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Regulation of the Cardiac Na^+-Ca^{2+} Exchanger by Ca^{2+}

Mutational Analysis of the Ca^{2+}-binding Domain

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ABSTRACT The sarcolemmal Na^+-Ca^{2+} exchanger is regulated by intracellular Ca^{2+} at a high affinity Ca^{2+} binding site separate from the Ca^{2+} transport site. Previous data have suggested that the Ca^{2+} regulatory site is located on the large intracellular loop of the Na^+-Ca^{2+} exchange protein, and we have identified a high-affinity $^{45}Ca^{2+}$ binding domain on this loop (Levitsky, D. O., D. A. Nicoll, and K. D. Philipson. 1994. *Journal of Biological Chemistry.* 269:22847–22852). We now use electrophysiological and mutational analyses to further define the Ca^{2+} regulatory site. Wild-type and mutant exchangers were expressed in *Xenopus* oocytes, and the exchange current was measured using the inside-out giant membrane patch technique. Ca^{2+} regulation was measured as the stimulation of reverse Na^+-Ca^{2+} exchange (intracellular Na^+ exchanging for extracellular Ca^{2+}) by intracellular Ca^{2+}. Single-site mutations within two acidic clusters of the Ca^{2+} binding domain lowered the apparent Ca^{2+} affinity at the regulatory site from 0.4 to 1.1–1.8 μM. Mutations had parallel effects on the affinity of the exchanger loop for $^{45}Ca^{2+}$ binding (Levitsky et al., 1994) and for functional Ca^{2+} regulation. We conclude that we have identified the functionally important Ca^{2+} binding domain. All mutant exchangers with decreased apparent affinities at the regulatory Ca^{2+} binding site also have a complex pattern of altered kinetic properties. The outward current of the wild-type Na^+-Ca^{2+} exchanger declines with a half time (t_h) of 10.8 ± 3.2 s upon

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Ca^{2+} removal, whereas the exchange currents of several mutants decline with t_h values of 0.7–4.3 s. Likewise, Ca^{2+} regulation mutants respond more rapidly to Ca^{2+} application.

Study of Ca^{2+} regulation has previously been possible only with the exchanger operating in the reverse mode as the regulatory Ca^{2+} and the transported Ca^{2+} are then on opposite sides of the membrane. The use of exchange mutants with low affinity for Ca^{2+} at regulatory sites also allows demonstration of secondary Ca^{2+} regulation with the exchanger in the forward or Ca^{2+} efflux mode. In addition, we find that the affinity of wild-type and mutant Na^+-Ca^{2+} exchangers for intracellular Na^+ decreases at low regulatory Ca^{2+}. This suggests that Ca^{2+} regulation modifies transport properties and does not only control the fraction of exchangers in an active state.

INTRODUCTION

Na^+-Ca^{2+} exchange activity of the cardiac sarcolemmal membrane has an important role in controlling cytoplasmic Ca^{2+}, primarily as a Ca^{2+} extrusion mechanism (for reviews see Blaustein, DiPolo, and Reeves, 1991; Philipson and Nicoll, 1992). The cardiac exchanger was first found to be regulated by cytoplasmic Ca^{2+} in whole-cell recordings of the outward exchange current of intact myocytes (Kimura, Noma, and Irisawa, 1986). The regulation has been studied in single ventricular cells (Miura and Kimura, 1989; Noda, Shepherd, and Gadsby, 1988) and characterized in detail using giant patches of excised membranes (Hilgemann, 1990; Hilgemann, Collins, and Matsuoka, 1992a).

The canine cardiac Na^+-Ca^{2+} exchange protein has been cloned and expressed in *Xenopus* oocytes (Nicoll, Longoni, and Philipson, 1990). The protein is modeled to consist of two groups of transmembrane segments separated by a large intracellular loop. The loop comprises more than half of the exchanger protein by itself, but is not essential for transport. Deletion of a portion of the loop, however, completely eliminates secondary Ca^{2+} regulation (Matsuoka, Nicoll, Reilly, Hilgemann, and Philipson, 1993). The data suggest that the large cytoplasmic loop is involved in Ca^{2+} regulation and demonstrate that the binding site responsible for Ca^{2+} regulation can be functionally separated from the site which mediates Ca^{2+} transport.

Levitsky, Nicoll, and Philipson (1994) subsequently identified and characterized a region of the cytoplasmic loop of the exchanger which could bind $^{45}Ca^{2+}$ with high affinity. Several single-site mutations within this region markedly reduced Ca^{2+} binding affinity. The number of Ca^{2+} ions binding in this region was not quantified but could be more than one. It was hypothesized that this Ca^{2+} binding site, identified by biochemical techniques, was also the functionally important Ca^{2+} regulatory site.

To test this hypothesis, we performed electrophysiological studies of Na^+-Ca^{2+} exchangers with mutations in the putative Ca^{2+}-regulatory region. We demonstrate that the $^{45}Ca^{2+}$ binding region is indeed responsible for cytoplasmic Ca^{2+} regulation and we characterize the altered kinetic properties of Ca^{2+} binding mutants. In addition, we present evidence that both the Ca^{2+} influx and Ca^{2+} efflux modes of the exchanger are regulated by intracellular Ca^{2+}.

METHODS

Preparation of Mutant Na^+-Ca^{2+} Exchangers

Mutation reactions were performed as previously described (Levitsky et al., 1994) and the mutated cassettes were subcloned into full-length exchanger clones. Capped RNA was synthesized with T3 mMessage mMachine (Ambion, Austin, TX) after linearization with Hind III. Unincorporated nucleotides were removed on ChromaSpin-100 DEPC-H_2O columns (Clontech Laboratories, Palo Alto, CA).

Electrophysiology

Na^+-Ca^{2+} exchange currents were measured in inside-out giant excised patches as described previously (Matsuoka et al., 1993). In brief, RNA (46 nl) was injected into *Xenopus* oocytes and membrane currents were measured 3–6 d later. For electrophysiological studies, oocytes were placed in a hypertonic solution containing (in millimolar) KOH (100), MES (100), HEPES (20), EGTA (5), $Mg(OH)_2$ (5), K-aspartate (100), pH = 7.0 adjusted with MES to permit removal of the vitellin layer. Oocytes were then moved to a second solution containing KOH (100), MES (100), HEPES (20), EGTA (5), $Mg(OH)_2$ (5) or $MgCl_2$ (5), pH 7.0 for seal formation. Glass pipettes (inner diameter 20–35 μM) were coated with a parafilm/light mineral oil (Sigma Chemical Co., St. Louis, MO) mixture (Collins, Somlyo, and Hilgemann, 1992). *n*-Decane (Sigma Chemical Co.; 1–5%) was occasionally added to the mixture to facilitate seal formation.

TABLE I

Experimental Solutions

	Cytoplasmic solution	Pipette solution A	Pipette solution B
	mM	*mM*	*mM*
NaOH	—	—	140
CsOH	20	20	20
NaOH + CsOH	100	—	—
EGTA	10	—	10
$CaCO_3$	0–10	8	—
$Mg(OH)_2$	1–1.5	2	4
TEA-OH	20	20	20
HEPES	20	20	10
Ouabain	—	0.25	0.25
$Ba(OH)_2$	—	2	2
MES	100	100	140
N-methyl-D-glucamine	—	100	—

Membrane currents were measured using an Axopatch-1C or -1D amplifier (Axon Instruments, Inc., Foster City, CA) and recorded by Axotape software (acquisition rate 30 Hz). Experiments were carried out at 30°C.

Experimental Solutions

Experimental solutions were similar to those previously described (Matsuoka et al., 1994) and are listed in Table I. The pH of all solutions was adjusted to 7.0 with MES. Pipette solutions A and B were used for outward and inward Na^+-Ca^{2+} exchange current measurements, respectively. To minimize proteolysis (see Results), protease inhibitors (leupeptin, 2 μg/ml, aprotinin,

1 μg/ml and pepstatin, 2 μg/ml; Boehringer Mannheim Corp., Indianapolis, IN) were added to the initial bathing solution and cytoplasmic solutions. Outward Na^+-Ca^{2+} exchange currents were well regulated by cytoplasmic Ca^{2+} during the experimental protocol (20–30 min). Free Ca^{2+} and Mg^{2+} concentrations were calculated using MAXC software (Bers, Patton, and Nuccitelli, 1994). The free Mg^{2+} concentration was 1 mM for all cytoplasmic solutions. To suppress the endogenous Ca^{2+} activated Cl^- current, 0.1 mM niflumic acid (Sigma Chemical Co.; 200 mM stock solution in DMSO) was added to the pipette solutions and chloride was replaced with MES in all cytoplasmic and pipette solutions.

For measurements of outward Na^+-Ca^{2+} currents, background Ca^{2+}-sensitive currents were first assessed by applying 1 μM cytoplasmic Ca^{2+} in the absence of Na^+. Current amplitudes were typically 0–10 pA and ran down quickly. For inward current measurements, background Ca^{2+}-sensitive currents were estimated by applying 1 μM Ca^{2+} in the presence of 100 mM Na^+. Na^+ (100 mM) has previously been reported to completely suppress the inward Na^+-Ca^{2+} exchange current activated by 1 μM Ca^{2+} (Hilgemann, Matsuoka, Nagel, and Collins, 1992*b*). Patches were usually formed in Cl^- free bath solution to avoid possible Cl^- contamination of the pipette solution during seal formation. Background Ca^{2+}-sensitive currents were typically 0–5 pA and they declined rapidly.

RESULTS

Outward Na^+-Ca^{2+} Exchange Currents from Wild-Type and Mutant Exchangers

Levitsky et al. (1994) demonstrated the presence of a high affinity Ca^{2+}-binding domain encompassed by amino acids 371 to 508 of the cytoplasmic loop of the Na^+-Ca^{2+} exchanger. Mutations of two acidic segments within this domain affected $^{45}Ca^{2+}$ binding. Fig. 1 shows the amino acid sequences of these two acidic segments. Both segments have a motif of three consecutive aspartic acid residues (DDD) and the mutants analyzed below are shown. Correlations between the effects of mutations on $^{45}Ca^{2+}$ binding (Levitsky et al., 1994) and on exchange currents are presented in Table II and in the Discussion.

Fig. 2 shows representative outward Na^+-Ca^{2+} exchange currents from the wild-type and three mutant exchangers. The pipette contained solution A (8 mM Ca^{2+} and 0 mM Na^+). Outward current was activated by applying 100 mM Na^+ to the cytoplasmic surface of the excised oocyte membrane. Currents at different levels of cytoplasmic regulatory Ca^{2+} (0, 0.3, 1, and 10 μM) are superimposed. For the wild-type exchanger, outward exchange current has a transient component due to Na^+-dependent, or I_1, inactivation as previously reported (Hilgemann, 1990). Stimulation of current by regulatory Ca^{2+} is modeled as the removal of a second type of inactivation (I_2) which is Na^+ independent, though I_1 inactivation can also be modulated by Ca^{2+} (see Fig. 12 below). Both types of inactivation can be removed by treatment with chymotrypsin which puts the exchanger into an activated, or deregulated, state. In this study, we are primarily concerned with the I_2 form of regulation (activation of exchange current by Ca^{2+}). I_1 inactivation occurs over several seconds after Na^+ application (Fig. 2), and I_2 can be assessed by measuring peak current just after Na^+ application before the occurrence of substantial I_1 inactivation.

With increasing cytoplasmic Ca^{2+}, the amplitude of peak and steady state currents increased for both wild-type and all mutant exchangers. The mutant E509L showed characteristics similar to those of the wild-type exchanger. Peak current at 1 μM Ca^{2+}

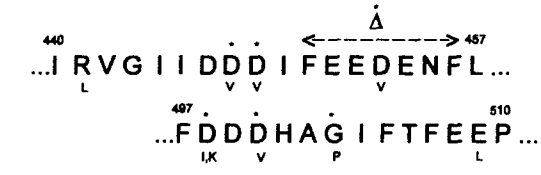

FIGURE 1. Amino acid sequences of two acidic segments in the large cytoplasmic loop of the Na^+-Ca^{2+} exchanger. Amino acids indicated by small capitals represent point mutations; e.g., arginine at position 441 to leucine (R441L). A deletion mutant (Δ450–56) is denoted by the dashed line. Mutants marked with asterisks had altered Ca^{2+} regulation.

was ~80% of the current at 10 μM Ca^{2+} and activation by Na^+ was rapid. Mutant exchangers R441L and D453V also had unaltered behavior (not shown).

In contrast, altered kinetic characteristics were observed for mutants D448V and D500V. Upon Na^+ application, the outward current was activated slowly at 0.3 and 1 μM Ca^{2+}. The activation became faster as Ca^{2+} concentration increased and became almost instantaneous between 10 and 100 μM. In these mutants, current amplitude at 1 μM Ca^{2+} was about half of that at 10 μM Ca^{2+}, and the Na^+-dependent inactivation was slower than that of the wild-type Na^+-Ca^{2+} exchanger. The mutants D447V, D498K, D498I, D447V + D498I, and Δ450–456 all exhibited these aberrant properties (not shown). The level of expression of the wild-type and mutant exchangers was variable in different batches of oocytes. Mutant D500V tended to generate higher exchange currents (e.g., see Fig. 2) than other exchangers. We are uncertain as to whether this is due to an increase in the number of functional exchangers or whether mutant D500V molecules have an inherently higher exchange activity. In any case, the characteristics of different exchangers did not vary with level of expression.

TABLE II

Properties of Ca^{2+} Regulation

Exchanger	K_h (μM)	t_h (s) upon Ca^{2+} removal	t_h (s) upon Ca^{2+} addition	$^{45}Ca^{2+}$ binding
GROUP A				
Wild-type	0.4 ± 0.2 (6)	10.8 ± 3.2 (4)	7.5 ± 1.5 (4)	High
R441L	0.5 ± 0.3 (3)	14.2 ± 0.9 (2)	8.8 ± 1.1 (2)	High
D453V	0.2 ± 0.0 (2)	9.8 ± 1.6 (4)	9.4 ± 3.6 (3)	High
G503P	0.3 ± 0.3 (3)			High
E509L	0.4 ± 0.1 (2)	11.9 ± 3.3 (4)	9.2 ± 4.5 (3)	High
GROUP B				
D447V	1.8 ± 0.6 (3)	1.1 ± 0.7 (5)	2.0 ± 0.3 (4)	Low
D448V	1.7 ± 0.7 (3)	4.3 ± 1.0 (4)	11.0 ± 5.8 (3)	Low
Δ450-456	1.6 ± 0.2 (3)	0.9 ± 0.6 (5)	1.3 ± 0.6 (4)	Low
D498I	1.3 ± 0.4 (7)	1.9 ± 0.8 (4)	5.1 ± 2.4 (5)	Low
D498K	1.4 ± 0.6 (3)	1.1 ± 0.4 (3)	3.8 ± 0.9 (2)	Low
D500V	1.7 ± 0.3 (3)	0.7 ± 0.1 (5)	1.2 ± 0.3 (3)	
D447V+ D498I	1.1 ± 0.6 (3)	1.2 ± 0.3 (6)	2.4 ± 0.8 (5)	Low

All values are given as means ± SD. The number of experiments (n) is given in parentheses. $^{45}Ca^{2+}$ binding data are from Levitsky et al. (1994).

In the absence of cytoplasmic Ca^{2+}, a small but significant outward current was activated by 100 mM Na^+ for all exchangers. In water-injected control oocytes, 100 mM Na^+ induced only a small inward shift of current (0–3 pA; not shown). The Ca^{2+}-insensitive current, therefore, is due to exchange activity. The Ca^{2+}-insensitive component was more substantial in our previous report (Matsuoka et al., 1993). Here, we included protease inhibitors in the cytoplasmic (bath) solutions (see Methods) which may have reduced partial deregulation which occurred in the prior study.

The extent of the initial (I_1) inactivation of outward exchange current was somewhat variable. Nevertheless, one trend was quite clear. All mutant exchangers with altered Ca^{2+} regulation had a reduced transient component. This is seen for mutants D448V and D500V in Fig. 2 and for D498I in Fig. 10. The inactivation of the current from the wild-type exchanger in Fig. 2 is smaller than usual (compare with the results shown in Fig. 10 or with results from the wild-type–like mutant E509L in Fig. 2). The I_1 inactivation process of the wild-type Na^+-Ca^{2+} exchanger expressed in

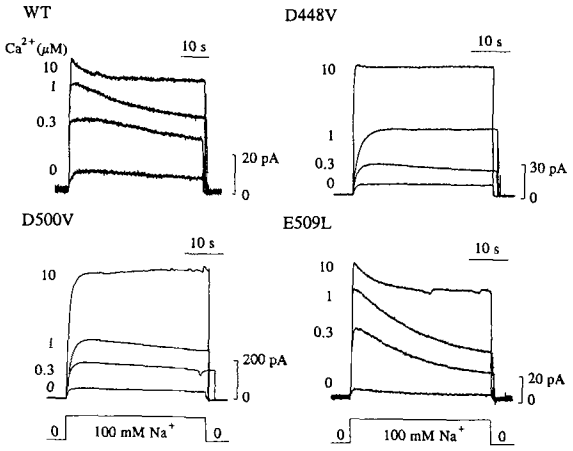

FIGURE 2. Representative outward currents from the wild-type (WT), D448V, D500V, and E509L Na^+-Ca^{2+} exchangers. Solution A (8 mM Ca^{2+}) was present in the pipette. Currents were activated by 100 mM Na^+ at four different cytoplasmic Ca^{2+} concentrations (0, 0.3, 1, and 10 μM) as indicated.

oocytes is less pronounced than that observed for the native exchanger in sarcolemmal patches (Hilgemann et al., 1992*b*). Perhaps this suggests that there are extrinsic factors present in the cardiac cells which modulate exchanger function.

Cytoplasmic Ca^{2+} Dependence of Wild-Type and Mutant Na^+-Ca^{2+} Exchangers

Fig. 3 shows representative cytoplasmic Ca^{2+} dependencies of outward Na^+-Ca^{2+} exchange currents for the wild-type, D448V, D500V, and E509L exchanger mutants. Again, the transported Ca^{2+} is at the extracellular surface within the patch pipette and only Ca^{2+} binding at the intracellular regulatory site is being varied. Data were fit to the Hill equation and normalized to maximum current. Current amplitudes always declined at the highest Ca^{2+} levels as Ca^{2+} competes with Na^+ at the transport site (Matsuoka and Hilgemann, 1992). For the wild-type, D448V, D500V, and E509L exchangers the K_h (Ca^{2+} concentration at half maximal activation) values were 0.3, 1.1, 1.4 and 0.3 μM and the corresponding Hill coefficients were calculated as 0.9, 1.5, 1.0, and 1.1, respectively.

A summary of the K_h values of the wild-type and mutant exchangers is shown in Fig. 4. Mutants could be assigned to two groups based upon apparent affinities for regulatory Ca^{2+} (Fig. 4 and Table II, column 2). Exchangers in group A, which includes the wild-type exchanger, had relatively high apparent affinities of 0.5 μM Ca^{2+} or less. Mutant exchangers in group B had lower apparent affinities of 1.1 μM Ca^{2+} or higher. It is notable that only single site mutations of aspartic acid residues of the DDD motifs induced a decrease in apparent Ca^{2+} affinity. As shown in Fig. 3, high Ca^{2+} levels inhibited exchange currents due to competition with Na^+ at the transport site. This inhibition tends to negate the activation of exchange by Ca^{2+} at the regulatory site at higher Ca^{2+} levels. The net effect is to lower the K_h values; the effect will be greatest for the group B mutants for which inhibition may begin to occur before the Ca^{2+} regulatory site is saturated. Thus, the affinity for regulatory Ca^{2+} for group B mutants may be substantially lower than the observed K_h values.

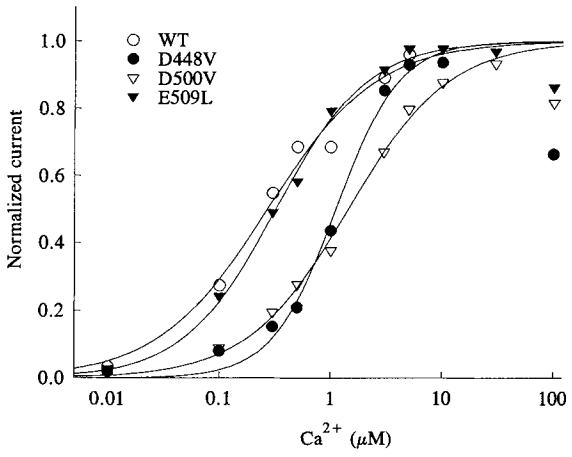

FIGURE 3. Representative dependencies on cytoplasmic Ca^{2+} of outward currents for the wild-type and various mutant exchangers. Data are from wild-type (*open circles*), D448V (*closed circles*), D500V (*open triangles*), and E509L (*closed triangles*). Current amplitudes were measured at peak current and the Ca^{2+}-insensitive current was subtracted. Data were normalized to the maximal current and the solid curves represent fits to the Hill equation. As shown by the data points, current declined at 100 μM Ca^{2+} due to competition between Na^+ and Ca^{2+} at transport sites (see text). These data were not included in the analysis.

Mutant exchanger G503P was distinctive in that the outward exchange current displayed a large Ca^{2+}-insensitive component which constituted 80–100% of the total current. In this case, the Ca^{2+}-sensitive component, when present, still had an apparent Ca^{2+} affinity similar to that of the wild-type exchanger.

Time Course of Current Change on Removing and Applying Cytoplasmic Ca^{2+}

Mutations which affect the apparent affinity for Ca^{2+} binding might also affect the kinetics of Ca^{2+} binding and regulation. We therefore measured the time courses of the changes in outward exchange currents upon removing and applying cytoplasmic regulatory Ca^{2+}.

Fig. 5 shows representative current traces of the decline in outward current upon removal of cytoplasmic Ca^{2+} for the wild-type, D448V, D500V, and E509L exchangers. The outward currents were first activated by 100 mM Na^+ in the presence of 1

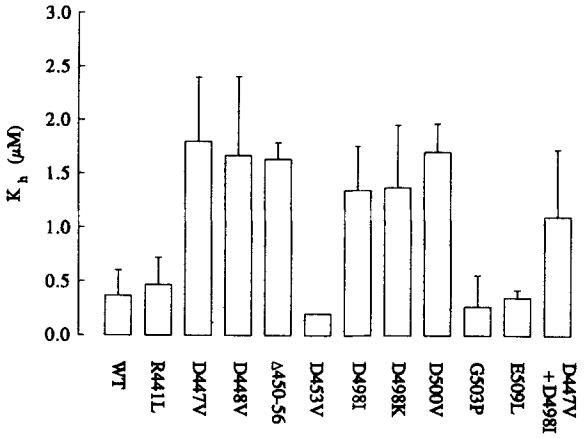

FIGURE 4. Summary of K_h values for regulatory Ca^{2+} for the wild-type and various mutant Na^+-Ca^{2+} exchangers. Data are shown as means ± SD.

μM Ca^{2+} . When current was at steady state level, cytoplasmic Ca^{2+} was removed as indicated. Half times (t_h) for the current changes were 12.4, 3.4, 0.8, and 9.6 s, respectively. The data could not easily be fit to a single exponential, and fast and slow components appear to exist. The origin of the different components is unknown, but perhaps reflects the presence of multiple active exchanger states.

Fig. 6 (*top*) and Table II (column 3) summarize the t_h values for the current decline for wild-type and mutant exchangers. For mutant exchangers D447V, Δ450–56, D500V, and D447V + D498I, the current changes were sufficiently fast as to possibly be limited by the rapidity of the solution change. There is a strong inverse correlation for the different exchangers between the apparent affinity for regulatory Ca^{2+} (K_h)

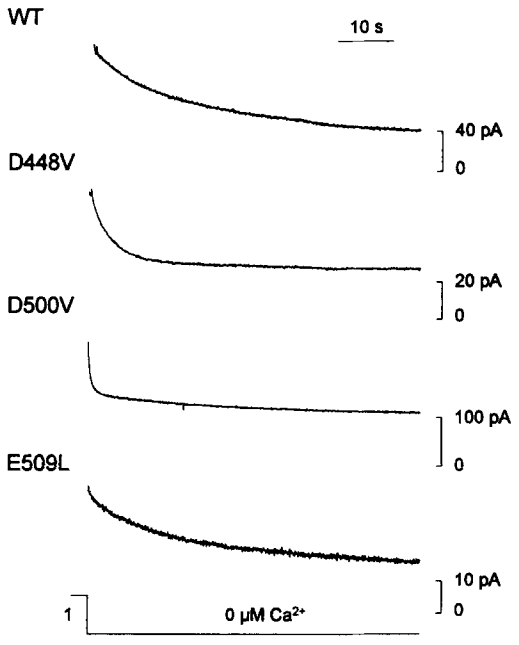

FIGURE 5. Representative current traces obtained upon removal of regulatory (cytoplasmic) Ca^{2+}. The outward Na^+-Ca^{2+} exchange current was first activated by 100 mM Na^+ in the presence of 1 μM Ca^{2+} (not shown). After currents reached steady state levels, cytoplasmic Ca^{2+} was removed resulting in the decay of current as shown. Currents are from the wild-type, D448V, D500V, and E509L exchangers, as indicated.

and the t_h values for current upon Ca^{2+} removal (Table II). The data are consistent with the idea that an enhanced off rate contributes to the lower Ca^{2+} affinity of mutants with altered Ca^{2+} regulation.

Fig. 6 (*bottom*) and Table II (column 4) summarize the t_h values for the increase in exchange current upon applying 1 μM Ca^{2+}. Na^+ (100 mM) was already present in the bath medium when the regulatory Ca^{2+} was added. Exchangers that responded rapidly to the addition of regulatory Ca^{2+} (Fig. 6, *bottom*) also responded rapidly to the removal of regulatory Ca^{2+} (Fig. 6, *top*) with the exception of mutant D448V.

Regulation of Inward Na^+-Ca^{2+} Exchange Currents by Cytoplasmic Ca^{2+}

Manifestation of Ca^{2+} regulation of Na^+-Ca^{2+} exchange is most readily observed for outward currents where the exchanger operates in the "reverse" mode. In this case, the transported Ca^{2+} and the regulatory Ca^{2+} are on opposite sides of the membrane and regulatory effects of Ca^{2+} are apparent. With the exchanger in the "forward"

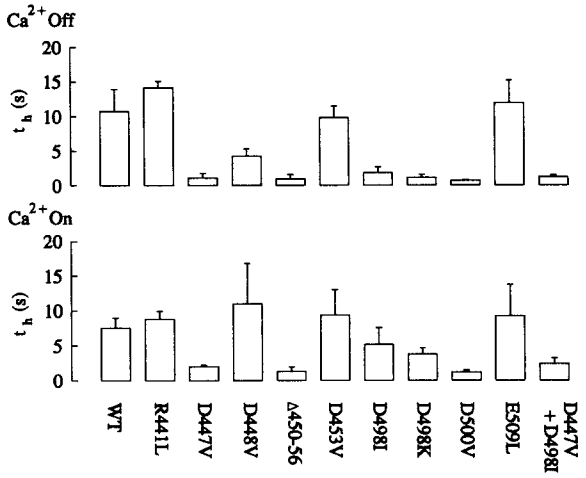

FIGURE 6. Summary of half times (t_h) of current decay (Ca^{2+} Off) and current development (Ca^{2+} On) of wild-type and mutant Na^+-Ca^{2+} exchangers during the removal and reapplication of 1 μM regulatory Ca^{2+}. Pipette Ca^{2+} and bath Na^+ were present at all times; only the level of regulatory Ca^{2+} was changed. Data are shown as means ± SD.

mode, however, the transported and regulatory Ca^{2+} are at the same membrane surface. Changes in the Ca^{2+} level will alter both transport and regulation and the dual effects are not easily separated. Thus, it is not known whether the forward (Ca^{2+} efflux) mode of the exchanger is Ca^{2+} regulated. The Ca^{2+} regulation mutants afforded an opportunity to investigate this possibility.

Fig. 7 shows typical inward exchange currents for the wild-type and the double mutant (D447V + D498I) exchangers. Within the pipette was solution B (140 mM Na^+ and 0 mM Ca^{2+}). Currents were activated with increasing levels of cytoplasmic Ca^{2+}. The time course of current activation was similar for wild-type and mutant D447V + D498I, as shown, and also for mutants D447V and D500V (not shown). Inward currents for other mutants were not examined. The results contrast with those obtained with outward currents (Fig. 2) where activation of currents with Na^+ was significantly slower for mutants D447V, D500V, and D447V + D498I.

The Ca^{2+} dependence of the inward current of the wild-type Na^+-Ca^{2+} exchanger

is shown in Fig. 8 (*top*). The K_h was 6.9 μM and the Hill coefficient was 1.1 (n = 6). The K_h value might reflect either regulatory or transport Ca^{2+} dependencies. However, the apparent affinity at the regulatory site was estimated above (Fig. 4) to be 0.4 μM Ca^{2+} and the K_h value for inward current would be dominated by a lower Ca^{2+} affinity at the transport site. α-chymotrypsin treatment eliminates Ca^{2+} regulation (Hilgemann, 1990) and allows Ca^{2+} dependence for transport to be measured in the absence of regulation. After α-chymotrypsin treatment, the K_h for Ca^{2+} decreased only slightly to 5.6 μM (n = 3). These data are consistent with measurements from giant membrane patches of guinea pig ventricular cells (Hilgemann et al., 1992*a*).

For the mutant exchanger D447V + D498I, however, the apparent affinity for Ca^{2+} activation of inward Na^+-Ca^{2+} exchange current was much lower than for the wild-type exchanger. The K_h was 46 μM with a Hill coefficient of 1.1 (Fig. 8, *bottom*,

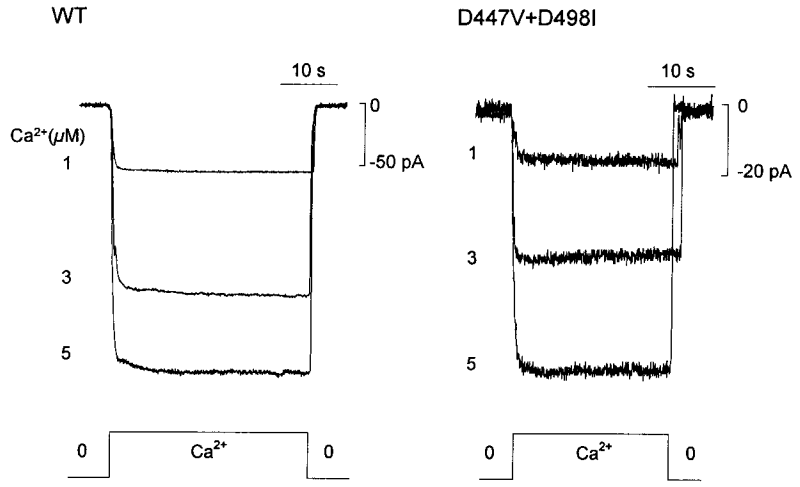

FIGURE 7. Inward currents from the wild-type (WT) and double mutant (D447V + D498I) Na^+-Ca^{2+} exchangers. Solution B (140 mM Na^+) was present in the pipette. Currents were activated by increasing cytoplasmic Ca^{2+} from 0 to 1, 3 or 5 μM. Note the fast activation of current by Ca^{2+} for both exchangers.

n = 4). This K_h value is 6.7-fold higher than that observed for the wild-type exchanger. After α-chymotrypsin treatment, the K_h value decreased to 5.0 μM (n = 3) with a Hill coefficient of 0.9. That is, the K_h value became similar to that of the wild-type exchanger.

For the mutant, the K_h value for activation at the regulatory site was estimated from outward exchange currents (Fig. 4) to be higher than for the wild-type exchanger. As pointed out above, the K_h values for Ca^{2+} to activate the outward current are underestimated for mutants of group B, because of competition of Ca^{2+} with Na^+ at the transport site. This may at least partially account for the discrepancy between the K_h values for regulatory Ca^{2+} obtained from outward currents (1.1 μM) and from inward currents (46 μM) for the mutant D447V + D498I. Indeed, in the study of Levitsky et al. (1994), it appeared that the double mutant D447V + D498I had an

especially low affinity for $^{45}Ca^{2+}$ at the regulatory site. Thus, for D447V + D498I, we interpret the low affinity for Ca^{2+} activation of inward current to be due to the low affinity for Ca^{2+} binding at the regulatory site. After α-chymotrypsin treatment, Ca^{2+} regulation is removed and activation of inward current by Ca^{2+} reflects only affinity at the transport site. The data demonstrate that the inward (forward) mode of Na^+-Ca^{2+} exchange current is regulated by Ca^{2+}. This conclusion applies to mutant D447V + D498I but presumably applies also to the wild-type exchanger.

Activation of Outward Exchange Current by Na^+

One difference between mutants in group B and the wild-type exchanger is the slow activation of the outward current upon application of cytoplasmic Na^+ (Fig. 2). As shown in Fig. 9, α-chymotrypsin treatment eliminates this slow activation. The

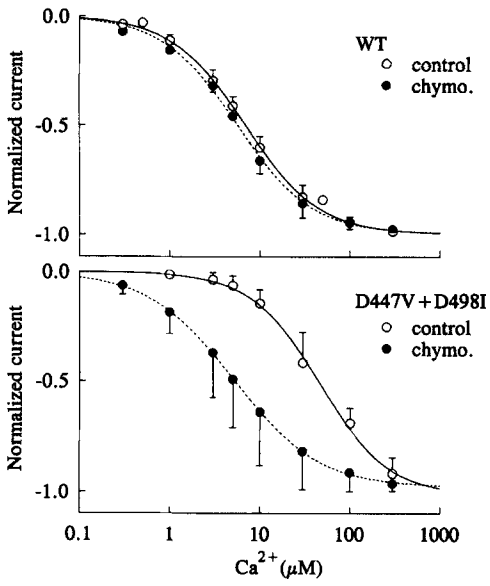

FIGURE 8. Ca^{2+} dependence of inward currents from wild-type (WT) and D447V + D498I. Current amplitudes were normalized to the fit maximal current using the Hill equation and are shown as means ± SD. Currents were measured under control conditions (*open circles*) and after treatment with 1–2 mg/ml α-chymotrypsin (*closed circles*).

outward current for mutant D447V + D498I was first activated by 100 mM Na^+ in the presence of cytoplasmic Ca^{2+}. Slow current activation by Na^+ was similar to that shown for mutants D448V and D500V (Fig. 2). After α-chymotrypsin treatment, the current was activated immediately by 100 mM Na^+ with no requirement for cytoplasmic Ca^{2+}. α-chymotrypsin had the same effect on mutants D500V and D498I (not shown). The results suggest that the slow activation by Na^+ is related to the abnormal Ca^{2+} regulation. We therefore further investigated Na^+ effects on the mutant exchangers.

The cytoplasmic Na^+ dependence for outward exchange current was examined at different levels of cytoplasmic regulatory Ca^{2+}. For both the wild-type and a mutant exchanger, Ca^{2+} levels above and below the K_h value were chosen. In the top of Fig. 10, the cytoplasmic Na^+ dependence of the wild-type exchanger was studied at 1 (*left*) and 0.01 μM Ca^{2+} (*right*) for the same excised patch. At 1 μM Ca^{2+}, the current

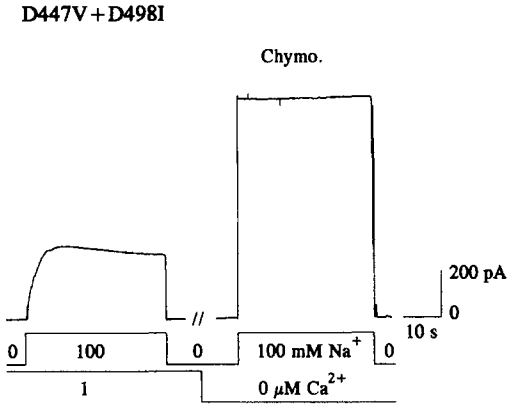

FIGURE 9. The effect of α-chymotrypsin on the double mutant, D447V + D498I. The outward current was first activated by 100 mM Na^+ in the presence of 1 μM Ca^{2+} (*left*) before 2 mg/ml α-chymotrypsin was applied to the cytoplasmic surface. The right panel shows the current after α-chymotrypsin treatment. Note the immediate activation by 100 mM Na^+ and the loss of Ca^{2+} regulation.

activated by 50 mM Na^+ was ~80% of the current activated by 100 mM Na^+, and the Na^+-dependent inactivation became less pronounced as the Na^+ concentration was decreased. These tendencies are consistent with data from giant membrane patches from guinea pig ventricular cells (Hilgemann et al., 1992*b*). At 0.01 μM Ca^{2+}, current

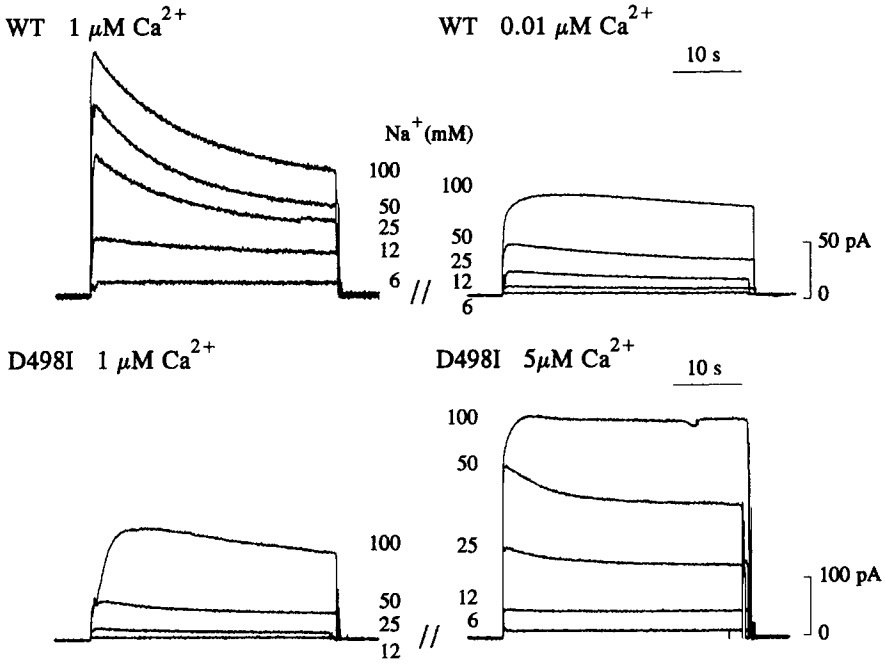

FIGURE 10. Outward currents for the wild-type (*top*) and D498I Na^+-Ca^{2+} exchangers (*bottom*) at different Na^+ and Ca^{2+} concentrations. Currents were activated by different levels of cytoplasmic Na^+ as indicated. For the wild-type exchanger, currents were measured at 1 (*left*) and 0.01 μM Ca^{2+} (*right*). Note the slow development of outward current activated by 100 mM Na^+ at the lower Ca^{2+} concentration. For D498I, currents were measured at 1 (*left*) and 5 μM Ca^{2+} (*right*). At 1 μM Ca^{2+}, currents in D498I are similar to those of WT at 0.01 μM Ca^{2+}. At higher Ca (5 μM), currents for D498I appear similar to WT at 1 μM Ca^{2+}.

activation by 100 mM Na^+ was slower than at 1 μM Ca^{2+}. Currents at low Na^+ and Ca^{2+} for this patch were relatively large as the exchangers were partially deregulated. The partial deregulation does not appear to have affected the results, however, as similar results were obtained with several other patches (see below).

In the bottom of Fig. 10, the Na^+ dependence of a group B mutant, D498I, was determined at 1 (*left*) and 5 μM Ca^{2+} (*right*). The activation by 100 mM Na^+ at 1 μM Ca^{2+} is slow, similar to other mutants in group B (Figs. 2 and 9). This tendency is similar to that for wild-type exchange currents at 0.01 μM Ca^{2+}. The same patch was then exposed to 5 μM Ca^{2+} and Na^+ dependence was examined in the lower right panel. Current activation by 100 mM Na^+ was faster than at 1 μM Ca^{2+}.

We found that the cytoplasmic Na^+ dependence varied with the level of regulatory Ca^{2+}. In Fig. 11, peak current amplitudes are plotted against Na^+ concentration at

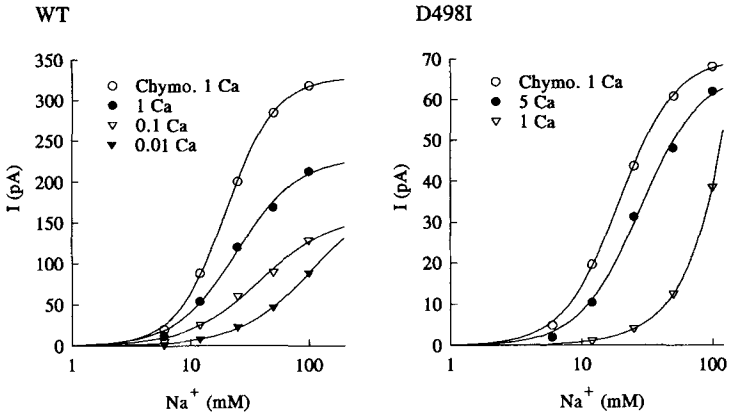

FIGURE 11. Na^+ dependence of the outward current from the wild-type (*left*) and D498I (*right*) Na^+-Ca^{2+} exchangers. Current amplitudes were measured at the current peak. For the wild-type exchanger, the current was measured at 1 μM Ca^{2+} (*closed circles*), 0.1 μM Ca^{2+} (*open triangles*), 0.01 μM Ca^{2+} (*closed triangles*), and at 1 μM Ca^{2+} after α-chymotrypsin treatment (*open circles*). For the D498I mutant, the current was measured at 1 μM Ca^{2+} (*open triangles*), 5 μM Ca^{2+} (*closed circles*) and at 1 μM Ca^{2+} after α-chymotrypsin treatment (*open circles*). Data are fit to the Hill equation.

different Ca^{2+} levels and also after α-chymotrypsin treatment. The K_h values for Na^+ increased as the level of regulatory Ca^{2+} decreased. For the wild-type exchanger, the K_h values were 109, 39, and 25 mM Na^+ at 0.01, 0.1, and 1 μM Ca^{2+}, respectively. After α-chymotrypsin treatment, the K_h values for Na^+ were 20 mM in the presence of 1 μM Ca^{2+} and 18 mM in the absence of Ca^{2+} (not shown). Similar results were obtained with patches containing the wild-type (n = 3) and two group A mutants, D453V (n = 3) and R441L (n = 4). For mutant exchanger D498I, the K_h values for Na^+ were 510, 28, and 20 mM at 1 μM Ca^{2+}, 5 μM Ca^{2+} and after α-chymotrypsin treatment, respectively. Hill coefficients for all curves in Fig. 11 ranged from 1.4 to 2.1. Consistent results were obtained from another patch containing D498I and from the other group B mutant exchangers, D448V (n = 3) and D500V (n = 3). K_h values for Na^+ were obtained by curve fitting and values greater than 100 mM are unlikely

to be quantitatively accurate as no Na^+ levels above 100 mM were used in the experiments. Nevertheless, the tendency of the K_h value for Na^+ to increase as Ca^{2+} was decreased was always qualitatively clear.

DISCUSSION

Intracellular Ca^{2+} regulates activity of the cardiac Na^+-Ca^{2+} exchanger at a binding site separate from the Ca^{2+} transport site. Experimentally Ca^{2+} regulation is most easily studied in inside-out giant excised patches with the exchanger in the reverse mode (extracellular Ca^{2+} exchanging for intracellular Na^+). For optimal exchange activity, Na^+ and Ca^{2+} must be on opposite sides of the patch membrane but trace Ca^{2+} must also be in the bath medium. Regulation of exchanger activity by Ca^{2+} is demonstrated in Fig. 2. The stimulatory effect of regulatory Ca^{2+} has been modeled in detail as the removal of a form of inhibition referred to as I_2 inactivation (Hilgemann et al., 1992*a*). The effects of regulatory Ca^{2+} are complicated by the fact

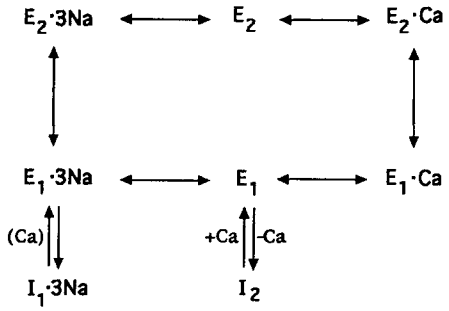

FIGURE 12. Transport cycle and regulation of the Na^+-Ca^{2+} exchanger. The ion binding sites alternately face the intracellular or extracellular medium in the E_1 or E_2 states, respectively. The reaction mechanism is consecutive with three Na^+ ions or one Ca^{2+} ion being translocated in separate reaction steps. The exchanger can enter two inactivated states, I_1 and I_2. The binding of three Na^+ ions at the cytoplasmic surface leads to the E_1·3Na state from which a fraction of the exchangers enters the I_1 inactivated state. The population of I_1 exchangers is modulated by Ca^{2+} as indicated by Ca in parentheses. The presence of Ca^{2+} will tend to decrease occupancy of the I_1 state. In contrast, I_2 (Na^+-independent) inactivation is directly controlled by the binding of Ca^{2+} at a regulatory site. For detailed analyses of I_1 and I_2 inactivation, see Hilgemann et al., 1992*a*,*b*; Matsuoka and Hilgemann, 1994.

that Ca^{2+} also has a modulatory influence on a second form of inactivation, I_1 or Na^+-dependent inactivation (Hilgemann et al., 1992*a*). Thus, for example, higher levels of regulatory Ca^{2+} tend to decrease the magnitude of I_1 inactivation. This is shown schematically in Fig. 12.

The cloning of the Na^+-Ca^{2+} exchanger (Nicoll et al., 1990) has facilitated the study of Ca^{2+} regulation. Analysis of mutants expressed in *Xenopus* oocytes initially suggested that the large intracellular domain of the exchanger was involved in Ca^{2+} regulation (Matsuoka et al., 1993). Subsequently, using biochemical techniques, we found that a region of the intracellular loop bound $^{45}Ca^{2+}$ with high affinity (Levitsky et al., 1994). The Ca^{2+} binding region extended from amino acid 371 to amino acid 508. Mutations of certain aspartic acid residues within two acidic segments (Fig. 1 and Table II) markedly decreased Ca^{2+} binding. Here, we address the question of whether the biochemically determined Ca^{2+} binding site is also the functionally

important Ca^{2+} regulatory site. Additionally, we examine the functional consequences of mutations within this region.

The data demonstrate that the Ca^{2+} binding region is the site of Ca^{2+} regulation. There is an excellent correlation between effects of mutations on Ca^{2+} binding and on Ca^{2+} regulation. As shown in Table II, all exchangers with reduced Ca^{2+} binding also had reduced affinity for Ca^{2+} regulation. We conclude that we have identified the Ca^{2+} binding site responsible for secondary Ca^{2+} regulation of the Na^+-Ca^{2+} exchanger.

Outward Na^+-Ca^{2+} exchange current declines and becomes inactivated when regulatory Ca^{2+} is removed from the bath (intracellular) medium (Figs. 5 and 6; Table II). Mutation of the Ca^{2+} binding region altered the kinetics of the inactivation. For mutants with decreased Ca^{2+} affinity (group B), inactivation was much more rapid. This is consistent with a more rapid dissociation of Ca^{2+} from a binding site with decreased Ca^{2+} affinity. However, the time courses are relatively slow (e.g., t_h = 10.8 s for the wild-type exchanger) and the rate limiting step is perhaps more likely to be a slow conformational change subsequent to the Ca^{2+} dissociation step; mutants with altered Ca^{2+} binding sites may undergo these conformational changes more rapidly. In any case, the correlation between apparent Ca^{2+} affinities and inactivation rates is quite striking.

Outward Na^+-Ca^{2+} exchange currents can also be activated by the application of regulatory Ca^{2+} to an intracellular bathing medium which already contains Na^+ (Fig. 6, bottom; Table II). Again, altered kinetics are induced by mutation of the Ca^{2+} binding site. Group B exchangers activate more rapidly than group A exchangers. Here, a possible interpretation is even more complex as the rate of net Ca^{2+} binding will depend on both the association and dissociation rate constants, and the rate of exchanger activation probably again depends on slow conformational changes. An increase in the Ca^{2+} dissociation rate constant would by itself lead to an increase in the rate of net Ca^{2+} binding in the absence of any change in the Ca^{2+} association rate constant.

Modulation of I_1 (Na^+-dependent) inactivation by Ca^{2+} may also play a role. That is, for the wild-type exchanger, Ca^{2+} has a direct regulatory effect by relieving I_2 inactivation but also decreases the extent of Na^+-dependent (I_1) inactivation over a period of several seconds (Hilgemann et al., 1992*a*,*b*), presumably due to a slow conformational change of the protein. Mutations of the Ca^{2+} binding site could alter the kinetics of activation and inactivation of exchange currents by modulating I_1 inactivation. Indeed, Na^+-dependent inactivation was consistently much less pronounced in exchanger mutants with reduced Ca^{2+} affinity. This is seen, for example, in Figs. 2 and 10 where the transient component of current for the wild-type exchanger is almost absent for group B mutants D448V, D498I, and D500V. Since the response of exchangers in the I_1 inactivated state to Ca^{2+} is slow, the response to Ca^{2+} of mutants with diminished Na^+-dependent inactivation (I_1) would be more rapid and this may contribute to the observed effects. The results suggest that the modulation of I_1 inactivation by Ca^{2+} is due to the same Ca^{2+} binding site which controls I_2 inactivation. Overall, regulation of the exchanger system displays a high degree of complexity.

The activation of outward current upon application of Na^+ to exchangers preincu-

bated with regulatory Ca^{2+} was altered for mutants in group B; activation was slower than that for the wild-type exchanger (Fig. 2). We hypothesize that the slow activation is related to abnormal Ca^{2+} regulation. Perhaps, for mutants with low Ca^{2+} affinity, Na^+ is able to interact with the Ca^{2+} regulatory site to produce the slow activation but further work is needed to clarify this mechanism. In contrast, activation of inward current by Ca^{2+} was rapid for both mutant and wild-type exchangers (Fig. 7). In this case, no Na^+ is present at the intracellular surface. No Na^+-dependent inactivation will occur and the rapid response is consistent with the interpretation that Ca^{2+} regulation is slowed by I_1 inactivation.

Interestingly, we have recently cloned a second Na^+-Ca^{2+} exchanger isoform (NCX2; Li, Matsuoka, Hryshko, Nicoll, Bersohn, Burke, Lifton, and Philipson, 1994) with some properties similar to the low Ca^{2+} affinity mutants of NCX1 described here. The affinity of NCX2 for regulatory Ca^{2+} was low ($K_h \cong 1.5$ μM) and NCX2 also showed rapid inactivation kinetics upon the removal of regulatory Ca^{2+}. Several splicing isoforms of NCX1 also exist (Kofuji, Lederer, and Schulze, 1994; Lee, Yu, and Lytton, 1994) for which Ca^{2+} regulation has not yet been characterized.

Although it has been clear that intracellular Ca^{2+} has a strong modulatory effect on reverse Na^+-Ca^{2+} exchange, it has never been clear as to whether Ca^{2+} also regulates the forward or Ca^{2+} efflux mode of exchange (intracellular Ca^{2+} exchanging for extracellular Na^+). The problem has been that in the forward mode an alteration of intracellular Ca^{2+} directly affects the level of Ca^{2+} at both the transport and regulatory sites. The availability of mutants with low affinity for Ca^{2+} regulation allowed us to address this problem. For the wild-type exchanger, the apparent affinities for regulatory and transported Ca^{2+} are ~0.4 and 7 μM, respectively, and the activation of inward current (Fig. 8) is thus controlled by the availability of Ca^{2+} at the transport site. For a mutant with especially low affinity for Ca^{2+} such as D447V + D498I (Levitsky et al., 1994), however, the activation curve is limited by the availability of Ca^{2+} at the regulatory site (Fig. 8, *bottom*). This interpretation is confirmed by the results obtained after α-chymotrypsin treatment, which removes Ca^{2+} regulation and leaves the exchanger in an activated state. After α-chymotrypsin, the Ca^{2+} dependence of inward exchange current is only a function of the Ca^{2+} level at the transport site. As would be excepted, the Ca^{2+} activation curve for the wild-type exchanger is unaffected by α-chymotrypsin, as the Ca^{2+} dependence was already dominated by the Ca^{2+} level at the transport site. For mutant exchanger D447V + D498I, however, the apparent affinity for Ca^{2+} activation shifts from that at the regulatory site to that at the transport site. After α-chymotrypsin treatment, the wild-type and mutant exchangers are indistinguishable (Fig. 8). We conclude that forward mode Na^+-Ca^{2+} exchange is modulated by Ca^{2+} at the regulatory site. In vivo, with Ca^{2+} at the submicromolar level, secondary Ca^{2+} regulation may exert an important influence on Ca^{2+} extrusion.

We investigated the Na^+ dependence of the exchanger at different levels of regulatory Ca^{2+} for wild-type and mutant exchangers (Figs. 10 and 11). This led us to the finding that at low intracellular Ca^{2+} the apparent affinity for intracellular Na^+ decreased markedly for both wild-type and mutant exchangers. The mechanism for this effect is unclear. Exchange activity is diminished at low regulatory Ca^{2+} and, perhaps, the rate limiting step of the exchanger transport mechanism is altered. Such

an alteration by itself could modify the apparent affinity for Na^+ (Hilgemann, Nicoll, and Philipson, 1991). Alternatively, the result could indicate that Ca^{2+} regulation modifies intrinsic transport characteristics of the exchanger. Previously, Hilgemann et al. (1992*a*) had modeled Ca^{2+} regulation to only modulate the number of exchangers in an active state.

The physiological implication of the altered Na^+ affinity is that at low diastolic Ca^{2+} levels the exchanger will tend to inactivate for multiple reasons. The low Ca^{2+} will inhibit Ca^{2+} efflux by limiting the Ca^{2+} level at the Ca^{2+} transport site and will inhibit both influx and efflux by causing Ca^{2+} to dissociate from the Ca^{2+} regulatory site. Additionally, Ca^{2+} influx will be further inhibited by the decrease in apparent Na^+ affinity at the intracellular Na^+ transport site. These mechanisms may decrease unnecessary ion transport during diastole.

In summary, we have unequivocally identified the region of the Na^+-Ca^{2+} exchange protein responsible for Ca^{2+} regulation. In addition, we demonstrate that both the Ca^{2+} influx and the Ca^{2+} efflux modes of the exchanger are regulated by intracellular Ca^{2+}. Mutants with altered binding of regulatory Ca^{2+} have distinctive and complex kinetic patterns which are not fully interpretable. Future mutational analysis and modeling may improve understanding of the exchanger regulatory processes.

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