

**The Functional Role of Ionic Regulation
of the Na⁺/Ca²⁺ Exchanger
Assessed in Transgenic Mouse Hearts**

**A Thesis Presented to the University of Manitoba
in Partial Fulfillment of the Requirement
for the Degree of:**

MASTER OF SCIENCE IN PHYSIOLOGY

**By
KRISTA DAWN MAXWELL**

**Department of Physiology
Faculty of Medicine
University of Manitoba**

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The Functional Role of Ionic Regulation of the Na⁺/Ca²⁺ Exchanger Assessed in Transgenic Mouse Hearts

BY

Krista Dawn Maxwell

**A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University
of Manitoba in partial fulfillment of the requirements of the degree**

of

MASTER OF SCIENCE

KRISTA DAWN MAXWELL©1999

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LIST OF ABBREVIATIONS

BSA, bovine serum albumin

Ca(NO₃)₂, calcium nitrate

Ca(OH)₂, calcium hydroxide

CaCl₂, calcium chloride

CaCO₃, calcium carbonate

CsOH, cesium hydroxide

EGTA, ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetra-acetic acid

HEPES, (N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid])

K₂HPO₄, potassium hydrophosphate

KCl, potassium chloride

KH₂PO₄, potassium phosphate

KOH, potassium hydroxide

LiOH, lithium hydroxide

MES, (2-[N-morpholino] ethanesulfonic acid)

Mg(OH)₂, magnesium hydroxide

MgCl₂, magnesium chloride

MgSO₄, magnesium sulfate

MOPS, (3-[N-morpholino] propanesulfonic acid)

NaCl, sodium chloride

NaH₂PO₄, sodium dihydrophosphate

NaHCO₃, sodium bicarbonate

NaOH, sodium hydroxide

NH₃SO₃, sulfamic acid

NMG-MES, N-methyl-D-glucamine-(2-[N-morpholino] ethanesulfonic acid

TEA-OH, tetraethylammonium hydroxide

Abstract

Sodium-calcium exchange is the principal mechanism for cellular Ca^{2+} efflux following a cardiac contraction. On a beat-to-beat basis, the $\text{Na}^+/\text{Ca}^{2+}$ exchanger removes a similar quantity of Ca^{2+} from cardiac cells as that entering through L-type Ca^{2+} channels. As such, the $\text{Na}^+/\text{Ca}^{2+}$ exchanger plays a critical role in controlling cardiac contractility. The exchanger is an electrogenic transporter, which uses the energy within the Na^+ electrochemical gradient to accomplish transsarcolemmal Ca^{2+} transport. In exchange for 3 Na^+ , one Ca^{2+} is removed, resulting in a net movement of one positive charge *per* exchange cycle. Electrophysiological analyses of the canine cardiac exchanger, NCX1.1, have established that, in addition to transporting Na^+ and Ca^{2+} , the exchanger is also regulated by these ions. As well, structure-function studies have identified regions within the large cytoplasmic loop of the exchanger that are functionally important in mediating these ionic regulatory processes. However, there is little to no information concerning the role of these regulatory processes in cardiac physiology. To begin determining whether or not ionic regulation of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger is physiologically relevant, transgenic mice overexpressing either the wild-type canine cardiac exchanger, NCX1.1, or a deletion mutant in which ionic regulation has been nearly abolished, were examined. The initial characterization was carried out on *Xenopus laevis* oocytes expressing these two different exchangers to define their regulatory properties. Since these $\text{Na}^+/\text{Ca}^{2+}$ exchangers originated from a cloned canine exchanger and were being expressed in a mouse model system, the native regulatory processes of both dog and mouse $\text{Na}^+/\text{Ca}^{2+}$ exchangers were then examined to determine whether or

not species differences existed. Using the giant excised patch clamp technique, ventricular myocytes isolated from each species were studied. The native exchangers from both species were found to exhibit similar Na^+ and Ca^{2+} regulatory phenotypes and exchange currents were stimulated in the presence of regulatory Ca^{2+} over a similar concentration range. Ionic regulation was then characterized for cardiomyocytes obtained from control and transgenic mice using the above approach. While normal ionic regulation was observed for control and transgenic mice overexpressing NCX1.1, ionic regulation in mice overexpressing $\Delta 680-685$ was nearly abolished. This indicated that transgene expression was able to overwhelm the native regulatory processes. To determine the functional consequences of overexpressing NCX1.1 or $\Delta 680-685$, post-rest force development in papillary muscles from the transgenic mice was examined. This contractile paradigm provides insight into the interplay between the sarcolemmal and sarcoplasmic reticular Ca^{2+} removal mechanisms. Results were obtained from muscles stimulated over a range of frequencies and rest intervals. Post-rest potentiation, the ratio of the contraction after the rest interval compared to the one prior, was greater for the $\Delta 680-685$ transgenic mouse muscles at all intervals and frequencies. Therefore, through the ablation of ionic regulation and the consequences observed with respect to excitation-contraction coupling, a significant physiological role for ionic regulation of $\text{Na}^+/\text{Ca}^{2+}$ exchange currents is suggested.

Literature Review

Role of Na⁺/Ca²⁺ Exchange in Cardiac Excitation-Contraction Coupling

Cell survival is dependent on many factors and calcium plays a pivotal role. In most tissues, Ca²⁺ also plays a major role in cellular signaling. For example, in cardiac muscle, the intracellular Ca²⁺ concentration varies from 0.1-10 μM while the extracellular Ca²⁺ concentration remains close to 1.25 mM. Maintaining this large concentration gradient, as well as controlling its temporal fluctuations, is very important and several mechanisms have evolved to regulate Ca²⁺ homeostasis.

The Na⁺/Ca²⁺ exchange process, first identified in cardiac muscle in the 1960's (118), is an important contributor to cellular Ca²⁺ homeostasis. In cardiac muscle, Na⁺/Ca²⁺ exchange is regarded as the primary mechanism for transsarcolemmal Ca²⁺ removal and as a result, plays a significant role in maintaining Ca²⁺ homeostasis and proper cell functioning. Calcium moves down its large concentration gradient into the cell in order for a contraction to occur. This is followed by the interplay between a variety of Ca²⁺ removal mechanisms, which lower cytoplasmic Ca²⁺ in order for muscle relaxation to occur prior to the next contraction (15).

Upon depolarization of the cardiac sarcolemma, voltage-gated L-type Ca²⁺ channels are activated allowing Ca²⁺ to enter the myocyte. This pool of Ca²⁺, termed trigger Ca²⁺, then binds to ryanodine receptors to initiate additional Ca²⁺ release from the sarcoplasmic reticulum (SR) (19; 29; 36; 97). This process, first identified in cardiac

muscle by Fabiato, is referred to as calcium-induced calcium release (44). It is facilitated by the close physical relationship between the L-type Ca^{2+} channels, $\text{Na}^+/\text{Ca}^{2+}$ exchangers and the SR Ca^{2+} release channels (34; 35; 120; 129). An inappropriate coupling of the L-type Ca^{2+} channels to ryanodine receptors has been associated with diseases such as hypertrophy and heart failure whereby EC coupling becomes defective, highlighting the importance of this close proximity (48). However, while L-type Ca^{2+} channels are responsible for the initial increase in intracellular Ca^{2+} (12; 36; 117), it is the SR which releases the majority of Ca^{2+} required for cardiac contraction. As such, it is often referred to as activator Ca^{2+} .

Relaxation of the myocyte depends on the lowering of Ca^{2+} to diastolic levels of ~ 100 nM which is carried out by the SR Ca^{2+} -ATPase, sarcolemmal Ca^{2+} -ATPase and the $\text{Na}^+/\text{Ca}^{2+}$ exchanger. The SR Ca^{2+} -ATPase uses energy from ATP hydrolysis to transport Ca^{2+} against a large concentration gradient (66). In general, the SR Ca^{2+} -ATPase resequesters the same amount of Ca^{2+} back into the SR as was released previously to initiate contraction. It has been reported that SR Ca^{2+} uptake is responsible for $\approx 75\%$ of relaxation in rabbit ventricular myocytes (65) and similar results were obtained for rabbit ventricular muscle (18). Recent examinations of the role of the SR Ca^{2+} -ATPase in mouse have also been carried out. Transgenic mice overexpressing the SR Ca^{2+} -ATPase were shown to have altered myocardial contractility including enhanced relaxation (5, 55). The importance of the SR Ca^{2+} -ATPase was further demonstrated in mouse when, upon removal of one functional copy of the SR Ca^{2+} -ATPase isoform 2

gene, relaxation was found to be impaired (106). In general, mouse and rat are similar with respect to showing a considerable dependence on the SR for relaxation.

In addition to the SR Ca^{2+} -ATPase, relaxation of the myocyte also depends upon two sarcolemmal (SL) mechanisms for Ca^{2+} efflux. First, the SL Ca^{2+} -ATPase transports Ca^{2+} to the extracellular side of the membrane. However, in comparison to the amount transported by the $\text{Na}^+/\text{Ca}^{2+}$ exchanger, the SL Ca^{2+} -ATPase is not considered to be of major significance to Ca^{2+} extrusion (16; 136). For example, the rate of muscle relaxation in rabbit ventricular muscles, solely dependent upon SL Ca^{2+} -ATPase, was 1200% slower compared to muscles where the SR Ca^{2+} -ATPase and $\text{Na}^+/\text{Ca}^{2+}$ exchange mechanisms were fully functional (18). Further evidence that the SL Ca^{2+} -ATPase plays a minor role comes from the finding that there is a lack of intracellular Ca^{2+} increase upon the loss of Ca^{2+} pump function (32). Therefore, it appears that the SL Ca^{2+} -ATPase may be responsible for simply fine tuning Ca^{2+} concentrations, but does not provide a major pathway for Ca^{2+} efflux.

The major pathway for sarcolemmal Ca^{2+} extrusion during cell relaxation is the $\text{Na}^+/\text{Ca}^{2+}$ exchanger. This transport process can greatly affect cardiac contractility through its influence on both diastolic and systolic Ca^{2+} levels. The importance of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger is exemplified by considering the effects of cardiac glycosides (*ie.* digoxin). Through the inhibition of the Na^+/K^+ ATPase, these therapeutic agents operate by increasing cytoplasmic Na^+ levels. This slight rise (~ 1 -2 mM) in intracellular Na^+ is sufficient to alter the activity of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger and produce a positive inotropic effect (43; 113).

The amount of Ca^{2+} removed by the $\text{Na}^+/\text{Ca}^{2+}$ exchanger has been found to vary considerably between different species as well as with different stages of development (10; 136). $\text{Na}^+/\text{Ca}^{2+}$ exchange is responsible for the majority of Ca^{2+} efflux across the SL in immature hearts, which have not completed development of the T-tubular systems, and the SR. Similarly, late fetal rabbit and newborn rat hearts were found to have an eight or six fold greater amount of $\text{Na}^+/\text{Ca}^{2+}$ exchange protein, respectively, when compared to adult hearts of rabbit or rat (21). Transcript levels for the $\text{Na}^+/\text{Ca}^{2+}$ exchanger measured from rat and rabbit myocytes have been found to peak near birth and decrease postnatally (21).

$\text{Na}^+/\text{Ca}^{2+}$ exchange contributes to relaxation through Ca^{2+} extrusion (9; 30; 103) but also has been found to play a role in systolic functioning. Upon initial depolarization of the SL, the Na^+ concentration increases as Na^+ enters voltage-dependent Na^+ channels. It is postulated that this increased cytoplasmic Na^+ is able to induce reverse mode $\text{Na}^+/\text{Ca}^{2+}$ exchange activity. As a result, Ca^{2+} is brought into the cell and contributes to Ca^{2+} -induced Ca^{2+} release during E-C coupling (80; 102). This second pathway for Ca^{2+} influx was suggested following studies whereby Ca^{2+} transients and cardiac contractions were observed despite blockage of the L-type Ca^{2+} channels by either Ca^{2+} channel blockers or positive potentials (62; 80). Further support for this mechanism comes from the finding that $\text{Na}^+/\text{Ca}^{2+}$ exchange proteins are located close to L-type Ca^{2+} channels and ryanodine receptors (129) and therefore have access to the mechanisms for triggering release of Ca^{2+} from the SR.

The precise role of reverse mode $\text{Na}^+/\text{Ca}^{2+}$ exchange in Ca^{2+} -induced Ca^{2+} release has been controversial. Rather than the sharp increase in the Ca^{2+} transient observed in response to Ca^{2+} flux through L-type Ca^{2+} channels, reverse mode Ca^{2+} entry results in a slower inward current and Ca^{2+} transient (89). These findings have led to suggestions that diminish the physiological importance of reverse mode $\text{Na}^+/\text{Ca}^{2+}$ exchange as a mechanism to initiate Ca^{2+} -induced Ca^{2+} -release (24; 31; 124; 127). Furthermore, the activation of β -adrenergic receptors or protein kinase A in rat heart myocytes, yields a process referred to as slip mode conductance whereby voltage-dependent Na^+ channels allow the passage of both Na^+ and Ca^{2+} ions (121). Calcium entry *via* this route is able to trigger release of Ca^{2+} from the SR and may be more powerful than reverse mode $\text{Na}^+/\text{Ca}^{2+}$ exchange (121). Finally, upon studying transgenic mice overexpressing the $\text{Na}^+/\text{Ca}^{2+}$ exchange protein, it was observed that physiological membrane potentials were unable to initiate SR Ca^{2+} release (2). Thus, a definitive account for the precise role of $\text{Na}^+/\text{Ca}^{2+}$ exchange in initiating cardiac E-C coupling has not yet been established.

Stoichiometry of the $\text{Na}^+/\text{Ca}^{2+}$ Exchanger

The $\text{Na}^+/\text{Ca}^{2+}$ exchanger is the primary mechanism for Ca^{2+} efflux from cardiac cells against a large opposing electrochemical gradient. This transport requires energy, which is obtained from the Na^+ electrochemical gradient. In order to maintain cellular Ca^{2+} homeostasis, the $\text{Na}^+/\text{Ca}^{2+}$ exchanger removes 1 Ca^{2+} ion in exchange for three Na^+ ions. Previously, a wide range of possible stoichiometries had been presented.

Examination of squid axon determined the stoichiometry to be in the region of 2-5 $\text{Na}^+/\text{Ca}^{2+}$ (6). Similarly, others proposed a value of 2.5 (126). However, considering the charge differences between these two ions and the amount of Ca^{2+} being brought into the cell by other transporters, a ratio of two Na^+ to one Ca^{2+} would not be adequate to support the low resting levels of Ca^{2+} found in myocytes. Furthermore, it can be shown theoretically that a stoichiometry of four Na^+ to one Ca^{2+} would produce an intracellular Ca^{2+} concentration that is far too low to support cell functioning. As a result, a 3:1 ratio was suggested and has become established through several studies involving the analyses of Na^+ and Ca^{2+} fluxes in vesicles and myocytes (72; 114). Similar stoichiometries have been obtained for tissues other than cardiac muscle (111; 140). Despite an abundance of research supporting a 3:1 stoichiometry, a recent abstract measuring reversal potential of $\text{Na}^+/\text{Ca}^{2+}$ exchange current suggested that the ratio is in fact 4:1 (92). To date however, the stoichiometry of 3:1 remains the accepted coupling ratio for this transport mechanism.

The transport stoichiometry of 3 Na^+ in exchange for 1 Ca^{2+} results in a net movement of one positive charge per exchange cycle (78) and enables the electrophysiological measurement of $\text{Na}^+/\text{Ca}^{2+}$ exchange activity. Exchange is thought to occur as part of a multi-step pathway whereby these ions are transported across the membrane consecutively rather than simultaneously (61; 71; 101). Hilgemann originally proposed an eight-state transport model (61). It was suggested that 3 Na^+ ions bind to a transport site followed by a state of occlusion. Translocation of Na^+ to the other side of the membrane occurs where the binding site releases Na^+ and then becomes available for

Ca^{2+} binding. Calcium binds, is occluded, and is then transported to the opposite side of the membrane and is released (61).

Topological Arrangement of the $\text{Na}^+/\text{Ca}^{2+}$ Exchanger

$\text{Na}^+/\text{Ca}^{2+}$ exchange activity has been identified in many tissues including heart, brain, and kidney, and has been isolated from numerous species including dog, squid, rat, *etc.* In cardiac tissue, initial suggestions for the molecular weight varied from 33 kD (52) to 82 kD (88; 128). The cloning of the cardiac $\text{Na}^+/\text{Ca}^{2+}$ exchanger revealed the actual weight to be 108 kD (99) while molecular weights for $\text{Na}^+/\text{Ca}^{2+}$ exchangers in rod outer segments and rat brain were found to be 130 kD (115) and ~108 kD (46), respectively.

With respect to protein structure, the $\text{Na}^+/\text{Ca}^{2+}$ exchanger was initially thought to consist of 970 amino acids. Furthermore, hydropathy analysis predicted a model of the exchanger consisting of 12 transmembrane spanning regions (99). Three different regions of the exchanger were identified including: 1) a hydrophobic N-terminal segment having 6 transmembrane spanning segments, 2) a large hydrophilic loop, 3) a hydrophobic region containing 6 C-terminal transmembrane segments. The large hydrophilic loop was predicted to exist on the intracellular side of the membrane (99).

Since the original model for the cardiac $\text{Na}^+/\text{Ca}^{2+}$ exchanger was proposed in 1990, this topological model has undergone numerous revisions. A signal sequence composed of 32 amino acids was identified (77) at the NH_2 terminal end and was thought to be a possible cleavage site. Subsequent studies revealed that this cleavage site was

situated between amino acids 32 and 33, and that the protein sequence began at this cleavage site (41). It is interesting to note that in addition to the cardiac exchanger, a cleavable signal has also been identified in the rod photoreceptor (115) and brain (84) exchangers despite the fact that most membrane transporters do not possess a such a signal sequence (41).

Glycosylation studies have assisted in the identification of the correct orientation of the exchanger since glycosylation of amino acids identifies them as being located extracellularly. With respect to the cardiac exchanger, glycosylation only occurs at position 9 (Asn) of the N-terminus (64) indicating that this portion of the exchanger is extracellular. Identification of the signal sequence and the glycosylation studies resulted in the modification of the model so that the $\text{Na}^+/\text{Ca}^{2+}$ exchanger protein was believed to consist of 11 transmembrane spanning regions having 5 on the N terminus and 6 on the C terminus. Further investigation showed that amino acid sequences, termed α repeats, were not only located between transmembrane segments 2 and 3 and between segments 8 and 9, but were also extracellular (121).

Recently, with the advent of molecular approaches, newer topological models of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger have been proposed (100). Through construction of a cysteineless $\text{Na}^+/\text{Ca}^{2+}$ exchanger, further examination of the topology was carried out. Of the revisions suggested, most were found on the C-terminal end of this transporter. The N-terminus remains situated on the extracellular side as previously confirmed by glycosylation and signal peptide cleavage studies. Some proposed changes to the C-terminal end include the relocation of the first C-terminal segment (segment 6) to the

large intracellular loop. It is also proposed that segment 9 does not span the membrane at all and that the C-terminal α repeat crosses the membrane only once rather than twice like the $\alpha 1$ repeat (100). These alterations result in the α repeats being located on the opposite sides of the membrane. A further examination, specifically aimed at the $\alpha 1$ and $\alpha 2$ repeats, was carried out using cysteine substituted NCX1. It was predicted that the connecting loop in the $\alpha 1$ region is found within the membrane rather than on the extracellular side and that it is involved in cation binding and transport (67). In this topological model, segment 6 remains in the intracellular loop while segment 9 is enveloped in the membrane resulting in an exchanger which is comprised of only 9 transmembrane spanning regions (67).

Regulation of $\text{Na}^+/\text{Ca}^{2+}$ Exchange Activity

Ca^{2+} -Dependent Regulation

Calcium is not only transported by the $\text{Na}^+/\text{Ca}^{2+}$ exchanger but also contributes to the regulation of this protein. First identified in the squid giant axon preparation (7), Ca^{2+} regulation has now been observed for all cloned exchangers and isoforms studied to date.

The exchange of three Na^+ for one Ca^{2+} can occur in either direction depending upon the prevailing electrochemical gradient. Irrespective of the direction of transport however, the transport process is stimulated by the presence of cytoplasmic Ca^{2+} . That is, cytoplasmic Ca^{2+} stimulates exchange activity by acting on a site distinct from the transport site. For forward $\text{Na}^+/\text{Ca}^{2+}$ exchange, where cytoplasmic Ca^{2+} exchanges for

extracellular Na^+ , regulatory Ca^{2+} and transported Ca^{2+} are present on the same membrane surface (i.e. cytoplasmic). This makes it difficult to distinguish between the influence of Ca^{2+} on transport vs. regulation. In contrast, if one examines reverse or outward $\text{Na}^+/\text{Ca}^{2+}$ exchange currents, where cytoplasmic Na^+ exchanges for extracellular Ca^{2+} , the ability to examine the influence of cytoplasmic regulatory Ca^{2+} is greatly simplified as transported and regulatory Ca^{2+} are now segregated to opposite membrane surfaces. For this reason, regulation of $\text{Na}^+/\text{Ca}^{2+}$ exchange activity by cytoplasmic Ca^{2+} is typically measured using outward or reverse $\text{Na}^+/\text{Ca}^{2+}$ exchange current measurements. The vast majority of information on this regulatory process has been obtained using the giant excised patch clamp technique. These studies have revealed that low concentrations of regulatory Ca^{2+} (e.g. 300 nM) are able to initiate exchange activity.

Once Ca^{2+} regulation became well characterized, studies proceeded to determine the protein regions of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger which were involved in this process. The cloning of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (99) allowed the manipulation of the protein structure using molecular biological techniques. It was soon established that the large cytoplasmic loop, which accounts for $\sim 1/2$ of the protein's mass, was involved in Ca^{2+} regulation. This information was obtained through mutagenesis studies whereby the large intracellular loop was deleted from the canine cardiac exchanger, NCX1.1. The resultant mutant exchanger was able to transport Ca^{2+} but was no longer regulated by this ion (94). Later, it was determined that a protein site capable of binding regulatory Ca^{2+} was located within the cytoplasmic loop (83; 93; 94). Using fusion protein approaches, the Ca^{2+}

binding site has been found to lie near the center of the cytoplasmic loop, span 138 amino acids, and have 2 acidic clusters within this region (83).

In mammalian exchangers, cytoplasmic Ca^{2+} is essential for initiating $\text{Na}^+/\text{Ca}^{2+}$ exchange activity and $\text{Na}^+/\text{Ca}^{2+}$ exchange current is severely depressed in the absence of regulatory Ca^{2+} regardless of the ionic gradient favoring exchange. $\text{Na}^+/\text{Ca}^{2+}$ exchange proteins appear to enter an inactive state in the absence of regulatory Ca^{2+} where $\text{Na}^+/\text{Ca}^{2+}$ exchange is not possible (59; 60). The application of regulatory Ca^{2+} then eliminates this inactivation by producing a conformational change in the protein, allowing a progressively larger fraction of the exchanger population to participate in current production (59; 60).

Calcium regulation has been examined, not only for mammalian exchangers and their isoforms, but also for the *Drosophila* $\text{Na}^+-\text{Ca}^{2+}$ exchanger, CALX1.1. The Ca^{2+} binding region for CALX1.1 has been shown to be ~75% identical to the Ca^{2+} binding site from mammalian exchangers (63; 84). However, while regulatory Ca^{2+} stimulated exchange activity for NCX1.1, it produced the opposite effect in CALX1.1. That is, for CALX1.1, the presence of regulatory Ca^{2+} inhibited exchange activity. Despite the contrasting responses (inhibitory vs stimulatory), regulation of exchange activity occurs for both NCX1.1 and CALX1.1 over the same Ca^{2+} concentration range.

Studies of Ca^{2+} regulation of $\text{Na}^+-\text{Ca}^{2+}$ exchange, made *in vitro*, have contributed to our knowledge of the kinetics and gating of $\text{Na}^+/\text{Ca}^{2+}$ exchange proteins but details about the physiological relevance of these processes are lacking. It has been suggested that perhaps Ca^{2+} regulation is necessary to maintain proper Ca^{2+} concentrations within a

cell, although there is currently no evidence for this proposition (57). The purpose of this thesis was to further study the physiological significance of this regulatory process through the examination of transgenic mice devoid of this ionic regulatory mechanism.

Na⁺-Dependent Regulation

Na⁺/Ca²⁺ exchange activity is also regulated by cytoplasmic Na⁺ in addition to transporting this ion (62). Application of Na⁺ initiates Na⁺/Ca²⁺ exchange activity, which manifests as a rapid rise in current to a peak level followed by a slow, time-dependent decay to a lower steady-state current level (62). This decay of current is termed Na⁺-dependent inactivation or I₁ inactivation. This inactivation results from binding of Na⁺ at the cytoplasmic surface of the Na⁺/Ca²⁺ exchanger which may either lead to exchange with Ca²⁺, or to the entry of the exchanger into an inactive state (62). Steady-state current levels therefore reflect the balance between active and inactive exchangers. As the concentration of Na⁺ applied to the intracellular side increases, exchangers will become increasingly loaded with Na⁺ resulting in an augmentation of both peak current and the extent of Na⁺-dependent inactivation (decay to steady-state) (59; 60).

The protein regions involved in Na⁺-dependent inactivation of the Na⁺/Ca²⁺ exchanger have been investigated. Mutagenesis studies have identified a 20 amino acid sequence located at the amino terminus of the large intracellular loop that is involved in this process. This region was originally thought to be involved in regulation since it was found to have a similar structure to calmodulin binding sites which often represent autoinhibitory domains (43). However, a physiological role for this 20 amino acid

sequence still had to be identified. To aid in this determination, a 20 amino acid peptide corresponding to the sequence was created. Application of the peptide to patches of membrane during examination of $\text{Na}^+/\text{Ca}^{2+}$ exchange activity resulted in a reversibly inhibited exchange current. This was observed for all $\text{Na}^+/\text{Ca}^{2+}$ exchangers examined (73; 85) and was termed the eXchange Inhibitory Peptide (XIP).

Specific amino acids within the XIP peptide were shown to be critical in maintaining its inhibitory function (55; 137). However, since it was still unknown whether endogenous and exogenous XIP bind to the same site in the exchanger, information about the XIP binding site was actively investigated. Mutant exchangers, having deletions in the area of the large intracellular loop, no longer demonstrated regulation by Ca^{2+} and were not inhibited by the addition of XIP (94). This finding supported the belief that both the XIP region and its binding site play an important role in regulation of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger.

A more recent examination of this regulatory region has been carried out through mutagenesis studies. The endogenous XIP region was studied using point mutations and deletions in order to examine the consequences on Na^+ -dependent inactivation. Measurement of $\text{Na}^+/\text{Ca}^{2+}$ exchange activity of these mutants revealed two different phenotypes. While the Na^+ -dependent inactivation was accelerated for one group of mutants, this property was eliminated for another highlighting the importance of the XIP region in Na^+ regulation (45). Finally, it has been suggested, through the study of cardiac sarcolemmal vesicles, that the XIP binding region may be located in loop "f" of the exchanger and consist of amino acids 445-455 (51).

The *Drosophila* Na⁺/Ca²⁺ Exchanger

Na⁺/Ca²⁺ exchange proteins have been identified in several species other than mammals. These include squid (6), barnacle (110) and the fruit fly, *Drosophila melanogaster*. The *Drosophila* exchanger, referred to as CALX, was cloned (119; 123) and found to have a 49% sequence identity to NCX1, the mammalian exchanger. Further examination, using hydropathy analysis, produced a model of the CALX exchanger which contained a large hydrophilic loop near the center of the protein (119). Also, an alternative splice site corresponding to the splice site in NCX1 was located within the large intracellular loop of CALX1.

Having identified the topological and sequence similarities between NCX1 and CALX1, the next process was to compare their regulatory properties. Previously, Na⁺/Ca²⁺ exchange was found to be regulated by Na⁺ producing a Na⁺-dependent inactivation, and by Ca²⁺ whereby exchange current was stimulated. To study these regulatory properties for CALX1, *Xenopus* oocytes expressing CALX1.1 were characterized using the giant excised patch clamp technique (63). Similar to NCX1, CALX1 was found to be regulated by both Na⁺ and Ca²⁺. However, the presence of regulatory Ca²⁺ produced an opposite effect for CALX, compared to that for all other Na⁺/Ca²⁺ exchangers (63). That is, CALX Na⁺/Ca²⁺ exchange current was inhibited by regulatory Ca²⁺ over exactly the same range of Ca²⁺ concentrations which stimulated Na⁺/Ca²⁺ exchange activity in NCX1 and NCX2 (84; 93; 94). To date, CALX1 is a completely unique exchanger with respect to the inhibition that occurs in the presence of regulatory Ca²⁺, while still demonstrating conventional Na⁺-dependent inactivation.

Alternatively-Spliced Isoforms of the Na⁺/Ca²⁺ Exchanger

Several Na⁺/Ca²⁺ exchangers, including CALX1 and NCX1, have been found to undergo alternative splicing. These alternatively spliced isoforms are created through alternative exon expression in the major cytoplasmic loop of the exchanger. While the role of alternative splicing remains unknown, it may possibly constitute a mechanism for producing unique regulatory phenotypes. Many of the alternatively spliced isoforms are expressed in a tissue-specific manner. Alternatively spliced isoforms have been identified for NCX1 and NCX3 (76; 81; 98; 110) as well as for CALX1 (119; 123).

Alternative-splicing for NCX1 occurs in a 110 amino acid region located near the C-terminus of the large cytoplasmic loop. This alternative splice site consists of two mutually exclusive exons, A and B, along with four cassette exons, C, D, E, and F (76). These exons may be arranged in various ways allowing 32 possible isoforms (76). However, to date, identification of only 12 splice variants of NCX1 has been observed (75; 76; 81; 110). These studies have shown that the mutually exclusive exon A is primarily associated with cardiac, brain and skeletal tissue (i.e. excitable tissue) whereas exon B is found in all rat tissues except heart (110).

The observation of tissue specific expression of alternatively spliced isoforms is consistent with the idea that different tissues may require particular functional properties of the Na⁺/Ca²⁺ exchange protein, considering the major differences in Ca²⁺ signalling and trafficking. For example, cardiac contractility is regulated by NCX1.1 whereas NCX1.3, which is prominent in kidney, controls Ca²⁺ reabsorption and is involved in whole body Ca²⁺ homeostasis, in general (20; 25; 116). We have observed substantial

differences in the ionic regulatory properties of alternatively spliced $\text{Na}^+/\text{Ca}^{2+}$ exchangers from both *Drosophila melanogaster* and mammalian NCX1 (42; 103). While there is no comprehensive understanding of how these particular regulatory attributes are appropriate for a particular tissue, these differences are highly suggestive that ionic regulation serves an important functional role.

Comparison of amino acid sequences reveal that NCX3 has a deletion of 37 amino acids corresponding to the position of the alternative splice site in NCX1. This deletion is equivalent to exons D, E, and F in NCX1 therefore leaving only the mutually exclusive exons, A and B, as well as exon C to form any isoforms. To date, there have only been three alternatively-spliced isoforms identified for NCX3 obtained from brain and skeletal muscle from rat. There is no information on whether or not functional differences exist between these isoforms (110).

A region corresponding to the NCX1 alternative splice site has been identified in the large cytoplasmic loop of the *Drosophila* exchanger, CALX (119). Two alternatively-spliced isoforms have been identified for *Drosophila*, CALX1.1 and CALX1.2 (119; 123). These studies indicated that these two isoforms differed only by five amino acids (119). The two *Drosophila* isoforms, CALX1.1 and CALX1.2, were studied with respect to their ionic regulatory properties (104). Na^+ -dependent inactivation occurred to a greater extent for CALX1.2, resulting in lower steady-state current levels. There was also a greater stability of the I_1 inactive state, based on the rate of recovery from inactivation (104). Ca^{2+} -dependent regulation was also examined and found to be substantially different between these isoforms. While both CALX1.1 and

CALX1.2 exhibited negative regulation by cytoplasmic Ca^{2+} (63), CALX1.1 was inhibited to a much greater extent (104). However, a higher concentration of Ca^{2+} was required to produce this inhibitory effect indicated that both affinity and efficacy differ between these alternative splicing variants (104).

The Technology of Transgenics

Transgenic animals are created by integrating foreign DNA into the somatic cells of animals. This DNA may be in the form of a gene whose function is known or unknown. Also, genes which are not normally part of that animal's genome or mutant genes, may be integrated. This technology allows the physiological effects of this integration to be examined. The approach has been successfully employed to address a wide variety of biological questions including studies of viral diseases, developmental regulation, cardiovascular functioning, and autoimmune diseases.

While the term "transgenic" was not introduced until 1981 (49), the first animals produced using this technology occurred prior to this (69). These initial experiments involved the introduction of SV40 viral DNA into the blastocyst cavity of early mouse embryos by microinjection (69). Production of transgenic animals progressed to where early embryos were exposed to infectious retroviruses (68). Several other approaches regarding transgenics involve transfection of DNA into teratocarcinoma cells (embryonic stem cells) which is followed by the transfer of selected cells to 3.5 day old blastocysts (26; 96). Blastocysts may then be implanted into pseudopregnant foster mothers (26). Another alternative is for nuclei from teratocarcinoma cells to be transplanted into

fertilized eggs from which the pronuclei have been removed (95). However, the most common approach to the production of transgenic animals is for recombinant DNA to be microinjected directly into the pronuclei of fertilized eggs (50). While DNA integration has been achieved in pigs, sheep and rabbits (53), the predominant animal used is the mouse largely as a result of economic considerations.

In our study the transgenic mice were produced through microinjection of DNA into mouse eggs. This method was adapted from a technique for injecting nucleic acid into amphibian eggs (49) and several initial investigators used it successfully (27; 133). Since then, it has become the most widely used method for transgenic production having the advantages that size or sequence of DNA to be introduced is not restricted and that most genes are expressed in a predictable manner. However, even though the mouse genome is easily manipulated, there are limitations to this technique. First, many technical difficulties are encountered due to the small size of the mouse. Second, the mouse has a short cardiac cycle which is 1/10 the length of humans. Consequently, information obtained using transgenic mice may not necessarily be applicable for the investigation of human pathologies and caution must be used in extrapolating these results to other animal models.

The microinjection technique allows a cloned DNA sequence to be introduced directly into the mouse genome. These microinjected eggs are then implanted into the oviducts of a pseudopregnant mouse. These original transgenic mice are referred to as “founder” mice and they have foreign DNA present in every cell. Their offspring often

continue to carry the gene which indicates that it is incorporated into the germ line as well as the somatic cells (49).

To establish which mice are transgenic, the presence of the inserted foreign DNA must be confirmed. In adult mice, a tail clipping provides enough DNA for Southern Blot analysis to be completed. However, in fetal mice younger than 13 days of gestation, there is not enough DNA present for a Southern blot analysis. Therefore, presence of the transgene must be confirmed using other methods such as marking the transgene with a bacteriophage DNA sequence (38). While these newly introduced genes are usually transcribed properly, their levels of transcription may be unpredictable from one transgenic mouse to another (33; 105). While these differences usually do not correlate with copy number, the levels can be readily examined through the completion of mRNA or protein assays.

Transgenic animals have provided new opportunities for the advancement of knowledge in a large number of research areas. For example, while knowledge of SR Ca^{2+} -ATPase and $\text{Na}^+/\text{Ca}^{2+}$ exchange function has continually increased, it was believed that novel physiological insight into the roles of these transporters could be obtained through the use of this technology. Transgenic mice overexpressing either the SR Ca^{2+} -ATPase or the $\text{Na}^+/\text{Ca}^{2+}$ exchanger were produced. Initially, a study was carried out whereby cardiac contractility was examined as a function of SERCA2 expression. In this instance, the rat SR Ca^{2+} -ATPase gene was overexpressed in mice. Examinations of both cardiac myocytes and papillary muscles revealed an accelerated Ca^{2+} decline, as well as enhanced contraction and relaxation (54). While mRNA levels had increased for

SERCA2, they had also increased for other Ca^{2+} handling proteins such as calsequestrin and the $\text{Na}^+/\text{Ca}^{2+}$ exchanger. However, protein levels were only increased for SERCA2 suggesting that increased expression of SERCA2 was responsible for the altered contractility and Ca^{2+} transients (54). In this study, SERCA2 was overexpressed using a promoter which was not tissue specific. To prevent any possible alteration to Ca^{2+} homeostasis as a result of non-specific expression, a second study using the cardiac tissue-specific α -myosin heavy chain promoter was subsequently carried out (5). Regardless of the promoter employed, the results support the initial study linking SERCA2 overexpression to enhanced cardiac contractility.

Transgenic mouse technology has been useful for studying $\text{Na}^+/\text{Ca}^{2+}$ exchange and its physiological relevance. Transgenic mice overexpressing the cardiac $\text{Na}^+/\text{Ca}^{2+}$ exchanger cardio-specifically have been produced and several different aspects of $\text{Na}^+/\text{Ca}^{2+}$ exchange function have been examined. While $\text{Na}^+/\text{Ca}^{2+}$ exchange is widely accepted as the primary mechanisms for Ca^{2+} efflux, there is little consensus as to its role in Ca^{2+} influx (28; 82; 86; 124). To determine if $\text{Na}^+/\text{Ca}^{2+}$ exchange is in fact excluded from the association of Ca^{2+} with the DHP/ryanodine receptors as previously reported (1; 125), transgenic mice overexpressing the $\text{Na}^+/\text{Ca}^{2+}$ exchanger were studied. It was found that while myocytes from these transgenic mice had increased $I_{\text{Na/Ca}}$, $\text{Na}^+/\text{Ca}^{2+}$ exchange did not provide enough Ca^{2+} influx to trigger a further release of Ca^{2+} from the SR (1). Despite this, overexpression of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger was shown to effect Ca^{2+} handling by increasing SR Ca^{2+} content specifically, allowing the rate of SR Ca^{2+} release and uptake to be increased (131).

Further examination of the role of the inward mode of $\text{Na}^+/\text{Ca}^{2+}$ exchange was carried out using the Na^+ channel agonist, BDF 9148. Upon application of BDF 9148, Na^+ channels would allow a greater quantity of Na^+ to move into the cell as this agent augments Na^+ channel opening. In response to this increase in Na^+ levels, the $\text{Na}^+/\text{Ca}^{2+}$ exchanger would operate in reverse mode, transporting Na^+ out of the cell in exchange for Ca^{2+} . This augmentation in Ca^{2+} influx was evaluated through the examination of electrically-stimulated atria, revealing that transgenic mice has a significantly greater inotropic response compared to controls (11). Calsequestrin, phospholamban, and SR Ca^{2+} -ATPase protein levels were measured and found to be unchanged in these transgenic mice. These results suggested that reverse mode $\text{Na}^+/\text{Ca}^{2+}$ exchange was responsible for the increased force production under the setting of augmented Na^+ influx (11).

Overexpression of $\text{Na}^+/\text{Ca}^{2+}$ exchange has been examined for effects other than those affecting Ca^{2+} handling protein levels. Cross *et al.*(37) studied the effects of ischemia on both male and female transgenic mice and found that overexpression of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger contributed to an increase in ischemia/reperfusion injury. However, this effect was less significant in female mice possibly due to the female hormone, estrogen, which is thought to play a role in combating cardiac disease in females (37). Continued efforts along these lines should be valuable towards increasing our understanding of the role of this transporter in both physiological and pathophysiological settings.

Post-Rest Potentiation

In cardiac muscle, rest potentiation refers to the augmentation of contractile amplitude following an interruption in electrical stimulation that is longer than the basic stimulation interval. While the exact cause of this phenomenon has not been determined, the majority of evidence suggests that an increase in the amount of Ca^{2+} accumulated within and released from the SR is responsible for this phenomenon (3; 13). Thus, during a rest interval, the SR becomes increasingly loaded with Ca^{2+} and upon re-stimulation, the amount of Ca^{2+} released will be increased to produce the potentiated post-rest contraction.

Rapid cooling or caffeine contracture measurements have been prominent methods used to study the cause of rest potentiation. These experimental procedures, which are able to assess SR Ca^{2+} content, indicated that post-rest potentiation was associated with increased SR Ca^{2+} loading during rest (8; 14). These techniques have verified the augmentation of SR Ca^{2+} release in both single myocytes and intact muscle preparations. Rest potentiation has been observed in many species including cat (74), rat (13), and rabbit (56). However, for species in which SR Ca^{2+} release is thought to be less prominent, rest potentiation is absent. Instead, rest decay is observed for these animals including frog and rabbit ventricle (4; 13).

Many pharmacological examinations have been conducted to verify that post-rest potentiation is dependent on a functional SR. Ryanodine, which is able to deplete the SR Ca^{2+} stores, was shown to abolish post-rest potentiation in ferret (91), rat (40), and canine (22) myocardium. Similarly, ryanodine was shown to completely inhibit post-rest

potentiation in failing and non-failing human myocardium (108). Furthermore, in failing human myocardium, where SR Ca^{2+} accumulation is reduced and $\text{Na}^+/\text{Ca}^{2+}$ exchange activity is increased, post-rest potentiation and Ca^{2+} transients were found to be decreased compared to control (108).

While there is an abundance of evidence supporting the role of the SR and augmented intracellular Ca^{2+} release in post-rest contractile force potentiation, other mechanisms for Ca^{2+} removal may also play a role. $\text{Na}^+/\text{Ca}^{2+}$ exchange has been shown to be very important for Ca^{2+} extrusion and therefore, cell relaxation (14). Thus, as additional Ca^{2+} is removed from the cell, less Ca^{2+} will be available for production of a subsequent contractile event. If the prevalence of transsarcolemmal Ca^{2+} removal is increased, rest decay may be observed rather than rest potentiation. Conversely, inhibition of $\text{Na}^+/\text{Ca}^{2+}$ exchange has been shown to slow rest decay in rabbit myocardium (130) since more Ca^{2+} is available for SR uptake. In human dilated cardiomyopathy an increased $\text{Na}^+/\text{Ca}^{2+}$ exchange activity is observed along with a decreased SR Ca^{2+} pump activity. Therefore, in this setting, less Ca^{2+} will be available for a post-rest contraction, resulting in a rest decay (108). An increased $\text{Na}^+/\text{Ca}^{2+}$ exchange activity and a decreased inotropic state have also been documented in studies involving congestive heart failure (70; 139) as well as in myocardial hypertrophy (139).

Post-rest behavior has also been found to be affected by stimulation frequency, length of rest interval and inotropic agents. An increased rate of stimulation allows more Ca^{2+} to enter the cell via L-type Ca^{2+} channels (109). Therefore, more Ca^{2+} is available for loading of the SR, which typically results in enhanced post-rest potentiation (108). In

contrast, however, others have not found a direct relationship between stimulation frequency and post-rest behavior (107). The length of the rest interval has also been found to play a role in post-rest contractility. Animal models which exhibit post-rest potentiation at shorter intervals will often show rest decay as the rest intervals are lengthened (108). Also, post-rest contraction may become independent of rest interval once this interval becomes too long. Once this occurs, the contractile event upon re-stimulation is referred to as the “rested state contraction” (74).

Inotropic agents, such as ouabain, histamine or simply altered Ca^{2+} concentration, have been found to affect post-rest behavior in humans (4). In rat heart muscle, studies using inotropic agents revealed that post-rest potentiation was accelerated but was not greater than the effect observed in controls or upon increased rest interval length (112). These results suggested that during post-rest potentiation, the SR is loaded to capacity and the application of positive inotropic agents will not be able to further enhance the post-rest contraction (112).

Rest potentiation is a useful means of assessing the competition between the SR and sarcolemma for the removal of Ca^{2+} from the cytoplasm. In general, it is thought that if a greater amount of Ca^{2+} is removed *via* the sarcolemma, then the contraction following a rest interval will not be potentiated. However, if the SR is a better competitor, Ca^{2+} will not be removed from the cell. This will result in more Ca^{2+} being available for the next contraction resulting in a potentiated beat (15). Contractions may also be affected by other agents such as Ca^{2+} channel blockers. For example, if Ca^{2+} influx is blocked, potentiation will be observed (23). This is also the case if there is a lower Ca^{2+}

concentration outside the cell (23). However, a contraction may also appear to be potentiated when in fact it is the steady-state level of contraction which is lower.

Many studies examining contractile behavior have been carried out on rats (10; 17; 79; 122). Despite having considerable differences in heart rate, it had previously been assumed that the contractile behavior of mouse cardiac muscle most resembled that of rat. Our study was carried out on left ventricular papillary muscles from transgenic mice and it was believed that these previous evaluations of rat would be used as a paradigm. However, recent studies have shown that mouse and rat differ substantially in terms of their physiology (48). For example, in comparison to rat, mouse cardiac muscle was found to be more tolerant to elevated extracellular Ca^{2+} concentrations (47). As such, it appears that rest potentiation in mouse may occur through a similar mechanism as that proposed for canine and ferret muscle whereby increased SR Ca^{2+} loading is not necessary to augment SR Ca^{2+} release (17). Thus, with the recent discrepancies in the literature, it has been more difficult to evaluate the mouse cardiac contractile behavior observed in our study.

Prior Studies in Our Laboratory

Structure-function studies have identified protein regions within the large cytoplasmic loop of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger that are functionally important in mediating the two ionic regulatory processes. Although these regions associated with Na^+ and Ca^{2+} -dependent regulation are highly conserved among cloned exchangers, it was unknown whether structure-function relationships characteristic of NCX1.1 could be applied to any

other exchanger subtypes or alternatively-spliced isoforms. In order to resolve this question, we examined the $\text{Na}^+/\text{Ca}^{2+}$ exchanger from *Drosophila*, CALX1.1 (42). CALX1.1 is regulated by Na^+ and Ca^{2+} , but is unique among all cloned exchangers in that μM concentrations of regulatory Ca^{2+} produce an inhibition of exchange current rather than a stimulation. This inhibitory response provided an excellent opportunity to study the role of these protein regions more closely.

We examined a series of point mutations and deletion mutants for CALX1.1. Both wild-type and mutant CALX1.1 exchangers were expressed in *Xenopus laevis* oocytes and characterized electrophysiologically using the giant excised patch clamp technique. Mutations within the putative regulatory Ca^{2+} binding site were able to produce either a reduction or an elimination of the inhibitory effect of Ca^{2+} . Similarly, alterations to the segment mediating Na^+ -dependent regulation, referred to as the eXchange Inhibitory Peptide or XIP region, resulted in modified responses. The results obtained from CALX1.1 mutants were analogous to those observed for analogous NCX1.1 mutants. This indicated that the function of both the regulatory Ca^{2+} binding site and the XIP region were conserved between CALX1.1 and NCX1.1, irrespective of the fact that these exchangers exhibit opposite responses to regulatory Ca^{2+} (42).

We then evaluated the reasons for these opposite responses to regulatory Ca^{2+} . That is, we postulated that if both the Na^+ - and Ca^{2+} -dependent regulatory domains served equivalent functions in CALX1.1, then it should be possible to identify protein domains involved in the transduction of the regulatory Ca^{2+} binding signal. To carry this out, chimeric exchangers containing segments from both NCX1.1 and CALX1.1 were

constructed towards identifying the transduction domain (42). One chimera consisted of the regulatory Ca^{2+} binding site and flanking sequences from NCX1.1 spliced into a CALX1.1 parent transporter. Upon application of regulatory Ca^{2+} , this chimera was stimulated suggesting that this portion of NCX1.1 contains the necessary functional domains to produce positive regulation by Ca^{2+}_i (41).

These studies highlight the high degree of sequence and function conservation between different $\text{Na}^+/\text{Ca}^{2+}$ exchangers. More recently, we have identified important functional alterations in alternatively spliced mammalian NCX1.1 isoforms (42). These exchangers, NCX1.3 and NCX1.4, show prominent expression in kidney and brain, respectively, and differ only in the expression of the A or B mutually exclusive exon. We have identified large differences in their regulatory responses to both Na^+ and Ca^{2+} . Thus, while we do not know the physiological role of ionic regulation of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger, the following facts are highly suggestive that a role may be discovered shortly: 1) domains which play a prominent role in ionic regulation show a higher degree of sequence conservation than that observed when comparing overall sequence. 2) alternatively splice isoforms differ with respect to ionic regulation, possibly reflecting different Ca^{2+} homeostasis requirements in different tissues. For these reasons, we investigated the functional consequences of ablating ionic regulation in transgenic mouse hearts, as a first step towards understanding the physiological significance of ionic regulation of $\text{Na}^+/\text{Ca}^{2+}$ exchange.

METHODS

Preparation of *Xenopus laevis* Oocytes

Anaesthetic solution was prepared by dissolving 250 mg/l ethyl p-aminobenzoate (Sigma) in deionized water. *Xenopus laevis* were placed in the mixture and after 30 min oocytes were surgically removed. Oocytes were initially washed in Solution A consisting of (in mM): 88 NaCl, 15 HEPES, 2.4 NaHCO₃, 1.0 KCl, 0.82 MgSO₄; pH 7.6. Oocytes were then gently teased apart were placed in 5 ml Solution A plus 80 mg collagenase. After incubation of the oocytes for 40-45 min at room temperature with gentle agitation, they were then washed free of the collagenase in Solution B (Solution A plus 0.41 mM CaCl₂ plus 0.3 mM Ca(NO₃)₂ containing 1 mg/ml BSA) and transferred to 5 ml of 100 mM K₂HPO₄ containing 1 mg/ml BSA. Following incubation for 11-12 min at room temperature with gentle agitation, oocytes were washed several times in Solution B plus BSA. Once defolliculated stage V and VI oocytes were selected, they were incubated at 16 °C overnight in Solution B, prior to injection of cRNA the following day.

Isolation of Murine Cardiac Myocytes

Adult mouse ventricular myocytes were isolated as described previously (134) with minor modifications. Following a heparin injection (10 IU *i.p.*), mice were anaesthetized with 5% isoflurane: 95% O₂, and euthanized by cervical dislocation. The hearts were rapidly excised and placed in oxygenated Ca²⁺- free Tyrode's solution composed of (in mM): 137 NaCl, 10 D-glucose, 5.4 KCl, 5 HEPES, 0.5 NaH₂PO₄: pH

7.4 (37 °C) with NaOH, at 22 °C. The aortae were then cannulated and attached to a perfusion apparatus. Hearts were then perfused with oxygenated solutions at a flow rate of 2 ml/min. Perfusion began with Ca²⁺-free Tyrode's solution for 5 min, followed by 10 min of Ca²⁺-free Tyrode's solution containing 1.25 mg/ml collagenase (Type 2: Worthington), 0.063 mg/ml protease (Type XIV: Sigma) and 0.94 mg/ml fatty acid-free BSA (Sigma). Finally, hearts were perfused for 10 min with KB solution consisting of (in mM): 70 L-glutamic acid, 25 KCl, 20 taurine, 10 KH₂PO₄, 10 HEPES, 10 D-glucose, 3 MgCl₂, 0.5 EGTA; pH 7.4 (37 °C) with KOH. Upon removal from the perfusion apparatus the ventricles were teased apart and cells dispersed by trituration. Cells were washed several times in KB and stored at 4 °C until use.

Miscellaneous

Each *Xenopus* oocyte was injected with ≈5 ng of cRNA encoding either NCX1.1 or Δ680-685 as described previously (42). Exchange activity was typically measured 4-6 days post-injection. To giant excised patch clamp experiments in myocytes, cells were placed in a hypotonic solution to induce membrane “blebbing” (Figure 1). The solution consisted of (in mM): 67.5 KCl, 9.0 D-glucose, 6.75 HEPES, 4.5 EGTA, 0.9 MgCl₂, pH 7.2 (22°C) with KOH at 4 °C for several hours prior to experiments (59).

Sarcolemmal Membrane “Blebs”

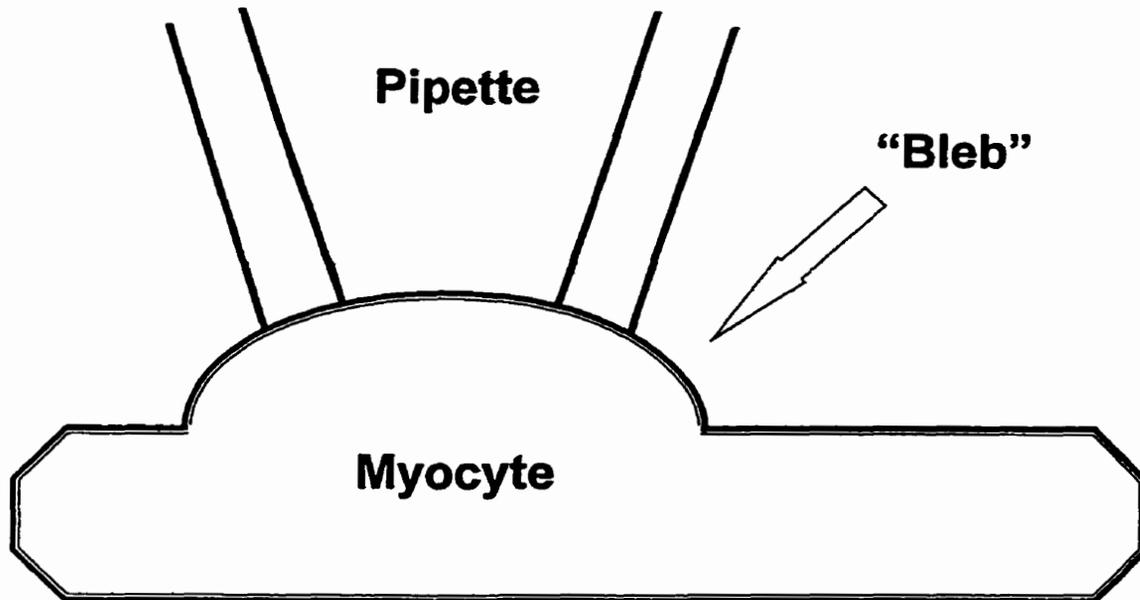


Figure 1. Sarcolemmal Membrane “Blebs”. Myocytes obtained from either control or transgenic mice were placed in a hypotonic solution. This causes the membrane of the myocyte to form a “bleb”. Borosilicate glass pipettes are then brought to the surface of the membrane. A light suction is applied to form a $G\Omega$ seal, and the pipette gradually removed excising a patch of membrane containing hundred of thousands of exchangers. Current is then initiated by solution changes on the cytoplasmic surface.

Isolation of Canine Cardiac Myocytes

This procedure was carried out by Dr. Anton Lukas and his associates (90). The myocytes were generously provided for these experiments.

Transgenic Mice

All transgenic mice overexpressing either the wild-type $\text{Na}^+/\text{Ca}^{2+}$ exchanger or the deletion mutant, $\Delta 680-685$, were provided by Dr. K.D. Philipson and were prepared as described earlier (2). Briefly, transgenic mice overexpressing either the wild-type or $\Delta 680-685$ deletion mutant were produced by injecting the transgene construct under the direction of the α -myosin heavy chain promoter into the pronuclei of eggs from superovulated mice. These eggs were then transplanted into oviducts of pseudo-pregnant mice. To confirm the integration of the transgene in the offspring, genomic DNA was extracted from tail clippings and subjected to Southern blot analysis. This process was carried out by the UCLA Transgenic Core Facility.

Electrophysiological Analyses

The giant excised patch clamp technique was used to measure outward $\text{Na}^+/\text{Ca}^{2+}$ exchange currents (58; 63; 132) in both oocytes and myocytes. Borosilicate glass pipettes with a final inner diameter of $\sim 20\text{-}30\ \mu\text{m}$ were brought to the surface of the membrane, a light suction was applied to form a $G\Omega$ seal, and then the pipette was gradually removed to excise a patch of membrane. Outward currents were elicited by rapid application of $100\ \text{mM}\ \text{Na}^+_i$ plus $1\text{-}10\ \mu\text{M}\ \text{Ca}^{2+}$ to the intracellular surface of the patches. Extracellular Ca^{2+} (transport Ca^{2+}) remained constant at $8\ \text{mM}$. The general experimental configuration is shown in Figure 2. Axon Instruments hardware (Axopatch 200a) and software (Axotape) were used for data acquisition and analysis. Cytoplasmic solutions contained (in mM): $100\ (\text{Li}^+ \text{ or } \text{Na}^+)\text{-aspartate}$, $20\ \text{TEA-OH}$, $20\ \text{MOPS}$, $20\ \text{CsOH}$, $10\ \text{EGTA}$, $1.02\text{-}1.5\ \text{Mg}(\text{OH})_2$; $2.04\text{-}3.0\ \text{NH}_3\text{SO}_3$, $0\text{-}9.96\ \text{Ca}(\text{OH})_2$; $0\text{-}19.92\ \text{NH}_3\text{SO}_3$; pH 7.0 ($37\ ^\circ\text{C}$) with MES or LiOH. Extracellular (i.e. pipette) solution contained (in mM): $100\ \text{NMG}$: MES, $30\ \text{HEPES}$, $30\ \text{TEA-OH}$, $16\ \text{NH}_3\text{SO}_3$, $8\ \text{CaCO}_3$, $6\ \text{KOH}$, $0.25\ \text{ouabain}$, $0.1\ \text{flufenamic acid}$, $0.1\ \text{niflumic acid}$; pH 7.0 ($37\ ^\circ\text{C}$) with MES. All experiments were conducted at $37 \pm 1\ ^\circ\text{C}$.

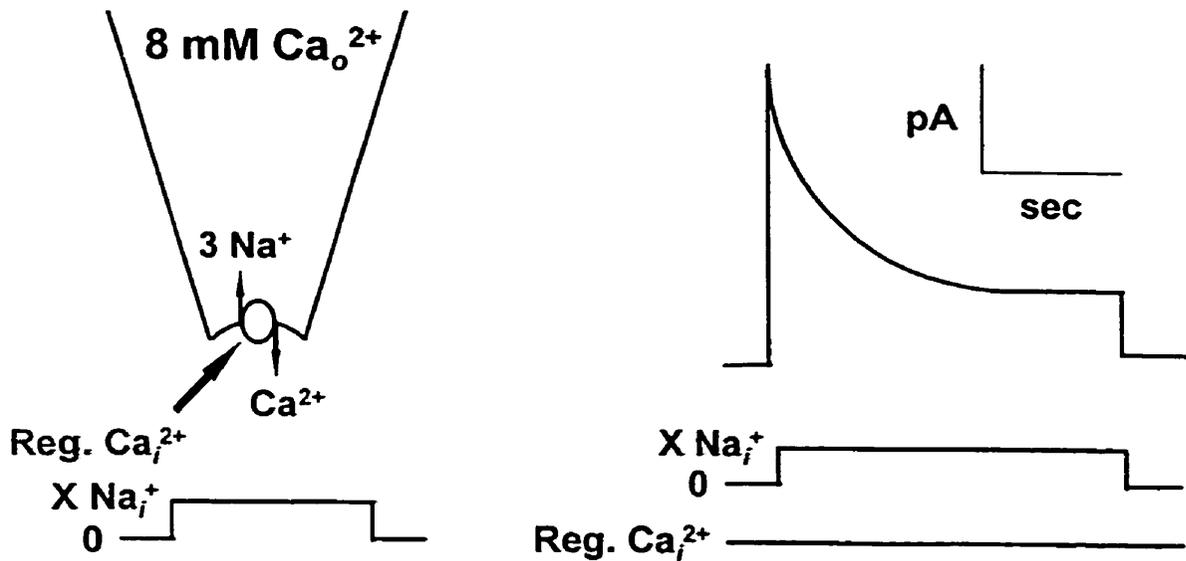


Figure 2. Typical $\text{Na}^+/\text{Ca}^{2+}$ Exchange Current. The *left panel* is a depiction of a pipette tip along with a patch of membrane expressing $\text{Na}^+/\text{Ca}^{2+}$ exchangers. In this orientation, the extracellular side of the membrane is located within the pipette. With 8 mM transport Ca^{2+} on the extracellular side and 100 mM Na^+ on the cytoplasmic surface, $\text{Na}^+/\text{Ca}^{2+}$ exchange operates in reverse mode bringing Ca^{2+} to the intracellular side of the membrane. This reverse mode of $\text{Na}^+/\text{Ca}^{2+}$ exchange transport must be examined in order to distinguish between the two pools of Ca^{2+} (*i.e.* transported and regulatory). The net movement of charge is in the outward direction (*i.e.* to the extracellular side), and is referred to as outward exchange current. The *right panel* shows a typical outward exchange current. Upon application of Na^+ to the cytoplasmic surface, exchange is initiated. This is characterized by a rapid rise in current to a peak level followed by a slow, time-dependent decay to a lower steady-state level and is termed Na^+ -dependent inactivation.

Contractility Measurements

Hearts were rapidly excised from either strain control or transgenic mice which had been euthanized by cervical dislocation. Under oxygenated Tyrode's solution (as above) containing 30 mM 2,3-butanedione monoxime (BDM) and 0.5 mM Ca^{2+} at 22 °C, the left ventricular papillary muscles were tied at both ends with 9.0 nylon suture. Muscles were then placed in an ≈ 0.25 ml bath where one end was attached to a permanently-mounted glass hook while the other end was attached to a hook bound to a capacitive force transducer mounted to a micro-manipulator. The experimental configuration is illustrated in Figure 3. Papillary muscles were constantly bathed in temperature regulated ($37 \pm 1^\circ\text{C}$) 2.0 mM Ca^{2+} Tyrode's solution at a flow rate of $\approx 6-7$ ml/min. Muscles were electrically-stimulated at 3 Hz and stretched to approximately half of L_{max} . Equilibration of the muscles occurred for 60 min and then the muscles were stretched to their optimal length for force production. Force interval studies were then carried out whereby electrical stimulation of the muscles was interrupted for a pre-determined amount of time ranging from 1-60 sec. Upon resumption of stimulation, resultant contractions were monitored until steady-state was again established.

Statistical Analyses

All data reported are means \pm SEM. Statistical comparisons for all oocyte and myocyte data were performed using the two-tailed student's T-test. Significant differences were defined by a probability of less than 0.05. Data obtained from papillary muscle preparations were also statistically compared. Due to the technical difficulty associated with these muscle preparations and the limited availability of these transgenic mice, the number of data points were not consistent among all groups examined. In order to account for smaller data sets, the critical p value was reduced using the Bonferroni correction factor.

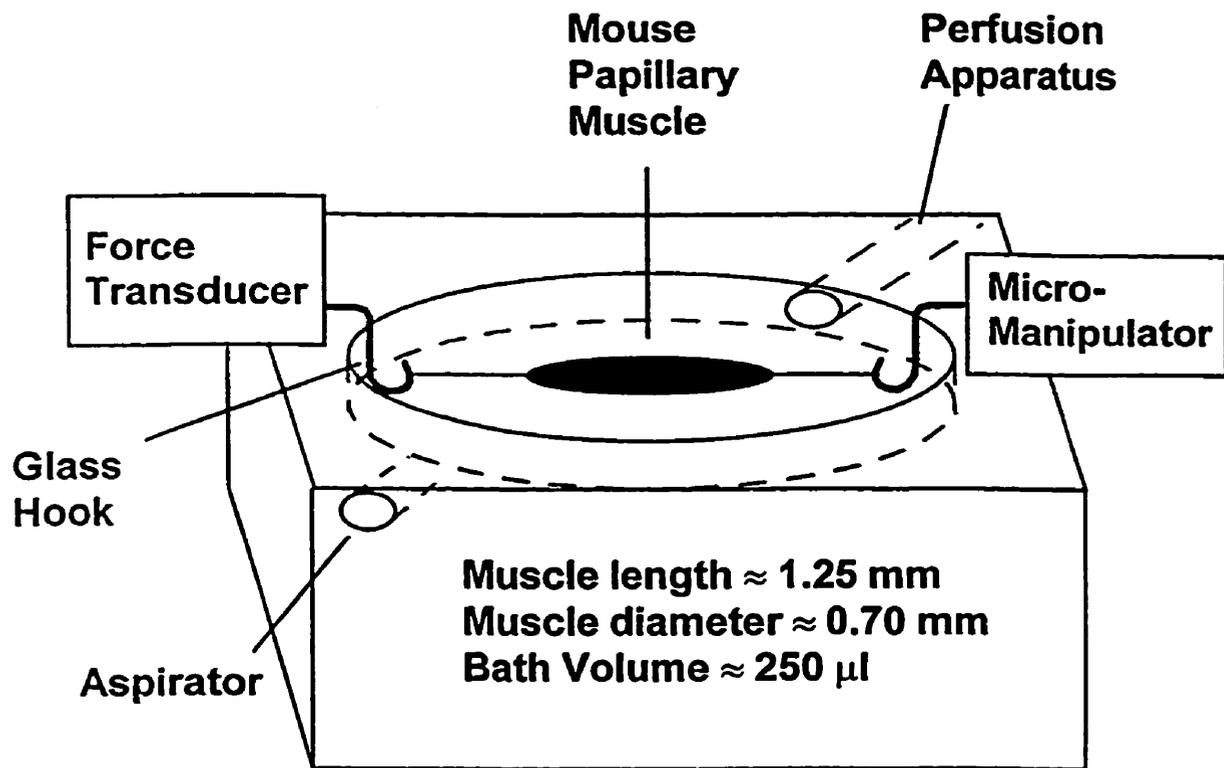


Figure 3. Perfusion Apparatus. To study the physiological effects of Na^+ and Ca^{2+} regulation of $\text{Na}^+/\text{Ca}^{2+}$ exchange, a perfusion chamber device was constructed to study mouse papillary muscles. Each papillary muscle, measuring approximately 1.2 mm in length and 0.7 mm in width, is hung between two hooks. One hook is attached to a force transducer while the second hook is attached to a micro-manipulator. The muscle is perfused with temperature-regulated solution and is electrically-stimulated to produce contractions.

RESULTS

Ionic Regulation in *Xenopus laevis* Oocytes

To assess the physiological significance of ionic regulation, we examined two Na^+ - Ca^{2+} exchangers, the wild-type canine $\text{Na}^+/\text{Ca}^{2+}$ exchanger, NCX1, and a deletion mutant, $\Delta 680-685$. To document their ionic regulatory phenotypes, outward exchange current was measured from *Xenopus laevis* oocytes expressing either of these two exchangers. As presented in earlier figures, outward currents are initiated by applying 100 mM Na^+ to the intracellular side of the patch. This exchanges for the 8 mM transport Ca^{2+} in the pipette. Figure 4 reveals the effect of varying regulatory Ca^{2+} concentrations for both NCX1.1 and $\Delta 680-685$. The first panel (wild type NCX1.1) shows the progressive increase in outward exchange current in response to increasing intracellular Ca^{2+} .

Outward $\text{Na}^+/\text{Ca}^{2+}$ exchange current consists of two major parameters referred to as peak and steady-state current. Figure 5 summarizes the effect of different concentrations of regulatory Ca^{2+} on these two outward current parameters using pooled data from *Xenopus* oocyte patches. Outward currents were activated as described for the previous figure and values from both NCX1.1 and $\Delta 680-685$ were normalized according to their respective values obtained at 3 μM Ca^{2+}_i . For NCX1.1, very little peak current is observed in the absence of regulatory Ca^{2+} . However, in the presence of either 1 or 3 μM Ca^{2+} , there is a large increase in peak current. This stimulation is reduced at the highest Ca^{2+}_i concentration studied (10 μM) due to competition between Na^+ and Ca^{2+} at the

intracellular transport site (59; 132). Steady-state $\text{Na}^+/\text{Ca}^{2+}$ exchange currents for NCX1.1 also show a marked increase in response to increased levels of regulatory Ca^{2+}_i . This is a consequence of a direct stimulation of exchange current through alleviation of I_2 inhibition and the progressive alleviation of Na^+ -dependent (I_1) inactivation. Both of these processes enhance steady state current, and largely offset the inhibitory effect of Ca^{2+} competing at the intracellular transport site. On the other hand, $\Delta 680-685$ shows an insensitivity to regulatory Ca^{2+} . Whether Ca^{2+}_i was present or not, the deletion mutant exhibited high levels of both peak and steady-state current. However, current decreased at concentrations of $10 \mu\text{M}$ as a result of competition between Ca^{2+} and Na^+ , similar to that observed for NCX1.1. The near maximal peak and steady-state currents provide evidence that both Na^+ - and Ca^{2+} -dependent regulation is eliminated for the deletion mutant exchanger $\Delta 680-685$. Thus, if ionic regulation plays a physiological role, it is reasonable to believe that the activity of this mutant exchanger would not be affected by the ionic fluxes found within an intact mammalian cardiomyocyte.

Xenopus Oocytes

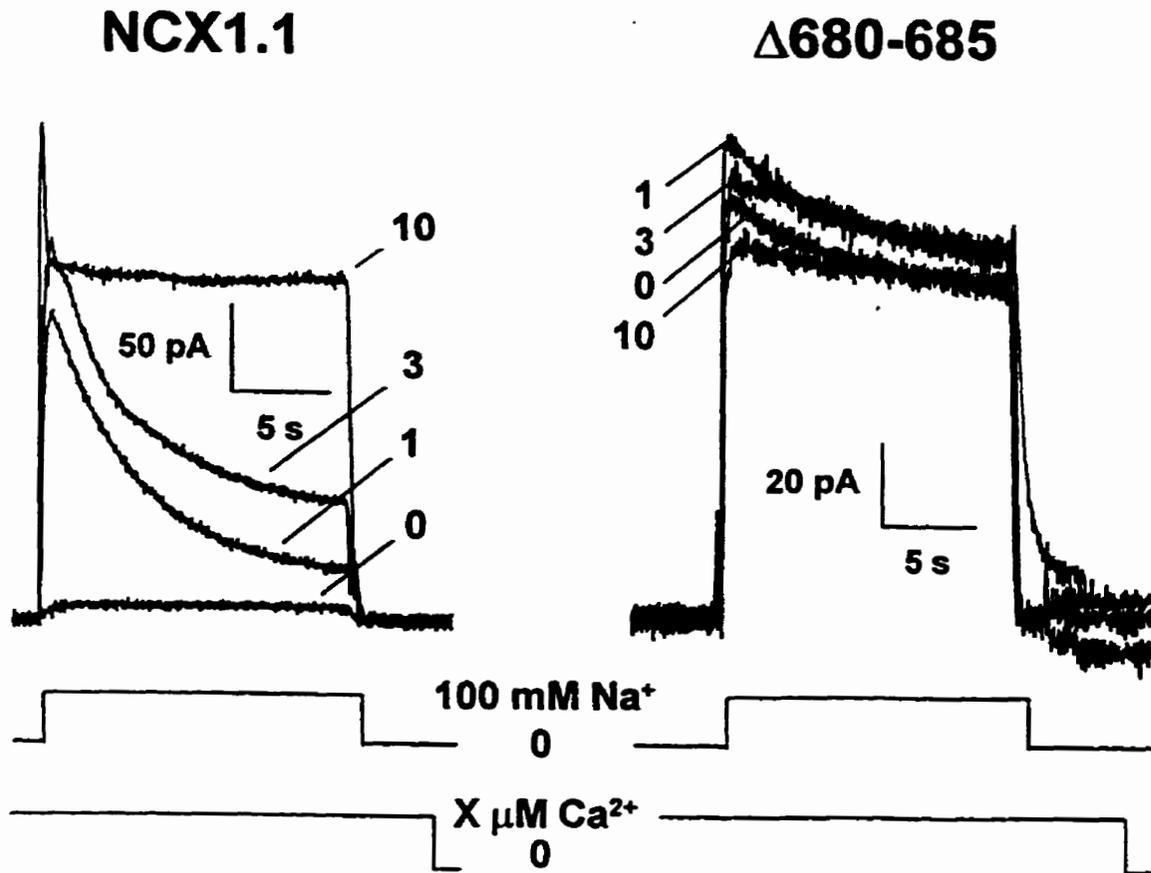


Figure 4. Effects of Regulatory Ca^{2+} on Cloned $\text{Na}^+/\text{Ca}^{2+}$ Exchangers. Outward $\text{Na}^+/\text{Ca}^{2+}$ exchange currents obtained from *Xenopus laevis* oocytes expressing either NCX1.1 or $\Delta 680-685$ are shown. Pipette Ca^{2+}_o (i.e. extracellular) was constant at 8 mM while regulatory Ca^{2+} was varied as indicated. Activation of currents was carried out by applying 100 mM Na^+ to the cytoplasmic surface of the patch. NCX1.1 shows an increased exchange activity in response to increased Ca^{2+} concentrations while $\Delta 680-685$ is not regulated.

Xenopus Oocytes

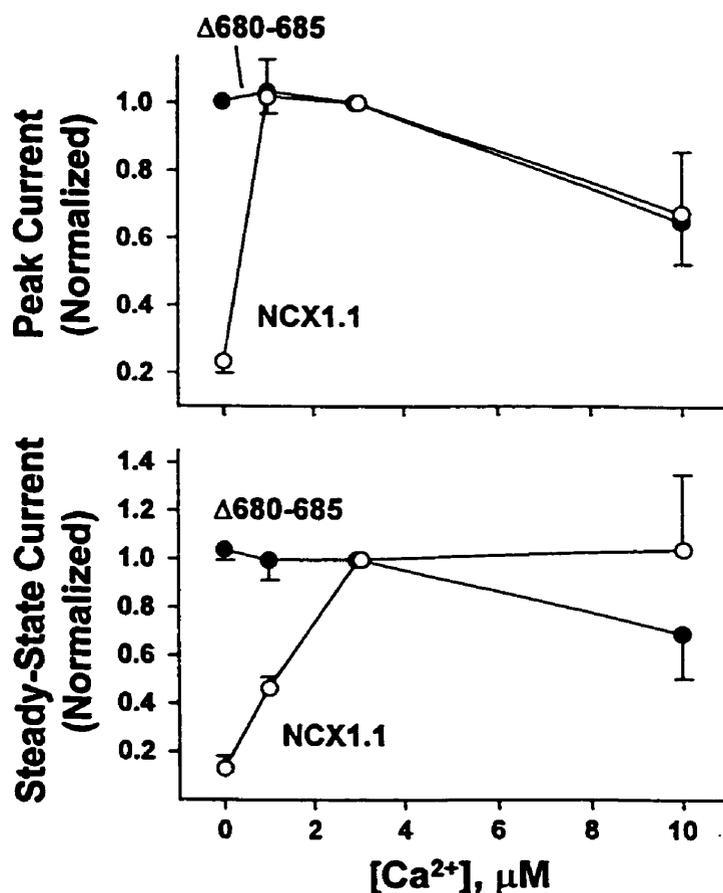


Figure 5. Pooled Data for Cloned Na⁺/Ca²⁺ Exchangers. Peak and steady-state outward Na⁺/Ca²⁺ exchange currents are shown graphically. Data for NCX1.1 and Δ680-685 were obtained as described under Figure 4. Current levels for peak and steady-state were normalized to the values obtained at 3 μM regulatory Ca²⁺_i, respectively. Data are mean ± SEM of 4 - 15 measurements at each [Ca²⁺]_i taken from 15 NCX1.1 patches, and 3 measurements at each [Ca²⁺]_i taken from 3 Δ680-685 oocyte giant patches.

Ionic Regulation in Dog and Mouse Myocytes

Since we employed transgenic mice overexpressing canine cardiac exchangers, it was important to determine if any species differences were present with respect to the two ionic regulatory mechanisms. To conduct this species comparison, $\text{Na}^+/\text{Ca}^{2+}$ exchange activity was measured for both mouse and dog isolated ventricular myocytes. The giant excised patch clamp technique was used to examine the electrophysiological properties of $\text{Na}^+/\text{Ca}^{2+}$ exchange currents. However, unlike the expression system of *Xenopus* oocytes, ventricular myocytes must be placed in a hypotonic solution prior to experimentation to induce a membrane “bleb”. This protrusion of the membrane is required for giant patching as the pipette diameter often exceeds the width of the myocyte. The large patch size is required in order to obtain sufficient electrical signal. Outward $\text{Na}^+/\text{Ca}^{2+}$ exchange currents were initiated using the same protocols as previously described in Figure 4. Figure 6 shows ionic regulation for myocyte membrane “blebs” from both dog and mouse. For both species, peak and steady-state currents are progressively increased upon application of additional regulatory Ca^{2+} . This behavior is similar to the response observed for NCX1.1 expressed in *Xenopus* oocytes (Figure 4). Since dog and mouse exhibited similar regulatory properties, it seems reasonable to assume that overexpression of regulated canine cardiac exchangers in transgenic mice might lead to similar regulatory responses. In other words, if ionic regulation contributes to the normal regulation of $\text{Na}^+/\text{Ca}^{2+}$ exchangers, then overexpressing regulated exchangers might be relatively benign in the transgenic mice.

Pooled results for data obtained from dog and mouse myocytes are shown in Figure 7 with respect to the effects of regulatory Ca^{2+} on both peak and steady-state

currents. Upon normalizing the currents to the values obtained at 3 μM regulatory Ca^{2+} , examination of steady-state currents revealed an identical response to increasing intracellular Ca^{2+} for the two species. Peak currents were also similar between dog and mouse increasing with increased regulatory Ca^{2+} . We observed a substantial deviation between these two species at 10 μM regulatory Ca^{2+} , an effect for which we have no explanation. Overall, however, the similarities found for both peak and steady-state currents seemed sufficiently large to expect that expression of the canine $\text{Na}^+/\text{Ca}^{2+}$ exchanger in mice would not result in a major change in the ionic regulatory phenotype. This expectation appears to be borne out by all previous results with transgenic mice overexpressing NCX1.1.

Myocytes

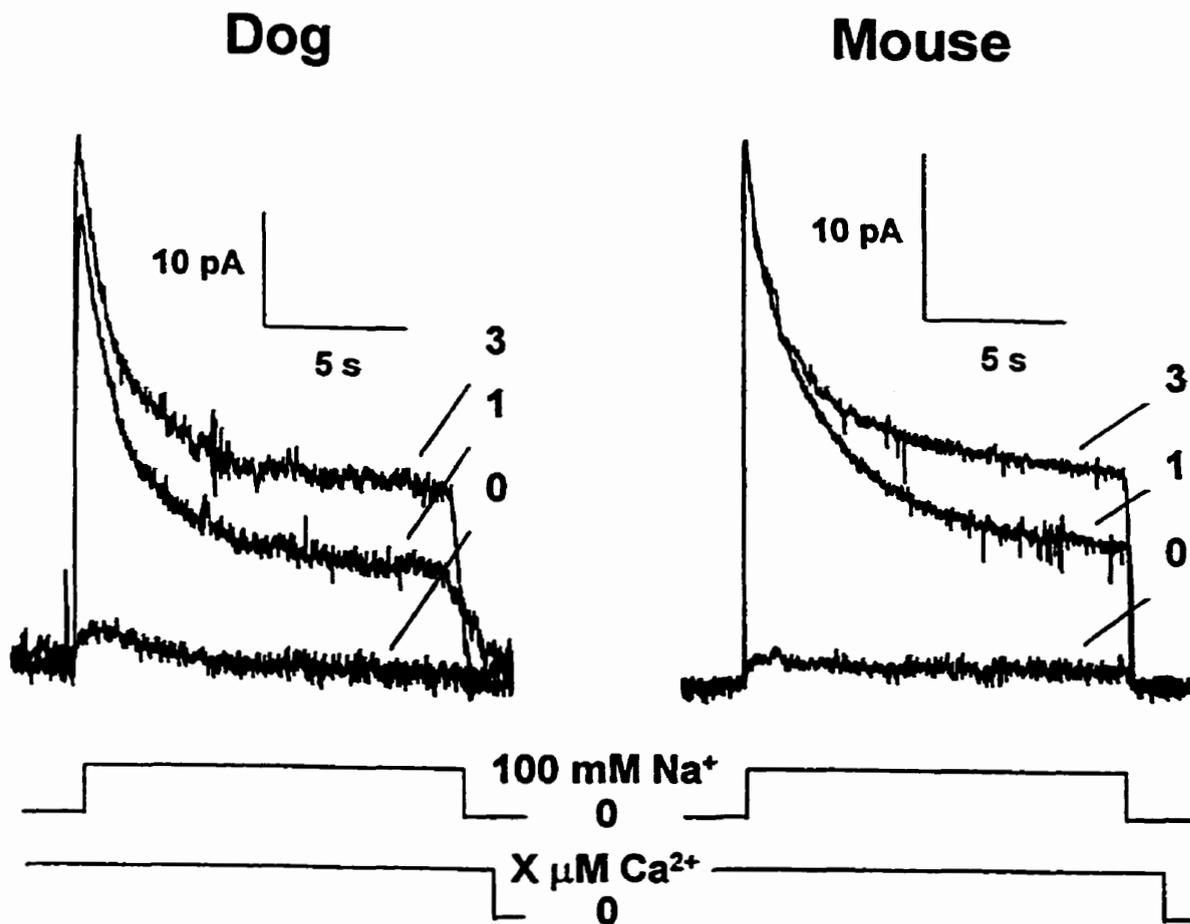


Figure 6. Examination of Dog and Mouse Exchange Activity. Na⁺/Ca²⁺ exchange activity was obtained from both dog and mouse ventricular myocytes. These ventricular myocytes were incubated in hypotonic buffer at 4 °C for several hours (as described under Methods) to induce the formation of sarcolemmal membrane “blebs”. Na⁺/Ca²⁺ exchange currents were elicited using the same method described earlier (see Figure 4). A stimulatory effect was observed for both species as shown in the representative traces.

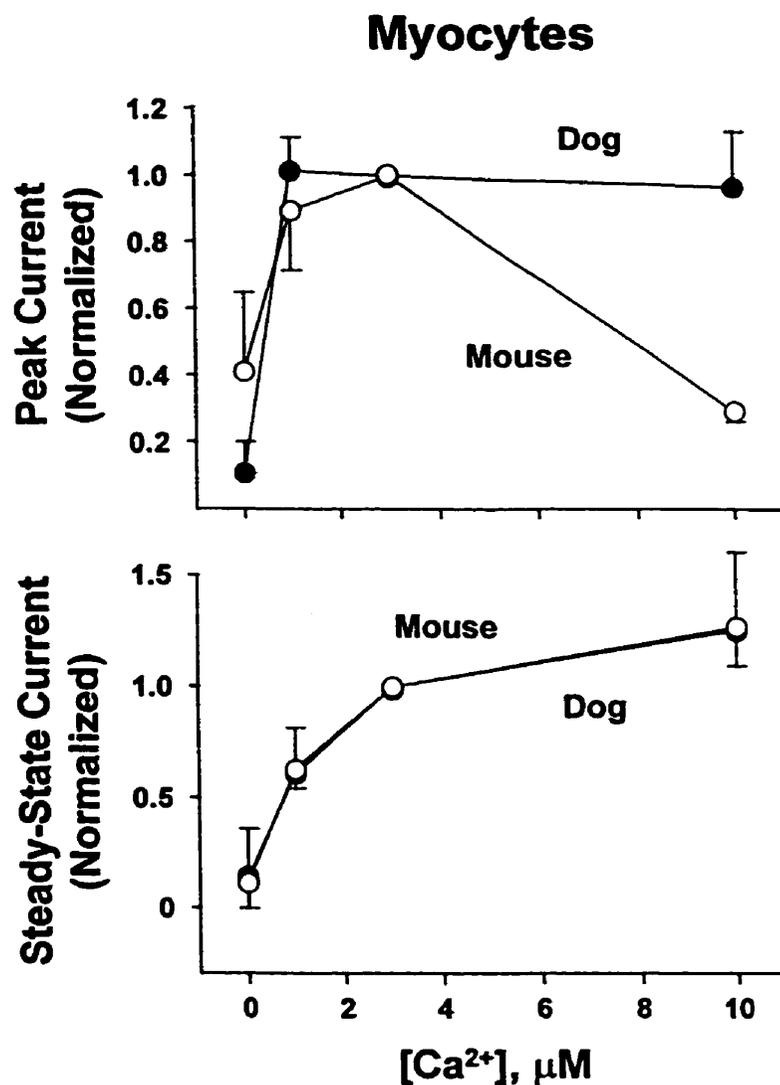


Figure 7. Pooled Data for Dog and Mouse Exchangers. The top panel shows peak outward exchange current for both NCX1.1 and $\Delta 680-685$ normalized to their respective values obtained at $3 \mu M Ca^{2+}$. Below, steady-state current which has also been normalized is illustrated. As the Ca^{2+} concentration is increased, both dog and mouse exchange activity is increased. Data are mean \pm SEM of 2 - 5 measurements at each $[Ca^{2+}]_i$ taken from 4 canine myocyte patches, and 3 - 5 measurements at each $[Ca^{2+}]_i$ taken from 5 control mouse patches.

Ionic Regulation in Transgenic Mice Overexpressing NCX1.1 or $\Delta 680-685$

Having documented the ionic regulatory phenotypes for NCX1 and $\Delta 680-685$ in *Xenopus* oocytes, and determining that major species differences did not exist between canine and murine $\text{Na}^+/\text{Ca}^{2+}$ exchangers, we began examination of myocytes from transgenic mice. Figure 8 depicts the $\text{Na}^+/\text{Ca}^{2+}$ exchange electrophysiological properties of myocyte membrane “blebs” from mice overexpressing NCX1.1 and the deletion mutant, $\Delta 680-685$. These records represent the first giant excised patch recordings from control and transgenic mice. Examination of the $\text{Na}^+/\text{Ca}^{2+}$ exchange currents was carried out using similar conditions to those described for Figure 4. Upon application of intracellular Na^+ , in the presence of regulatory Ca^{2+} , the transgenic NCX1.1 mouse myocyte patches exhibit a large increase in current followed by a slow decay to steady-state. This response is similar to that observed in dog and mouse myocytes (Figure 6) as well as in oocytes expressing NCX1.1 (Figure 4). Studies of exchange current from $\Delta 680-685$ yielded a phenotype that was insensitive to regulatory Ca^{2+} similar to that observed in oocytes overexpressing $\Delta 680-685$. Therefore, it is apparent that while overexpression of NCX1.1 did not alter ionic regulation, $\Delta 680-685$ overexpression resulted in a phenotype in which ionic regulation was nearly abolished. That is, mutant overexpression is able to largely overwhelm the native $\text{Na}^+/\text{Ca}^{2+}$ exchange ionic regulatory properties. This result verifies that our transgenic mice expressing mutant exchangers will exhibit altered electrophysiological properties of $\text{Na}^+-\text{Ca}^{2+}$ exchange. Prior to obtaining this result, there was no way to determine whether $\text{Na}^+-\text{Ca}^{2+}$ exchange regulation was, in fact, altered.

Figure 9 illustrates pooled data obtained from membrane “blebs” from both NCX1.1 and $\Delta 680-685$ mouse myocytes. For NCX1.1, ionic regulation was apparent since both peak and steady-state currents increased in response to increased regulatory Ca^{2+} . This response is nearly identical to that observed in oocytes expressing NCX1.1 and in control mouse myocytes. On the other hand, $\Delta 680-685$ did not exhibit major alterations in exchange current whether in the presence or absence of regulatory Ca^{2+} . This response is similar to that observed for *Xenopus* oocytes expressing $\Delta 680-685$. Overall, it appears that overexpression of $\Delta 680-685$ dominates the ionic regulatory profile of $\text{Na}^+/\text{Ca}^{2+}$ exchange in these mouse myocytes.

Transgenic Mouse Myocytes

NCX1.1

$\Delta 680-685$

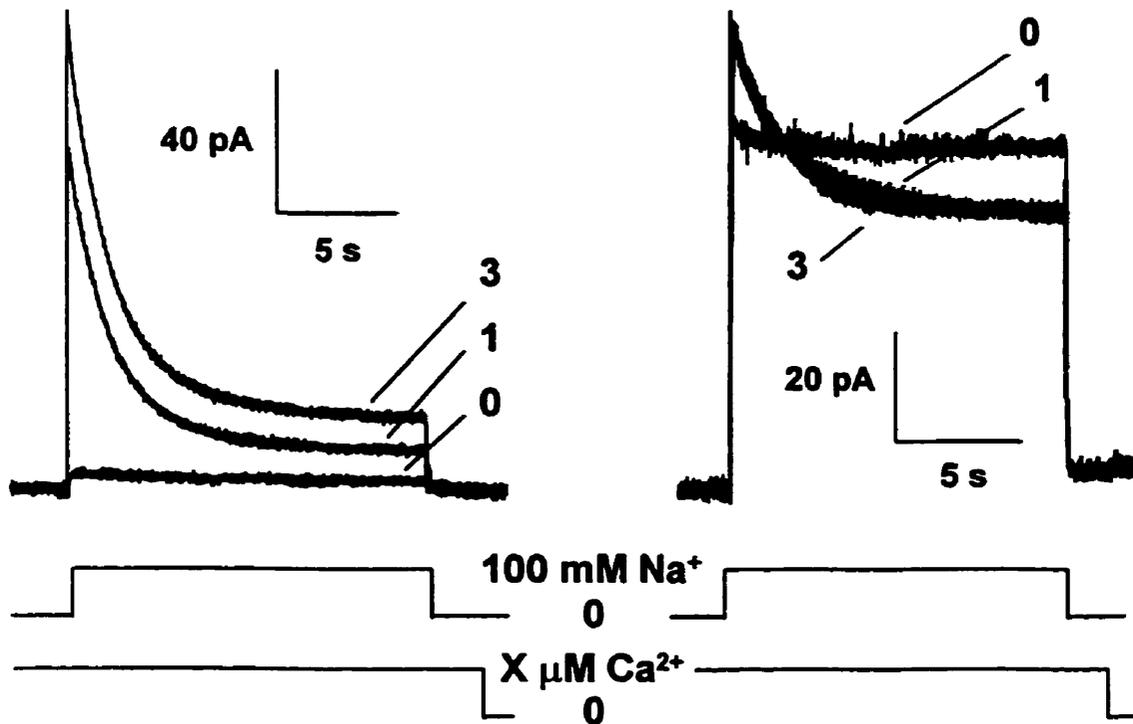


Figure 8. Effects of Regulatory Ca^{2+} on Transgenic Mice Overexpressing NCX1.1 or $\Delta 680-685$. Outward Na^+/Ca^{2+} exchange currents were compared for NCX1.1 and $\Delta 680-685$ sarcolemmal membrane “blebs” obtained from transgenic myocytes. Currents were obtained in the presence of the indicated Ca^{2+} concentrations. As the Ca^{2+} concentration was increased, NCX1.1 was stimulated. On the other hand, the deletion mutant showed an insensitivity to regulatory Ca^{2+} .

Transgenic Mouse Myocytes

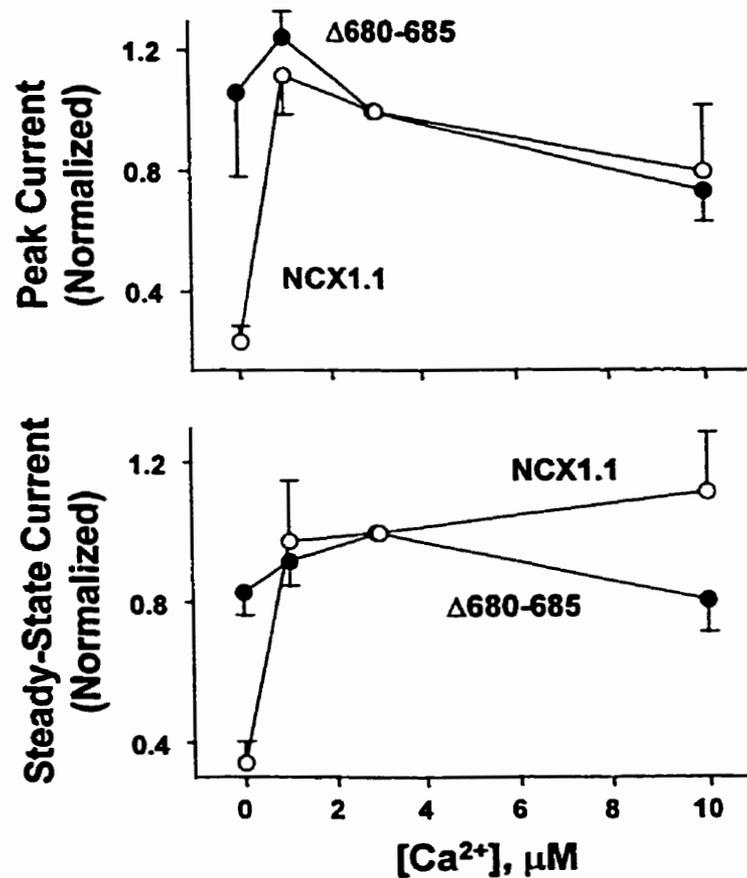


Figure 9. Pooled Data for Transgenic Mice Overexpressing NCX1.1 or Δ680-685.

Pooled data illustrating the outward Na⁺/Ca²⁺ exchange currents for transgenic mice overexpressing either NCX1.1 or Δ680-685 were examined. Data were normalized to the respective peak and steady-state current values obtained at 3 μM regulatory Ca²⁺_i. NCX1.1 exchange current increased with increased regulatory Ca²⁺ while Δ680-685 current levels were only slightly altered. Data are mean ± SEM of 7 - 9 measurements at each [Ca²⁺]_i taken from 9 NCX1.1 patches, and 6 - 11 measurements at each [Ca²⁺]_i taken from 11 Δ680-685 giant patches.

Effects of Regulatory Ca^{2+} on Transgenic Mouse Myocytes

The effects of regulatory Ca^{2+} on outward $\text{Na}^+/\text{Ca}^{2+}$ exchange currents were examined in patches obtained from control, transgenic NCX1.1 and transgenic $\Delta 680-685$ myocytes using a different experimental protocol. These experiments are referred to as “on-off-on” protocols to describe the influence of regulatory Ca^{2+} on steady state current production. Here, outward currents are initiated by the application of Na^+ in the presence of regulatory Ca^{2+} (on). Once steady-state currents were achieved, regulatory Ca^{2+} was removed for ≈ 32 s (off) and then reapplied (on). The results of this protocol are depicted in Figure 10. Note that for both the control and transgenic NCX1.1 exchanger records, the removal of regulatory Ca^{2+} results in a substantial inhibition of current. This result highlights the extent to which Ca^{2+} regulation can influence $\text{Na}^+-\text{Ca}^{2+}$ exchange currents. Specifically, in the absence of regulatory Ca^{2+} , exchange currents are almost completely eliminated despite an enormous electrochemical gradient favoring exchange. Transgenic $\Delta 680-685$ exchangers however, did not respond in a similar fashion. Instead, removal of regulatory Ca^{2+} did not greatly affect steady-state current, revealing an insensitivity to the presence or absence of regulatory Ca^{2+} . The slight decrease to steady-state current levels is likely attributable to the background expression of the native mouse exchanger which we have shown to be regulated by Ca^{2+}_i . Overall, we anticipate that under physiological conditions, control and transgenic NCX1.1 exchangers would be regulated by cytoplasmic Ca^{2+} fluxes whereas transgenic $\Delta 680-685$ exchangers, which are unresponsive to changes in Ca^{2+}_i concentrations, would remain active at all times.

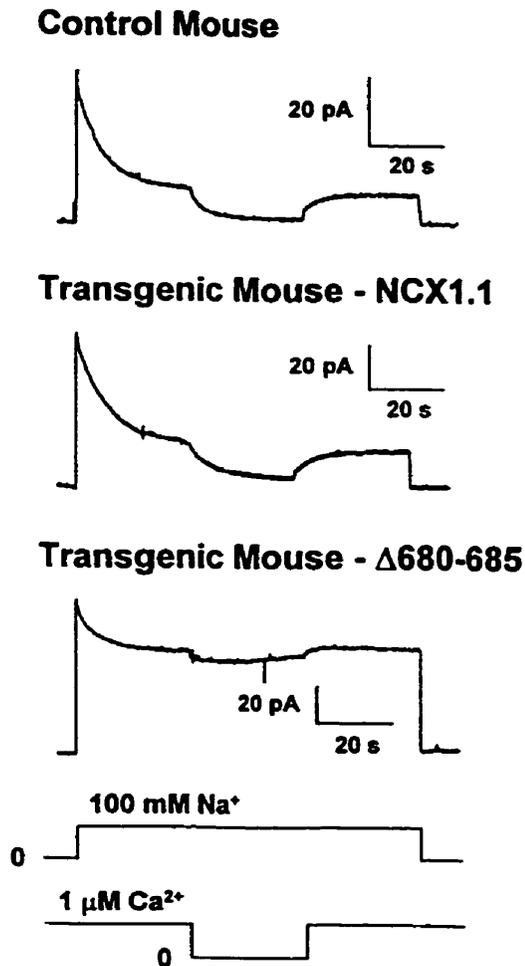


Figure 10. Response to Removal of Regulatory Ca²⁺. Outward Na⁺/Ca²⁺ exchange currents are shown for control and transgenic (NCX1.1 and Δ680-685) sarcolemmal membrane “blebs” upon the removal and reapplication of regulatory Ca²⁺. Na⁺/Ca²⁺ exchange current was activated through the application of 100 mM Na⁺ and 1 μM regulatory Ca²⁺. Regulatory Ca²⁺ was then removed for 32 sec and reapplied while the patch was continuously perfused with 100 mM Na⁺. Both control and transgenic NCX1.1 exchange currents were substantially decreased upon removal of regulatory Ca²⁺. However, the absence of regulatory Ca²⁺ did not greatly affect the exchange activity of Δ680-685.

Examination of the Physiological Consequences

After confirming the electrophysiological characteristics of myocytes from transgenic mice expressing either NCX1.1 or $\Delta 680-685$, and establishing that similar phenotypes occurred between these and the cloned exchangers expressed in *Xenopus* oocytes, we proceeded to investigate the physiological consequences of these interventions. This was conducted by investigating a paradigm of cardiac excitation-contraction coupling known as post-rest potentiation in isolated papillary muscles. The muscles were electrically stimulated over a range of frequencies (2-6 Hz) and the resulting contractile force was measured. Once steady-state force levels were achieved, rest intervals (1-60 s) were randomly imposed to study Ca^{2+} handling behavior. In particular, post-rest behavior provides insight into the competitive interplay between sarcolemmal and sarcoplasmic reticulum Ca^{2+} removal mechanisms. Representative force tracings obtained for a 5 s rest interval from both NCX1.1 and $\Delta 680-685$ muscles at a stimulation rate of 3 Hz are illustrated in Figure 11. Both preparations exhibit post-rest potentiation followed by a gradual recovery to steady-state for this intervention. However, we observed considerable differences in the characteristics of this potentiation as described below.

The potentiation fraction is defined as the ratio of force produced by the first contraction after the rest interval (post-rest beat) compared to the force of steady-state contractions prior to the rest period. Figure 12 shows the potentiation fractions obtained from papillary muscles of both NCX1.1 and $\Delta 680-685$ mice over stimulation frequencies

of 3-6 Hz. The results for three different rest intervals are shown (3, 5 and 30 s). Post-rest potentiation for muscles obtained from $\Delta 680-685$ mice was greater at all rest intervals and for all frequencies examined in comparison to NCX1.1 muscles. This finding indicates that the Ca^{2+} handling properties of these muscles are altered, presumably due to the consequences of eliminating ionic regulation of the $\text{Na}^+-\text{Ca}^{2+}$ exchanger. Possible explanations for these differences are provided in the Discussion. The statistical significance was measured using student t-tests. In order to reduce the possibility of a Type I error, the Bonferroni correction factor was incorporated. The majority of data points were found to be significant to $p < .01$ whereas a few data points only reached significance levels of $p < .05$.

Post-rest potentiation for both NCX1.1 and $\Delta 680-685$ transgenic mouse lines is depicted in Figure 13. Various rest intervals (1-60 s) were imposed for muscles stimulated at a basic stimulation frequency of 4 Hz. Both transgenic lines exhibit greater potentiation at shorter rest intervals followed by a gradual decline as the rest interval is lengthened. We found that it was very common to observe spontaneous contractions as the rest interval was increased (i.e. 15-60 s). In these cases, we did not include the trial in our data analysis. However, this observation raises the possibility that prolonged rest intervals show decreased contractile force due to a progression towards a spontaneous contractile event. Furthermore, we frequently observed very small spontaneous contractions raising the possibility that these could also occur below our detection threshold. This pattern of behavior is common in rat cardiac muscle which is thought to exhibit a progressive increase in SR Ca^{2+} during diastole. Importantly, we observed that

muscles from $\Delta 680-685$ transgenic mice produced greater post-rest potentiation at all rest intervals examined compared to that from NCX1.1 transgenic mice.

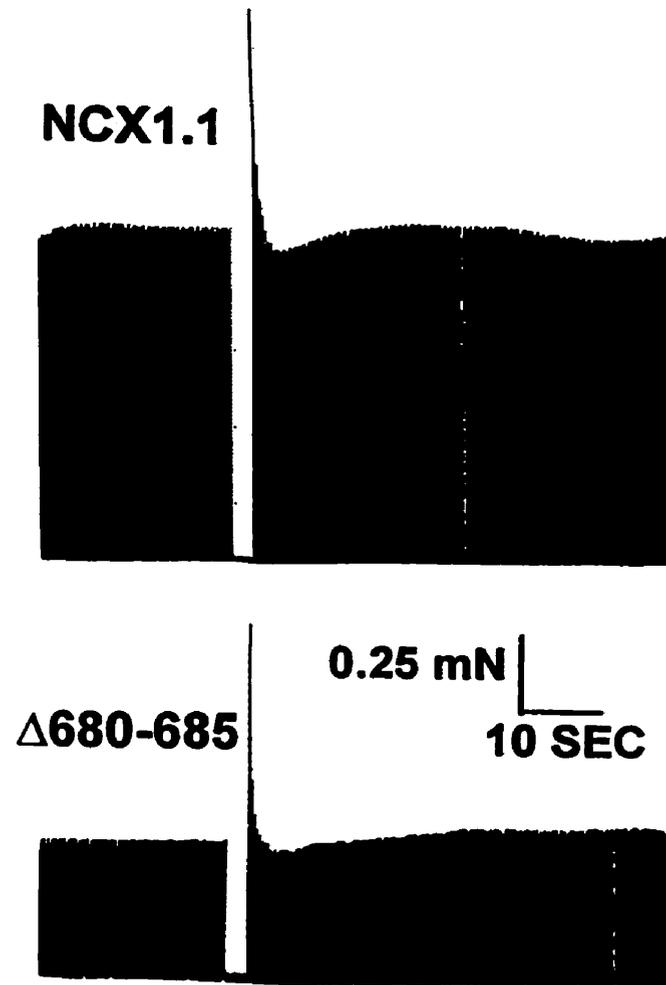


Figure 11. Post-Rest Potentiation in Papillary Muscles. Papillary muscles from transgenic mice overexpressing NCX1.1 and $\Delta 680-685$ $\text{Na}^+/\text{Ca}^{2+}$ exchangers were studied to examine the physiological relevance of ionic regulation. Representative traces illustrating post-rest potentiation following a 3 s rest interval are compared for both exchangers at an electrical stimulation rate of 3 Hz.

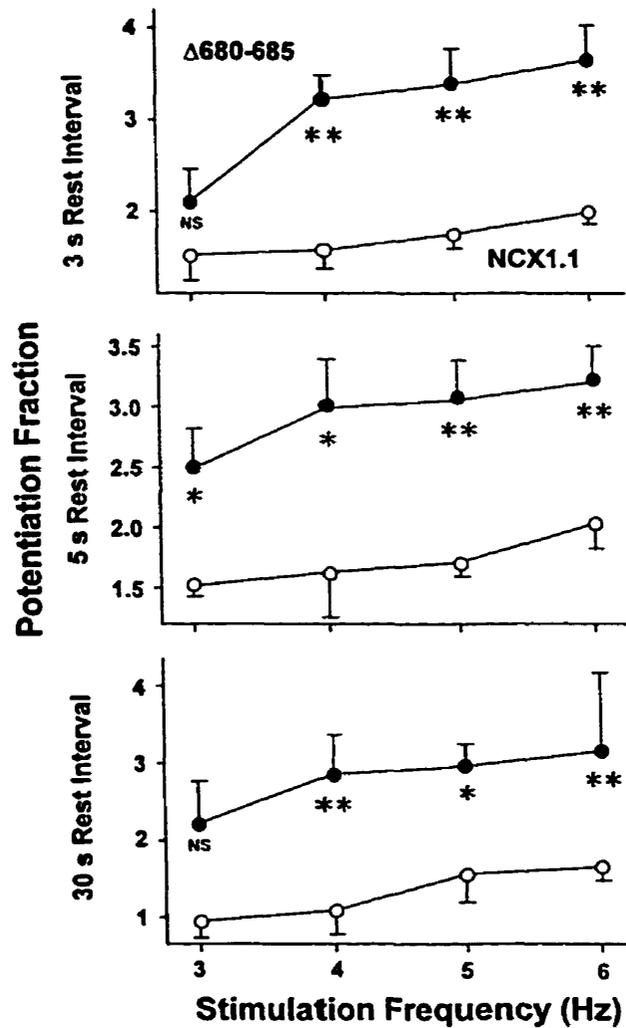


Figure 12. Potentiation Fraction vs Stimulation Frequency. Papillary muscles from transgenic mice overexpressing NCX1.1 and $\Delta 680-685$ $\text{Na}^+/\text{Ca}^{2+}$ exchangers were studied at various stimulation frequencies (2-6 Hz) and rest intervals (1 - 60 s). Data obtained at rest intervals of 3, 5, and 30 s is depicted graphically. At all rest intervals examined, $\Delta 680-685$ had a greater potentiation fraction than NCX1.1. Data are mean \pm SEM of 4 - 9 (NCX1.1) measurements and 4 - 8 ($\Delta 680-685$) measurements at each stimulation frequency obtained from papillary muscles from each group. ** $p \leq 0.01$, * $p \leq 0.05$

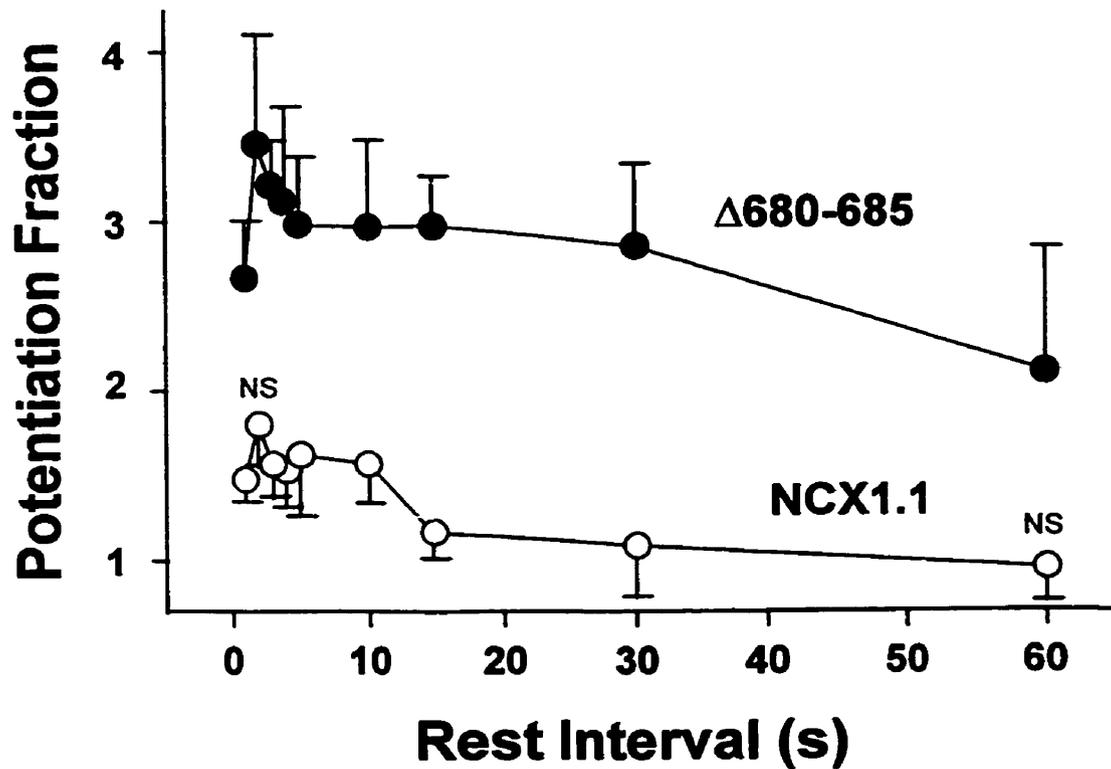


Figure 13. Potentiation Fraction vs Rest Interval. Potentiation fraction for papillary muscles from transgenic mice overexpressing NCX1.1 and $\Delta 680-685$ $\text{Na}^+/\text{Ca}^{2+}$ exchangers, as a function of rest interval, was examined. Data from randomly imposed rest intervals ranging from 1 - 60 s at a stimulation frequency of 4 Hz are illustrated. A greater rest potentiation was observed for $\Delta 680-685$ compared to NCX1.1 at all rest intervals. Data are mean \pm SEM of 3 - 7 measurements (NCX1.1) and 3 - 8 measurements ($\Delta 680-685$) at each rest interval from 8 papillary muscles for each transgenic category. All points are $p \leq 0.05$ unless otherwise indicated.

DISCUSSION

Overview

Regulation of $\text{Na}^+/\text{Ca}^{2+}$ exchange activity by intracellular Ca^{2+} has been known since early experiments revealed this phenomenon in the 1970's. This process was first identified through examination of the squid giant axon where it was found that a stimulation of $\text{Na}^+/\text{Ca}^{2+}$ exchange activity occurred upon application of cytoplasmic Ca^{2+} (7; 39). Subsequently, regulation of this type was identified for the cardiac exchanger. With the advent of the giant, excised patch clamp technique, it was also discovered that the $\text{Na}^+/\text{Ca}^{2+}$ exchanger is regulated by Na^+ (59, 61). Through the application of this technique, along with the cloning of the exchanger in 1990, our understanding of both Na^+ and Ca^{2+} regulation at the molecular level greatly increased. However, it was still unknown whether these regulatory mechanisms served any relevant physiological role. Therefore, we studied these processes using transgenic mice and have concluded that ionic regulation of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger is an important physiological process.

Examinations of the wild-type cardiac $\text{Na}^+/\text{Ca}^{2+}$ exchanger, NCX1, have identified protein regions with important roles in the ionic regulation of $\text{Na}^+/\text{Ca}^{2+}$ exchange. Within the large intracellular loop of the NCX1 exchanger, a 138 amino acid segment has been closely associated with Ca^{2+} dependent regulation. Similarly, a region at the N-terminus of the cytoplasmic loop is associated with I_1 inactivation. Many mammalian exchangers have been studied to date and it has been found that ionic regulation plays an important role for all exchangers examined. The amino acid

sequences for several exchangers have also been compared. While the sequences are similar among the majority of exchangers, compared to overall sequence homology, a much higher degree of similarity has been observed within the regions involved in these regulatory processes.

The protein regions responsible for Na^+ regulation have been studied extensively. Historically, after the exchanger was cloned in 1990, a region was identified which showed similarity to a calmodulin binding domain. In several proteins, these domains serve auto-inhibitory functions which are relieved upon calmodulin binding. A peptide was created duplicating this sequence and it was found that the intracellular application of this peptide produced a strong inhibition of $\text{Na}^+/\text{Ca}^{2+}$ exchange currents (85; 94). As such, this peptide became known as XIP or eXchange Inhibitory Peptide. Subsequently, structure-function studies targeted the endogenous XIP region and it was found that these mutations could dramatically alter the rate and extent of I_1 inactivation. Thus, it appears that exogenous XIP simply mimics the endogenous regulatory process of Na^+ -dependent inactivation.

Evaluations of ionic regulation for the *Drosophila* exchanger, CALX1.1, have shown that it is also regulated by Ca^{2+} , but in an opposite manner than NCX1 (64). Mutagenesis studies followed by electrophysiological analyses were carried out on the Ca^{2+} binding site to determine if the role of this site was conserved between NCX1 and CALX1. Both NCX1 and CALX1 exhibited a reduction in the regulatory Ca^{2+} affinity suggesting that the Ca^{2+} binding site may be conserved for all exchangers (42). However, the finding of a conserved functional role for both Na^+ and Ca^{2+} regulation in the

Drosophila Na⁺/Ca²⁺ exchanger did not provide an explanation for the opposite responses to regulatory Ca²⁺. We therefore carried out an examination involving chimeric exchangers. One particular chimera produced a phenotypic conversion between NCX1 and CALX1 supporting the idea of a separate transduction domain involved in the regulatory Ca²⁺ response (42).

While our understanding of ionic regulation and the regions of conserved sequence have improved over the years, the physiological significance of both Na⁺ and Ca²⁺-dependent regulation is still unknown. With the improvements in molecular biological techniques, we are now able to use transgenic mice to investigate the importance of regulation.

The consequences of overexpressing the canine cardiac Na⁺/Ca²⁺ exchanger, NCX1.1 and a deletion mutant of NCX1.1 (Δ 680-685), which was devoid of ionic regulation, were examined in transgenic mouse hearts. While the major goal was to determine if this overexpression would lead to alterations in E-C coupling, we first had to establish whether ionic regulation of Na⁺/Ca²⁺ exchange would be reduced or eliminated with the overexpression of Δ 680-685. Therefore, using electrophysiological techniques, it was revealed that the overexpression of Δ 680-685 was associated with a marked reduction in ionic regulation of Na⁺/Ca²⁺ exchange. It was also found that the regulatory phenotype observed in *Xenopus* oocytes overexpressing Δ 680-685 was similar to that derived from cardiomyocytes obtained from transgenic mice overexpressing this mutant exchanger. Physiological analyses were carried out using papillary muscles from these transgenic mice. It was observed that this overexpression of Δ 680-685 lead not only to

altered cardiac contractile properties resulting from altered ionic regulation, but also resulted in different Ca^{2+} handling properties.

Role in Excitation-Contraction Coupling

An elevation of cytoplasmic Ca^{2+} (e.g. 1-10 μM) stimulates a cardiac contraction by activating the myofilaments. In order for cardiac relaxation to occur, cytoplasmic Ca^{2+} must be returned to diastolic levels of ~ 100 nM. Trans-sarcolemmal Ca^{2+} removal is primarily carried out by the $\text{Na}^+/\text{Ca}^{2+}$ exchanger. In general, the $\text{Na}^+/\text{Ca}^{2+}$ exchanger removes the same quantity of extracellular Ca^{2+} which enters cardiac cells *via* L-type Ca^{2+} channels during systole (103). Since cardiac function must be capable of a wide range of contractile outputs, Ca^{2+} entry *via* the L-type Ca^{2+} channels fluctuates. To prevent either insufficient Ca^{2+} levels or Ca^{2+} overload, Ca^{2+} efflux and Ca^{2+} influx must be balanced. Even though a dynamic response of Ca^{2+} efflux must be present, it is unknown how Ca^{2+} influx and Ca^{2+} efflux are coupled. Therefore, a major goal of our study was to establish whether ionic regulation of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger played a role in this coupling and to gain insight into the role of ionic regulation in general.

$\text{Na}^+/\text{Ca}^{2+}$ exchange extrudes the greatest quantity of Ca^{2+} from cardiac cells. However, determining the exact magnitude of Ca^{2+} fluxes mediated by $\text{Na}^+/\text{Ca}^{2+}$ exchange on a beat to beat basis has been hindered by several factors. For example, while the sarcolemmal Ca^{2+} -ATPase is generally thought to be a much smaller contributor to diastolic Ca^{2+} removal compared to $\text{Na}^+/\text{Ca}^{2+}$ exchange, any activity by this

transport mechanism would reduce the Ca^{2+} load presented to the exchanger (724,730}. Another factor which creates difficulties for this assessment is the finding that under certain experimental conditions and/or in particular species, the $\text{Na}^+/\text{Ca}^{2+}$ exchanger may be involved in Ca^{2+} influx (80; 87; 135). If this reverse mode of $\text{Na}^+/\text{Ca}^{2+}$ exchange occurs, an even greater amount of Ca^{2+} would be present in the cell that must also be removed by the $\text{Na}^+/\text{Ca}^{2+}$ exchanger to maintain Ca^{2+} homeostasis.

Another major difficulty in studying the role of $\text{Na}^+/\text{Ca}^{2+}$ exchange stems from the lack of highly specific inhibitors for either $\text{Na}^+/\text{Ca}^{2+}$ exchange or the Ca^{2+} pump. Secondly, most results examining the contribution of these Ca^{2+} efflux pathways cannot be generalized due to the substantial differences which exist between species. Therefore, while it is known that $\text{Na}^+/\text{Ca}^{2+}$ exchange is a major mechanism for maintaining cellular Ca^{2+} homeostasis, there remains a great deal that is still unknown.

Ionic Regulation

The heart must be capable of operating over a wide inotropic range. In order to maintain a balance between Ca^{2+} entry and efflux, the $\text{Na}^+/\text{Ca}^{2+}$ exchanger must also be capable of operating over the same dynamic range as Ca^{2+} influx. However, it is unknown how $\text{Na}^+/\text{Ca}^{2+}$ exchange is coupled to changes in Ca^{2+} entry. Several possibilities could provide this dynamic response. For example, recruitment of individual $\text{Na}^+/\text{Ca}^{2+}$ exchangers could be regulated to respond to changes in Ca^{2+} concentrations or the activity of the entire population of exchangers could be modified. It is also possible that

active regulation of the population of $\text{Na}^+/\text{Ca}^{2+}$ exchangers may not be required at all. For example, if the capacity for Ca^{2+} efflux by $\text{Na}^+/\text{Ca}^{2+}$ exchange is very large, then fluxes could be solely determined by prevailing electrochemical gradients. That is, there could simply be a population of tonically active exchangers which generally exceeds the normal requirements for Ca^{2+} efflux, and no active regulation occurs. The ability of the $\text{Na}^+/\text{Ca}^{2+}$ exchange system to match Ca^{2+} entry could result from any of these possible mechanisms. Our working hypothesis is that ionic regulation of $\text{Na}^+/\text{Ca}^{2+}$ exchange is involved in this coupling.

The canine cardiac $\text{Na}^+/\text{Ca}^{2+}$ exchanger, NCX1, is regulated by both Na^+ and Ca^{2+} (58-60). When NCX1.1 is expressed in *Xenopus* oocytes, the exchange activity is increased by cytoplasmic Ca^{2+} . That is, the exchanger is largely inactive at diastolic Ca^{2+} levels (100 nM), whereas at greater Ca^{2+} concentrations within the same range thought to occur during normal cardiac excitation-contraction coupling (1-10 μM), a stimulation of exchange activity occurs. This response can occur very rapidly. When applied simultaneously with Na^+ , this Ca^{2+} -dependent activation can be observed within solution switching time (e.g. ~200 ms) and probably occurs much faster. It has also been found that Ca^{2+} -dependent regulation occurs for both inward and outward exchange currents (59; 72). On the other hand, Na^+ -dependent regulation refers to an inactivation of exchange activity in response to intracellularly bound Na^+ . The initial peak of $\text{Na}^+/\text{Ca}^{2+}$ exchange current slowly decays to steady-state as exchangers become inactive (42; 93). It is unknown whether intracellular Na^+ levels would ever be sufficiently high to induce this response physiologically.

The deletion of amino acids 680-685 in NCX1 produces a mutant exchanger, $\Delta 680-685$, which exhibits an insensitivity to regulatory Ca^{2+} and a reduction of Na^{+} -dependent inactivation (in the absence or presence of regulatory Ca^{2+} , the outward currents behave as though a high concentration of regulatory Ca^{2+} is always present). While the precise mechanisms for this absence of regulation are not known, it is possible that the deletion of amino acids 680-685 alters the ability of the exchanger to form or sustain ion-dependent inactive states. As such, this mutant exchanger provided us with a valuable tool to determine whether these regulatory mechanisms were important within the heart through the examination of the transgenic $\Delta 680-685$ mouse line.

Isoforms

Six exons make up the alternatively spliced region of the mammalian NCX1 $\text{Na}^{+}/\text{Ca}^{2+}$ exchanger. Through alternative-splicing of these exons, numerous tissue-specific variants of NCX1 can be generated. There are two mutually-exclusive exons, A and B, which are expressed in a tissue specific manner. Exon A is preferentially expressed in heart, brain and skeletal muscle whereas exon B is found in all rat tissue except heart (110).

To assess the role of the mutually exclusive exons A and B, ion transport and regulation of $\text{Na}^{+}/\text{Ca}^{2+}$ exchange was examined for two alternatively-spliced isoforms. The expression of exons AD has been found in brain splice variants while exons BD are found in kidney. These exchangers are referred to as NCX1.4 and NCX1.3 respectively.

We found that the isoforms NCX1.3 and NCX1.4 differ appreciably in terms of their I_1 and I_2 regulatory properties. With respect to Na^+ -dependent regulation, NCX1.3 exhibited a more pronounced inactivation than that of NCX1.4. This suggests that the expression of exon B in NCX1.3 is able to produce an I_1 inactive state of the exchanger which is more stable than that found in NCX1.4 when exon A is expressed. In terms of I_2 regulation, regulatory Ca^{2+} was found to produce both stimulatory and inhibitory effects on NCX1.3 $\text{Na}^+/\text{Ca}^{2+}$ exchange currents. Differences were observed for NCX1.3 and NCX1.4 in that regulatory Ca^{2+} was able to eliminate I_1 inactivation for NCX1.4 while only modest effects were observed on Na^+ -dependent inactivation of NCX1.3. These results reveal that particular characteristics of ionic regulation are controlled by the expression of these particular exons, suggesting that tissue-specific requirements for Ca^{2+} homeostasis may be met through this alternative splicing.

The consequences of alternative-splicing has been examined with respect to the *Drosophila* $\text{Na}^+/\text{Ca}^{2+}$ exchanger, CALX1. The splice variants, CALX1.1 and CALX1.2, which differ by only 5 amino acids (119; 123), both exhibit Na^+ and Ca^{2+} -dependent regulation although the nature of these regulatory processes are substantially different. Na^+ -dependent inactivation was observed for both isoforms. However, the extent of inactivation was greater for CALX1.2, as well as the rate of recovery. Upon examination of Ca^{2+} -dependent regulation, both isoforms were found to be negatively regulated by Ca^{2+} , a feature characteristic of *Drosophila* exchangers. Specifically, Ca^{2+} inhibited exchange currents for CALX1.1 to a greater extent compared to CALX1.2. However, much higher concentrations of regulatory Ca^{2+} were required to exert this inhibitory

effect for CALX1.1. Therefore, although the Ca^{2+} binding region is highly conserved between CALX1.1 and CALX1.2, these isoforms exhibited very different responses in the presence of regulatory Ca^{2+} . This supports the belief that Ca^{2+} -dependent regulatory properties may be modified through alternative splicing.

Transgenics

The overexpression of the canine NCX1.1 exchanger in transgenic mice has now been examined by several investigators. These transgenic mice were found to exhibit several altered characteristics compared to control mice. The overexpression of NCX1.1 was found to produce enhanced $\text{Na}^+/\text{Ca}^{2+}$ exchange currents, as well as enhanced relaxation rates of Ca^{2+} transients and contractions (2; 131; 138). While alterations in other SR Ca^{2+} handling proteins were suspected, levels of the SR Ca^{2+} handling proteins were found to be unaltered in mice overexpressing NCX1.1 (131). These transgenic mice were also found to have a greater inotropic response to the Na^+ channel agonist, BDF 9148 (11) and revealed an increased susceptibility to ischemia-reperfusion injury, specifically in males (37). Overall, however, while the cardio-specific overexpression of NCX1.1 produced detectable experimental changes, it is noteworthy that normal cardiac function appeared to be largely unaltered.

Consequences of Overexpressing either $\Delta 680-685$ or NCX1.1

In our study, we examined the consequences of overexpressing either NCX1.1 or $\Delta 680-685$. Our evaluation of transgenic mice overexpressing the canine NCX1.1 yielded ionic regulatory profiles similar to those observed for native $\text{Na}^+/\text{Ca}^{2+}$ exchange currents. Current traces obtained from these transgenic mice not only exhibited both Na^+ and Ca^{2+} -dependent regulation, but were qualitatively similar to traces obtained from control mice. If Na^+ and Ca^{2+} -dependent regulatory mechanisms play a significant role in the normal functioning of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger, then we predicted that overexpression of NCX1.1 would not remarkably alter cardiac function, a result borne out by previous investigations. Two possible explanations for this finding are suggested. First, if the number of active exchangers required for normal cardiac functioning is controlled by intact regulatory mechanisms, then overexpressing regulated exchangers should not alter exchanger recruitment. Consequently, normal cardiac function will be preserved. Secondly, if there is already a large reserve population of $\text{Na}^+/\text{Ca}^{2+}$ exchangers in excess of that required, then increasing this excess population should also be relatively benign. Both of these possibilities are compatible with existing experimental evidence.

Cardiac overexpression of $\Delta 680-685$ in transgenic mice produces a phenotype whereby both Na^+ and Ca^{2+} -dependent regulatory mechanisms are largely ablated. While regulatory Ca^{2+} was necessary to stimulate exchange activity in myocytes from control and transgenic mice overexpressing NCX1.1, substantial $\text{Na}^+/\text{Ca}^{2+}$ exchange activity was observed for $\Delta 680-685$ even in the complete absence of regulatory Ca^{2+} . Assuming the

direction of transport of $\text{Na}^+/\text{Ca}^{2+}$ exchange (*i.e.* forward or reverse) is determined by the electrochemical gradients, we hypothesize that the recruitment of exchangers to carry out this transport is controlled by ionic regulatory mechanisms such as Ca^{2+} regulation. According to this scenario, upon an increase in intracellular Ca^{2+} , additional $\text{Na}^+/\text{Ca}^{2+}$ exchangers are recruited to maintain Ca^{2+} homeostasis through increased Ca^{2+} efflux. With the overexpression of NCX1.1, physiological cardiac function should not be affected since diastolic Ca^{2+} levels would merely inactivate these additional exchangers. However, since $\Delta 680-685$ exchangers are not regulated by Ca^{2+} , regardless of the cytoplasmic Ca^{2+} concentration, these mutant exchangers would be constitutively active. Our electrophysiological results indicate that the expression of native NCX1.1 exchangers in the $\Delta 680-685$ transgenic mouse line is overwhelmed by the mutant exchanger population. Thus, even though the native exchangers might behave normally and respond to fluctuating Ca^{2+} levels, the overall effect is dominated by the mutant exchangers. That is, due to the inability of these mutant $\text{Na}^+/\text{Ca}^{2+}$ exchangers to become inactivated, the $\Delta 680-685$ myocytes would exhibit a more prominent efflux and/or influx of Ca^{2+} . Future studies employing the examination of diastolic Ca^{2+} levels and Ca^{2+} transient kinetics will be very useful in testing these hypotheses.

To assess the competition between the SR and the sarcolemma for Ca^{2+} removal from the cytoplasm, we used rest potentiation as our experimental paradigm. One difficulty with this approach is that fact that very little information exists on this contractile paradigm for the mouse. In contrast, rat has been extensively investigated. The contractile behavior of the mouse was previously thought to resemble rat whereby

Ca^{2+} accumulated during systole *via* reverse $\text{Na}^+/\text{Ca}^{2+}$ exchange (13; 14; 17) is released from the SR following a rest period. However, recent evidence has suggested that increased SR loading is not required for the augmentation of SR Ca^{2+} release in mice. In this regard, the mouse might more closely resemble ferret and canine muscle, whereby potentiation occurs due to augmented release without an absolute requirement for augmented SR Ca^{2+} content (17). This issue has not been resolved and hampers interpretation due to the paucity and conflicting nature of existing contractile studies in the mouse.

Our results revealed that a greater rest potentiation was observed in papillary muscles from $\Delta 680-685$ mice compared to those from NCX1.1. Most importantly, this indicates that Ca^{2+} handling properties are altered in these mice. If ionic regulation of $\text{Na}^+/\text{Ca}^{2+}$ exchange did not play a role in cardiac Ca^{2+} homeostasis, then the behavior exhibited from transgenic NCX1.1 and $\Delta 680-685$ mice would have been comparable. We attribute these functional alterations to post-rest contractile behavior in $\Delta 680-685$ mice to the impairment of ion-dependent regulatory properties. Both forward and reverse modes would be affected by this mutation.

Steady-state contractile force results from the balance between Ca^{2+} entry, efflux and release (13). In general, steady-state contractions are very sensitive to reductions in Ca^{2+} influx or lowered intracellular Ca^{2+} compared to post-rest contractions. These post-rest contractions are controlled primarily from SR Ca^{2+} release. We propose that non-regulated $\text{Na}^+/\text{Ca}^{2+}$ exchange is able to compete more effectively with the SR for cytoplasmic Ca^{2+} removal, since the mechanism to recruit and inactivate exchangers has

been impaired and renders them constitutively active. Consequently, we expect that this would reduce steady-state contractile force. A reduction in steady state force alone is sufficient to account for our results. This would ultimately produce the appearance of augmented post-rest contractile force, as the post-rest beat is less affected than steady state contraction. Lowering extracellular Ca^{2+} or adding Ca^{2+} channel blockers leads to the appearance of augmented post-rest contractions by this exact mechanism. A second possibility is that mouse cardiac muscle behaves similarly to rat. In rat, reverse $\text{Na}^+/\text{Ca}^{2+}$ exchange leads to a progressive increase in SR Ca^{2+} during diastole. If a similar mechanism occurs in mouse, then deregulated reverse $\text{Na}^+/\text{Ca}^{2+}$ exchange would be expected to further increase intracellular Ca^{2+} during rest (131). While this explanation is compatible with results, we remain conservative in forwarding this possibility simply because excitation-contraction coupling is very poorly described for mouse. One recent study suggests that Ca^{2+} handling properties of the mouse differ substantially from rat (47), although it is premature to view this as dogma. Overall, our results support a physiological role of ionic regulation of $\text{Na}^+/\text{Ca}^{2+}$ exchange as revealed by the alterations in contractile behavior exhibited by transgenic mice overexpressing a deregulated form of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger. While the exact mechanism will require additional studies, a body of evidence is growing indicating the ionic regulation of $\text{Na}^+/\text{Ca}^{2+}$ exchange is a physiologically meaningful phenomenon.

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