β-Adrenergic regulation of a novel isoform of NCX: sequence and expression of shark heart NCX in human kidney cells

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Janowski E, Day R, Kraev A, Roder JC, Cleemann L, Morad M. β-Adrenergic regulation of a novel isoform of NCX: sequence and expression of shark heart NCX in human kidney cells. Am J Physiol Heart Circ Physiol 296: H1994–H2006, 2009. First published April 24, 2009; doi:10.1152/ajpheart.00038.2009.—The function, regulation, and molecular structure of the cardiac Na+/Ca2+ exchangers (NCXs) vary significantly among vertebrates. We previously reported that β-adrenergic suppression of amphibian cardiac NCX,1,2 is associated with specific molecular motifs. Here we investigated the bimodal, cAMP-dependent regulation of spiny dogfish shark (Squalus acanthias) cardiac NCX, exploring the effects of molecular structure, host cell environment, and ionic milieu. The shark cardiac NCX sequence (GenBank accession no. DQ 068478) revealed two novel proline/alanine-rich amino acid insertions. Wild-type and mutant shark NCXs were cloned and expressed in mammalian cells (HEK-293 and FlpIn-293), where their activities were measured as Ni2+-sensitive Ca2+ fluxes (fluor 4) and membrane (Na+/Ca2+ exchange) currents evoked by changes in extracellular Na+ concentration and/or membrane potential. Regardless of Ca2+ buffering, β-adrenergic stimulation of cloned wild-type shark NCX consistently produced bimodal regulation (defined as differential regulation of Ca2+-influx and -efflux pathways), with suppression of the Ca2+-influx mode and either no change or enhancement of the Ca2+-efflux mode, closely resembling results from parallel experiments with native shark cardiomyocytes. In contrast, mutant shark NCX, with deletion of the novel region 2 insertion, produced equal suppression of the inward and outward currents and Ca2+ fluxes, thereby abolishing the bimodal nature of the regulation. Control experiments with nontransfected and dog cardiac NCX-expressing cells showed no cAMP regulation. We conclude that bimodal β-adrenergic regulation is retained in cloned shark NCX and is dependent on the shark’s unique molecular motifs.

sodium/calcium exchanger; cAMP; bimodal regulation; cloning; cardiac electrophysiology

The sodium/calcium exchanger (NCX) proteins are widely distributed in the biosphere, where they are generally found to exchange 1 Ca2+ for 3 Na+ across the sarcosomal membrane, thereby generating net electrical current in the opposite direction of the Ca2+ flux (2, 6, 37). NCX flux is modulated by the membrane potential (Vm) and the intra- and extracellular concentrations of the transported ions, which contribute to the driving force (Vm – ENa,Ca, where Na+/Ca2+ exchange equilibrium potential (ENa,Ca) = 3ENa – 2ECa for 3 Na+/1 Ca2+ exchange) of Na+/Ca2+ exchange current (INa,Ca) flux and allosteric regulation (19, 20). NCX activity varies during the cardiac cycle: first, it contributes some Ca2+ entry during depolarization, and then it is involved in efflux of Ca2+ during repolarization and diastole (3, 4, 6, 7). In contrast, with other Ca2+-transporting proteins and ion channels, the electrogenic activity and Ca2+ flux of NCX contribute to shape the action potential and control the strength of the heartbeat and its relaxation through tidal changes in cytosolic Ca2+ (24, 27, 28).

NCX activity in the mammalian heart has been reported to be either unaltered (10, 19-21, 25) or enhanced (16, 34) by β-adrenergic stimulation. In sharp contrast, NCX activity of ventricular myocytes of frog (26) and shark (29) hearts is downregulated by β-adrenergic/cAMP stimulation. Furthermore, although the β-adrenergic stimulation of frog NCX suppresses INa,Ca equally in both directions (14), suppression of NCX activity in the shark is confined to the Ca2+-influx mode with no change, or even enhancement, of the Ca2+-efflux mode of NCX (“bimodal regulation”) (43).

In an attempt to understand the molecular basis underlying the differential β-adrenergic regulation, we found that the cloned frog cardiac NCX had a unique insertion of 9 amino acids (27 bp, “exon X”) with a Walker A motif, or P loop (22), which conferred the unique cAMP suppressive effect to the clone (38). Interestingly, this regulation could be conferred onto a chimeric dog exchanger that incorporated the critical Walker A motif of the frog NCX (17), indicating that, despite the species-related differences in ionic milieu, heart rate, temperature, or the presence (dog) or absence (frog) (33) of sarcoplasmic reticulum (SR), the distinctive NCX regulatory characteristics depended on the presence of a unique amino acid sequence.

In this report, we have similarly explored the unique bimodal regulation of shark INa,Ca by cloning and expressing the shark NCX in mammalian expression systems (HEK-293 and FlpIn-293 cells). The cloned shark NCX revealed novel molecular motifs that may be responsible for its regulatory properties. To test this theory, we created a mutant shark whose unique region 2 insert was deleted. Using fluorescent Ca2+ imaging and electrophysiology, we characterized the expression and function of the shark and mutant shark clones and compared their regulatory characteristics with those of native shark cardiomyocytes. The mutant and mutant shark NCX clones remained physiologically functional in eukaryotic cells; however, although the shark NCX clone retained the essentials of its bimodal β-adrenergic regulation, the mutant shark lost the bimodal nature of its regulation. The finding that the deleted shark-specific insert affected the modality of cAMP-dependent regulation suggests that, similar to the frog, the

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specific β-adrenergic regulation of the shark NCX is based on its molecular structure.

METHODS

Sequencing Shark Cardiac NCX

Total RNA was obtained from the heart of the dogfish shark with use of TRIzol (Invitrogen/GIBCO, Carlsbad, CA) and subjected to RT using oligo(dT) primers (Invitrogen). PCR was carried out with primers based on DNA sequences for NCX from squid and vertebrates (trout, frog, and mammals). Initially, PCR produced a 171-bp ampli-

con (41). Using a series of degenerate primers predicted from multiple sequence alignments of NCX from other organisms, this sequence was subsequently extended in both directions to obtain a stretch encoding 720 amino acids that, nevertheless, lacked unconserved extreme ends of the protein (13).

The missing 5' end was provided from expressed sequence tags posted from Mount Desert Island Biological Laboratory. One of the underlying clones (Sa_mx0_45h08/CX196474.1) was generously pro-

vided by Dr. Towle (42) and sequenced completely to provide the first 1,780 bp of NCX, including apparent 5'-untranslated sequences within 90 bp upstream from the putative start codon (see Supplemental Fig. 1 in the online version of this article). In a parallel approach to completing the sequence, a cDNA library was constructed, and several clones were identified by screening with unique sequences from the original shark NCX1. Although multiple clones with long region 2 inserts were obtained, they terminated within a very narrow area, falling ~200 bp short of the 5'-terminal expressed sequence tag clone Sa_mx0_45h08. Four clones provided sequences with ~600 bp past the stop codon (bp 3175–3177), including the poly(A) tail of the mRNA.

Reconstruction of the Full-Length cDNA

An expression construct 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). The NCX sequence nucleotides 1–1780 were derived from the cDNA clone Sa_mx0_45h08. Nucleotides 1992–3845 were derived from a cDNA clone obtained from 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 obtained in our initial direct PCR product-sequencing effort (see Supplemental Fig. 1).

Initial attempts to propagate the construct-containing bacterial strain for large-scale DNA isolation failed, inasmuch as it displayed rapid plasmid loss on growth at 37°C in liquid medium containing appropriate antibiotic. It was subsequently found that the construct in the bacterial strain STBL2 (Invitrogen) could be stably grown on rich solid medium at 30°C for 48 h. The medium was prepared from autoclaved 1.6% tryptone-1% yeast extract-1.2% agar, cooled to ~50°C, and supplemented with 0.1 M Tris-HCl (pH 7.5), 20 mM MgCl2, 50 mM glucose, and 200 μg/ml ampicillin. Bacterial outgrowth as “fat streak” from 10–20 plates provided sufficient cell yield for plasmid DNA purification with use of Qiagen columns (Qiagen, Valencia, CA). Unusual strain behavior was probably due to ectopic expression of the shark insert, despite the presence of transcription terminator sequence in the vector (pCMV-SPORT6.1).

Mutant Shark Development

The role of the region 2 insert was tested by construction of a mutant shark (shark 2), where the second A/P-rich insert had been eliminated by deletion of bp 1624–1785 coding for amino acids 512–565. This was accomplished using PCR to insert an endonuclease Not I site at bp 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 by the G-C-rich PCR system (Roche Diagnostics, Indianapolis, IN) was performed using reverse primer (5’-ATAAGAATGCCGCGCTCAGGCCC- AGCCGAGGGGTCTC-3’) to insert the new Not I site. The forward primer for cloning (5’-GGTGACGGCATCTACTTTCG-3’) was 5’ to an endonuclease BsrBI restriction site, which occurs upstream of the second A/P-rich shark sequence at bp 1345. The resulting PCR product was then inserted into the rest of the shark sequence after digestion with BsrBI/Not I. The final mutant shark expression construct was bidirectionally sequenced for verification.

Transient and Stable Expression of Shark Cardiac NCX in HEK-293 Cells

On the day before transient transfection, HEK-293 cells were split onto coverslips in antibiotic-free medium. The cells were transfected with shark, mutant shark, dog, or empty vector pCMV-SPORT6.1 using Fugene 6 (Roche Diagnostics) or Lipofectamine 2000 (Invitrogen) protocols according to the manufacturers’ instructions. The cells were cultured for 24–48 h before they were used in experiments.

Cell lines with stable expression of shark (S cells) and mutant shark (MS cells) NCX constructs were created using FlpIn-293 cells (Invitrogen), which are derived from HEK-293 cells. Lipofectamine cotransfection of FlpIn-293 cells with a Flp-In expression vector and the Flp recombinase vector pOG44 resulted in targeted integration of the expression vector to the same locus in every cell. To prepare the Flp-In expression vector, recombinant cDNAs for shark and mutant shark NCX were cut out of the vector pCMV-SPORT6.1 (Invitrogen) and inserted into the FlpIn pRT expression vector in two pieces: 3' Apa I to the middle Not I site and 5' EcoR V to the middle Not I site. The resulting plasmids were sequenced for verification. Transfected cells were selected by culturing in the presence of hygromycin B (200 μg/ml) for 2 wk; then the colonies were separated, cultured, and subjected to selection on the basis of NCX expression. Stable cells expressing the dog NCX were generously provided by Dr. Donald Hilgemann. All stable cells were cultured at 37°C, and experiments were carried out at room temperature (22–24°C).

Confocal Ca2+ Imaging

Imaging of intracellular Ca2+ was performed with confocal fluo-

rescence microscopy (Nikon Eclipse TE300; Nikon, Tokyo, Japan), as described previously (43) and used within 2–8 h. Brie

fly, dogfish sharks (2–7 kg) were immobilized by complete spinal pithing. Hearts were removed and mounted on a Langendorff apparatus. The two major coronary vessels and aorta were cannulated and perfused with oxygenated Ca2+-free elasmobranch solution containing (in mM) 270 NaCl, 4 KCl, 3 MgCl2, 0.5 KH2PO4, 0.5 Na2SO4, 350 urea, 10 HEPES, and 5 glucose (pH 7.2) at 30°C for 10–15 min. The heart was then perfused for 15 min with Ca2+-free elasmobranch solution containing 1 mg/ml collagenase (type A, Boehringer, Mannheim, Germany) and 0.2 mg/ml protease (type XIV, Sigma, St. Louis, MO) and washed free of enzyme with 0.2 mM CaCl2-containing elasmobranch solution for 10 min. The ventricle was then cut free of
β-ADRENERGIC REGULATION OF A NOVEL ISOFORM OF NCX

the cannula and gently agitated in 0.2 mM Ca\(^{2+}\)-containing solution to disperse the cells. Cell yields varied greatly, between 20% and 80%, depending on the efficacy of the coronary perfusion.

Voltage-Clamp Recording of I_{NaCa} in Freshly Dissociated Shark Ventricular Cardiomyocytes and HEK-293 and FlpIn-293 Cells Expressing Shark NCX

Whole cell currents were measured with a 3- to 5-MΩ pipette attached to the input of a patch-clamp amplifier (model 8900, Dagan Instruments, Minneapolis, MN). After the whole cell voltage-clamp configuration was established, a “ramp-clamp” protocol (see Fig. 7, A and B), initiated by short-step depolarization from −60 to +80 mV, a ramp down to −120 mV, and a recovery to −60 mV [to activate NCX1-generated current (I_{NaCa})], was applied to measure the voltage dependence of I_{NaCa}. During voltage-clamp procedures, K\(^+\) currents were suppressed by omission of K\(^+\) from the external solution and inclusion of tetraethylammonium (TEA) in the internal solution. The Ca\(^{2+}\) dependence of I_{NaCa} was used in the external solution to block NaCa during brief intervals. Changes in the baseline current were often measured during the ramp-clamp protocol before, during, and after Na\(^{2+}\) or Ca\(^{2+}\) exposure. In addition to activation of the NCX via manipulation of the voltage, NCX was also activated by alteration of extracellular Na\(^+\) and Ca\(^{2+}\) concentrations ([Na\(^+\)]\(_i\) and [Ca\(^{2+}\)]\(_i\)) of some test solutions (see Solutions and Chemicals). Collected current recordings were analyzed using ORIGIN software (OriginLab, Northampton, MA). Transfected HEK-293 and FlpIn-293 cells were cultured on 25-mm coverslips, which served as an exchangeable bottom in the perfusion chamber, which, in turn, was used for voltage-clamp and Ca\(^{2+}\)-imaging procedures. Dissociated shark myocytes were placed in a chamber on the stage of an inverted microscope and superfused with 2 mM Ca\(^{2+}\)-containing clasmobranch solution. Some experimental details varied depending on the cell type and the use of Ca\(^{2+}\)-imaging.

Solutions and Chemicals

Stably transfected FlpIn-293 cells were dialyzed with a pipette solution containing (in mM) 40 KCl, 60 K-aspartate, 10 or 20 TEA-Cl, 10 or 20 mM NaCl, 0.1 Mg-ATP, 10 HEPS (titrated to pH 7.2 with KOH), and Ca\(^{2+}\) buffers yielding an estimated intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) of 100 nM. High-Ca\(^{2+}\)-buffering was provided by 0.1 mM BAPTA, 5 mM EGTA, and 2.66 mM CaCl\(_2\). Low-Ca\(^{2+}\)-buffering in experiments with concomitant Ca\(^{2+}\)-imaging, we used 0.1 mM BAPTA, 0.1 mM K\(_2\)-EGTA, and 0.02 mM CaCl\(_2\).

The basic external Tyrode solution contained (in mM) 140 NaCl, 5.4 TEA-Cl, 5 CaCl\(_2\), 1 MgCl\(_2\), 10 HEPES, and 10 glucose (titrated to pH 7.4 with NaOH). For activation of the Ca\(^{2+}\)-influx mode in the solution-exchange experiments, the Na\(^+\) concentration was lowered from 140 to 5 mM by KCl substitution for the high extracellular K\(^+\) concentration ([K\(^+\)]\(_o\))-low [Na\(^+\)]\(_o\) experiments; or by TEA-Cl substitution for the low [Na\(^+\)]\(_o\) experiments; [Na\(^+\)], was maintained at 140 mM, while [K\(^+\)], was increased from 5 to 40 mM by addition of KCl for the high [K\(^+\)]\(_o\)-normal [Na\(^+\)]\(_o\) depolarizing experiments. For activation of the Ca\(^{2+}\)-efflux mode in the solution-exchange experiments, Na\(^+\) concentration was increased from 140 to 210 mM, while [Ca\(^{2+}\)]\(_o\) declined from 5 to 0.5 mM. CaCl\(_2\) (10 μM) or dibutyl cAMP (DBCAMP, 10 or 100 μM; Calbiochem, San Diego, CA) was used as β-adrenergic agonist. An electronically controlled multibarreled puffing system was used for rapid exchange of solution (9).

For the dissociated shark cardiomyocytes, the pipette solution contained (in mM) 200 KCl, 50 NaCl, 300 urea, 10 HEPS (titrated to pH 7.2 with KOH), 5 Na\(_2\)ATP, 5 MgCl\(_2\), 10 TEA, 10 mM EGTA, and 6 CaCl\(_2\), yielding an estimated [Ca\(^{2+}\)]\(_i\) of ~200 nM. Isoproterenol or epinephrine (5 μM) was used as β-adrenergic agonist. [Na\(^+\)], of some test solutions was increased from 250 to 450 mM by urea replacement or lowered to 10 mM by Cs\(^{+}\) substitution.

Statistical Analyses

Values are means ± SE (n = number of observations). Statistical analysis was carried out using paired and unpaired Student’s t-test. Differences were considered statistically significant when P < 0.05.

RESULTS

Sequence of Shark Cardiac NCX

We deduced the cDNA sequence of shark cardiac NCX to determine its relationship to the known sequences of mammalian and frog NCX1.1 and to explore whether it contained any novel molecular motifs that might be relevant to the differential cAMP-dependent regulation observed in these species. A full-length clone was reconstructed from two cDNA clones and a PCR product (see METHODS). Sequencing was repeated on a number of partial clones from cardiac tissues derived from several individual animals and from different cardiac regions (see Supplemental Fig. 1A) to reduce the probability that we might have missed uncommon gene products. The completed sequence (GenBank accession no. DQ 068478) encodes a 1,034-amino acid polypeptide.

The deduced sequence of amino acids of shark cardiac NCX was aligned (Clustal W2) with representative sequences from other animals, with the dog cardiac NCX1 (30) used as reference for the numbering of amino acids; e.g., the cytoplasmic loop encompasses amino acids R\(_{239}\)–A\(_{667}\) (see Supplemental Fig. 1). In Fig. 1A, shark NCX is compared with dog, frog, and tunicate Ciona intestinalis NCX sequences (see Supplemental Fig. 2). The genomic tunicate sequence was included as a benchmark for chordate evolution. The alignments (Fig. 1A; also see Supplemental Fig. 2) show that the shark sequence displays a distinct pattern of similarities to and differences from other chordate sequences. The putative transmembrane-spanning α-helices at the amino (TM1–TM5) and carboxy (TM6–TM9) terminals are highly conserved, and the same is true for the core regions of two Ca\(^{2+}\)-binding domains (CBD1 and CBD2), each of which includes seven β-strands (Fig. 1A, a–f) in antiparallel pleated sheets (5, 18, 31). The shark sequence has “cardiac” characteristics, since it has sequence homology with coding that is provided by exons C–F in terrestrial vertebrates. We find no trace of these exons in tunicates or nonchordates. The cytoplasmic regulatory loop (Fig. 1A) coded by the shark cardiac mRNA shows features that may be relevant to cAMP-mediated regulation: 1) the P loop (GxgxGKS) that is coded by exon X in frog (22) is absent; 2) a potential PKA site (RKAVS\(_{357}\)) is present, as in dog and frog, but not in tunicate; and 3) unexpectedly, two conspicuous alanine/proline-rich block insertions of 10 (region 1: L\(_{277}\):PAAEAGETAT) and 54 (region 2: A\(_{469}\)PGQGADSSAHAPATAPAHPSPKMVALAGAAACGDANDAASVSSAPAPT) amino acids are found at locations where vertebrate NCX sequences are of somewhat variable composition and length. The longer insertion (region 2) is within CBD1 at the equivalent position where exons C–F provide a stretch of variable length within CBD2. To test whether this longer insert is essential to the bimodal adrenergic regulation of shark NCX, we constructed a mutant shark (shark 2) with deletion of bp 1624–1785 coding for amino acids 512–565.
Similarities between NCXs from different species are summarized in Fig. 1. The phylogram (Fig. 1E), which quantifies the relationships of the different species at the protein level, reproduces the accepted evolutionary pattern, except the shark sequence appears to diverge from the chordate lineage that leads to mammals after the divergence of the bony fishes, tilapia, trout, and zebra fish. We explored whether different molecular motifs might show different degrees of conservation, which might reveal conservation dictated by changing functional constraints. We found (see Supplemental Table 1) that the degree of conservation was somewhat different for different segments (mammals vs. shark: CBD1 > α1 ~ α2 ~ CBD2), but not enough to alter the branching pattern of the phylogram. This attests to a gradual genetic drift in the structurally conserved regions of cardiac NCX.

**Verification of Functional Activity of Shark and Mutant Shark Cardiac NCX in HEK-293 and FlpIn-293 Cells**

A full-length cDNA of shark NCX and mutant shark NCX was constructed and expressed transiently or stably in HEK-293 and FlpIn-293 cells, respectively (see METHODS). Functional expression was first verified in experiments with non-dialyzed cells (Figs. 2–4), where Ni^{2+}-sensitive inward Ca^{2+} fluxes were activated by a change in the driving force for NCX ($V_m - E_{NaCa}$), with use of KCl to depolarize the $V_m$ and/or alterations in $[Na^+]_o$ to shift the equilibrium potential ($E_{NaCa} = 3E_{Na} - 2E_{Ca}$ for 3 Na⁺:1 Ca²⁺ exchange).

The level of transient expression was verified initially using Ca²⁺ imaging of multiple HEK-293 cells incubated with fluo-4-AM (Fig. 2). Imaging with ratiometric normalization (Fig. 2C vs. 2B and Fig. 3C vs. 3B) allowed identification of responsive cells with substantial Ca²⁺ influx in multiple cells transiently transfected with shark NCX could be activated to some degree by KCl depolarization (trial 3, high [K⁺], normal [Na⁺]).

Depolarization in the presence of low [Na⁺], produced a stronger Ca²⁺ influx (trial 1, high [K⁺]-low [Na⁺]), which was used to screen data [normalized fluorescence ($\Delta F/F_0$) >1] before statistical analysis. Routinely, experiments were bracketed with control runs to ensure the sustained viability of the cells (trial 5) and included testing for sensitivity to Ni^{2+} (trials 2 and 4). In transiently transfected cells, application of 5 mM Ni^{2+} blocked only about half of the Ca²⁺ influx activated by low [Na⁺], alone (50%: $\Delta F/F_0 = 1.09 \pm 0.20$ and 0.54 ± 0.12
for control and Ni\(^{2+}\), respectively, \(n = 12\) each, \(P = 0.0006\) or in combination with high \([K^+]_o\) (45%; \(\Delta F/F_0 = 1.90 \pm 0.13\) and \(1.05 \pm 0.09\) for control and Ni\(^{2+}\), respectively, \(n = 24\) each, \(P < 0.0001\); Fig. 4, A and C). As summarized in Fig. 4, the Ni\(^{2+}\)-sensitive Ca\(^{2+}\) influx responses to low \([Na^+]_o\), were comparable for the shark (0.56 \pm 0.11, \(n = 12\)), dog (0.88 \pm 0.20, \(n = 26\)), and mutant shark (0.38 \pm 0.07, \(n = 6\)) NCX but were much larger than those of cells transfected with the empty vector (0.040 \pm 0.04, \(n = 23\); Fig. 4, A and C, red bars).

These measurements of Ni\(^{2+}\)-sensitive Ca\(^{2+}\) fluxes verify functional transient expression of shark and mutant shark NCX in mammalian HEK-293 cells. They indicate, as expected, that Ca\(^{2+}\) influx via shark NCX is activated more effectively with Na\(^+\) withdrawal than with KCl depolarization. In addition, they show that shark NCX Ca\(^{2+}\) influx is comparable to that produced by the dog cardiac NCX under similar conditions.

To achieve more uniform expression and facilitate the study of NCX regulation, we created two lines of FlpIn-293 cells with stable expression of the cloned shark (S cells) or mutant shark (MS cells) NCX (see METHODS). As illustrated in Fig. 3, the level of expression was again evaluated by measurement of the Ca\(^{2+}\) influx in response to low \([Na^+]_o\). The color-coded traces in Fig. 3E show changes in the normalized Ca\(^{2+}\) signal (\(\Delta F/F_0\)) in the cells identified in Fig. 3, A–D. Using bracketed
measurements, we recorded the Ca\(^{2+}\) influx under control conditions before (trial 1) and after (trial 3) testing the Ni\(^{2+}\) sensitivity (trial 2). Application of low [Na\(_o\)] produced a higher percentage (78–79%) of Ni\(^{2+}\)-sensitive Ca\(^{2+}\) influx, yielding ΔF/ΔF\(_0\) = 1.12 ± 0.10 (\(n = 42\)) in the S cells, ΔF/ΔF\(_0\) = 0.96 ± 0.13 (\(n = 31\)) in the MS cells, and no detectable Ni\(^{2+}\) sensitivity in the untransfected FlpIn-293 cells (Fig. 4, B and D, red bars).

Although fluorescence imaging of Ca\(^{2+}\) signals in multiple nondialyzed fluo 4-AM-stained cells is useful for verifying and comparing levels of expression, detailed investigation of NCX regulation also calls for voltage-clamp experiments that allow measurements of I\(_{NaCa}\) and dialysis of an internal solution with increased [Na\(_o\)] (0.5 mM) produced inward currents of 0.1 mM K\(_5\)-fluo 4 and 0.02 mM Ca\(^{2+}\). S cells (Fig. 5, C and D) were voltage clamped at -60 mV and dialyzed with a solution containing 0.1 mM K\(_5\)-fluo 4 and 0.02 mM Ca\(^{2+}\) to weakly buffer [Ca\(^{2+}\)] in 100 nM, with the intention of activating NCX without completely abolishing changes in Ca\(^{2+}\)-dependent fluorescence. Rapid (1 s) application of solution with increased [Na\(_o\)] (210 mM) and reduced [Ca\(^{2+}\)] (0.5 mM) produced inward currents of -2.81 ± 0.72 μA/μF.

Fig. 5. Activation of Ca\(^{2+}\) fluxes by changes in [Na\(_o\)] produces matching changes in Na\(^+/Ca\(^{2+}\) exchange current (I\(_{NaCa}\); A and C) and Ca\(^{2+}\) signals (B and D) in weakly Ca\(^{2+}\)-buffered voltage-clamped cells with stable expression of shark NCX (S cells; A and B) and mutant shark NCX (MS cells; C and D). Ca\(^{2+}\)-influx and -efflux modes were activated, respectively, by reduction of [Na\(_o\)] (from 140 to 5.4 mM, by TEA-Cl substitution) or elevation of [Na\(_o\)] (from 140 to 210 mM, by Na\(^+\) addition) during reduction of [Ca\(^{2+}\)] (from 5 to 0.5 mM) for 1-s intervals. Insets (a–f): fluorescence images at the indicated times and [Na\(_o\)]. [Voltage-clamped FlpIn-293 cells were held at -60 mV and dialyzed with internal solution, where intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) was buffered at 100 nM by 0.1 mM BAPTA and 0.1 mM K\(_5\)-fluo 4 and 0.02 Ca\(^{2+}\); ratiometric normalization of images and color scale as in Fig. 2.]

AJP-Heart Circ Physiol • VOL 296 • JUNE 2009 • www.ajpheart.org
pA/pF (n = 15) for the S cells (Fig. 5A) and -2.70 ± 0.64 pA/pF (n = 14) for the MS cells (Fig. 5C) that were accompanied by efflux of Ca$^{2+}$, as judged from the accompanying gradual decline in Ca$^{2+}$-dependent fluorescence (Fig. 5, B and D). Conversely, a reduction in [Na$^{+}$]$_o$ to 5.4 mM produced an outward current of 1.90 ± 0.51 pA/pF (n = 15) for S cells (Fig. 5A) and 1.91 ± 0.46 pA/pF (n = 15) for MS cells (Fig. 5C) accompanied by a gradual increase in fluorescence indicative of Ca$^{2+}$ influx (Fig. 5, B and D). The currents subsided or reversed direction after the cells were returned to standard solution, and the Ca$^{2+}$-dependent fluorescence started to decline slowly to baseline levels. These results establish a link between the cumulative $I_{NaCa}$ and the Ca$^{2+}$ signals.

We generally observed that the currents resulting from high and low [Na$^{+}$]$_o$ crossed each other during reequilibration (Fig. 5, A and C). This is consistent with reversal of $I_{NaCa}$ during the declining phase of the Ca$^{2+}$ transients (Fig. 5, B and D). Similarly, a gradual decline in outward current was often observed during exposure to low [Na$^{+}$]$_o$ (Fig. 5C). This finding is consistent with a slight reduction in the driving force of NCX caused by elevation of [Ca$^{2+}$] (Fig. 5D) (14). It appears less certain whether changes in driving force may explain the conspicuous, but quite variable, inward tail currents (-1.30 ± 0.92 pA/pF, n = 15) that were frequently observed when normal [Na$^{+}$]$_o$ was reintroduced after exposure to low [Na$^{+}$]$_o$ (Fig. 5A).

In the experiments illustrated in Fig. 6, we examined whether such tail currents might reflect [Ca$^{2+}$]-dependent activation of $I_{NaCa}$ (19) or might be an artifact of the expression system. For this purpose, we compared voltage-clamp experiments where high concentrations of Ca$^{2+}$ buffer were dialyzed into freshly dissociated shark cardiomyocytes (Fig. 6A) and S cells (Fig. 6B). The different ionic milieus of mammals and sharks called for the use of somewhat different Ca$^{2+}$-buffering solutions, and the Ca$^{2+}$-dependent fluorescence (Fig. 5, D) that were accompanied by efflux of Ca$^{2+}$ as indicated by the gradual increase in fluorescence (Fig. 5, B) were larger in shark cardiomyocytes [4.13 ± 0.89 pA/pF (n = 8) and -5.15 ± 1.68 pA/pF (n = 6)] than in S cells [1.74 ± 0.43 pA/pF and -0.62 ± 0.19 pA/pF (n = 13 each)]. Low- and high-Ca$^{2+}$-buffering conditions produced similar, statistically insignificant, peak inward and outward current magnitudes after 1-s solution exchanges in S and MS cells. However, although the inward tail currents were present in Ca$^{2+}$-buffered cells, the increased buffering, despite considerable scatter, appeared to have some suppressive effect (-1.30 ± 0.92 vs. -0.62 ± 0.19 pA/pF), suggesting a degree of Ca$^{2+}$-dependent activation. This notion is supported by the findings that the inward tail currents were larger when 1) the Ca$^{2+}$ signals were larger, 2) the outward $I_{NaCa}$ decreased significantly in amplitude during the preceding exposure to low [Na$^{+}$]$_o$, and 3) the duration of this exposure was increased (1–5 s; data not shown).

In summary, generation of NCX current by S and MS cells in a mammalian system is supported by Ni$^{2+}$-sensitive Ca$^{2+}$ fluxes and the simultaneously measured $I_{NaCa}$, which, in turn, has properties similar to those found in shark cardiomyocytes.

**Regulation of Cloned Shark NCX by β-Adrenergic Stimulation**

The bimodal characteristics of β-adrenergic regulation of shark NCX were identified initially (43) on the basis of voltage-clamp measurements of $I_{NaCa}$ in freshly dissociated ventricular cells with minimal Ca$^{2+}$ buffering. Here we tested whether the expressed shark NCX displays similar regulation independent of Ca$^{2+}$ buffering or alterations in the reversal potential ($E_{NaCa}$). Using nondialyzed S cells, we measured the Ni$^{2+}$-sensitive Ca$^{2+}$ influx before (Fig. 3E, trials 1–3) and after (Fig. 3F, trials 4–6) the cells had been exposed to 10 μM DBcAMP for 300 s. The stability of the cells was evaluated by monitoring the baseline fluorescence before each low...
[\text{Na}^+]_o\) trial (Fig. 3E) and during the incubation with DBcAMP. As shown in Fig.-4, B and D, DBcAMP reduced the Ni\textsuperscript{2+}-sensitive NCX Ca\textsuperscript{2+} influx by 75% in S cells (\(\Delta F/F_0 = 1.12 \pm 0.10\) and 0.28 \(\pm 0.05\) for control and DBcAMP, respectively, \(n = 20, P = 0.0025\)) and by 58% in MS cells (\(\Delta F/F_0 = 0.96 \pm 0.06\) and 0.40 \(\pm 0.15\) for control and DBcAMP, respectively, \(n = 3, P = 0.04\)). Control FlpIn-293 cells showed little Ni\textsuperscript{2+}-sensitive Ca\textsuperscript{2+} influx or effect of DBcAMP (\(\Delta F/F_0 = 0.02 \pm 0.04\) and 0.01 \(\pm 0.02\) for control and DBcAMP, respectively, \(n = 9\)).

The effects of \(\beta\)-adrenergic modulation of S and MS cells were compared in parallel experiments, where changes in \([\text{Na}^+]_o\) were used to activate \(I_{\text{NaCa}}\) in the outward or inward direction in voltage-clamped FlpIn-293 cells held at -60 mV (Fig. 7). As summarized in Fig. 7B, which shows data from high- and low-Ca\textsuperscript{2+}-buffering experiments, DBcAMP reduced outward \(I_{\text{NaCa}}\) of S cells by 29 \(\pm 4\%\) (\(n = 21, P < 0.0001\)) but variably increased inward \(I_{\text{NaCa}}\) by 22 \(\pm 18\%\) (\(n = 23\)). In contrast, \(I_{\text{NaCa}}\) of MS cells was reduced in the outward (23 \(\pm 8\%, n = 17, P = 0.0078\); Fig. 7D) and inward (20 \(\pm 7\%, n = 19, P = 0.0134\)) directions, whereas changes in \(I_{\text{NaCa}}\) of dog NCX were insignificant: inward current changed 1 \(\pm 6\%\), and outward current changed 2 \(\pm 5\%\) (\(n = 17\)).

After separation of the data on the basis of Ca\textsuperscript{2+} buffering, we found that dialysis of extra Ca\textsuperscript{2+} buffers in S cells slightly altered the effects of DBcAMP, so that the suppression of outward \(I_{\text{NaCa}}\) was increased from 25 \(\pm 8\%\) (\(n = 8\)) to 31 \(\pm 5\%\) (\(n = 13\)), whereas the enhancement of inward \(I_{\text{NaCa}}\) was reduced from 42 \(\pm 35\%\) (\(n = 10\)) to 6 \(\pm 18\%\) (\(n = 13\)), for low- and high-Ca\textsuperscript{2+} buffering, respectively. Thus, although the enhancement of inward \(I_{\text{NaCa}}\) appears to be most pronounced in cells with low-Ca\textsuperscript{2+} buffering, bimodal regulation was invariably seen as predominant suppression of outward \(I_{\text{NaCa}}\).
Detailed current-voltage (I-V) relations were constructed from four current traces (Fig. 8, B, C, E, and G) that were recorded immediately before (a), during (b and c), and after (d) rapid application of 5 mM Ni\(^{2+}\). From these traces, we obtained \(I_{\text{NaCa}}\) as the Ni\(^{2+}\)-sensitive membrane current \([a - b - c + d]/2\]. By relating the current measured at different times to simultaneous values of \(V_m\) during the ramp-clamp protocol, we constructed the I-V relation shown as “control” in Fig. 8, B (shark cardiomyocyte), B (S cell), and H (MS cell). The same procedure was used to measure I-V relations 2.5 min \((e-h)\) in Fig. 8, C, E, and G) and 4 min \((i-l)\) in Fig. 8, C, E, and G) after \(\beta\)-adrenergic stimulation. The I-V relations in Fig. 8D show that, in a native shark myocyte, epinephrine suppresses outward \(I_{\text{NaCa}}\) at +60 mV (Fig. 8F), leaving inward \(I_{\text{NaCa}}\) essentially unchanged from −120 mV to the only slightly altered \(E_{\text{NaCa}}\) (−58 to −54 mV). The I-V relations in Fig. 8F for S cells show similar effects of DBcAMP: the outward \(I_{\text{NaCa}}\) was significantly reduced at positive potentials without significant changes of inward \(I_{\text{NaCa}}\) from −120 mV to the stable \(E_{\text{NaCa}}\) (−60 mV). One noticeable difference is the dip in the control I-V relation of the shark cardiomyocyte at ~0 mV (Fig. 8E), which is thought to represent residual \(I_{\text{Ca}}\) since it disappeared in cardiomyocyte experiments, where 1–5 \(\mu\)M nifedipine was added to the perfusate and was never observed in the FlpIn-293 cell lines, probably reflective of the absence of significant \(I_{\text{Ca}}\) in HEK-293 cells. Interestingly, the same experiment in MS cells revealed a suppression of outward and inward \(I_{\text{NaCa}}\). Although comparison of \(E_{\text{NaCa}}\) of the shark (−62 ± 6 mV, \(n = 8\)) with \(E_{\text{NaCa}}\) of the mutant shark (−46 ± 8 mV, \(n = 14\)) reveals a trend toward a slightly more positive reversal potential for the mutant shark, the difference between the two was not significant (\(P = 0.1517\)).

The ramp-clamp experiments uncovered a degree of variability similar to that of experiments with altered \([Na^+]_o\) (cf. Figs. 8 and 9 with Fig. 7), where we found that the inward \(I_{\text{NaCa}}\) in some shark cardiomyocytes and S cells was actually enhanced by, rather than insensitive to, \(\beta\)-adrenergic stimulation (cf. Fig. 9, A and C and Fig. 8, D and F). The relative change of \(I_{\text{NaCa}}\) at different potentials was found to vary smoothly with \(V_m\) and could be approximated by Boltzmann distribution \([\exp(-0.12 \times FV_m/RT)]\) (where \(F\) is Faraday’s constant, \(R\) is gas constant, and \(T\) is absolute temperature Kelvin), except near \(E_{\text{NaCa}}\), where it was poorly defined. In contrast, the mutant shark NCX displayed the characteristic suppression pattern of outward and inward \(I_{\text{NaCa}}\) (Figs. 8H and 9F).

Results from 7 native shark cardiomyocytes, 8 S cells, and 14 MS cells are quantified in Fig. 9, B, D, and F. The results from the shark cardiomyocytes showed that, on average, epinephrine did
not change $I_{\text{NaCa}}$ at $-120$ mV [ratio of epinephrine current ($I_{\text{Epi}}$) to control current ($I_{\text{Control}}$) was $1.04 \pm 0.19$, $n = 7$] but reduced the current at $+60$ mV by nearly one-half ($I_{\text{Epi}}/I_{\text{Control}} = 0.60 \pm 0.04$, $n = 7$). $E_{\text{NaCa}}$ did not significantly change (from $-52 \pm 5$ to $-51 \pm 4$ mV, $n = 9$). An enhancement of $I_{\text{NaCa}}$ at $-120$ mV was observed in two of seven cells.

Similarly, in the S cells, on average, DBcAMP did not change $I_{\text{NaCa}}$ at $-120$ mV [ratio of cAMP current ($I_{\text{cAMP}}$) to $I_{\text{Control}} = 0.97 \pm 0.49$ (SE), $n = 8$] but did significantly reduce $I_{\text{NaCa}}$ at $+80$ mV ($I_{\text{cAMP}}/I_{\text{Control}} = 0.7 \pm 0.04$, $P = 0.0002$, $n = 8$). $E_{\text{NaCa}}$ was not significantly altered ($-62$ vs. $-63$ mV, $n = 8$). Enhancement of $I_{\text{NaCa}}$ at $-120$ mV was observed in three of eight cells.

In contrast, DBcAMP in the MS cells reduced $I_{\text{NaCa}}$ at $-120$ mV ($I_{\text{Epi}}/I_{\text{Control}} = 0.76 \pm 0.06$, $n = 14$) and reduced the current at $+80$ mV by nearly one-half ($I_{\text{Epi}}/I_{\text{Control}} = 0.62 \pm 0.05$, $n = 14$). Again, $E_{\text{NaCa}}$ did not change significantly ($-46$ vs. $-44$ mV, $n = 14$, $P = 0.1517$).

The experiments with adrenergic agonists show that shark NCX displays a degree of bimodal regulation that 1) does not depend critically on Ca$^{2+}$ buffering, 2) is reflected in Ca$^{2+}$ signals and $I_{\text{NaCa}}$, and 3) is present whether the Ca$^{2+}$-influx and -efflux modes are activated by changing [Na$^+$], or $V_m$, and 4) have remarkably similar effects on the voltage dependence of $I_{\text{NaCa}}$ in the mammalian expression system and native shark cardiomyocytes. Loss of bimodal regulation in the MS cells suggests that the mutant’s deleted unique molecular motif plays a critical role in cAMP-mediated regulatory activity.

**DISCUSSION**

Here we cloned the shark cardiac NCX, expressed the shark clone in mammalian cell lines, and studied its regulation. The bimodal characteristics of the cloned shark’s cAMP-mediated regulation resembled those of freshly dissociated shark ventricular cardiomyocytes, persisted with different degrees of intracellular Ca$^{2+}$ buffering, and were changed to unimodal inhibition after deletion-mutation of a unique shark region 2 insert.

Cells from sharks and mammals differ greatly with respect to osmolarity (~900 vs. 300 mosM) and [Na$^+$], (~60 vs. ~5–8 mM), and these values, as well as [Ca$^{2+}$], are unlikely to change significantly during the relatively brief (3–5 min) applications of β-adrenergic agonists, especially in cells that are dialyzed with Ca$^{2+}$-buffered internal solutions via low-resistance (~5 Ω) patch pipettes. These observations suggest that bimodal regulation of shark NCX is an inherent property of this protein and is unlikely to be mediated by the conventional types of [Ca$^{2+}$]-dependent activation (19) or [Na$^+$]-dependent inactivation (20). Our findings extend previous studies of mammalian and amphibian cardiac NCXs that have shown gain or loss of β-adrenergic regulatory properties after insertion and deletion of specific molecular motifs (25).

**Characteristic Features of the Amino Acid Sequence of Shark Cardiac NCX**

The present study was undertaken to explore whether the bimodal cAMP-dependent regulation of shark NCX (43)
depends on specific molecular motifs, such as the 29-bp block insertion encoding a P loop in frog NCX (17), or unique cellular environmental factors, such as osmolarity, ionic concentration, and AKAPs. The determined sequence of shark NCX may be characterized as “cardiac type,” since it includes a close equivalent corresponding to the stretch of amino acids coded by exons A, C, D, E, and F of NCX1.1 of higher vertebrates. Redundant sequencing of shark cardiac mRNAs in cloned and PCR-derived fragments from several samples of shark heart (see Supplemental Fig. 1) produced no signs of a P loop (nucleotide-binding Walker A sequence, GxxxxxGKT) similar to that of frog cardiac NCX. In fact, the current genomic information suggests that the P loop of frog derives from extension of exon E, and although such an extension could also produce a P loop in some other species (Homo sapiens, Pan troglodytes, Macaca mulatta, Bos taurus, and Rattus norvegicus), this has not been observed and would generally corrupt the reading frame. Inasmuch as shark tissue collection is typically restricted to the summer season, a remote possibility remains that a shark NCX variant with a P loop may appear when the animals are not readily accessible. The P-loop mechanism has only been observed in frog (17, 38).

The most conspicuous features of shark cardiac NCX, which set it apart from cardiac-specific isoforms of other organisms, are the two A/P-rich block insertions in the long cytoplasmic loop. The longer insert (region 2) is added to CBD1 at a location corresponding to the location of the variable spliced exons (A–F) within CBD2 (Fig. 1). This provides some similarity between exon X and the shark region 2 insert, since both may extend a flexible linker sequence (11, 35) and provide or modulate specific binding sites. More extensive experiments are required to determine whether and to what extent the shark region 2 insert and the variably spliced region may share a common functionality.

The shorter shark insert (region 1) is found at a location where NCX of C. intestinalis also has some additional amino acids compared with higher vertebrates (Fig. 1; see Supplemental Fig. 2). The structure of this region of NCX is not known, but sequence similarity suggests that the shark region 1 insert extends an unstructured loop that originates from the first (segment A) α-helix of a catenin-like domain (44) and circumscribes its second (segment B) α-helix (Fig. 1, C and D).

In light of these observations, it is possible that the long cytoplasmic loop is organized as an assemblage of modular components with additional flexible linker sequences, which could provide a degree of flexibility that allows binding of regulatory moieties (e.g., A-kinase-anchoring proteins and PKA catalytic unit) or impedes the activity of phosphatases, thus explaining difficulties in crystallizing the entire NCX molecule (15).

Expression of Shark and Mutant Shark Cardiac NCX in Mammalian Cell Lines

The expression of full-length cDNAs of shark and mutant shark NCX in HEK-293 and FlpIn-293 cells was verified and compared with each other as well as appropriate controls. The two mammalian expression systems were shown to have only small endogenous Ca²⁺-influx pathways, consistent with their greatly reduced Ca²⁺ transients (Fig. 4). To quantify the NCX-generated currents and Ca²⁺ fluxes, millimolar concentrations of Ni²⁺ or Cd²⁺ were used to block NCX rapidly and reversibly (Figs. 2, 3, and 8, C, E, and G; see Supplemental Fig. 3). The Ni²⁺-insensitive residual Ca²⁺ fluxes were larger in HEK-293 (Figs. 2 and 4) than in FlpIn-293 (Figs. 3 and 4) cells, regardless of the type of NCX transfected, inasmuch as similar results were found in dog, shark, and mutant shark NCXs.

Normalization of  𝐈_{NaCa} relative to the membrane capacitance showed that the level of stable expression of shark and mutant shark NCX in FlpIn-293 cells was comparable to that found in freshly dissociated cardiomyocytes (2–3 pA/pF; Fig. 8, D, F, and H and Fig. 9, A, C, and E). Successful expression of shark NCX is also supported by the similarity of regulatory effects of adrenergic agonists in mammalian expression systems and native shark cardiomyocytes (Figs. 8 and 9), as well as by the large inward tail currents following brief intervals of partial Na⁺ withdrawal (Figs. 5A and 6B).

Inward Tail Currents

Our finding that readmission of normal extracellular Na⁺, after 1–2 s of partial Na⁺ deprivation, produced such large inward  𝐈_{NaCa} in cells with heterologous expression of NCX (Figs. 5A and 6B) or in native shark cardiomyocytes (Fig. 6A) is novel. These currents are larger than the inward currents that are typically measured at hyperpolarizing potentials and are of a magnitude that could provide sufficient Ca²⁺ efflux to cause rapid relaxation. We estimate that a charge transfer via  𝐈_{NaCa} of ~200 pF in shark cells (~10 μm diameter, ~100 μm long) with a volume of ~8 pl in 1 s would change the total intracellular Ca²⁺ content by ~125 μM [(200 × 10⁻⁹ charge/s × 1 s)/(8 × 10⁻¹² liters × 2 × 10⁸ charge/M)], which is enough to saturate the myofilaments (40) or significantly alter  𝐸_{NaCa} in shark cells with low concentrations of Ca²⁺ buffers (43). In FlpIn-293 cells, Ca²⁺ fluxes of that order of magnitude are consistent with the observed Ca²⁺ signals (Fig. 5) and the strong suppression of tail currents on addition of 5 mM EGTA to the internal solution (Fig. 5 vs. Fig. 6B). It is surprising, therefore, that the tail currents remained strong in shark cardiomyocytes with high concentrations of Ca²⁺ buffers (Fig. 6B vs. Ref. 43), where effective dialysis was supported by the stability of the measured  𝐸_{NaCa} during β-adrenergic stimulation. This suggests that the tail currents may reflect changes in  𝐸_{NaCa} and Ca²⁺-dependent activation of  𝐈_{NaCa} (19), as well as an enhancement of the Ca²⁺-efflux mode similar to that observed with β-adrenergic stimulation (Fig. 8).

Adrenergic Regulation of Shark and Mutant Shark NCX

cAMP-dependent regulation of shark NCX was evoked in shark cardiomyocytes by β-adrenergic agents (epinephrine or isoproterenol). However, in HEK-293 cells, which have been reported to lack β-receptors and to have stunted β-adrenergic responses, cAMP or its nonhydrolyzable analog (DBcAMP) was used to activate PKA directly. As mentioned above, sharks and mammals provide distinctly different ionic milieus and may have different baseline PKA and phosphatase activities. Also, it is possible that mammalian expression systems may not incorporate shark NCX into the cytoskeleton in the same way as in shark cardiomyocytes or express A-kinase-anchoring proteins, which are effective across the vertebrate species.
Under these conditions, it is significant that the cAMP-dependent regulation of the expressed shark NCX was indistinguishable from that found in its native environment (Figs. 8 and 9).

In critical tests of ß-adrenergic modulation (Figs. 7–9), we used internal dialysis to impose fixed [Na\(^{+}\)], and [Ca\(^{2+}\)]; to abolish or minimize the allosteric intracellular effects of these ions (19, 20). In fact, significant Na\(^{+}\)-lag effects are unlikely to occur during brief (10 s) activation of NCX constructs, even in nondialed mammalian cells (Figs. 1–4) or in the intact shark myocardium, where baseline [Na\(^{+}\)] is ~50 mM. The possibility of significant changes in [Ca\(^{2+}\)]; was excluded by use of high-Ca\(^{2+}\) buffers, brief voltage-clamp depolarization (Fig. 8A), and screening of the data based on the stability of ENaCa (Fig. 8, C, E, and G and Fig. 9, A, C, and E). It is unlikely, therefore, that the effects of cAMP-dependent regulation are mediated by changes in [Ca\(^{2+}\)];, and [Na\(^{+}\)].

The rundown of NCX during experiments is a concern, in part, because we rarely achieved complete washout of the cAMP-dependent effects. However, the absence of cAMP regulatory effects on dog NCX under the same experimental conditions (Fig. 7) suggests that the cAMP-dependent effects are specific to the shark NCX and are not a result of rundown or the experimental paradigm. In addition, any potential rundown does not detract from the observation that the efflux mode of shark NCX is suppressed significantly more than the influx and efflux are specific to the shark NCX and are not a result of rundown or the experimental paradigm. In addition, any potential rundown does not detract from the observation that the efflux mode of shark NCX is suppressed significantly more than the influx and efflux. However, the absence of cAMP regulatory effects on dog NCX under the same experimental conditions (Fig. 7) suggests that the cAMP-dependent effects are specific to the shark NCX and are not a result of rundown or the experimental paradigm. In addition, any potential rundown does not detract from the observation that the efflux mode of shark NCX is suppressed significantly more than the influx and efflux are specific to the shark NCX and are not a result of rundown or the experimental paradigm. In addition, any potential rundown does not detract from the observation that the efflux mode of shark NCX is suppressed significantly more than the influx and efflux.

Physiological Implications of Bimodal Regulation of NCX

The cardiac NCX in shark and frog operates at low temperature and heart rate in cells where ICa is also under ß-adrenergic control but the SR is nonfunctional (26, 29, 33). Thus excitation-contraction coupling is controlled predominantly by sarcolemmal Ca\(^{2+}\) effluxes, of which the influx is mediated by the Ca\(^{2+}\) channel and NCX, but the efflux is controlled by the NCX alone to generate relaxation. In contrast, tidal Ca\(^{2+}\) effluxes in the more rapidly beating adult mammalian myocardium are generated primarily by the SR, where the ICa, generated by NCX during relaxation may cause depolarization and arrhythmias (39); this liability may be expected to increase when NCX is required to operate very fast or to remove a large fraction of the activator Ca\(^{2+}\) from the cytosol. These facts may explain the adaptive value of the large SR-recirculation fraction (>90%) (1) in the very rapidly beating hearts of rodents and the prevalence of arrhythmias in cardiac diseases where SR function is compromised and the expression of NCX is increased (32, 36).

Conversely, cAMP-dependent enhancement of inward INaCa in shark may be essential to achieve rapid Ca\(^{2+}\) influx and diastolic relaxation of the shark ventricle when the heart rate is increased by adrenergic stimulation of ICa. A simultaneous adrenergic-induced suppression of Ca\(^{2+}\) influx mode of NCX may serve to limit NCX-mediated Ca\(^{2+}\) influx during depolarization, so that force development is controlled primarily by the phosphorylated L-type Ca\(^{2+}\) channel during the initial part of the plateau of the cardiac action potential. Together, these two Ca\(^{2+}\)-transporting proteins may provide a regulation that is similar to that which, in mammals, is achieved with smaller transsarcolemmal Ca\(^{2+}\) fluxes and Ca\(^{2+}\)-induced Ca\(^{2+}\) release from the SR.

We found that the degree of bimodal regulation of shark cardiac NCX was quite variable, and the underlying causes remain to be determined. However, it is possible that although the Ca\(^{2+}\)-influx and -efflux modes of shark cardiac NCX are governed by a single reversal potential, they may be regulated independently. Under stressful conditions, this might provide the means by which the cell prevents Ca\(^{2+}\) overload and triggering of aberrant excitation by inward INaCa. Intriguingly, similar flexible control mechanisms may be latent or inducible in mammalian hearts.

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