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Immunological Crossreactivity between the Retinal Na^+-Ca^{2+},K^+ and the Cardiac Na^+-Ca^{2+} Exchanger Proteins

Hartmut Porzig, Regula Gutknecht
Pharmakologisches Institut der Universität Bern, Friedbühlstr. 49, CH 3010 Bern, Switzerland

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Abstract. We have used a series of monoclonal antibodies (mAbs) to determine the degree of microscopic structural homology between the retinal Na^+-Ca^{2+},K^+ and the cardiac Na^+-Ca^{2+} exchange proteins. Sets of mAbs were raised separately to partially purified preparations of either the retinal or the recombinant myocardial exchanger. Each panel of mAbs was then screened for crossreactivity with the respective heterologous exchanger using enzyme-linked immunoassay and immunoblotting techniques. Out of 43 anti-retinal exchanger mAbs, we found 3 detecting the cardiac exchanger on immunoblots, while 4 out of 36 anti-cardiac exchanger mAbs reacted with the retinal exchanger. The strength of the crossreactions was generally weak and suggested that only low affinity epitopes were available on the heterologous proteins. For two crossreacting anti-retinal mAbs the apparent binding affinities to the cardiac exchanger were lower by more than two orders of magnitude. The overall low degree of epitope sharing among the two sets of mAbs confirms that in spite of their obvious functional and topological similarities, microscopic structural homologies between the two proteins are scarce.

Key words: Na^+-Ca^{2+} exchanger—Na^+-Ca^{2+},K^+ exchanger—Monoclonal antibodies—Epitope sharing—Immunological crossreactivity—Structural homology

Correspondence to: H. Porzig

Introduction

In many tissues sodium-calcium exchange across the cell membrane plays an important role in maintaining cellular calcium homeostasis (for review *see* DiPolo & Beaugé, 1989; Reeves, 1990, 1992). Retinal rods and cardiac myocytes are particularly rich in this transport activity and, therefore, have been used as primary sources for the isolation and characterization of the exchanger protein (Cook & Kaupp, 1988; Philipson, Longoni & Ward, 1988; Durkin et al., 1991). Detailed analysis has shown that the functional properties of the exchangers from these two tissues differ in several important aspects. Most characteristically, the retinal protein catalyzes an exchange of 4 Na ions for 1 Ca and 1 K ion, while only Ca^{2+} (and no K^+) is exchanged for Na^+ in a 1:3 ratio by the cardiac protein (Reeves & Hale, 1984; Cervetto et al., 1989; Schnetkamp, Basu & Szerencsei, 1989). Most recently, molecular cloning has revealed that the two proteins derive from different genes and show only very limited sequence homology (Nicoll, Longoni & Philipson, 1990; Reiländer et al., 1992). In spite of their differing primary sequences the two proteins show remarkably similar hydropathy plots. Moreover, the basic property of utilizing the sodium gradient as an energy source to mediate an energetically uphill electrogenic Ca transport is common to both proteins. Therefore, they may well share some biochemical and structural properties that are not obvious from primary sequence data. We have tested this hypothesis by studying the behavior of the two proteins during purification in the same chromatographic systems. Moreover, we have raised monoclonal antibodies

(mAbs[1]) against both the retinal and the cardiac exchanger in order to test crossreactivity. We report that the two proteins, in spite of their functional and structural similarities, seem to share surprisingly few epitopes. All crossreacting mAbs had a much higher affinity to the homologous than to the heterologous protein, suggesting a significant decrease in binding specificity.

Materials and Methods

Preparation of Partially Purified Retinal Na^+-Ca^{2+},K^+ Exchanger

Retinal rod outer segments (ROS) were prepared from dark-adapted bovine retinae as described by Schnetkamp and Daemen (1982) and stored at −25°C until used.

The exchanger protein was purified from ROS membranes essentially as described by Cook and Kaupp (1988). Purification was monitored by measuring Na^+-dependent $^{45}Ca^{2+}$ influx (*see below*) after reconstitution of the transport protein into asolectin vesicles. The vesicles were prepared by mixing the chromatographic fractions with equal volumes of CHAPS buffer A (700 mM NaCl, 20 mM $MgCl_2$, 20 mM EGTA, 6 mg/ml CHAPS, 10 mg/ml asolectin) and then dialyzing for 48 hr against a solution containing (mM) 160 NaCl, 20 MOPS, 0.1 DTT pH 7.4. The transport activity copurified with a 215–230 kD band in SDS-PAGE (*see* Fig. 1).

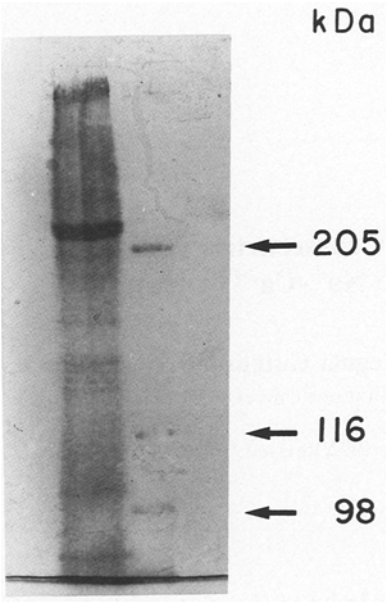

Fig. 1. SDS-PAGE separation of partially purified retinal Na^+-Ca^{2+},K^+ exchanger. The exchanger forms a major protein band at 215–230 kD on a 5% gel after successive ion exchange and reactive dye chromatography (~25 μg total protein/lane). Such preparations were used as antigen for immunizations, as described in Materials and Methods. Left lane: exchanger fraction; right lane: molecular weight markers.

Preparation of Cardiac Membrane Vesicles

Local conditions made it impossible to collect fresh bovine hearts within a few minutes after killing of the animal. Therefore, equine hearts obtained from a local slaughterhouse were used for the preparation of myocardial membrane vesicles according to the methods of Kuwayama and Kanazawa (1982) and Slaughter, Sutko and Reeves (1983). The homogenized tissue suspended in 250 mM mannitol, 70 mM Tris (pH 7.4) was digested at 37°C with DNAse type I (20,000 U/ml, Philipson & Ward, 1987). Further homogenization and centrifugation yielded a crude membrane preparation which was suspended in NaCl-MOPS buffer (160 mM NaCl, 20 mM MOPS, pH 7.4) and then layered on top of a buffered sucrose solution (600 mM sucrose, 10 mM MOPS/Tris, 0.5 mM EGTA, pH 7.4). A purified membrane vesicle preparation was collected from the interface between buffer and sucrose solution after ultracentrifugation for 80 min in a swing-out rotor at 126,000 × g_{max}. Membrane markers (β-adrenoceptors, L-type calcium channels) were enriched about 10-fold over the value in the homogenate.

Partial Purification of the Cardiac Na^+-Ca^{2+} Exchanger

Purified sarcolemmal vesicles were solubilized after alkaline extraction (Philipson et al., 1987) in detergent buffer B containing (mM) 65 NaCl, 65 KCl, 5 $CaCl_2$, 2 DTT, 20 MOPS, 3–5 mg/ml phospholipid, protease inhibitors leupeptin (5 μg/ml), pepstatin (5 μg/ml) and aprotinin (10 μg/ml) and either 8 mg free deoxyBIGCHAP or 7 mg free decylmaltoside per mg protein, pH 7.4. The solubilized protein was run through an ion exchange column (Fractogel EMD-TMAE-650) equilibrated in buffer B, containing 7 mg/ml detergent. Unlike the ROS exchanger, the cardiac exchanger was not retained on the ion exchange column if solubilized in a zwitterionic rather than a nonionic detergent. The column was eluted using a linear gradient between 65 mM NaCl/65 mM KCl and 350 mM NaCl/350 mM KCl in the same buffer modified by changing the detergent to CHAPS (8 mg/ml). The active fraction, reduced to a volume of 0.4–0.6 ml by ultrafiltration, was further purified by successive FPLC Superose 12 (Pharmacia) gel filtration and Fractogel AF red column chromatography. The dye column was eluted with buffer C (900 mM NaCl, 900 mM KCl, 20 mM PIPES, 5 mM $CaCl_2$, 2 DTT, proteinase inhibitors as in buffer B, pH 6.4). Unlike the ROS exchanger, the cardiac exchanger was retained on the active dye column and appeared in the high salt elution buffer. Exchange activity was checked

[1] Abbreviations used; mAb(s), monoclonal antibodies; ROS, rod outer segments; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; MOPS, 3-[N-morpholino]propanesulfonic acid; PIPES, piperazine-N-N′-bis[2-ethane sulfonic acid]; deoxy-BIGCHAP, N,N-bis(3-D-gluconamidopropyl)deoxycholamide; PEG, polyethyleneglycol; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; DMEM, Dulbecco's modified minimal essential medium; FCS, fetal calf serum; ECL, enhanced chemoluminescence; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline.

after reconstitution of the chromatographic fractions into asolectin vesicles by dialysis for 48 hr in a buffer containing (mM) 100 Na_2HPO_4, 50 NaCl, 1 EGTA, 0.5 DTT.

Determination of Na-Activated Ca Fluxes in Lipid Vesicles

Na-loaded lipid vesicles were incubated at room temperature in the presence of 160 mM KCl, 1 μM valinomycin and 100 μM $^{45}Ca^{2+}$ (specific activity 100 μCi/mM Ca). $^{45}Ca^{2+}$ influx was measured by a filtration assay using Whatman GF/F glassfibre filters presoaked with 30 mM polyethyleneimine (Cheon & Reeves, 1988). The assay was started by adding vesicle suspension (20 μl) into 500 μl incubation medium. The uptake was terminated by adding 100 μl samples of the incubation mixture into ice-cold stopping solution (140 mM KCl, 20 mM Tris, 10 mM EGTA, pH 7.4).

Preparation of mAbs to the Retinal Exchanger

We used nitrocellulose transblots of the exchanger as antigen to immunize BALB/c mice (Diano, LeBivic & Hirn, 1987). After SDS-PAGE separation of a partially purified preparation in a 6% gel, the proteins were blotted onto nitrocellulose (4 hr, 1.8 A) according to the method of Towbin, Staehelin and Gordon (1979), except that the transfer buffer contained no methanol. The characteristic band at 215–230 kD was excised, homogenized in PBS using a micro Duall® tissue grinder (Kontes) and applied intraperitoneally. Assuming a transblot efficiency of 50%, about 10 μg protein/mouse was injected with each application. Two booster injections were given two and eight weeks after the first immunization. Immune responses were routinely stimulated with muramyldipeptide (100 μg/mouse). Spleen cell fusion to NSO plasmocytoma cells and isolation of hybridomas followed standard methods (Harlow & Lane, 1988). The hybridomas were screened by ELISA (Streptavidin HyBRL screen, GIBCO/BRL, Basel, Switzerland) against a lipid vesicle preparation containing the partially purified retinal exchanger. ELISAs were performed on nitrocellulose filter plates (Millipore, type HA) and the Millititer Filtration System (Millipore, Volketswil, Switzerland) to avoid loss of antigen during washing.

Preparation of mAbs to the Cardiac Na^+-Ca^{2+} Exchanger

mAbs were raised against the recombinant canine myocardial exchanger as expressed in Sf9 insect cells after infection with a recombinant baculovirus (Li et al., 1992). The exchanger constituted approximately 2–10% of the nonsoluble cell protein. For the first immunization, the cell homogenate, after alkaline extraction, was suspended in complete Freund's adjuvant. Incomplete Freund's adjuvant was used for the two subsequent booster injections. Hybridomas, which were prepared according to the same procedure described above for the retinal exchanger, were screened against equine myocardial membrane vesicles.

mAb Purification

All antibodies from ELISA-positive hybridoma supernatants (including IgM) were affinity-purified on protein G-sepharose columns as suggested for protein A beads in Harlow and Lane (1988). However, the salt concentration and the pH of the supernatant were not modified before application to the column. The resultant mAb preparations were adjusted to about 200 μg/ml and appeared 80–90% pure if compared to pure mouse IgG in an ELISA titration assay. Isotyping of purified mAbs was done with the INNOLIA kit (Innogenetics, Antwerp, Belgium). Protein was determined according to the method of Peterson (1977) using gammaglobulin as a standard.

Immunoblotting

Immunolabeling of protein transblots after SDS-PAGE on 6% gels was performed using 100 μg/ml of the primary antibodies. Bound mAb was detected with the Streptavidin HyBRL Screen kit. Horseradish peroxidase activity was measured either with diaminobenzidine as substrate or with ECL assay from Amersham, UK.

Materials

Separation materials for gel chromatography and protein G-sepharose were purchased from Pharmacia, Zurich, Switzerland. Ion exchange gels and reactive dye-coupled gels were obtained from Merck ABS, Dietikon, Switzerland. Asolectin and detergents (CHAPS, deoxyBIGCHAP, decylmaltosid) were from Sigma, St. Louis, MO. All other chemicals (analytical grade) were from Merck, Darmstadt, Germany or from Fluka, Buchs, Switzerland. NS0 plasmocytoma cells, originally isolated in C. Milstein's laboratory, were obtained through Z. Eshhar, Dept. of Immunology, Weizmann Institute, Rehovot, Israel.

Results

Generation and Characterization of Antibodies to the ROS Exchanger Protein

We performed three separate immunizations of mice with the partially purified ROS Na^+-Ca^{2+},K^+ exchanger protein prepared according to the method of Cook and Kaupp (1988). Together, the immunizations yielded a total of 43 mAbs reacting in an ELISA screening test with partially purified ROS exchanger reconstituted into asolectin vesicles.

To test for specific antigen recognition, we first screened the antibodies for their capacity to label the exchanger band in Western blots of partially purified ROS proteins. Only eight mAbs reacted specifically with one or several bands on these blots. In each case, a band with an apparent molecular weight slightly above 200 kD appeared most prominently labeled. However, our antigen preparation (Fig. 1) still contained variable (but minor) amounts of various other proteins. Therefore, it was essential to establish that our mAbs reacted with the correct target band rather than with a contaminant. Of par-

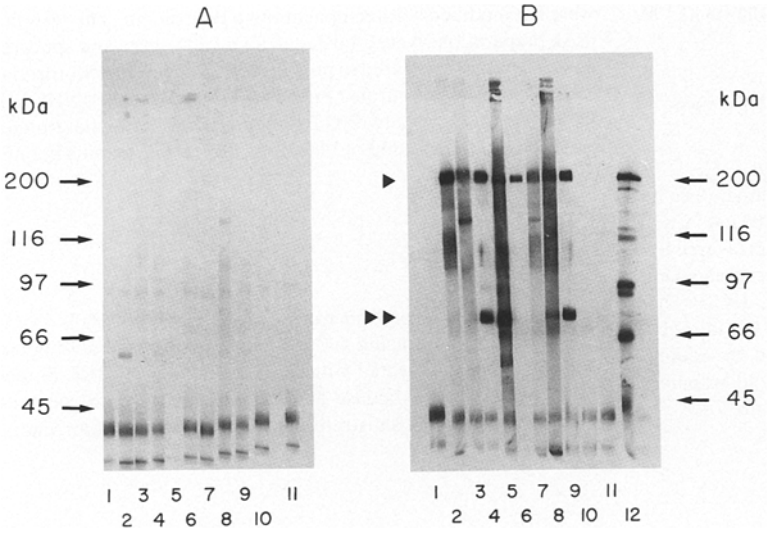

Fig. 2. Immunostaining of ROS membrane protein fractions with eight different mAbs using ECL detection. (*A*) Immunolabeling of Western blots obtained after SDS-PAGE separation of the low ionic strength fraction (~30 μg protein/lane) from ion exchange chromatography. This fraction had no exchange activity but contained most of the "rim" protein. (*B*) Immunolabeling of Western blots from the active fraction (~15 μg protein/lane) after ion exchange and reactive dye chromatography. (▶) Main exchanger band, (▶▶) accessory immunoreactive band (proteolytic fragment of the exchanger?). Lane coding for primary antibodies: *1*, 10% FCS in DMEM (negative control); *2*, O7C1; *3*, O9D8; *4*, P17D2; *5*, P8G10; *6*, Q12G1; *7*, P15E1; *8*, P15D4; *9*, Q13F10; *10*, *11*, negative controls with FCS; *12*, biotinylated marker proteins. Concentrations of primary antibodies: 100 μg/ml.

ticular concern was a protein that migrated in close vicinity to the exchanger (apparent MW about 205 kD) and had been identified as the "rim protein" by Haase et al. (1990). It formed the major high molecular weight band of ROS proteins prior to ion exchange chromatography. The 205 kD protein was essentially absent from most antigen preparations (Fig. 1), but remained visible in others. The "rim" protein was largely separated from the exchanger on the cation exchange column where it was eluted with significantly lower ionic strength than the Na^+-Ca^{2+} exchanger [$I < 0.2$ *vs.* $I > 0.4$, (Cook & Kaupp, 1988)]. Therefore, we have used our panel of antibodies to immunostain transblots obtained from both the low ionic strength peak of the ion exchange column and the final eluate of the reactive dye column. The exchange activity was enriched 50- to 100-fold in the purified sample, but was absent from the ion exchange eluate. Figure 2*B* shows that all of the eight mAbs stained specifically a band with an apparent MW > 200 kD in the purified sample (▶). Some mAbs reacted with an additional prominent band at about 77 kD (▶▶). In contrast, no specific labeling of a high molecular weight band was visible in the protein fraction that contained most of the "rim protein" (Fig. 2*A*), even though the corresponding protein band was clearly visible in a silver-stained control SDS-PAGE run of this fraction. We concluded from these findings that the eight mAbs were directed against the exchanger protein.

As the most stringent test for antigen recognition, we tested the capacity of the mAbs to immunoprecipitate the exchanger out of a detergent-solubilized preparation of ROS proteins according to the method of Porzig (1991). The only way to quantify a change in exchanger concentration after exposure of the mAb-exchanger complex to protein G-sepharose, was by measuring its function following reconstitution into lipid vesicles. This test does not work for mAbs recognizing epitopes that are inaccessible in the functional protein. Only one of the eight immunoblot-positive mAbs (P8G10) removed most of the exchange activity (~70%) from a CHAPS-solubilized mixture of ROS membrane proteins (Porzig, 1991). The remaining mAbs most likely recognized epitopes that were not accessible or were not present in the solubilized functional exchanger.

Characterization of mAbs Directed against the Myocardial Exchanger Protein

The recombinant cardiac Na^+-Ca^{2+} exchanger as expressed in Sf9 insect cells after infection with a recombinant baculovirus (Li et al., 1992) was used as antigen to produce a library of specific mAbs in mice. A detailed analysis of these antibodies and their epitopes will be published elsewhere (Porzig et al., 1993). Out of 36 antibodies, 12 reacted specifically with Western blots of the recombinant protein and also recognized a protein of the predicted size in equine and dog cardiac membranes. The specific reaction of two representative mAbs with the recombinant cardiac exchanger is illustrated in Fig.

3, top. These particular mAbs are shown below to crossreact with ROS protein (*compare* Fig. 6).

Crossreactions of the anti-ROS Exchanger mAbs with the Cardiac Exchanger Protein

Of the eight mAbs that labeled the ROS exchanger in immunoblots, three showed limited crossreactivity with myocardial proteins either in immunoblots or in an ELISA assay which used cardiac membrane proteins as antigen. A specific crossreaction with the cardiac Na^+-Ca^{2+} exchanger was confirmed by analyzing immunoblots of the recombinant exchanger (Fig. 3, middle). All three antibodies labeled several, though partially different, well-defined bands in transblots of proteins from insect cells infected with the recombinant (*R*) baculovirus. Except for a faint unspecific labeling with mAb Q13F10, no reaction was seen with proteins from insect cells infected with wild type (*WT*) virus. Surprisingly, all mAbs reacted very weakly with the mature exchanger at ~120 kD. The reaction was almost invisible on nitrocellulose blots (Fig. 3, middle) but could be detected clearly with the more sensitive ECL assay (Fig. 3, bottom). A possible explanation for this observation is presented in the discussion.

In ELISA titrations we measured the apparent binding affinities of the crossreactive mAbs to their epitopes in cardiac and ROS membranes. Partially purified membrane proteins from both tissues reconstituted into phospholipid vesicles were used as antigen. The results illustrated in Fig. 4 showed that apparent binding affinities for two of the 3 mAbs were significantly lower in the myocardial prepration than in their original target protein from ROS.

Reid et al. (1990) and Achilles et al. (1991) have described a monoclonal antibody to the ROS exchanger which has a defined epitope within an acidic sequence on the carboxyterminal side of the large hydrophilic loop extending between the hydrophobic domains 5 and 6. When this mAb (PMe 1B3) was reacted with a transblot of the recombinant cardiac exchanger, it labeled three distinct bands in the 116,

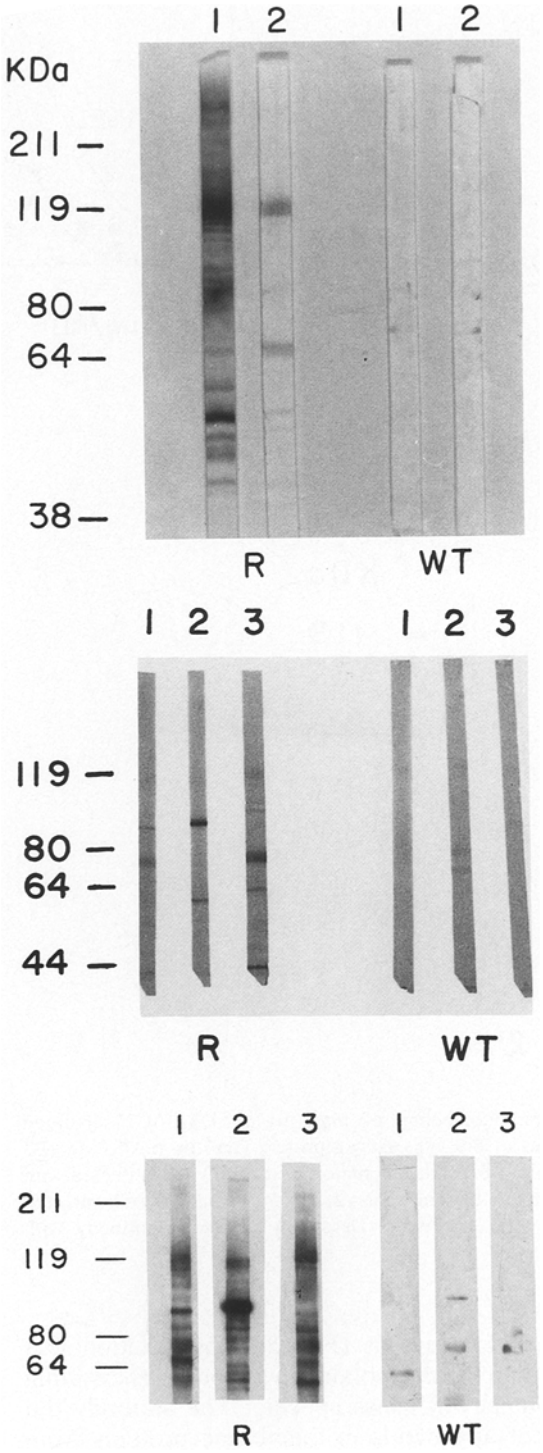

Fig. 3. Immunodetection of the recombinant cardiac exchanger on Western blots of SDS-PAGE-separated Sf9 cell membrane proteins by crossreacting anti-ROS and anti-cardiac mAbs. Top: Anti-cardiac mAbs. Lane *1*, R2H4; *2*, R16D11. Middle: Anti-ROS mAbs. Lane *1*, P15D4; *2*, Q13F10; *3*, P15E1. (*R*) Transblots from Sf9 cells infected with recombinant baculovirus. (*WT*) Transblots from Sf9 cells infected with wild type virus. Bottom: Anti-ROS mAbs, same line identification as in middle panel. Western blots of recombinant (*R*) and of wild-type (*WT*) virus-infected Sf9 cells were developed with the more sensitive ECL system. Note the labeling of the mature exchanger at ~120 kD. The arrow points to a prominent band recognized by mAb Q13F10 which is also visible on the nitrocellulose transblots. The transblots in middle and bottom panels are from different experiments.

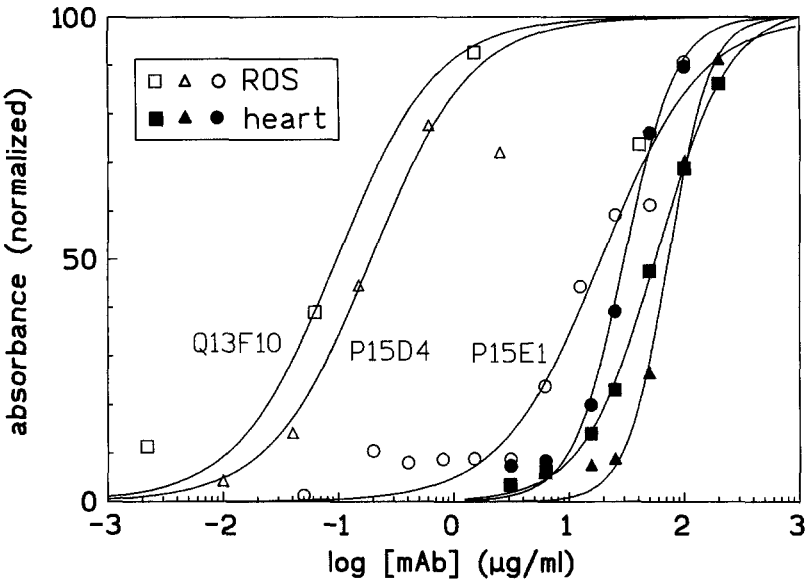

Fig. 4. ELISA titration curves reflecting binding of cross reacting anti-ROS mAbs to partially purified exchanger preparations from ROS or equine cardiac cells. Open symbols: mAb reaction with ROS exchanger (active fraction after ion exchange chromatography, 3 µg/well). Filled symbols: mAb reaction with equine cardiac exchanger (membrane preparation after alkaline extraction, 17 µg/well). Color development was stopped after 20 min by adding concentrated H_2SO_4. The reaction mixture (100 µl) was taken from each well, diluted 1 : 10 and measured photometrically at 490 nm. All titration curves represent nonlinear least square fits of the data normalized to the individual maximal responses. Half-maximal binding of mAbs Q13F10, P15D4 and P15E1 to the ROS preparation was observed with 0.1, 0.2 and 18.9 µg/ml, respectively. These values changed to 58, 73, and 30 µg/ml, respectively, in cardiac membranes.

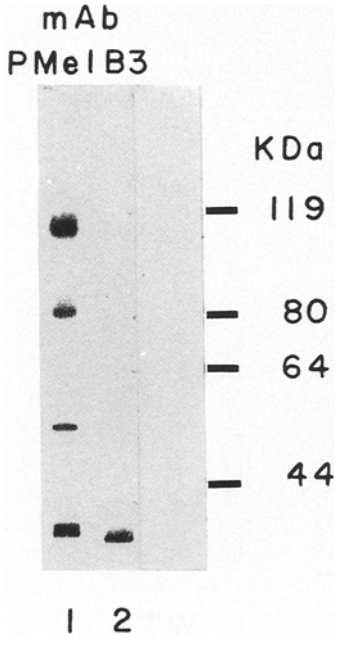

Fig. 5. Immunolabeling of blots from SDS-PAGE-separated membranes of Sf9 cells (20 µg protein/lane) by mAb PMe 1B3 (Reid et al., 1990).Lane *1*: material from Sf9 cells infected with the recombinant baculovirus; *2*, material from Sf9 cells infected with the wild type virus. Detection of second antibody with ECL assay.

80 and 50 kD regions (Fig. 5). The reaction was weaker than the one obtained with our crossreacting mAbs but nevertheless specific: The antibody did not detect any bands in membrane proteins from wild-type baculovirus-infected Sf9 insect cells. This labeling pattern is distinctly different from those shown by our own mAbs. Therefore, if the results with mAb PMe 1B3 are typical for a crossreacting antibody with a "large loop" epitope, we expect our mAbs to bind to some different part of the protein.

Crossreaction of Anti-Cardiac Exchanger mAbs with ROS Membrane Proteins

To detect possible crossreacting antibodies in our panel of mAbs raised against the recombinant myocardial exchanger protein, the antibodies were screened in an ELISA against a partially purified preparation of the ROS exchanger. The screening included all mAbs which gave a positive response with equine myocardial membrane proteins regardless of whether they detected the exchanger protein on immunoblots. Of 36 antibodies, 4 (all IgM) showed a positive, albeit weak, reaction with the retinal protein. Three of them labeled a distinct protein band in the 215 kD region. However, in two cases the labeling was rather weak. The reaction with mAb R16D11, yielding the strongest response, is documented in Fig. 6 (lane *1*).

Crossreactions of Anti-ROS and Anti-Cardiac Exchanger mAbs with Brain Synaptosomal Proteins

Recent evidence has suggested that a polyclonal antiserum to the cardiac exchanger detects a protein of similar immunological reactivity in brain synaptosomes (Yip, Blaustein & Philipson, 1992). The anti-

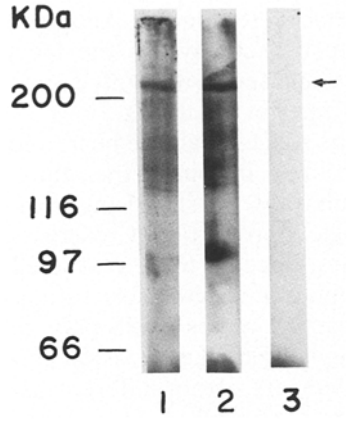

Fig. 6. Immunolabeling of partially purified ROS exchanger (25 μg protein/lane), after SDS-PAGE separation on a 7% gel, with crossreacting anti-cardiac mAb R16D11 (lane *1*, 100 μg/ml) and crossreacting anti-ROS mAb Q13F10 (lane *2*, 50 μg/ml). Lane *3*: negative control, 10% FCS in DMEM replacing the specific antibody. Detection of second antibody with ECL assay.

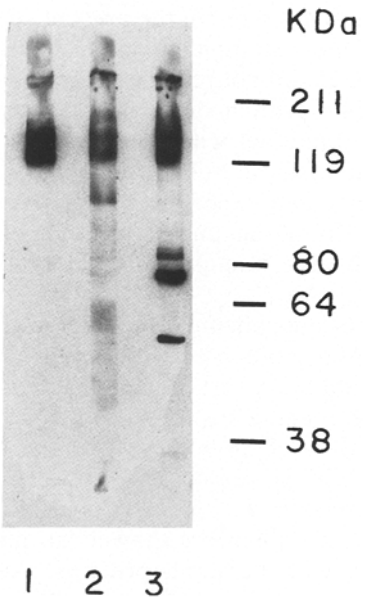

Fig. 7. Immunolabeling of proteins from bovine brain synaptic vesicles with anti-cardiac and anti-ROS mAbs. Western blots were prepared after SDS-PAGE separation on a 7% gel (20 μg protein/lane). Lane *1*, R3F1 (10 μg/ml, anti-cardiac, noncross-reacting with ROS exchanger); *2*, P15D4 (100 μg/ml, anti-ROS, crossreacting with cardiac exchanger); *3*, Q13F10 (100 μg/ml, anti-ROS, crossreacting with cardiac exchanger). Detection of second antibody with ECL assay.

body labeled specifically prominent bands in the 160, 120, and 70 kD range in both sarcolemmal membrane and brain synaptosome preparations. Therefore, we have screened bovine brain synaptosomes with our mAbs to the recombinant cardiac exchanger or with the crossreacting antibodies to the ROS protein. As shown in Fig. 7, both types of antibodies consistently detected the same bands at 160 and 120 kD. Only crossreacting mAb Q13F10, but not P15D4 labeled an additional protein at ~70 kD.

Discussion

The close functional relationship between the retinal and the cardiac exchanger (Nicoll, Barrios & Philipson, 1991) has led most workers in the field to test for immunological crossreactivity between the two proteins. The results were surprisingly variable. While some authors found significant, though weak, positive crossreactivity (Vemuri et al., 1990; Durkin et al., 1991), others reported negative results (Leser, Nicoll & Applebury, 1991). However, since all previous studies used polyclonal antibodies, the true extent of epitope sharing between the two proteins is difficult to judge. By contrast, in the present study we have used a series of monoclonal antibodies raised against both types of exchangers to assess more systematically the degree of crossreactivity.

Overall, our study has revealed a low degree of epitope sharing between the two proteins. This surprising result could perhaps be ascribed to the very limited homology between their primary sequences (Nicoll et al., 1990; Reiländer et al,. 1992). However, the extent of crossreactivity that has been observed in earlier studies with polyclonal antibodies does not necessarily require the presence of epitopes with linear sequence homology. Judging from the similarities in the hydrophobicity plots, the two exchanger proteins seem to share their overall transmembrane topology (Nicoll & Philipson, 1991; Reiländer et al., 1992). Also, our observation that both proteins can be partially purified using the same chromatographic techniques, suggests similarities in their molecular properties. Consequently, the two proteins might be expected to share subtle structural details on the secondary or tertiary level which could be detected by mAbs. Indeed, our results with the mAb PMe 1B3 prepared to the retinal exchanger (Reid et al., 1990), confirm this hypothesis. This antibody with a known epitope on the large cytoplasmic loop showed crossreactivity even though there is no primary sequence homology with the analogous loop of the cardiac exchanger. However, this loop also has an acidic region rich in glutamic acid residues which may show some structural relationship to the epitope of mAb PMe 1B3. Still, our

results indicate that even such secondary structural homologies are exceedingly rare. In the present series of 36 mAbs to the recombinant cardiac exchanger, a majority of 25 did not recognize the partially denatured cardiac protein on Western blots and, hence, is likely to interact with structural epitopes. However, none of these mAbs crossreacted significantly with the retinal exchanger. All crossreacting mAbs were from the minority group of mAbs which gave positive immunoblots with the cardiac protein.

Extensive attempts at localizing the epitopes for the crossreacting mAbs more precisely, using an expression cDNA sublibrary of the cardiac exchanger, failed. The screening system in this assay relied on specific reactions of antibodies with partial sequences of the protein expressed as fusion proteins with β-galactosidase in *E. coli* (Porzig et al., 1993). However, all candidate mAbs showed strong background reactions with bacterial proteins. At high concentrations, the crossreacting anti-ROS mAbs interacted with a synthetic peptide corresponding to the 12 C-terminal residues of the cardiac exchanger (Porzig, 1991). Yet, additional experiments showed that the specificity of this interaction was probably too low to account for the observed crossreactivity (H. Porzig, *unpublished results*). Nevertheless, our present results suggest that the crossreacting anti-ROS mAbs do not bind to the long hydrophilic loop of the cardiac exchanger but have determinants on other parts of the protein. All mAbs reacting with loop epitopes yielded a specific banding pattern in immunoblots of the recombinant exchanger from Sf9 cell membranes that was shared by none of the crossreacting mAbs (Porzig et al., 1993).

At least two of the anti-ROS exchanger mAbs showed a marked decrease in apparent binding affinities when reacting with cardiac proteins (Fig. 4). Most likely, this shift reflects a significant loss of complementarity in the antigenic determinants. In addition, these mAbs show a variable tendency of low affinity crossreactions with proteins unrelated to the cardiac exchanger. This is best explained by assuming that only relatively unspecific features of the mAb epitopes on the retinal exchanger are recognized on the cardiac protein. An even lower degree of structural similarity may also explain crossreactions with other proteins. A similar mechanism may account for our finding that the three crossreacting mAbs all seem to interact more strongly with (proteolytic?) fragments of the exchanger than with the mature protein itself (*compare* Fig. 3, middle and bottom panels). The mobility of epitopic domains may be higher in the fragments than in the intact protein. This could well help to form a tighter fit between the antibodies and their binding sites on the polypeptide chain in case of suboptimal complementarity of the antigenic determinant. Antigenic recognition is known to be sensitive to the local mobility of antigenic sites and seems to involve some degree of induced fit (Tainer et al., 1985).

Together our findings suggest that the heart and the retinal exchanger proteins, in spite of their functional and structural similarities, are surprisingly distinct in their immunological properties. Crossreactivity, as far as it occurs, appears to be restricted to epitopes with a minimum of specific structuring.

Specific noncrossreacting mAbs could play an essential part in mapping individual expression patterns for Na^+-Ca^{2+} exchangers within different tissues, in particular the brain, and may help to define their functional roles. It is still controversial whether the brain synaptosomal Na^+-Ca^{2+} exchanger belongs to the cardiac, to the retinal or to some still undefined third class of exchangers. Two groups working with rat or with bovine brain synaptosomes have identified 70 and 36 kD proteins, respectively, that copurified with the exchange activity (Barzilai, Spanier & Rahamimoff, 1987; Rahamimoff et al., 1991; Michaelis et al., 1992). Polyclonal antibodies generated to these two proteins immunoprecipitated the exchange activity from a preparation of solubilized membrane proteins. Moreover, a polyclonal antibody to the retinal exchanger labeled a 70 kD protein in rat brain synaptosomes (Rahamimoff et al., 1991). It is not entirely clear whether these synaptosomal proteins represent new types of exchangers or could have been generated by proteolysis from cardiac or retinal type proteins.

In our experiments, crossreacting anti-ROS as well as noncrossreacting anti-cardiac exchanger mAbs prominently labeled the same 160/120 kD bands. This confirms the results of Yip et al. (1992) with a polyclonal antibody and is compatible with the presence of a cardiac-type exchanger in brain synaptosomes. By contrast, the antibody of Barzilai et al. (1987) to the 70 kD rat synaptosomal protein did not react with the 160/120 kD bands and hence, seems to detect a separate protein. Similarly, the antibody of Michaelis et al. (1992) to the 36 kD protein did not detect any protein in the 160/120 kD region. These observations suggest that immunologically distinct exchanger proteins may coexist in brain. It is a matter of dispute whether one of these different exchanger types is related to the retinal exchanger. Our experiments do not support such a relationship. All antibodies to the retinal protein from various sources detect a protein in the 215–230 kD region (Reid et al., 1990; this paper). A protein of that size has not been labeled specifically by any of the antibodies reported to react with the brain exchanger. (The band above the 211 kD marker on

Fig. 7 is probably nonspecific since it was labeled also by anticardiac mAb R3F1 which did not cross-react with the retinal exchanger.) Finally, functional studies have shown that Na^+-Ca^{2+} exchange kinetics in synaptosomes have many properties in common with the cardiac transport system (Blaustein, 1988). On the other hand, a recent study suggested that at least part of the Na^+-activated Ca^{2+} transport in rat brain synaptic vesicles may be coupled to K^+ cotransport (Dahan, Spanier & Rahamimoff, 1991). If alternative explanations for these observations can be excluded, a K^+/Ca^{2+} cotransport coupled to the Na^+gradient would strongly suggest the presence of a retinal type exchanger in synaptosomes. Ongoing cloning studies trying to isolate exchanger isoforms from brain will probably solve these problems in the near future.

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References

Achilles, A., Friedel, U., Haase, W., Reiländer, H., Cook, N.J. 1991. Biochemical and molecular characterization of the sodium-calcium exchanger from bovine rod photoreceptors. *Ann. NY Acad. Sci.* **639:**234–244

Barzilai, A., Spanier, R., Rahamimoff, H. 1987. Immunological identification of the synaptic plasma membrane Na^+-Ca^{2+}exchanger. *J. Biol. Chem.* **262:**10315–10320

Blaustein, M. 1988. Calcium and synaptic function. *In:* Calcium in Drug Action. P.F. Baker, editor. *Hdb. Exp. Pharmacol.* **83:**275–394. Springer-Verlag, Berlin

Cervetto, L., Lagnado, L., Perry, R.J., Robinson, D.W., McNaughton, P.A. 1989. Extrusion of calcium from rod outer segments is driven by both sodium and potassium gradients. *Nature* **337:**740–743

Cheon, J., Reeves, J.P. 1988. Site density of the sodium-calcium exchange carrier in reconstituted vesicles from bovine cardiac sarcolemma. *J. Biol. Chem.* **206:**2309–2315

Cook, N.J., Kaupp, U.B. 1988. Solubilization, purification, and reconstitution of the sodium-calcium exchanger from bovine retinal rod outer segments. *J. Biol. Chem.* **263:**11382–11388

Dahan, D., Spanier, R., Rahamimoff, H. 1991. The modulation of rat brain Na^+-Ca^{2+} exchange by K^+. *J. Biol. Chem.* **266:**2067–2075

Diano, M., LeBivic, A., Hirn, M. 1987. A method for the production of highly specific polyclonal antibodies. *Anal. Biochem.* **166:**224–229

DiPolo, R., Beaugé, L. 1990. Calcium transport in excitable cells. *In:* Intracellular Calcium Regulation. F. Bronner, editor. pp. 381–413. Wiley-Liss, New York

Durkin, J.T., Ahrens, D.C., Pan, Y.C.E., Reeves, J.P. 1991. Purification and amino-terminal sequence of the bovine cardiac sodium calcium exchanger—evidence for the presence of a signal sequence. *Arch. Biochem. Biophys.* **290:**369–375

Haase, W., Friese, W., Gordon, R.D., Müller, H., Cook, N.J. 1990. Immunological characterization and localization of the Na^+/Ca^{2+}-exchanger in bovine retina. *J. Neurosci.* **10:** 1486–1494

Harlow, E., Lane, D. 1988. Antibodies. A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Kuwayama, H., Kanazawa, T. 1982. Purification of cardiac sarcolemmal vesicles: High sodium pump content and ATP-dependent, calmodulin-activated calcium uptake. *J. Biochem. (Tokyo)* **91:**1419–1426

Leser, G.P., Nicoll, D.A., Applebury, M.L. 1991. Distinctive properties of the purified Na-Ca exchanger from rod outer segments. *Ann. NY Acad. Sci.* **639:**222–233

Li, Z., Smith, C.D., Smolley, J.R., Bridge, J.H.B., Frank, J.S., Philipson, K.D. 1992. Expression of the cardiac Na^+-Ca^{2+} exchanger in insect cells using a baculovirus vector. *J. Biol. Chem.* **267:**7828–7833

Michaelis, M.L., Nunley, E.W., Jayawickreme, C., Hurlbert, M., Schueler, S., Guilly, C. 1992. Purification of a synaptic membrane Na^+/Ca^{2+} antiporter and immunoextraction with antibodies to a 36-kDa protein. *J. Neurochem.* **58:**147–157

Nicoll, D.A., Barrios, B.R., Philipson, K.D. 1991. Na^+-Ca^{2+} exchangers from rod outer segments and cardiac sarcolemma: comparison of properties. *Am. J. Physiol.* **260:**C1212–C1216

Nicoll, D.A., Longoni, S., Philipson, K.D. 1990. Molecular cloning and functional expression of the cardiac sarcolemmal Na^+-Ca^{2+} exchanger. *Science* **250:**562–565

Nicoll, D.A., Philipson, K.D. 1991. Molecular studies of the cardiac sarcolemmal sodium-calcium exchanger. *Ann. NY Acad. Sci.* **639:**181–188

Peterson, G.L. 1977. A simplification of the protein assay method of Lowry et al. which is more generally applicable. *Anal. Biochem.* **83:**346–356

Philipson, K.D., Longoni, S., Ward, R. 1988. Purification of the cardiac Na^+-Ca^{2+} exchange protein. *Biochim. Biophys. Acta* **945:**298–306

Philipson, K.D., McDonough, A.A., Frank, J.S., Ward, R. 1987. Enrichment of Na^+-Ca^{2+} exchange in cardiac sarcolemmal vesicles by alkaline extraction. *Biochim. Biophys. Acta* **899:**59–66

Philipson, K.D., Ward, R. 1987. Modulation of Na^+-Ca^{2+} exchange and Ca^{2+} permeability in cardiac sarcolemmal vesicles by doxylstearic acids. *Biochim. Biophys. Acta* **897:**152–158

Porzig, H. 1991. Characterization of monoclonal antibodies cross-reacting with myocardial and retinal sodium-calcium exchange proteins. *Ann. NY Acad. Sci.* **639:**202–209

Porzig, H., Li, Z., Nicoll, D.A., Philipson, D.K. 1993. Mapping of the cardiac sodium-calcium exchanger with monoclonal antibodies. *Am. J. Physiol. (in press)*

Rahamimoff, H., Dahan, D., Furman, I., Spanier, R., Tessari, M. 1991. Molecular and mechanistic heterogeneity of the Na^+-Ca^{2+} exchanger. *Ann. NY Acad. Sci.* **639:**210–221

Reeves, J.P. 1990. Sodium-calcium exchange. *In:* Intracellular Calcium Regulation. F. Bronner, editor. pp 305–347. Wiley-Liss, New York

Reeves, J.P. 1992. Molecular aspects of sodium-calcium exchange. *Arch. Biochem. Biophys.* **292:**329–334

Reeves, J.P., Hale, C.C. 1984. The stoichiometry of the cardiac sodium-calcium exchange system. *J. Biol. Chem.* **259:** 7733–7739

Reid, D.M., Friedel, U., Molday, R.S., Cook, N.J. 1990. Identi-

fication of the sodium-calcium exchanger as the major ricin-binding glycoprotein of bovine rod outer segments and its localisation to the plasma membrane. *Biochemistry* **29:** 1601–1606

Reiländer, H., Achilles, A., Friedel, U., Maul, G., Lottspeich, F., Cook, N.J. 1992. Primary structure and functional expression of the Na/Ca,K-exchanger from bovine rod photoreceptors. *EMBO J.* **11:**1689–1695

Schnetkamp, P.P.M., Basu, D.K., Szerencsei, R.T. 1989.Na^+-Ca^{2+} exchange in bovine rod outer segments requires and transports K^+. *Am. J. Physiol.* **257:**C153–C157

Schnetkamp, P.M., Daemen, F.J.M. 1982. Isolation and characterization of osmotically sealed bovine rod outer segments. *Methods Enzymol.* **81:**110–116

Slaughter, R.S., Sutko, J.L., Reeves, J.P. 1983. Equilibrium calcium-calcium exchange in cardiac sarcolemmal vesicles. *J. Biol. Chem.* **258:**3183–3190

Tainer, J.A., Getzoff, E.D., Paterson, Y., Olson, A.J., Lerner, R.A. 1985. The atomic mobility component of protein antigenicity. *Annu. Rev. Immunol.* **3:**501–535

Towbin, H., Staehelin, T., Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose: Procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76:**4350–4354

Vemuri, R., Haberland, M.E., Fong, D., Philipson, K.D. 1990. Identification of the cardiac sarcolemmal Na^+-Ca^{2+} exchanger using monoclonal antibodies. *J. Membrane Biol.* **118:**279–283

Yip, R.K., Blaustein, M.P., Philipson, K.D. 1992. Immunologic identification of Na/Ca exchange protein in rat brain synaptic plasma membrane. *Neurosci. Lett.* **136:**123–126