Rapid Interaction of FRCRCFa with the Cytosolic Side of the Cardiac Sarcolemma Na^+-Ca^{2+} Exchanger Blocks the Ion Transport without Preventing the Binding of either Sodium or Calcium†

Daniel Khananshvili,* David Baazov, Evelyne Weil-Maslansky, Gilat Shaulov, and Brenda Mester

The Department of Physiology and Pharmacology, Sackler School of Medicine, Tel-Aviv University, Ramat-Aviv 69978, Israel

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ABSTRACT: Positively charged cyclic hexapeptide Phe-Arg-Cys-Arg-Cys-Phe-$CONH_2$ (FRCRCFa) represents a group of conformationally constrained peptides that block the cardiac sarcolemma Na^+-Ca^{2+} exchanger [Khananshvili et al. (1995b) *J. Biol. Chem. 270*, 16182−16188]. Here, we study the kinetic mechanisms of FRCRCFa-induced inhibition of Na^+-Ca^{2+} exchange and its partial reaction, the $Ca^{2+}-Ca^{2+}$ exchange. The Na_i-dependent ^{45}Ca uptake and Ca_i-dependent ^{45}Ca uptake were measured by adding the EGTA quench in the semirapid mixer. The reverse mode of Na^+-Ca^{2+} exchange (Na_o-dependent Ca efflux) was monitored ($t = 10-5000$ ms) in the stopped-flow machine by measuring extravesicular free calcium with a fluorescence probe fluo-3. Saturating concentrations of FRCRCFa inhibit completely the forward and reverse modes of exchange, suggesting that the inside-out vesicles contribute to most (if not all) of the exchange activity. A short time exposure ($t = 10-20$ ms) of FRCRCFa with the vesicles is enough to reach a rapid equilibrium between FRCRCFa and a putative inhibitory site at the extravesicular (cytosolic) side of the membrane. A lower limit for the second-order rate constant of FRCRCFa binding can be estimated as a k_{on} of $>10^6$ M^{-1} s^{-1}. A possible competition between FRCRCFa and either Na^+ or Ca^{2+} has been tested at the extravesicular (cytosolic) side of the membrane. At different extravesicular Ca_o concentrations of 13−250 μM, FRCRCFa inhibits the Na^+-Ca^{2+} and $Ca^{2+}-Ca^{2+}$ exchanges with an IC_{50} of 11−16 μM, suggesting no competition between FRCRCFa and Ca^{2+}. At different extravesicular Na_o concentrations of 40−160 mM, FRCRCFa inhibits Na_o-dependent Ca efflux with an IC_{50} of 12−18 μM, suggesting that FRCRCFa and Na^+ do not compete for binding at the extravesicular side. A mild proteolytic treatment of vesicles activates the Na_i-dependent ^{45}Ca uptake, but has a little effect on the FRCRCFa-induced inhibition. Thus, the "inhibitory site" is still functional after the proteolytic treatment of the inside-out vesicles. In conclusion, a rapid (<20 ms) interaction of extravesicular (cytosolic) FRCRCFa with the exchanger prevents the ion translocation through the exchanger, while the inhibitory peptide does not interact with the ion transport sites of the exchanger at the cytosolic side of the membrane.

Ion transport through the cardiac sarcolemma Na^+-Ca^{2+} exchanger involves multiple steps and intermediates, the identities of which are poorly understood (Khananshvili & Weil-Maslansky, 1994; Khananshvili et al., 1995a, 1996; Khananshvili, 1996; Hilgemann, 1996; Matsuoka et al., 1996). Despite the complexity of partial exchange reactions, the basic ion transport mechanism can be described as separate movements of Na^+ and Ca^{2+} ions through the exchanger (Khananshvili, 1990, 1991a,b; Niggli & Lederer, 1991; Hilgemann et al., 1991; Li & Kimura, 1991; Khananshvili & Weil-Maslansky, 1994). The binding of Na^+ and Ca^{2+} are weakly voltage-sensitive, while a primary response of Na^+-Ca^{2+} exchange to voltage may arise within the rate-limiting and voltage-sensitive movement of $3Na^+$ (Khananshvili, 1991a,b; Hilgemann et al., 1991; Matsuoka & Hilgemann, 1992; Khananshvili & Weil-Maslansky, 1994; Hilgemann, 1996). On the basis of kinetic data, it was proposed that the $E{\cdot}Na_3$ species might be positively charged and thus may respond to voltage changes while the E·Ca species may carry very little or no charge [for review, see Khananshvili (1996)]. According to this proposal, $2Na^+$ or Ca^{2+} may interact with a common ion transport domain that carries a negative charge, while the third Na^+ ion can interact with a distinct uncharged domain (Khananshvili, 1996). Site-directed mutagenesis suggests that a putative ion transport domain of the cardiac Na^+-Ca^{2+} exchanger (NCX1) contains at least two negatively charged residues, Glu-113 and Glu-199 (Nicoll et al., 1996). A regulatory loop of NCX1contains some charged sequences, the function of which is unknown (Nicoll et al., 1990, 1996; Philipson et al., 1996).

It was found recently that micromolar concentrations of positively charged cyclic hexapeptide Phe-Arg-Cys-Arg-Cys-Phe-$CONH_2$ (FRCRCFa) inhibits Na^+-Ca^{2+} exchange, when exposed to the extravesicular (cyctosolic) side of the isolated sarcolemma vesicles (Khananshvili et al., 1995b). The interest in FRCRCFa has been further boosted by the finding that nanomolar concentrations of cytosolic FRCRCFa result in a complete inhibition of Na^+-Ca^{2+} exchange currents in the intact patches of cardiac cells (Hobai et al., 1996). It is striking that some unknown cellular factors can increase the inhibitory potency of FRCRCFa at least 500−1000-fold. It has also been shown that high concentrations of FRCRCFa have no detectable effect on the major ion channel currents,

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* To whom correspondence should be addressed. Fax: 972-3-640-9113. Telephone: 972-3-640-9961.

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L-type Ca^{2+} current, or inward rectifier and delayed rectifier K^+ currents (Hobai et al., 1996). In general, one may expect that the positively charged FRCRCFa may interact with the ion transport or regulatory sites of the exchanger that carry negative charge(s). Here, we examine the FRCRCFa-induced effects on both the forward and reverse modes of Na^+-Ca^{2+} and $Ca^{2+}-Ca^{2+}$ exchanges with a goal to resolve the kinetics of FRCRCFa interaction with a putative inhibitory site at the cytosolic side of the membrane. The stopped-flow technique has been introduced to follow the kinetics of Na_o-dependent Ca efflux by monitoring the extravesicular free calcium with a fluorescence probe, fluo-3. We conclude that a fast (<20 ms) interaction of FRCRCFa with the cytosolic side of the membrane drives the exchanger to the inactive state, while FRCRCFa does not interact with the ion tranport sites of the exchanger.

MATERIALS AND METHODS

Materials. A calibration kit for free calcium, BAPTA,[1] and fluo-3 were obtained from Molecular Probes (Eugene, OR) or from Teflabs (Austin, TX). Poly(ethyleneimine), EGTA, calcium atomic absorption standard, α-chymotrypsin (type I-S), deoxyribonuclease I (type DN-25 from bovine pancreas), soybean chymotrypsin inhibitor, and protease inhibitors (phenylmethanesulfonyl fluoride, pepstatin, leupeptin, and aprotenin) were from Sigma (St. Louis, MO). Chelex-100 was from Bio-Rad Laboratories (Hercules, CA). $^{45}CaCl_2$ (0.8−1.5 Ci/mmol) was from New England Nuclear/DuPont (Boston, MA). The scintillation cocktail Opti-Fluor for radioactivity counting was from Packard (Groningen, The Netherlands). The glass microfiber filters (GF/C Whatman) were from Tamar (Jerusalem, Israel).

Methods. Large scale preparations of sarcolemma vesicles (50−200 mg) were obtained from fresh calf hearts (0.7−1.6 kg) by using a cocktail of protease inhibitors: phenylmethanesulfonyl fluoride (0.2 mM), pepstatin, leupeptin, and aprotenin (1 μg/mL each) (Khananshvili et al., 1993, 1995a,b). The isolated sarcolemma vesicles (5−14 mg of protein/mL) were equilibrated with 20 mM Mops/Tris (or 5 mM Bis-Tris propane), at pH 7.4 and 0.25 M sucrose and stored at −70 °C.

The Na-loaded or Ca-loaded preparations were obtained by incubating the cardiac sarcolemma vesicles with either sodium (160 mM) or calcium (250 μM) at 4 °C for 14−18 h. The initial rates ($t = 0.5-2$ s) of Na_i- or Ca_i-dependent ^{45}Ca uptake were measured at 37 °C (Reeves, 1988; Khananshvili, 1990, 1991a; Khananshvili et al., 1993, 1995a,b). FRCRCFa was added to the assay medium 20−30 min before the initiation of ^{45}Ca uptake. The assay medium contained 20 mM MopsTris (pH 7.4), 0.25 M sucrose, 12−250 μM $^{45}CaCl_2$ ($2-6 \times 10^5$ cpm/nmol), plus 0−250 μM FRCRCFa. After the desired time, the reaction of ^{45}Ca uptake was quenched by rapid injection of 5 mL of cold quenching buffer [20 mM MopsTris (pH 7.4), 5 mM EGTA, and 160 mM KCl]. The timing between the initiation and termination of mixing was electronically controlled by a GraLab-625 timer (Dimco-Gray Co., Centervile, OH). The timer was programed to trigger a rapid injection (20−50 mL/min) of quenching buffer from the peristaltic pump (Perifill IQ 200, Zinsser-Analytic, Frankfurt, Germany) to the assay medium. The IC_{50}, K_d, and their standard errors (±SE) were calculated (GraFit v3.0, Erithacus Software Ltd., U.K.) by statistical + robust weighting. The IC_{50} values were calculated according to four-parameter logistic equations (duplicates or triplicates of FRCRCFa concentrations were taken for analysis of dose−response curves). The concentrations of ^{45}Ca in the assay medium and the specific radioactivity (counts per minute of ^{45}Ca per nanomol Ca_T) were corrected for ambient calcium as outlined before (Khananshvili, 1990; Khananshvili & Weil-Maslansky, 1994; Khananshvili et al., 1995a). Concentrations of free calcium in solutions were measured with Arsenaso III (Bauer, 1981).

Before the proteolytic treatment of sarcolemma vesicles, they were loaded with 160 mM NaCl at 37 °C for 1 h. The Na-loaded vesicles were pretreated with chymotrypsin (1 μg of chymotrypsin per 200 μg of vesicular protein) at 37 °C for 20 min. Proteolytic treatment was terminated by adding 5 μg of soybean trypsin inhibitor per microgram of chymotrypsin. After the proteolytic treatment, the vesicles were kept at 4 °C. The exchange activities of chymortypsin-treated vesicles were measured no later than 1−2 h after the proteolytic treatment. Protein was measured as described before (Markwell et al., 1978).

For stopped-flow experiments, the vesicles (2.3−4.7 mg of protein/mL) were loaded with 250 μM $CaCl_2$ plus 10 mM KCl at 4 °C for 14−18 h. Before the experiment, the Ca-loaded vesicles were mixed thoroughly with valinomycin (in ethanol) at room temperature to give a final concentration of 1 μM (final concentration of ethanol was <0.2%). The Ca-loaded and valinomycin-treated vesicles were placed in syringe A (2.3−4.3 mg of protein/mL). Syringe B contained 0.4−1.0 mM BAPTA, 6 μM fluo-3, 10 mM KCl, plus varying concentrations of NaCl and choline chloride ([Na] + [ch-Cl] = 320 mM). The nonspecific Ca efflux from the vesicles was measured by substituting NaCl with KCl in syringe B. FRCRCFa was always included in syringe B. The SFM-3 stopped-flow machine (BioLogic, Grenoble, France) is equipped with an MOS-200 optical system, three thermostated syringes, and two mixers (each syringe is driven independently by an individual stepped motor and microprocessor controller). Fluo-3 was excited at a λ_{ex} of 475 nm with a monochromator, connected to the observation chamber via fiber optics, and emission was monitored at a λ_{em} of >495 nm (GG-495 long path filter) with a Hamamatsu R-376 photomultiplier (a high-voltage power supplier was fixed at 900−950 V). A 10 mm TC-100/15 light path cuvette (40 μL volume) was most appropriate for obtaining optimal signals (1−3 V on output). The mixing volume, flow rate and duration, dead time, etc., were optimized by the MPF program (BioLogic). The kinetic analysis of stopped-flow data was performed with the BioKin 0.14 program, equipped with 'Pad'-Laplace' and 'Simplex' modes (BioLogic).

Customer services for peptide synthesis have been provided by Chiron Mimotopes Peptide Systems Ltd. (Clayton Victoria, Australia). An intramolecular disulfide bond in FRCRCFa has been formed by oxidation of cysteine in the parent linear peptide. FRCRCFa was purified with HPLC to 80−95% purity, and the formation of an intramolecular S−S bond has been confirmed for each batch by ion spray mass spectrometry. Stock solutions of FRCRCFa were

[1] Abbreviations: Mops, 3-(*N*-morpholino)propanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; Bis-Tris propane, 1,3-bis[[tris(hydroxymethyl)methyl]amino]propane; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; fluo-3, *N*-[[2-[2-[20[bis(carboxymethyl)amino]-5-(2,7-dichloro-6-hydroxy-3-oxy-3*H*-xanthen-9-yl)phenoxy]ethoxy]-4-methylphenyl]-*N*-(carboxymethyl)glycine; BAPTA, 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid.

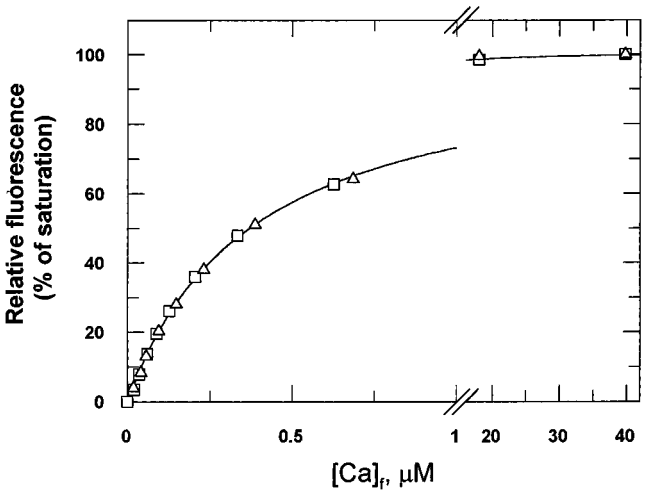

FIGURE 1: Titration of fluo-3 with calcium in the presence or absence of FRCRCFa. The assay buffer contained 20 mM Mops/Tris (pH 7.4), 160 mM KCl, 0.5 mM BAPTA, and 3 μM fluo-3 with (□) or without (△) 100 μM FRCRCFa. Formation of the Ca·fluo-3 complex was controlled by addition of 25 mM $CaCl_2$ to 2 mL of assay medium to give various concentrations (50−600 μM) of total calcium. Fluo-3 was excited at a λ_{ex} of 460 nm, and the fluorescence emission spectra were recorded from 480 to 620 nm by using a conventional spectrofluorimeter (SLM8000). Data were plotted as observed signals at 528 nm. Concentrations of free calcium were determined by using a commercially available calibration kit (see Materials and Methods). The line was computed according to the equation for a single binding site [$F = F_{max}[Ca]_f/([Ca]_f + K_d)$]. An optimal fit to the experimental points gives a K_d of 0.374 ± 0.007μM.

prepared with deionized water to give concentrations of 2−10 mM (pH ~5.8−7.0) and stored in small aliquots at −20 °C. Similar inhibitory potencies of FRCRCFa-induced inhibition were observed for the 7 different batches of FRCRFa and 25 preparations of sarcolemma vesicles. No decrease of inhibitory potency has been detected within at least 6 months of FRCRCFa storage at −20 °C (freezing−thawing of FRCRCFa should be avoided).

RESULTS

Assay of Free Extravesicular Calcium by Fluo-3 in the Presence or Absence of FRCRCFa. Our initial goal was to record continuously the reverse mode of Na^+-Ca^{2+} exchange (Na_o-dependent Ca efflux) by measuring fluorescence changes of fluo-3 in the stopped-flow machine. To ensure the validity of the fluorescence dye for our measurements, the binding affinity of calcium to fluo-3 was measured in the presence or absence of 100 μM FRCRCFa. As can be seen from Figure 1, FRCRCFa has no detectable effect on the dissociation constant ($K_d = 0.37 \pm 0.01$ μM) of Ca·fluo-3 formation. A possible effect of FRCRCFa on the rate constant of calcium dissociation from the Ca·fluo-3 complex has been tested by mixing ($t = 12$ ms) the Ca·fluo-3 complex (syringe A) with a large excess of BAPTA (syringe B). Within experimental error, the rate constant of calcium dissociation ($k_{off} = 208\ s^{-1}$) from Ca·fluo-3 is similar in the absence (trace a in Figure 2A) or presence (trace b in Figure 2A) of 100 μM FRCRCFa. The effect of extravesicularly added FRCRCFa was tested on the nonspecific Ca efflux. In these experiments, the Ca-loaded vesicles (syringe A) were mixed ($t = 40$ ms) with KCl buffer in the presence or absence of 100 μM FRCRCFa (syringe B) and fluorescence changes of fluo-3 were monitored. As shown in Figure 2B, FRCRCFa has no effect on the nonspecific Ca efflux. All these data

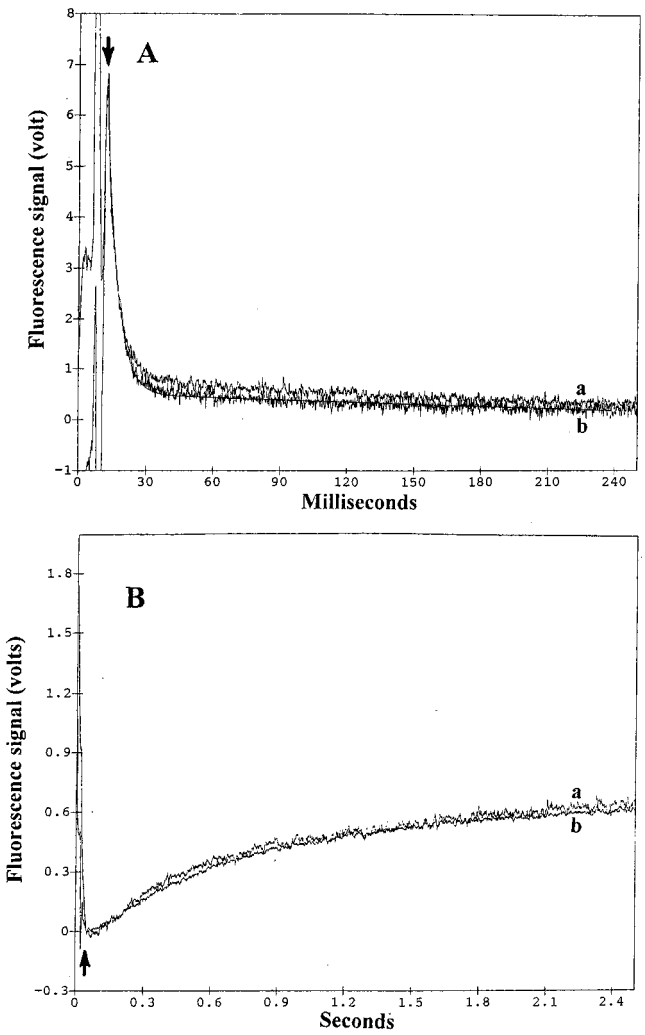

FIGURE 2: Stopped-flow assay of calcium in the presence or absence of FRCRCFa. (A) The stopped-flow kinetics of calcium dissociation from the Ca·fluo-3 complex was measured in the absence (trace a) or presence (trace b) of 100 μM FRCRCFa. Equal volumes (20 μL) of syringe A [20 mM Mops/Tris (pH 7.4), 0.25 M sucrose, 6 μM fluo-3, and 250 μM $CaCl_2$] and syringe B [20 mM Mops/Tris (pH 7.4), 320 mM KCl, and 1 mM BAPTA with or without 200 μM FRCRCFa] were mixed for 12 ms. The solid line represents a fitted curve with a rate constant of k_{off} 208 s^{-1}. (B) The nonspecific Ca efflux was monitored in the sarcolemma vesicles in the presence (trace a) or absence of (trace b) 100 μM FRCRCFa. The sarcolemma vesicles [20 mM Mops/Tris (pH 7.4) and 0.25 M sucrose] were loaded with 250 μM $CaCl_2$ plus 10 mM KCl at 4 °C for 14−18 h, and before the experiment, the loaded vesicles were treated with 1 μM valinomycin (see Materials and Methods). The nonspecific Ca efflux was initiated in the stopped-flow machine by mixing ($t =$ 40 ms) the equal volumes (30 μL) of syringe A [20 mM Mops/Tris (pH 7.4), 0.25 M sucrose, 10 mM KCl, 3 mg of protein/mL, 250 μM $CaCl_2$, and 1 μM valinomycin] and syringe B [20 mM Mops/Tris (pH 7.4), 320 mM KCl, 1 mM BAPTA, and 6 μM fluo-3 with or without 200 μM FRCRCFa]. Each trace represents the average of four or five mixing experiments. The arrow indicates the electrically synchronized stop.

suggest that FRCRCFa and the preparation of isolated cardiac vesicles do not alter the assay of free extravesicular calcium by fluo-3.

Stopped-Flow Kinetics of Na_o-Dependent Ca Efflux in the Cardiac Sarcolemma Vesicles. To observe the kinetics of Na_o-dependent Ca efflux, the Ca-loaded and valinomycin-treated vesicles were mixed with NaCl/BAPTA/fluo-3 buffer in the stopped-flow machine and the concentrations of free extravesicular calcium were monitored by fluo-3. In these experiments, the small volumes (30 μL) of the Ca-loaded

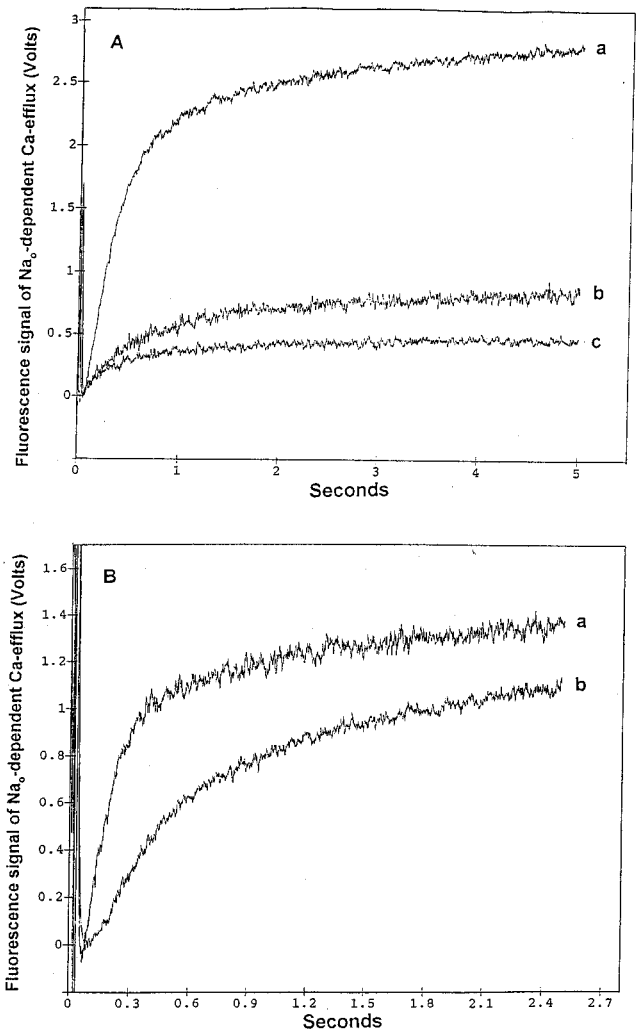

FIGURE 3: Stopped-flow kinetics of Na_o-dependent Ca efflux in the cardiac sarcolemma vesicles. (A) The Ca-loaded and valinomycin-treated vesicles were obtained as described in Figure 2. The Na_o-dependent Ca efflux (trace a) was initiated in the stopped-flow machine by mixing ($t = 20$ ms) the equal volumes (30 μL) of syringe A [20 mM Mops/Tris (pH 7.4), 0.25 M sucrose, 4.4 mg of protein/mL, 250 μM $CaCl_2$, 10 mM KCl, and 1 μM valinomycin] and syringe B [20 mM Mops/Tris (pH 7.4), 320 mM NaCl, 10 mM KCl, 1 mM BAPTA, and 6 μM fluo-3]. The nonspecific Ca efflux was measured with 320 mM KCl (trace b) or choline chloride (trace c) in syringe B instead of 320 mM NaCl. Each trace represents the average of four to six mixing experiments. (B) The sarcolemma vesicles were loaded with $CaCl_2$ and KCl as described in panel A and treated with (trace a) or without (trace b) valinomycin. Sixty microliters of Ca-loaded vesicles (Mops/Tris/sucrose buffer, 6.3 mg of protein/mL, 250 μM $CaCl_2$, and 10 mM KCl) was mixed ($t = 40$ ms) with the assay buffer to give final concentrations of 160 mM NaCl, 10 mM KCl, 3 μM fluo-3, and 0.5 mM BAPTA. Each trace represents the average of four mixing experiments.

vesicles (syringe A) and NaCl/BAPTA/fluo-3 buffer (syringe B) were mixed ($t = 20$ ms) to give final concentrations of 2.2 mg of protein/mL and 160 mM NaCl (Figure 3A, trace a). These experiments were designed in such a way that after 10−15 ms of mixing of the Ca-loaded (250 μM) vesicles with NaCl/BAPTA/fluo-3 medium the concentrations of extravesicular free calcium decrease to 0.1−0.2 μM. The observed signal of Na_o-dependent Ca efflux (Figure 3A, trace a) is much larger than the nonspecific Ca efflux with extravesicular potassium (Figure 3A, trace b) or choline chloride (Figure 3A, trace c). The observed plateau values of Na_o-dependent Ca efflux are apparent, thereby reflecting a number of factors. First, the initial rates of Na_o-dependent Ca efflux decline with decreasing concentrations of intravesicular calcium (slow rates of Ca efflux can be indeed observed at $t = 3-20$ s). Second, the "sensitivity" of fluo-3 to free calcium is reduced progressively, because the concentrations of extravesicular calcium increase due to Ca efflux (note that fluo-3 has a K_d of 0.37 μM, while the initial concentrations of extravesicular free calcium after mixing are ~0.1−0.2 μM). Third, with 175 μM $CaCl_2$ (after the mix), the buffering capacity of BAPTA is limited to 0.3−0.5 mM. Lower concentrations of BAPTA result in a rapid saturation of fluo-3 by the released calcium, while higher concentrations of BAPTA increase the Ca-buffering capacity and thus diminish considerably the amplitude of observed signals. Despite these limitations, fluo-3 is still a better choice for stopped-flow experiments because it has a reasonably high affinity for calcium, while the rate constants for calcium binding/dissociation are faster as compared to those for other calcium probes.

If the observed Na_o-dependent Ca efflux signals represent the Na^+-Ca^{2+} exchange, they might be voltage-sensitive. This because one positive charge is transferred inside the vesicles per each cycle of exchange ($3Na^+$ per Ca^{2+}). Thus, it is expected that the inside-positive potential, generated by Na^+-Ca^{2+} exchange, might inhibit the Na_o-dependent Ca efflux. This possibility was tested by measuring the Na_o-dependent Ca efflux in valinomycin-treated and valinomycin-untreated vesicles, under conditions in which the concentrations of extravesicular and intravesicular potassium are equal ($[K]_o = [K]_i = 10$ mM). As expected, the initial rate of Na_o-dependent Ca efflux is faster in the valinomycin-treated vesicles (Figure 3B, trace a) as compared to that in the valinomycin-untreated vesicles (Figure 3B, trace b).

Stopped-flow kinetics of Na_o-dependent Ca efflux was monitored in the presence of various, fixed concentrations of extravesicular sodium (the intravesicular calcium was always saturating). The Ca-loaded and valinomycin-treated vesicles (syringe A) were mixed ($t = 20$ ms) with the assay buffer (syringe B) to give 0−160 mM NaCl, while $[Na]_o$ + $[\text{choline chloride}]_o = 160$ mM (Figure 4A). The initial rates of Na_o-dependent Ca efflux increase with increasing concentrations of extravesicular sodium, while the observed signal reaches a saturation at 160 mM NaCl, exhibiting a typical sigmoidal curve with a Hill coefficient n of 2.0 ± 0.3 (Figure 4B). These data confirm the validity of experimental protocols that can be used for the assay of Na_o-dependent Ca efflux.

FRCRCFa Induced Inhibition of Na_o-Dependent Ca Efflux. The time course ($t = 40-5000$ ms) of Na_o-dependent Ca efflux has been monitored at a saturating Na_o concentration of 160 mM (Figure 5) in the absence (trace a) or presence of 10 μM (trace b) or 100 μM FRCRCFa (trace c). The Ca-loaded and valinomycin-treated vesicles (syringe A) were mixed ($t = 40$ ms) with the assay buffer (syringe B) to give final concentrations of 160 mM NaCl. These data show that even with saturating concentrations of extravesicular sodium FRCRCFa inhibits effectively the Na_o-dependent Ca efflux. Comparable results were obtained under similar conditions when the vesicles and FRCRCFa were mixed with a t of 20 or 10 ms. These data support the idea that the inside-out vesicles contribute to most of the Na^+-Ca^{2+} exchange activity. Thus, FRCRCFa interacts with a cytosolic side of the membrane.

The dose−response curves of FRCRCFa-induced inhibition of Na_o-dependent Ca efflux were titrated at varying

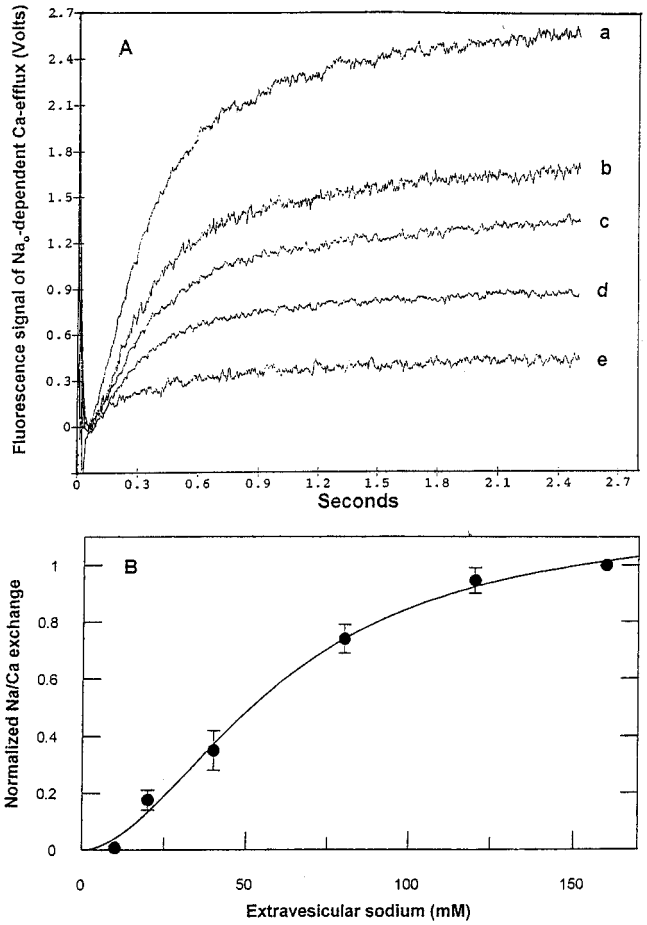

FIGURE 4: Stopped-flow kinetics of Na_o-dependent Ca efflux at various fixed concentrations of extravesicular sodium. (A) The sarcolemma vesicles [20 mM Mops/Tris (pH 7.4) and 0.25 M sucrose] were loaded with $CaCl_2$ and KCl and pretreated with valinomycin as described in Figure 2. Syringe A contained Mops/Tris/sucrose (pH 7.4), 4.1 mg of protein/mL, 250 μM $CaCl_2$, 10 mM KCl, and 1 μM valinomycin, while syringe B contained the assay buffer [20 mM Mops/Tris (pH 7.4), 10 mM KCl, 1 mM BAPTA, and 6 μM fluo-3] plus different concentrations of NaCl and choline chloride ([Na] + [chl-Cl] = 320 mM). Thirty microliters from syringes A and B mixed (t = 20 ms) to give 160 mM (trace a), 60 mM (trace b), 40 mM (trace c), 20 mM (trace d), and 0 mM NaCl (trace e). Each trace represents the averaging of five mixing experiments. (B) The experiments were done as described in panel A to give final concentrations of 0, 10, 20, 40, 80, and 160 mM NaCl. The initial rates of Na_o-dependent Ca efflux were normalized to a signal size at 160 mM NaCl and plotted vs. concentrations of extravesicular sodium. The Hill coefficient and its standard error were calculated as $n = 2.0 \pm 0.3$. Bars indicate standard deviations from the mean for data collected from three independent experiments. Each trace represents the average of four to seven mixing experiments that were done on the same day of experiment.

concentrations of extravesicular sodium ($[Na]_o$ + [choline chloride]$_o$ = 160 mM), while the concentration of intravesicular calcium was kept saturating. The Ca-loaded and valinomycin-treated vesicles (syringe A) were mixed with the contents of syringe B to give varying concentrations of FRCRCFa (0−200 μM) (Figure 6A). With a nonsaturating concentration of extravesicular sodium (40 mM), FRCRCFa inhibits the Na_o-dependent Ca efflux with an IC_{50} of 13.9 ± 2.3 μM (Figure 6B). If extravesicular sodium and FRCRCFa compete with each other for binding, it is expected that the IC_{50} values must increase several-fold with increasing concentrations of extravesicular sodium. In order to test this possibility, the Na_o-dependent Ca efflux was measured at various fixed concentrations of extravesicular NaCl (40, 80,

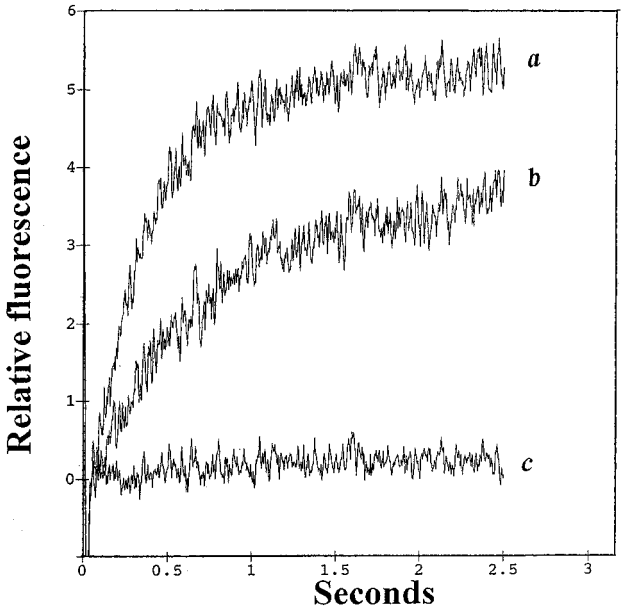

FIGURE 5: Inhibition of Na_o-dependent Ca efflux by FRCRCFa. The Ca-loaded vesicles were obtained as in Figure 2. The Na_o-dependent Ca efflux was monitored in the absence (trace *a*) or in the presence of 10 μM (trace *b*) or 100 μM FRCRCFa (trace *c*). Equal volumes (60 μL) of syringe A (Ca-loaded vesicles) and syringe B (assay buffer ± FRCRCFa) were mixed (t = 40 ms) to give 160 mM NaCl. The nonspecific Ca^{2+} efflux was assayed with KCl in syringe B (instead of NaCl) and subtracted from traces a−c. Other experimental conditions were similar to those described in Figures 2−4.

or 160 mM) and varying concentrations of FRCRCFa (0−200 μM). As can be seen from Figure 6B, within experimental error, the IC_{50} values are $[Na]_o$-independent, meaning that extravesicular sodium and FRCRCFa do not compete for binding at the extravesicular side of the membrane. The present data also show that, in a wide range of FRCRCFa concentrations (10−250 μM), rapid equilibrium ($t \leq$ 10−40 ms) is achieved for the inhibitory effect. Since under given conditions the stopped-flow setup cannot measure the reactions with a $t_{1/2}$ of <3−5 ms, one may estimate a lower limit for the second-order rate constant for FRCRCFa binding as $k_{on} > 10^6$ M^{-1} s^{-1}.

Effect of FRCRCFa on the Na_i- or Ca_i-Dependent ^{45}Ca Uptake. The Na_i-dependent ^{45}Ca uptake was initiated by diluting (25-fold) the Na-loaded (160 mM) vesicles in the assay medium with or without 200 μM FRCRCFa, and ^{45}Ca uptake was quenched at t = 0.5−180 s by adding the EGTA buffer in the semirapid mixer. Like the Na_o-dependent Ca efflux, the saturating concentrations of FRCRCFa suppress the time course of Na_i-dependent ^{45}Ca uptake (Figure 7A), suggesting that the inhibitory peptide inhibits both the forward and reverse modes of exchange. To test a possible competition of extravesicular calcium and FRCRCFa, the IC_{50} values of Na_i-dependent ^{45}Ca uptake were measured at varying concentrations of extravesicular FRCRCFa and calcium. If FRCRCFa competes with extravesicular calcium, a several-fold increase in the IC_{50} values is expected to be observed by increasing the concentrations of extravesicular calcium from 0.3K_m to 4K_m (Segel, 1993). The initial rates of Na_i-dependent ^{45}Ca uptake were measured at four fixed concentrations of extravesicular ^{45}Ca (13, 33, 63, or 150 μM), while the concentrations of FRCRCFa in the assay medium were varied (0−150 μM) and the concentration of intravesicular sodium was always 160 mM (Figure 7B). The observed IC_{50} values ranged from 12 to 18 μM, suggesting

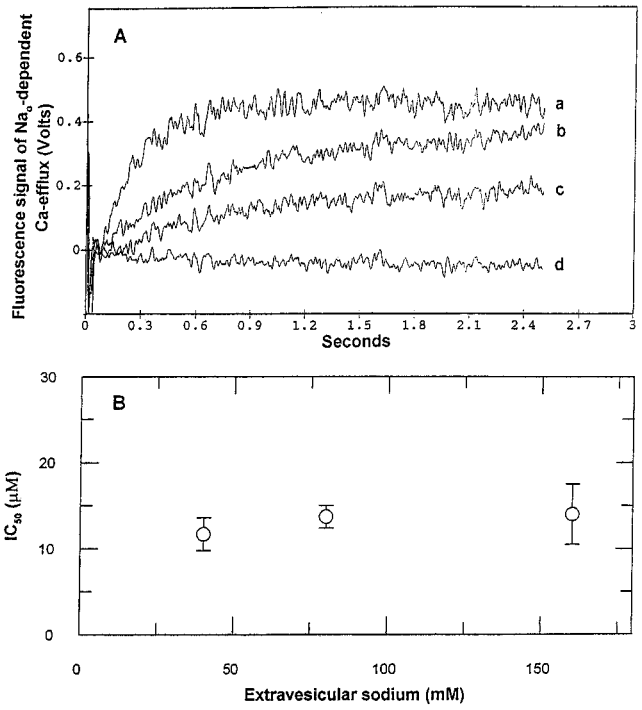

FIGURE 6: FRCRCFa-induced inhibition of Na_o-dependent Ca efflux at varying $[Na]_o$ concentrations. (A) The Ca-loaded and valinomycin-treated vesicles were obtained as described in Figure 1. Thirty microliters from of syringe A and B were mixed ($t = 40$ ms) to give varying concentrations of FRCRCFa: 0 μM (trace a), 20 μM (trace b), 50 μM (trace c), or 200 μM (trace d). Syringe A contained Mops/Tris/sucrose buffer (pH 7.4), 4.4 mg of protein/mL, 250 μM $CaCl_2$, 10 mM KCl, and 1 μM valinomycin. Syringe B contained 20 mM Mops/Tris (pH 7.4), 10 mM KCl, 1 mM BAPTA, 6 μM fluo-3, 80 mM NaCl, 240 mM choline chloride, plus 0−400 μM FRCRCFa. The nonspecific Ca efflux was measured with 320 mM choline chloride (0 mM NaCl) in syringe B and subtracted from the traces. Each trace was obtained by averaging of five mixing experiments. (B) The IC_{50} values of the FRCRCFa-induced inhibition of Na_o-dependent Ca efflux were measured at 40, 80, or 160 mM of extravesicular sodium. At each fixed concentration of extravesicular sodium, the initial rates of Na_o-dependent Ca efflux were plotted vs FRCRCFa concentrations and the IC_{50} values ± SE were estimated from the dose−response curves (bars indicate ±SE).

no boost of IC_{50} with increasing concentrations of extravesicular Ca^{2+} (Figure 7C). A similar picture was observed for Ca_i-dependent ^{45}Ca uptake, suggesting that the IC_{50} is $[Ca]_o$-independent for $Ca^{2+}-Ca^{2+}$ exchange too (Figure 8). Thus, in the case of Na^+-Ca^{2+} exchange as well as for its partial reaction, $Ca^{2+}-Ca^{2+}$ exchange, FRCRCFa and Ca^{2+} do not compete for binding at the extravesicular (cytosolic) side of the membrane.

Dose Response of Na_i-Dependent ^{45}Ca Uptake to FRCRCFa in the Chymotrypsin-Treated Vesicles. If a putative FRCRCFa site is located on the intracellular loop of the exchanger, one may expect that proteolytic treatment of vesicles may affect the potency of FRCRCFa-induced inhibition of Na^+-Ca^{2+} exchange. To test this possibility, the cardiac sarcolemma vesicles were treated with α-chymotrypsin and then a dose response of Na_i-dependent ^{45}Ca uptake was examined in chymotrypsin-treated vesicles. It is well-known that under fixed experimental conditions mild proteolytic pretreatment of sarcolemma vesicles activates Na^+-Ca^{2+} exchange (Philipson & Nishimoto, 1982; Khananshvili et al., 1993). Indeed, chymotrypsin pretreatment of sarcolemma vesicles activates Na^+-Ca^{2+} exchange by 80−100% (Figure 9), suggesting that the exchanger activity is modified by the proteolytic treatment. However, after the chymotrypsin treatment, the Na^+-Ca^{2+} exchange remains sensitive to FRCRCFa (Figure 9), meaning that a putative inhibitory site is still "functional" after the proteolytic digestion of extravesicular (cytosolic) domains.

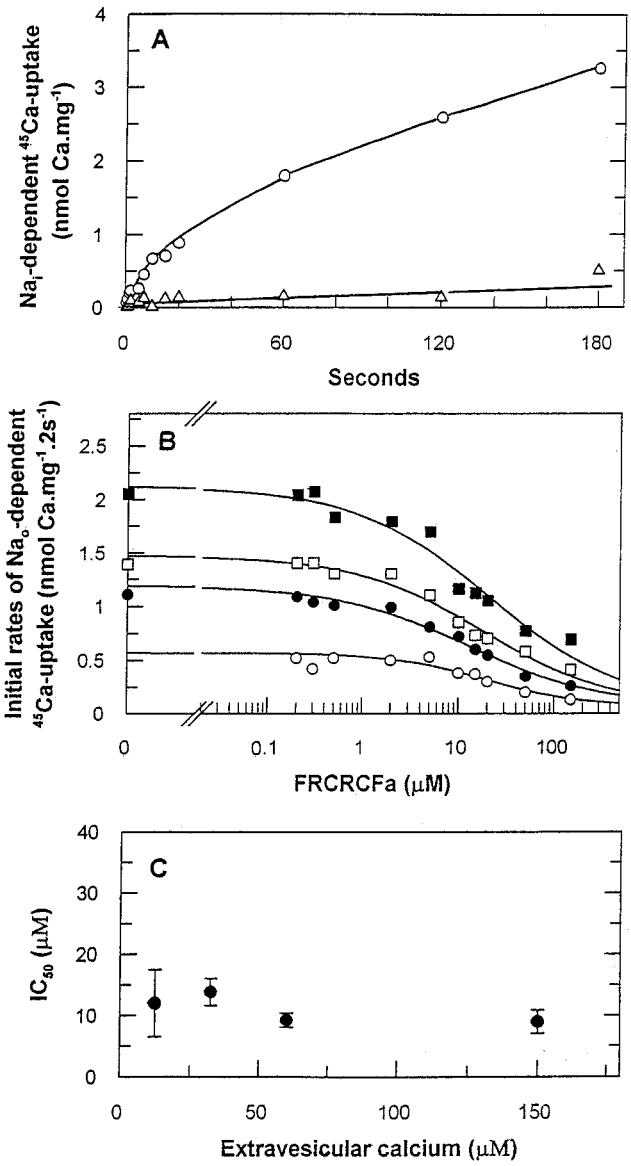

FIGURE 7: FRCRCFa-induced inhibition of Na_i-dependent ^{45}Ca uptake with varying $^{45}Ca_o$ concentrations. (A) The time ourse of Na_i dependent ^{45}Ca uptake was assayed in the absence (○) or presence (△) of 200 μM FRCRCFa in the assay medium. The Na-loaded (160 mM) vesicles were diluted 25-fold in the assay medium [20 mM Mops/Tris (pH 7.4), 0.25 M sucrose, and 13 μM $^{45}CaCl_2$]. The ^{45}Ca uptake was measured by filtration on GF/C filters (see Materials and Methods). The blanks were taken with 160 mM NaCl in the assay medium and subtracted from each time point. (B) The Na-loaded vesicles (160 mM) were rapidly diluted 50-fold in the assay medium, and the Na_i-dependent ^{45}Ca uptake ($t = 2$ s) was measured by using a semirapid mixing device as described in Materials and Methods. The assay medium [20 mM Mops/Tris (pH 7.4) and 0.25 M sucrose] contained either 13 μM (○), 33 μM (●), 63 μM (□), or 150 μM (■) $^{45}CaCl_2$ plus 0−150 μM FRCRCFa. The lines were computed to fit the experimental points as outlined in Materials and Methods. (C) The IC_{50} ± SE values were plotted vs extravesicular concentrations of calcium (bars indicate ± SE).

DISCUSSION

A simple model suggests that one Ca^{2+} or two Na^+ ions may interact with the negatively charged ion transport domains (e.g., E113 and E199) of NCX1, while the third Na^+ may associate with a distinct domain (Khananshvili,

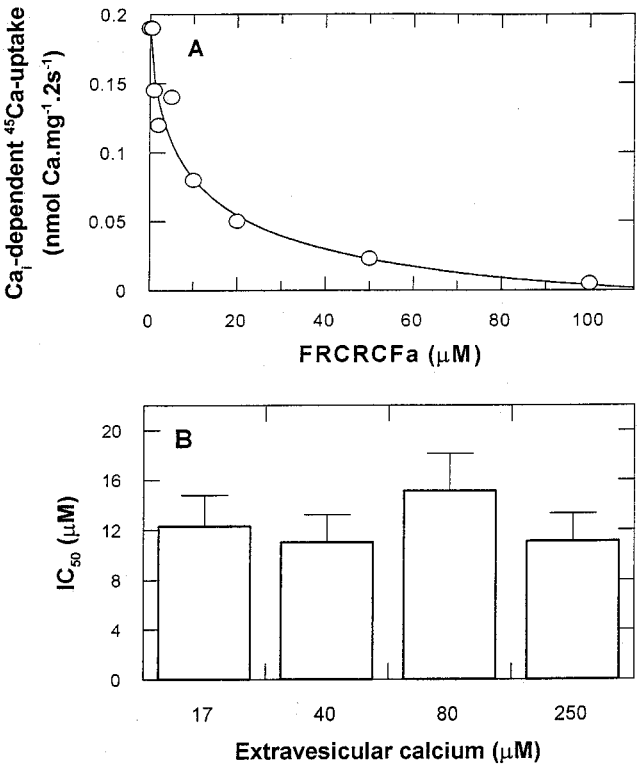

FIGURE 8: FRCRCFa induced inhibition of Ca_i-dependent ^{45}Ca uptake. (A) The Ca-loaded (250 μM) vesicles (see Materials and Methods) were diluted in the assay medium [20 mM Mops/Tris (pH 7.4), 0.25 M sucrose, and 17 μM $^{45}CaCl_2$] containing 0−100 μM FRCRCFa. The $IC_{50} \pm$ SE value was estimated as 11.2 ± 2.2 μM. (B) The vesicles were loaded with 250 μM $CaCl_2$ as shown in panel A and diluted in Mops/Tris/sucrose buffer with either 17, 40, 80, or 250 μM $^{45}CaCl_2$ plus 0−200 μM FRCRCFa. The initial rates (t = 2 s) of Ca_i-dependent ^{45}Ca uptake were measured as described in Materials and Methods. The IC_{50} values and their standard errors (indicated by bars) were estimated from the dose−response curves as described in Figure 6 and 7.

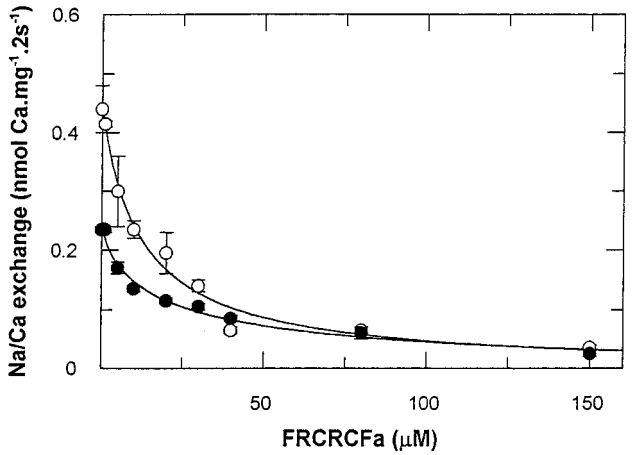

FIGURE 9: Dose−response curve of FRCRCFa-induced inhibition of Na_i-dependent ^{45}Ca uptake in chymotrypsin-treated vesicles. The Na-loaded vesicles (2310 μg of protein) were mixed with 11.6 μg of α-chymotrypsin and incubated at 37 °C for 20 min. The proteolytic treatment was arrested by adding 58 μg of soybean trypsin inhibitor. After the proteolytic treatment the vesicles were stored at 4 °C and the Na_i-dependent ^{45}Ca uptake was measured. The chymotrypsin-untreated vesicles (control) were obtained under similar conditions by mixing first α-chymotrypsin and soybean trypsin inhibitor together and then adding the Na-loaded vesicles. The initial rates of Na_i-dependent ^{45}Ca uptake (t = 2 s) were measured by diluting the chymotrypsin-treated (○) or untreated (●) vesicles in the semirapid mixer (see Materials and Methods). The standard assay medium [20 mM Mops/Tris (pH 7.4) and 0.25 M sucrose] contained 12 μM $^{45}CaCl_2$ and 0−150 μM FRCRCFa. Each point represents a mean of duplicate measurements (bars indicate ± the standard deviation of the mean). The $IC_{50} \pm$ SE values were estimated as 14.1 ± 3.4 μM (○) 18.1 ± 1.1 μM (●).

1996). Since FRCRCFa contains two guanidinium groups of Arg, one may ask the following. Can FRCRCFa interact with either Ca^{2+} or Na^+ binding/transport sites of the exchanger? Here, we examine FRCRCFa-induced effects on the forward and reverse modes of Na^+-Ca^{2+} exchange and its partial reaction, the $Ca^{2+}-Ca^{2+}$ exchange, with a goal of resolving the kinetics of FRCRCFa interaction with a putative inhibitory site. The present experimental data suggest that FRCRCFa binding to the inhibitory site moves the exchanger in a completely inactive state rather than interferes with the ion binding or decelerates ion translocation through the exchanger.

Stopped-Flow Kinetics of Na_o-Dependent Ca Efflux. The initial rate of Na_i-dependent ^{45}Ca uptake can be precisely measured in vesicles (Reeves, 1988; Khananshvili, 1990; Khananshvili et al., 1995a,b). This is not the case for Na_o-dependent ^{45}Ca efflux. Although there is no problem following a time course of Na_o-dependent ^{45}Ca efflux by quenching the ^{45}Ca efflux from the ^{45}Ca-loaded vesicles (Reeves, 1988; Khananshvili et al., 1993), the assay of initial rates (5−10% of ^{45}Ca released vs 90−95% of intravesicular ^{45}Ca remained) is practically impossible. To overcome this problem, we introduce here the stopped-flow technique for continuous monitoring of Na_o-dependent Ca efflux (Figure 3A).

The control experiments show that FRCRCFa and/or vesicles do not interrupt with the steady-state (Figure 1) or stopped-flow measurements (Figure 2) of free extravesicular calcium in the assay medium. The Na_o-dependent Ca efflux can be conveniently monitored by mixing small volumes (20−40 μL) of Ca-loaded vesicles with NaCl (Figure 3A). The validity of this signal as the specific Na_o-dependent Ca efflux has been confirmed by various tests. The rates of Na_o-dependent Ca efflux are inhibited by the inside-positive potential, generated by the electrogenic activity of Na^+-Ca^{2+} exchange (Figure 3B). The initial rates of Na_o-dependent Ca efflux increase with increasing concentrations of extravesicular Na^+, showing a sigmoidal curve with a Hill coefficient n of 2.0 ± 0.3 (Figure 4).

FRCRCFa-Induced Inhibition of Na^+-Ca^{2+} Exchange Is Rapid and Complete. A short time exposure (t = 20−40 ms) of FRCRCFa to the vesicles is enough to produce complete inhibition of Na_o-dependent Ca efflux (Figure 5). Similar results were obtained when the vesicles and FRCRCFa were mixed for 10 ms (not shown). These data suggest that rapid equilibrium is achieved between FRCRCFa and a putative inhibitory site, while a lower limit for the second-order rate constant of FRCRCFa binding can be estimated as $k_{on} > 10^6\ M^{-1}\ s^{-1}$. It is not clear at this moment whether the peptide-exchanger interaction is diffusion limited. Further systematic experimentation is necessary to resolve this point. Saturating concentrations of FRCRCFa result in complete inhibition of both the forward (Figure 8) and reverse (Figure 6) modes of Na^+-Ca^{2+} and $Ca^{2+}-Ca^{2+}$ (Figure 7) exchange under conditions in which the extravesicular sodium (40−160 mM) or calcium (13−250 μM) varies in a wide range. The present data are consistent with a previous claim that in the preparation of isolated cardiac sarcolemma vesicles the inside-out vesicles contribute to most, if not all, of the Na^+-Ca^{2+} exchange activity (Li et al., 1991; Khananshvili et al., 1993, 1995a,b; Khananshvili

& Weil-Maslansky, 1994). In conclusion, a rapid interaction (<20 ms) of FRCRCFa with the cytosolic side of the membrane results in complete inhibition of the exchanger.

FRCRCFa Prevents the Ion Translocation through the Exchanger without Competing with Extravesicular (Cytosolic) Sodium or Calcium. FRCRCFa inhibits the forward and reverse modes of Na^+-Ca^{2+} exchange (Na_o-dependent Ca efflux and Na_i-dependent Ca influx) with a similar value for IC_{50} of 12−18 μM (Figures 6 and 7). These values of IC_{50} are also valid for $Ca^{2+}-Ca^{2+}$ exchange (Figure 8A), suggesting that the inhibitory mechanism may involve the same inhibitory site for FRCRCFa binding. Still the question is how the FRCRCFa peptide interacts with the exchanger activity. We tested here the capability of FRCRCFa to compete with either Na^+ or Ca^{2+} at the extravesicular (cytosolic) side of the membrane. If FRCRCFa interacts with the ion-transporting site(s), a competitive inhibition is expected between the extravesicular (cytosolic) FRCRCFa and Na^+ (or Ca^{2+}). In order to test this possibility, the dose responces of FRCRCFa-induced inhibition of Na_o-dependent Ca efflux have been examined at various fixed concentrations of extravesicular Na^+. The IC_{50} does not increase with increasing concentrations of extravesicular Na^+ (Figure 6B), suggesting that Na^+ and FRCRCFa do not compete for binding at the extravesicular (cytosolic) side of the membrane.

A possible competition between the extravesicular calcium and FRCRCFa was tested for Na_i-dependent ^{45}Ca uptake (Figure 7B) and Ca_i-dependent ^{45}Ca uptake (Figure 8B). The IC_{50} values were measured at varying concentrations of extravesicular Ca^{2+} (13−250 μM) and fixed concentrations of intravesicular sodium (160 mM NaCl) or calcium (250 μM $CaCl_2$). Under these concentrations, FRCRCFa inhibits Na_i-dependent ^{45}Ca uptake (Figure 7B) and Ca_i-dependent ^{45}Ca uptake (Figure 8B) with an IC_{50} of 12−18 μM. These data suggest that extravesicular FRCRCFa does not compete with extravesicular calcium. In conclusion, extravesicular FRCRCFa can suppress the ion translocation through the exchanger without preventing the ion binding at the transport site(s) at the cytosolic side of the membrane. It is possible that FRCRCFa blocks specific conformational transitions that occur after ion binding (e.g., alternative exposure of binding sites and/or ion occlusion).

Proteolytic Pretreatment of the Exchanger Does Not Prevent FRCRCFa-Induced Inhibition. The cardiac Na^+-Ca^{2+} exchanger (NCX1) is a single polypeptide with a large intracellular regulatory loop (Nicoll et al., 1990). Since FRCRCFa does not compete with either sodium or calcium at the cytosolic side of the membrane, it may interact with intracellular loops of the exchanger. It is established that a mild proteolytic treatment of the cytosolic side of the membrane activates Na^+-Ca^{2+} exchange with an accompanying loss of regulatory activities of the exchanger (Philipson & Nishimoto, 1982; Matsuoka & Hilgemann, 1992; Matsuoka et al., 1993; Nicoll et al., 1996; Philipson et al., 1996). To test the possible involvement of a large intracellular loop in the FRCRCFa−exchanger interaction, a dose response of FRCRCFa-induced inhibition was examined in the chymotrypsin-treated and -untreated vesicles. Addition of α-chymotrypsin to vesicles activates the Na_i-dependent ^{45}Ca uptake by 80−100%, although this proteolytic treatment does not prevent the FRCRCFa-induced inhibition of Na^+-Ca^{2+} exchange (Figure 9). These data suggest that a putative inhibitory site is still operating after the proteolytic treatment of vesicles. One possible explanation for these data is that FRCRCFa can be attracted with negatively charged parts of intracellular domains that can "survive" the proteolytic treatment. Two negatively charged sequences of NCX1 may be involved in a primary attraction of FRCRCFa on the intracellular surface of the exchanger. The first sequence, EDDDDDECGEE (amino acids 723−733), is located in the close vicinity of transmembrane segment six. The second sequence (amino acids 56−96) contains six negatively charged amino acids and is a major part of the short intracellular loop that connects transmembrane segments one and two.

A possible interaction of positively charged FRCRCFa with negatively charged lipid membrane cannot be excluded at this moment. Although the positively charged cyclic hexapeptide structure, like FRCRCFa, is too small to be barred into the membrane, lipids can still mediate some specific interactions between the peptide and receptor (Schwyzer, 1995). Further systematic study is necessary to examine the possible involvement of lipids in the FRCRCFa-induced inhibition of Na^+-Ca^{2+} exchange.

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