J. Membrane Biol. 118, 279–283 (1990)

The Journal of
Membrane Biology

Identification of the Cardiac Sarcolemmal Na^+-Ca^{2+} Exchanger Using Monoclonal Antibodies

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Summary. We have previously partially purified the sarcolemmal Na^+-Ca^{2+} exchange protein and produced rabbit polyclonal antibodies to the exchanger (Philipson, K.D., Longoni, S., Ward, R. 1988. *Biochim. Biophys. Acta* **945**:298–306). We now describe the generation of three stable murine hybridoma lines which secrete monoclonal antibodies (MAb's) to the exchanger. These MAb's immunoprecipitate 50–75% of solubilized Na^+-Ca^{2+} exchange activity. The MAb's appear to be reactive with native conformation-dependent epitopes on the Na^+-Ca^{2+} exchanger since they do not react on immunoblots. An indirect method was used to identify Na^+-Ca^{2+} exchange proteins. A column containing Na^+-Ca^{2+} exchanger immobilized by MAb's was used to affinity purify the rabbit polyclonal antibody. The affinity-purified polyclonal antibody reacted with proteins of apparent molecular weights of 70, 120, and 160 kDa on immunoblots of sarcolemma. The data provide strong support for our previous association of Na^+-Ca^{2+} exchange with these proteins.

Key Words Na^+-Ca^{2+} exchange · cardiac sarcolemma · monoclonal antibodies · polyclonal antibodies · apparent molecular weights

Introduction

The Na^+-Ca^{2+} exchange system of cardiac sarcolemma is a highly active transporter and is important in the Ca^{2+} movements which accompany cardiac excitation-contraction coupling. The properties of Na^+-Ca^{2+} exchange have been examined in detail using isolated membrane vesicles (for a review, *see* ref. [17]). The exchanger protein is present in sarcolemma in low abundance [4, 15], and no specific marker for the exchanger is available other than transport. The cardiac exchanger has been difficult to isolate to homogeneity, since transport function must be maintained throughout membrane solubilization, protein fractionation, and membrane reconstitution for assay of Na^+-Ca^{2+} exchange activity.

Nevertheless, several candidate proteins have been proposed. These include proteins with molecular weights of 33 [13, 18], 82 [10], and 220 kDa [9]. We have recently suggested that proteins of 70 and 120 kDa are associated with sarcolemmal Na^+-Ca^{2+} exchange activity [15]. The 70-kDa protein may be an active proteolytic fragment of the 120-kDa protein. On nonreducing gels, only one band was present at a molecular weight of 160 kDa [15]. Additionally, the exchanger has been isolated from brain [2, 3] and rod outer segments [5, 14] with reported apparent molecular weights of 70 and 220 kDa, respectively. Antibodies to the 220-kDa exchanger protein of rod outer segments demonstrate weak immunoreactivity with the 70- and 120-kDa bands of canine cardiac sarcolemma (K.D. Philipson, N.J. Cook, and R.S. Molday, *unpublished observations*).

We report here on the production of MAb's[1] to the cardiac Na^+-Ca^{2+} exchange protein and use these hybridoma antibodies to further support the association of Na^+-Ca^{2+} exchange activity with the 70-, 120-, and 160-kDa proteins.

Materials and Methods

MAb's and ascites fluid were produced using previously described protocols [7, 8, 12]. For immunization, about 1.5 μg of purified Na^+-Ca^{2+} exchange proteins from canine cardiac sarcolemma [15] were emulsified with Freund's complete adjuvant and injected subcutaneously at multiple sites in the backs of two male BALB/c mice. Two weeks later, this protocol was repeated using Freund's incomplete adjuvant. After three more weeks, antigen (0.15 μg in 0.1 ml 140 mM NaCl) was injected into the tail vein. On the third day, the mice were sacrificed and spleens were removed. Hybridoma cells were derived from polyethylene-in-

[1] *Abbreviations:* MAb = monoclonal antibody; RIA = radioimmunoassay; SDS-PAGE = sodium dodecylsulfate-polyacrylamide gel electrophoresis; PBS = phosphate-buffered saline; and BSA = bovine serum albumin.

duced fusion of murine myeloma S194/5.XX0.Bu-1 with the splenic cells (1 : 10). Cells were plated in 96-well microtiter plates in selective medium, screened for antibody production after 14 days, and cloned by limiting dilution.

Solid-phase radioimmunoassays were conducted in flexible, round bottom polyvinylchloride microtiter plates (Costar). The wells were coated with canine heart sarcolemma (5 μg protein in 50 μl) for 2 hr at 37°C. The wells were postcoated for 10 min with PBS containing 3% BSA (PBS/BSA) and washed three times with PBS/BSA. For assay, culture supernatant (50 μl) was added and allowed to react 18 hr at 4°C. After washing the wells with PBS/BSA, antibody binding was detected by a 6-hr incubation at 37°C with ^{125}I-labeled sheep antibody to murine immunoglobulin in PBS/BSA (Amersham, 50 μl of 0.8 μCi/ml). After washing, the radioactivity in individual wells was determined by gamma counting. Isotyping was done using the ScreenType test kit (Boehringer Mannheim).

The immunoprecipitation assay was performed as follows: 50 μl of protein A Sepharose (Sigma) or murine anti-IgG agarose (Sigma) was incubated with 1.0 μl of ascites fluid in 0.5 ml MAPS II Binding Buffer (Bio-Rad) for 60 min at room temperature. The ascites fluid was omitted for controls. The beads were washed five times with MAPS buffer and 75 μl of solubilized sarcolemma was added for 15 min at 4°C. (The sarcolemma was solubilized by adding 37.5 μl of 140 mM NaCl, 10 mM MOPS/Tris (pH 7.4), and 20 mM decylmaltoside to 37.5 μl of sarcolemma (3 mg/ml) and centrifuging (Beckman Airfuge, 10 min) to remove nonsolubilized material.) After the 15-min incubation, the beads were spun and the supernatant was reconstituted for assay of Na^+-Ca^{2+} exchange activity [15]. Reconstitution, as described previously [15], was performed by adding one-quarter volume of 50 mg/ml asolectin, 7.5% Triton X-100, 2.5 M NaCl, 100 mM MOPS/Tris (pH 7.4) and using Bio-Beads SM-2 (Bio-Rad) to remove detergent. In preliminary experiments, anti-IgM beads were of equal efficacy to either the anti-IgG or protein A beads.

In some experiments using anti-IgG agarose, the immobilized MAb/exchanger complex in the pellet, after removal of supernatant for reconstitution, was washed five times with MAPS buffer and then incubated with 1.5 μl of rabbit polyclonal antibody [15] in 0.5 ml of MAPS buffer for 60 min at room temperature. The beads were washed five times and bound antibodies were eluted with 0.1 M glycine (pH 2.5). The eluted material was neutralized and used for reactions with immunoblots.

Sarcolemmal proteins were separated by SDS-PAGE (7.5%) and transferred onto nitrocellulose for 30 min at 100 V in a Bio-Rad Mini Trans-Blot apparatus. Prior to SDS-PAGE, the sarcolemma was extracted with an alkaline solution (pH 12) to remove extrinsic membrane proteins and to reduce the amount of nonexchanger protein [16]. Each nitrocellulose strip used for immunoreactions contained about 10 μg of protein. Immune complexes were detected using goat anti-rabbit IgG conjugated to horseradish peroxidase with 3,3'-diamino-benzidine as substrate [11].

Results

MAb's were produced from mice immunized with our preparation [15] of purified sarcolemmal Na^+-Ca^{2+} exchange proteins. Positives were initially selected by the ability of cell culture supernatants to react with crude sarcolemma using solid-phase RIA. The immunoglobulin products secreted by three of these hybridomas were subsequently found to immunoprecipitate Na^+-Ca^{2+} exchange activity (*see below*). These three hybridomas were subcloned by limited dilution and are designated MAb's A10, F12, and H7. We had planned to initially select MAb's by their reaction on immunoblots to the 70- and 120-kDa bands of sarcolemma. However, none of the MAb's showed any immunoreaction on blots and we used the strategy of selection by immunoprecipitation. MAb's A10, F12, and H7 were all of the IgM subtype and, in general, showed identical characteristics.

Table 1. Immunoprecipitation of Na^+-Ca^{2+} exchange activity by immobilized monoclonal antibodies

MAb	Activity immunoprecipitated (%)	
	MAb immobilized with Protein A Sepharose	Anti-IgG Agarose
A10	74.2 ± 3.2	76.2 ± 7.4
F12	58.8 ± 7.2	44.1 ± 11.4
H7	72.5 ± 3.4	77.2 ± 5.8

Immobilized MAb was first prepared by specific antibody adsorption with protein A Sepharose or anti-murine IgG. Solubilized sarcolemma was incubated with the immobilized MAb and the Na^+-Ca^{2+} exchange activity remaining in solution was then reconstituted for Ca^{2+} transport assay. Controls were treated in the same manner except for the absence of MAb. Control Na^+-Ca^{2+} exchange activities of the reconstituted vesicles were 21.5 ± 1.5 and 24.3 ± 2.2 nmol Ca^{2+}/mg protein/sec when protein A Sepharose and anti-murine IgG agarose were used, respectively. *See* Materials and Methods for details. Data are means ± SEM. n = 4–13.

Failure to react on immunoblots demonstrates the sensitivity of the MAb's to epitope conformation. This sensitivity is also demonstrated by the reduced reactivity of the MAb's to sarcolemma after solubilization of the sarcolemma with detergents. We dotted onto nitrocellulose either native sarcolemmal vesicles or sarcolemma solubilized with Triton X-100, Chaps, or decylmaltoside at low concentrations (1%, 12.5 mM, or 10 mM, respectively). The immunoreactivity of the MAb's as detected visually by ELISA reactions was greatly reduced after solubilization (*not shown*). In contrast, the reactivity of the polyclonal antibody to the cardiac exchanger, which we previously described [13], was unaffected by detergent solubilization.

All three MAb's were able to immunoprecipitate Na^+-Ca^{2+} exchange activity as shown in Table 1. MAb F12 consistently immunoprecipitated about 50% of activity while MAb's A10 and H7 removed about 75% of activity. We found that MAPS buffer was necessary to facilitate the initial binding of the

Table 2. Binding of polyclonal antibodies blocks binding of MAb's to sarcolemma

Volume of polyclonal antibody prebound to sarcolemma (μl)	MAb bound (o.d.)
0	0.123
0.001	0.100
0.01	0.084
0.1	0.079
1.0	0.056
10.0	0.030

Microtiter wells were coated with sarcolemmal membranes (5 μg in 50 μl) for 2 hr at 37°C. Rabbit polyclonal antibodies (as indicated) were allowed to bind to the sarcolemma for 1 hr at 37°C. Murine MAb A10 (1 : 500 dilution of ascites fluid) was added for 1 hr at 37°C. Bound MAb was then quantitated using horseradish peroxidase-conjugated goat anti-murine IgG with 5-aminosalicylic acid as substrate. Wells were washed five times with PBS plus 1% BSA between each step. Results are from a single experiment though a second experiment gave almost identical results.

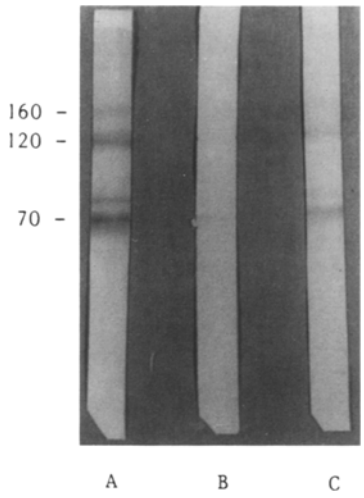

Fig. 1. Immunoreactions of affinity-purified polyclonal antibody to Na^+-Ca^{2+} exchanger. Lane *A*: Control in which polyclonal antibody (1 : 2000 dilution), before affinity purification, is reacted to blotted sarcolemmal proteins. Lanes *B* (control, no MAb) and *C*: Affinity-purified polyclonal antibody reacted with sarcolemmal proteins. *See* text and Materials and Methods for details

MAb's to either the protein A Sepharose or the anti-IgG agarose. Using a combination of the three MAb's did not increase the percentage of exchange activity which could be removed from solution. The experiments shown in Table 1 were conducted with 1.0 μl of ascites fluid though volumes as small as 0.1 μl of ascites fluid produced comparable results. In control experiments, no exchange activity was immunoprecipitated (n = 3) by a control MAb, MDAlys, an IgG_{2a} antibody which reacts with malondialdehyde-modified low density lipoprotein [8]. Also, MAb A10 did not immunoprecipitate Na^+,K^+-ATPase activity (n = 1).

We have previously developed rabbit polyclonal antibodies which immunoprecipitate Na^+-Ca^{2+} exchange activity [15]. Binding of polyclonal antibody to sarcolemma blocked subsequent binding of MAb to the sarcolemma (Table 2). This result is consistent with the epitope for the MAb's being on the Na^+-Ca^{2+} exchange protein.

Because the MAb's did not react with SDS-solubilized exchanger on immunoblots, we devised an alternative strategy to identify the Na^+-Ca^{2+} exchange protein. MAb's were immobilized with anti-mouse IgG agarose and the exchanger proteins in solubilized sarcolemma were bound to the immobilized MAb's. Next, rabbit polyclonal antibodies [15] were bound to the immobilized exchanger. The bound rabbit antibodies were eluted with glycine (pH 2.5), neutralized, and applied to immunoblots of sarcolemmal proteins using an anti-rabbit IgG secondary antibody for detection. Thus, only those polyclonal antibodies which had bound to protein immobilized by the murine antibodies were selected for immunoblot analysis. Essentially, the experiment is designed to produce affinity-purified polyclonal antibodies.

Figure 1 shows results from such an experiment. In Lane *A*, polyclonal antibodies were reacted directly with sarcolemmal proteins. The pattern is similar to that seen previously [15]. Lane *C* shows the immunoreaction of polyclonal antibodies after affinity purification by the MAb/exchanger column. A similar pattern is obtained with the major reactions appearing at 70 and 120 kDa. The result confirms the association of sarcolemmal proteins of 70 and 120 kDa with Na^+-Ca^{2+} exchange activity. Interestingly, the minor reaction bands of the polyclonal antibody at 74 and 160 kDa also appear to be associated with the exchanger. (The 160-kDa band is more clear in a similar experiment shown in Fig. 2, lane *F*.) Lane *B* is a parallel control in which MAb was omitted from the column. The control should correct for any nonspecific binding of polyclonal antibody to the column or for any polyclonal antibody which attached to nonspecifically bound proteins. Another control used MAb MDAlys, in place of MAb H7, and no binding of polyclonal antibody to this column could be detected (*not shown*).

Figure 2, lanes *D*, *E*, and *F* show an experiment identical to that in Fig. 1 with qualitatively similar results but showing variability that was encountered. For the control reaction with polyclonal antibody, the most intense band is at 120 kDa, rather than at 70 kDa; this variability may be due to minor differences in protein transfer conditions. The major

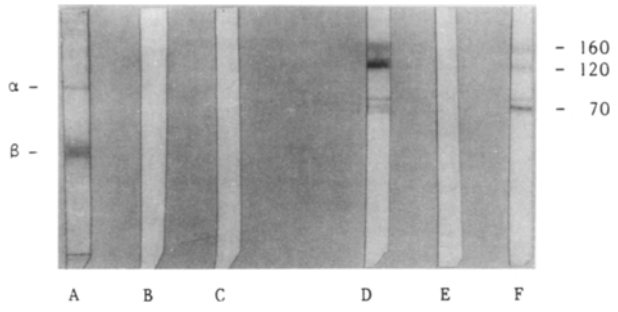

Fig. 2. Immunoreactions of affinity-purified polyclonal antibodies with sarcolemmal membrane proteins. Lanes *D*, *E*, and *F* represent an experiment identical to that shown in Fig. 1 using polyclonal antibody to the Na^+-Ca^{2+} exchanger. Lanes *A*, *B*, and *C* represent a control experiment in which polyclonal antibodies to Na^+K^+-ATPase were used in place of polyclonal antibodies to the Na^+-Ca^{2+} exchanger but using an otherwise identical protocol. Reactions of the Na^+,K^+-ATPase antibody (1 : 2500 dilution) with blotted sarcolemmal proteins are shown before (lane *A*) and after (lane *C*) processing by the MAb/exchanger column. Lane *B* represents a control, analogous to that in Fig. 1, lane *B*, in which no MAb was prebound to the anti-mouse IgG column. Lanes *B* (control, no MAb) and *C* show that no Na^+,K^+-ATPase antibody is artifactually affinity purified by the MAb/exchanger column

reaction of the affinity-purified polyclonal antibody (lane *F*) is again at 70 kDa with less intense reactions at 120 and 160 kDa. Figure 2, lanes *A*, *B*, and *C* show a control experiment in which a polyclonal antibody to Na^+-K^+-ATPase (courtesy of Dr. J. Kyte) was used in place of polyclonal antibody to the Na^+-Ca^{2+} exchanger. Lane *A* shows control reactions of the antibody to the α and β subunits of the Na^+-K^+-ATPase in sarcolemma. Lane *C* demonstrates that none of the Na^+-K^+-ATPase antibody was affinity purified by MAb/exchanger complexes. The data in Figs. 1 and 2 were obtained with MAb H7, though similar results were obtained with MAb A10.

Discussion

We have described three murine hybridoma antibodies which are able to immunoprecipitate Na^+-Ca^{2+} exchange activity. We assume the MAb's are directed against an epitope on the exchanger protein itself. The immunoreactive site appears to be conformationally dependent since the MAb's do not bind to immunoblots following SDS-PAGE and cannot be used for immediate identification of the Na^+-Ca^{2+} exchanger protein. Also, the reactivity of the MAb's to the exchanger decreases after membrane solubilization which may explain why only 50–75% of activity can be immunoprecipitated. The reduced reactivity after detergent solubilization limits the usefulness of the MAb's for direct purification of the exchanger protein by immunoaffinity chromatography.

We have previously proposed that sarcolemmal exchange activity is associated with 70- and 120-kDa proteins under reducing conditions. These proteins appear as a single band on nonreducing SDS-PAGE at 160 kDa [15]. The proteins were not isolated to homogeneity, however, and possibly Na^+-Ca^{2+} exchange activity was associated with a minor component in our preparation. The polyclonal antibody [15] to this preparation reacted with the 70- and 120-kDa proteins and immunoprecipitated exchange activity, but also reacted weakly with other protein bands (*see* Figs. 1*A* and 2*D*). In the present study, we affinity purify the polyclonal antibody and validate that, indeed, the 70-, 120-, and 160-kDa proteins are associated with Na^+-Ca^{2+} exchange activity. We also demonstrate the specificity of our polyclonal antibody for the exchanger proteins since the reactions of the affinity-purified polyclonal antibody with sarcolemmal proteins are identical to those of the unpurified antibody.

In summary, the data supporting association of cardiac Na^+-Ca^{2+} exchange activity with proteins of apparent molecular weights of 70, 120, and 160 kDa are as follows: (*i*) Our initial protein isolation and polyclonal antibody data [15]. (*ii*) The results presented here using monoclonal and polyclonal antibodies. (*iii*) The weak cross-reactivity of antibodies to the rod outer segment exchanger with the cardiac 70- and 120-kDa proteins. (*iv*) Preliminary data from another laboratory [1] also associates canine cardiac sarcolemmal activity with 70- and 120-kDa proteins.

Previously, we provided evidence that the 70-kDa protein was a proteolytic fragment of the 120-kDa protein. The 120-kDa protein itself may be derived from the 160-kDa protein due to the action of an endogenous protease. This would explain the continued presence of variable amounts of 160-kDa protein even under reducing conditions and is consistent with a preliminary report associating exchange activity with a 150-kDa sarcolemmal protein [6]. Experiments using polyclonal antibodies eluted off of specific sarcolemmal proteins following transfer of the proteins to nitrocellulose (ref. [15], and K.D. Philipson, *unpublished observations*) have demonstrated that the 70-, 120-, and 160-kDa proteins are immunologically related to one another. The detailed relationships of the exchanger proteins to one another are still not clear, but we are now confident that the 70-, 120-, and 160-kDa proteins are all associated with Na^+-Ca^{2+} exchange activity.

This research was supported by the U.S. Public Health Services grants HL 27821 (KDP) and HL 30568 (MEH), the Laubisch Foundation (MEH, KDP), and the American Heart Association,

Greater Los Angeles Affiliate (KDP). We thank L. Green for secretarial assistance.

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Received 8 March 1990; revised 25 May 1990

Note Added in Proof

Subsequent to submitting this article, we cloned the Na^+-Ca^{2+} exchange protein (Nicoll, D.A., Longoni, S., Philipson, K.D. 1990. *Science, in press*). Antibodies were prepared against a synthetic peptide with an amino acid sequence corresponding to a region of the exchanger. These antibodies confirm the association of 70-, 120-, and 160-kDa proteins with the Na^+-Ca^{2+} exchanger.