Cloning and functional expression of the cardiac Na^+-Ca^{2+} exchanger of the spiny dogfish shark (*Squalus acanthias*)

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The Na^+-Ca^{2+} exchangers (NCXs) of eukaryotic cells consist of two groups of homologous clusters of α-helical transmembrane-spanning segments (TMs, Fig. 1) separated by a long cytoplasmic loop that is thought to have several regulatory and structural functions. We have investigated molecular determinants of β-adrenergic regulation of cardiac NCX (NCX1) of dog (no change) and frog (uniform suppression) and have recently focused on shark NCX1, which has unique bimodal regulation with stronger suppression in the Ca^{2+}-influx than in the efflux mode[6]. Here we report on: a) the completion of the sequencing of shark NCX1 (DQ 068478), b) the assembly of expression constructs for native and mutant shark NCX1, c) the transient expression of these NCXs in a mammalian cell line (HEK 293), d) functional verification of expression by confocal imaging of Ni^{2+}-sensitive intracellular Ca^{2+} (Ca_i-) transients, and e) preliminary measurements of adrenergic regulation. In a companion article in this volume of the MDIBL Bulletin, we present new evidence for bimodal regulation of NCX in freshly dissociated cardiac ventricular shark cardiomyocytes including the finding that the Ca^{2+}-efflux mode, in some cells, appears to be enhanced by epinephrine.

Based on mRNA isolated at MDIBL, we have previously reported a 2688 bp sequence coding 896 aa corresponding to 87% of the NCX molecule as found in other vertebrate species[1]. This approach was continued to cover 2993 bp (Fig. 1). The missing 3' end was provided from ESTs posted from MDIBL (DT, Fig. 1 [5]). One of the underlying clones (Sa_mx0_45h08/CX196474.1) was generously provided by Dr. Towle and sequenced completely to provide the first 1780 bp of NCX including suspected core promoter sequences within 90 bp upstream from the suspected start codon (Fig. 1, AK). This full length protein sequence confirmed the presence of two A/P rich inserts[1] that may be essential to the unique bimodal regulation of shark NCX1. The presence of the second insert was evident also in the sequence from a new isolation of mRNA (LY) so that it was present in a total of 3 different mRNA samples and therefore unlikely to be an artifact of the isolation procedure. In a parallel approach to completing the sequencing, a cDNA library was constructed and several clones were positively identified by screening with unique sequences from the original shark NCX1[1]. In 7 of these clones (SH 03, 05, 06, 07, 08, 09, 12, Fig. 1) we still found no evidence for anything corresponding to "exon X", which in frog completes a nucleotide-binding P-loop required for cAMP-mediated regulation[2,4]. Four clones provided sequences with ~600 bp past the stop-codon (bp 3175-3177), including the poly-A tail of the mRNA. In summary the continued sequencing: a) provided the missing 3' and 5' ends, b) verified the presence of the unique shark-inserts (I and II, Fig. 1), c) firmly established a few ambiguous aa's in the provisional sequence[1], d) confirmed that exon X of the frog heart is not expressed in the shark, and e), in spite of isolation from multiple tissue samples (including our cDNA library from shark heart and the equalized library from the entire animal[5]), showed no indication of other isoforms (NCX2, NCX3).

An expression construct (S-NCX) for the native shark NCX containing the full-length NCX cDNA (including a complete 3'-untranslated region was made in the vector pCMV-SPORT6.1 (Invitrogen,

Carlsbad, CA). The NCX sequence nucleotides 1-1780 were derived from the cDNA clone Sa_mx0_45h08[5]. Nucleotides 1992-3845 were derived from another cDNA clone, obtained from our shark heart cDNA library in lambda ZAP-XR. The sequence of the remaining gap was added from RT-PCR products generated directly from shark heart RNA as used in our initial sequencing effort

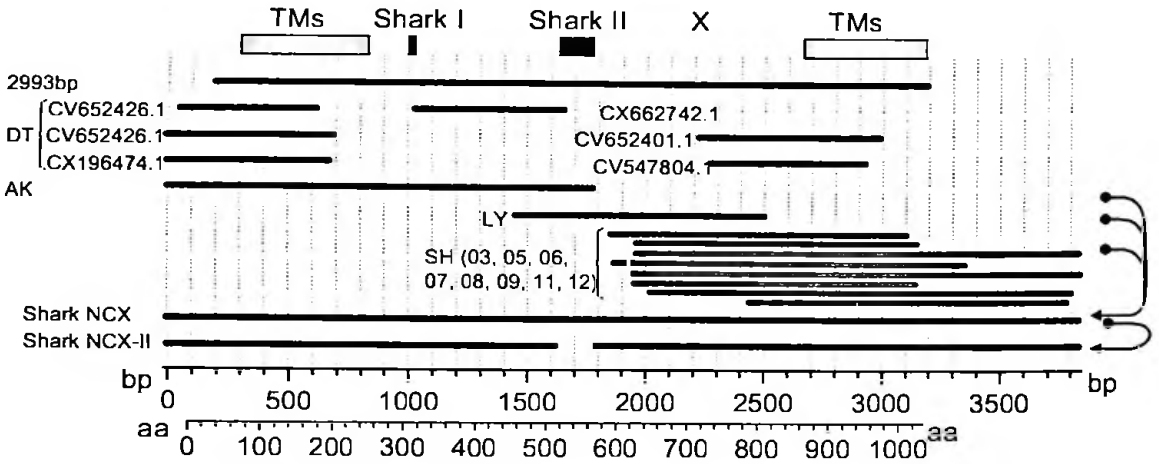

Fig. 1. Verification of the sequence of shark cardiac NCX and construction of two expression constructs corresponding to native NCX (S-NCX) and a mutant NCX with deletion of the second shark insert (S-NCX-II) . The two-letter labels refer the people who did the sequencing (DT: Towle and Smith; AK: Kraev; LY: Lee; SH: Laney and Williams).

To test if long inserts are essential to the bimodal adrenergic regulation of NCX we constructed a mutant (Shark NCX-II) where the second A/P rich insert had been eliminated by deleting bp 1624-1785 coding for aa 512-565. This was accomplished by using PCR to insert an endonuclease Not I site at 1622-1629; a Not I site is naturally present at bp 1780-1787 downstream of the unique sequence, allowing convenient cloning of the resulting PCR fragment. PCR was performed using reverse primer 5'-ATAAGAATGCGGCCGCGCTCAGGCCCACGCGCAGGTTGCT-3' to insert the new Not I site. The forward primer for cloning (5'-GGTCAGCCGCATCTACTTCG-3') was 5' to an endonuclease BstB1 restriction site, which occurs upstream of the second A/P-rich shark sequence. The resulting PCR product was then inserted into the rest of the shark sequence following digestion with BstB1/Not I. This approach was successful as verified by bi-directional sequencing of the expression construct.

The day before transfection, Hek 293 cells were split onto coverslips in antiobiotic free media. The cells were transfected with either shark NCX (Shark NCX), mutant shark NCX (Shark NCX-II) or dog NCX (Dog NCX[3]) using either fugene 6 (Gugent L.L.C.) or lipofectamine 2000 (Invitrogen) protocols according to the manufacturers instructions. 24 hours post-transfection, the cells were loaded with the calcium fluorescent indicator Fluo-4AM and subjected to two-dimensional confocal fluorescence microscopy (Noran Odyssey, acoustoptical laser scanner; Zeiss Axiovert 135; Olympus WPlanApox60UV NA: 1.20; 4 frames/sec). HEK cells were cultured at 37 °C and experiments were carried out at room temperature (22-24 °C)

Figure 2 compares Ca_i-transient measured in multiple cultured HEK 293 cells subjected to transient transfection (lipofectamine) with dog or shark NCX, or transfecting agent without plasmid (Control). The cells were first perfused with normal mammalian Tyrode's solution (140 mM NaCl, 5.4 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM glucose and 10 mM Hepes titrated to pH 7.4 with NaOH) and then exposed for 10 sec to an isotonic depolarizing solution with high $[K^+]_o$ (140 mM) and low $[Na^+]_o$ (5.4 mM). Many of the cells in the cultures transfected with dog (panel A) or shark (panel B) NCX responded with large fluorescence increases suggesting influx of Ca^{2+} *via* expressed NCX molecules. These Ca_i-transients were somewhat larger with dog ($\Delta F/F_0$ = 4.0±0.3, n=16) than with shark NCX

($\Delta F/F_0$ = 1.7±0.2, n=12), but in either case much larger than in control cells. Both the amplitude and the rate of rise of the Ca_i-transients were mostly blocked by 5 mM Ni^{2+} (Fig. 2, lower graphs; suppression of dF/dt: shark 56±6 % *vs.* dog 70±2 %),). These findings indicate that our construct for expression of native shark NCX is effective and generates Ni^{2+}-sensitive Ca^{2+} fluxes that are comparable to those of dog NCX[3] as also measured previously[2].

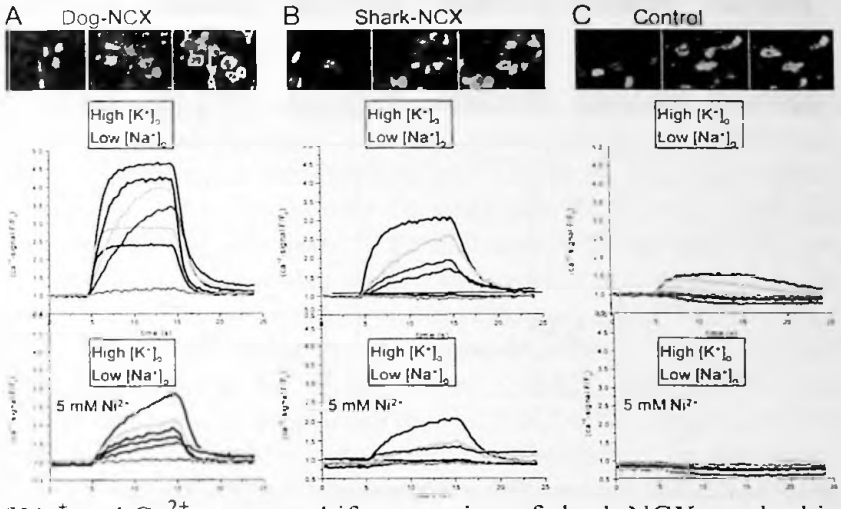

Figure 2. Comparison of Ca_i-transients in HEK 293 cells transfected with Dog-NCX (Panel A), or Shark-NCX (Panel B) or subjected to transfecting agent, but no plasmid (Control, panel C). From top to bottom each panel shows images (maps of selected cells, baseline fluorescence (F_0), or increase in fluorescence (ΔF)), and graphs showing time course of normalized fluorescence (F/F_0) during 10 sec exposure to depolarizing solution in the absence (middle) and presence (bottom) of 5 mM $NiCl_2$.

Since the driving force of NCX is known to depend not only on the membrane potential, but also on the intra- and extreellular concentrations of Na^+ and Ca^{2+}, we tested if expression of shark NCX resulted in Ca_i-transients with such properties. Figure 3 shows that the Ni^{2+}-sensitive Ca^{2+}-influx in multiple cells transfected with shark NCX could be activated to some degree by depolarization (Panel A top, High $[K^+]$), and somewhat more by withdrawal of Na^+ (Panel B top, "0" $[Na^+]_o$, but that the synergistic effects, as expected, were the strongest (Panel A bottom, High $[K^+]$ Low $[Na^+]_o$). Routinely such experiments included testing the sensitivity to Ni^{2+} (middle graphs in Fig. 3) and parallel recordings with control cells and cells expressing dog NCX (data not shown).

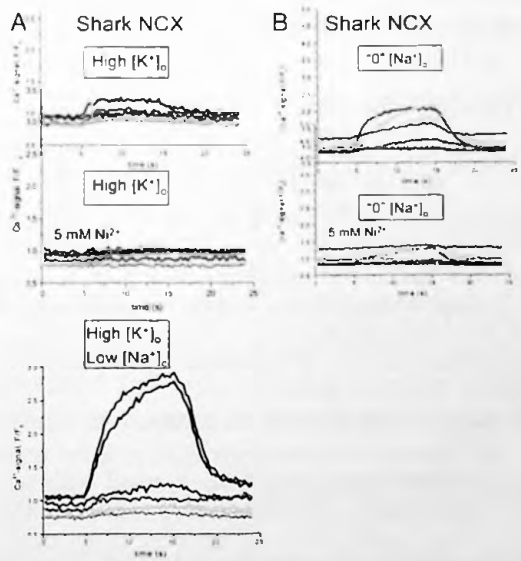

Figure 3: Synergistic effects of depolarization (high $[K^+]_o$) and Na^+-withdrawal on Ca_i-transients in HEK 293 cells transfected with Shark NCX. A: Ca_i-transients in cells exposed for 10 sec first to solution with high $[K^+]_o$ (100 mM KCl, hypertonic) in the absence (top) and presence (middle) of 5 mM Ni^{2+} and then to an isotonic solution with low $[Na^+]_o$ (5.4 mM) and high K^+ (140 mM, bottom). (lipofectamine, 080505 n16-18.) B: Ca_i-transients in cells exposed to solution without added Na^+ ("0" $[Na^+]_o$, Li^+-substitution, isotonic) but with normal $[K^+]_o$ (5.4 mM) in the absence (top) and presence (middle) of 5 mM Ni^{2+}. (lipofectamine, 81105n4-5.)

The expression of the mutant shark NCX (Shark NCX-II) was verified in similar experiments (not shown). The rationale for the construction of this mutant was that the deleted "second insert" (II in Fig. 1) is the most notable characteristic of shark NCX and possibly might be associated with its equally conspicuous bimodal regulation. In preliminary experiments we therefore compared the effects of 8Br-cAMP, a non-hydrolyzable cAMP analogue, on Ca^{2+} influx in cells transfected with either native (Fig. 4 A) or mutant (Fig. 4B) shark NCX. The plotted traces represent the Ni^{2+}-sensitive components measured from the difference between the Ca_i-transients recorded in the absence and presence of 5 mM Ni^{2+} (Cf. Figs. 2 and 3). The change in the fluorescence signals, measured after 5 min treatment with 8Br-cAMP, suggest that the early Ca^{2+} influx was enhanced in cells transfected with native NCX, while the final amplitude of Ca_i-transients

was suppressed in cells transfected with the mutant. Additional experiments will be required to establish and fully characterize these intriguing differences.

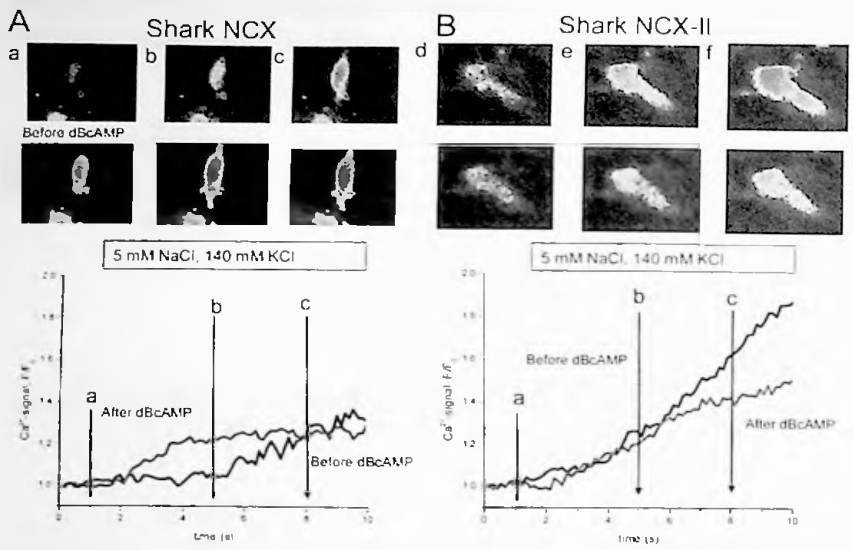

Figure 4. Effect of 10 μM 8Br-cAMP on the Ni^{2+}-sensitive Ca^{2+} influx in HEK 293 cells transfected with native (Shark NCX, panel A) and mutant (Shark-NCX-II, panel B) shark NCX. Each trace is corrected by subtraction of the fluorescence that persists in the presence of 5 mM Ni^{2+}. The traces represent recordings before (black) and after (red) 5 min of exposure to 10 μM of 8Br-cAMP. (fusgen transfection; Shark-NCX: 101905 n09-15; Shark NCX-II: 102105 n01-07)

The most significant results presented here are that sequence of shark cardiac NCX has been carefully established (Fig. 1) and reported to Genbank (DQ 068478). Further, expression constructs we produced and successful expression was ascertained functionally based on fluorescence measurement of Ca^{2+} influx that was: a) blockable by Ni^{2+}, b) activated by KCl-depolarization, c) synergistically activated by depolarization and Na^{2+}-withdrawal, and d) absent in non-transfected cells, and therefore highly characteristic of NCX. It may also be noted that prior to this work, it was by no means certain that shark NCX that normally functions at near molar osmolarity would also transport Ca^{2+} in the environment of mammalian cells where ionic concentrations and osmolarity (300 mosM) are generally much reduced. In short, shark NCX is now available to be studied in virtual isolation in mammalian cell lines using available molecular, electrophysiological and Ca^{2+}-imaging techniques. It is likely that additional insight may be gained by: a) achieving stable transfection, b) studying the rates of Ca^{2+}-efflux, c) voltage-clamping the host cells, d) characterizing cAMP-mediated regulation, and e) systematically studying the effects of different mutations.

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