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Membrane topology of the rat brain Na^+–Ca^{2+} exchanger

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Abstract

To provide experimental evidence for the topology of the Na^+–Ca^{2+} exchanger protein NCX1 in the membrane, indirect immunofluorescence studies using site specific anti-peptide antibodies and Flag-epitope insertion into chosen locations of the protein were carried out. Anti-peptide antibodies AbO-6 and AbO-8 were raised against peptide segments present in a large hydrophilic loop of about 500 amino acids, which separates the hydrophobic amino terminal part of the protein from the hydrophobic carboxy terminal. AbO-10 was raised against the C-terminal tail of the protein. All three antibodies bound to the exchanger protein expressed in transfected cells, in rat brain synaptic plasma membrane and in dog sarcolemmal preparations. The antibodies bound only to those NCX1 isoforms that contained the epitope against which they were raised. Detection of the exchanger protein in transfected cells in situ required the addition of permeabilizing agents suggesting an intracellular location of the epitopes to which AbO-6, AbO-8 and AbO-10 bind. The Flag epitope was inserted into ten putative extramembraneous segments along the exchanger protein. For topology studies, only the Flag-mutants that retained Na^+–Ca^{2+} exchange activity in whole HeLa cells, were used. Immunofluorescence studies indicated, that the N-terminal of the protein is extracellular, the first hydrophilic loop separating transmembrane helices 1 and 2 as well as the C-terminal, are intracellular. © 1998 Elsevier Science B.V.

Keywords: Na^+–Ca^{2+} exchanger; Topology; Antibody; Epitope tagging

1. Introduction

The Na^+–Ca^{2+} exchanger is involved in the regulation of the intracellular $[Ca^{2+}]$ in all excitable and in many non-excitable cells [1]. It is encoded by at least three different genes NCX1 [2], NCX2 [3] and NCX3 [4]. In addition, an NCKX ($Na^+/Ca^{2+}+K^+$) transporter [5] is expressed in rod outer segment and probably also in the brain [6,7].

Elucidation of the topology of membrane proteins is important for understanding the structural basis of their mechanism of action. Cloning of the Na^+–Ca^{2+} exchanger genes and deduction of their primary structures followed by hydropathy analysis [8] suggested, that their putative topology is similar. Based on hydropathy analysis and using a window of 20–21 amino acids, the proteins of the cloned exchangers could be organized into 12 transmembrane (TM) helices [2–5]. The 6 TM helices at the amino terminal side are separated by a very large hydrophilic loop from the 6 TM helices at the carboxy terminal side.

Microsequencing of the N-terminal amino acid segment of the purified bovine exchanger NCX1 indicated, that the first amino acid of the protein

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corresponds to amino acid number 33 of the cloned gene [9]. Based on this and on the presence of a consensus signal peptidase cleavage site it has been suggested, that the first putative TM helix is a cleavable signal peptide [9].

An 11 TM helical model of the Na^+–Ca^{2+} exchanger predicts that the N- and C-terminal ends of the protein face opposing sides of the membrane. The extracellular location of the amino terminal, is suggested by the location of the signal peptidase cleavage site [9] and by the presence of a glycosylation site at N9 [10]. The intracellular location of the large hydrophilic loop is supported by the presence of consensus internal Ca^{2+} and calmodulin binding sites [2,11] and phosphorylation sites [12]. Selective binding of [^{3}H] labeled monoclonal antibodies to cell homogenates and not to intact monolayer cultures of cardiomyocytes, provided experimental evidence for the intracellular location of the carboxy terminal part of this loop [13].

Recently, locations of transmembrane helices were successfully predicted with a method based on the neural network system [14–16]. This approach resulted in 95% accuracy in predicting secondary structure and topology of integral membrane proteins. Topological analysis of the rat brain Na^+–Ca^{2+} exchanger carried out by the network algorithm, predicted a different topological model than that derived from hydropathy analysis [8]. The major difference between the two models (see Fig. 1) is in the number of transmembrane helices and location of the N- and C-terminals. In this paper, we are describing studies designed to test directly the putative topological models of the Na^+–Ca^{2+} exchanger in situ. Immunocytochemical localization using site specific antibodies and epitope tagging was used.

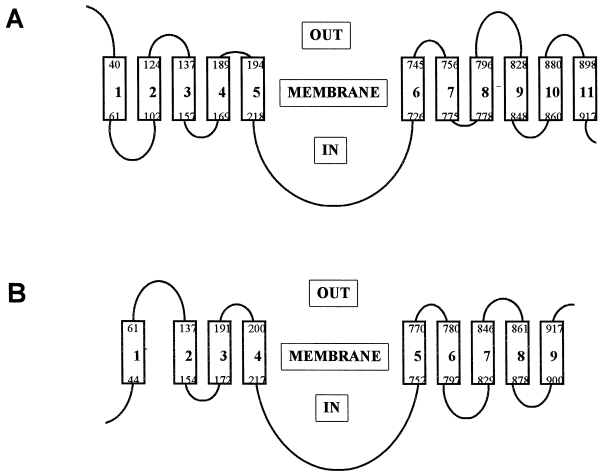

Fig. 1. The topological models of the Na^+–Ca^{2+} exchanger: (A) The topological model of the rat brain exchanger RBE-2, resulting from hydropathy analysis using a window of 20 amino acids. The extracellular orientation of the N-terminal is predicted by the cleavage of the initial 32 amino acids that constitute the putative signal peptide [9]. (B) The best topological model of the rat brain exchanger RBE-2 predicted by the neural network program PHD [16]. The second best model predicted by the PHD program is an 8 TM helical model in which the stretch of amino acids between C780 and G797 (TMS 6 of the 9 TM helical model) is part of the extracellular loop. Consequently the C-terminal is predicted to be intracellular.

2. Materials and methods

2.1. Site directed mutagenesis

The FLAG epitope tag, DYKDDDDK (Eastman Kodak, New Haven, CT), was introduced into Na^+–Ca^{2+} exchanger encoded by the rat brain *rbe-2* [17] gene using site directed mutagenesis [18]. The antisense primers were 54–60 bases long. They contained 24 bases corresponding to the Flag epitope and 15–18 bases corresponding to the sequence of *rbe-2*, 3′ and 5′ of the insertion point. Whenever possible, a new *Hin*dIII restriction site was introduced for selecting mutants. In order to create this site, amino acids valine or isoleucine, following the lysine of the FLAG, were replaced by leucine (details are shown in Fig. 7). To ensure that no other mutation beyond the planned occurred, fragments of 300–600 bases containing the desired mutation were subcloned into the corresponding sites of a wild type *rbe-2*. Plasmid preparation was carried out by Wizard plasmid preparation kit (Promega, Madison, WI). Sequencing was carried out by the dideoxy method [19], using the Sequenase II kit (Amersham, UK). FC mutant (see Fig. 7) was constructed by ligating *Hin*dIII–*Stu*I fragment which contains *rbe*-2 lacking its last eight amino acids into pFLAG-CTS (obtained gratefully from Dr. Robert Ferrari, Eastman Kodak) upstream and in-frame to the FLAG sequence. The clone was excised and inserted into the expression vector pET downstream to T7 promoter, resulting in *rbe-2* gene

in which the last eight amino acids were replaced with the Flag sequence.

2.2. Expression of Na^+–Ca^{2+} exchanger clones in HeLa-cells

Transient expression of the cloned rat brain or heart exchanger genes *rbe-1*, *rbe-2*, *rhe-1* and the epitope-tagged *rbe-2* in HeLa-cells, was done essentially as described [17,20]. Briefly, 60–80% confluent HeLa-cells were infected with the recombinant vaccinia virus VTF-7 [21] and transfected with plasmid DNA using DOTAP (Boehringer-Mannheim, Germany). Na^+–Ca^{2+} exchange activity was examined 16–18 h post transfection, either in whole cells or in reconstituted membranes, as previously described [17,20].

2.3. Preparation of native membrane fractions

Rat brain synaptic plasma membranes were prepared as described [22]. Canine heart sarcolemmal membranes were prepared as described [23,24].

2.4. Preparation of anti-peptides antibodies

Peptides corresponding to chosen hydrophilic segments in RBE-2 (Fig. 2) were custom-synthesized and coupled to three different carrier proteins: Bovine serum albumin (BSA), Keyhole limpet hemocyanine (KLH) and Thyroglobulin using glutaraldehyde [25]. Primary immunizations of rabbits was done in a solution of the coupled peptide with complete Freund's adjuvant (Sigma, Israel). The booster injections were done in incomplete adjuvant, at intervals of 4 weeks. In every second injection a different carrier was used. Rabbits were routinely bled 7–10 days after a given boost, starting after the second one. Immunoreactivity of the sera was determined by enzyme-linked immunosorbent assay (ELISA) as described [25]. Microtiter plates were coated with the appropriate peptide antigen and incubated with serum (diluted 10^2–10^5). Antibody binding was detected by secondary anti-rabbit antibody conjugated to alkaline phosphatase which catalyzed a colorimetric reaction. Partial purification of the antisera was done by preadsorption on HeLa cells [26].

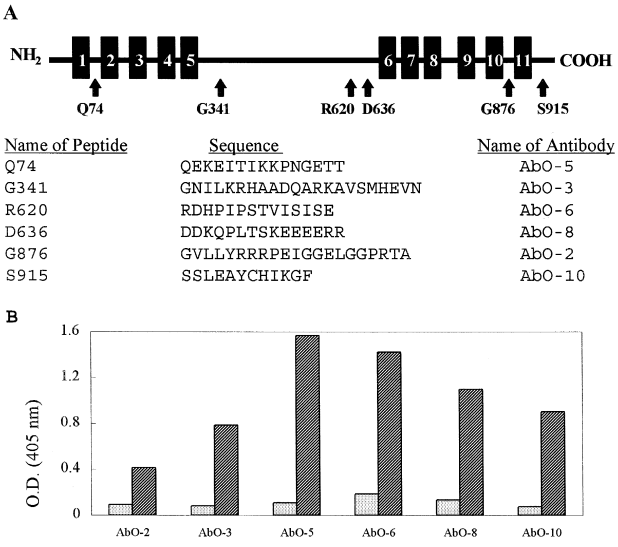

Fig. 2. The preparation of anti-peptide antibodies. (A) Schematic presentation of the Na^+–Ca^{2+} exchanger protein based on the hydropathy plot. Dark boxes represent the putative transmembrane helices (numbered from 1 to 11) and the dark line represents the hydrophilic segments of the protein connecting the TM domains. The location of the sequences chosen for antibody production is marked by arrows. The name of each peptide starts with its first amino acid followed by its number in the linear sequence of the mature protein (after the cleavage of the signal peptide). The name of the corresponding antibody is also listed. (B) Antibody binding to the corresponding peptide tested by ELISA. Dotted columns represent the binding of the preimmune sera and stripped columns represent the antisera. Each well contained 500 ng of the appropriate peptide and the sera were diluted $1{:}10^4$.

2.5. Immunoblotting

For Western-blot analysis of the Na^+–Ca^{2+} exchanger, proteins from HeLa cells expressing the exchanger genes, or membranes from rat brain or dog heart were used. The protein extracts were boiled 5 min with Leammli sample buffer and were analyzed by 7% SDS-PAGE [27]. Transfer to nitrocellulose, treatment with blocking solution (3% BSA in PBS) and incubation with an appropriate dilution of primary antibody (purified sera diluted in blocking solution), was done by standard protocols [25]. ^{125}I-Protein A was used to detect antigen–antibody complexes.

2.6. Immunoprecipitation

Post transfection (14 h) cells grown in 12-well plates with methionine (Met)-free medium for 30

min, were washed and labeled with ^{35}S-Met (25 μCi/ml) in Met-free medium for 2 h. The labeled medium was removed, the cells were washed and incubated with unlabeled methionine containing medium for 1 h. Cells were lysed by RIPA (150 mM NaCl, 100 mM Tris-HCl pH 8, 1 mM EDTA, 0.5% deoxycholate, 1% Triton X-100) and the lysate derived from each well was incubated overnight at 4°C with 10 μl antiserum bound to 10 mg protein A-Sepharose beads. The beads were washed four times with RIPA, once with high salt solution (100 mM NaCl, 20 mM Tris-HCl pH-8, 1 mM EDTA) after which the proteins were eluted by boiling with Leammli sample buffer and analyzed by SDS-PAGE.

2.7. Indirect immunofluorescence

HeLa cells grown on polylysine coated coverslips (4×10^4 cells), were infected and transfected as described. Post transfection (18 h) cells were washed twice with PBS and fixed with 4% paraformaldehyde. For permeabilization, cells were incubated for 5 min in 0.25% Triton X-100 or Tween-20, Nonidate P-40 or, Digitonin. Permeabilized and non-permeabilized cells were incubated with an appropriate dilution of the primary antibody (in PBS containing 10% newborn calf serum (NCS)) for 3 h at 25°C or overnight at 4°C. To detect the FLAG epitope, the anti-FLAG monoclonal antibodies (mAb) (Kodak IBI) were used. Depending on the primary antibody, Fluorescein isothiocyanate (FITC)-conjugated goat anti rabbit or anti mouse (Jackson ImmunoResearch Laboratories, West Grove, PA) secondary antibodies were used. These antibodies were partially purified by preadsorption on HeLa cells [26], and diluted 1:1000 in PBS containing 10% NCS. Immunofluorescence microscopy was performed using epifluorescence optics (Zeiss Axioscop).

2.8. Secondary structure analysis

Secondary structure analysis was derived from the hydropathy analysis of Kyte and Doolittle [8] over a window of 20 amino acids using the Genetics Computer Group (GCG, Madison, WI) software and by the PHD Profile fed neural network systems from HeiDelberg [14–16] with the generous help of Dr. Burkhard Rost from EMBL Heidelberg.

3. Results

3.1. Putative topology of the Na^+–Ca^{2+} exchanger

Fig. 1 shows the putative topological models of the Na^+–Ca^{2+} exchanger. In the model shown in Fig. 1A, the length of transmembrane helices is based on hydropathy analysis over a window of 20 amino acids [20]. The extracellular orientation of the N-terminal relative to the plasma membrane is based on the location of the signal peptidase cleavage site [9] and the glycosylation site at N9 [10]. The model shown in Fig. 1B is the best model predicted by the network system (see Section 2). The network system does not identify the amino acid segments corresponding to transmembrane segment (TMS) 2 and TMS 6 of the hydropathy analysis based model (Fig. 1A) as helical transmembrane segments. Hence, the total number of TM helices decreases to 9. This model also predicts, that the orientation of the N-terminal is intracellular and that of the C-terminal is extracellular. The network system also predicts a 'second best model' (not shown) in which the protein is organized in 8 TM helices. In that model, the stretch of amino acids between 780 and 797 (TMS 6 of Fig. 1B) is predicted to be an extramembraneous loop, since the probability of its assignment as a helical transmembrane region (HTM) is similar to its assignment as a non-HTM region. The 8 TM helical model predicts that both the N- and C-terminals are intracellular. To determine the membrane topology of the protein, immunocytochemical analysis was carried out.

3.2. Preparation and characterization of site specific antibodies against the Na^+–Ca^{2+} exchanger

Based on the amino acid sequence of the cloned rat Na^+–Ca^{2+} exchanger gene [17,20], extramembraneous segments of high immunogenecity were chosen, corresponding peptides were synthesized and used to immunize rabbits. Fig. 2A shows the location of the sequences chosen for antibody production on a schematic presentation of the Na^+–Ca^{2+} exchanger based on the original topological model [2]. Peptides G341, R620 and D636, are localized within the large hydrophilic loop of the exchanger protein, which is cytoplasmic in all three putative models. Q74 is

within the first hydrophilic loop separating TMS 1 and TMS 2. G876 is localized within the hydrophilic segment separating TMS 10 and TMS 11 (Fig. 1A) or TMS 8 and 9 (Fig. 1B) or TMS 7 and 8 of the second best model of the network system (not shown). Peptide S915 corresponds to the C-terminal extramembraneous tail of the Na^+–Ca^{2+} exchanger. Antibody production was tested by ELISA using the respective immunizing peptides as the antigen (Fig. 2B). The sera of all the immunized rabbits, contained antibodies against the appropriate peptide antigen. Their affinity to the immunizing peptides, determined at dilutions between 10^{-2}–10^{-5}, was similar.

To find out whether these antibodies recognize the Na^+–Ca^{2+} exchanger protein, extracts from RBE-2 expressing HeLa cells were examined by Western analysis. Three of the antibodies, AbO-6, AbO-8 and AbO-10 that were prepared against peptides R620, D636 and S915 respectively (see Fig. 2), bound in a specific manner to a protein of about 130 kDa (Fig. 3), which fits the calculated molecular mass of the glycosylated Na^+–Ca^{2+} exchanger. The specific immunoreactive band was present only when the cells were transfected with *rbe-2* (Fig. 3, lanes 4, 8, 12), and not when the cells were transfected with the plasmid vector pBluescript KS (Fig. 3, lanes 3, 7, 11). No protein bands were detected, when the appropriate preimmune sera were used (Fig. 3, lanes 1, 2, 5, 6, 9, 10). Although antibodies AbO-2, AbO-3 and AbO-5 recognized in a similar manner to AbO-6, AbO-8 and AbO-10 the antigenic peptides against which they were raised, they did not bind to the exchanger protein. They failed to reveal the exchanger protein by ELISA or Western analysis, they did not immunoprecipitate the protein from transfected cells nor did they bind to the proteins in situ, as tested by immunofluorescence.

In order to confirm that the antibodies recognize also the exchanger protein present in excitable membrane preparations, we tested their ability to bind to proteins derived from brain and heart membranes, exhibiting Na^+–Ca^{2+} exchange activity. In Fig. 4, we compare by Western analysis the binding of the anti-peptide antibodies AbO-6 (A), AbO-8 (B) and AbO-10 (C) to the Na^+–Ca^{2+} exchanger present in native membranes. In sarcolemmal membranes and in synaptic membranes the anti-peptide antibodies bind to proteins of about 140 kDa. This band appeared in native membranes, only when serum from immunized rabbits was used. No specific bands were visible when preimmune sera were used. For comparison, the binding of all the three anti-peptide antibodies to *rbe-2* transfected cell extracts is also shown (Fig. 4).

The three rat isoforms of the Na^+–Ca^{2+} exchanger gene NCX1 are identical, except in a segment of amino acids within the large hydrophilic loop of the protein [17,20]. AbO-8 and AbO-10 were raised against conserved peptide segments, present in all three rat isoforms. AbO-6 was raised against a peptide segment which is present in RHE-1 and RBE-2, but missing in RBE-1. The ability of the anti-peptide antibodies to distinguish between the different Na^+–Ca^{2+} exchanger isoforms was tested by immunoprecipitation of the exchanger protein from HeLa cells transfected with each one of the three isoforms (Fig. 5A). Antibodies AbO-8 and AbO-10 immunoprecipitated proteins of about 130 kDa molecular mass from HeLa cells transfected with each one of the three isoforms. AbO-6 however, immunoprecipitated proteins only, when the transfection of the cells

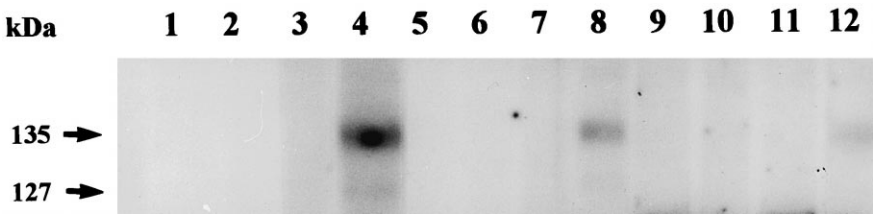

Fig. 3. Binding of the anti-peptide antibodies to the Na^+–Ca^{2+} exchanger protein expressed in HeLa cells. Proteins (100 μg in each lane) from *rbe-2* and from mock transfected HeLa cells extract were analyzed by Western blot. pBluescript-KS transfected cells: Lanes 1, 3, 5, 7, 9, 11; *rbe-2* transfected cells: lanes 2, 4, 6, 8, 10, 12. Proteins in lanes: 1, 2, 5, 6, 9, 10 were treated with preimmune sera; in lanes 3, 4 with AbO-6; in lanes 7, 8 with AbO-8; and in lanes 11, 12 with AbO-10. All sera were diluted 1:500. Antibody–antigen complexes were detected by ^{125}I-protein A.

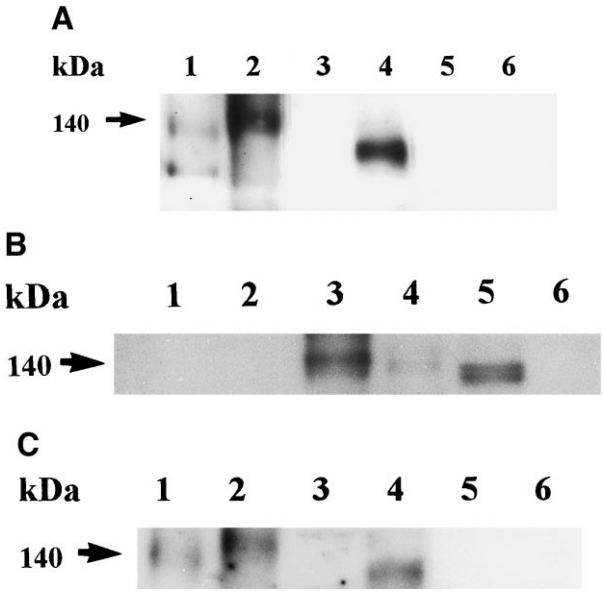

Fig. 4. Binding of the anti-peptide antibodies to rat brain synaptic plasma membranes and dog sarcolemma. Western-blot analysis was carried out with membrane proteins derived from rat brain synaptosomes and dog heart sarcolemma using antibodies AbO-6, AbO-8 and AbO-10. Binding of these antibodies to HeLa cells transfected with *rbe-2* (positive control) and with KS (negative control) was tested as well. (A) Binding of AbO-6 to rat brain synaptic membrane proteins (lane 1), to cardiac sarcolemmal membrane proteins (lane 2), and to proteins from HeLa cells transfected with pBluescript KS (lane 3) and *rbe-2* (lane 4). In lanes 5 and 6 the binding of the corresponding preimmune serum to rat brain membranes and to sarcolemmal membranes respectively is shown. (B) Binding of AbO-8 to brain membrane proteins (lane 4), heart proteins (lane 3), and proteins from pBluescript KS transfected cells (lane 6) and *rbe-2* transfected cells (lane 5). Binding of the preimmune serum to brain membrane proteins (lane 2) and to heart proteins (lane 1) is also shown. (C) Binding of AbO-10 to brain membrane proteins (lane 1), heart proteins (lane 2), and proteins from pBluescript KS transfected cells (lane 3) and *rbe-2* transfected cells (4). The binding of the corresponding preimmune serum to brain membrane proteins (lane 5) and to heart proteins (lane 6) is shown as well. In each lane, 50 μg of protein was layered and the sera were diluted 1:1000. ^{125}I-protein A was used for detection of the antibody–antigen complexes.

was carried out with *rhe-1* or with *rbe-2*, but not with *rbe-1*, as expected. AbO-6 and AbO-8 did not immunoprecipitate any proteins from mock transfected (with the plasmid vector pBluescript KS) HeLa cell extracts. The 95 kDa band, which is revealed when AbO-10 was used is present also when serum from this rabbit prior to immunization was used (not shown). This HeLa cell related protein, recognized by the non-immunized rabbit, is not detected in any of the native membrane preparations. Fig. 5B shows the Na^+ gradient dependent Ca^{2+} uptake activity expressed in whole HeLa cells, transfected with the three Na^+–Ca^{2+} exchanger isoforms. No transport activity is detected in mock transfected HeLa cells.

3.3. Topological studies of the Na^+–Ca^{2+} exchanger using the anti-peptide antibodies

Location of the epitope recognized by the anti-peptide antibodies relative to the plasma membrane, was tested, by indirect immunofluorescence (see Section 2). Fig. 6 shows an experiment, in which HeLa cells were transfected with *rbe-2* and exposed to the three anti-peptide antibodies, AbO-6, AbO-8 and

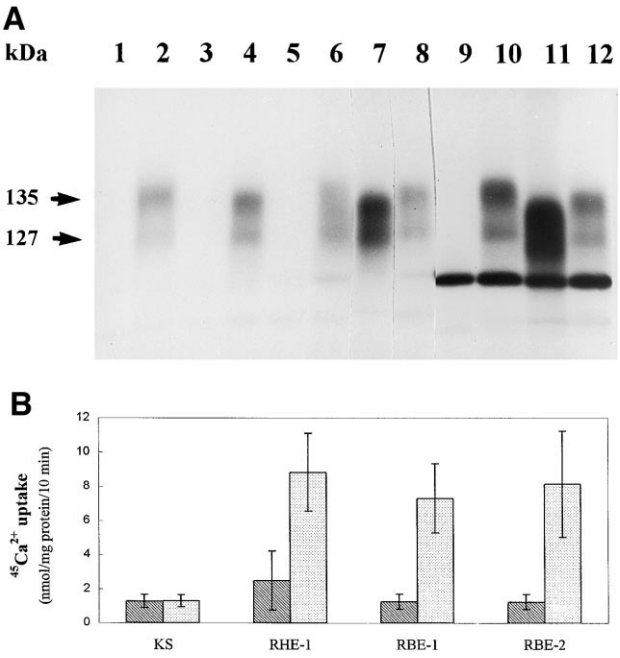

Fig. 5. The isoform specificity of the anti-peptide antibodies. (A) HeLa cells transfected with the three rat exchanger isoforms and mock transfected with the vector were metabolically labeled with ^{35}S-Methionine, immunoprecipitated and analyzed by SDS-PAGE as described in Section 2. Transfection with pBluescript KS: lanes 1, 5, 9; with *rhe-1*: lanes 2, 6, 10; with *rbe-1*: lanes 3, 7, 11; and with *rbe-2*: lanes 4, 8, 12. Antibodies used for immunoprecipitation: AbO-6: lanes 1, 2, 3, 4; AbO-8: lanes 5, 6, 7, 8; AbO-10: lanes 9, 10, 11, 12. (B) Ca^{2+} uptake with (dotted columns) and without (stripped columns) Na^+ gradient, by HeLa cells transfected with the three rat exchanger isoforms: *rhe-1*, *rbe-1*, *rbe-2*, and mock transfected with the vector KS.

AbO-10. Exposure of the cells to the antibodies in the absence of permeabilizing agents (Fig. 6, left side panel) did not reveal a fluorescent signal with any one of the antibodies, above the background. Phase microscopy showed, that this background fluorescence was associated with broken cells only. In 10 different experiments it did not exceed 2% of the total number of cells. Permeabilization of the transfected cells (Fig. 6, right side panel) with Triton X-100, revealed bright fluorescent staining in cells expressing the exchanger protein. No fluorescence was revealed when the primary antibody was omitted (not shown) or, in mock transfected cells (Fig. 6, panels A and B). Similar results were also obtained when Tween 20, Digitonin or Nonidate P-40 were used to permeabilize the cells. The finding that none of the antipeptide antibodies detected the exchanger protein without permeabilization suggests, that their corresponding epitopes are not accessible from the extracellular face of the membrane.

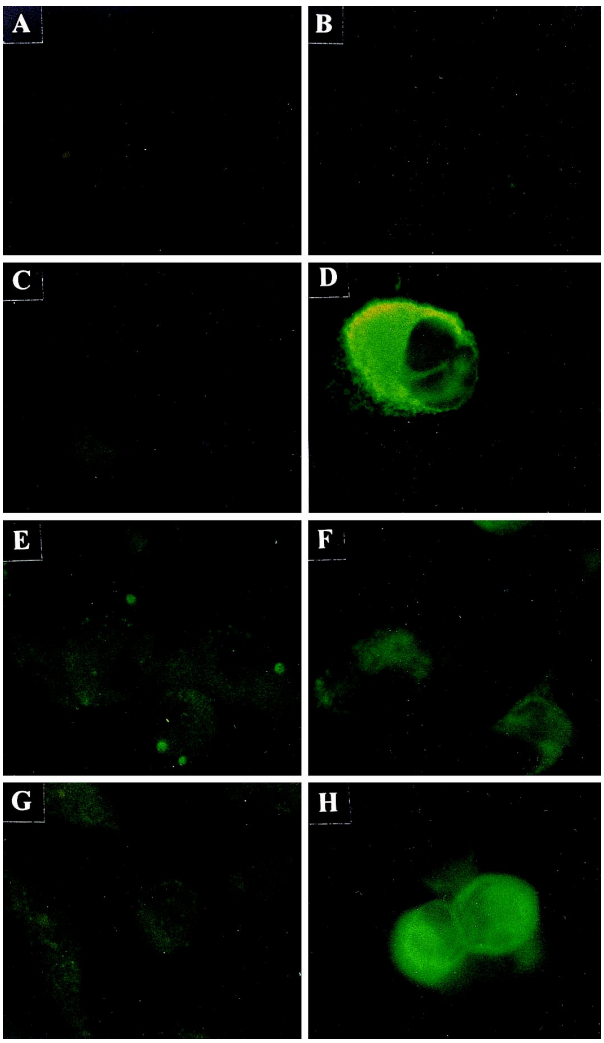

Fig. 6. The immunofluorescent labeling of RBE-2 expressed in HeLa cells. Transfection and treatment of the cells was carried out as described in Section 2. The right side panel shows labeling of cells permeabilized with Triton X-100 and the left side panel shows the labeling of non-permeabilized cells. Bound antibodies were detected with FITC- conjugated goat anti-rabbit antibodies. In A and B the control mock transfected cells (with pBluescript) are shown. The following anti-peptide antibodies were used: AbO-10: A, B, G, H; AbO-6: C and D; AbO-8: E and F.

3.4. Topological studies of the Na^+–Ca^{2+} exchanger using Flag epitope tagging

In order to obtain more information about the topology of the Na^+–Ca^{2+} exchanger protein, the method of epitope tagging was used. The Flag-epitope, consisting of eight hydrophilic amino acids, was introduced into the N-terminus of the protein (FN1 and FN2), the C-terminus (FC) and into selected putative extramembraneous loops of RBE-2 (see Fig. 7). Ten mutants containing the Flag epitope were constructed. Fig. 7 also shows the intracellular (in) or extracellular (out) location of the epitopes in the different topological models. Prior to usage of the Flag-tagged mutants for studying the topology of the Na^+–Ca^{2+} exchanger protein in the plasma membrane, we had to ensure that introduction of the epitope did not impair functional conformation. This was done by measuring Na^+–Ca^{2+} exchange activity in whole HeLa cells transfected with the mutant exchangers. The transport activity of the mutants was compared to that of the wild type exchanger RBE-2, as shown in Table 1. It can be seen, that only four out of the 10 Flag-tagged mutant exchangers: FN1, FN2, FE77 and FC, retained the ability to carry out Na^+–Ca^{2+} exchange. Insertion of the epitope into the N-terminal (FN1 and FN2 mutants), resulted in about 80% activity as compared to the wild type exchanger RBE-2. FE77 exhibited about 50% activity and the FC mutant 140% relative to RBE-2. Expression of Na^+–Ca^{2+} exchange activity in whole cells by these four Flag-tagged mutant proteins not only indicated that they retained functional conformation, but also that they were targeted to the plasma membrane.

Transfection of HeLa cells with all the other Flag-tagged mutants did not result in functional expression in whole cells. To rule out the possibility that these non-functional Flag mutants were defective in their protein synthesis, we have determined the amount of exchanger protein formed. Proteins derived from the Na^+–Ca^{2+} exchanger, were immunoprecipitated with the site specific antibody AbO-10. Immunodetection

B

			8 9 11
FN1	33 D P S F G ***D Y K D D D D*** *K* I A		
	5′ gac cca tct ttt ggg *gactacaaggacgacgac* gat aaa att gct 3′		i i o
FN2	4 A E T G G ***D Y K D D D D K*** E T T E C		
	5′ gca gaa aca gga gga *gactacaaggacgacgatgacaag* gaa acc act gaa tgt 3′		i i o
FE77	73 S Q E K E ***D Y K D D D D K*** L T I K K		
	5′ cc tct caa gaa aag gag *gactacaaggacgacgatgacaag ctt* acc ata aag aaa cc 3′		o o i
FD130	126 F T A G D ***D Y K D D D D K*** L R P S T		
	5′ ttc acc gca ggg gac *gactacaaggacgacgatgacaag ctt* ggt ccc agc aac 3′		o o o
FK162	158 G E T R K ***D Y K D D D D K*** L K H L R V		
	5′ gga gag acg agg aag *gactacaaggacgacgatgacaag ctt* aaa cat ctg cgt gtg 3′		i i i
FE749	745 V P P T E ***D Y K D D D D K*** Y W N G W		
	5′ gtc cca cct aca gaa *gactacaaggacgacgatgacaag* tat tgg aat ggc tgg 3′		i i o
FK807	803 T F A S K ***D Y K D D D D K*** L A A T Q D		
	5′ aca ttt gcc agc aaa *gactacaaggacgacgatgacaag ctt* gca gct acc cag gac 3′		o i o
FK854	850 G E Q F K ***D Y K D D D D K*** L S P G T L		
	5′ ggg gaa cag ttc aaa *gactacaaggacgacgatgacaag ctt* tcc cct ggc acg cta 3′		i o i
FE889	885 E I G G E ***D Y K D D D D K*** L G G P R T		
	5′ gaa ata gga ggt gag *gactacaaggacgacgatgacaag ctt* ggt ggg ccc cgg act 3′		o i o
FC	916 S L E G S V ***D Y K D D D D K***		i o i

Fig. 7. Construction of the Flag epitope tagged Na^+–Ca^{2+} exchanger mutants. (A) Schematic presentation of the Na^+–Ca^{2+} exchanger based on the hydropathy plot. Numbered boxes represent the 11 TM helices. Dark lines represent the extramembraneous hydrophilic segments. Arrows indicate the sites of the epitope tag insertion. (B) Oligonucleotides used for mutagenesis. The nucleotide and the translated amino acid sequences of the Flag tag are presented in italic letters. The sequences flanking the tag are shown for each mutant. The number preceding the position of the first amino acid of each primer corresponds to its location in the linear sequence of the protein. The underlined nucleotide sequences indicate the restriction sites that were created and the underlined amino acids are the corresponding altered amino acids. The following changes in amino acid sequences were done: I → L: FE77 and FK162; V → L: FK807 and FK854. The names of the mutants start with F (Flag) which is followed by the name of the amino acid at the site of the insertion and its position in the exchanger protein, except for the N-terminal (FN-Flag in the N-terminal) and C-terminal (FC-Flag in the C-terminal) mutants.

Table 1
The Na^+–Ca^{2+} exchange activity measured in HeLa cells expressing the Flag-tagged mutants

Mutant	Activity (SD)
RBE-2 (w.t.)	100
FN1	78 (20)
FN2	93 (17)
FE77	55 (22)
FD130	ND
FK162	ND
FE749	ND
FK807	ND
FK854	ND
FE889	ND
FC	139 (21)

Na^+ gradient dependent Ca^{2+} uptake was measured in whole HeLa cells expressing each one of the Flag mutants as described in Section 2. The exchange activity, is presented relative to that obtained in cells expressing RBE-2. The standard deviation (SD) is shown in brackets. The 100% activity corresponds to 6.88 nmoles $^{45}Ca^{2+}$/mg protein/10 min. (ND = not detectable).

of the FC-mutant, where the Flag epitope replaced the last 8 amino acids of the wild type exchanger, was done with antibody AbO-8. Fig. 8 demonstrates, that transfection with all the Flag-mutants resulted in the production of a 130 kDa protein, which fits the calculated molecular mass of the Na^+–Ca^{2+} exchanger. Moreover, using AbO-10, which recognizes the C-terminus of the protein, for immunoprecipitation, also indicates that the synthesis of the protein proceeded to completion.

To examine whether the non-functional Flag mutants were defective only in targeting to the plasma membrane, we have solubilized the membranes of the transfected cells and reconstituted their proteins. Reconstitution of the non-functional Flag mutants, did not rescue their transport activities.

Topology studies were done only with the four mutants that retained Na^+–Ca^{2+} exchange activity. The location of the Flag epitope in these mutant proteins respective to the plasma membrane was determined by indirect immunofluorescence, in a similar manner to that done with the anti-peptide antibodies. Intact or permeabilized HeLa cells expressing the mutants were incubated with the anti-Flag monoclonal antibody, and the binding of which was examined by exposure to FITC conjugated anti-mouse secondary antibody. The immunofluorescence obtained with intact cells is shown in the left side panel of Fig. 9 and that obtained in permeabilized cells, is shown in the right side panel. In panel A, it can be seen that cells transfected with FN2, the mutant containing the epitope in the N-terminal tail, stained around the cell periphery in the absence of permeabilization. None of the other mutants (FE77 and FC) expressing cells, stained under these conditions (panels C and E). Permeabilization of the cells resulted in diffuse fluorescent staining in the periphery and within the cells. These results indicate that the amino terminus of the exchanger protein is extracellular. In contrast to FN2, the insertion of the Flag epitope into the N-terminal at G37 (mutant FN1), results in different staining pattern. FN1 transfected cells stain only in the presence of a cell permeabilizing agent (not shown).

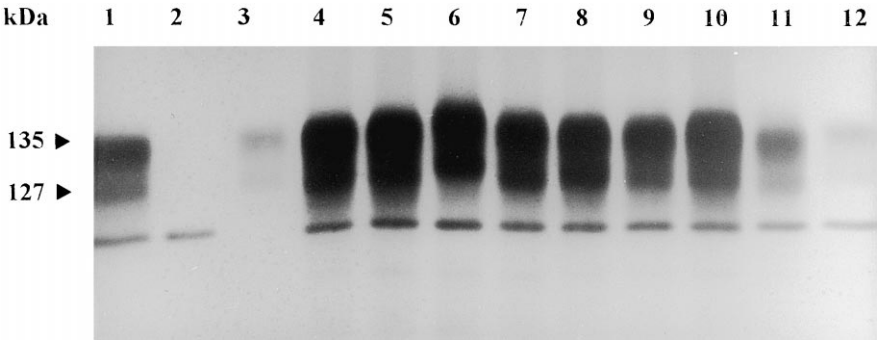

Fig. 8. The immunoprecipitation of the epitope-tagged RBE-2 expressed in HeLa cells. Proteins from cells transfected with the Flag-tagged mutants were precipitated by the anti-peptide antibody AbO-10 except of the FC mutant which was immunoprecipitated by AbO-8. Cells transfected with the wild-type *rbe-2* and mock transfected with pBluescript were used as controls. 1: RBE-2; 2: KS; 3: FC; 4: FE889; 5: FK854; 6: FK807; 7: FE749; 8: FK162; 9: FD130; 10: FE77; 11: FN1; 12: FN2.

4. Discussion

In this work, we used indirect immunofluorescence microscopy of cells expressing the Na^{+}–Ca^{2+} exchanger RBE-2 or its Flag-epitope tagged mutants, to study the membrane topology of the protein.

Of the six antipeptide antibodies prepared against chosen extramembraneous segments, three (AbO-6, AbO-8 and AbO-10) detected in a specific manner the exchanger protein. As shown in Fig. 4, the apparent molecular mass of the specific protein band, which is revealed by the antipeptide antibodies in transfected cells, is of somewhat higher mobility than that in native membranes. This can result from either a different glycosylation pattern in heart, brain and transfected HeLa cells, or from differences in conformation of the denatured proteins derived from different cells. There is also a difference of about 3 kDa between the exchanger protein identified in rat brain synaptic membranes and in dog sarcolemma. This fits

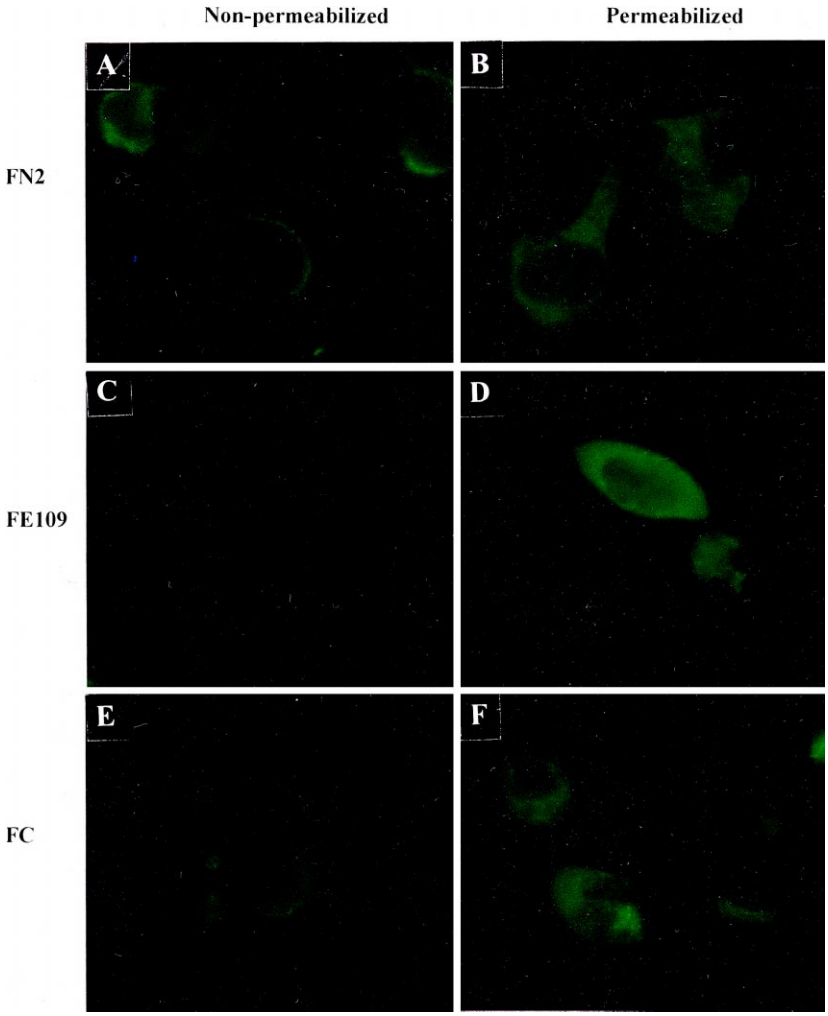

Fig. 9. The in situ detection of epitope-tagged RBE-2 by immunofluorescence. HeLa cells expressing the epitope tagged Na^{+}–Ca^{2+} exchanger isoform RBE-2 were treated with the anti-Flag monoclonal antibody (diluted 1:300). The right side panel shows labeling of cells permeabilized with 0.25% Triton X-100 and the left side panel shows the labeling of non-permeabilized cells. Bound antibodies were detected with FITC-conjugated goat anti-mouse antibodies (diluted 1:1000). The epitope tags in constructs FE77 (C and D) and FC (E and F) were detected only in permeabilized cells, while the tag in FN1 (A and B) was revealed both in permeabilized and in non-permeabilized cells.

the expected difference in molecular mass, based on the splice variant that is expressed in each one of these tissues [28].

To date, three different genes were found to code for the mammalian Na^+–Ca^{2+} exchanger: NCX1 [2], NCX2 [3] and NCX3 [4]. NCX1 and probably NCX3 [29] are alternatively spliced. NCX1 splicing can lead to the expression of over 32 isoforms [31]. In the brain and in the kidney, multiple isoforms of the NCX1 gene are expressed [28,30]. AbO-8 was raised against a peptide segment which is present in all three brain (rat and rabbit) and kidney isoforms. AbO-6, however, was prepared against a segment that was present in one brain (RBE-2 or NaCa5) and one kidney (NaCa7) isoform [17,28]. In the brain, in addition to the NCX1 gene, NCX2 and NCX3 are also expressed. Although these gene products have about 70% homology to NCX1, they do not exhibit any sequence, which is homologous to those against which AbO-6 and AbO-8 were raised. Hence, we assume that these two antibodies will not be able to recognize the gene products of NCX2 and NCX3. We do not know whether AbO-10 will be able to bind to the exchanger proteins derived from NCX2 or NCX3, but the sequence homology in the C-terminal of the three genes suggests that this might be possible. Based on sequence homology, none of our antibodies should recognize the NCKX gene products. It is not known whether each isoform of NCX1 or any other exchanger genes are expressed in different cells or, if the different gene products co-localize in the same cells. No information is available whether their relative expression pattern changes during development or in different physiological and pathological conditions. The combined usage of our three antibodies should provide useful tools to answer these questions.

Immunocytochemical studies with the three antipeptide antibodies AbO-6, AbO-8 and AbO-10 show that the detection of the fluorescent signal derived from RBE-2 transfected cells in situ depends on the presence of detergent. This suggests that the epitopes to which the antibodies bind are not accessible from the extracellular face of the membrane. Two of these antipeptide antibodies (AbO-6 and AbO-8), were raised against protein segments in the large loop (see Fig. 2) of the exchanger protein. This loop is predicted to be intracellular by all models that we have considered. This also fits the identification of amino acids 219–238 [2] as a consensus sequence for calmodulin binding, the presence of a phosphorylation site between amino acids 240–475 [12] and the localization of an internal Ca^{2+} regulatory site, in the amino acid segment 440–510 [32]. Taken together with the intracellular location of amino acids 620–650 as revealed by our immunofluorescence studies (see Fig. 6) and the binding studies with monoclonal antibodies [13] to cardiomyocyte homogenates and not to intact cells, the hydrophilic stretch of amino acids starting at R219 and ending either at S725 (Fig. 1A) or at N752 (Fig. 1B), is intracellular.

The studies with AbO-10 (Fig. 6, panels G and H) and with cells expressing FC, the epitope tagged C-terminal mutant of RBE-2 (Fig. 9 panels E and F) provide experimental evidence, that the carboxy terminal extramembraneous tail of the exchanger is cytoplasmic. Hence, an even number of TM segments has to separate the large cytoplasmic loop and the C-terminal tail of the protein. This requirement fits the hydropathy analysis based model (Fig. 1A) in which 6 TM helices separate the large cytoplasmic loop and the C-terminal tail and the 8 TM helical model (the second best) of the network system (see the legend to Fig. 1B), in which 4 TM helices are identified between N752 and the C-terminal. Our immunofluorescence studies with the FN2 mutant of RBE-2 show that the amino terminal of the exchanger protein is accessible by the anti-Flag antibody without permeabilizing the transfected cells in situ (Fig. 9) indicating that the exchanger protein is an N_{out}–C_{in} transporter. This fits only the model based on hydropathy analysis (Fig. 1A). Moreover, the extracellular location of the N-terminal also suggests that an uneven number of transmembrane segments should separate the amino terminal and the large intracellular loop. We do not know why detection of fluorescent signal using FN1 tagged RBE-2 expressing cells depended on cell permeabilization. It is possible that due to the proximity of G37 to the membrane, or to the glycosylation site at N9, the epitope is not accessible to the antibody and detergent treatment is needed to reveal it.

Both network system based topological models HTM 8 or HTM 9 (see Fig. 1) identify only 4 TM segments between the amino terminal and the large cytoplasmic loop. They also predict, that the N-terminal of the protein is intracellular. This prediction does

not fit the experimental evidence provided by the FN2 mutant of RBE-2, the presence of the signal peptidase cleavage site between A(-1) and D1 [9] and the glycosylation site at N9 [10]. This also does not fit the immunofluorescence studies with FE77 tagged RBE-2 expressing cells that suggest that the Flag epitope inserted after E77 is intracellular (see Fig. 1). Combination of the experimental evidence obtained with FN2, FN1 and FE77 mutants show, that the amino terminal tail of the protein is extracellular, it is followed by a hydrophobic stretch of amino acids identified in the three models as TMS1 and an intracellular hydrophilic loop that connects to TMS2.

Insertion of the Flag epitope into all other locations except the N- and C-terminals and the extramembraneous loops connecting TMS1 and TMS2, impaired functional expression. Hence, these mutants could not be used to solve inconsistencies among topological models. Since transfection of the cells with these mutant exchangers resulted in the synthesis of protein it is possible, that the introduction of the Flag epitope either impaired the functional conformation of the protein or, interrupted a linear segment, the sequence of which is of importance to Na^+–Ca^{2+} exchange. In an attempt to clarify the inconsistency between the experimental evidence and the topological predictions of the network program, we have submitted for analysis the complete amino acid sequence derived from the open reading frame of the cloned *rbe-2* (including the signal peptide). This, however, resulted in identical topological predictions to those obtained for the signal peptide truncated protein. Fischbarg et al. [33] compared the predicted structures of several transport proteins and channels by hydropathy analysis over windows of 21 and 7 amino acids, by the neural network program (PHD) and by their own program UCFP. They suggest that many inconsistencies originate in the assumption that TMS of membrane proteins are α helices, when they are in fact organized as antiparallel β barrels. If this applies to the structure of the Na^+–Ca^{2+} exchanger as well, it might provide an explanation for the different topological models.

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