Determinants of cardiac Na\(^+\)/Ca\(^2+\) exchanger temperature dependence: NH\(_2\)-terminal transmembrane segments

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Marshall, Christian, Chadwick Elias, Xiao-Hua Xue, Hoa Dinh Le, Alexander Omelchenko, Larry V. Hryshko, and Glen F. Tibbits. Determinants of cardiac Na\(^+\)/Ca\(^2+\) exchanger temperature dependence: NH\(_2\)-terminal transmembrane segments. Am J Physiol Cell Physiol 283: C512–C520, 2002. First published March 20, 2002; 10.1152/ajpcell.00558.2001.—The cardiac Na\(^+\)/Ca\(^2+\) exchanger (NCX) in trout exhibits profoundly lower temperature sensitivity in comparison to the mammalian NCX. In this study, we attempt to characterize the regions of the NCX molecule that are responsible for its temperature sensitivity. Chimeric NCX molecules were constructed using wild-type trout and canine NCX cDNA and expressed in Xenopus oocytes. NCX-mediated currents were measured at 7, 14, and 30°C using the giant excised-patch technique. By using this approach, the differential temperature dependence of NCX was found to reside within the NH\(_2\)-terminal region of the molecule. Specifically, we found that ~75% of the Na\(^+\)/Ca\(^2+\) exchange differential energy of activation is attributable to sequence differences in the region that include the first four transmembrane segments, and the remainder is attributable to transmembrane segment five and the exchanger inhibitory peptide site.

myocardial contractility; excitation-contraction coupling; salmonid; calcium handling

The Na\(^+\)/Ca\(^2+\) exchanger (NCX) is an integral membrane protein that plays an important role in the regulation of Ca\(^2+\) concentration in the cytosol. Utilizing the Na\(^+\) electrochemical gradient, the NCX transports Ca\(^2+\) across the membrane with a stoichiometry of three Na\(^+\) ions to one Ca\(^2+\) ion. Although the NCX is present in many cell types, the cardiac-specific mammalian isoform (NCX1.1) has been the most extensively characterized, where it serves as the prime mechanism of Ca\(^2+\) extrusion from the cardiomyocyte (1, 2, 5). Active transporters such as NCX1.1, which are involved in ion translocation in the mammalian heart, are highly temperature dependent. For example, it has been demonstrated that the Q\(_{10}\) value for NCX1.1 is in the range of 2.2–4.0 (12, 16) in mammals. Cardiac function in active salmonid species such as rainbow trout (Oncorhynchus mykiss) is distinguished by the ability of the heart to maintain adequate contractility under hypothermic conditions that are cardioplegic to mammals. Studies of Na\(^+\)/Ca\(^2+\) exchange in trout sarcoplasmic vesicles have shown that >75% of NCX activity is maintained after reducing the temperature from 21 to 7°C, whereas in canines that value is diminished to <10% (30). This behavior of NCX was observed in both the native membranes and when exchangers were reconstituted into asolectin vesicles, which suggests that the differential temperature dependencies between isoforms are due to differences in primary structure. The recent cloning of the trout cardiac NCX (33) provides us with a molecular model for further investigation of the temperature dependence of the NCX molecule (7).

The wild-type trout exchanger NCX-TR1.0 has a predicted topology similar to that of the mammalian NCX1.1 based on hydropathy analysis and sequence identity. At the amino acid level, the NCX-TR1.0 shows ~75% overall identity to NCX1.1. However, sequence identity between these exchangers is significantly higher in regions of the molecule known to be functionally important such as the o-repeats (~92%), the exchanger inhibitory peptide (XIP) site (85%), and regulatory Ca\(^2+\) binding domains (~86%) (33). Based on these comparisons, the two isoforms appear similar from a molecular perspective despite exhibiting very different temperature dependencies. In a recent study (7), we characterized in detail the temperature dependencies of NCX1.1 and TR-NCX1.0 wild-type exchangers expressed in oocytes by measuring outward currents using the giant excised-patch technique. The peak outward current of NCX1.1 exhibited typical mammalian temperature sensitivities with a Q\(_{10}\) value.

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of 2.4, whereas the NCX-TR1.0 peak current was relatively temperature insensitive with a Q10 value of 1.2 (7). Furthermore, it was found that the disparities in temperature dependence between these two exchanger isoforms are unlikely due to either differences in inactivation kinetics or NCX regulatory mechanisms (7).

The purpose of this study was to delineate the regions of the NCX molecule that are responsible for its temperature sensitivity. The strategy used involved the construction of chimeric NCX molecules using cDNA derived from canine and trout NCX wild-type cDNA. Outward currents for each chimeric construct were measured in *Xenopus* oocytes over a temperature range of 7–30°C using the giant excised-patch technique. By using this approach, the majority of the differential temperature dependence of the NCX isoforms was found to reside within the region of the molecule that includes the four transmembrane (TM) segments.

**METHODS**

**Construction of chimeras.** The strategy used for the construction of the chimeras is shown in Fig. 1. Initially, three chimeras were constructed and tested. Wild-type dog and trout exchanger cDNA were cut twice at homologous places into three domains: an NH2-terminal domain that comprises the first five TM segments and the XIP site; the intracellular loop, which contains the Ca2+-binding domain and alternative splicing site; and a COOH-terminal domain that includes the final four TM segments. For the trout NCX-TR1.0 (33), a silent mutation was introduced through silent mutation to form the construct TDD. A fourth construct called TTMN was produced to isolate the effects of differences within the XIP site and TM segment 5 (TM5) regions on the differential temperature dependence. For this construct, a StuI site was introduced through silent mutation into the cDNA of NCX-TR1.0 at nucleotide 719 as well as the chimeric DTT at nucleotide 695, relative to the start codon, respectively. The cDNA was then digested with StuI and BclI. A fragment from NCX-TR1.0 cDNA spanning from amino acid 240 to 279 was inserted into the corresponding region of treated DTT from amino acid 232 to 271 and generating chimera DTTT. All constructs were confirmed by sequencing.

**Expression of chimeras in Xenopus oocytes.** Expression of chimeric exchangers in *Xenopus* oocytes was carried out as described previously (7, 23). In brief, chimeric cDNA was prepared from XL1-Blue Escherichia coli (Stratagene) by using a QIAprep miniprep kit (Qiagen, Mississauga, ON) and was then linearized with *HindIII*. Chimera cRNA was synthesized by using the T3 mMessage mMachine in vitro transcription kit (Ambion, Austin, TX) and run on a 1% agarose gel to assess purity. Oocytes were prepared as described by Longoni et al. (18) and injected with 5 ng of cRNA. Exchange activity was measured 3–4 days after injection (see Assay of Na+)/Ca2+ exchange activity).

**Assay of Na+/Ca2+ exchange activity.** Outward Na+/Ca2+ exchange currents were measured by using the giant excised-patch technique as described previously (7, 23). Briefly, oocytes were placed in a solution containing (in mM) 100 KOH, 100 2-N-morpholinooctanesulfonic acid (MES), 20 HEPES, 5 EGTA, and 5 MgCl2, pH 7.0 at room temperature with MES. Gigaohm seals were formed via suction by using borosilicate glass pipettes (inner diameter of ~20–30 μm), and membrane patches were excised by movements of the pipette tip. The pipette solution contained (in mM) 100 N-methylglucamine (NMG)-MES, 30 HEPES, 30 tetraethylammonium (TEA)-OH, 16 sulfamic acid, 8 CaCO3, 6 KOH, 0.25 ouabain, 0.1 niflumic acid, and 0.1 flufenamic acid; pH 7.0 with MES. Outward Na+/Ca2+ exchange currents were activated by switching from Li+ to Na+–based bath solutions containing (in mM) 100 (Na+ or Li+)–aspartate, 20 MOPS, 20 TEA-OH, 20 CsOH, 10 EGTA, 0–7.3 CaCO3, and 1.0–1.13 Mg(H2)3; pH 7.0 with MES or LiOH. Mg2+ and Ca2+ were adjusted to yield free concentrations of 1.0 mM and either 0 or 1 μM, respectively, with MAXC software (3). All experiments were conducted first at room temperature (22–23°C), and then exchange currents were measured at different temperatures (30, 14, and 7°C) by heating or refrigerating the bath solu-
tions. Axon Instruments hardware and software were used for data acquisition and analysis.

Inactivation kinetics and temperature-dependence parameters. To explore further the differences in chimeric and wild-type exchangers, we examined the inactivation kinetics and temperature-dependence parameters for all constructs. For the inactivation kinetics, we calculated the inactivation rate constant (λ) and the ratio of steady-state current to peak current (Fss) for each trace. The λ values were obtained by fitting current-time traces to a single exponential (7, 12). The Fss was calculated as the ratio of steady-state current over fitting current (12). The energy of activation (Eact) and temperature coefficient (Q10), were calculated for each chimera as indices of temperature dependence. The Eact was estimated from the equation

$$\ln \left( \frac{I}{I_{303}} \right) = \frac{E_{act}(303 - T_i)}{R(303 \times T_i)}$$

where \(I/I_{303}\) is the normalized current, \(R\) is the universal gas constant, and \(T_i\) is the experimental temperature (in K). To allow for statistical analysis of data obtained from different patches, exchange current was normalized to that obtained at 30°C (or ~303 K). The Q10 values for the chimeric exchangers were estimated for each patch by averaging results calculated for the three pairs of temperatures by using the equation

$$Q_{10} = \frac{I_{T_2}}{I_{T_1}}^{10/T_2 - T_1}$$

where \(I_{T_1}\) and \(I_{T_2}\) are the currents at the corresponding experimental temperatures \(T_1\) or \(T_2\).

Data analysis and statistics. Statistical significance of the results was determined by unpaired Student’s t-test and one-way ANOVA using Microcal Origin and GraphPad software. Unless indicated otherwise, a value of \(P < 0.05\) was considered significantly different.

RESULTS

Temperature dependence of chimeric proteins. The goal of this study was to determine the domains of the NCX molecule responsible for the unique temperature dependencies observed between mammalian and salmonid NCX. To accomplish this, four different chimeras were constructed from the cDNA of the temperature-sensitive canine NCX1.1 and the relatively temperature-insensitive trout NCX-TR1.0. We examined the temperature dependence of NCX current for chimeric and wild-type exchangers expressed in Xenopus oocytes. Representative current traces are shown in Fig. 2. Canine NCX1.1 and trout NCX-TR1.0 currents were qualitatively similar at 30°C, and both exchangers exhibited decreased peak and steady-state currents with decreasing temperature (Fig. 2, A and B).

However, this decrease in current was much more pronounced in the canine NCX1.1, in which the exchanger current at 7°C was <10% of that measured at 30°C. In contrast, trout NCX-TR1.0 activity remained relatively high at ~60% of its activity at 7°C compared to that at 30°C (7).

To examine the mechanisms of the differential temperature dependence of the NCX molecule, the temperature dependence of four chimeric NCX exchangers was examined. Initially three chimeras, DTD, DTT, and TDD, were constructed and the outward currents were measured at 7, 14, and 30°C (Fig. 2, C-E). All three NCX chimeras displayed a typical outward NCX current with an initial peak current, which then decayed to a steady-state level. However, the temperature dependence of the chimeric exchangers varied, and based on the current traces at the different temperatures, each chimera can be qualitatively labeled as having either a predominately trout- or dog-temperature phenotype. In this regard, it is clear that DTD, which includes the canine NH2-terminal TM segments and trout loop, displays characteristics similar to that of canine NCX1.1 outward exchange-current-temperature dependence (Fig. 2C). At 7°C, DTD maintained only ~10% of the peak and steady-state currents measured at 30°C. DTT, in which the canine portion includes only the five NH2-terminal TM segments and the XIP site, also displayed a temperature dependence similar to the canine NCX1.1 (Fig. 2D). In contrast, currents from TDD exhibited a temperature phenotype similar to wild-type trout NCX-TR1.0 (Fig. 2D). Specifically, at 7°C, TDD maintained ~50% of its peak and steady-state currents measured at 30°C. The TDD construct includes the five NH2-terminal TM segments and the XIP site with the rest of the exchanger cDNA being derived from dog. From these results, it appears that the region responsible for the differential temperature dependencies of the NCX molecule is localized within the NH2-terminal portion of the molecule, a region that includes the XIP site. To determine whether the XIP site had any effect on the temperature dependence of the NCX, a fourth chimera was constructed and designated DTTT. In this construct the NH2-terminal end including the first seven amino acids within TM5 are canine, and the rest of the exchanger was salmonid wild type. The temperature dependence of DTTT was clearly more similar to that of the dog wild-type exchanger (Fig. 2F). At 7°C, DTTT was attenuated to ~20% of the peak and steady-state current measured at 30°C. From these results, differences in the sequences within the TM5 and XIP regions appear to play a rather minor role in the temperature dependence of the NCX molecule.

Inactivation kinetics and temperature-dependence parameters. To investigate the temperature dependence of the NCX molecule in a more quantitative manner, the effect of temperature on the Na+ dependent inactivation of the chimeric exchangers was examined. The inactivation rate constant, λ, and Fss values for each current trace were determined. The temperature dependence of λ for NCX currents from the chimeric exchangers is shown in Fig. 3. In general, the λ values decreased monotonically with temperature for all constructs. For the chimeras DTD and DTTT, there is a slight inflection point at 14°C as the mean λ value for this temperature is less than the λ...
value at 7°C; however, these differences are not statistically significant.

Based on the absolute value of λ, constructs can be placed into two distinct groups depending on the isoform origin of the NH₂-terminal portion of the exchanger. The λ values for the trout wild type (data not shown) and TDD are significantly (P < 0.01) lower at all temperatures compared with DTD, DTT, and DTTT and canine wild type. From the one-step I₁ inactivation model (12), the Fss value characterizes the extent of I₁ inactivation because 1–Fss is the fraction of inactivation. For a given exchanger construct, there were no statistically significant differences between Fss values determined at the three different temperatures (Table 1).

The average values of Q₁₀ for peak and steady-state currents for the chimeras are shown in Table 2. Again, the canine wild type, DTD, DTT, and DTTT can be grouped together as having Q₁₀ values in the range of 2.0–2.7, whereas the Q₁₀ values for TDD and the trout wild type are significantly different and fall within the range of 1.2–1.3.

Previously, it was reported that the E₂ values for peak and steady-state currents were 53 ± 1 and 66 ± 9 kJ/mol for canine NCX1.1 and 7 ± 2 and 6 ± 0.1 kJ/mol for NCX-TR1.0, respectively (7). Arrhenius plots of Na⁺/Ca²⁺ exchange peak and steady-state currents for the chimeric exchangers are shown in Fig. 4.

We calculated the E₂ values for the four chimeras, and these values together with the E₂ values of wildtype dog and trout are summarized in Fig. 5. From these E₂ values, it is clear that DTD and DTT exhibited the canine phenotype, whereas TDD was troutlike with respect to temperature dependence. The chimera DTTT is clearly closer to the canine than trout phenotype in this regard, as the E₂ calculated from either the peak or steady-state currents is ~75% of the canine wild type. Statistical significance of E₂ values between all exchangers is reported in Table 3.

![Fig. 2. Giant patch recordings from Xenopus oocytes expressing wild-type and chimeric exchangers. Representative current traces for wild-type dog (A), trout (B), and chimeric (C-F) Na⁺/Ca²⁺ exchangers obtained from inside-out giant membrane patches. Currents were induced by the rapid application of 100 mM Na⁺ to the cytoplasmic surface of the patch. Regulatory Ca²⁺ (1 μM) and transport Ca²⁺ (8 mM) were present in the bath and pipette solutions, respectively.](image-url)
DISCUSSION

In this study, we attempt to elucidate this temperature dependence discrepancy at the molecular level by using chimeric proteins derived from wild-type trout and dog exchangers. The use of chimeric proteins in studies involving NCX (6, 13, 20) and the thermostability of proteins (25, 27) has proven useful in comparing functional differences between isoforms. Qualitative examination of outward exchange currents for the chimeras DTD, DTT, and TDD over a range of temperatures placed the region responsible for NCX temperature dependence within the NH2-terminal TM segments and the XIP site. A fourth chimera, DTTT, revealed that the differences within TM5 and the XIP regions have an effect on the temperature dependence of the NCX molecule, but the contribution is relatively small compared with the first four TM segments. This is consistent with earlier findings that deregulation of both wild-type trout and dog exchangers with chymotrypsin treatment had no significant effect on the temperature dependencies of the NCX isoforms (7). However, with the use of chimeras it is possible to more closely examine the relative contribution to the temperature dependence of the NCX by different regions of the molecule. For instance, at 7°C, the chimeras DTD and DTT (in which the NH2-terminal end including TM5 and the XIP site are dog in origin) maintain only ~10% activity of the peak current measured at 30°C. The activity of the chimera DTTT at 7°C is relatively higher, retaining ~20% of peak current measured at 30°C. From this observation, it appears that differences in sequence in TM5 and XIP do contribute to the temperature dependence of the NCX molecule, albeit in a relatively minor capacity.

The effect of temperature on the inactivation kinetics of NCX currents was characterized using λ and Fss. As a general trend, λ decreased with temperature for all chimeras measured (see Fig. 3), which is consistent with what was found in wild-type trout and dog NCX values (7). However, it should be noted that the DTT chimera had abnormally high λ values with large SEs at all temperatures, a phenomenon that cannot currently be explained. The inactivation rates of DTD, DTT, and DTTT were consistently faster than that of TDD over the temperature range of 7–30°C and were not significantly different than canine wild type values. This supports further the conclusion that sequence differences in the NH2-terminal region of the NCX molecule are responsible for temperature-dependence disparities between isoforms and that the XIP site and TM5 have only a small effect on the temperature dependence of the NCX. Fss values were higher for TDD compared with the other chimeras, which again indicates that the Na+-dependent inactivation was slower for this construct. However, for each chimera there is no statistically significant difference between Fss values determined over the temperature range of 7–30°C. This indicates that an increase in the inactivation rate with temperature is accompanied by an increase in the rate of recovery from inactivation. The measures of λ and Fss to characterize the inactivation kinetics of these chimeras support the idea that the differential temperature dependencies of the NCX isoforms are not related to Na+-dependent inactivation. However, it is apparent that the NH2-terminal region of the NCX is a

Table 2. Q10 values for chimeric NCX

<table>
<thead>
<tr>
<th>Exchanger</th>
<th>Peak current estimate</th>
<th>Steady-state current estimate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog WT</td>
<td>2.4 ± 0.4</td>
<td>2.6 ± 0.4</td>
</tr>
<tr>
<td>DTD</td>
<td>2.3 ± 0.3</td>
<td>2.5 ± 0.6</td>
</tr>
<tr>
<td>DTT</td>
<td>2.7 ± 0.2</td>
<td>2.5 ± 0.1</td>
</tr>
<tr>
<td>DTTT</td>
<td>2.0 ± 0.1</td>
<td>2.2 ± 0.1</td>
</tr>
<tr>
<td>TDD</td>
<td>1.3 ± 0.1*</td>
<td>1.2 ± 0.0*</td>
</tr>
<tr>
<td>Trout WT</td>
<td>1.2 ± 0.1*</td>
<td>1.1 ± 0.1*</td>
</tr>
</tbody>
</table>

Values are means ± SE; *P < 0.05 vs. Dog WT, DTD, DTT, and DTTT.

Table 1. Temperature dependence of Fss values for chimeric NCX

<table>
<thead>
<tr>
<th>Exchanger</th>
<th>7°C</th>
<th>14°C</th>
<th>30°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog WT</td>
<td>0.28 ± 0.05</td>
<td>0.24 ± 0.04</td>
<td>0.17 ± 0.02</td>
</tr>
<tr>
<td>DTD</td>
<td>0.24 ± 0.03</td>
<td>0.24 ± 0.03</td>
<td>0.19 ± 0.04</td>
</tr>
<tr>
<td>DTT</td>
<td>0.37 ± 0.05</td>
<td>0.23 ± 0.04</td>
<td>0.28 ± 0.03</td>
</tr>
<tr>
<td>DTTT</td>
<td>0.42 ± 0.06</td>
<td>0.38 ± 0.07</td>
<td>0.40 ± 0.04</td>
</tr>
<tr>
<td>TDD</td>
<td>0.56 ± 0.07</td>
<td>0.55 ± 0.08</td>
<td>0.48 ± 0.03</td>
</tr>
<tr>
<td>Trout WT</td>
<td>0.21 ± 0.04</td>
<td>0.23 ± 0.08</td>
<td>0.19 ± 0.03</td>
</tr>
</tbody>
</table>

Values are means ± SE. Fss, ratio of steady-state to peak current; WT, wild type; NCX, Na+/Ca2+ exchanger.
Temperature dependence as mammalian or troutlike. Conceived in an attempt to classify the temperature dependence phenotype exhibited by the chimeric exchangers. Similar to other mammalian species (15, 16), the exchanger activity of DTD and DTT is highly temperature dependent with $Q_{10}$ values in the range of 2.3–2.7. With values in the range of 48–65 kJ/mol, the chimeras DTD and DTT exhibit $E_{\text{act}}$ values typical of other mammalian NCX values such as dog, trout, rabbit, and guinea pig (12). Conversely, the chimera TDD is relatively temperature insensitive and exhibits $Q_{10}$ (1.2–1.3) and $E_{\text{act}}$ values (14–15 kJ/mol) similar to those of the trout wild-type exchanger (7). By using these parameters, the chimera DTTT displays intermediate temperature dependencies. Even though not statistically significant, the $Q_{10}$ values for DTTT are slightly lower than those for canine NCX and the chimeras DTD and DTT. The $E_{\text{act}}$ values for DTTT are 39–40 kJ/mol, making them $\sim$75% of the values for canine wild type (53–66 kJ/mol; Ref. 7) but much higher than the $E_{\text{act}}$ values for the ectothermic species such as trout (6–7 kJ/mol; Ref. 7) and frog (21–25 kJ/mol; Ref. 4). These data indicate that the TM5 and XIP region play a relatively minor role in the temperature dependence of the NCX compared with the first four TM segments.

Figure 6 shows a sequence alignment comparing the NH$_2$-terminal regions of canine NCX1.1 and trout NCX-TR1.0. For convenience, the sequence alignment is split where the restriction cuts were made to make the DTTT chimera. The section of TM5 and the XIP site that is responsible for $\sim$25% of the temperature dependence disparity between the trout and dog NCX contains minor sequence differences. The two nonconservative substitutions in the trout XIP site (F to V and Q to R at XIP positions 5 and 18, respectively) are possible causes for the minor role played by this region. The structural effects of these substitutions are unknown but they are likely to make the trout XIP site less hydrophobic. The phenylalanine at position 5 (F5) of the XIP site has been found to be important in both the inactivation of the NCX (19) and inhibitory effects of the XIP peptide (10). Matsuoka et al. (19) found that making an F5E substitution increased the rate of inactivation sixfold and decreased $F_{\text{act}}$ twofold. This substitution, in which the aromatic and hydrophobic amino acid phenylalanine is replaced by the negatively charged and hydrophilic amino acid glutamic acid, appears to induce the conformational changes involved in inactivation more rapidly (19). In the trout XIP site, the F5V substitution observed converts the highly hydrophobic amino acid to a nonaromatic and less-nonpolar amino acid. The data from Matsuoka et al. indicated the importance of the F5, but it is not clear what the impact of the valine substitution is at this point. Related to this is the observation that the potency of the exogenous XIP on NCX inhibition is sensitive to mutations of the phenylalanine at position 5 (10). Exogenously added peptide with the XIP sequence has been shown to inhibit exchanger function, which is consis-

Determining factor in the phenotype of the NCX inactivation kinetics.

In the present study, we calculated $Q_{10}$ values (see Table 2) and $E_{\text{act}}$ values (see Fig. 5 and Table 3) for the chimeric exchangers in an attempt to classify the temperature dependence as mammalian or troutlike. Con-

**Fig. 4.** Arrhenius plots of NCX currents for chimeric exchangers. NCX peak (A) and steady-state (B) current values for wild-type dog (NCX1.1), trout (NCX-TR1.0), and various chimeric exchangers. Exchange currents were normalized to those obtained at 30°C (303K). For the peak currents, data points are mean values from 3–4 measurements from 3–4 patches, 5 measurements from 5 patches, 6 measurements from 6 patches, 5 measurements from 5 patches, 6 measurements from 6 patches, 5 measurements from 5 patches, 6 measurements from 6 patches, and 3–4 measurements from 4 patches for NCX1.1, NCX-TR1.0, DTD, DTT, DTTT, and TDD, respectively. For the steady-state currents, data points are averaged from 3–4 measurements from 3–4 patches, 5–7 measurements from 7 patches, 6 measurements from 6 patches, and 3–4 measurements from 4 patches for NCX1.1, NCX-TR1.0, DTD, DTT, DTTT, and TDD, respectively. Solid lines represent linear least-squares fit to the experimental values of currents. Fitting of energy of activation ($E_{\text{act}}$) for each exchanger value is presented in the table and figure.
tent with the hypothesis that the endogenous XIP site has an autoregulatory function (17, 20). Substitution of F5 with a charged group (glutamate) decreases inhibition by a factor of 5, whereas substitution with a conserved residue (tryptophan) has no effect on inhibition (10). Replacing the phenylalanine with an alanine or valine decreases the potency of XIP peptide inhibition by 50% and 30%, respectively (10). Presumably, the XIP binds to the exchanger through hydrophobic interactions, and aromatics like F5 are required for maximal inhibition. The valine substitution at this position is the same substitution found in the trout NCX, and this may have an effect on the inactivation kinetics of the exchanger. However, further testing with site-directed mutagenesis is needed to confirm this theory. Interestingly, the frog (Xenopus laevis) NCX (14) shows complete identity to the canine NCX in the XIP region, even though the bullfrog (Rana catesbeian) NCX has a much lower $E_{\text{act}}$ than the mammalian NCX isoform. Because the bullfrog NCX $E_{\text{act}}$ values are higher than the trout NCX $E_{\text{act}}$ values (21–25 vs. 6–7 kJ/mol, respectively), perhaps the amino acid substitutions in the XIP region may account for this difference. However, it must be pointed out that sequence differences in the XIP domain play a relatively minor role in the temperature dependence of the NCX molecule.

Although fixed amounts of cRNA of each construct were injected into the oocytes, the absolute value of the peak currents varied substantially. Typically, peak currents for NCX-TR1.0 were lower than those observed for NCX 1.1. The reasons for this difference are not clear but could include disparities in unitary activity, stability of mRNA, and/or stability/longevity/trafficking of the protein in the membrane. These differences in current magnitude are unlikely to alter our conclusions for several reasons. The focus of the present study is on an intrinsic property, specifically, the temperature dependence of outward $\text{Na}^+/\text{H}^+\text{Ca}^{2+}/\text{H}^+$ currents. The magnitude of these currents reflects both the number and unitary currents of exchangers within the patch. Although the number of NCX molecules within a patch is likely to vary considerably, possibly reflecting differences cited above and the heterogeneity

Table 3. Statistical significance of differences in $E_{\text{act}}$ values

<table>
<thead>
<tr>
<th>Exchanger Construct</th>
<th>DTD Peak</th>
<th>ss</th>
<th>DTT Peak</th>
<th>ss</th>
<th>DTTRT Peak</th>
<th>ss</th>
<th>TDD Peak</th>
<th>ss</th>
<th>Trout WT Peak</th>
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<tr>
<td>Dog WT</td>
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<td>NS</td>
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A comparison of $E_{\text{act}}$ values derived from peak and steady-state (ss) NCX current for all constructs measured. NS denotes not significant ($P > 0.05$), whereas statistical significance is depicted as follows: $* P < 0.01$, $\dagger P < 0.005$, $\ddagger P < 0.001$, and $\ddagger\ddagger P < 0.0001$. 

Fig. 5. $E_{\text{act}}$ for wild-type (WT) and chimeric exchangers as a function of topology. For the representation of topology, trout NCX-TR1.0 is shown in black and dog NCX 1.1 is shown in gray. $E_{\text{act}}$ from peak currents are shown in shaded bars and open bars represent $E_{\text{act}}$ from steady-state currents. $+ P < 0.05$, significant difference vs. trout wild-type exchanger; $* P < 0.05$, significant difference vs. dog wild-type exchanger.
of pipette and patch geometries, there is no evidence that differences of the number of exchangers within a patch alters its unitary properties. Second, the possibility of NCX dimerization has been raised (9, 31) but remains unproven and controversial. In our study, the putative potential of dimerization as an explanation of the temperature dependence of the NCX in the trout NCX-TR1.0 within the first four TM segments is responsible for its relatively high activity at low temperatures. An amino acid sequence comparison of this region between NCXs from mammalian species such as the dog (22) and from ectothermic species such as frog (14), trout (33), and squid (11) revealed no general trends regarding the aforementioned adaptive mechanisms that proteins use to function at low temperature. The number of prolines (7 for dog and trout, 8 for frog, and 6 for squid) and arginines (5 for dog and 6 for trout, frog, and squid) in this region are fairly well conserved between species. In addition, substitutions in loop regions between isoforms did not show an overall increase or decrease in polarity (data not shown), nor were there large differences in the number of aromatic residues (21 for dog and 20 for trout, frog, and squid). Another feature that may promote structural flexibility are glycine residues, which are thought to be destabilizing in helices and stabilizing in loop regions (26). Within the first four TM segments, the number of glycine residues is conserved between these four isoforms except in the NH2-terminal loop (6 for dog, 5 for trout, 3 for frog, and 2 for squid). From sequence analysis, there is little to discern the specific mechanisms enabling the trout NCX to function at low temperatures. In fact, it is unlikely that there is one region or a few amino acid substitutions that can totally account for the cold activity of trout NCX-TR1.0. A more likely explanation is that a series of amino acid substitutions in different regions of the molecule all play a role in the activity of the trout NCX at low temperatures.

In summary, we have placed the region responsible for the temperature dependence of the NCX in the NH2-terminal region of the molecule. The majority of the differential temperature dependence between canine and trout isoforms seems to reside in the first four TM segments, with a minor role played by TM5 and the XIP site. Further mutational analysis is needed to determine the specific amino acids involved in the temperature dependence of the molecule, but we speculate that a series of amino acid substitutions within the first four TM segments are responsible for the activity of the trout NCX at low temperatures.

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