INHIBITION OF Na^+-Ca^{2+} EXCHANGE BY KB-R7943

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LIST OF ABBREVIATIONS

λ, rate of current decay

BSA, bovine serum albumin

$Ca(NO_3)_2$, calcium nitrate

$CaCl_2$ calcium chloride

$CaCO_3$, calcium carbonate

Calx, *Drosophila* Na^+-Ca^{2+} exchanger

CHX, Ca^{2+}-H^+ exchanger

CICR, Ca^{2+}-induced Ca^{2+}-release

cRNA, complementary ribonucleic acid

CsOH, cesium hydroxide

DAD(s), delayed afterdepolarization(s)

DCB, (3',4'-dichlorobenzamil)

DMSO, dimethyl sulfoxide

EAD(s), early afterdepolarization(s)

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

FMRF-amide, molluscan cardioexcitatory peptide

FRCRCF-amide, analogue of molluscan cardioexcitatory peptide

F_{SS}, fraction of steady-state current remaining

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

I_1, Na^+-dependent inactivation

I_2, Ca^{2+}-dependent regulation

I_{K1}, inward rectifying potassium current

I_{TI}, transient inward current

IV, current-voltage relationship

K_2HPO_4, potassium phosphate

KB-R7943, (2-[2-[4-(4-nitrobenzyloxy)-phenyl]ethyl]isothiourea methesulfonate)

KCl, potassium chloride

KOH, potassium hydroxide

LiOH, lithium hydroxide

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

$Mg(OH)_2$, magnesium hydroxide

$MgSO_4$, magnesium sulfate

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

myristyl-FRCRCF-amide, analogue of molluscan cardioexcitatory peptide

NaCl, sodium chloride

$NaHCO_3$, sodium bicarbonate

NCKX, Na^+-Ca^{2+}+K^+ exchanger

NCX, Na^+-Ca^{2+} exchanger

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

PI, phosphatidylinositol

PIP_2, phosphatidylinositol 4,5-bisphosphate

PKA, protein kinase A

PKC, protein kinase C

PMA, phorbol 12-myristate 13-acetate

RT, room temperature

SEA0600, (2-4[-[(2,5-difluorophenyl)methoxy]phenoxy]-5-ethoxyaniline)

SL, sarcolemmal

SR, sarcoplasmic reticulum

TEA-OH, tetraethylammonium hydroxide

TM(s), transmembrane segment(s)

XIP, exchange inhibitory peptide

I. ABSTRACT

The cardiac Na^+-Ca^{2+} exchanger is an important protein involved in transsarcolemmal Ca^{2+} transport in the heart. On a beat-to-beat basis, the Na^+-Ca^{2+} exchanger removes nearly all of the Ca^{2+} entering the myocyte through L-type Ca^{2+} channels in exchange for Na^+. Calcium influx *via* Na^+-Ca^{2+} exchange is also possible, as the direction and activity of the exchanger is dependent on membrane potential, and Na^+ and Ca^{2+} gradients. While the physiological role of Ca^{2+} influx mediated by Na^+-Ca^{2+} exchange (*i.e.*, reverse-mode exchange) is controversial, reverse-mode Na^+-Ca^{2+} exchange is believed to play a major role in a variety of models of cardiac disease and injury.

It is difficult to precisely define the role of Na^+-Ca^{2+} exchange in both physiology and pathophysiology, since in addition to transporting Na^+ and Ca^{2+} the exchanger is also regulated by these ions. Furthermore, Na^+-Ca^{2+} exchange research has been severely hindered by the lack of highly selective and potent pharmacological agents. A recently released, novel isothiourea derivative, KB-R7943, was described as a preferential inhibitor of reverse-mode Na^+-Ca^{2+} exchange. A number of discrepancies in this assumption have recently appeared in the literature. For example KB-R7943 has been reported to be both competitive and non-competitive with respect to extracellular Ca^{2+}. KB-R7943 was also demonstrated to show no inhibitory preference for a particular mode under bi-directional ionic conditions. Finally, there is disagreement regarding its potency among Na^+-Ca^{2+} exchanger isoforms. However it

is widely employed in a number of studies investigating the role of Na^+-Ca^{2+} exchange that address physiological and pathophysiological end-points.

Therefore we addressed some controversial aspects of KB-R7943 using the giant, excised patch technique. Membrane patches were excised from *Xenopus laevis* oocytes expressing the cloned, cardiac Na^+-Ca^{2+} exchanger, NCX1.1. Forward, reverse, and combined forward-reverse-mode Na^+-Ca^{2+} exchange currents were analyzed in the presence and absence of KB-R7943. We found that KB-R7943 preferentially inhibited reverse-mode Na^+-Ca^{2+} activity. At 20 μM KB-R7943, reverse-mode Na^+-Ca^{2+} exchange activity was inhibited by > 90%, whereas forward-mode Na^+-Ca^{2+} exchange was only inhibited by < 20%. There was no evidence of purely competitive interactions between KB-R7943 and either extracellular Ca^{2+} or intracellular Na^+. Additionally, regulatory Ca^{2+} did not significantly alter KB-R7943's inhibitory potency. While we were not able to define the exact mechanism by which KB-R7943 exerts its effects on Na^+-Ca^{2+} exchange, our data suggests that KB-R7943 primarily interacts with the exchanger's transport machinery, with additional influences on its ionic regulatory properties. Using the consecutive eight-state model, we suggest that KB-R7943 may interact with a particular kinetic state ($E_1 3 N_i$).

II. INTRODUCTION

The Na^+-Ca^{2+} exchanger is an ion transport protein found in the plasma membrane of most tissues that was first described in the heart (1) and giant squid axon (2). The Na^+-Ca^{2+} exchanger catalyzes the transport of 1 Ca^{2+} in exchange for 3 Na^+ (3;4), with the direction and magnitude of Na^+-Ca^{2+} exchange driven by membrane potential and the concentration gradients of both Na^+ and Ca^{2+}. In addition to transporting Na^+ and Ca^{2+}, the Na^+-Ca^{2+} exchanger is regulated by these ions, and is also modulated by pH, ATP, phosphorylation, and lipid-protein interactions (see reviews (5-8)).

Currently, three genes have been identified that encode for Na^+-Ca^{2+} exchanger proteins in mammals, denoted as NCX1, NCX2, and NCX3 (9-11). Both NCX1 and NCX3 undergo alternative splicing, forming a variety of splice variants that are tissue specific (12;13). NCX1.1, the isoform expressed the in heart, was the first Na^+-Ca^{2+} exchanger to be cloned (9), and is the most studied to date. The mature NCX1.1 protein is 938 amino acids long (14) and is currently modeled to consist of 9 membrane-spanning segments with a large intracellular loop between transmembrane segments 5 and 6 (15-17). On this intracellular loop are regions important for ionic regulation (18), including the alternative splice site (19). Two re-entrant loops between transmembrane segments 2 and 3 and 7 and 8 (Calx-α motifs (20)), are believed to form part of the ion translocation pathway (15;21).

The physiological role of Na^+-Ca^{2+} exchange in the heart is well documented. It is generally accepted that on a beat-to-beat basis, the amount of Ca^{2+} brought into

the myocyte by L-type Ca^{2+} channels is removed by forward-mode Na^{+}-Ca^{2+} exchange (*i.e.*, Ca^{2+} efflux), thereby facilitating cardiac relaxation (reviewed in (6;7;22;23)). The precise significance of the Na^{+}-Ca^{2+} exchanger's role in cardiac function is currently being re-evaluated, mainly due to new evidence suggesting a larger role for the sarcolemmal (SL) Ca^{2+}-ATPase (24;25) and the proposal of a different stoichiometry (26) (*i.e.*, 4:1 instead of 3:1). Enhanced Ca^{2+} efflux *via* the SL-Ca^{2+}-ATPase would compete with Na^{+}-Ca^{2+} exchange-mediated Ca^{2+} efflux, while a 4:1 stoichiometry increases the driving force for Ca^{2+} extrusion by the Na^{+}-Ca^{2+} exchanger. Additionally, the relative importance of forward-mode Na^{+}-Ca^{2+} exchange in the heart varies from species to species (reviewed in (23)). Despite these quantitative discrepancies and species differences, however, Na^{+}-Ca^{2+} exchange undoubtedly plays an important role in Ca^{2+} homeostasis.

In the working heart, calcium influx *via* the Na^{+}-Ca^{2+} exchanger (*i.e.*, reverse-mode exchange) is also a possibility. However, the physiological relevance of this process is currently the subject of much debate (reviewed in (7;22)). Proposed roles for reverse-mode Na^{+}-Ca^{2+} exchange in the heart include directly triggering SR Ca^{2+} release (27-30), modulating the effectiveness of the L-type Ca^{2+} channel in inducing SR Ca^{2+} release (31-33), and increasing the content of Ca^{2+} in the SR (34-36). Other studies find reverse-mode Na^{+}-Ca^{2+} exchange in the heart to be negligible in normal physiology (37;38). Thus, the precise physiological role of reverse Na^{+}-Ca^{2+} exchange remains to be defined.

There is evidence that Na^{+}-Ca^{2+} exchange plays a significant role in a variety of models of cardiac injury or disease (39-45). A number of studies have shown

higher levels of Na^+-Ca^{2+} exchange expression and activity in several models of heart failure and/or cardiac hypertrophy (39;42;46-48), while others reported either no change or a decrease in Na^+-Ca^{2+} exchange levels (49-51). Whether increased Na^+-Ca^{2+} exchange activity is beneficial in heart failure is also under scrutiny (see commentary by (52)). For example, increased Na^+-Ca^{2+} exchange activity has been shown to contribute to arrhythmogenesis (39;40) since Na^+-Ca^{2+} exchange contributes to transient inward currents (I_{TI}) associated with delayed afterdepolarizations or oscillatory afterpotentials (53-55).

Part of the reason there is so much controversy surrounding the contribution of Na^+-Ca^{2+} exchange in physiology and pathophysiology is because the Na^+-Ca^{2+} exchanger may contribute to both Ca^{2+} influx and efflux, and differentiating between these two processes can be difficult to achieve experimentally. Potent and highly selective modulators of Na^+-Ca^{2+} exchange would be extremely useful in this regard, but unfortunately there is a lack of pharmacological agents currently available (see review (8)). However, in 1996, a novel isothiourea derivative, KB-R7943 (formerly No. 7943), was introduced (56;57). In the original reports it was described as a specific inhibitor of reverse-mode Na^+-Ca^{2+} exchange, with a > 90% inhibition of reverse-mode Na^+-Ca^{2+} exchange in the low micromolar (*i.e.*, 0.3 – 2.4 μM) range. Conversely, the same level of inhibition of forward-mode Na^+-Ca^{2+} exchange required 10 – 50 times higher concentrations. However while one study found KB-R7943 to be non-competitive with respect to transported ions (57), another reported competition between KB-R7943 and extracellular Ca^{2+} (56).

Since the original reports were released, controversy has surrounded KB-R7943, especially regarding its inhibitory potency and mode specificity. Recently, a study investigating Na^+-Ca^{2+} exchange under bi-directional ionic conditions found no apparent mode-specific inhibition (58). One study assessing the effect of KB-R7943 on NCX1, NCX2 and NCX3 found KB-R7943 to be 3-fold more potent for NCX3 than NCX1 and NCX2 (59), while another encountered no difference in inhibitory potency (60). Although some of the controversy surrounding KB-R7943 may partly stem from the variety of assay systems and differences in protocols being employed, there is currently no consensus regarding how KB-R7943 exerts its inhibitory effects on the Na^+-Ca^{2+} exchanger.

Despite these discrepancies, KB-R7943 has primarily been used as an inhibitor of reverse-mode Na^+-Ca^{2+} exchange in a variety of studies examining Na^+-Ca^{2+} exchange since 1996. For example, KB-R7943 reportedly shows renal protective effects in rats with ischemic acute renal failure (61), KB-R7943 prevented Ca^{2+} overload in rat ventricular myocytes (62), and KB-R7943 protected CA1 neurons in rat hippocampal slices against hypoxic/hypoglycemic injury (63). Not all studies employing KB-R7943 have demonstrated beneficial effects, however (64). Nonetheless, interest in KB-R7943 remains high, and is still widely used. A more detailed examination of KB-R7943 on Na^+-Ca^{2+} exchange would clearly be advantageous.

Using the giant, excised patch technique, we examined the effects of KB-R7943 on the NCX1.1 canine cardiac Na^+-Ca^{2+} exchanger, expressed in *Xenopus laevis* oocytes. Our results suggest KB-R7943 preferentially inhibited reverse-mode

Na^+-Ca^{2+} exchange currents. We also found that KB-R7943 is non-competitive with respect to transported ions. Finally, while the inhibitory mechanism primarily consisted of direct effects on the Na^+-Ca^{2+} exchanger's transport machinery, we detected additional influences on the exchanger's ionic regulatory properties.

III. LITERATURE REVIEW

1. Transport Properties of Na^+-Ca^{2+} Exchange

1.1. Electrogenicity and Stoichiometry

The Na^+-Ca^{2+} exchanger was suspected to be an electrogenic, or rheogenic (*i.e.*, current generating) transporter early on, with an estimated stoichiometry (*i.e.*, transport coupling ratio) of ≈ 3 Na^+ to 1 Ca^{2+} (2), though the data was considerably scattered (see Figures 13 and 14 in (2)). These experiments were based on measuring unidirectional movement of Na^+ and Ca^{2+} isotopes in Na^+-dependent Ca^{2+} efflux and Ca^{2+}-dependent Na^+ influx in giant squid axons. However considerable debate over the exact stoichiometry of Na^+-Ca^{2+} exchange and whether the exchange was indeed electrogenic, would ensue in the following decades because of two major technical difficulties: (1) Isolation of ion fluxes attributable to one protein transporting Na^+ and Ca^{2+} from other Na^+ and Ca^{2+} transporters in biological tissues; (2) The absence of specific, high affinity blockers of Na^+-Ca^{2+} exchange activity. To varying degrees, both problems persist to this day.

Many early studies, like the one described above (2), circumvented these difficulties by examining the voltage-dependence of the exchanger, investigating competition among ions, or by estimating the stoichiometry based on thermodynamic considerations (*e.g.*, (1;4;65-70)). Unfortunately, these studies provided only indirect information about the stoichiometry, and some of these reports tended to infer

electrogenicity based on observed voltage-dependencies. Although electrogenic mechanisms must, by definition, be voltage sensitive, Eisner and Lederer (66) pointed out that not all voltage-sensitive processes must be electrogenic. They described a simple carrier model of Na^+-Ca^{2+} exchange showing that both electrogenic and electroneutral Na^+-Ca^{2+} exchange may be influenced by membrane potential.

Reeves and Hale (4), who measured $^{45}Ca^{2+}$ fluxes in vesicles obtained from bovine heart preparations, conducted one of the first studies examining the stoichiometry of Na^+-Ca^{2+} exchange more directly. Their results were based on the notion that by adjusting the concentration of Na^+ and Ca^{2+} on both sides of the membrane, they could reduce the overall driving force to zero, thereby allowing the stoichiometry to be described by the membrane potential and the equilibrium potentials for Na^+ and Ca^{2+}. By treating the vesicles with valinomycin (an ionophore) in the presence of K^+, negative or positive membrane potentials were generated, and a stoichiometry of 2.97 Na^+ to 1 Ca^{2+} was calculated based on the magnitude of the Na^+ gradient compensating for changes in membrane potential.

The first clear measurement of Na^+-Ca^{2+} exchange current was demonstrated by Kimura et al. (71), providing compelling evidence that Na^+-Ca^{2+} exchange was a electrogenic process (*i.e.*, evidence that the stoichiometry was >2:1), since electroneutral exchange (*i.e.*, a 2:1 stoichiometry) would not produce a net current. In the years that followed, a number of studies would take advantage of being able to measure Na^+-Ca^{2+} exchange current. As it is possible to predict the reversal potential of Na^+-Ca^{2+} exchange for each stoichiometry, measurements of Na^+-Ca^{2+} exchange current reversal potential under known ionic conditions could used to determine the

stoichiometry of the Na^+-Ca^{2+} exchanger. These studies measured the reversal potential of Na^+-Ca^{2+} exchange currents using whole-cell voltage clamp in combination with intracellular perfusion, and a stoichiometry of 3:1 was reported (*i.e.*, the reversal potential was altered with changes in Na^+ and Ca^{2+} levels, as expected for this stoichiometry) (72;73). Further studies also concluded that the Na^+-Ca^{2+} exchanger was electrogenic, and had a stoichiometry of 3:1 (3;71;74-78), which is the currently accepted stoichiometry.

The stoichiometry of Na^+-Ca^{2+} exchange forms the basis of how the Na^+-Ca^{2+} exchange process is interpreted biophysically and also helps define what role Na^+-Ca^{2+} exchange plays in the physiological setting. For example, most studies assume a 3:1 stoichiometry when assessing Ca^{2+} efflux and influx via Na^+-Ca^{2+} exchange (*e.g.*, (2;79;80)). Evaluation of Na^+-Ca^{2+} exchange transport rates (*i.e.*, the number of ions transported per exchanger per unit time) based on exchanger partial reactions also assume a stoichiometry of 3:1 (81-84).

A recent study reported a 4:1 stoichiometry for Na^+-Ca^{2+} exchange when examining reversal potentials obtained from "macro" patches excised from guinea-pig ventricular myocytes (26). The authors suggested the stoichiometry may even vary with changes in ionic conditions. Should this new stoichiometry prove true, a rather extensive re-evaluation of the physiological role of Na^+-Ca^{2+} exchange would be necessary. Exchanger turnover rates (discussed below) would be halved, and reverse-mode exchange would seem less likely to occur (*i.e.*, reverse exchange would only occur at much higher $[Ca^{2+}]_i$ because of the change in the exchanger's reversal

potential). More evidence will be required before the vast body of research supporting a 3:1 stoichiometry is set aside, however.

1.2. Transport Mechanism

Two major transport schemes have been used to describe the Na^+-Ca^{2+} exchange transport cycle both mechanistically (*i.e.*, physical model) and kinetically (*i.e.*, mathematical description): 1) The "consecutive" or "Ping-Pong" transport mechanism, is a *two step* process where first Na^+ binds and is transported across the membrane, and then Ca^{2+} binds and is transported, and *vice versa.* 2) The "simultaneous" transport mechanism describes the situation where both Na^+ and Ca^{2+} must bind before they can be transported across the membrane (*i.e.*, they are transported in the *same* step) (Figure 1).

Early on, Blaustein and Santiago (85) suggested Na^+-Ca^{2+} exchange was consistent with the simultaneous model since increasing saturation of intracellular Ca^{2+} did not affect Ca^{2+} efflux mediated by extracellular Na^+ application. Additionally, extracellular Na^+ facilitated Ca^{2+} efflux regardless of the presence of the alkali metal ions present intracellularly. Conversely, Ca^{2+}-Ca^{2+} exchange required alkali metal ions present both intra- and extracellularly. Therefore, both the internal and external ion-binding sites must be loaded simultaneously before exchange can occur, otherwise unidirectional (*i.e.*, exchange without "coupling" of a counter ion) exchanger-mediated transport would occur. However, this study did not account for several possibilities. For example, the possibility that "uncoupled" exchangers cannot

cycle was not taken into consideration. Furthermore, this study, which largely relied on ion competition, did not perform experiments under "zero-trans conditions" (*i.e.*, the concentration of the counter-ion should be zero on the opposing side of the membrane), as was later suggested by Läuger (86). An ion competition experiment similar to Blaustein and Santiago (85), repeated under zero-trans conditions, could be used to determine whether Na^+-Ca^{2+} exchange proceeded *via* a simultaneous or consecutive model based on mathematical descriptions of these two processes (refer to equations 18 and 41 in (86); Basically, for the consecutive model, the half-saturation concentration of intracellular Ca^{2+} (K_d) should decrease as extracellular Na^+ concentration decreases, whereas for the simultaneous model, K_d should remain unchanged as extracellular Na^+ concentration decreases, and vice versa). Years later, two separate groups repeated these experiments under zero-trans conditions and found that their data was more consistent with a consecutive transport model (87;88).

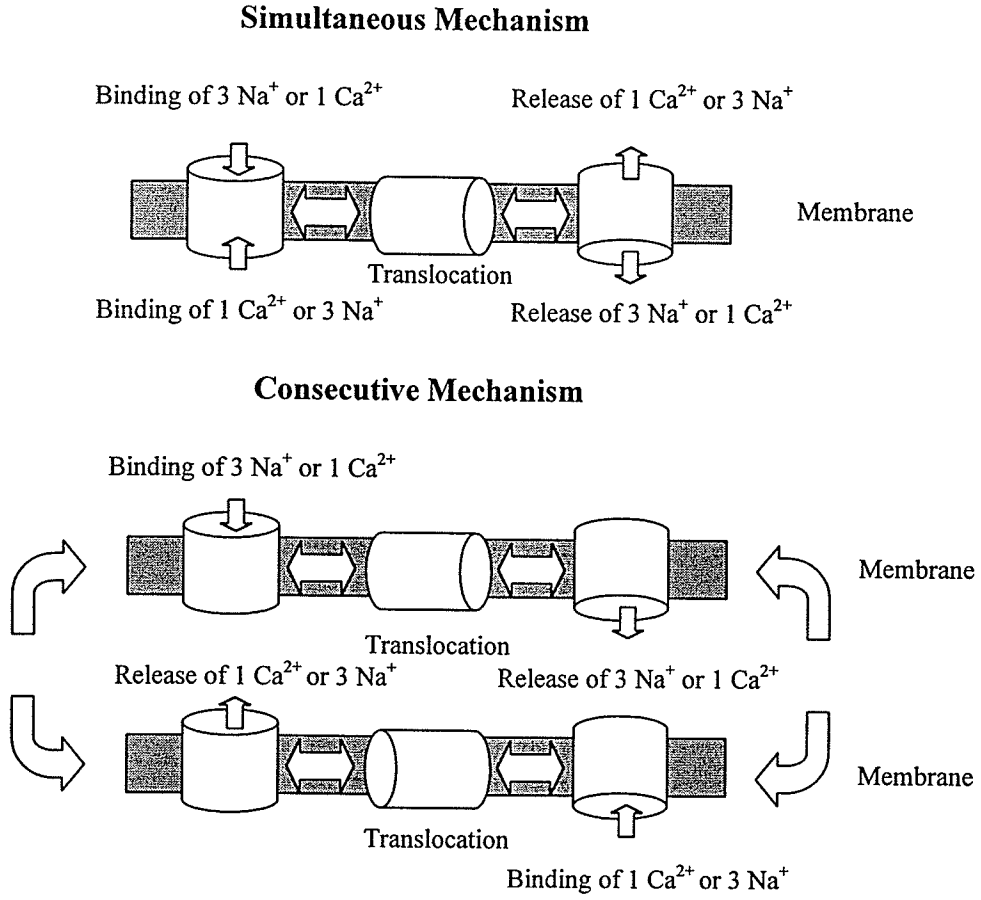

Figure 1. ***Comparison of Transport Mechanisms Proposed for Na^+-Ca^{2+} Exchange.*** The simultaneous mechanism requires ion binding on both sides of the exchanger before transport of both ions occurs in a *single* step, whereas the consecutive mechanism involves *two* steps, *i.e.*, the binding and transport of either Na^+ or Ca^{2+} in one step, followed by the binding and transport of Ca^{2+} or Na^+ in the second step.

In addition to ion competition experiments, studies looking at partial transport reactions for either Na^+ or Ca^{2+} (82) provided support for a consecutive transport mechanism, or, at least a mechanism that moves charge in more than one partial step (84). In giant, excised patch experiments, membrane patches were initially exposed to low pipette Na^+ or Ca^{2+} in the absence of cytoplasmic Ca^{2+} or Na^+. In the consecutive model, all ion-binding sites would orient themselves to the cytoplasmc surface. Upon rapid application of the counter ion to the cytoplasmic surface of the patch, Hilgemann et al., found that the K_d of Na^+ approached zero as the K_d of Ca^{2+} approached zero, as predicted by the consecutive model. Interestingly, Hilgemann et al. also reported that charge movement was only associated with Na^+ translocation and/or Na^+ unbinding, suggesting that these are the electrogenic step(s) (82). Niggli and Lederer obtained similar results supporting a consecutive reaction mechanism using flash photolysis experiments (*i.e.*, intracellular "caged" Ca^{2+} is released upon stimulation), however, charge movement was associated with Ca^{2+} translocation (84). Kappl and Hartung also reported that charge movement occurred during Ca^{2+} translocation (89), which was also demonstrated in the squid exchanger (90). Later it would be shown that translocation of either ion could involve charge movement (81).

The currently accepted consecutive transport mechanism is modeled to contain eight kinetic states (Figure 2): E_1 and E_2, which represent "empty" exchangers with their ion binding sites oriented to the intra- and extracellular surfaces, respectively; E_2C_o and E_23N_o, the extracellular Ca^{2+} and Na^+ bound states, respectively; E_oC and E_o3N, transitional states occluded with either Ca^{2+} or Na^+, respectively; E_1C_i and E_13N_i, intracellular Ca^{2+} and Na^+ bound states, respectively

(82). In addition to transporting Na^+, the Na^+-Ca^{2+} exchanger is also regulated by Na^+. This process is referred to as Na^+-dependent inactivation, or I_1, because the amount of current decay during reverse-mode Na^+-Ca^{2+} exchange increases as the Na^+ concentration rises. In order to account for I_1, an inactive state was also modeled (91). Forward-mode Na^+-Ca^{2+} exchange (*i.e.*, Ca^{2+} efflux) cycles counter-clockwise in Figure 2, whereas reverse-mode exchange cycles clockwise.

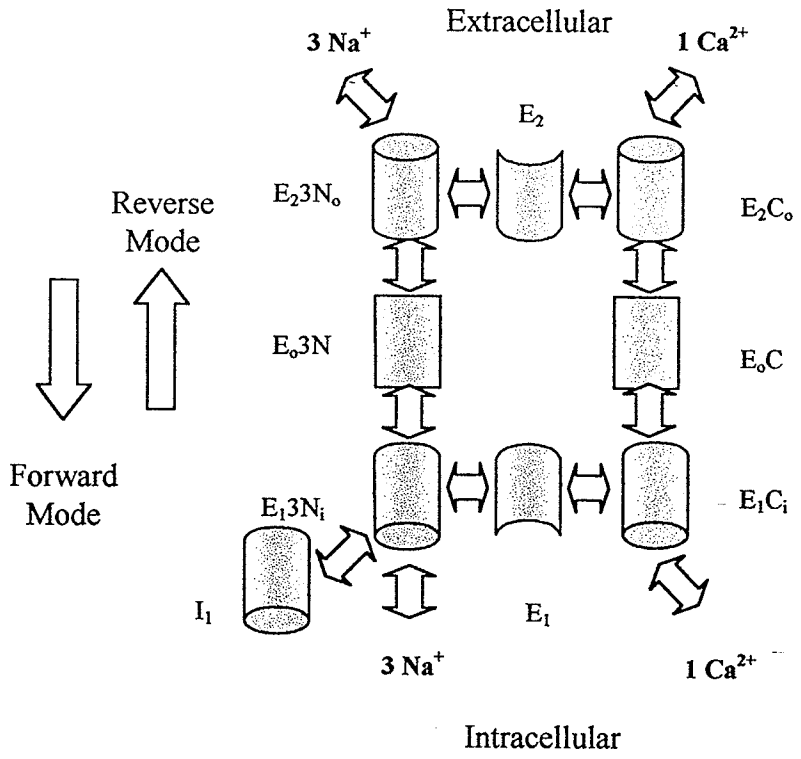

Figure 2. ***The Consecutive Eight-State Model for Na^+-Ca^{2+} Exchange.*** Eight distinct, kinetic entities are modeled in this transport scheme, as well as one inactive state (I_1). E_1 and E_2 represent empty states, E_o3N and E_0C are Na^+ and Ca^{2+} occluded states, E_23N_o and E_2C_o correspond to extracellular Na^+ and Ca^{2+} bound states, while E_13N_i and E_1C_i stand for intracellular Na^+ and Ca^{2+} bound states. Cycling in the clockwise direction represents reverse-mode (*i.e.*, outward) exchange whereas counter-clockwise cycling refers to forward-mode (*i.e.*, inward) exchange.

1.3. Turnover Rate and Exchanger Density

Obtaining transporter turnover rates (*i.e.*, the number of ions transported per exchanger per unit time) for ion channels is relatively easy, because the magnitude of current that passes through one ion channel protein (*i.e.*, its elementary current) is readily detectable using conventional electrophysiological techniques. In comparison, *directly* measuring turnover rates for the Na^+-Ca^{2+} exchanger is not feasible using the same approaches as ion channels because the electrical signal generated by the Na^+-Ca^{2+} exchanger is much smaller ($\approx$ 1000-fold). To make matters worse, there is no direct way to distinguish how many exchangers are contributing to a given Na^+-Ca^{2+} exchange current.

A number of investigators have estimated turnover rates of the Na^+-Ca^{2+} exchanger *indirectly*, such as Cheon and Reeves who took observed ion flux and the calculated density of Na^+-Ca^{2+} exchanger protein in sarcolemmal vesicles, to estimate a turnover rate of $\approx$ 1000 sec^{-1} (92). Hilgemann et al. used charge translocation experiments and an assumed surface area of 25 x 10^3 μm^2 to calculate a model dependent turnover rate of $\approx$ 5000 sec^{-1} (82). Others have reported estimated turnover rates as low as $\approx$ 75 sec^{-1} or more than 2500 sec^{-1} (84). Hilgemann conducted the closest approximation of elementary Na^+-Ca^{2+} exchange current in 1996 (93). In this study, current "noise" associated with exchanger inactivation reactions were analyzed, and a turnover rate $\approx$ 5000 s^{-1} was calculated.

As mentioned earlier, there is no way to determine how many Na^+-Ca^{2+} exchanger proteins contribute to a measured Na^+-Ca^{2+} exchange current since these

currents are generated by an unknown number of exchangers. However, several investigators have estimated Na^+-Ca^{2+} exchange current densities, with ranges obtained recently spanning 250 - 1235 exchangers per μm^2 (84;94). Using the giant, excised patch technique, a density of 300 - 400 exchanger proteins per μm^2 was reported (82;93). Much like the estimates of Na^+-Ca^{2+} exchange turnover rates, estimates of exchanger density also have a large range. This is understandable considering the absence of tools, such as radioligands, available to count exchangers. Precise knowledge of Na^+-Ca^{2+} exchanger turnover rates and density would provide substantial insight into the role of Na^+-Ca^{2+} exchange in physiology and pathophysiology. For example, in the heart, Na^+-Ca^{2+} exchange activity is currently described as either increasing or decreasing, but how this is achieved is completely unknown. Once exchanger turnover rates and densities are known, we could begin to calculate how many exchangers would be required to perform specific roles in physiology, such as Ca^{2+} efflux in the heart, and in turn, this may provide insight into possible recruitment and regulation strategies of Na^+-Ca^{2+} exchange in physiology and pathophysiology.

1.4. Temperature Dependence

Mammalian Na^+-Ca^{2+} exchange activity is markedly reduced at low temperatures, with Q_{10}s $\approx$ 2 – 4, depending on the temperature range used (71;89;95;96). This characteristic reduction in Na^+-Ca^{2+} exchange activity due to decreased temperature is especially evident in rapid cooling contractures of cardiac

muscle (used to evaluate competition between the sarcoplasmic reticulum Ca^{2+}-ATPase and the Na^{+}-Ca^{2+} exchanger) where the low temperature inhibits the removal of Ca^{2+} *via* the exchanger but still allows the release of Ca^{2+} from the sarcoplasmic reticulum, thereby increasing intracellular Ca^{2+} high enough to initiate a contracture (97;98).

Interestingly, and in contrast to the mammalian cardiac Na^{+}-Ca^{2+} exchanger, the rainbow trout cardiac Na^{+}-Ca^{2+} exchanger is much less sensitive to temperature, with a Q_{10} of ≈1.2 (96). The trout Na^{+}-Ca^{2+} exchanger was recently cloned (99) and the canine NCX1.1 and trout NCX1.1 exchange activity was compared at 7, 14, and 30 °C using the giant, excised patch technique (100). Canine NCX1.1 exchange currents were nearly abolished at 7 °C, whereas trout NCX1.1 exchange currents were much less affected. Structure-function studies searching for location of the temperature-sensitivity on the exchanger molecule reveal that replacing the first third of the canine exchanger with trout renders the chimera relatively insensitive to temperature (100).

2. Molecular Biology of the Na^+-Ca^{2+} Exchanger

2.1. The Exchanger Superfamily

The exchanger superfamily is large and continues to grow. Since most of these proteins have been identified based on BLAST (101) searches of the GenBank database, very little is known about these proteins. Therefore, many are only proposed to be membrane transporters, based on sequence homology to proteins we know more about. All of these proteins have Calx-α motifs and span the membrane multiple times, indicated by hydrophobicity plots. Using the above information and ClustalW analysis (102), Philipson and Nicoll divided the exchanger superfamily into four categories: (1) NCX family; (2) NCKX family; (3) Bacterial family; (4) CHX family (6) (see Table 1). Relative to the NCX family, very little is known about the NCKX, Bacterial, and CHX families, hence the latter families will only be mentioned briefly.

Table 1. ***The Exchanger Superfamily.*** The Exchanger Superfamily consists of four families: (1) The Na^+-Ca^{2+} exchanger (NCX) family, (2) the Na^+-Ca^{2+}+K^+ exchanger (NCKX) family, (3) the Bacterial family, and (4) the Ca^{2+}-H^+ exchanger (CHX) family. Listed below are known genes and the species from which the gene originated (Adapted from Philipson and Nicoll (6)).

FAMILY	SPECIES	GENE
NCX	*Canis* sp.	NCX1
	Rattus norvegicus	NCX2
	R. norvegicus	NCX3
	Drosophila melanogaster	Calx
	Loligo opalescens	NCX-SQ1
	Caenorhabditis elegans	CEJ001181
	C. elegans	CENACAEX
	Arabidopsis thaliana	ATAC002535
NCKX	*Bos Taurus*	NCKX1
	Rattus norvegicus	NCKX2
	R. norvegicus	NCKX3
	Caenorhabditis elegans	CEF35C12
	C. elegans	CEC35A5
	Schizosaccharmoyces pombe	SPZC3A12.06c
	Arabidopsis thaliana	AC0001061
	Caenorhabditis elegans	C13D9.7
	C. elegans	C13D9.8
	C. elegans	C07A9.11
	C. elegans	C07A9.4
Bacterial	*Esherichia coli*	YRBG ECOLI
	Treponema pallidum	TP1034
	Synechosystis sp.	
	Aquifex aeolicus	aq 066
	Borrelia burgdorferi	BB0164
	Pyrococcus horikoshii	PH0473
	Methanococcus jannaschii	MJ0091
	M. thermoautotrophicum	MTH1155
	M. thermoautotrophicum	MTH1073
CHX	*Saccharomyces cerevisiae*	VCX1
	Arabidopsis thaliana	AF049236

2.1.1. NCXK Family

The NCKX family comprises exchangers similar to the Na^+-Ca^{2+}+K^+ exchanger, initially cloned from retinal tissue. Currently, there are three mammalian NCKX exchangers, NCKX1 (103), NCKX2 (104), and NCKX3 (105), along with eight others (six from *Caenorhabditis elegans*, one from *Schizosaccharomyces pombe*, and one from *Arabidopsis thaliana*). All share similar sequence identity, including a pair of Calx-α motifs and a large intracellular loop. The mammalian NCKX1 exchanger, which has been cloned and expressed, exchanges four Na^+ for 1 Ca^{2+} and 1 K^+ (106).

2.1.2. Bacterial Family

The bacterial family includes nine members, of which almost nothing is known. All proteins in this family are much smaller than exchangers found in the NCX and NCKX families (*i.e.*, ≈ 400 residues for bacterial exchangers *vs.* > 900 residues for NCX and NCKX exchangers). However, despite their small size (owing to the lack of a large hydrophilic loop), there is extensive sequence similarity beyond the Calx-α motifs throughout the members of this family.

2.1.3. CHX Family

The CHX family contains proteins believed to exchange Ca^{2+} for H^+ (*e.g.*, *Saccharomyces cerevisiae* (107;108). Interestingly, the *Arabisopsis thaliana* protein is much larger than the *S. cerevisiae* protein, containing four Calx-α motifs. Additionally, both *S. cerevisiae* and *A. thaliana* are members of another large family of proteins of which two proteins are known to be Ca^{2+}-H^+ exchangers (109;110).

2.1.4. NCX Family

The last family of exchangers, the NCX family, contains the most extensively studied Na^+-Ca^{2+} exchange proteins. The mammalian exchangers NCX1 (9), NCX2 (10), and NCX3 (11), the *Loligo opalescens* exchanger (NCX-SQ1) (90), and the *Drosophila melanogaster* exchanger (Calx) (152;166), have all been shown to exchange Na^+ for Ca^{2+}, and are similar in sequence and functionality. While two NCX proteins isolated from *Caenorhabditis elegans* are homologous to other NCX proteins, suggesting that they act as Na^+-Ca^{2+} exchangers, the remaining NCX family member, isolated from *Arabidopsis thaliana*, is considerably different in sequence and therefore may differ in function. Since the NCX1 isoform of the Na^+-Ca^{2+} exchanger has received the most intense study, and due to the nature of the research presented in this thesis, the remainder of the literature review will refer to the NCX1 exchanger protein found in the heart, NCX1.1, unless indicated otherwise.

2.2. Gene Structure

NCX1, NCX2, and NCX3 are localized on human chromosomes 2p21-23 (111), 19q13.2 (10), and 14q21-31 (11), respectively. The NCX1 gene itself has also been characterized with respect to its intron-exon organization (112) (Figure 3). While the gene contains 12 exons, the majority of the NCX1 protein is coded into a single exon of 1.8 kb. Additionally, three alternative 5' exons are under control of tissue-specific promoters (113-115). Further investigation into the cardiac-specific promoter reveal a minimum promoter region of ~ 200 base pairs, containing a GATA 4 transcriptional element highly sensitive to mutation (116). Recently, analysis of NCX1 gene expression indicated that the CArG element must be present as well, and that α-adrenergic up-regulation of NCX1 expression (117) is sensitive to mutations of an E-box (118). Up-regulation of Na^{+}-Ca^{2+} exchange expression has also been demonstrated to occur upon treatment with β-adrenergic factors (117), calcineurin (119), and thyroid hormone (120).

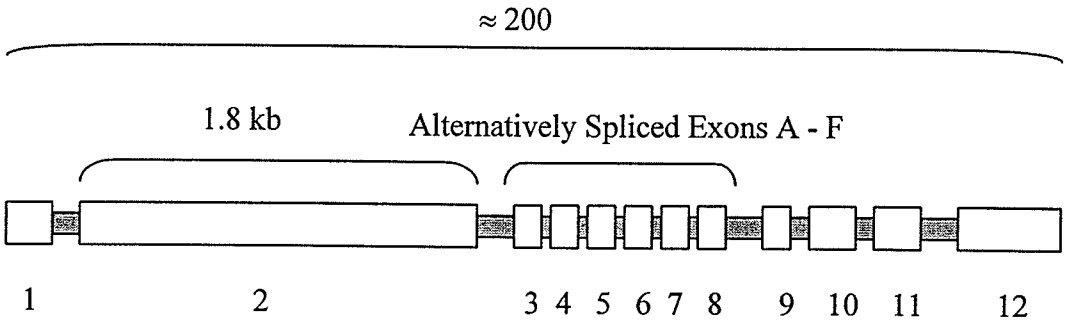

Figure 3. ***Organization of the NCX1 Gene.*** The NCX1 gene is organized into 12 exons, with two-thirds of the NCX1 transcript encoded by exon 2 (illustration not to scale). Exons 3 – 8 are alternatively spliced exons (also known as A – F). Exons 3 and 4 are mutually exclusive (Adapted from Kraev et al. (112)).

2.3. *Topology of the Na^+-Ca^{2+} Exchanger*

NCX1.1 is currently modeled to contain 9 transmembrane segments (TMs) with a large intracellular loop between TMs 5 and 6 (Figure 4). While early work using hydropathy analysis provided the initial topology of 11 TMs (9), more elegant experiments using methods like cysteine substitution mutagenesis have begun to provide a more accurate topology of the exchanger protein (16;121). First, a cysteine-less, but still functional, Na^+-Ca^{2+} exchanger was produced. Then a series of mutants were constructed with cysteines specifically inserted on the proposed membrane surfaces of the exchanger based on the current model. After treatment of the mutant exchangers with sulfydryl agents from the intra- or extracellular side, exchange activity was analyzed. If exchange activity was decreased or eliminated, the accessible cysteine was modeled to be present on the susceptible surface of the membrane. However, if exchange activity was unchanged, the cysteine was probably inaccessible to the particular sulfhydryl agent because it was located inside, or on the opposite side, of the plasma membrane. Other methods, such as epitope mapping (17), have confirmed previous work (14;122) regarding the location of the amino terminus on the extracellular side of the membrane and the carboxy terminus on the intracellular side.

Using cysteine substitution, a number of refinements to the 11 TM model were made (16). First, the old TM 6 was moved into the intracellular loop. Second, TM 9 (which forms part of the second Calx-α motif) does not span the membrane, but rather forms a P-loop-like structure, or reentrant loop, similar to K^+ channels (123).

At its center is a GIG sequence, similar to the GYG sequence in K^+ channel P-loops. Further work investigating specific mutations in the first Calx-α motif suggests that this repeat forms a reentrant loop opposite in orientation to that of the second Calx-α motif (15;16;124). This would suggest that the putative ion translocation pathway of the Na^+-Ca^{2+} exchanger is more similar to aquaporin water channels, which have repeated domains on opposite sides of the membrane (125), rather than K^+ channels, which have repeated domains on the same side of the membrane (123).

Recently, a controversial aspect of Na^+-Ca^{2+} exchanger topology has surfaced in the literature. Two separate groups have demonstrated the moderate activity of "half-exchangers," *i.e.*, the first 5 TMs and nearly all the intracellular loop (126;127). The first report also suggested that dimerization of these half-exchangers may occur (126). A truncated exchanger has also been identified and cloned from the BALB/c mouse heart, but it's only missing 30 amino acids at the C-terminus due to the presence of a premature termination site (128). However, another group was unable to detect Na^+-Ca^{2+} exchange activity after expression of half exchangers (129). The presence of functional and/or dimerized half-exchangers is inconsistent with the current topological model. Specifically, the dimerization of half-exchangers would mean both Calx-α motifs oriented towards the extracellular side of the membrane, which is in contrast to native Na^+-Ca^{2+} exchangers that are modeled to contain oppositely oriented Calx-α motifs.

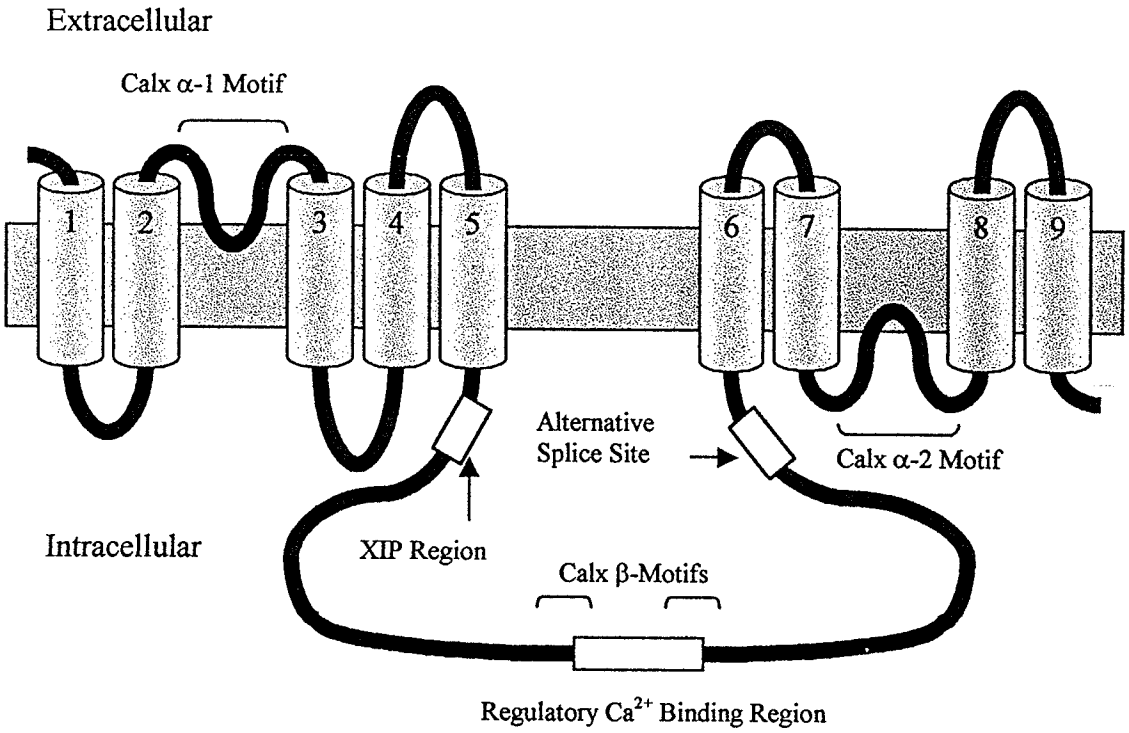

Figure 4. ***Topology of the Na^+-Ca^{2+} Exchanger.*** The Na^+-Ca^{2+} exchanger is currently modeled to span the membrane 9 times, with a large intracellular loop between transmembrane segments (TMs) 5 and 6. There are 2 reentrant loops, called Calx-α motifs, located between TMs 2 and 3, and TMs 7 and 8 (Adapted from Philipson and Nicoll (6)).

Progress has also been made on the three-dimensional structure of the Na^+-Ca^{2+} exchanger (130) (Figure 5). By employing similar techniques to those mentioned above, mutants were created by introducing cysteine pairs to a cysteine-less exchanger. Each cysteine was placed on either the N-terminus half of the exchanger (TMs 1 - 5) or the C-terminus half of the exchanger (TMs 6 - 9). Mutants were expressed in cells and exchanger proteins were analyzed for disulfide bond formation using gel mobility shift assays. It appears that TM 7 is in close proximity to TM 3 on the intracellular side, but is also near TM 2 on the intracellular side. Additionally, TMs 2 and 8 are connected. Since the first and second Calx-α motifs span TMs 2 - 3 and 7 - 8, respectively, this is the first evidence that shows that these two regions are close together, further suggesting the Calx-α motifs form part of the putative ion translocation pathway.

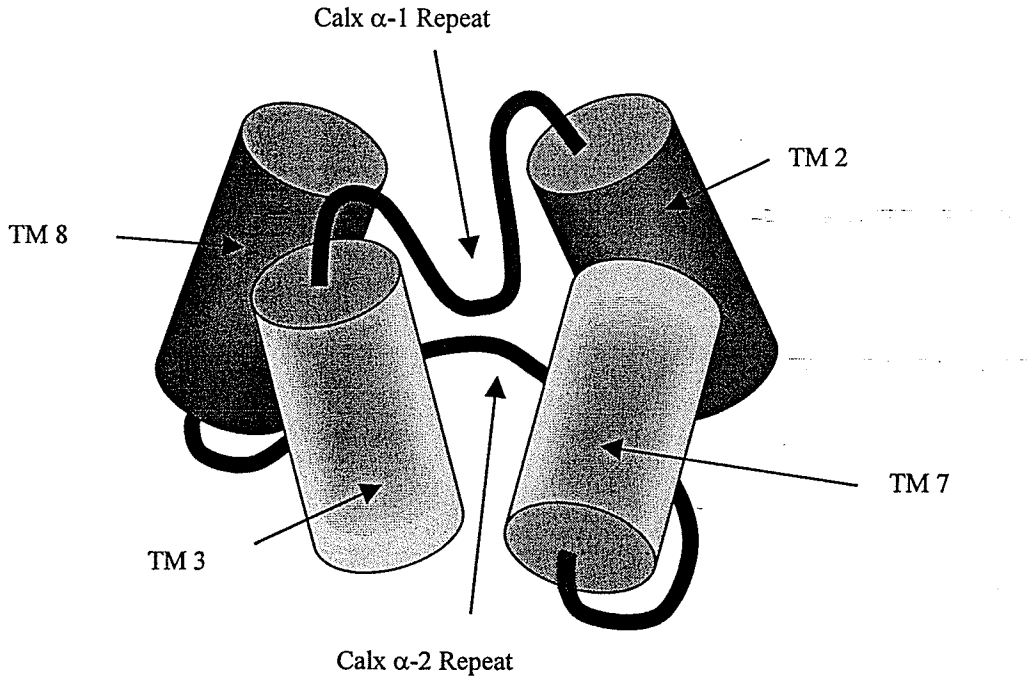

Figure 5. ***Putative 3-Dimensional Arrangement of the Na^{+}-Ca^{2+} Exchanger.*** The 3-dimensional topology of the transmembrane segments (TMs) comprising the Calx α-motifs currently are modeled to be oppositely oriented, possibly forming the ion translocation pathway (Adapted from Qui et al. (130)).

2.3.1. The Calx-α Motifs

Calx-α motifs were first identified in the *Drosophila* Na^+-Ca^{2+} exchanger (20). There are two motifs per exchanger, each comprising of ≈ 40 amino acids, and are conserved in all members of the NCX family as well as in other related cation exchangers (6;20). The first and second Calx-α motifs are sometimes referred to in the literature as the α-1 and α-2 repeats, respectively. As mentioned previously, the Calx-α motifs are in close proximity to one another and oppositely oriented according to current topological models, suggesting that these residues make up part of the ion translocation pathway (Figure 4 and 5).

A number of mutagenesis studies have identified many key residues in the Calx-α motifs, whose mutations significantly alter the transport properties of the Na^+-Ca^{2+} exchanger (Figure 6). One study found that substitution of residues with carboxyl- or hydroxyl-containing amino acids within TMs 2, 3, and 7 (*i.e.*, the Calx-α motifs) either eliminated or significantly decreased $^{45}Ca^{2+}$ uptake in *Xenopus* oocytes expressing these mutants (131). The same study also observed that mutation of two conserved glycines, Gly138 (between TMs 2 - 3) and Gly837 (between TMs 7 - 8), altered the current-voltage relationship of the exchanger.

Mutations in the Calx-α motifs modify the ionic selectivity of the Na^+-Ca^{2+} exchanger. One report demonstrated an increase in affinity for cytoplasmic Na^+ after mutation of Thr103 (TM 2), and even observed Li^+ transport (121). A 6-fold reduction in the apparent affinity for extracellular Ca^{2+} was reported when three conserved aspartic acid residues located in the Calx-α motifs were mutated (Asp130,

Asp825, and Asp829). A 8-fold reduction in inhibition of Na^+-Ca^{2+} exchange current mediated by Ni^{2+} was observed when mutation of Asn125, Thr127, and Val820 occurred (132). Smilarly, mutation of Val820, Gln826, and Gly833 in the second Calx-α motif altered and/or eliminated the apparent affinity of KB-R7943, a normally potent inhibitor of reverse-mode Na^+-Ca^{2+} exchange (21). Lastly, simultaneous mutation of Val820 and Gln826 change the degree of stimulation of Na^+-Ca^{2+} exchange activity by externally applied Li^+ (132). The above studies provide compelling evidence that the Calx-α motifs are integral parts of the ion translocation pathway.

2.3.2. The Intracellular Loop

As noted in the previous section, a large (≈ 550 amino acids), hydrophilic intracellular domain is located between TMs 5 and 6 (Figure 4). The intracellular loop does not seem to be necessary for transport, since a mutant lacking a large portion of this loop (Δ240 - 679) retains exchange activity (18). Further study of this mutant revealed the absence of regulation by intracellular Ca^{2+}, intracellular Na^+ (18), or protein kinase C (PKC) (133), and therefore it is believed the intracellular loop is primarily involved with regulation of Na^+-Ca^{2+} exchange. Treating the intracellular surface of the Na^+-Ca^{2+} exchanger with proteolytic enzymes also renders native Na^+-Ca^{2+} exchangers insensitive to intracellular Ca^{2+} and Na^+ (18), as well as protons (134;135). Although the precise point(s) of cleavage are unknown, it is generally assumed that digestion primarily occurs on the intracellular loop, since exchange

activity responds similarly to the Δ240 - 679 mutant. A number of regions involved in the regulation of the Na^+-Ca^{2+} exchanger located on the intracellular loop have been identified, and are discussed in detail below.

2.3.2.1. eXchange Inhibitory Peptide (XIP) Region

The XIP region, located near the N-terminus of the intracellular loop (219 – 238) (Figure 4), comprises a sequence of 20 basic and hydrophobic residues identified based on its homology to calmodulin-binding domain (9). Mutations and deletions in this region have been shown to accelerate (*e.g.,* F223E) or eliminate (*e.g.,* K229Q) Na^+-dependent inactivation (or I_1) (136). Furthermore, mutations in the XIP region that eliminate Na^+-dependent inactivation also render the Na^+-Ca^{2+} exchange activity insensitive to modulation by ATP, PIP_2, or PIP_2 antibody (137). Additionally, certain XIP mutants no longer react to ATP depletion, which results in inhibition of exchange activity, or to activation of PKC *via* phorbol ester (138). Also, a synthetic peptide with the same sequence as the endogenous XIP region, called XIP, has been shown to completely inactivate reverse-mode Na^+-Ca^{2+} exchange activity when applied to the intracellular surface of giant, excised patches ($K_i \approx 0.1$ μM) (139). Based on these observations, and combined with the fact that this region is highly conserved throughout the NCX1 family (140), it was proposed that the XIP region may function as an autoinhibitory domain. At present, the putative receptor site interacting with the XIP region remains unidentified.

2.3.2.2. Regulatory Ca^{2+} Binding Region

Near the centre of the intracellular loop is the high affinity regulatory Ca^{2+} binding region (amino acids 371 – 508) (Figure 4), which was identified by expressing this region in fusion proteins and assaying $^{45}Ca^{2+}$ binding (141). The regulatory Ca^{2+} binding region contains two conserved clusters of acidic amino acids that when mutated, significantly lower the affinity for regulation by intracellular Ca^{2+}. These data strongly suggest Ca^{2+}-dependent regulation, or I_2, is mediated by this region (142). There is also evidence that the regulatory Ca^{2+} binding region interacts with the Calx-β motifs (discussed below).

2.3.2.3. Calx-β Motifs

In addition to a pair of Calx-α motifs, the Na^+-Ca^{2+} exchanger also contains a pair of Calx-β motifs, spanning ≈ 70 amino acids, on its intracellular loop (Figure 4). The functional significance of these regions is unknown, but they are highly conserved in the NCX family and were, like the Calx-α motifs, first described in the *Drosophila* Na^+-Ca^{2+} exchanger as Calx-β motifs (20). The first Calx-β motif nearly overlaps the N-terminal half of the high affinity Ca^{2+}-regulatory site (discussed above) while the second Calx-β motif is found at the C-terminal side of the Ca^{2+}-regulatory site. Interestingly, Calx-β motifs have been identified in integrin β4 and related proteins (143). Based on a recent study demonstrating that limited proteolysis of the Ca^{2+}-regulatory site only occurs in the absence of Ca^{2+}, it has been suggested

that the Ca^{2+}-regulatory site and the second Calx-β motif may form a folded structure in the presence of Ca^{2+} (144) Conformational changes were also detected when Ca^{2+} binding to fusion proteins containing the Ca^{2+}-regulatory site resulted in large mobility shifts during SDS-PAGE (141).

2.3.2.4. The Alternative Splice Site

A number of splice variants of NCX1 have been identified (12;19;113;145-148). The alternative splice site, a region located near the C-terminus of the large intracellular loop (Figure 4), is encoded by six small exons (designated A through F), of which two are mutually exclusive (A and B). For example, the cardiac isoform, NCX1.1, is comprised of exons ACDEF. Exchangers containing the A exon are generally found in excitable tissues, while other tissues express exchangers containing the B exon (12;19;146;147). Currently, 15 alternatively spliced isoforms of NCX1 have been identified; although it's possible another 17 have yet to be found.

While the presence of tissue-specific alternative splicing suggests that cells express particular types of Na^{+}-Ca^{2+} exchanger proteins based on the specific requirements of the tissue or cell, the physiological significance of this process remains unknown. However, electrophysiological characterization of NCX1.1, NCX1.3 (Kidney splice variant - BD), and NCX1.4 (Brain splice variant - AD) using the giant, excised patch technique demonstrated very distinct patterns of ionic regulation (140). NCX1.4 showed a similar phenotype to NCX1.1. That is, increasing concentrations of intracellular Ca^{2+} alleviated I_1. In contrast, NCX1.3 was insensitive

to intracellular Ca^{2+}. Note that the only difference between the two isoforms is the presence of the A or B exon. Similar differences in ionic regulation as a result of alternative splicing were also found in the two splice variants of the *Drosophilia* Na^{+}-Ca^{2+} exchanger, CALX1.1 and CALX1.2 (149). Consequences of alternative splicing are not limited to changes in ionic regulation, however as Ruknudin et al. demonstrated that NCX1.1 was regulated by protein kinase A (PKA), while NCX1.3 was unaffected (150). How alternative splicing mediates its effects on the regulatory properties of the Na^{+}-Ca^{2+} exchanger remains unknown.

3. Regulation of Na^+-Ca^{2+} Exchange

3.1. Ionic Regulation

3.1.1. Na^+-Dependent Inactivation (I_1)

In addition to transporting Na^+, the Na^+-Ca^{2+} exchanger is also regulated by Na^+. First identified in giant, excised patch experiments, the process of Na^+-dependent inactivation, or I_1, describes the progressive decay of outward (*i.e.*, reverse-mode) Na^+-Ca^{2+} exchange current observed in response to raising the cytoplasmic Na^+ concentration (Figure 6) (81;91;93;95). Similar cytoplasmic Na^+ affinities for peak and steady-state exchange currents imply that I_1 originates from the 3-Na^+-loaded state of the exchanger. Current-voltage relationships measured throughout an outward Na^+-Ca^{2+} exchange current trace were super-imposable once scaled, suggesting that I_1 reduces the number of active exchangers (*i.e.*, exchangers are either active or inactive) (91). Hilgemann and colleagues proposed that the I_1 mechanism, therefore, may act to control the availability of active exchangers (81;91). The physiological significance of this mechanism remains unknown, although I_1 has been identified in intact guinea-pig cardiac myocytes (151). These experiments were performed under very non-physiological conditions, however (*i.e.*, $[Na^+_i] > 30$ mM).

Proteolysis of membrane patches *via* α-chymotrypsin treatment is commonly employed to study I_1-less Na^+-Ca^{2+} exchange activity (95). Presumably, α-

chymotrypsin cleaves unidentified portions of the Na^+-Ca^{2+} exchanger important for I_1, such as the intracellular loop, since absence of the loop results in an exchanger with no I_1 mechanism (18). Mutations in the XIP region, located on the intracellular loop, either alter or eliminate I_1 inactivation, although how the XIP region interacts with the rest of the exchanger molecule to produce these effects is unknown. Further complicating matters is that these mutations also alter Ca^{2+}-dependent regulation (see below), just as mutations in the regulatory Ca^{2+} binding region affect I_1 (142). This indicates that these ionic regulatory mechanisms interact with one another (136), frustrating efforts to isolate a particular form of ionic regulation. Additionally, the I_1 mechanism is enhanced at low pH (91;134), and alleviated by regulatory Ca^{2+}, ATP, and PIP_2, (discussed below) (90;152-154).

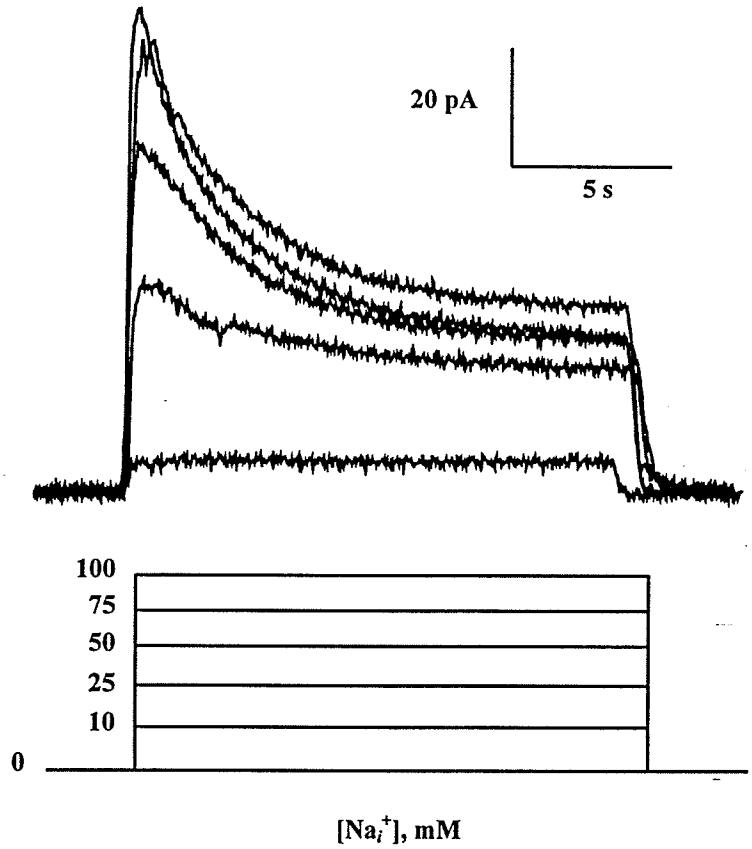

Figure 6. ***Na$^+$-Dependent Inactivation (I_1).*** The above figure shows a typical outward (*i.e.*, reverse-mode) Na^+-Ca^{2+} exchange current trace obtained using the giant, excised patch technique. Current decay (*i.e.*, inactivation) increases as the cytoplasmic Na^+ concentration is raised.

3.1.2. Ca^{2+}-Dependent Regulation (I_2)

Intracellular Ca^{2+}, or regulatory Ca^{2+}, also regulates the Na^+-Ca^{2+} exchanger. Contrary to cytoplasmic Na^+, which inactivates Na^+-Ca^{2+} exchange currents, regulatory Ca^{2+} alleviates I_1-inactivation and stimulates Na^+-Ca^{2+} exchange current. This process was first observed in squid giant axons, which described the stimulatory effect of non-transported, cytoplasmic Ca^{2+} on Na^+-Ca^{2+} exchange (155). Subsequently, this mechanism has been characterized in both native cells and in a variety of cell types expressing the cloned cardiac exchanger, using many different approaches (60;71;78;142;156-160). Giant, excised patch experiments have also been used to study Ca^{2+}-dependent regulation, or I_2, in detail (95;142;152). Note that like I_1 inactivation, α-chymotrypsin treatment eliminates I_2 regulation (18), and mutations in the regulatory Ca^{2+} binding region markedly reduce cytoplasmic Ca^{2+} affinities (142). Figure 7 depicts a typical outward Na^+-Ca^{2+} exchange current trace showing the stimulatory effect of applying increasing amounts Ca^{2+} to the cytoplasmic side of a patch in the presence of Na^+. At higher concentrations of Ca^{2+}_i (*i.e.*, > 10 μM), Na^+-Ca^{2+} exchange activity decreases, since regulatory Ca^{2+} begins to compete with transported Na^+ at these concetrations.

While there is general agreement that intracellular Ca^{2+} activates Na^+-Ca^{2+} exchange activity, there is considerable discrepancy regarding apparent regulatory Ca^{2+} affinities among different experimental conditions, with $K_{1/2}$'s ranging from 0.022 - 0.4 μM (136;142;152;157;159;161). These disparities among apparent regulatory Ca^{2+} affinities hinder the elucidation of the physiological significance of I_2

regulation. That is, a low apparent affinity would mean that this mechanism would be saturated, even at diastolic Ca^{2+} levels, whereas a higher apparent affinity would suggest a physiological role for I_2 in modulating Na^{+}-Ca^{2+} exchange activity, assuming intracellular Ca^{2+} oscillates between $\approx$ 0.1 and $\approx$ 1.0 μM (162). A recent study conducted by Weber et al. supports a physiological role for I_2 in intact native ferret cardiomyocytes, but not in intact mouse cardiomyocytes, suggesting that ultimately, the significance of I_2 may be species specific (163).

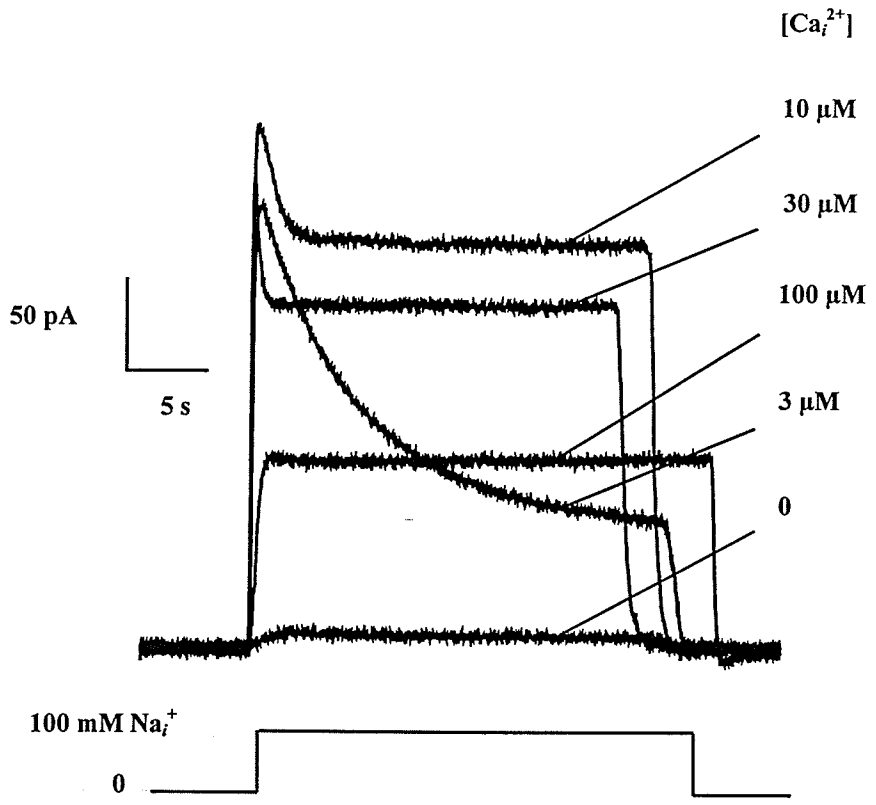

Figure 7. ***Ca^{2+}-Dependent Regulation (I_2).*** Illustrated above is a typical outward (*i.e.*, reverse-mode) Na^+-Ca^{2+} exchange current trace obtained from giant, excised patch experiments. Increasing cytoplasmic Ca^{2+}, or regulatory Ca^{2+}, stimulates Na^+-Ca^{2+} exchange current, and alleviates I_1 inactivation. At concentrations > 10 μM, regulatory Ca^{2+}, Na^+-Ca^{2+} exchange activity decreases presumably due to competition between regulatory Ca^{2+} and cytoplasmic Na^+.

3.2. pH

The Na^+-Ca^{2+} exchanger has been shown to be sensitive to changes in pH in cardiac sarcolemmal vesicles (164;165), and giant patch experiments (91;134;135;166). The giant, excised patch experiments demonstrated that under normal physiological pH ($\approx$ 7.2), Na^+-Ca^{2+} exchange activity was approximately half-maximally activated (135). Alkalinization of the bath solution increased Na^+-Ca^{2+} exchange activity, whereas decreasing the pH inhibited exchanger activity. Furthermore, inhibition of exchange activity at acidic pH levels was shown to consist of two components (134). The first, a slowly developing inhibition, is dependent on the presence of cytoplasmic Na^+, possibly involving I_1, whereas the second component, a rapid inhibition did not depend on the presence of Na^+. Treatment of membrane patches with α-chymotrypsin (used to remove ionic regulatory influences in Na^+-Ca^{2+} exchange activity) increased exchanger activity at pH < 7.2, essentially negating the inhibitory effect of acidic pH, further suggesting the interaction of protons with the intracellular loop, and therefore possibly I_1 and I_2 (135).

3.3. Phosphorylation

3.3.1. Protein Kinase C (PKC) and Protein Kinase A (PKA)

Phosphorylation of the squid giant axon Na^+-Ca^{2+} exchanger has been shown to stimulate Na^+-Ca^{2+} exchange activity (reviewed in (22)). DiPolo and Beaugé

reported that phosphorylation of the exchanger by a Ca^{2+}-dependent kinase reaction resulted in an increased affinity for intracellular Ca^{2+} and extracellular Na^{+}, thereby stimulating exchange (167). Furthermore, a 13 kDa cytosolic protein, isolated from the axoplasm and brain of the squid was reported to be involved in ATP-dependent regulation of the squid exchanger (168). More recently, the squid giant axon Na^{+}-Ca^{2+} exchanger, NCX-SQ1, has been cloned and a putative protein kinase C phosphorylation site was identified that is unique among exchangers (90). Compared to the squid exchanger, however, there is a lack of a consensus regarding studies investigating phosphorylation of other mammalian Na^{+}-Ca^{2+} exchangers.

A number of phospholipase C-activating agonists, such as phenylephrine, endothelin 1, angiotensin II and certain growth factors, are known to stimulate Na^{+}-Ca^{2+} exchange activity in native cardiomyocytes, cells transfected with the cardiac exchanger, and cardiac sarcolemmal vesicles (133;169-171). Note that selective inhibitors of PKC block the stimulatory effects of these agonists. Phorbol esters (*e.g.*, phorbol 12-myristate 13-acetate, or PMA) that activate PKC or phosphatase inhibitors (*e.g.*, okadaic acid) have also been used to mimic these agonists (59;60;169), implicating these agonists in PKC mediated activation of Na^{+}-Ca^{2+} exchange activity.

The first direct evidence of PKC mediated phosphorylation of mammalian Na^{+}-Ca^{2+} exchangers came from studies conducted by the Shigekawa group in aortic smooth muscle (77) and cardiac tissue (169). In both studies, Na^{+}-Ca^{2+} exchange activity was up-regulated in response to PKC-dependent phosphorylation. Further analysis would reveal multiple phosphorylation sites (serine residues). When these sites were replaced by alanine in a later study (133), the exchanger still exhibited

responsiveness to PMA, suggesting that direct phosphorylation of the exchanger was not required for PKC-dependent activation of the exchanger. However, removal of the intracellular loop eliminated PKC-dependent regulation for both NCX1 and NCX3, and in certain XIP mutants (138).

Contrary to the previous mentioned studies, Collins et al. found no evidence of protein kinase involvement in the regulation of mammalian cardiac Na^+-Ca^{2+} exchangers by ATP, using the giant, excised patch technique (172). In this study, outward Na^+-Ca^{2+} exchange currents were examined in the presence of a large number of kinase inhibitors, phosphatases, and phosphatase inhibitors. More recently, Condrescu et al. reported inhibition of outward Na^+-Ca^{2+} exchange current *via* phosphatase inhibitors calyculin A and okadaic acid, whereas inward exchange activity was relatively unaffected in cells expressing the bovine cardiac Na^+-Ca^{2+} exchanger (173). Furthermore, the preferential inhibitory effect on Na^+-Ca^{2+} exchange activity mediated by these phosphatase inhibitors was conserved in a mutant with the majority of the intracellular loop missing, and the authors concluded that direct phosphorylation of the exchanger was unlikely, but that phosphorylation by kinases other than PKC may be possible.

PKA stimulation of the Na^+-Ca^{2+} exchanger has also been demonstrated. Interestingly, the effect of PKA on Na^+-Ca^{2+} exchange varies among alternatively spliced isoforms of NCX1. For example, NCX1.4 was stimulated 40% upon PKA activation, whereas no stimulation was observed for NCX1.3 (174). PKA-dependent regulation of the frog Na^+-Ca^{2+} exchanger was reported, however, the effect is inhibitory rather than stimulatory (175;176). More detailed examination of the frog

exchanger revealed a unique 9 amino acid exon, not present in mammalian exchangers. Removal of this exon eliminated PKA-mediated inhibition of Na^+-Ca^{2+} exchange activity.

Some of the discrepancies among studies investigating PKC or PKA-mediated phosphorylation of Na^+-Ca^{2+} exchange may be explained by differences in species, isoforms, and experimental protocols. The myriad of proteins in these systems affected by PKC and PKA may also add to the complexity of the situation. Finally, prolonged PKC and PKA activation are known to decrease exchanger expression levels (177;178).

3.3.2. Phosphatidylinositol 4,5-bisphosphate (PIP_2)

Giant, excised patch experiments have shown millimolar levels of ATP to stimulate Na^+-Ca^{2+} exchange activity (90;152;172). Hilgemann and Ball showed that this stimulatory response was due to ATP-induced production of the signaling lipid phosphatidylinositol 4,5-bisphosphate (PIP_2) from phosphatidylinositol (PI) (153). In their experiments, cytoplasmic application of PIP_2 duplicated the stimulatory effect of ATP, and was eliminated upon treatment of a PI-specific phospholipase C. Furthermore, the stimulatory effect could be restored upon PI application to the membrane. A PIP_2-specific phospholipase and an anti-PIP_2-antibody also reversed the stimulatory effects of ATP, as did aluminum, which binds to PIP_2 with high affinity (90). Stimulation of exchange current by ATP occurs through the elimination of I_1 (152).

Therefore, a subsequent study investigated the possible interaction between PIP_2 and the XIP region of the Na^+-Ca^{2+} exchanger, in which mutations of this region alter I_1 characteristics (137). Iodinated exogenous XIP peptide was shown to bind with high affinity to immobilized phospholipid vesicles containing low PIP_2 concentrations, implicating a possible interaction between the highly basic endogenous XIP region and relatively acidic PIP_2. Further indirect evidence that PIP_2 binds the Na^+-Ca^{2+} exchanger is based on experiments examining mutations made in the endogenous XIP region of the exchanger. In each case that XIP mutations showed altered I_1 properties, the functional response of the Na^+-Ca^{2+} exchanger to PIP_2 was also eliminated (137).

3.4 Other Modulators of Na^+-Ca^{2+} Exchange Activity

In addition to PIP_2, other studies have shown Na^+-Ca^{2+} exchange activity to be sensitive to the lipid environment (179-186). Specifically, Na^+-Ca^{2+} exchange activity is sensitive to the presence of negatively charged phospholipids. In studies that incorporate negatively charged amino acids (such as phosphatidylserine) into cardiac sarcolemmal vesicles, an increase in exchange activity was observed (179;183;184). Treatment of cardiac sarcolemmal vesicles with phospholipase C (185) and D (186) (thereby increasing the relative composition of negatively charged phospholipids present in the plasma membrane) also stimulated Na^+-Ca^{2+} exchange activity. While the mechanism by which negatively charged phospholipids stimulate Na^+-Ca^{2+} exchange activity is unknown, direct application of phosphatidylserine to deregulated

(*i.e.*, α-chymotrypsin treated) giant, excised cardiac sarcolemmal patches augmented Na^+-Ca^{2+} exchange current, suggesting that phosphatidylserine may interact with the exchange mechanism (180). However, prolonged treatment of patches with phosphatidylserine eliminated I_1, so interaction with the ionic regulatory mechanisms and phosphatidylserine is also possible (180). In addition to lipids, Na^+-Ca^{2+} exchange activity is also sensitive to cholesterol and fatty acid content. Increasing the cholesterol content in reconstituted proteoliposomes (187) and cardiac sarcolemal vesicles increases Na^+-Ca^{2+} exchange activity (188). A variety of fatty acids have also been shown to stimulate Na^+-Ca^{2+} exchange activity in cardiac sarcolemmal vesicles (183).

4. Pharmacology of Na^+-Ca^{2+} Exchange

Electrophysiologists must be able to isolate the current generated by a particular transporter they are interested in from other ionic currents. The use of highly selective and potent pharmacological blockers is a standard method for dissemination of function, such as tetrodotoxin (TTX) for the voltage-dependent Na^+ channel. While there are no highly specific pharmacological modulators of Na^+-Ca^{2+} exchange currently available, a variety of relatively non-specific "blockers" have been identified that have inhibitory properties. These agents fall under three categories: 1) Tri- and Divalent Cations; 2) Peptides; and 3) Organic Inhibitors (for recent reviews see (5))(7;8;22).

4.1. Tri- and Divalent Cations

A number of tri- and divalent cations have been used (e.g., La^{3+} and Ni^{2+}) and continue to be used to block Na^+-Ca^{2+} exchange activity. Unfortunately, these cations either interfere with other transporters (*e.g.*, La^{3+}; (189;190)), act as substrates for the exchanger (*e.g.*, Ba^{2+} and Sr^{2+}; (71;157;191;192)), or are relatively weak inhibitors (*e.g.*, Mg^{2+}; (193), but also see (194;195)), and are therefore considered relatively non-specific. Consequently, care must be taken when employing any of these cations, and other steps are usually required in order to overcome these limitations, especially since many of these cations (*i.e.*, La^{3+}, Cd^{2+}, Co^{2+}, Mn^{2+}, Ni^{2+}) must be used in the low millimolar range for ≈100% inhibition of Na^+-Ca^{2+} exchange current (71).

Ni^{2+} is widely utilized as a Na^+-Ca^{2+} exchange blocker. (*e.g.*, (71;73;84)). However, to be effective, Ni^{2+} concentrations must be in the 2 - 5 mM range, and this necessitates the suppression of other membrane currents before Na^+-Ca^{2+} exchange current can reliably be isolated (*i.e.*, the "Ni^{2+}-sensitive current"). Interestingly, while La^{3+} has been employed as a Na^+-Ca^{2+} blocker in the past (2;71;196;197), recently it has been used to *isolate* Na^+-Ca^{2+} exchange current (194;198) since its affinity for other Ca^{2+} transporters is much higher (189;190).

It is also worthy to mention that these cations are believed to mediate their effects by interacting at different binding sites of the exchanger and/or compete with the transport of Na^+ and/or Ca^{2+}. However, very little is known about how this achieved. A case in point would be recent experiments conducted in cells expressing the human cardiac Na^+-Ca^{2+} exchanger: Although Ni^{2+} was found to completely inhibit Na^+-Ca^{2+} activity, it was at the same time found to be rapidly transported by the exchanger with a supposed electroneutral stoichiometry (199;200). As mentioned earlier, not all cations are used as non-specific inhibitors. For example, cations like Sr^{2+} and Ba^{2+}, have also been employed in studies investigating the transport mechanism of Na^+-Ca^{2+} exchange since they can act as substrates (*i.e.*, as a substitute for Ca^{2+}) for the exchanger, although transport rates are generally reduced (71;85;157;191;192). It is interesting to note that externally applied monovalent cations other than Na^+, specifically Li^+ and K^+, can actually stimulate Na^+-Ca^{2+} exchange activity in certain tissues (85;154;193;201-204).

4.2. Peptides

4.2.1. The eXchange Inhibitory Peptide (XIP)

Shortly after the Philipson group cloned the cardiac Na^+-Ca^{2+} exchanger (9), they identified a portion of the intracellular loop interspersed with hydrophobic residues similar to a calmodulin-binding domain. Furthermore, the endogenous XIP sequence is highly conserved among Na^+-Ca^{2+} exchanger isoforms (*e.g.*, NCX1, NCX2, and NCX3) (60). To investigate whether this domain might be autoinhibitory, a peptide (XIP) based on the endogenous XIP sequence was synthesized and applied to the intracellular side of the membrane. XIP inhibited Na^+-Ca^{2+} exchange activity in cardiac sarcolemmal vesicles (139), as well as in a number of other preparations (10;29;205-208), with a relatively high potency (IC_{50} ranges from 0.15 - 1.5 μM).

Despite its relatively high potency and specificity relative to inhibitory cations, XIP has seen limited use in Na^+-Ca^{2+} exchange research for several reasons. Since XIP is based on a calmodulin binding sequence, it can be expected to interfere with the actions of endogenous calmodulin in intact cells. Furthermore, XIP has been shown to cross-react with other calmodulin binding proteins like the sarcolemmal Ca^{2+}-ATPase (209;210). Additionally, XIP can only inhibit Na^+-Ca^{2+} exchange when applied to the cytoplasmic side of the exchanger (139). Therefore, in intact cell preparations, XIP must be internally dialyzed, before it can be used as a Na^+-Ca^{2+} exchange inhibitor.

4.2.2. Other Peptides

Based on earlier work examining the effects of opiate agonists and antagonists on Na^+-Ca^{2+} exchange (211), a number of peptides have been identified with similar potency and specificity to XIP. FMRF-amide, the molluscan cardioexcitatory peptide (and analogues), known to bind to opiate binding sequences, was subsequently shown to inhibit Na^+-Ca^{2+} exchange in sarcolemmal vesicles (211;212) (IC_{50}'s ranged from 1-1000 μM), squid giant axons (213), and pancreatic β-cells (214). Additionally, a number of positively charged cyclic hexapeptides, such as FRCRCF-amide and analogues, have also exhibit inhibitory effects on Na^+-Ca^{2+} exchange (215;216) with IC_{50}s ranging from 0.023 - 10 μM. However, another study found greatly reduced potency of FRCRCF-amide in NCX1, NCX2, and NCX3 (60) (*i.e.*, at 50 μM FRCRCF-amide resulted in only 32 - 41% inhibition *vs.* a K_D of 22.7 nM in (216)). However as in the case of XIP, these peptides must be applied to the intracellular surface in order for inhibition of Na^+-Ca^{2+} exchange to occur. One notable exception is myristyl-FRCRCF-amide, a cell permeant analogue of FRCRCF-amide. Myristyl-FRCRCF-amide was recently demonstrated to inhibit Na^+-Ca^{2+} exchange in rabbit ventricular myocytes (217), although at a much reduced potency.

4.3. Organic Inhibitors

4.3.1. Amiloride Derivatives

Amiloride and it's derivatives, such as Benzamil and DCB (3',4'-dichlorobenzamil), have also been used to inhibit Na^+-Ca^{2+} exchange activity. The IC_{50}'s for these compounds are variable, ranging from 1 mM for amiloride, to 100 μM for Benzamil in rat anterior pituitary cells (218), to 17 and 36 μM in guinea pig (219) and bovine sarcolemmal vesicles (220), respectively. Unfortunately, many of these amiloride analogues also block other membrane transporters, often at similar concentrations (*e.g.*, L-type Ca^{2+} channels; (221)). Therefore, interpretation of studies employing these compounds must be done cautiously. However, much like studies utilizing Ni^{2+}, a number of investigators have successfully used DCB to isolate Na^+-Ca^{2+} exchange currents after blocking other ion channels (84;222).

The mechanism of inhibition of Na^+-Ca^{2+} exchange by amiloride analogues is probably complex. For example, DCB has been shown to block both Na^+-dependent Ca^{2+} uptake and Na^+-dependent Ca^{2+} efflux, and competitively inhibits Na^+-Na^+ exchange, while Ca^{2+}-Ca^{2+} exchange was still active at physiological K^+ concentrations (220). Based on these results, the authors suggested that at low concentrations, amiloride analogues preferentially inhibit a binding site exclusive for Na^+, while at higher concentrations, they affect a site common for Na^+, Ca^{2+}, and K^+. Moreover, studies have shown DCB to preferentially inhibit forward-mode Na^+-Ca^{2+} exchange (54;57).

4.3.2. Isothiourea Derivatives: KB-R7943

KB-R7943 (2-[2-[4-(4-nitrobenzyloxy)-phenyl]ethyl]isothiourea methesulfonate), formerly No. 7943 (Figure 8) was first introduced in 1996 by two different groups (56;57). Both groups observed preferential inhibition of reverse-mode Na^+-Ca^{2+} exchange (*i.e.*, Ca^{2+} influx) by KB-R7943 in guinea pig ventricular myocytes (56) as well as rat cardiomyocytes and other cells expressing the cardiac Na^+-Ca^{2+} exchanger, NCX1.1 (57), with IC_{50}'s ranging from 0.3 - 2.4 μM for reverse-mode exchange *vs.* 17 - 30 μM for forward-mode exchange. Under bi-directional conditions, however, KB-R7943 was shown to inhibit both modes equally in guinea pig ventricular myocytes (58).

Some studies have investigated the inhibitory effects of KB-R7943 on other Na^+-Ca^{2+} exchanger isoforms. Shigekawa and colleagues reported a 3-fold higher inhibition of Na^+-dependent $^{45}Ca^{2+}$ uptake in NCX3 transfected fibroblasts in the presence of KB-R7943 *vs.* cells transfected with NCX1 and NCX2 (59), IC_{50}'s ranging from 1.5 - 4.9 μM. In contrast, however, Philipson's group found very little difference in the inhibition of Na^+-dependent $^{45}Ca^{2+}$ uptake (30 - 45% inhibition at 10 μM KB-R7943) in membrane vesicles obtained from cells expressing the same isoforms (60). Furthermore, KB-R7943's inhibitory potency was far less than previous reports. In giant excised patches obtained from *Xenopus laevis* oocytes expressing the *Drosophila* Na^+-Ca^{2+} exchanger, CALX1.1, preferential inhibition of reverse-mode exchange was conserved, albeit at a reduced potency (223). With regard

to alternatively spliced isoforms, preliminary evidence from our laboratory suggests that KB-R7943 inhibits the brain (NCX1.4) and kidney (NCX1.3) isoforms with a similar potency (unpublished data).

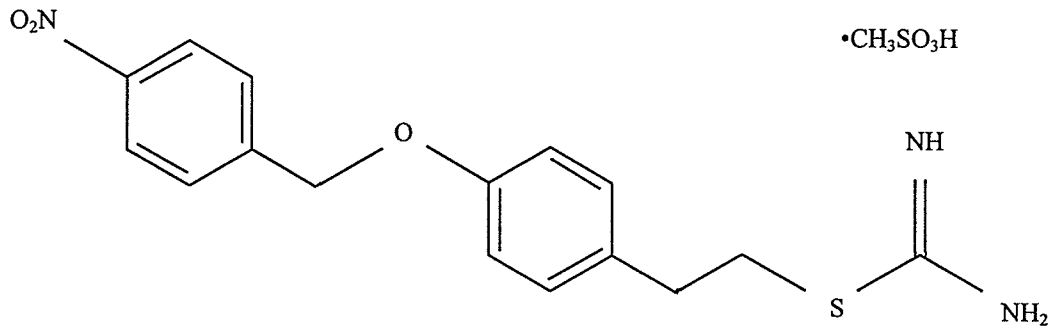

Figure 8. ***Chemical Structure of KB-R7943 (No. 7943),***

(2-[2-[4-(4-nitrobenzyloxy)phenyl]ethlyl]isothiourea methanesulphonate).

The mechanism(s) of KB-R7943's action is unknown. While one of the original reports found KB-R7943 to be non-competitive with respect to Na^+ and Ca^{2+}, the other study found it to be competitive with extracellular Ca^{2+} (59). This was demonstrated again in yet another study by the same group (224). It has also been suggested that KB-R7943 and extracellular Ca^{2+} involve mixed-type competition (*i.e.*, KB-R7943 is both competitive and non-competitive with extracellular Ca^{2+}) (59). Although it is certainly feasible that KB-R7943 may mediate some of its effects *via* competition with extracellular Ca^{2+}, evidence presented in this thesis, combined with results obtained in other studies that observed preferential inhibition of reverse-mode Na^+-Ca^{2+} exchange (57), (62)contradict this possibility.

The location of a KB-R7943 binding site has not been confirmed. However, mutational analysis of the second Calx-α motif of the cardiac Na^+-Ca^{2+} exchanger (*i.e.*, the putative reentrant membrane loop) demonstrated up to a ≥ 30-fold reduction in KB-R7943 potency for mutations at Val820, Gln826, and especially Gly833 (21), suggesting that this region may be part of the KB-R7943 receptor. Since KB-R7943 has been shown to inhibit reverse-mode Na^+-Ca^{2+} exchange when administered externally, (21;56), a simple explanation would be that this region may indeed form part of the KB-R7943 receptor. However, this does not account for evidence demonstrating that KB-R7943 can mediate its effects when applied to the cytoplasmic side of inside-out giant patches excised from *Xenopus laevis* oocytes expressing NCX1.1 (225) or when applied intracellularly in one of the original reports (57). In contrast, application of KB-R7943 to the inside of intact cells using pipettes did not result in inhibition of Na^+-Ca^{2+} exchange current (21).

KB-R7943 does not affect the Na^+-Ca^{2+}+K^+ exchanger (21), the Na^+-H^+ exchanger, sarcolemmal Ca^{2+}-ATPase, sarcoplasmic reticulum Ca^{2+}-ATPase, or the Na^+-K^+-ATPase at concentrations up to 30 μM (57). However, it was reported that KB-R7943 inhibits voltage-sensitive Na^+ currents, L-type Ca^{2+} channels, and inward rectifier K^+ currents in guinea pig myocytes with IC_{50}'s of 14, 8 and ≈ 7 μM, respectively (56). Another study demonstrated that 5 μM KB-R7943 did not alter steady-state twitches, Ca^{2+} transients, Ca^{2+} load in the sarcoplasmic reticulum, or rest potentiation, but did prolong the late low plateau of the action potential, indicating a moderate inhibition of K^+ currents (62). However, other studies report that KB-R7943, up to 10 μM, did not alter resting membrane potential or other action potential characteristics (57;226). Additionally, in isolated guinea pig atria, 10 and 30 μM KB-R7943 did not affect spontaneous beating rate and developed tension (227). Despite these discrepancies, it is probably safe to say that KB-R7943 can be employed as a relatively specific inhibitor of reverse-mode Na^+-Ca^{2+} exchange at concentrations < 10 μM in the heart.

As noted briefly above, in addition to a variety of intact cell, vesicle, and patch clamp studies, KB-R7943 has been utilized in a variety of physiological and pathophysiological animal heart models. The contribution KB-R7943 has made to physiological and pathophysiologcial models in the heart will be discussed later. However, it is worthwhile to briefly mention the effects KB-R7943 has in other systems. For example, KB-R7943 has shown to be protective in rats with ischemic acute renal failure when treated before or after a period of ischemia (61). Additionally, KB-R7943 protects CA1 neurons in rat hippocampal slices against

hypoxic/hypoglycemic injury (63). KB-R7943 has also been used to examine Na^+-Ca^{2+} exchange in other tissues, such the liver. Ikari et al., demonstrated that PKC mediates the up-regulation of Na^+-Ca^{2+} exchange in rat hepatocytes (228). Hoyt et al. reported that reverse Na^+-Ca^{2+} exchange contributes to glutamate-induced Ca^{2+} increases in cultured rat forebrain neurons (229).

4.3.3. Other Organic Molecules

Several organic molecules, including some antiarrhythmic agents (*e.g.*, quinacrine (221) and bepridil (230)), local (*e.g.*, tetracaine (231)) and general anesthetics (*e.g.*, halothane (232)), as well as other Ca^{2+} channel blockers (*e.g.*, verapamil (233)), have been demonstrated to inhibit Na^+-Ca^{2+} exchange. Ascorbic acid (234;235), chlorpromazine (236), quinidine (237), neomycin (238), polymyxin B (239), and harmaline (240) have also been described as weak inhibitors of Na^+-Ca^{2+} exchange. These compounds primarily exert their effects on other molecules, however, at concentrations much lower than required to be effective inhibitors of Na^+-Ca^{2+} exchange.

A novel ethoxyaniline derivative, SEA0600 (2-4[-[(2,5-difluorophenyl)methoxy]phenoxy]-5-ethoxyaniline), was recently introduced in a report comparing the inhibitory profiles of SEA0600 and KB-R7943 on Na^+-dependent Ca^{2+} uptake *via* Na^+-Ca^{2+} exchange in cultured rat neurons, astrocytes, and microglia (241). SEA0600 was reported to have an IC_{50} ranging from 5 - 33 nM *vs.* 2 - 4 μM for KB-R7943. Furthermore, these investigators found negligible affinities for

SEA0600 on Ca^{2+}-, Na^{+}-, and K^{+}-channels, the norepinephrine transporter, as well as 14 other receptors. SEA0600 had no effect on the Na^{+}-H^{+} exchanger, Na^{+}-K^{+}-ATPase, Ca^{2+}-ATPase, and 5 other enzymes. Finally, SEA0600 protected the brain from damage in their postischemic model. While the use of SEA0600 is promising, a thorough examination of its inhibitory effects will be required.

5. Physiological Role of the Cardiac Na^+-Ca^{2+} Exchanger

5.1. Forward-Mode Na^+-Ca^{2+} Exchange

During a cardiac action potential, Ca^{2+} is brought into the myocyte *via* L-type Ca^{2+} channels. This Ca^{2+} triggers the release of even more Ca^{2+} from the sarcoplasmic reticulum (SR) *via* ryanodine receptors (*i.e.*, Ca^{2+}-induced Ca^{2+}-release, or CICR; (242)), raising cytosolic Ca^{2+} levels high enough to activate the myofilaments, thereby initiating a contraction (243-246). In order to facilitate relaxation and diastolic filling, cytosolic [Ca^{2+}] must be reduced. There are four major transporters capable of reducing intracellular Ca^{2+} in the heart: (1) SR Ca^{2+}-ATPase, (2) sarcolemmal (SL) Na^+-Ca^{2+} exchanger, (3) SL Ca^{2+}-ATPase, and (4) mitochondrial Ca^{2+} uniporter (Figure 9). Of these, it is generally accepted that the two most important transporters for reducing cytosolic Ca^{2+} are the SR Ca^{2+}-ATPase, which brings Ca^{2+} back into the SR, and the Na^+-Ca^{2+} exchanger (operating in its forward-mode (*i.e.*, Ca^{2+} efflux)), which transports Ca^{2+} out of the cardiomyocyte (reviewed in (6;7;22;23;162;247-249)).

A number of studies have shown Na^+-Ca^{2+} exchange to be a major Ca^{2+} efflux mechanism in the heart. Bridge et al. (75) published a classic demonstration of the Na^+-Ca^{2+} exchanger's role in transsarcolemmal efflux. In this study, the integration of charge carried inward by L-type Ca^{2+} channel current was double that of the integration of charge transported outward by the Na^+-Ca^{2+} exchanger. Assuming a 3:1 stoichiometry for Na^+-Ca^{2+} exchange, their results indicated the Na^+-Ca^{2+} exchanger

removes the same amount of Ca^{2+} brought into the myocyte via L-type Ca^{2+} channels. Niggli and Lederer presented similar results when comparing the current amplitudes generated *via* Na^{+}-Ca^{2+} exchange and L-type Ca^{2+} channels in flash photolysis of "caged" Ca^{2+} experiments (250). Using indo-1 (Ca^{2+} fluorescence indicator) loaded guinea pig myocytes, Crespo et al. used voltage clamp protocols to demonstrate that ≈ 15% of the rate of the declining Ca^{2+}_{i} transient was due to the Na^{+}-Ca^{2+} exchanger, with the rest due to re-uptake *via* the SR Ca^{2+}-ATPase (3). Other studies, including caffeine-evoked contractions (251) and pharmacological and ion-substitution experiments using rapid cooling contractions (used to assess SR Ca^{2+} content and the contribution of other Ca^{2+} transporters relative to the SR) (97;98;252), also demonstrated a major role for forward-mode Na^{+}-Ca^{2+} exchange in facilitating relaxation. Furthermore, contribution of the SL Ca^{2+}-ATPase to Ca^{2+} efflux was not significant in these studies.

Currently, however, the precise quantitative relationship of the Na^{+}-Ca^{2+} exchanger in this role is being re-evaluated. Specifically, there is increasing evidence that the SL Ca^{2+}-ATPase plays a bigger role than previously described (24;25;253). For example, in rat ventricular myocytes, Choi and Eisner (24) demonstrated that, in the absence of SR Ca^{2+} uptake (*via* the SR Ca^{2+}-ATPase), SL Ca^{2+}-ATPase-mediated Ca^{2+} efflux is ≈ 25% that of Ca^{2+} efflux mediated by Na^{+}-Ca^{2+} exchange. While this is much higher than previously reported in rats (≈ 14%) and rabbits (≈ 7%) (253) and in rabbit (≈ 6%) and ferret (≈ 14%) (25), the transport rate for the SL Ca^{2+}-ATPase is still far to slow (i.e., ≈ 15 - 30 times slower than the Na^{+}-Ca^{2+} exchanger (23)) to be a major contributor to cardiac relaxation on a beat-to-beat basis. It is important to note,

that like the SL Ca^{2+}-ATPase, the contribution of Na^{+}-Ca^{2+} exchange-mediated Ca^{2+} efflux also varies from species to species (reviewed in (162)). For example, Na^{+}-Ca^{2+} exchange was found to remove ≈ 28% of Ca^{2+} from the cytosol in rabbit, while only ≈ 7% in rat (253).

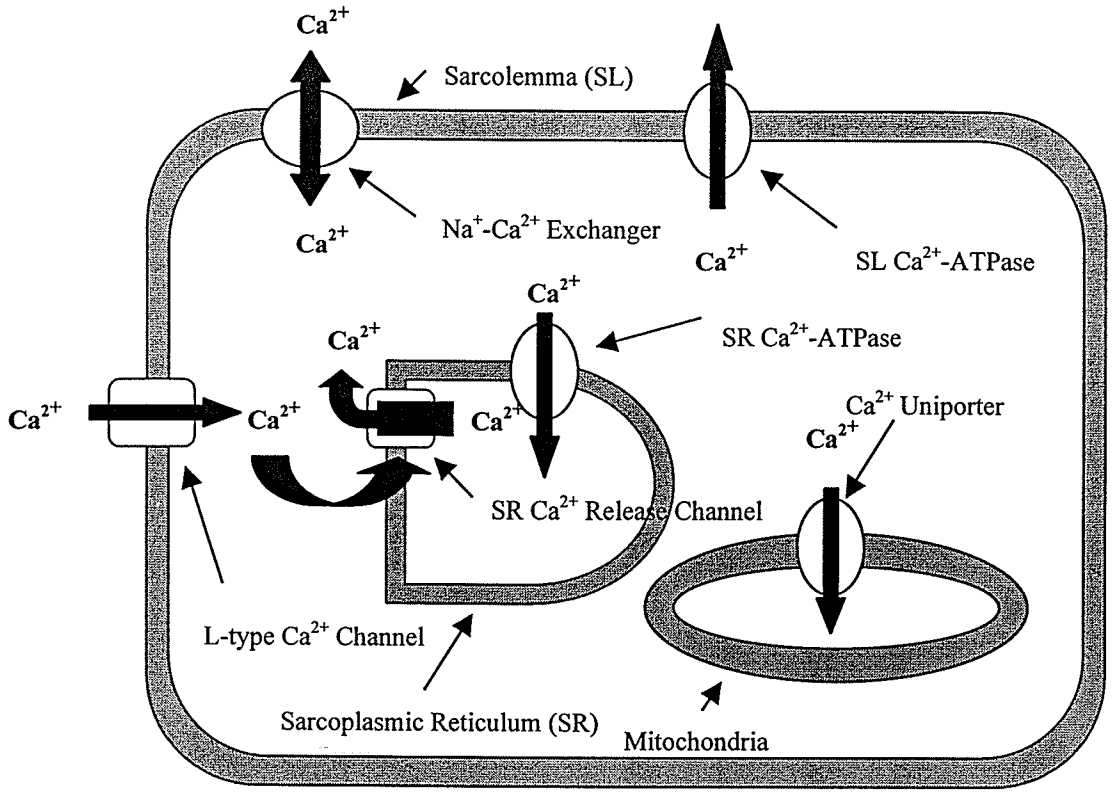

Figure 9. ***Ca^{2+} Transporters in the Mammalian Cardiac Myocyte.*** Of the 6 major Ca^{2+} transporters in the cardiac myocyte, 4 are involved in lowering Ca^{2+} in the cytoplasm: (1) The SR Ca^{2+}-ATPase, (2) The Na^{+}-Ca^{2+} Exchanger, (3) The SL Ca^{2+}-ATPase, and (4) The Ca^{2+}-Uniporter. In mammalian cardiomyocytes, the SR Ca^{2+}-ATPase and the Na^{+}-Ca^{2+} exchanger are the primary transporters involved in lowering cytosolic Ca^{2+}.

5.2. *Reverse-Mode Na^+-Ca^{2+} Exchange*

During the upstroke of the action potential, Ca^{2+} influx mediated by reverse-mode Na^+-Ca^{2+} exchange is thermodynamically favored (68;249). However, as cytoplasmic Ca^{2+} increases *via* L-type Ca^{2+} channels inducing sarcoplasmic reticulum (SR) Ca^{2+} release, Ca^{2+} influx mediated by reverse-mode Na^+-Ca^{2+} exchange becomes greatly reduced. Furthermore, the contribution of reverse-mode Na^+-Ca^{2+} exchange to Ca^{2+} influx is small relative to L-type Ca^{2+} channels (37;38), and some studies failed to observe a significant role for reverse Na^+-Ca^{2+} exchange in CICR (254;255). There is evidence to suggest that Na^+-Ca^{2+} exchange mediated Ca^{2+} influx can contribute to direct activation of myofilaments, resulting in a contraction, but only when cytoplasmic Na^+ is elevated or when subjected to very large or very long depolarizations (41). Likewise, studies have shown that Ca^{2+} entry *via* Na^+-Ca^{2+} exchange can trigger Ca^{2+} release from the SR, but only at high (*i.e.*, non-physiological) cytoplasmic Na^+ (35;256-258). However, it has been suggested that local intracellular Na^+ concentrations may be higher than cytosolic [Na^+] due to Na^+ influx *via* Na^+ channels at the onset of the action potential, and the influence by the Na^+-K^+-ATPase (*i.e.*, Na^+-K^+-ATPase-mediated Na^+ influx), thereby raising local [Na^+] high enough to stimulate reverse Na^+-Ca^{2+} exchange (28;256;259;260).

Another proposed role for reverse-mode Na^+-Ca^{2+} exchange includes modulating the effectiveness of the L-type Ca^{2+} channel in inducing SR Ca^{2+} release (31-33). According to these studies, reverse Na^+-Ca^{2+} exchange would act to amplify the extent that L-type Ca^{2+} channels could trigger SR Ca^{2+} release by raising local

cytoplasmic Ca^{2+}. Additionally, reverse Na^{+}-Ca^{2+} exchange has been suggested to modify the content of Ca^{2+} in the SR (34-36). For example, Nuss and Houser observed that reverse Na^{+}-Ca^{2+} exchange not only was capable of activating the contractile proteins, but could also induce and adjust SR Ca^{2+} release, suggesting that Ca^{2+} entry that occurs by reverse Na^{+}-Ca^{2+} exchange may be important for normal SR Ca^{2+} loading. SR Ca^{2+} loading mediated by reverse Na^{+}-Ca^{2+} exchange has been used to explain the inotropic effect of cardiac glycosides. Ouabain, which inhibits the Na^{+}-K^{+}-ATPase, raises cytoplasmic Na^{+}, and thereby increases intracellular Ca^{2+} *via* reverse Na^{+}-Ca^{2+} exchange. The elevated cytoplasmic Ca^{2+} levels lead to a greater amount of Ca^{2+} in the SR, resulting in a larger release of Ca^{2+} during each contraction (261;262), creating the inotropic effect (263-265). However, most aspects surrounding both the mechanism of action of cardiac glycosides and the physiological relevance of reverse-mode Na^{+}-Ca^{2+} exchange remain controversial.

6. Na^+-Ca^{2+} Exchange and Cardiac Pathophysiology

6.1. Alterations of Na^+-Ca^{2+} Exchange in Cardiac Hypertrophy and Failure

Cardiac hypertrophy is often associated with a conversion to a fetal pattern of gene expression in a variety of animal models. This includes changes in actin and myosin isoform expression patterns, an increase in atrial natriuretic factor (see reviews (266;267)), and a decrease in the SR Ca^{2+}-ATPase (268;269). There are several conflicting reports regarding changes in Na^+-Ca^{2+} exchange levels in failing myocytes, with some demonstrating an increase in Na^+-Ca^{2+} exchange protein, transcript, and activity (42;47;270-272), while others found no change in protein (50) or transcript levels (49), or a decrease in activity (273;274) Additionally, several animal models of heart failure and hypertrophy have demonstrated an increase in Na^+-Ca^{2+} exchange levels and activity (39;275-277).

There is also considerable debate surrounding the issue of whether increased Na^+-Ca^{2+} exchange expression occurs in the failing heart. Some reports suggest that increased Na^+-Ca^{2+} exchanger expression compensates for the down-regulation of other Ca^{2+} transporters in the failing myocardium, such as the SR Ca^{2+}-ATPase (12;45;278;279), while others have demonstrated that elevated levels of Na^+-Ca^{2+} exchange activity can be detrimental, due to a negative inotropic effect at increased heart rates (280;281), depletion of sarcoplasmic reticulum Ca^{2+} stores (282;283), and generation of delayed afterdepolarizations (DADs; and thereby inducing arrhythmias) (39;283;284).

6.2. Arrhythmogenesis

Arrhythmias, a major contributor to sudden cardiac death during heart failure, can be elicited by reentrant or nonreentrant mechanisms. Using three-dimensional mapping, Pogwizd (285) (see also (286)) demonstrated that virtually all ventricular tachycardias in nonischemic heart failure and roughly half of those in ischemic heart failure are due to nonreentrant mechanisms, which are generated by abnormal automaticity (287) or triggered activity. In regard to the latter, the "trigger" for arrhythmogenesis involves either early afterdepolarizations (EADs) or delayed afterdepolarizations (DADs). DADs arise in response to spontaneous Ca^{2+} release from the SR and a Ca^{2+}-activated, transient, depolarizing inward current (I_{TI}) (288-290).

Most studies have shown that the major transporter contributing to I_{TI} and subsequent DAD generation is the Na^{+}-Ca^{2+} exchanger (39;40;53-55;291-293). Further implication of Na^{+}-Ca^{2+} exchange's involvement in arrhythmogenesis was demonstrated by three studies employing KB-R7943. Watano et al. (227) reported that KB-R7943 suppressed ouabain-induced arrhythmias in guinea pigs, while Elias et al. (225) found that KB-R7943 suppressed reperfusion arrhythmias in intact rabbit hearts. Mukai et al. (226) reported that KB-R7943 attenuated reoxygenation-induced arrhythmias in guinea pig papillary muscles. Recent evidence (283) demonstrates that changes in the levels of Na^{+}-Ca^{2+} exchange, inward rectifying K^{+} current (I_{K1}), and β-adrenergic receptors all greatly increase the incidence of triggered arrhythmias in a

rabbit model of heart failure. Furthermore, up-regulation of the Na^+-Ca^{2+} exchanger seemed to be the critical link between contractile dysfunction and arrhythmogenesis in this model. However, Lu et al. (64) failed to observe any beneficial effect of KB-R7943 in ischemia/reperfusion-induced arrhythmias in anesthetized rats (*i.e., in vivo*). This study suggests that preventing Ca^{2+} loading *via* the Na^+-Ca^{2+} exchanger is unimportant for preventing ischemia/reperfusion-induced arrhythmias.

6.3. Reperfusion Injury

Reverse Na^+-Ca^{2+} exchange is generally believed to contribute to Ca^{2+} loading during ischemia and reperfusion injury (294-297). Ischemia results in the production of H^+ from anaerobic glycolysis and ATP degradation, activating Na^+ influx via the Na^+-H^+ exchanger (298;298). The increased cytoplasmic [Na^+] then putatively stimulates reverse Na^+-Ca^{2+} exchange, raising intracellular Ca^{2+}. Due to the incredible complexity of the conditions of ischemia and reperfusion, the precise mechanism by which Ca^{2+} overload is reached is still the subject of much debate.

KB-R7943 has been employed in studies investigating the role of Na^+-Ca^{2+} exchange in ischemia/reperfusion injury. For example, Ladilov et al. reported that KB-R7943 significantly reduced the cytosolic Ca^{2+} accumulation upon anoxic treatment in isolated cardiomyocytes, and upon reoxygenation, hypercontracture was reduced. Furthermore, in Langendorff-perfused rat hearts, KB-R7943 significantly decreased reoxygenation-induced release of lactate dehydrogenase and creatine

kinase (299). In addition, Nakamura et al. also reported cardioprotective effects in a perfused rat heart model of ischemia/reperfusion (300).

IV. METHODS

1. Preparation of *Xenopus laevis* Oocytes

Xenopus laevis were anesthetized in 250 mg/l ethyl *p*-aminobenzoate (Sigma) in deionized ice-water for 30 minutes before sacrificing. Surgically removed oocytes were washed in Solution A containing (in mM): 88 NaCl, 15 HEPES, 2.4 $NaHCO_3$, 1.0 KCl, 0.82 $MgSO_4$; pH 7.6 at room temperature (RT). Follicles surrounding the oocytes were teased apart to facilitate digestion before they were transferred to 5 ml of Solution A containing ≈ 3500 U/ml collagenase (Type II; Worthington). Following 45-60 minutes of incubation and gentle agitation at RT, the oocytes were washed several times in Soultion B containing (in mM): 88 NaCl, 15 HEPES, 2.4 $NaHCO_3$, 1.0 KCl, 0.82 $MgSO_4$, 0.41 $CaCl_2$, 0.3 $Ca(NO_3)_2$, 1 mg/ml BSA (Fraction V; Sigma); pH 7.6 at RT, then transferred to 5 ml of 100 mM K_2HPO_4; pH 6.5 at RT, containing 1 mg/ml BSA. After incubation for 12 minutes with gentle agitation at RT, the oocytes were washed in Solution B. Defolliculated stage V-VI oocytes were selected and incubated at 18 °C in Solution B (*minus* BSA) until cRNA injection the next day.

2. Synthesis of NCX1.1 cRNAs

Hind III (*New England* Biolabs) was used to linearize complementary DNA (encoding NCX1.1) residing in pBluescript II SK(+) (Stratagene). Complementary

RNA was synthesized using T3 mMessage mMachine *in vitro* transcription kits (Ambion) according to manufacturer's instructions. Oocytes were injected (46 nl) with ≈ 5 ng of cRNA encoding NCX1.1, and stored in Solution B (*minus* BSA) at 18 °C. Electrophysiological measurement of Na^+-Ca^{2+} exchange activity was obtained 3-5 days post-injection.

3. Measurement of Exchange Activity

The giant, excised patch clamp technique (95;192;225) (Figure 10) was used to measure Na^+-Ca^{2+} exchange current. Oocytes were placed in a hypo-osmotic solution containing (in mM): 250 Sucrose, 67 KCl, 10 D-Glucose, 10 HEPES, 5 $MgCl_2$, 2.5 EGTA; pH 7.0 at RT (with KOH) for ≈ 15 minutes, to facilitate separation of the vitellin layer from the oocyte. After removal of the vitellin layer with forceps, oocytes were placed in a solution containing: (in mM): 100 KOH, 100 MES, 20 HEPES, 5 EGTA, 5 $MgCl_2$; pH 7.0 at RT (with MES). Borosilicate glass pipettes were pulled (Sutton) and polished to a final inner diameter of ≈ 20 - 30 μm (Narshige) and coated with a Parafilm™:Mineral oil mixture to enhance patch stability and reduce electrical noise. Pipettes were filled with the desired pipette solution (see below), placed into the pipette holder (Axon Instruments), and maneuvered onto the animal pole of the oocyte with a micromanipulator (Narshige). Gigaohm seals were formed via gentle suction, and membrane patches (inside-out configuration) were excised by progressive movements of the pipette tip. Pipettes containing patches were then maneuvered in front of the perfusion tip of a custom-built, computer-controlled,

20-channel solution switcher, allowing rapid (*i.e.*, ≈ 200 ms) solution changes of the bath (*i.e.*, intracellular) solution. Axon Instruments hardware (Axopatch 200a) and software (Axotape 2.0; pClamp 6.0) were used for data acquisition and analysis, and Origin software was used for curve-fitting and statistical analysis.

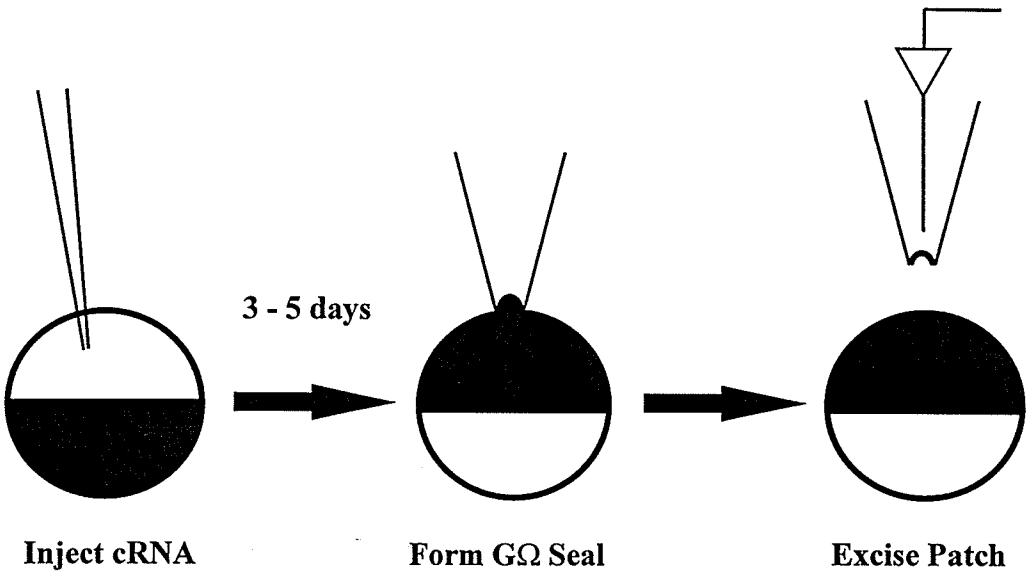

Figure 10. ***The Giant, Excised Patch Technique.*** Complimentary RNA encoding Na^{+}-Ca^{2+} exchanger protein was injected into *Xenopus laevis* oocytes (*left panel*). After 3 – 5 days to allow for protein expression, gigaohm seals were formed by applying gentle suction to the pipette tip (*middle panel*). Excision of the patch (inside-out configuration, *i.e.*, the cytoplasmic face of the patch is on the outside) was achieved by mechanical manipulation of the pipette tip (*right panel*).

Outward (*i.e.*, reverse-mode) Na^+-Ca^{2+} exchange currents (Figure 11) were generated by switching from Li^+- to Na^+-based bath solutions containing (in mM): 0-100 Na^+-aspartate *or* 100 Li^+-aspartate, and 20 CsOH, 20 MOPS, 20 TEA-OH, 10 EGTA, 0 - 0.01 $CaCO_3$, 1.0 - 1.5 $Mg(OH)_2$; pH 7.0 at 30 °C (with MES or LiOH, respectively). Free concentrations of 1.0 mM Mg^{2+} and 0 - 30 μM Ca^{2+} were calculated using MAXC software (301). For outward Na^+-Ca^{2+} exchange currents, pipettes contained (in mM): 100 NMG-MES, 30 HEPES, 30 TEA-OH, 16 sulfamic acid, 8.0 $CaCO_3$, 6 KOH, 0.25 ouabain, 0.1 niflumic acid, 0.1 flufenamic acid; pH 7.0 at RT (with MES). Inward (*i.e.*, forward-mode) Na^+-Ca^{2+} exchange currents (Figure 12) were activated by switching between Ca^{2+}-free and Ca^{2+}-containing, Li^+-based bath solutions, as described above. For inward currents, pipettes contained (in mM): 100 Na-MES, 20 CsOH, 20 TEA-OH, 10 EGTA, 10 HEPES, 4 $Mg(OH)_2$, 0.25 ouabain, 0.1 niflumic acid, 0.1 flufenamic acid; pH 7.0 at RT (with MES). Outward and inward currents activated on the same patch were generated using the same solutions used for pure inward and pure outward currents (described above). Pipette solution for combined inward and outward current measurements contained (in mM): 100 Na-MES, 20 CsOH, 20 HEPES, 20 TEA-OH, 4 sulfamic acid, 2 CaCO3, 0.25 ouabain, 0.1 niflumic acid, 0.1 flufenamic acid; pH 7.0 at RT (with MES). All experiments were conducted at 30 ± 1 °C. KB-R7943 (generously donated by Nippon Organon K.K.) was dissolved in DMSO to form a 20 mM stock solution (concentration of DMSO never exceeded 0.1%). All data are mean ± SEM, unless indicted otherwise.

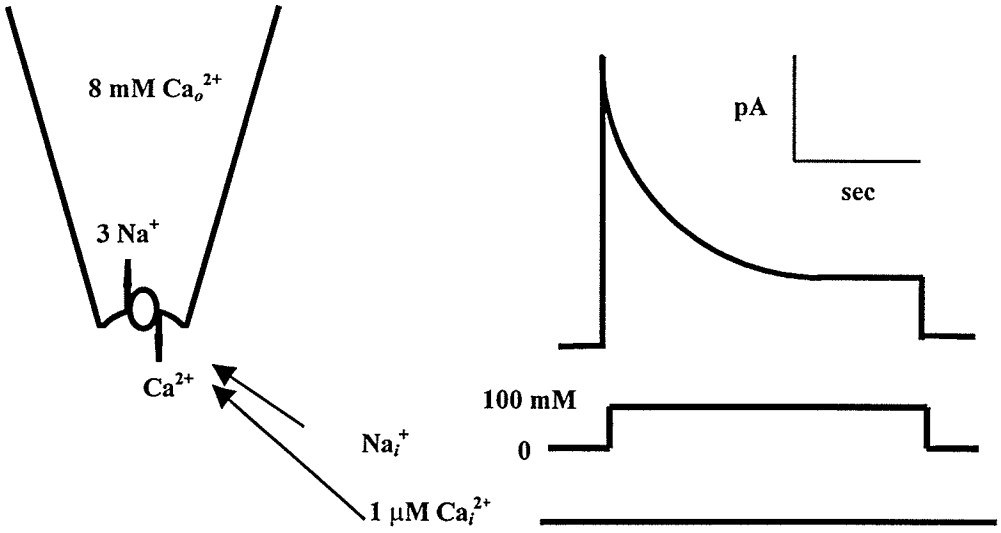

Figure 11. ***Generation of a Typical Outward (i.e. Reverse-Mode) Na^+-Ca^{2+} Exchange Current.*** In this configuration, pipettes contain 8 mM Ca^{2+} (*left panel*). Upon bathing the pipette in 100 mM Na^+ and 1 μM Ca^{2+}, Na^+-Ca^{2+} exchange was initiated, in which 3 Na^+ enters the pipette in exchange for 1 Ca^{2+}. Therefore, 1 positive charge associated with Na^+ translocation moves into the pipette (*i.e.*, out of the cell), generating an *outward* current. A typical outward (*i.e.* reverse-mode) Na^+-Ca^{2+} exchange current obtained using the giant, excised patch technique is represented in the *right panel*.

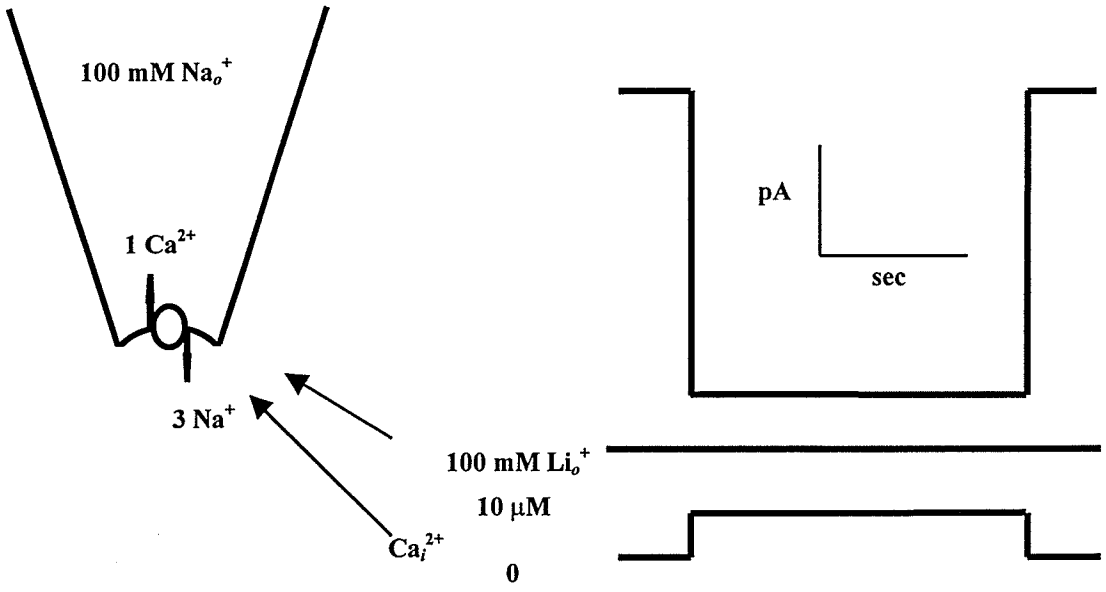

Figure 12. ***Generation of a Typical Inward (i.e. Forward-Mode) Na^+-Ca^{2+} Exchange Current.*** In this configuration, pipettes contain 100 mM Na^+ (*left panel*). Upon bathing the pipette in 100 mM Li^+ and 10 μM Ca^{2+}, inward Na^+-Ca^{2+} exchange was initiated, bringing 1 Ca^{2+} into the pipette in exchange for 3 Na^+. Therefore, 1 positive charge associated with Na^+ translocation moves out of the pipette (*i.e.*, into of the cell), generating an *inward* current. A typical inward (*i.e.* forward-mode) Na^+-Ca^{2+} exchange current obtained using the giant, excised patch technique is represented in the *right panel*.

V. RESULTS

1. KB-R7943 and Na^+-Ca^{2+} Exchange Currents

1.1. KB-R7943 and Outward (i.e. Reverse-Mode) Na^+-Ca^{2+} Exchange Currents

We first examined the effects of KB-R7943 on pure outward (*i.e.*, reverse-mode) Na^+-Ca^{2+} exchange currents. Outward Na^+-Ca^{2+} exchange currents were obtained from membrane patches excised from *Xenopus laevis* oocytes expressing the cloned, canine cardiac exchanger NCX1.1. Figure 13A illustrates the inhibitory effects of KB-R7943 on a representative outward Na^+-Ca^{2+} exchange current trace. Switching the bath solution, which is applied to the cytoplasmic surface of the patch, from 100 mM Li^+ to 100 mM Na^+ activated outward exchange currents. As a requirement for activation of the Na^+-Ca^{2+} exchanger, 1 μM Ca^{2+} (*i.e.*, regulatory Ca^{2+}) was present throughout the trace. Pipettes contained 8 mM Ca^{2+} (*i.e.*, transport Ca^{2+}). The control trace shows a typical outward Na^+-Ca^{2+} current waveform: Current rapidly rises to a peak level before gradually decreasing to a steady-state level. This process is referred to as Na^+-dependent inactivation, or I_I (91;95). Application of KB-R7943 to the patch led to a dose-dependent inhibition of both peak and steady-state outward Na^+-Ca^{2+} exchange currents. Figure 13B represents pooled data describing the concentration dependency effect of KB-R7943 on outward Na^+-Ca^{2+} exchange. KB-R7943 was estimated to have an inhibitory potency (IC_{50}) of 2.8 ±1.0 μM and 0.6

± 0.1 μM for peak and steady-state outward exchange currents, respectively (mean ± SD).

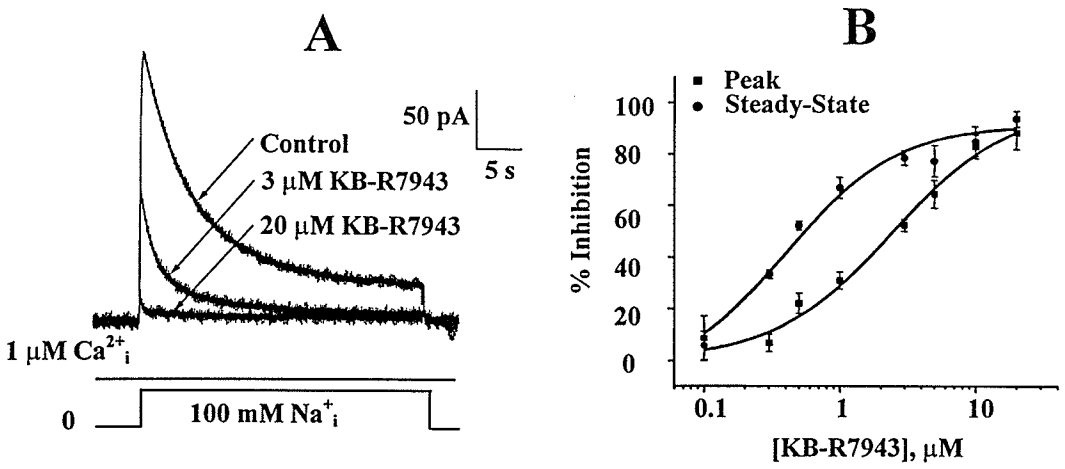

Figure 13. ***The Inhibitory Effects of KB-R7943 on Outward Na^+-Ca^{2+} Exchange Activity.*** Panel *A* shows representative outward, NCX1.1-mediated exchange currents activated by applying 100 mM Na^+ to the cytoplasmic surface of the patch in the continuous presence of 1 μM regulatory Ca^{2+}. The pipette (*i.e.*, extracellular) Ca^{2+} concentration was constant at 8 mM. The three overlapping recordings show outward currents obtained prior to KB-R7943 application (Control), and following the addition of 3 and 20 μM KB-R7943. The inhibitory potency (*i.e.*, IC_{50}) of KB-R7943 to inhibit peak and steady-state outward currents was determined from the pooled data shown in panel *B*. *Per cent* inhibition derived from 3-17 patches are shown. Data obtained in the *presence* of KB-R7943 were normalized according to the peak or steady-state, outward current level obtained in the same patch in the *absence* of KB-R7943.

1.2. KB-R7943 and Inward (i.e., Forward-Mode) Na^+-Ca^{2+} Exchange Currents

Figure 14 illustrates the effects of KB-R7943 on a representative pure inward (*i.e.*, forward-mode) Na^+-Ca^{2+} exchange current trace. Switching from a 100 mM Li^+ to a 100 mM Li^+ plus 10 μM Ca^{2+} solution activated inward Na^+-Ca^{2+} exchange currents. In contrast to outward exchange currents, inward exchange currents do not inactivate, hence the square current waveform (91;142). For pure inward currents, patch pipettes contained 100 mM Na^+. Inhibition of inward exchange current by KB-R7943 was substantially less potent when compared to its inhibitory effect on outward exchange current (Figure 13A). Inhibition of forward exchange current in the presence of 10 μM KB-R7943 was only 13.5 ± 1.3% and when the concentration of KB-R7943 was increased to 20 μM, inhibition was still below 20%. Since KB-R7943 was dissolved in DMSO, which affects patch stability at higher concentrations, we didn't attempt to estimate an IC_{50} by utilizing concentrations of KB-R7943 greater than 20 μM.

1.3. KB-R7943 and combined inward-outward Na^+-Ca^{2+} exchange currents

Next, we tested the effects of KB-R7943 on Na^+-Ca^{2+} exchange under conditions that both inward and outward exchange currents could be activated on the *same population of exchangers*. Outward currents were generated by application of 100 mM Na^+ plus 1 μM Ca^{2+} to the cytoplasmic surface of the patch, while inward currents were activated by switching from a 100 mM Li^+ *plus* 1 μM Ca^{2+} solution to a

100 mM Li^+ *plus* 10 μM Ca^{2+} solution. The pipette contained 100 mM Na^+ and 2 mM Ca^{2+}. Figure 15 shows a typical combined inward-outward Na^+-Ca^{2+} exchange current trace obtained in the absence and presence of 10 μM KB-R7943. Control outward and inward Na^+-Ca^{2+} exchange currents exhibited their usual characteristics (*i.e.*, I_1 for outward exchange currents, and a square waveform associated with inward exchange currents). Treatment of outward Na^+-Ca^{2+} exchange activity with 10 μM KB-R7943 resulted in a substantial inhibition of outward exchange current, similar to what was seen for pure outward exchange current (Figure 13A). Additionally, 10 μM KB-R7943 had little effect on inward exchange current (≈ 16% inhibition), analogous to what was observed under pure inward conditions (Figure 14). Therefore, these data would suggest a definite inhibitory preference of outward (*i.e.*, reverse-mode) Na^+-Ca^{2+} exchange by KB-R7943 under conditions where both outward and inward exchange currents are possible.

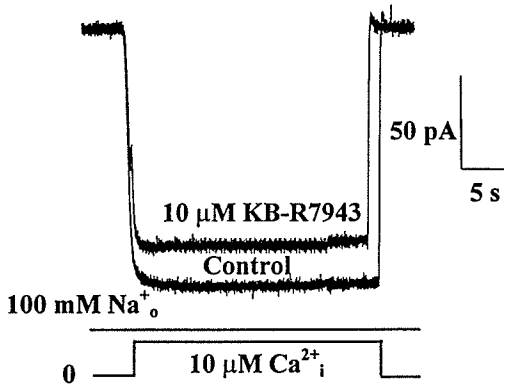

Figure 14. ***The Inhibitory Effects of KB-R7943 on Inward Na^+-Ca^{2+} Exchange Activity*** Representative inward, NCX1.1-mediated exchange current traces, obtained in the absence (Control) and presence of 10 μM KB-R7943, are shown. Inward currents were activated by rapidly applying 10 μM Ca^{2+} to the cytoplasmic surface of the patch. The pipette solution contained 100 mM Na^+. Similar data were obtained with three patches.

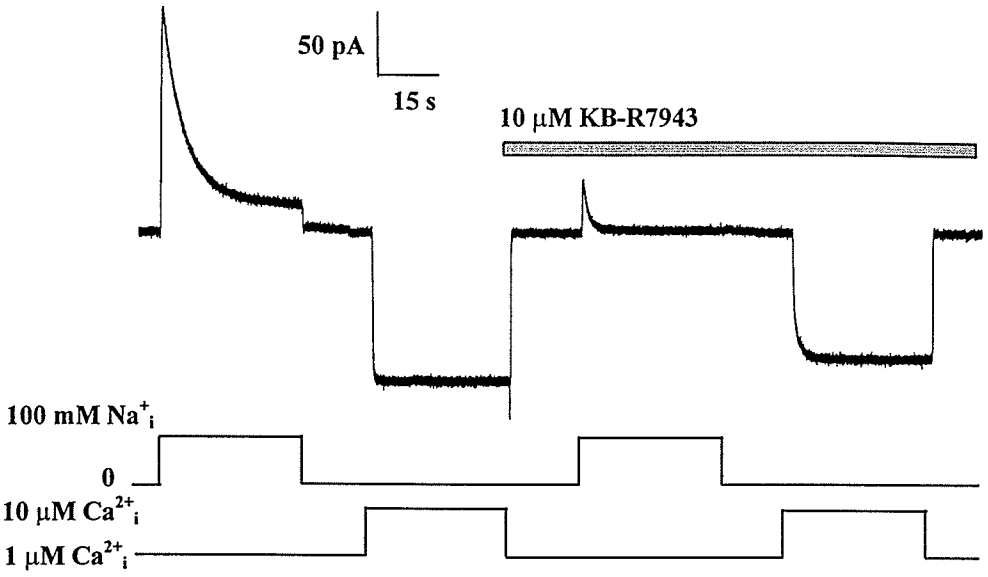

Figure 15. ***The Inhibitory Effects of KB-R7943 on Combined Inward-Outward Na^+-Ca^{2+} Exchange Activity.*** A representative NCX1.1-mediated exchange current recording is shown where outward *and* inward current measurements were acquired from the same patch. Here, pipette solutions contained 100 mM Na^+ and 2 mM Ca^{2+}. Outward currents were initiated by rapid application of 100 mM Na^+, in the presence of 1 μM regulatory Ca^{2+} (as described under Figure 1), to the cytoplasmic surface of the patch. Inward currents were generated by switching from 100 mM Li^+, 1 μM Ca^{2+} to 100 mM Li^+, 10 μM Ca^{2+}-containing solutions on the cytoplasmic surface of the patch. The first two current transients (*i.e.*, outward followed by inward) are control recordings, whereas the second two transients were obtained in the presence of 10 μM KB-R7943.

2. Interaction of KB-R7943 with Na^+-Ca^{2+} Exchange Ionic Regulatory Mechanisms

2.1. KB-R7943 and Regulatory Ca^{2+}

Progressive increases in regulatory Ca^{2+} stimulate outward Na^+-Ca^{2+} exchange activity when applied to the cytoplasmic side of the membrane. This process is referred to as Ca^{2+}-regulation or I_2 (95;152). Conversely, inward Na^+-Ca^{2+} exchange currents do not exhibit this behavior since the regulatory Ca^{2+} binding site is saturated ($K_d \approx 0.3$ μM) under conditions that we use to detect inward exchange currents ($K_d \approx$ 7 μM) (142). Therefore, it seemed possilbe that KB-R7943's inhibitory preference for outward Na^+-Ca^{2+} exchange might be explained by competitive interaction with regulatory Ca^{2+}, since this competition would be much more pronounced during outward Na^+-Ca^{2+} exchange, when the regulatory Ca^{2+} present is much lower (*i.e.*, 1 μM Ca^{2+}) than during inward Na^+-Ca^{2+} exchange when regulatory Ca^{2+} is much higher (*i.e.*, 10 μM Ca^{2+}). To investigate a possible competitive interaction between regulatory Ca^{2+} and KB-R7943, we examined pure outward Na^+-Ca^{2+} exchange currents elicited at a 1 and 10 μM regulatory Ca^{2+}. Figure 16 shows the effects of 3 μM KB-R7943 on a representative pure outward Na^+-Ca^{2+} exchange current trace at both 1 and 10 μM regulatory Ca^{2+}. At 1 μM and 10 μM regulatory Ca^{2+}, KB-R7943 substantially inhibited outward steady-state exchange current, suggesting that inhibitory effects and transport mode selectivity of KB-R7943 do not involve competitive interactions with the Ca^{2+} regulatory mechanism.

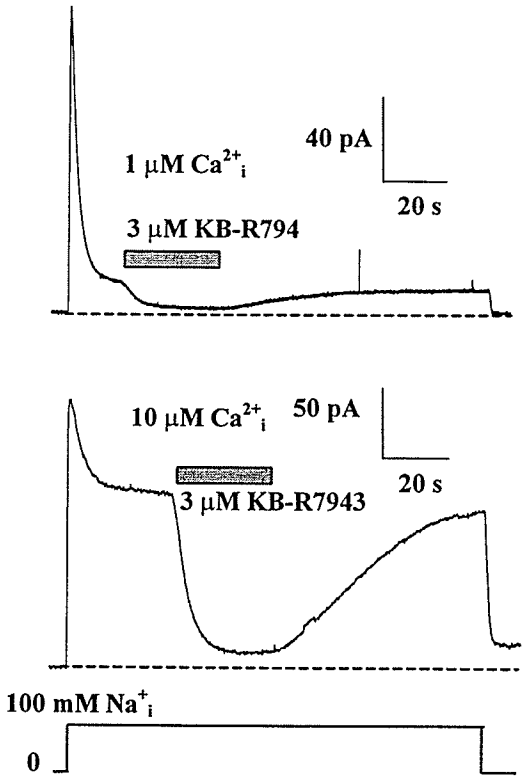

Figure 16. ***The Effects of Regulatory Ca^{2+} Concentration on KB-R7943-Mediated Inhibition of Outward Na^+-Ca^{2+} Exchange Activity.*** The effects of 3 μM KB-R7943 application on outward, NCX1.1-mediated exchange currents, obtained at 1 μM (*upper panel*) and 10 μM (*lower panel*) regulatory Ca^{2+}, are shown. Regulatory Ca^{2+} was applied, and outward currents activated, as described under Figure 1, until steady-state levels of exchange activity were attained. KB-R7943 was then applied for the indicated period and then allowed to wash out. Pipette solutions contained 8 mM Ca^{2+}. The data are representative of recordings from three patches at 1 μM Ca^{2+} and two patches at 10 μM Ca^{2+}.

2.2. *KB-R7943 and Na^+-Dependent Inactivation (I_1)*

In order to investigate the effects of KB-R7943 on outward Na^+-Ca^{2+} exchange current in the absence of ionic regulation (*i.e.*, I_1 and I_2), membrane patches were treated with α-chymotrypsin for ≈ 1 minute. Following this limited proteolysis, outward Na^+-Ca^{2+} exchange current appears fully activated, and I_1 and I_2 are virtually absent (95). Figure 17 illustrates a representative outward exchange current trace before proteolysis (Panel A) and after proteolysis (Panel B) in the absence and presence of 20 μM KB-R7943. While inhibition of outward Na^+-Ca^{2+} exchange current was still substantial for α-chymotrypsin-treated membrane patches, percent inhibition was only 76 ± 2%, whereas percent inhibition exceeded 95% for control membrane patches. Therefore, it appears the majority of the inhibitory effect mediated by KB-R7943's on outward exchange currents does not require the exchanger's ionic regulatory mechanisms to be functional. Rather, KB-R7943 seems to primarily exert its inhibitory effects *via* interaction with the transport machinery (see Discussion) of the exchanger.

3. Effects of KB-R7943 on Transported Na^+ and Ca^{2+}

3.1. KB-R7943 and Transported Ca^{2+}

Previous studies have reported that the inhibitory characteristics of KB-R7943 on Na^+-Ca^{2+} exchange were due to competition with extracellular Ca^{2+} (56;224), while others found KB-R7943 to be non-competitive with respect to extracellular Ca^{2+} (57). In order to provide insight into this dilemma, we measured the current-voltage (*IV*) relationships of exchange activity in α-chymotrypsin treated patches (*i.e.*, under conditions in which ionic regulation is absent and the characteristics of pure transport can be observed) in the absence and presence of 20 μM KB-R7943. The protocol we employed to obtain the *IV* relationships is illustrated in Figure 18A. Current-voltage relationships were measured when no exchange current was present (*a*), during pure, de-regulated outward exchange currents (*b*), and in the presence of 20 μM KB-R7943 (*c*). Then, in order to eliminate any possible leak-current contamination, *IV* relationship *a* was subtracted from *IV* relationships *b* and *c*. The leak-subtracted *IV* relationships are shown in Figure 18B. In comparison to the control IV relationship (*i.e., b - a*), the *IV* relationship measured in the presence of KB-R7943 (*i.e., c - a*) is depressed, but when scaled, the control and KB-R7943 *IV* relationships superimpose (dotted line). This would suggest that KB-R7943 does not compete with extracellular Ca^{2+} for several reasons. Firstly, lowering extracellular Ca^{2+}, which is analogous to introducing a purely competitive inhibitor, results in a reduction of voltage dependence of the *IV* relationship (*i.e.*, the *IV* becomes flat)

because the Ca^{2+} transport process becomes rate-limiting (302). Since the majority of the electrogenicity of the Na^+-Ca^{2+} exchanger resides in the Na^+ transport partial reaction (82), the *IV* relationship flattens and does not simply scale up. Our *IV* relationship data suggests that KB-R7943 reduces the number of active exchangers, rather than purely competing with extracellular Ca^{2+}.

Furthermore, since KB-R7943 is highly lipophilic and is applied to the cytoplasmic side of the patch, its seems improbable that KB-R7943 would have time to cross the membrane and reach a competitive equilibrium with extracellular Ca^{2+} in the pipette, given the rates at which inhibition develops and subsides (*e.g.*, see Figure 16). Additionally, I_1 is reduced in response to a lower extracellular Ca^{2+} levels, and the fraction of non-inactivating current actually increases (91). We did not find evidence of this however, and in fact, KB-R7943 showed the greatest potency for steady-state currents (see Figure 13). We cannot exclude the possibility that KB-R7943 may diffuse across the membrane to mediate its effects, however.

We also examined the effects of KB-R7943 on pure outward Na^+-Ca^{2+} exchange activity in the presence of low extracellular Ca^{2+} (*i.e.*, pipettes contained 0.5 mM Ca^{2+} instead of the usual 8 mM) (Figure 19). If KB-R7943 did compete with extracellular Ca^{2+}, the inhibitory potency would be expected to increase at a lower level of extracellular Ca^{2+}. However, we did not find evidence supporting this, since application of 3 μM KB-R7943 resulted in an average percent inhibition of 54 ± 4%, very close to the percent inhibition observed with 8 mM Ca^{2+} (*i.e.*, 52 ± 3%). These data also suggest that KB-R7943 does not purely compete with extracellular Ca^{2+}.

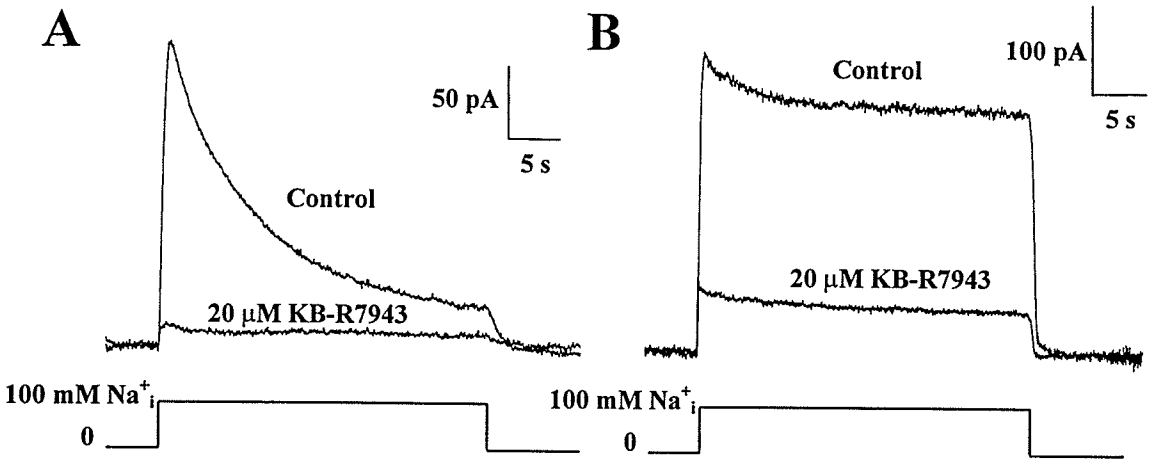

Figure 17. ***The Effects of α-Chymotrypsin Treatment on KB-R7943-Mediated Inhibition of Outward Na^+-Ca^{2+} Exchange Activity.*** Representative outward, NCX1.1-mediated current recordings, obtained in the absence (Control) and presence of 20 μM KB-R7943, prior to (*A*) and following (*B*) limited proteolysis of the cytoplasmic surface of the patch with α-chymotrypsin (1 mg/ml) for ≈ 1 min. Currents were activated in the presence of 1 μM regulatory Ca^{2+}, as described under Figure 1. Pipette solutions contained 8 mM Ca^{2+}. Comparable data were obtained from three patches in each group (*A and B*).

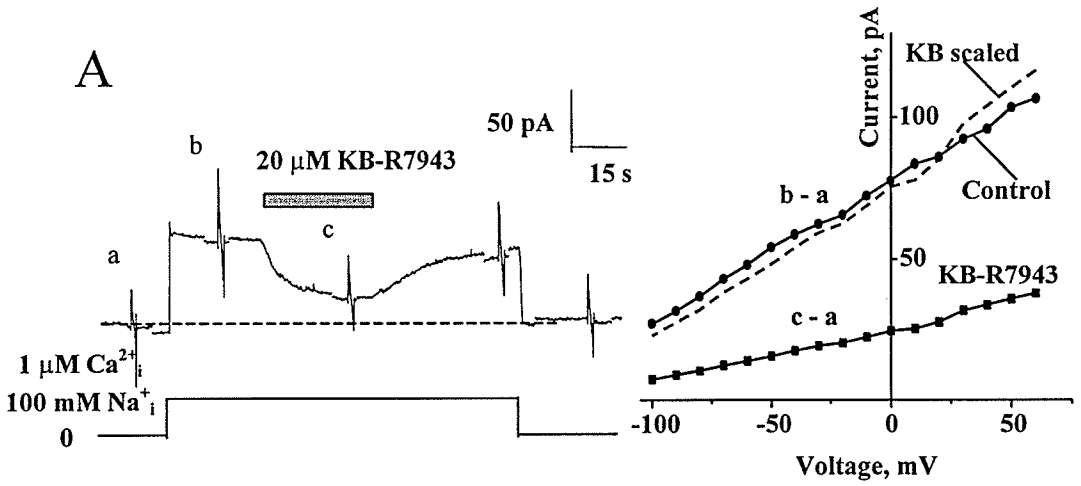

Figure 18. The Effects of KB-R7943 on the Current-Voltage Relationships of Outward Na^+-Ca^{2+} Exchange Activity Following Limited Proteolysis with α-Chymotrypsin. Representative outward, NCX1.1-mediated exchange activity, following treatment with α-chymotrypsin (as described under Figure 5), is illustrated in panel A. Current was activated in the presence of 1 μM regulatory Ca^{2+}, as described in Figure 1. Pipette solutions contained 8 mM Ca^{2+}. KB-R7943 (20 μM) was applied for the period indicated and then allowed to wash out. Current-voltage (*IV*) recordings (evident as spikes) were obtained prior to current activation (point a), during control outward currents (point b), and during KB-R7943 application (point c). The leak-subtracted *IV* relationships [*i.e.*, b - a (Control) and c - a (KB-R7943)] are shown in panel B. The dashed line was obtained by scaling the *IV* relationship obtained in the presence of KB-R7943 (*i.e.*, c - a). Comparable data were obtained from two additional patches.

Furthermore, since KB-R7943 is highly lipophilic and is applied to the cytoplasmic side of the patch, its seems improbable that KB-R7943 would have time to cross the membrane and reach a competitive equilibrium with extracellular Ca^{2+} in the pipette, given the rates at which inhibition develops and subsides (*e.g.*, see Figure 16). Additionally, I_1 is reduced in response to a lower extracellular Ca^{2+} levels, and the fraction of non-inactivating current actually increases (91). We did not find evidence of this however, and in fact, KB-R7943 showed the greatest potency for steady-state currents (see Figure 13).

We also examined the effects of KB-R7943 on pure outward Na^{+}-Ca^{2+} exchange activity in the presence of low extracellular Ca^{2+} (*i.e.*, pipettes contained 0.5 mM Ca^{2+} instead of the usual 8 mM) (Figure 19). If KB-R7943 did compete with extracellular Ca^{2+}, the inhibitory potency would be expected to increase at a lower level of extracellular Ca^{2+}. However, we did not find evidence supporting this, since application of 3 μM KB-R7943 resulted in an average percent inhibition of 54 ± 4%, very close to the percent inhibition observed with 8 mM Ca^{2+} (*i.e.*, 52 ± 3%). These data also suggest that KB-R7943 does not purely compete with extracellular Ca^{2+}.

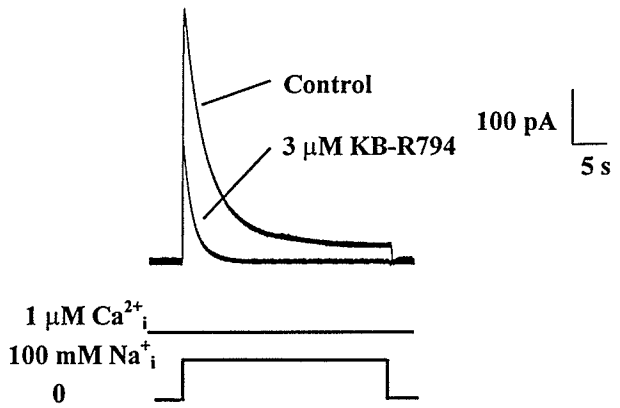

Figure 19. ***The Effects of KB-R7943 on Outward Na^+-Ca^{2+} Exchange Activity at 0.5 mM Pipette Ca^{2+}.*** Representative outward Na^+-Ca^{2+} exchange current traces with 0.5 mM pipette Ca^{2+} were activated upon application of 100 mM Na^+ and 1 μM regulatory Ca^{2+} (Control). Application of 3 μM KB-R7943 reduced outward exchange currents by an average of 54 ± 4 % in 4 determinations from 2 patches, virtually identical to inhibition when pipette Ca^{2+} is 8 mM (*i.e.*, 52 ± 3 %).

3.2. KB-R7943 and Transported Na^+

We also looked at whether the inhibitory mechanism of KB-R7943 on Na^+-Ca^{2+} exchange involved competition with intracellular Na^+. Pure outward exchange currents, activated with various concentrations of Na^+, were treated with 3 μM KB-R7943. The pooled data in Figure 20 shows no significant change in the exchanger's affinity for intracellular Na^+ in the absence (K_D = 34.1 ± 3.6 mM; mean ± SD) or presence of KB-R7943 (K_D = 34.0 ± 1.6 mM; mean ± SD). This strongly suggests that the inhibitory mechanism of KB-R7943 does not involve competition with intracellular Na^+. In fact, for pure outward exchange currents generated with 100 mM Na^+ and 1 μM regulatory Ca^{2+}, 3 μM KB-R7943 doubled the rate of current decay (λ) ($\lambda_{Control}$ = 0.23 ± 0.02 s^{-1} vs. $\lambda_{KB\text{-}R7943}$ = 0.40 ± 0.05 s^{-1}) and reduced steady-state current levels (represented by fraction of steady-state current remaining, or F_{SS}) by about half ($F_{SS(Control)}$ = 0.14 ± 0.01 vs. $F_{SS(KB\text{-}R7943)}$ = 0.06 ± 0.01). It is attractive to suggest that these data indicate at least part of KB-R7943's effects may be due to interactions with I_1. However, since both λ and F_{SS} represent a composite of I_1 and I_2, as well as their own interactions between them, it is difficult to verify whether KB-R7943 targets one particular ionic regulatory mechanism, or both. Hence, we can only suggest that KB-R7943 *may* exert some of its effects through interactions with the I_1 process.

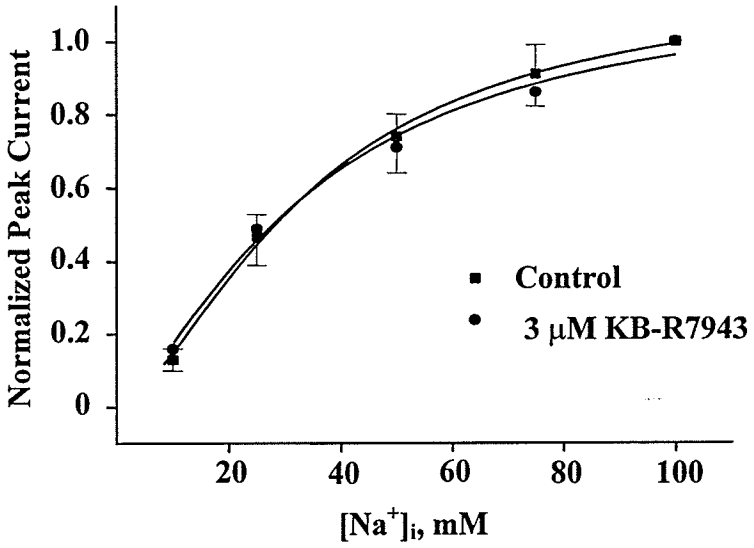

Figure 20. ***The Effects of KB-R7943 on the Na^+-Dependence of Peak, Outward Na^+-Ca^{2+} Exchange Activity.*** The relationship between peak outward, NCX1.1-mediated exchange currents and cytoplasmic Na^+ concentration, in the absence (Control) and presence of 3 μM KB-R7943, is presented. Data were normalized to the peak, outward current values obtained at 100 mM Na^+, in the absence or presence of KB-R7943. Outward currents were activated, and levels of regulatory and pipette Ca^{2+} were as described in Figure 1.

VI. DISCUSSION

We investigated the effects of KB-R7943 on the cloned, cardiac Na^+-Ca^{2+} exchanger, NCX1.1, using the giant, excised patch technique. We observed that KB-R7943 preferentially inhibited reverse-mode Na^+-Ca^{2+} exchange. Additionally, we found no evidence suggesting pure competition with transported ions (*i.e.*, Na^+ and Ca^{2+}). While some interaction with the exchanger's ionic regulatory properties was observed, KB-R7943 mediated the majority of its effects on the exchanger's transport mechanism (see below).

Our primary goal was to interpret KB-R7943's mechanism of inhibition and resolve several discrepancies that have surfaced in the literature since it was introduced. We felt that a better understanding of how KB-R7943 mediated its effects was warranted because of its increasing popularity as a selective inhibitor of Na^+-Ca^{2+} exchange. Furthermore, the giant, excised patch technique, in combination with cloned, Na^+-Ca^{2+} exchanger cRNA expressed in *Xenopus laevis* oocytes, provides us with several advantages over other studies previously conducted. First, this method permits the study of Na^+-Ca^{2+} exchange currents in near isolation. Secondly, we can examine pure forward and reverse-mode exchange currents, alone or combined. Finally, the giant, excised patch technique allows the investigation of Na^+-Ca^{2+} exchange currents at a greater resolution than other methods (*i.e.*, $^{45}Ca^{2+}$ uptake in sarcolemmal vesicles), allowing the opportunity to characterize the ionic regulatory properties of the exchanger. However, in order to measure appreciable Na^+-Ca^{2+} exchange current, ionic conditions employed are not the

same as those utilized in other studies, so direct comparison of results among various studies must be done cautiously.

1. Preferential Inhibition of Reverse Na^+-Ca^{2+} Exchange by KB-R7943

Similar to the results obtained from earlier reports (56;57), we observed preferential inhibition of reverse (*i.e.*, outward) Na^+-Ca^{2+} currents by KB-R7943 under conditions of pure reverse and combined forward-reverse configurations (*i.e.*, inward-outward) (Figures 13 - 15). Note, that under the combined configuration, both forward and reverse Na^+-Ca^{2+} exchange can be induced on the *same population* of exchangers, with identical KB-R7943 and extracellular ion concentrations. Under these conditions, KB-R7943 nearly eliminated outward exchange currents at concentrations as low as 10 μM, while inward currents, in pure inward or combined inward-outward configurations, were inhibited to a lesser degree. These results differ from that of Kimura et al. (58). In their study, ionic conditions remained constant and transport was initiated by changing membrane voltage. No mode preference was observed for KB-R7943 when examining Na^+-Ca^{2+} exchange under those bi-directional transport conditions.

As mentioned previously, KB-R7943 has been employed in many studies based on a variety of physiological models. For example, a number of reports have utilized KB-R7943 in the heart. As the Na^+-Ca^{2+} exchanger is the major transporter facilitating Ca^{2+} efflux (*i.e.*, forward exchange) in the heart (see reviews (6;22;303)), if KB-R7943 had any effect on forward exchange, a large positive inotropic effect would result. However, these studies either reported no inotropic effects (62;227;300) or a decrease in contractile

force (304-306). In our laboratory, we observed a negative inotropic effect in response to treatment of KB-R7943 to intact rabbit hearts (225). Taken together, these studies suggest that KB-R7943 does preferentially inhibit reverse Na^+-Ca^{2+} exchange, since positive inotropy was not observed.

2. Mechanism of Inhibition of Na^+-Ca^{2+} Exchange by KB-R7943

The current generally accepted kinetic model of Na^+-Ca^{2+} exchange is the consecutive transport mechanism (Figure 2) In this model, the orientation of ion binding sites alternate between facing the inside and outside of the cell (82;84;307). The basic steps involved are as follows: An "empty" exchanger becomes "occluded" when either Na^+ or Ca^{2+} binds to it. Once occluded, the exchanger orients the ion binding site to the opposite membrane surface, becoming "de-occluded," releasing the bound ions. At first glance, it is not obvious how KB-R7943's mode-specific inhibitory preference fits into this model without involving competitive interactions or the regulatory properties of the exchanger. Based on our results, it is unlikely that KB-R7943 simply reduced the number of exchangers contributing to the measured Na^+-Ca^{2+} current because both modes of transport would be affected equally. Although it has been suggested that preferential inhibition mediated by KB-R7943 could be explained by competition with extracellular Ca^{2+} (56;224), in our study we found no evidence of competitive interactions between KB-R7943 and transported Ca^{2+} or Na^+ (Figures 18 – 20). Additionally, KB-R7943 did not seem to interact with regulatory Ca^{2+} (*i.e.*, *via* Ca^{2+}-dependent regulation, or I_2)

(Figure 16) since inhibition of outward exchange currents by KB-R7943 was substantial, regardless of the concentration of regulatory Ca^{2+} present.

A requirement of a consecutive transport mechanism is that each kinetic entity, or "state," must be visited during each complete exchange cycle (82;84;307). Depending on the direction of transport, which is based on relative ions concentrations and membrane voltage, the time an exchanger molecule spends in a particular state, or how quickly it exits a particular state will vary. During reverse mode exchange, for example, the exchanger population may contain a larger proportion of a particular exchange state in comparison to forward mode exchange. Within this framework, the mode selectivity exhibited by KB-R7943 may be explained if KB-R7943 interacted with a particular exchanger state and based on our results, the $E_1 3N_i$ exchanger state is a good candidate (Figure 2). Prior to initiation of outward exchange currents in giant, excised patch experiments, all exchangers have their binding sites oriented intracellularly (*i.e.*, the E_1 state). When Na^+ is applied to the intracellular surface of the patch, ion binding occurs and the $E_1 3N_i$ state accumulates. At this point, an exchanger can exit from this state in one of three different was: 1) occlusion followed by de-occlusion of Na^+ extracellularly, thereby generating outward exchange current, 2) unbinding of Na^+, and/or 3) entry into an inactive state (*i.e.*, I_1). If KB-R7493 prevented or inhibited the forward rate for occlusion of $E_1 3N_i$, the result would be accumulation of the $E_1 3N_i$ and I_1 states, and outward current levels would decrease. On the other hand, $E_1 3N_i$ cannot accumulate during forward exchange because the only way it can form is *via* de-occlusion of $E_o 3N$ transitional state. Additionally, since Na^+ is being transported from the extracellular side (*i.e.*, in giant, excised patch inward experiments, the pipette contains 100 mM Na^+ and

zero Ca^{2+}) of the membrane to the intracellular side of the membrane (*i.e.*, since bath Na^+ concentration is essentially zero while Ca^{2+} concentration is 10 μM), and therefore the most likely event is the exit from $E_1 3N_i$ *via* the release of bound Na^+ and the subsequent binding of Ca^{2+}. Therefore, if KB-R7943 does indeed specifically target $E_1 3N_i$, the simplest explanation would be that it less opportunity to interact with $E_1 3N_i$ during forward mode exchange compared to reverse. While speculative, this explanation reasonably accounts for much of our data.

Similarly, the lack of mode selectivity for KB-R7943 under ionic conditions that allowed bi-directional transport (58) can also be explained using the consecutive transport mechanism scheme described above. As mentioned previously, the direction of Na^+-Ca^{2+} exchange is dependent on the concentrations of ions and the membrane voltage. However, the voltage dependence of the Na^+-Ca^{2+} exchanger is relatively weak (302), so ionic conditions primarily determine the relative populations of particular exchanger kinetic states. Since the concentrations of ions were kept constant on either side of the membrane the resulting distribution of exchanger states would be nearly identical for either mode of transport. Under these conditions it is expected that both forward and reverse exchange would be inhibited equally by KB-R7943. Therefore, perhaps the ionic conditions, in ours and other studies, are responsible for the observed preferential inhibition of Na^+-Ca^{2+} exchange by KB-R7943 (*i.e.*, reverse and forward exchange modes and their associated populations of particular exchange transport states may be responsible for KB-R7943's mode selectivity). In this regard, KB-R7943 can inhibit forward and reverse mode Na^+-Ca^{2+} exchange equally, but the presence of the relative

populations of exchanger states as dictated by experimental conditions will determine the effect of KB-R7943.

In order to investigate pure Na^+-Ca^{2+} exchange transport in giant, excised patches in the absence of ionic regulatory mechanisms (*i.e.*, I_1 and I_2), investigators routinely apply α-chymotrypsin to the cytoplasmic side of the patch (192;302). After about one minute of proteolysis, native ionic regulatory properties of Na^+-Ca^{2+} exchange currents are no longer observed, and exchangers appear to be fully activated (Figure 17) (95). Inhibition of outward exchange current when KB-R7943 was applied to α-chymotrypsin-treated patches was reduced, suggesting the ionic regulatory properties of the exchanger are not required for the majority of KB-R7943's effect (Figure 17). Yet, because of this reduction in potency, interaction between KB-R7943 and ionic regulatory properties of the exchanger cannot be ruled out. This is to be expected, since we speculated that KB-R7943 may target the $E_1 3N_i$ state, from which I_1 originates. Peak and de-regulated (*i.e.*, α-chymotrypsin treated) exchange currents do not become inactivated, as they do not enter I_1. However, over time, outward Na^+-Ca^{2+} exchange current decays from a peak to a steady-state level, reflecting entrance of more exchangers into the I_1 inactive state. Since KB-R7943 inhibits steady-state exchange currents more potently than peak exchange currents (Figure 13), the increased potency during steady-state may reflect a decrease in the number of $E_1 3N_i$ exchangers, as over time, more exchangers enter I_1. What is clear is that our data do not support a simple mechanism of inhibition represented by pure competitive interactions between KB-R7943 and transported Ca^{2+} or Na^+. Rather, KB-R7943's inhibitory effects are probably due to interactions with a specific transport state that is ultimately affected by ionic conditions present in the experimental model being

utilized. Further insight into KB-R7943's inhibitory mechanism will require the use of native (*i.e.*, Calx) or mutant Na^+-Ca^{2+} exchangers (*i.e.*, XIP mutants) with altered transport and/or ionic regulatory properties.

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