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Inhibition of the Cardiac Sarcolemma Na^+/Ca^{2+} Exchanger by Conformationally Constrained Small Cyclic Peptides

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SUMMARY

Positively charged cyclic peptides (three to seven amino acids) have been tested for their inhibitory effects on Na^+/Ca^{2+} exchange in the cardiac sarcolemma vesicles. The lead structure of Phe-Arg-Cys-Arg-Cys-Phe-$CONH_2$ (FRCRCFa) has been systematically modified for identification of important pharmacophores. In cyclic peptides (intramolecular S—S bond), the carboxyl terminal is locked with amide ($CONH_2$), and positive charge is retained by one or two arginines, ornithines, or lysines. Thirty-five different cyclic peptides show IC_{50} values in the range of 2–800 μM, suggesting that some specific structure-activity relationships may determine the inhibitory effects. Shortening of the FRCRCFa length to four amino acids decreases the inhibitory potency by 10–80-fold. The substitution of Arg2 or Arg4 in FRCRCFa with lysine or ornithine decreases the inhibitory potency by 5–12-fold, suggesting that both arginines are beneficial for inhibition. The substitution of Phe1 in FRCRCFa by 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid produces a potent inhibitor (IC_{50} = 2–4 μM). The *N*-myristoylated FRCRCFa exhibits an inhibitory potency (IC_{50} = 8–10 μM) similar to that of the parent FRCRCFa peptide, thereby arousing a new possibility for the development of a cell-permeable blocker of the Na^+/Ca^{2+} exchanger. D-Arg4 or D-Cys5 substitutions in FRCRCFa do not alter the inhibitory effect, whereas the L-to-D substitutions of other amino acids in FRCRCFa reduce the inhibitory potency by 4–5-fold. Thus, the L-to-D substitutions of Arg4 and/or Cys5 have a potential to increase the peptide stability to proteolytic degradation. The insertion of proline outside of the ring of FRCRCFa diminishes the inhibitory potency by 3–6-fold, whereas proline introduction into the ring decreases the inhibitory potency by 16–20-fold. The replacement of Cys3 and Cys5 in FRCRCFa with β,β-dimethylcystein has no significant effect on the inhibitory potency, suggesting that the S—S bond is not exposed to the interface of the peptide/receptor interaction. In conclusion, the current data support a proposal that the conformationally constrained Arg-Cys-Arg-Cys structure is obligatory for inhibition of Na^+/Ca^{2+} exchange, whereas hydrophobic additions at the carboxyl and amino ends have limited effects in increasing the inhibitory potency.

In biomedical research, a selective and potent inhibitor of the cardiac sarcolemma Na^+/Ca^{2+} exchanger is lacking. The development of new potential drugs is hampered because no lead structure is available. Recently, a new class of cyclic hexapeptides was designed, synthesized, and found to inhibit Na^+/Ca^{2+} exchange and its partial reaction, the Ca^{2+}/Ca^{2+} exchange, in the cardiac sarcolemma vesicles (1). The cyclic hexapeptide Phe-Arg-Cys-Arg-Cys-Phe-$CONH_2$ (FRCRCFa) is apparently most potent in inhibiting the Na^+/Ca^{2+} exchange while exhibiting a noncompetitive inhibition in regard to extravesicular (cytosolic) Ca^{2+} or Na^+ (1–3). FRCRCFa interacts rapidly (<20 msec) with a putative inhibitory site at the cytosolic side of the sarcolemma vesicles, suggesting that FRCRCFa may prevent some specific conformational transitions of ion/protein interactions (e.g., occlusion or alternative exposure of ion-binding sites) without altering the interaction of ions with the transport sites of the exchanger (1–3).

A demand for pharmacological targeting of the cardiac sarcolemma Na^+/Ca^{2+} exchanger stems from the unique role of this system. The cardiac sarcolemma Na^+/Ca^{2+} exchanger mediates a voltage-sensitive extrusion of 90–95% Ca^{2+} that has entered the cell via the Ca^{2+} channels during the action potential (4, 5). The interest in the cyclic peptide inhibitors has been further boosted by recent electrophysiological studies suggesting that nanomolar concentrations of cytosolic FRCRCFa result in complete inhibition of Na^+/Ca^{2+} exchange currents in the intact patches of the cardiac cells (6). It is striking that the inhibitory potency of FRCRCFa in intact cardiac cells (IC_{50} = 20 nM) is ≥500-fold higher than

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ABBREVIATIONS: MOPS, 3-(*N*-morpholino)propanesulfonic acid; TIC, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; Nal, 3-(1-naphthyl)alanine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Myr-FRCRCFa, *N*-myristoylated derivative of FRCRCFa; Nal1, 1-α-naphthylalanine; Nal2, 2-α-naphthylalanine.

the inhibitory effect of FRCRCFa (IC_{50} = 10–20 μM) in the isolated cardiac sarcolemma vesicles, suggesting that some cellular factors can enhance the inhibitory potency of FRCRCFa. High concentrations of FRCRCFa do not affect L-type Ca^{2+} current or inward rectifier and delayed rectifier K^+ currents, suggesting that FRCRCFa could be a reasonably selective inhibitor of Na^+/Ca^{2+} exchange (6). It is obvious that we are only at a very beginning of research and that more systematic studies are necessary to establish and to improve the selectivity, stability, and availability of cyclic peptide blockers.

The cardiac sarcolemma Na^+/Ca^{2+} exchanger contains 11 putative transmembrane segments; a large intracellular loop is situated between transmembrane segments five and six (7). The intracellular loop contains a putative calmodulin-binding site that is positively charged and exhibits an autoinhibitory potency (8). The synthetic analogues of this sequence, the XIP peptide (20 amino acids), inhibits Na^+/Ca^{2+} exchange in the isolated cardiac sarcolemma vesicles (IC_{50} = 1.5 μM) and in giant excised patches of cardiac cells (IC_{50} = 0.1 μM) (8). A significant disadvantage of all known peptide inhibitors of Na^+/Ca^{2+} exchange is that they are not available to the intracellular inhibitory site under most physiological conditions. A recent approach uses modification of peptides by attachment of a fatty acid (e.g., myristoyl) as a means to overcome the permeability barrier of the plasma membrane (9). In principle, this approach allows cell-permeable derivatives of positively charged short peptides (five to nine amino acids) to be constructed with two to four arginines.

We describe systematic studies on structure-activity relationships of positively charged small cyclic peptides (three to seven amino acids) that block the Na^+/Ca^{2+} exchange. The main strategy was to modify the molecular structure of FRCRCFa, with an aim of determining the contribution of different parts of the molecule to the inhibitory potency. The inhibitory potencies of various cyclic peptides were tested on exchange reactions by using the isolated cardiac sarcolemma vesicles (10–14). In this preparation, the inside-out vesicles contribute to most, if not all, of the Na^+/Ca^{2+} exchange activity (8, 12–14). Thus, in the isolated sarcolemma vesicles, the extravesicular side refers to the cytosolic side of the membrane; i.e., the added peptide inhibitors interact with a putative inhibitory site on the intracellular surface of the membrane.

Materials and Methods

Large-scale preparations of cardiac sarcolemma vesicles (100–200 mg of total membrane protein) were obtained from fresh calf ventricles and intraventricular septa in the presence of DNase and protease inhibitors (phenylmethanesulfonyl fluoride, leupeptin, aprotinin, pepstatin) as outlined previously (1, 10–14). The isolated sarcolemma vesicles (6–14 mg of protein/ml) were stored at −70° in 20 mM MOPS/Tris or 5 mM bis-Tris propane·HCl, pH 7.4, and 0.25 M sucrose. This preparation can be stored for ≥3-4 months without loss of exchange activities.

The Na_i-dependent ^{45}Ca uptake was measured by filtering the quenched solutions of cardiac sarcolemma vesicles through glass microfiber filters (GF/C Whatman) (15). The filters were presoaked in 0.3% polyethylenimine at 4° for 4–12 hr and washed with a cold filtration buffer (20 mM MOPS/Tris, pH 7.4, 160 mM KCl, 0.5 mM EGTA) before the experiment. The Na^+- or Ca^{2+}-loaded vesicles were obtained through incubation with NaCl (160 mM) at 4° for 16–18 hr or at 37° for 1 hr. The reaction of ^{45}Ca uptake was initiated by 25–50-fold dilution of Na^+- or Ca^{2+}-loaded vesicles (50–160 μg of total protein) in 0.5 ml of standard assay medium containing 20 mM MOPS/Tris, pH 7.4, 0.25 M sucrose, 16–27 μM $^{45}CaCl_2$ ($4–7 \times 10^5$ cpm/nmol), and various concentrations of a tested cyclic peptide (1, 10–14). "Blanks" were measured by diluting the Na^+- or Ca^{2+}-loaded vesicles in a standard assay medium with 160 mM NaCl. The cyclic peptides were added to the assay medium 10–20 min before the initiation of ^{45}Ca uptake. The reaction was electrically initiated by mixing the vesicles with the assay medium, and the ^{45}Ca uptake was quenched by automatic injection of a cold quenching buffer (20 mM MOPS/Tris, pH 7.4, 5 mM EGTA, and 160 mM KCl) (10–14). Quenched solutions were filtered on GF/C filters (Tamar, Jerusalem, Israel), and collected vesicles were washed (5 × 5 ml) on the filter with a cold filtration buffer (Tris/MOPS/KCl buffer with 0.5 mM EGTA). The timing of ^{45}Ca uptake was controlled by a timer that electronically triggers the injection (30–50 ml/sec) of quenching buffer from the high performance peristaltic pump (Perifill IQ 200; Zinsser-Analytic, Frankfurt, Germany) to the assay medium. The IC_{50} values (mean ± standard errors) were calculated with the use of GraFit version 3.0 (Erithacus Software, Staines, UK) according to four-parameter logistic equations by using statistical and robust weighting. The IC_{50} values were estimated from the dose-response curves for at least six different concentrations of the inhibitory peptide (each point represents the values of duplicate or triplicate measurements). The specific radioactivity values (in cpm/nmol) of ^{45}Ca and its concentration in the assay medium were corrected for ambient Ca^{2+} as described previously (10–14). Concentrations of ambient Ca^{2+} in solutions were measured with Arsenazo III (Sigma, St. Louis, MO) (16). Protein was measured as described previously (17).

Solid-phase peptide synthesis was provided by Chiron Mimotopes Peptide Systems (Clayton Victoria, Australia) and Neosystem Laboratory (Strasbourg, France). An intramolecular S—S bond was formed by oxidation of cysteine in the parent linear peptide. Because the efficiency of cyclization reaction is sequence dependent, after the oxidation step, the synthetic cyclic peptides were extensively purified with the use of high performance liquid chromatography to 70–95% purity, and the formation of intramolecular S—S bond was confirmed for each peptide through ion spray mass spectrometry. Stock solutions of cyclic peptides were prepared with deionized water (17–18 MΩ/cm) or dimethylsulfoxide to give final concentrations of 0.5–5 mM. The stock solutions were stored at −20° or −70°. No loss of inhibitory potency has been detected within ≥3 months. To retain the inhibitory potency, it is recommended that freezing and thawing of stock solutions be avoided.

Energy minima of the cyclic peptide structures were calculated with the use of Nemesis version 2.0 (Oxford Molecular, Oxford, UK), equipped with a COSMIC force field module and with the MM-XYZ mode of New Chem-X (Chemical Design, Oxon, UK). COSMIC force field calculates molecular energies by summing bond length, bond angle, torsion angle, and van der Waals and Columbic terms for all appropriate atoms. New Chem-X can optimize geometry by varying atomic x, y, and z coordinates through the use of the MM force field mode (an MM2-like force field, which is used in Chem-X).

Deoxyribonuclease I (type DN-25, obtained from a bovine pancreas), protease inhibitors (phenylmethanesulfonyl fluoride, pepstatin, leupeptin, aprotinin), and EGTA were obtained from Sigma Chemical (St. Louis, MO). $^{45}CaCl_2$ (10–30 mCi/mg) was purchased from DuPont-New England Nuclear (Boston, MA). 1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid and fluo-3 were from Molecular Probes (Eugene, OR) or from Teflabs (Austin, TX).

Results

In all experiments described below, the initial rates (t = 2 sec) of intracellular Na^+-dependent ^{45}Ca uptake were mea-

sured under standard assay conditions by using the semi-rapid mixing device (1, 10–14). In this way, the Na^+-loaded (160 mM NaCl) vesicles were rapidly diluted (25–50-fold) in the assay medium (MOPS/Tris/sucrose/$^{45}CaCl_2$) containing varying concentrations of cyclic peptides. The positively charged cyclic peptides with a different length (three to six amino acids) were tested for their effects on Na^+/Ca^{2+} exchange (Table 1). The three-to-five-amino acid peptides with one arginine exhibited very low inhibitory potency (IC_{50} = 130–800 μM), whereas at least five cyclic hexapeptides with Arg-Cys-Arg-Cys show IC_{50} values of 7–25 μM (Table 1). These data suggest that both arginine and conformational constrained structure of Arg-Cys-Arg-Cys are essential for exhibiting high inhibitory potency and that for effective inhibition the length of cyclic peptide inhibitors should be at least six amino acids, whereas hydrophobic moieties at the carboxyl and amino termini can facilitate the inhibitory potency.

To determine the role of the amino terminal Phe1, it was substituted in FRCRCFa with alternative hydrophobic moieties (Nal-1, Nal-2, and valine) or by 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid, the "conformationally constrained analog" of phenylalanine (18). Substitution of Phe1 with TIC produces a potent inhibitor exhibiting an IC_{50} value of 3.5 ± 1.2 μM (Fig. 1). The addition of TIC at the carboxyl terminal or substitution for both Phe1 and Phe6 by TIC is a little less effective (Table 1). In contrast to TIC substitutions, the Phe1-to-Trp1 replacement in FRCRCFa reduces the inhibitory potency (IC_{50} = 45.2 ± 5.2 μM), suggesting that the amino-terminal tryptophan decreases the inhibitory potency by 3–4-fold compared with the parent FRCRCFa peptide (Fig. 1). Substitutions of Phe1 by Nal-1, Nal-2, or valine do not significantly affect the IC_{50} values compared with the inhibitory potency of FRCRCFa (Table 1). These data suggest that although the hydrophobic moieties at the carboxyl or amino terminus, or both, have a potential to elevate the inhibitory potency, these structural modifications show no clear-cut correlations in terms of structure-activity relationships, and they may represent a general increase in hydrophobicity.

With a goal to develop a potential cell-permeable analog of FRCRCFa, Myr-FRCRCFa was synthesized and tested for its inhibitory effect on Na^+/Ca^{2+} exchange. As can be seen from Fig. 2, Myr-FRCRCFa inhibits the Na^+/Ca^{2+} exchange in the preparation of isolated cardiac sarcolemma vesicles with the IC_{50} of 8.2 ± 2.0 μM. Therefore, Myr-FRCRCFa exhibits inhibitory potency similar to that of the parent FRCRCFa peptide. These data suggest that the addition of myristoyl fatty acid to FRCRCFa could be beneficial in the development of more potent and a cell-permeable analogs of FRCRCFa.

To test the role of Arg2 and Arg4 in the peptide-induced inhibition, both arginine residues were replaced systemati-

TABLE 1

Effect of cyclic peptides with length of three to six amino acids and one or two arginines on the inhibition of Na^+/Ca^{2+} exchange

The initial rates (t = 2 sec) of Na^+/Ca^{2+} exchange (Na dependent ^{45}Ca uptake) were measured under standard experimental conditions with MOPS/Tris/sucrose buffer containing 13–27 μM $^{45}CaCl_2$ (see Materials and Methods). The dose response-curves were analyzed as in Figs. 2 and 3.

Peptide	C_{50}[a]
	μM
Phe-Arg-Cys-Arg-Cys-Phe-$CONH_2$	7–15
Phe-Cys-Arg-Cys-Phe-$CONH_2$	130–350
Cys-Arg-Arg-Cys-Phe-$CONH_2$	600–800
Phe-Cys-Arg-Cys-Val-$CONH_2$	>800
Phe-Cys-Arg-Cys-$CONH_2$	>400
Cys-Arg-Cys-Phe-$CONH_2$	>500
Phe-Arg-Cys-Arg-Cys-Tic-$CONH_2$	20–30
Tic-Arg-Cys-Arg-Cys-Tic-$CONH_2$	10–15
1-Nal-Arg-Cys-Arg-Cys-Phe-$CONH_2$	12–17
2-Nal-Arg-Cys-Arg-Cys-Phe-$CONH_2$	10–25
Val-Arg-Cys-Arg-Cys-Phe-$CONH_2$	10–20

[a] IC_{50} values were estimated in three to five independent experiments with six (or more) different concentrations of the specific cyclic peptide.

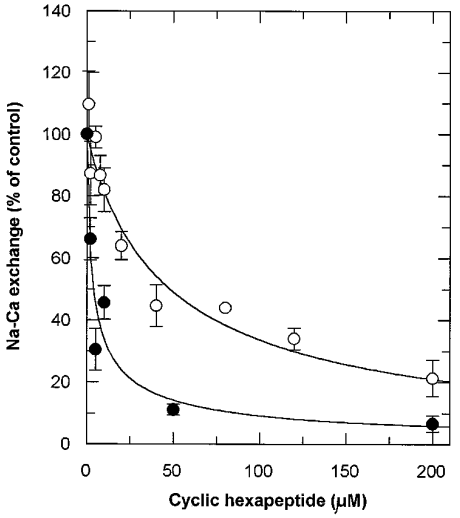

Fig. 1. Effect of Phe1-to-Trp- and Phe1-to-TIC-substituted FRCRCFa on the inhibition of Na^+/Ca^{2+} exchange. The initial rates (t = 2 sec) of Na_i-dependent ^{45}Ca uptake were measured under standard conditions with 23 μM $^{45}CaCl_2$ by using the semirapid mixing device (see Materials and Methods). The assay medium contained various concentrations of (○) Trp-Arg-Cys-Arg-Cys-Phe-$CONH_2$ or (●) TIC-Arg-Cys-Arg-Cys-Phe-$CONH_2$. *Points*, mean value from six independent measurements. *Extended lines*, standard deviations. One hundred percent of activity (control) represents the Na^+/Ca^{2+} exchange in the absence of any inhibitory peptide. *Curves*, computed to fit the experimental data as described Materials and Methods. The IC_{50} values (mean ± standard error) were computed as (●) 3.5 ± 1.2 μM and (○) 45.2 ± 5.2 μM.

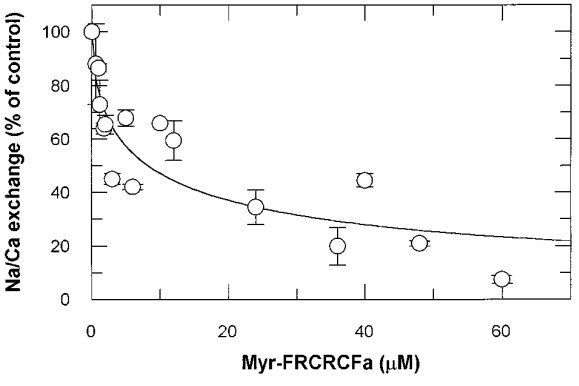

Fig. 2. Inhibitory effect of Myr-FRCRCFa on Na^+/Ca^{2+} exchange. The initial rates of Na_i-dependent ^{45}Ca uptake were measured as described in the legend to Fig. 1. Various concentrations of Myr-FRCRCFa were added to the assay medium before the experiment as described in Materials and Methods. *Points*, mean values from triplicate measurements. *Extended lines*, standard deviations. The control Na^+/Ca^{2+} exchange represents the activity in the absence of Myr-FRCRCFa. *Curve*, computed to fit the experimental points with an IC_{50} value of 8.2 ± 2.0 μM.

cally in the parent FRCRCFa by the other positively charged amino acids, lysine and ornithine. As can be seen from Fig. 3, all of these substitutions reduce the inhibitory potency of the parent cyclic peptide by 5–12-fold, suggesting that just a positive charge at these positions is not sufficient to exhibit the high inhibitory potency. Thus, the guanidinium groups of two arginines residues seem to be essential for determining the inhibitory potency, reinforcing the idea that Arg-Cys-Arg-Cys is a core pharmacophore for inhibitory effect.

Because two cysteines are an integral part of the Arg-Cys-Arg-Cys structure, we attempted to resolve their contribution to the inhibition of Na^+/Ca^{2+} exchange. For this goal, Cys3 and Cys5 were replaced in FRCRCFa by the conformationally constrained structural analog of cysteine, penicillinamine (β,β-dimethylcystein). The rationale for these substitutions is that they may affect, in general, a peptide helicity, transunnular interactions, and the diastereotopic environment around S—S bond (18–20). As can be seen from Fig. 4, Cys3-to-Pen3- and Cys5-to-Pen5-substituted cyclic peptides have IC_{50} values of 15–26 μM, indicating that these substitutions have a little (if any) effect on the inhibitory potency of the parent FRCRCFa peptide. In conclusion, a conformational constrained hydrophobicity and an increased hydrophobicity by methyl groups in the vicinity of S—S bond do not significantly affect the peptide/receptor interaction. These data support the idea that the role of Cys3 and Cys5 is to constrain FRCRCFa structure (e.g., to fix a proper orientation and/or distance between the guanidinium groups of two arginines).

To generate the cyclic peptides with diverse chirality, all six L-amino acids of FRCRCFa were replaced by analogous D-amino acids. These structural modifications may reverse some specific backbone turns, thereby modifying the cyclic peptide structure (18, 19). The introduction of D-amino acid substitutions may also improve resistance of cyclic peptide inhibitors to proteolytic enzymes (18). As can be seen from Table 2, substitutions by D-Phe1, D-Arg2, D-Cys3, and D-Phe6 reduce the inhibitory potency of the parent peptide by 4–5-fold, whereas substitutions by D-Arg4 and D-Cys5 have almost no effect on the inhibitory potency. This information can be exploited for designing peptide inhibitors with increased resistance to proteolysis.

Because proline can generate specific turns in the main chain (18, 19), this amino acid was introduced at different positions in FRCRCFa. As can be seen from Table 3, the introduction of proline in close vicinity of the carboxyl or amino terminus results in the IC_{50} of 30–60 μM, whereas insertion of proline inside the ring yields IC_{50} values as high as 200–400 μM (Table 3). Thus, allocation of proline outside of the ring declines the inhibitory potency by 3–6-fold, whereas the inclusion of proline inside the ring diminishes the inhibitory potency by 16–20-fold. The addition of proline to FRCRCFa expands the peptide structure to seven amino acids, so the extension of the ring and/or elongation of the

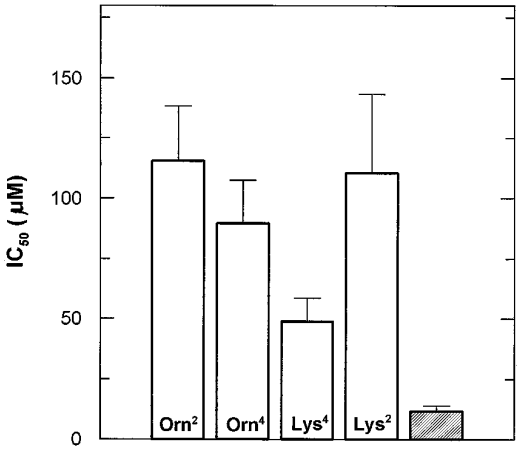

Fig. 3. Inhibitory potency of lysine- and ornithine-substituted FRCRCFa. The initial rates of Na^+/Ca^{2+} exchange were measured under standard conditions as described in the legend to Fig. 1. The assay medium contained various concentrations of the peptides Phe-Orn-Cys-Arg-Cys-Phe-$CONH_2$, Phe-Arg-Cys-Orn-Cys-Phe-$CONH_2$, Phe-Lys-Cys-Arg-Cys-Phe-$CONH_2$, or Phe-Arg-Cys-Lys-Cys-Phe-$CONH_2$. The IC_{50} values were estimated from the dose-response curves as described in Materials and Methods. *Extended lines*, standard deviation of IC_{50} value derived from three independent experiments. *Hatched bar*, IC_{50} value of the reference cyclic hexapeptide Phe-Arg-Cys-Arg-Cys-Phe-$CONH_2$ (FRCRCFa) estimated under the same experimental conditions.

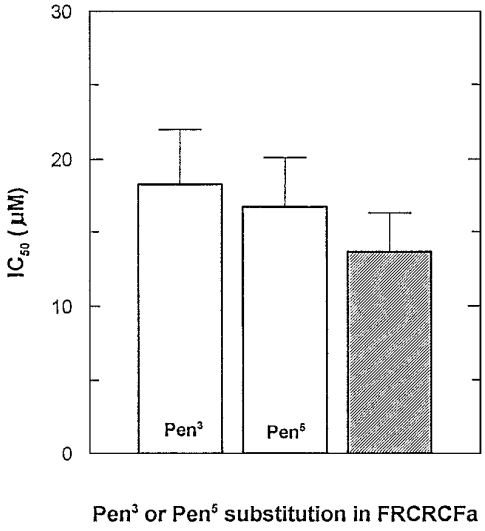

Fig. 4. Effects of Cys3-to-Pen3- and Cys5-to-Pen5-substituted FRCRCFa on the inhibition of Na^+/Ca^{2+} exchange. The initial rates of Na^+/Ca^{2+} exchange were measured under standard conditions (see legend to Fig. 1) in the presence of varying concentrations of Phe-Arg-Pen-Arg-Cys-Phe-$CONH_2$ or Phe-Arg-Cys-Arg-Pen-Phe-$CONH_2$. *Extended lines*, standard deviations. The IC_{50} values (mean ± standard error) were computed by dose-response curves as indicated in Materials and Methods. *Hatched bar*, IC_{50} value of the reference cyclic hexapeptide Phe-Arg-Cys-Arg-Cys-Phe-$CONH_2$ (FRCRCFa).

TABLE 2

Inhibition of Na^+/Ca^{2+} exchange by FRCRCFa with L→D-substituted amino acids

Peptide	IC_{50}[a]
	μM
D-Phe-Arg-Cys-Arg-Cys-Phe-$CONH_2$	30–37
Phe-**D-Arg**-Cys-Arg-Cys-Phe-$CONH_2$	47–68
Phe-Arg-**D-Cys**-Arg-Cys-Phe-$CONH_2$	42–50
Phe-Arg-Cys-**D-Arg**-Cys-Phe-$CONH_2$	17–25
Phe-Arg-Cys-Arg-**D-Cys**-Phe-$CONH_2$	16–23
Phe-Arg-Cys-Arg-Cys-**D-Phe**-$CONH_2$	37–41

[a] IC_{50} values of the peptide-induced inhibition of Na^+/Ca^{2+} exchange were estimated as described in Table 1.

TABLE 3

Effect of proline intercalation in FRCRCFa on inhibition of Na^+/Ca^{2+} exchange

The initial rates of Na^+/Ca^{2+} exchange were measured as outlined in Table 1 (see also Figs. 2 and 3 and Materials and Methods).

Peptide	IC_{50}[a]
	μM
Phe-**Pro**-Arg-Cys-Arg-Cys-Phe-$CONH_2$	30–40
Phe-Arg-Cys-**Pro**-Arg-Cys-Phe-$CONH_2$	200–400
Phe-Arg-Cys-Arg-Cys-**Pro**-Phe-$CONH_2$	42–62

[a] IC_{50} values were collected from three independent experiments.

carboxyl and amino termini by one additional amino acid can contribute to the inhibitory potency.

Discussion

The introduction of conformationally constrained cyclic peptides as a new class of blockers (1) opens new opportunities for pharmacological targeting of the Na^+/Ca^{2+} exchanger. Certainly, a substantial amount of work must be done on structure-activity relationships to identify an appropriate lead structure for improving the selectivity, availability, and stability of peptide-based inhibitors. Previously, we tested seven cyclic hexapeptides that have the same amino acid composition but differ in their amino acid sequence and intramolecular S—S cross-link (8–20 atoms in the ring) (1). Among these peptides, FRCRCFa (11 atoms in the ring) was a most potent inhibitor of Na^+/Ca^{2+} exchange, suggesting that the positively charged and conformationally constrained Arg-Cys-Arg-Cys structure might be a basal pharmacophore for inhibitory effect. For further resolution of structure-activity relationships, a number of new cyclic peptides have been synthesized and tested for their inhibitory potency. These cyclic peptides differ in their length (three to seven amino acids), and the positive charge is retained by one or two arginines, ornithines, or lysines. In addition, phenylalanine and cysteine were substituted in FRCRCFa with a goal of characterizing their contribution to the inhibitory potency.

Short cyclic peptides (three to five amino acids) that contain only one arginine show very low inhibitory potencies (Table 1). The substitution of Phe1 in FRCRCFa by TIC (analog of phenylalanine) results in the potent inhibition of the exchange with an IC_{50} value of 3.5 ± 1.2 μM (Fig. 1), suggesting 3–4-fold potentiation of inhibitory effect. The substitution for Phe1 by Nal-1 or valine had no significant effect on IC_{50} values (Table 1), whereas the Phe1-to-Trp1 substitution resulted in a 4-fold decrease in the inhibitory potency compared with FRCRCFa (Fig. 1). Although in some specific cases the addition of hydrophobic moieties to the carboxyl or amino terminus may increase the inhibitory potency, these effects seemed to have a limited capacity (at least in the scope of the current study) and showed no obvious structure-activity relationships. Further systematic research is necessary to resolve the structure-activity relationships at the carboxyl and amino ends of the cyclic peptide structure.

A significant disadvantage to the use of the arginine-containing peptide inhibitors is that they are positively charged and membrane impermeable under physiological conditions. A recent approach involves a fatty acid modification of an arginine-containing nanopeptide of phosphokinase C substrate peptides to overcome membrane impermeability (9). With acceptance of this general approach, myristoyl has been added to the amino terminus of FRCRCFa, and its effect was tested on Na^+/Ca^{2+} exchange. Myr-FRCRCFa inhibited the Na^+/Ca^{2+} exchange with an IC_{50} value of 8.2 ± 2.0 μM (Fig. 2). Thus, the inhibitory potency of Myr-FRCRCFa is similar to that of the parent FRCRCFa peptide. With the discovery of

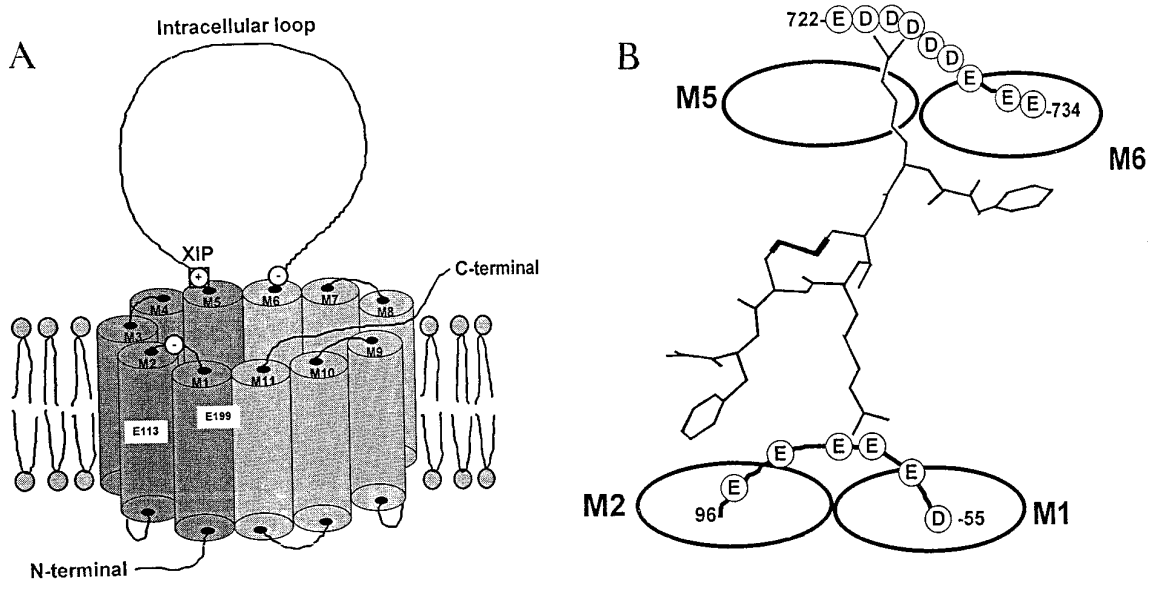

Fig. 5. Proposed interaction of FRCRCFa with the Na^+/Ca^{2+} exchanger. A, Na^+/Ca^{2+} exchanger topology. The exchanger (Na^+/Ca^{2+}) is described as a membrane protein with 11 transmembrane (*M*) segments and a large intracellular loop between transmembrane segments five and six. E113 and E199, two glutamic acids that presumably involve cation binding/translocation. The XIP domain (amino acids 219–238) is positively charged and may interact with negatively charged domains on the surface of the exchanger. B, Putative interactions of positively charged FRCRCFa with negatively charged domains at the cytosolic side of the Na^+/Ca^{2+} exchanger. The structure of FRCRCFa was drawn through the use of Nemesis and New Chem-X (for details, see Materials and Methods). For simplicity, hydrogen atoms are not shown. *Heavy line*, a peptide cyclization by S—S bond. It is proposed that FRCRCFa may interact with two negatively charged domains (amino acids 56–96 and 723–733) of the exchanger.

Myr-FRCRCFa, new possibilities arose to devise a cell-permeable analog of FRCRCFa.

The role of Arg2 and Arg4 has been tested by replacing arginine residues in FRCRCFa with lysine or ornithine. These substitutions have dramatic effects on the inhibitory potency, which is reflected in a 5–12-fold decrease in IC_{50} (Fig. 3). These data suggest that the guanidinium groups of two arginines are essential for an inhibitory effect of the cyclic hexapeptide, and they represent the inhibitory properties of conformationally constrained Arg-Cys-Arg-Cys pharmacophore. Because the diastereotopic environment and hydrophobicity around S—S bond can be considerably modified by the introduction of penicillinamine instead of cysteine (18, 19), Cys3 and Cys5 were replaced in FRCRCFa by penicillamine. It was concluded that the Cys-to-Pen substitutions have no significant effect on the inhibitory potency (Fig. 4). Thus, intramolecular S—S bond may not be exposed to the interface of the peptide/receptor interaction. These findings are consistent with a proposal that the side chains of two cysteines play little (if any) role in the interaction of FRCRCFa with a putative receptor. Therefore, S—S bond in the cyclic peptide inhibitors may play a role in conformational constrain and/or exposure (orientation) of other functional moieties (e.g., guanidinium, carbonyl, and amide groups). This point should be taken into account in the future design of the cyclic peptide blockers. The introduction of D-amino acid into the peptide structure may stabilize diverse chiral structures, thereby generating some specific turns in a peptide backbone (18). In this respect, all six L-amino acids were replaced in FRCRCFa by D-amino acid and tested for their inhibitory activity. As shown in Table 3, the substitutions with D-Phe1, D-Arg2, D-Cys3, and D-Phe6 reduced the inhibitory potency of the parent peptide by 4–5-fold, whereas no considerable change in inhibitory potency was observed with cyclic peptides with D-Arg4 and D-Cys5 substitutions. Therefore, these substitutions can potentially be used to increase the peptide resistance to proteolytic enzymes. Because cyclization enhances a conformational constrain and resistance to proteolysis (18–20), the D-substituted derivatives have a potential to provide even more stable peptide inhibitors.

Proline can generate specific turns in the main chain because it has less conformational freedom than any other natural amino acid residue (the proline side chain is fixed by a covalent bond to the main chain). Therefore, proline was included in FRCRCFa either inside or outside of the ring. The inclusion of proline outside of the ring of FRCRCFa decreases the inhibitory potency by 3–6-fold, whereas the introduction of proline inside Arg-Cys-Arg-Cys decreases the inhibitory potency by ≥16-20-fold (Table 3). Therefore, enlargement of the ring has a more drastic effect on the inhibitory potency than does elongation of the main chain outside of the ring.

Results of previous studies suggest that FRCRCFa does not compete with Na^+ or Ca^{2+} at the cytosolic side of the membrane (1–3), suggesting that FRCRCFa does not interact with ion-transport sites. Therefore, the conformationally constrained and positively charged blockers may interact with the negatively charged domains at a cytosolic side of the membrane. Two negatively charged sequences of the Na^+/Ca^{2+} exchanger may be involved in a primary attraction of peptide blockers on the intracellular surface of the exchanger protein molecule (Fig. 5). The first sequence, EDDDDDEGEE (amino acids 723–733), is part of a large regulatory loop and is located in close proximity to transmembrane segment six. It is possible that this segment interacts with positively charged XIP domains, thereby controlling some specific regulatory modes. The second sequence (amino acids 56–96) contains six negatively charged amino acids and represents a major part of the short intracellular loop that connects transmembrane segments one and two (Fig. 5). Systematic analysis of relevant mutants may resolve the mechanisms of proposed peptide/exchanger interaction.

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