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Biosynthesis and initial processing of the cardiac sarcolemmal Na^+–Ca^{2+} exchanger

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Based on the deduced amino-acid sequence of the cardiac Na^+-Ca^{2+} exchanger, there are six potential *N*-linked glycosylation sites and a potential cleaved signal sequence. To study the post-translational modifications of the exchanger, in vitro translation was examined in the presence and absence of canine pancreatic microsomes. Glycosylation, detected as endoglycosidase H induced shifts in molecular size, was examined for proteins having different numbers of potential N-linked glycosylation sites by using full and partial length RNA transcripts. In the presence of microsomes, the molecular mass of the full-length clone increased from 110 to 113 kDa. Endoglycosidase H treatment led to a reduction to 108 kDa, indicating that glycosylation increases the molecular mass by approx. 5 kDa and a signal sequence of approx. 2 kDa is cleaved during processing. Analysis of molecular-mass shifts obtained with partial transcripts suggested that glycosylation occurs at position N-9. This was confirmed by site-directed mutagenesis studies. A molecular mass of approx. 120 kDa was measured for Western blots of cardiac sarcolemmal membrane or oocytes expressing the wild-type exchanger. The molecular mass was reduced by approx. 10 kDa for the N9Y mutant or from exchanger obtained from a baculovirus-infected insect cell line where glycosylation does not occur. The giant excised patch technique was used to determine the functional consequences of glycosylation. Na^+-Ca^{2+} exchange current was examined in patches from oocytes expressing either the wild-type or N9Y mutant. The non-glycosylated mutant exhibited the same properties as the native exchanger with respect to voltage, sodium dependence, and the effects of chymotrypsin. The results indicate that glycosylation does not affect exchanger function in *Xenopus* oocytes and help to define exchanger topology.

Introduction

Cardiac muscle relaxation occurs upon reduction of myoplasmic Ca^{2+} levels. This Ca^{2+} is either sequestered into the sarcoplasmic reticulum or extruded from the cell. The primary mechanism for transsarcolemmal Ca^{2+} extrusion in cardiac muscle is Na^+-Ca^{2+} exchange [1]. Under normal conditions, Ca^{2+} extrusion by Na^+-Ca^{2+} exchange during relaxation is similar in magnitude to Ca^{2+} entry through Ca^{2+} channels during excitation. The Na^+-Ca^{2+} exchanger can also operate in the reverse direction and therefore can supply Ca^{2+} to the myoplasm under certain conditions [2–4]. Both modes of operation produce an electrical current due to the 3 Na^+ : 1 Ca^{2+} exchange stoichiometry. These currents can be measured in a variety of preparations allowing direct assessment of exchanger function [5–8].

With the cloning of the Na^+-Ca^{2+} exchanger [9], detailed structure-function studies have become possible. The Na^+-Ca^{2+} exchanger shows very little homology to any other proteins of known sequence. Even the rod outer segment $Na^+/Ca^{2+},K^+$ exchanger bears little resemblance to the cardiac exchanger at the amino-acid level [10]. Hydropathy analysis of the exchanger led to an initial model with 12 transmembrane regions and a large intracellular domain. However, the NH_2-terminal region possesses characteristics typical of a signal sequence [11] and, thus, it was postulated that this site might be cleaved during processing. Subsequent studies examining the N-terminal sequence of the purified bovine cardiac Na^+-Ca^{2+} exchanger show that the protein sequence begins at the predicted cleavage site [12].

Based on the primary amino-acid sequence, the

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exchanger possesses six potential *N*-linked glycosylation sites (N-X-T/S). The cardiac exchanger is glycosylated, since the protein can be immobilized on wheat germ agglutinin columns [13], and the rod outer segment (ROS) exchanger is extensively glycosylated [10]. However, no functional effects of glycosylation of the exchanger have been reported. Expression of the cloned exchanger in an insect cell line (Sf9) produces a functional exchanger which is not glycosylated [14].

In this study, we have examined the biosynthesis of the Na^+-Ca^{2+} exchanger to gain insight into the topological arrangement and initial processing of this protein. Knowledge of the location of *N*-linked glycosylation sites is useful in assigning protein domains to the extracellular surface. In particular, we have examined the extent and location of *N*-linked glycosylation and have confirmed that a signal sequence is cleaved during post-translational processing of the cloned protein. In addition, we have demonstrated that glycosylation does not affect function of the Na^+-Ca^{2+} exchanger.

Materials and Methods

In vitro translation. To produce RNA for in vitro translation, plasmid TB11 [9] was first linearized with various restriction enzymes as described in the text. RNA was then synthesized with T7 RNA polymerase following the instructions of the manufacturer (BRL). In vitro translation was performed using 1 μg RNA, reticulocyte lysate (Promega), dog pancreatic microsomes (Promega) and [^{35}S]methionine (NEN) as recommended by Promega. In the absence of microsomes, the translation product aggregated and would not enter the gel. The aggregation could be prevented by including 0.1% Triton X-100 in the reaction mixture. For treatment with endoglycosidase H, 3 μl translation product were first incubated with 3.2 μl 10% SDS at 80°C for 3 min. To this, 12 μl containing 50 mM Na_2HPO_4 (pH 6.85), 0.2% mercaptoethanol, 0.4 mM PMSF and 5 μl endoglycosidase H (1 mU/μl, Boehringer-Mannheim) were added. Samples were incubated at 30°C overnight, acetone precipitated, and used for SDS-PAGE. Controls received the same treatment except endoglycosidase H was omitted. Gels were infiltrated with PPO, dried and exposed to X-ray film.

Production of the mutant N9Y. Mutant N9Y was produced by the Kunkel method of site-directed mutagenesis [15]. Uracil containing ssDNA was generated from the *Eco*RI-*Sac*I subfragment of the exchanger TB11 clone (bases 1–897) and mutagenized using a phosphorylated oligonucleotide primer with the sequence 5′-CGCCGGTTTCATAGCCTTCTCCTTC-3′. The *Pst*I–*Pst*I fragment of the mutated DNA was subcloned into a full-length exchanger clone to yield the mutant N9Y. cRNA was synthesized from N9Y using T3 RNA polymerase, injected into *Xenopus* oocytes and the oocytes assayed for Na^+-gradient dependent $^{45}Ca^{2+}$ uptake as previously described [16].

Western blot analysis. Oocytes were examined for expression of exchanger protein by Western blot analysis. Oocytes were solubilized essentially as described by Colman [17]. Three oocytes were dissolved in 30 μl of homogenization buffer (1% Triton X-100, 100 mM NaCl, 20 mM Tris Cl (pH 7.8)) by sonication. The yolk protein, pigment granules and lipid pellicle were removed by centrifugation in an eppendorf microcentrifuge for 10 min at 11 000 rpm. 10 μl of the supernatant were removed, using a microcapillary pipet tip, for SDS-PAGE. Sarcolemmal and Sf9 proteins were prepared as previously described [13,14]. Proteins were size separated by SDS-PAGE and transferred to nitrocellulose. The primary antibody probe was a polyclonal antibody to the exchanger [13] at a dilution of 1:2000. Color development was performed as previously described [18].

Electrophysiological measurements. The giant excised patch-clamp technique was used as previously described [5]. Briefly, pipettes were pulled from capillary tubing (Corning 8161) to a diameter of 80–100 μm using a horizontal pipette puller (Sutter Instruments, Novato, CA, USA). Pipettes were then fire-polished to a final diameter between 20–30 μm and were coated with a Parafilm and mineral oil mixture. Pipettes contained (in mM): 100 Cs-Mes, 21.5 TEA-Mes, 20 Hepes, 8 $CaCl_2$, 6 KCl, 1 $MgCl_2$, 0.02 EGTA (pH 7.1). An Axopatch 1-D amplifier (Axon Instruments, Foster City, CA, USA) was used for electrical recordings. All current records were filtered at 1 kHz. Data were recorded directly onto a Graphtec chart recorder (Irvine, CA, USA) or were digitized and stored on computer using Axotape Software (Axon Instruments).

Na^+-Ca^{2+} exchange currents were recorded 3–5 days after injection of cRNA. Oocytes were placed in Petri dishes containing (in mM): 67 KCl, 250 sucrose, 9 $MgCl_2$, 10 Hepes, 2.5 EGTA (pH 7.4). This hypertonic solution was used to shrink the oocytes prior to removal of the vitellin layer. Gigaseals (typically 1–2 GΩ) were also formed in this solution. After seal formation, patches were excised and moved to a chamber allowing rapid solution changes (approx. 100 ms exchange time). All experiments were performed at 34°C. The normal superfusate contained (in mM): 100 Li-Mes (or Cs-Mes), 20 Hepes, 20 TEA-Cl, 1 $MgCl_2$, 10 Cs_2-EGTA (pH 7.1). For Ca^{2+} containing solutions, 7.5 mM $CaCl_2$ was added yielding a free [Ca^{2+}] of approx. 1 μM. Outward Na^+-Ca^{2+} exchange currents were generated by rapidly switching to a solution where Li^+ or Cs^+ was replaced by Na^+. Unless indicated otherwise, the exchanger was deregulated by a 1 min exposure to 1–2 mg/ml chymotrypsin on the cytoplasmic surface of the patch. This treatment eliminates important regulatory properties including the require-

ment for intracellular regulatory Ca^{2+} and slow inactivation of the exchanger current waveform (termed Na_i-induced inactivation [19,20]). Presumably, this occurs by removal or disruption of regulatory sites on the cytoplasmic surface of the exchanger [19,20]. Contamination of Na^+-Ca^{2+} exchange currents by the native Ca^{2+}-activated Cl^- current [21] in oocytes was found to be insignificant in the low free [Ca^{2+}] superfusate.

Results

Fig. 1A is a hypothetical model of the secondary structure of the Na^+-Ca^{2+} exchanger based on hydropathy analysis of its amino-acid sequence [9]. Of the six potential glycosylation sites (N-X-T/S), only three are modelled to be extracellular. In addition, a consensus sequence for a cleavable leader peptide is located between transmembrane regions 1 and 2. Cleavage at this site was predicted [9] based on the criteria of von Heijne [11]. These characteristics are shared by a large number of pre-proteins which undergo post-translational processing in the endoplasmic reticulum. Fig. 1B also shows the general strategy to determine the location of glycosylation sites. This general approach has previously been used to analyse the glycosylation of the Na^+/glucose co-transporter [22]. The expected truncated proteins obtained from the different truncated RNA transcripts are shown with the number of potential glycosylation sites indicated. These RNA transcripts were synthesized from cDNA digested with the restriction enzymes *Bam*HI, *Mbo*II, *Stu*I, *Xho*II as indicated.

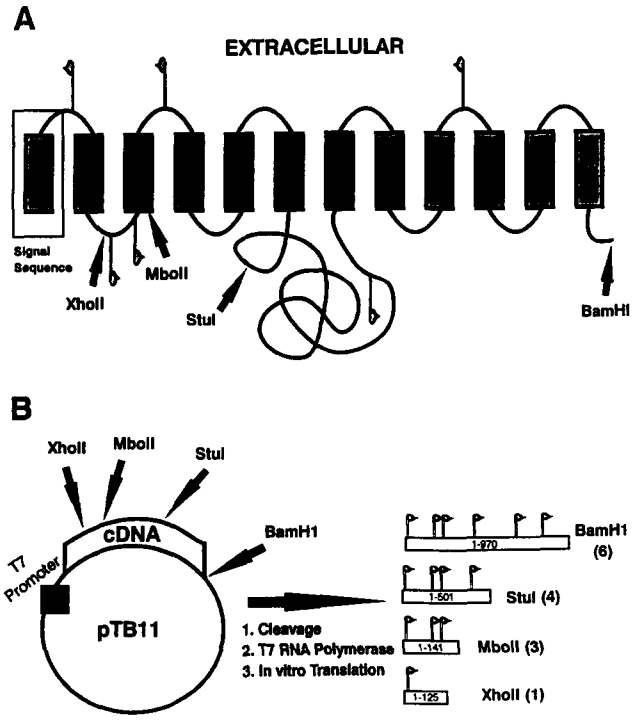

Fig. 1. (A) Original secondary structure model of the cardiac Na^+-Ca^{2+} exchanger showing the 12 transmembrane regions [9]. The six potential glycosylation sites (indicated by flags) and the signal-sequence-cleavage site are shown. (B) Illustration of the strategy for determining the location of glycosylation sites. The full-length exchanger clone was linearized by digestion at restriction enzyme sites at the 3′ end of the exchanger clone (*Bam*HI) or within the exchanger coding region (*Stu*I, *Mbo*II or *Xho*II). Full or partial-length exchanger transcripts were generated with T7 RNA polymerase and the transcripts were used in in vitro translation experiments to express truncated protein products containing the indicated number of potential *N*-linked glycosylation sites. The numbers in the rectangles give the lengths of the translation products in amino acids.

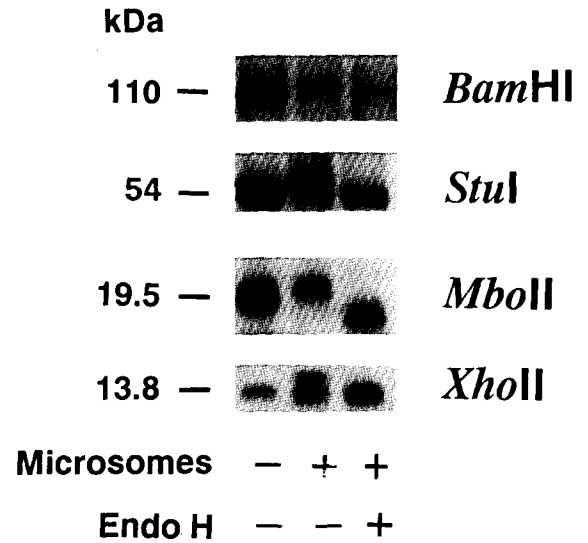

Fig. 2. Autoradiographs of four separate SDS gels showing the protein products obtained using in vitro translation. The transcripts used were obtained by restriction digestion as described in the text. The three lanes (from left to right) represent the protein products obtained without microsomes, with microsomes and with microsomes followed by endoglycosidase H treatment.

In vitro translation

To examine post-translational modification of the exchanger, in vitro translation was performed using the rabbit reticulocyte system with and without canine pancreatic microsomes in the presence of [^{35}S]methionine. Fig. 2 presents autoradiograms obtained using the full length transcript and the various truncated RNAs. Shown are proteins produced without microsomes (lane 1), with microsomes (lane 2) and with microsomes followed by endoglycosidase H treatment (lane 3). For the full-length clone (*Bam*HI), the addition of microsomes increased the molecular mass of the protein by approx. 3 kDa. Following treatment with endoglycosidase H, the molecular mass was reduced by approx. 2 kDa compared to the protein in the absence of microsomes. This indicates that, in the presence of microsomes, glycosylation added approx. 5 kDa to the exchanger while cleavage of a leader or signal peptide reduced the mass by approx. 2 kDa. Based on the

predicted location of cleavage for the signal sequence (between amino acids 32–33), an decrease of approx. 3 kDa is expected. These opposing effects resulted in the observed increase of approx. 3 kDa with microsomes.

The exchanger clone was truncated with different restriction enzymes to produce transcripts of differing lengths as shown schematically in Fig. 1B. The truncated protein products are shown in Fig. 2. In all cases, molecular mass increased in the presence of microsomes. It increased by about 3, 3, 2.5 and 2.7 kDa for the *Bam*HI, *Stu*I, *Mbo*II and *Xho*II proteins, respectively. Notably, the increase in molecular mass was similar for all proteins, despite the differences in the number of potential glycosylation sites. The protein produced from the *Xho*II-truncated product contains only 1 of the 6 potential *N*-linked glycosylation sites. Thus, this must be the one site at which glycosylation occurs. Additionally, treatment with endoglycosidase H led to a decrease in mass to below the unprocessed size for all cases. The decrease was approx. 2 kDa in all cases except when using the *Xho*II transcript. Here, the decrease was only approx. 0.8 kDa but the analysis is complicated by the apparent presence of unprocessed protein, even in the presence of microsomes, indicated by the two bands in the middle lane. Thus, the 0.8 kDa decrement may reflect the average of both processed and unprocessed protein. Incomplete processing by microsomes in in vitro translation assays is a frequent occurrence. Molecular-mass shifts in these experiments were small but readily reproducible.

Site-directed mutagenesis

Glycosylation of Asn-9 was verified by site-directed mutagenesis of the residue to a tyrosine. Note that position 9 refers to the ninth residue following the site of cleavage of the signal peptide [23]; this numbering system is different from that initially published [9]. Oocytes injected with N9Y cRNA expressed normal levels of exchanger activity as measured by Na^+-gradient-dependent $^{45}Ca^{2+}$ uptake (not shown). Exchanger proteins from oocytes, cardiac sarcolemma and Sf9 cells were examined by Western blot analysis. The sarcolemmal and Sf9 samples were prepared as previously described [13,14]. Oocytes were solubilized with Triton-X100, the insoluble material was removed by centrifugation, and the solubilized material was used for Western blots (Fig. 3). Cells injected with wild-type cRNA displayed normal weak reactions (Fig. 3, lane 2). Both sarcolemmal membranes and oocytes expressing wild-type exchanger had bands at about 120 and 160 kDa (lanes 1 and 2). The 160 kDa band may be an artifact due to sample preparation for SDS-PAGE [12]. As is frequently observed with glycosylated membrane proteins, the exchanger protein, whether expressed in native membranes or in oocytes, migrates as a slightly diffuse band, possibly due to heterogeneity in the

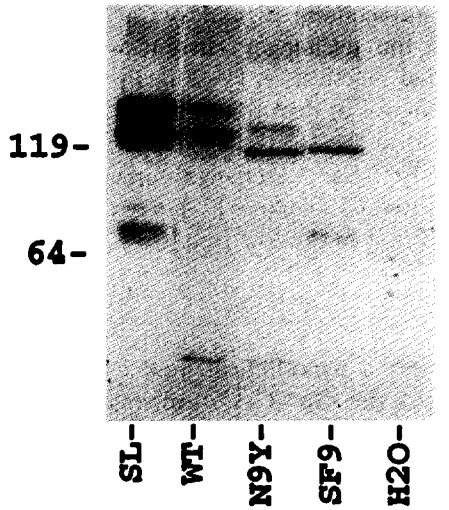

Fig. 3. Western blot analysis of sarcolemmal membrane protein, oocytes injected with either water or RNA encoding the wild-type or N9Y mutant exchanger protein and a baculovirus-infected insect cell line (Sf9) expressing the wild-type exchanger. Trans-blots were probed with a polyclonal antibody to the exchanger as detailed in Materials and Methods.

carbohydrate group. The exchanger from oocytes injected with N9Y cRNA exhibited an increased mobility on SDS-PAGE as seen by bands migrating at about 100 and 140 kDa (lane 3). Similar mobility is observed for the exchanger from Sf9 cells (lane 4) which is not glycosylated, as previously shown by Li et al. [14]. Note that the bands are less diffuse. No reaction was observed from cells injected with water (lane 5).

Electrophysiological measurements

We examined properties of the Na^+-Ca^{2+} exchange current to determine whether or not removal of glycosylation had functional consequences. The exchanger is known to possess regulatory mechanisms which include: (1) Na_i^+-induced inactivation which is manifest as a slow decay in current upon application of internal Na^+ [20] and (2) Ca_i^{2+}-induced activation of the Na^+-Ca^{2+} exchanger. That is, in addition to being transported, there also appears to be a cytoplasmic regulatory site for Ca^{2+}. This site must be occupied for exchange activity to occur. At very low cytoplasmic $[Ca^{2+}]$ ($\ll 1$ μM), outward Na^+-Ca^{2+}-exchange current cannot be activated.

Both of these regulatory properties can be eliminated by proteolysis of the cytoplasmic surface with chymotrypsin. This deregulation of the exchanger generally leads to a simpler waveform which does not decay and eliminates the requirement for cytoplasmic Ca^{2+}. In the absence of cytoplasmic Ca^{2+}, the problem of contamination of the Na^+-Ca^{2+} exchange current by the native Ca_i^{2+}-activated Cl^- conductance of oocytes is eliminated.

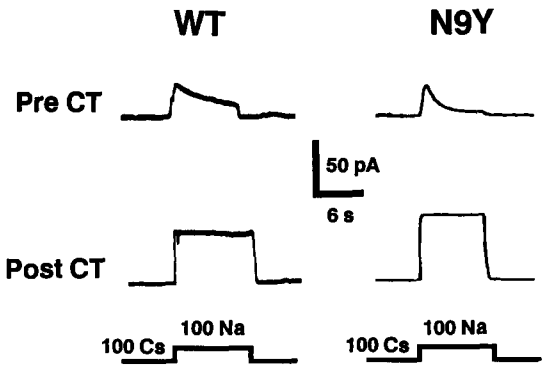

Fig. 4. Outward Na^+-Ca^{2+} exchange currents in giant excised patches from oocytes expressing the wild-type (WT) and N9Y mutant Na^+-Ca^{2+} exchange protein. Outward currents were elicited by replacing 100 mM Li^+ with 100 mM Na^+ at the cytoplasmic surface of the patch. Currents are shown before (pre-CT) and after (post-CT) treatment of the excised patch with 1 mg/ml chymotrypsin to remove secondary regulation. Ca^{2+} (1 μM) was present in the bath (cytoplasm) for the pre-CT case only. Prior to CT treatment, Na application in the absence of cytoplasmic Ca^{2+} did not elicit any current (not shown). After CT treatment, current could be elicited in the absence of cytoplasmic Ca^{2+} (secondary Ca^{2+} regulation) and the current waveform did not inactivate (Na^+-dependent inactivation).

Fig. 4 illustrates the outwardly directed Na^+-Ca^{2+}-exchange current in giant excised inside-out patches in response to rapidly replacing 100 mM Li^+ with 100 mM Na^+ on the cytoplasmic surface of the patch with 8 mM Ca^{2+}, 0 Na^+ solution in the pipette. Similar results were obtained when Cs^+ was used to replace Na^+. Before chymotrypsin treatment, no currents were observed in the absence of cytoplasmic Ca^{2+} for patches from oocytes expressing either the wild-type (WT) or N9Y mutant (not shown). In the presence of 1 μM free cytoplasmic Ca^{2+}, however, the expected outward (positive) current was elicited by Na_i^+ application in both cases (upper panels), indicating that the N9Y mutant retains secondary regulation by Ca_i^{2+} [19,20]. Note that after reaching an initial peak, the exchange current decays, indicating that Na_i^+-dependent inactivation [19,20] is also retained by the N9Y mutant. The small differences in the Na_i^+-induced inactivation rate were commonly observed both within and between oocyte populations but no consistent differences were noted between the wild-type and N9Y mutants. After briefly (approx. 1 min) treating the cytoplasmic surfaces of the patches with 1 mg/ml chymotrypsin (lower traces), both secondary Ca_i^{2+} regulation and Na_i^+ dependent inactivation were lost in patches expressing wild-type or N9Y mutant exchanger. Currents could be activated by 100 mM Na^+ in the absence of cytoplasmic Ca and the current waveform did not show substantial inactivation.

Fig. 5 illustrates the effects of different cytoplasmic Na^+ concentrations on the outward Na^+-Ca^{2+} exchange current in chymotrypsin-treated patches. Typical traces are shown for patches from oocytes expressing the wild-type or N9Y mutant exchange protein. No significant differences were apparent in the ability of Na^+ to activate exchange current between the two groups. Pooled data are shown in the graph and were fit to a binding isotherm to estimate the Na^+ affinity. For individual experiments, K_m values between 20–30 mM were obtained. These values are similar to that reported for a variety of preparations and measurement techniques [6,20]. A modest difference in the shape of the two curves is unlikely to be of significance. Thus, the presence or absence of glycosylation does not appear to alter the basic transport mechanism or binding-site affinity for Na_i^+.

The Na^+-Ca^{2+} exchanger is electrogenic with a 3:1 Na^+/Ca^{2+} stoichiometry [1,24]. We examined the ef-

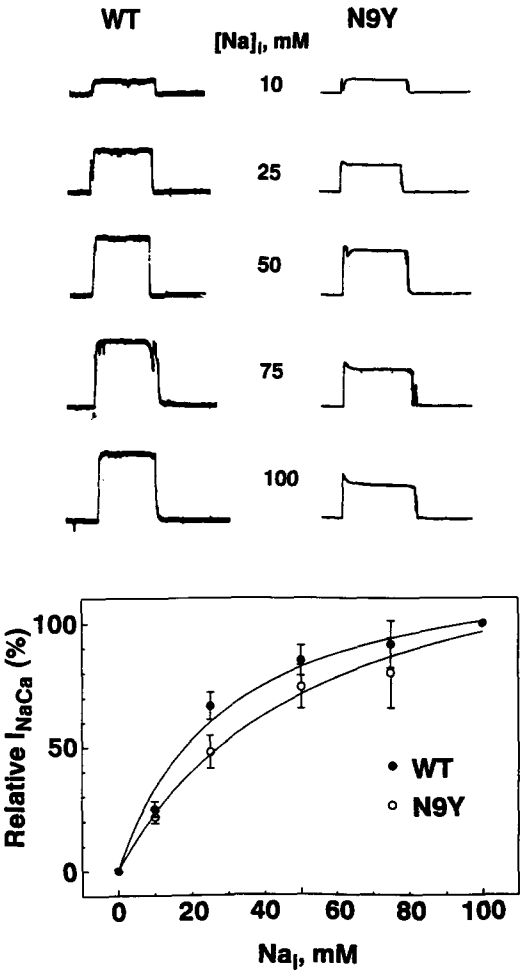

Fig. 5. Dependence of outward Na^+-Ca^{2+} exchange current on cytoplasmic Na^+ in the wild-type (WT) vs. N9Y mutant. Currents were activated by the application of various concentrations of Na^+ to the cytoplasmic surface of the patch as indicated. The total $Li^+ + Na^+$ concentration was 100 mM in all cases. Patches had been treated with 1 mg/ml chymotrypsin. Pooled data (mean ± S.E.; WT, $n = 3$; N9Y, $n = 3$) are fitted to a binding isotherm in the lower graph, yielding half maximal activation of the Na^+-Ca^{2+} exchange current at approx. 20–30 mM Na^+ in all cases.

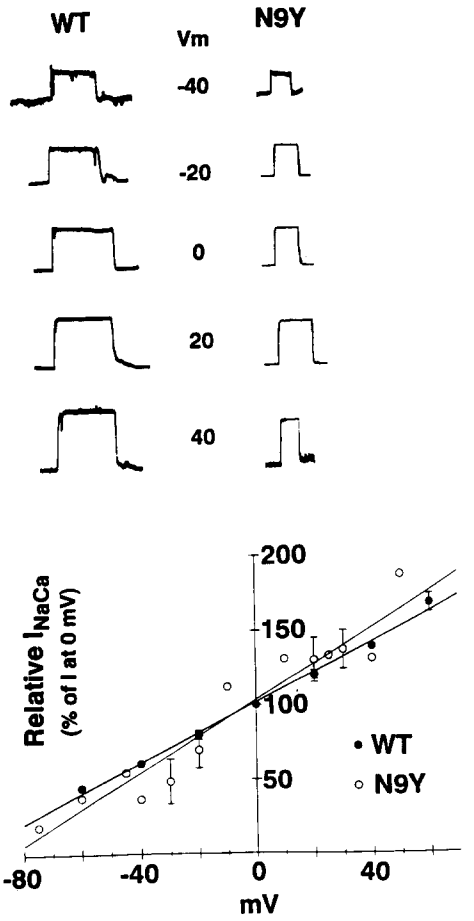

Fig. 6. Voltage dependence of outward Na^+-Ca^{2+} exchange current for the wild-type (WT) and N9Y mutant Na^+-Ca^{2+} exchanger. Patches were pretreated with 1 mg/ml chymotrypsin. Outward current was activated by replacing 100 mM Li^+ with 100 mM Na^+. The I/V relationship for pooled data (mean ± S.E.; WT, $n = 3$; N9Y, $n = 3$) is shown below. For each patch, current was normalized to the value obtained at 0 mV.

fects of transmembrane voltage on steady-state exchange currents as shown in Fig. 6. In all cases, currents were generated by replacing 100 mM Li^+ with 100 mM Na^+ in chymotrypsin-treated patches. At positive holding potentials, Na^+-Ca^{2+}-exchange currents were enhanced. Conversely, negative holding potentials led to a decrease in exchange currents. The steady-state current voltage relationship for the wild-type and mutant exchanger proteins are shown below. Again, there were no apparent differences between the two groups. Thus, removal of the glycosylation site does not appear to alter the voltage dependence of the Na^+-Ca^{2+} exchanger.

Discussion

The aim of the present study was to gain insight into the topology and initial processing of the Na^+-Ca^{2+} exchange protein. In particular, our intentions were to identify the extent and location of glycosylation using the cloned exchanger. Our results indicate that glycosylation occurs at a single site, N-9. We also verified that a leader peptide is cleaved during processing as predicted from the amino-acid sequence [9] and by NH_2-terminal amino-acid sequencing [12]. This was achieved by examining the in vitro expression of the exchanger with and without canine pancreatic microsomes. The effects of glycosylation were then examined on Na^+-Ca^{2+}-exchanger function using the giant patch clamp technique. Glycosylation does not appear to alter levels of activity of the Na^+-Ca^{2+} exchanger expressed at the cell surface in oocytes.

A common motif among transmembrane proteins is the presence of glycosylation at the extracellular surface. While the role of this glycosylation remains uncertain, it may be important for directing certain proteins to the extracellular surface. Based on the primary sequence of the exchanger, there are 6 potential *N*-linked glycosylation sites (N-X-T/S). Hydropathy analysis places three of these sites at intracellular locations. However, the reliability of hydropathy analysis is limited [25] and, thus, identifying the exact location of glycosylation provides important topological information. That is, sites of *N*-linked glycosylation define an extracellular segment of a protein. The partial transcripts used to produce the truncated exchanger proteins were selected to permit unequivocal assignment of glycosylation to any of these potential glycosylation sites. A similar approach has been used previously to study the Na^+-glucose cotransporter [22].

The assignment of a single glycosylation site to position N-9 on the Na^+-Ca^{2+} exchanger is based on the following observations: (i) A similar increase in M_r in the presence of microsomes was observed for all transcripts despite differences in the number of potential *N*-linked glycosylation sites. The shortest of these transcripts coded for a protein with only one potential glycosylation site: the one at position N-9. (ii) Endoglycosidase H treatment produced a similar decrease in M_r for protein products obtained with the full length clone and for all truncated partial transcripts. The one exception was the result obtained with the *Xho*II-truncated transcript. In this case, however, the data are complicated by the presence of some unprocessed protein even in the presence of microsomes. (iii) Site-directed mutagenesis techniques were used to produce the N9Y mutant. Compared to the wild-type or sarcolemmal exchanger, the molecular mass of this mutant was reduced by about 10 kDa to the size of the non-glycosylated exchanger produced by insect cells. Thus, similar to endoglycosidase H treatment of the wild-type exchanger, elimination of the putative glycosylation site by mutagenesis reduces the molecular mass. The difference in the extent of glycosylation between oocytes and the in vitro translation system is likely due to further glycosylation that occurs in the Golgi apparatus.

The cardiac Na^+-Ca^{2+} exchanger does not appear to be extensively glycosylated compared to the photoreceptor $Na^+/Ca^{2+},K^+$ exchanger. The calculated molecular mass of the photoreceptor exchanger is approx. 130 kDa based on the amino-acid sequence, whereas the purified protein has an apparent molecular weight of 210–220 kDa. Substantial glycosylation is thought to be responsible for this discrepancy [10]. The photoreceptor exchanger possesses six potential *N*-linked glycosylation sites in the loop region between the apparent signal sequence and the first transmembrane segment. In contrast, only one potential glycosylation site is present in the equivalent region for the cardiac exchanger and this is the one site which is actually glycosylated.

The possibility that the Na^+-Ca^{2+} exchanger possesses a cleavable leader sequence was initially proposed on the basis of the deduced amino-acid sequence [9]. Subsequently, sequencing of the NH_2-terminal region of the purified bovine cardiac Na^+-Ca^{2+} exchanger demonstrated that the initial amino-acids corresponded to the predicted cleavage site [12]. On the cardiac Na^+-Ca^{2+} exchanger, a potential cleavage site based on the criteria of von Heijne [11] occurs between amino acids 32 and 33. An alanine residue occurs at position 32 from the initiator methionine. Preceding the cleavage site is a hydrophobic core region and a region containing Arg and His residues. Similarly, the rod photoreceptor exchanger is thought to possess a cleavable signal sequence [10]. For this protein, analysis of the NH_2-terminal segment indicated that Asp-66 is the first amino acid. Thus residues 1–65 are postulated to represent a cleavable leader sequence for the rod photoreceptor exchanger.

The presence of a signal sequence on the cardiac Na^+-Ca^{2+} exchanger is surprising in light of the fact that most membrane transporters do not encode a cleaved signal sequence [12]. This information is also of considerable importance with respect to topographic modelling of the exchanger. Strong support for an extracellular location of the NH_2-terminal region is provided by the observation that *N*-linked glycosylation occurs only at position N-9. It is unlikely that a transmembrane domain could precede this glycosylation site, as it would be composed of eight relatively hydrophilic amino acids. Thus, cleavage of a signal sequence and knowledge of the location of glycosylation places the NH_2-terminus extracellular. Therefore, the Na^+-Ca^{2+} exchanger falls into the general class of transporters with a large hydrophilic domain and 11–12 transmembrane segments. In the case of the mature cardiac Na^+-Ca^{2+} exchanger, it is modelled that there are five transmembrane segments on the NH_2-terminal side of the large hydrophilic domain and six transmembrane segments on the COOH-terminal side. This tentative model may require refinement.

The giant excised patch technique of Hilgemann [5] was used to assess the functional consequences of preventing glycosylation. We have shown that glycosylation does not affect several major properties of Na^+-Ca^{2+} exchange function in the oocyte. No substantial differences were observed with respect to Na affinity, voltage dependence, or regulation by cytoplasmic Ca^{2+}. While more detailed measurements may reveal functional roles for glycosylation, it does not appear that glycosylation affects the basic transport mechanism. In particular, we did not find any evidence that the added polysaccharide is involved in concentrating ions near the transport sites. Also, the elimination of glycosylation did not alter the voltage sensitivity of the exchanger suggesting that glycosylation does not alter the electric field sensed by the voltage-dependent transport step. Thus, it seems likely that glycosylation does not have important functional consequences for Na^+-Ca^{2+} exchange.

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