TEMPERATURE DEPENDENCE

OF CLONED TROUT CARDIAC NA^{+}-CA^{2+} EXCHANGER

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Abstract

The cardiac Na^{+}-Ca^{2+} exchanger (NCX1) is an important regulator of cytosolic [Ca^{2+}] in contraction and relaxation. Studies with trout heart sarcolemmal vesicles have shown NCX to have high activity at 7°C and this unique property is likely due to differences in protein structure. In this study, we describe the cloning of a NCX (NCX-TR1) from a ZAP cDNA library constructed from rainbow trout (*Oncorhynchus mykiss*) heart RNA. The NCX-TR1 cDNA has an open reading frame that codes for a protein of 968 aa with a deduced molecular weight of 108 kDa. A hydropathy plot indicates the protein contains 12 hydrophobic segments (of which the first is predicted to be a cleaved leader peptide) and a large cytoplasmic loop. By analogy to NCX1, NCX-TR1 is predicted to have 9 transmembrane segments. The sequences demonstrated to be the XIP site and the regulatory Ca^{2+} binding site in the cytoplasmic loop of mammalian NCX1 are almost completely conserved in NCX-TR1. Both canine NCX1 and trout NCX-TR1 cRNA was injected into *Xenopus* oocytes and after 3-4 days currents were measured using the giant excised patch technique. NCX-TR1 currents measured at ~23°C demonstrated Na^{+}-dependent inactivation and

Ca^{2+}-dependent activation in a manner qualitatively similar to NCX1. Currents of the expressed exchangers were also studied as a function of temperature over the range of 7-30°C. Both peak and steady currents of canine NCX1 decreased with decreased temperature with Q_{10} values of 2.5 and 2.6, typical of mammalian ion transporters. In trout NCX-TR1 both peak and steady state currents decreased to a much lesser degree with Q_{10} values of 1.2 and 1.4. These studies demonstrate that the unique temperature profile of trout NCX-TR1 is due to differences in the protein structures. Current research is focusing on the molecular mechanisms of these differences.

Introduction

The Na^+-Ca^{2+} exchanger (NCX) is an integral membrane protein that plays an important role in the regulation of cytosolic Ca^{2+} concentration in many cell types. NCX transports Ca^{2+} across the membrane with a stoichiometry of three Na^+ to one Ca^{2+} and is therefore electrogenic. The Na^+-Ca^{2+} exchanger in the lower vertebrate heart has not been studied in great detail. Despite this, there is considerable circumstantial evidence in teleost and amphibian hearts that NCX plays a critical role in E-C coupling in these species (Tibbits et al. 1992a). In the absence of a substantial SR in teleosts, it is likely that the NCX is the primary means of reducing cytosolic Ca^{2+} in normal mode and may be an important source of contractile Ca^{2+} in reverse mode. Previous studies in our laboratory have strongly suggested that the differential temperature dependencies in the mammalian and teleost NCX are due to important differences in the primary structures of these isoforms (Tibbits et al. 1992b). The cloning of the trout cardiac NCX (NCX-TR1) described here (Tibbits et al. 1999) represents the first and crucial step in providing a mechanistic explanation of these important physiological differences.

Materials and Methods

Salmonid NCX was cloned by screening trout heart ZAP cDNA library with a probe produced by PCR conducted with a pair of degenerate primers designed to amplify a highly conserved fragment of NCX. A full-length clone (T6-1) was isolated and both strands of the cDNA were sequenced by the dideoxynucleotide chain termination method using ABI's AmpliTaq Dye Terminator Cycle Sequencing. T6-1 was subcloned from the original pBluescript into a modified vector, in which the multiple cloning site of pBluescript was removed and only Bam HI and Hind III sites were left. The 3' untranslated region of T6-1 was replaced with that of the Na^+-glucose cotransporter clone, which contains a

poly(A)$^+$ tail. The Hind III site at nucleotide 2947 of T6-1 was removed by silent mutation using the Quickchange™ Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA). The mutation was made in a 550 bp cassette generated by Aat II digestion. T6-1 was linearized with Hind III and cRNA was synthesized using T3 mMessage mMachine™ In Vitro Transcription Kit (Ambion Inc., Austin, TX). Oocytes were injected with 5 nl of cRNA and exchange activity was measured 3-4 days after injection as outward Na^+-Ca^{2+} exchange currents which were measured using the giant excised patch technique (Omelchenko et al. 1998). The pipette solution contained (in mM): 100 NMG-MES, 30 HEPES, 30 TEA-OH, 16 sulfamic acid, 8 $CaCO_3$, 6 KOH, 0.25 ouabain, 0.1 niflumic acid, 0.1 flufenamic acid; pH 7.0 with MES. Outward Na^+-Ca^{2+} exchange currents were activated by switching from Li^+_i- to Na^+_i-based bath solutions containing (in mM): 100 [Na^+ or Li^+]-aspartate, 20 MOPS, 20 TEA-OH, 20 CsOH, 10 EGTA, 0-7.3 $CaCO_3$, 1.0-1.13 $Mg(OH)_2$; pH 7.0 with MES or LiOH.

Results

The trout heart T6-1 clone has a nucleotide sequence of 3302 bp with an open reading frame of 2904 bp from base 72 to 2975 encoding a protein of 968 amino acids with a deduced molecular size of 108 kDa, designated as NCX-TR1. Like the hydropathy plot of mammalian NCX1 (Nicoll et al. 1990), NCX-TR1 has 12 hydrophobic segments and a long hydrophilic region forming an intracellular loop. The first hydrophobic segment is designated as a signal peptide by analogy to NCX1 and is between residues 32 and 33. The sequence comparison of the NCX-TR1 with that of dog NCX1 shows ~75% identity at the amino acid level and 69% at the nucleotide level. The hydrophobic segments are highly conserved.

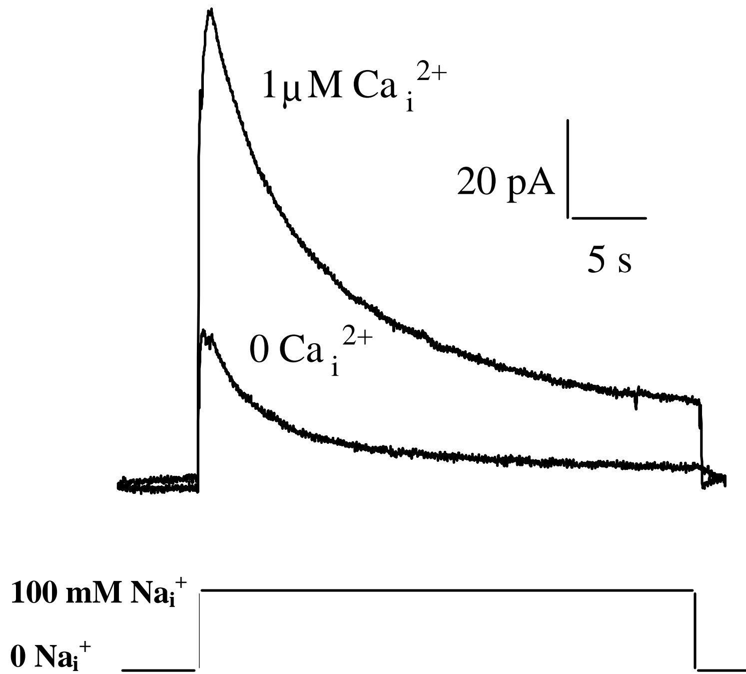

Figure 1. Outward Na^+-Ca^{2+} exchange currents for the NCX-TR1 exchanger expressed in Xenopus oocytes.

Currents were activated by the application of 100 mM Na^+ to the cytoplasmic surface of an excised patch of oocyte membrane. The pipette contained 8 mM Ca^{2+} (extracellular surface). As indicated on the overlapping current traces, records were obtained in the presence or absence of regulatory Ca^{2+} (1 μM) at the cytoplasmic surface. Outward Na^+-Ca^{2+} exchange currents show similar characteristics to other mammalian exchangers. That is, both peak and steady state outward currents were larger in the presence of regulatory Ca^{2+} demonstrating positive regulation of exchange current by Ca^{2+}_i. Also, in response to Na^+_i application, the current peaks and then slowly decays, indicative of Na^+_i-dependent inactivation.

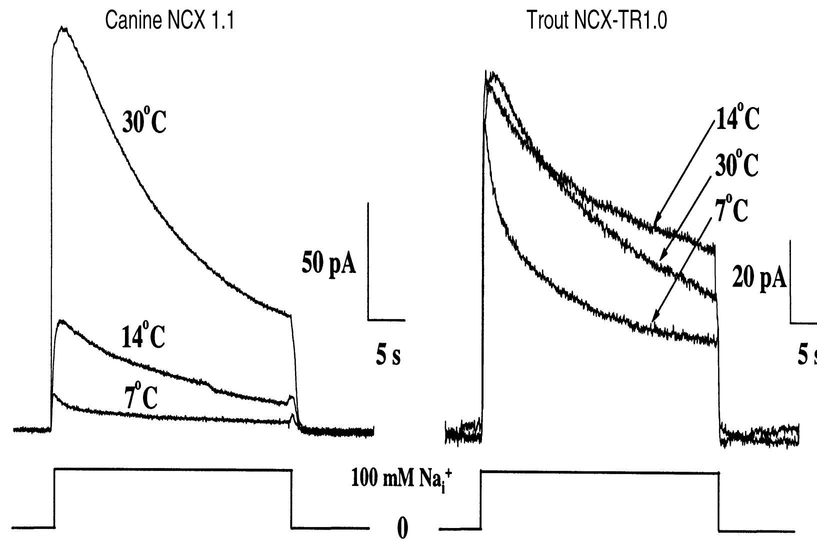

Figure 2. The temperature dependence of cloned canine and trout wild type Na^+-Ca^{2+} exchanger currents. Note differences at 7°C.

Discussion

We have cloned and expressed the trout heart Na^+-Ca^{2+} exchanger, NCX-TR1. The hydropathy plot is similar to mammalian NCX1, and predicts 12 hydrophobic segments and a large hydrophilic domain. The mammalian NCX1 has a cleavable signal peptide that is removed from the protein during biosynthesis in the endoplasmic reticulum. A potential cleavage site of NCX-TR1 is predicted to exist between amino acid 32 and 33 based on the sequence homology between mammalian NCX1 and NCX-TR1. Thus the topology of NCX-TR1 is not different to that proposed for NCX1 which is now modeled to have 9 transmembrane segments (Nicoll et al. 1999).

Sequence comparison, including the cleaved leader peptide, showed ~75% identity at the amino acid level to the dog NCX1 and 61% and 66% to rat NCX2 and NCX3, respectively. Like all NCXs, NCX-TR1 has the most divergent sequence at the N-terminus. Sequence identity becomes very high (85%) within the putative transmembrane segments consistent with their functional

significance for ion translocation. The last putative transmembrane segment is the least well-conserved transmembrane domain. Overall, the amino acid sequence of the intracellular loop sequence is 73% identical to NCX1, and as expected less conserved than the transmembrane segments. However, those regions within the loop with known functional importance are well conserved. The endogenous XIP site, consisting of 20 amino acids at the N-terminus of the loop, exhibits high conservation. There is strong evidence that the XIP site of NCX1 is involved in Na^+-dependent inactivation. The regulatory Ca^{2+} binding domains characterized by three consecutive aspartic acid residues have been found to be highly conserved in the NCX family and are completely conserved in NCX-TR1.

The high degree of conservation of the known regulatory components of the intracellular loop is reflected in the NCX-TR1 giant excised patch current records shown in Figure 1. The current decay or inactivation seen after peak current requires the presence of the XIP site in mammalian NCX1. The modulation of the NCX current by regulatory Ca^{2+} seen in both NCX1 and NCX-TR1 is dependent on the presence of the Ca^{2+} binding sites of the cytoplasmic loop. The regulation of the NCX-TR1 current by Ca^{2+} is positive making it similar to NCX1.

The gene coding for NCX is characterized by a cluster of six exons (A, B, C, D, E and F) coding for a variable region in the C-terminus of the large intracellular loop. Alternative splicing of these exons generates multiple tissue-specific variants of NCX. Exons A and B are mutually exclusive and are used in conjunction with the other four exons (C-F) to produce all NCX isoforms. Based on these findings, NCX-TR1 uses exon A and C, D and F. In comparison to NCX1.1 that is composed of exons ACDEF exon E is apparently missing from NCX-TR1 cDNA and this accounts for the 5 amino acid deletion in the alternate-splicing region.

In summary we have cloned the Na^+-Ca^{2+} exchanger from trout heart and have begun its characterization. When the cloned wild type NCX-TR1.0 is expressed in *Xenopus* oocytes it exhibits a temperature profile similar to that of the native molecule.

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