1. The total heart content of NaV1.5 was also reduced. Thus, like ankyrin, protein 4.1 controls the cellular accumulation of NaV1.5. In the 4.1R(AUG1 + 2) null, protein 4.1G was upregulated, possibly as a compensatory mechanism.

In this report, we have characterised further the 4.1 proteins in heart in relation to 4.1R. We find that the three 4.1 proteins common to human and mouse heart (4.1R, 4.1G and 4.1N) each have characteristic membrane associated distributions. We provide evidence for interaction with specific membrane proteins which, taken together with previous data, suggest that 4.1 proteins in heart are an intimate part of the systems that control ion transport, and, in particular, the Ca^{2+} homeostasis system.

Materials and methods

Animals

For all experiments, other than those with 4.1R-deficient mice, mouse material was from strain Balb/c. Adult cardiomyocytes were isolated as described by Gong et al. [28]. 4.1R-deficient mice, generated by homologous recombination [25], and their controls (C57bl/6) were used as described previously [23].

Antibodies

Antibodies to the four mammalian 4.1 proteins [23,29,30], βIIΣ1-, βIIΣ2-spectrin [31] and αII-spectrin [32,33] have been described and characterised elsewhere. In addition, rabbit polyclonal antibodies to exon 4 of 4.1R were kindly supplied by Dr N. Mohandas (New York Blood Center, New York, USA). A detailed list is given in Table 1S, Supplementary Information. Monoclonal anti-SERCA2 antibody (MA3-919), anti-Na^+/K^+-ATPase α1 subunit (MA3-929) were obtained from Affinity Bioreagents (Cambridge BioScience, Cambridge, UK). Mouse monoclonal anti-vinculin (V4505) was from Sigma (Poole, UK) and anti-NCX1 (R3F1) from Swant (Switzerland). Monoclonal anti-titin antibody directed against the I19 domain which marks the I-band immediately adjacent to the Z-line, was a gift from Dr Mathias Gautel (King's College, London). Rabbit polyclonal anti-β-catenin (C2206) was from Sigma and Mouse monoclonal anti-β-catenin (610154) was from BD Transduction Laboratories (Oxford, UK). Mouse monoclonal anti-connexin43 (mab3067) was from Millipore (Watford, UK). Mouse polyclonal anti-ZASP [34] was kindly given by Dr Georgine Faulkner (Padua, Italy).

Preparation of heart tissue homogenates

Mice were euthanised with CO_2. After dissection from the animal, the heart was finely chopped. In some cases to obtain tissue largely free of erythrocytes, the exposed beating heart was perfused with Ringer saline solution. The tissue was homogenised with 2–3 volumes of lysis buffer (100 mM NaCl, 50 mM Tris, 1 mM EGTA pH 7.4, containing 1% SDS, 1% Triton X-100, 1 mM phenylmethylsulphonylfluoride (PMSF) and a cocktail of protease inhibitors (PI; supplied by Sigma), leupeptin, chymostatin, aprotinin, pepstatin, antipain, bestatin, all used at a final concentration of 1 μg ml^{-1}.

Electrophoresis and immunoblotting

Tissue or cell homogenates as indicated in the text were solubilised for electrophoresis by addition of 2× SDS-PAGE sample buffer [35]. Proteins were either stained with Coomassie Brilliant Blue R or electrophoretically transferred to nitrocellulose for immunoblotting [23].