The Cardiac Na\(^{+}\)-Ca\(^{2+}\) Exchanger Binds to the Cytoskeletal Protein Ankyrin*

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Na\(^{+}\)-Ca\(^{2+}\) exchange is the major pathway of Ca\(^{2+}\) efflux during excitation-contraction coupling in cardiac muscle. The Na\(^{+}\)-Ca\(^{2+}\) exchanger is present in cardiac transverse tubules with an apparent high density (Frank, J. S., Mottino, G., Reid, D., Molday, R. S., and Philipson, K. D. (1992) J. Cell Biol. 117, 337-345). The mechanism for this localization is unknown but may involve interactions with the cytoskeleton. In the present study, we examined the interaction of the Na\(^{+}\)-Ca\(^{2+}\) exchanger with the cytoskeletal protein ankyrin. On immunoblots of isolated canine cardiac sarcolemma, an antibody raised against purified rabbit red blood cell-ankyrin (RBC-ankyrin) recognized a 220-kDa protein, which is the same size as RBC-ankyrin. Alkaline extraction of sarcolemma removed this protein. The Na\(^{+}\)-Ca\(^{2+}\) exchange protein, purified from recombinant baculovirus-infected insect cells, bound \(^{125}\)I-labeled-RBC-ankyrin with a \(K_d\) of 42 ± 3 nm. \(^{125}\)I-RBC-ankyrin was co-precipitated by antibodies to the Na\(^{+}\)-Ca\(^{2+}\) exchanger after preincubation with solubilized cardiac sarcolemma. Myocardial ankyrin could be localized to both surface and T-tubular sarcolemma by immunofluorescence techniques. These results demonstrate that the cardiac Na\(^{+}\)-Ca\(^{2+}\) exchanger binds ankyrin with high affinity. This interaction may be important for localizing the Na\(^{+}\)-Ca\(^{2+}\) exchanger to specific domains of the sarcolemma.

During excitation-contraction coupling of cardiac myocytes, transsarcolemmal Ca\(^{2+}\) influx through Ca\(^{2+}\) channels triggers Ca\(^{2+}\)-induced Ca\(^{2+}\) release from the SR to initiate contraction.

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The abbreviations used are: SR, sarcoplasmic reticulum; Caps, 3-cyclobexylaminol-1-propanesulfonic acid; Mops, 3-N-morpholino-propanesulfonic acid, PBS, phosphate-buffered saline; PVDF, polyvinylidine difluoride; RBC-ankyrin, red blood cell ankyrin; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin.
pl of antibodies with 100 pl of a 50% suspension of protein A-agarose for 1 h at room temperature followed by five washes with 140 mM NaCl and 10 mM Mops, pH 7.4. The immunoprecipitation reaction was allowed to proceed for 1 h at room temperature, and the agarose beads were then washed five times as above. The washed beads were then assayed for bound 125I-ankyrin.

**Immunofluorescence**—Isolated rat and guinea pig myocytes were prepared as described previously (Frank et al., 1992). The cells were fixed for 10 min in 1.5% buffered formaldehyde, quenched in 0.2% sodium borohydride, and then permeabilized by a 10-min exposure to 0.1% Triton X-100. Subsequently, the cells were incubated with 5% goat serum and 3% BSA in PBS solution for 45 min and then incubated in primary antibodies for 90 min. After four changes in PBS/BSA the cells were incubated for 1 h in fluorescence-labeled goat anti-rabbit antibody. The cells were washed several times in PBS and mounted on slides with mounting medium (90% glycerol, 2% 1,4-diazabicyclo-(2,2,2)-octane, and anti-bleaching agents).

**RESULTS**

**Ankyrin in Cardiac Sarcolemma**—Polyclonal antibodies raised against purified rabbit RBC-ankyrin reacted with cardiac sarcolemma on immunoblots. The antibodies recognize a sarcolemmal protein at ~220 kDa, the same size as purified rabbit RBC-ankyrin (Fig. 1). The antibodies also react weakly with a band at ~90 kDa. Cytoskeletal proteins can be removed from membranes by alkaline extraction (Steck and Yu, 1973). Treatment of sarcolemma with 10 mM Caps at pH 12 completely removed the 220-kDa protein band (Fig. 1). Fig. 2 shows immunofluorescent labeling of an isolated rat myocyte stained with the anti-RBC-ankyrin antibody. The immunolabeling occurs in a striation pattern that is consistent with localization at the transverse tubular membrane. Labeling appears to be less intense at the peripheral sarcolemma except at the intercalated discs, where labeling is intense. No labeling was seen using nonimmune antibodies. Similar results were seen with isolated guinea pig myocytes. These results demonstrate the presence of ankyrin in cardiac myocytes.

**Association of the Na"+-Ca"+ Exchanger with Ankyrin**—Na"+-Ca"+ exchanger protein was purified from recombinant baculovirus-infected Sf9 cells (Li et al., 1992). A 115-kDa protein band was eluted using a Bio-Rad Prep Cell and dialyzed against 10 mM NaCl, 10 mM Mops. The Na"+-Ca"+ exchanger protein was used to precipitate a Na"+-Ca"+ exchanger antibody with 100 mM NaCl, 100 mM Mops. The Na"+-Ca"+ exchanger was recognized strongly by anti-Na"+-Ca"+ antibodies (data not shown). Fig. 3A shows that 125I-RBC-ankyrin bound to the Na"+-Ca"+ exchanger protein with a KD of 41.6 ± 2.5 nM (n = 3). The nonspecific binding of 125I-RBC-ankyrin to the PVDF membrane was low (Fig. 3A). The binding of 125I-RBC-ankyrin to the Na"+-Ca"+ exchanger can be displaced by unlabeled ankyrin (Fig. 3B).

**Co-precipitation of Ankyrin with the Na"+-Ca"+ Exchanger**—The Na"+-Ca"+ exchanger of cardiac sarcolemma can be co-precipitated with anti-Na"+-Ca"+ exchanger polyclonal antibody (Philipson et al., 1988). After preincubation with solubilized cardiac sarcolemma, Na"+-Ca"+ ankyrin was co-precipitated by the polyclonal antibody. Ankyrin was not precipitated when nonimmune serum was used or when sarcolemma was absent (Fig. 4). Monoclonal antibody (C2C12) to the N2 Ca"+ exchanger (Frank et al., 1992) was also able to immunoprecipitate the ankyrin-Na"+-Ca"+ exchanger complex (not shown).

**DISCUSSION**

The Na"+-Ca"+ exchanger plays an important role in excitation-contraction coupling in cardiac muscle. The Na"+-Ca"+ exchanger is clearly the dominant Ca"+ efflux mechanism of myocardial cells (Bridge et al., 1990). A possible additional role in Ca"+ influx is controversial (Leblanc and Hume, 1990; Lederer et al., 1990; Sham et al., 1992). Applying rapid perfusion techniques to cultured cardiac myocytes, Langer and Rich (1992) have defined an intracellular Ca"+ compartment dependent upon Na"+-Ca"+ exchange. They suggested that this Ca"+ containing compartment is located in the diadic region of the myocytes. This model implies that exchange occurs across the sarcolemma of the transverse tubule. Frank et al. (1992) have
used antibodies to the cardiac Na\(^{+}\)-Ca\(^{2+}\) exchanger to immunolocalize the exchanger in isolated cardiac myocytes. The results showed strong labeling of the transverse tubular membrane and patchy labeling of the peripheral sarcolemma. It may be of much functional importance that the Na\(^{+}\)-Ca\(^{2+}\) exchanger is abundantly located in sarcolemma closest to the release sites of Ca\(^{2+}\). A second immunolocalization study, however, has described a different fluorescence pattern with a more uniform distribution of exchangers in the sarcolemma (Kieval et al., 1992). The mechanism for possibly localizing the exchanger to specific regions of the sarcolemma is unknown but would be likely to involve interactions with the cytoskeleton.

The cytoskeleton plays a major role in the regional localization of membrane proteins. Ankyrin is a peripheral membrane protein that links integral membrane proteins to other elements of the cytoskeleton. The present study demonstrates that the cardiac Na\(^{+}\)-Ca\(^{2+}\) exchanger binds to ankyrin with high affinity. This was shown by direct binding and by immunoprecipitation experiments. The interaction of the Na\(^{+}\)-Ca\(^{2+}\) exchanger with ankyrin may be responsible for any special localization of the exchanger. Flucher et al. (1990) reported that in skeletal muscle, the α1 and α2 subunits of the dihydropyridine receptor were co-localized in the triad junction with ankyrin. They suggested that the ankyrin might be involved in this organization of the triad. Using anti-RBC-ankyrin antibodies, we demonstrate the presence of ankyrin in cardiac sarcolemma by immunoblot and immunofluorescent techniques. The ankyrin is widely distributed in the sarcolemma but may be at higher levels in the transverse tubules. Such a distribution would be similar to what we observed previously for the distribution of the Na\(^{+}\)-Ca\(^{2+}\) exchanger (Frank et al., 1992). Its specific role in sarcolemmal organization needs to be defined in greater detail. Ankyrin is coded by multiple genes and several isoforms exist (Lambert et al., 1990; Otto et al., 1991). The molecular details of the cardiac form of ankyrin has not been resolved.

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