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Identification of Important Amino Acid Residues of the Na^+-Ca^{2+} Exchanger Inhibitory Peptide, XIP

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Abstract. The Na^+-Ca^{2+} exchanger plays an important role in cardiac contractility by moving Ca^{2+} across the plasma membrane during excitation-contraction coupling. A 20 amino acid peptide, XIP, synthesized to mimic a region of the exchanger, inhibits exchange activity. We identify here amino acid residues important for inhibitory function. Effects of modified peptides on Na^+-Ca^{2+} exchange activity were determined. Exchange activity was assessed as $^{45}Ca^{2+}$ uptake into Na^+-loaded cardiac sarcolemmal vesicles. We find that the entire length of XIP is important for maximal potency, though the major inhibitory components are between residues 5 and 16. Basic and aromatic residues are most important for the inhibitory function of XIP. Substitutions of arginine 12 and arginine 14 with alanine or glutamine dramatically decrease the potency of XIP, suggesting that these residues play a key role in possible charge-charge interactions. Substitutions of other basic residues with alanines or glutamines had less effect on the potency of XIP. All aromatic residues participate in binding with the exchanger, probably via hydrophobic interactions as indicated by tryptophan fluorescence. A tyrosine is required at position 6 for maximal inhibition and phenylalanine 5 and tyrosine 8 can only be replaced by other aromatic residues. Tyrosine 10 and tyrosine 13 can be replaced with other bulky residues. A specific conformation of XIP, with structural constrains provided by all parts of the molecule, is required for optimal inhibitory function.

Key words: Na^+-Ca^{2+} exchanger — Sarcolemma — Inhibitory peptide

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Introduction

Na^+-Ca^{2+} exchange is the major route by which Ca^{2+} exits from the cardiac cell during excitation-contraction coupling and is an important determinant of cardiac contractility. The Na^+-Ca^{2+} exchanger uses the energy of the Na^+ gradient to transport Ca^{2+} outward across the plasma membrane (sarcolemma) of the cardiac myocyte with a stoichiometry of three Na^+ for one Ca^{2+}. The cardiac exchanger (NCX1) was first cloned by Nicoll, Longoni & Philipson (1990). Subsequently, several isoforms of the exchanger have been cloned from other tissues such as kidney and brain and from diverse species including Drosophila and squid. All Na^+-Ca^{2+} exchangers show substantial sequence similarity and have a topology similar to that predicted for NCX1. The mature NCX1 protein has 938 amino acids and is modeled to have 11 transmembrane segments and a long hydrophilic loop which is located intracellularly between transmembrane segments 5 and 6 (Nicoll et al., 1990; Fig. 1). The exchanger contains a putative autoregulatory domain at the beginning of the large intracellular loop (Li et al., 1991; Fig. 1). A synthetic peptide with the same amino acid sequence as this region (endogenous XIP region; Fig. 1) inhibits exchanger activity (Li et al., 1991; Matsuoka et al., 1993). This peptide has been called exchanger inhibitory peptide or XIP.

XIP consists of 20 amino acid residues with several hydrophobic residues interspersed with basic residues: an arrangement characteristic of calmodulin binding sites and autoregulatory domains (O'Neil, Erickson-Vitanen & DeGrado, 1989; Table 1). Previous studies showed that the inhibition of exchange activity by XIP is noncompetitive with respect to both Na^+ and Ca^{2+} and that the apparent binding affinity of XIP to the exchanger is 0.15–1.5 μM depending on conditions (Li et al., 1991).

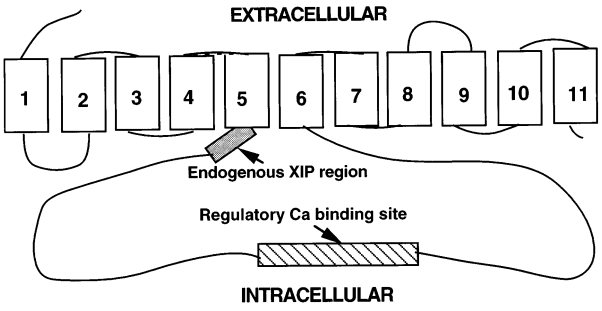

Fig. 1. Model of the cardiac Na^+-Ca^{2+} exchanger, NCX1. The endogenous XIP region and the regulatory Ca^{2+} binding site located in the intracellular loop between transmembrane segments 5 and 6 are indicated by arrows.

Kleiboeker, Milaneck & Hale (1992) have shown that ^{125}I-labeled XIP crosslinks to the exchanger in sarcolemmal membranes. In addition, Chin et al. (1993) showed that XIP inhibited outward Na^+-Ca^{2+} exchanger currents, but not currents through Ca^{2+} channels in whole cell patched cardiac myocytes. Shannon et al. (1994) have hypothesized that the endogenous XIP region of the exchanger interacts with membrane phospholipids. XIP is a relatively specific inhibitor of the exchanger, though it may also effect Ca^{2+} pumps (Enyedi & Penniston, 1993).

NCX1 has been expressed in *Xenopus* oocytes and has been analyzed by the giant excised patch technique. These studies have shown that, in addition to a Ca^{2+} transport site, the exchanger has an intracellular Ca^{2+} regulatory site. Binding of Ca^{2+} to the regulatory site activates exchanger activity. Mutants exchangers with deletions in the large intracellular loop are still capable of exchange activity but are no longer regulated by Ca^{2+}. Some deletion mutants are also not inhibited by XIP (Matsuoka et al., 1993). These studies suggest that the XIP-binding region and the regulatory Ca^{2+} site are both located on the large intracellular loop. Indeed, the Ca^{2+}-binding domain resides within amino acids 371–508 of the loop (Levitsky, Nicoll & Philipson 1994; Fig. 1). The XIP-binding region of the exchanger, however, has not yet been identified.

These studies suggest that the XIP-binding region of the exchanger may have an important regulatory role. However, there is no direct evidence that the endogenous XIP region of the intact protein interacts with the same site on the exchanger as exogenously added XIP. Nevertheless, identification of the XIP-binding region of the exchanger would provide useful information for understanding the possible regulatory role of the endogenous XIP region of the protein.

In this study, we attempt to identify amino acid residues of the XIP peptide which are essential for inhibitory function. We systematically synthesized XIP peptides with altered residues or with truncated termini to map the major inhibitory components of XIP. The goal is to better understand the nature of the interaction between XIP and its binding site. The inhibitory potencies of the modified XIP peptides were tested by measuring $^{45}Ca^{2+}$ uptake into Na^+-loaded cardiac sarcolemmal vesicles. In addition, tryptophan-containing peptides were synthesized to study the interaction between the exchanger and XIP by fluorescence techniques.

Materials and Methods

Peptide Synthesis

XIP peptides were synthesized by Bayer AG (Wuppertal, Germany) and were acetylated at the N-terminus and amidated at the C-terminus. The automated peptide synthesis was carried out according to a standard Fmoc/tBu protocol using TBTU or PyBOP as coupling reagents. Rink amide or Tentagel® S NH_2 resins were used. The Tentagel® S NH_2 resin was first reacted with an amide linker. The side-chain protecting groups were Arg(Pmc), Asn(Trt), Asp(OtBu), Cys(Trt), Gln(Trt), Glu(OtBu), His(Trt), Lys(Boc), Ser(tBu), Thr(tBu), Trp(Boc), and Tyr(tBu). Amino terminal acetylation was performed with acetic acid anhydride. The peptides were cleaved using trifluoroacetic acid containing various scavengers. The composition of the cleavage cocktail was adjusted to the amino acid sequence. The crude peptides were purified if necessary by preparative reversed-phase HPLC using different water-acetonitrile gradients containing 0.05% trifluoroacetic acid. The purity of the final products was assessed by analytical reversed-phase HPLC and the peptides were characterized by FAB-MS or ESI-MS.

sXIP was obtained from Dr. John Bridge.

Membrane Preparations

Cardiac sarcolemmal vesicles were prepared from canine ventricles as described by Frank et al. (1984).

Na^+-Dependent Ca^{2+} Uptake

Na^+-dependent Ca^{2+} uptake into sarcolemmal vesicles was measured as previously described in detail (Vemuri & Philipson, 1988). 5 μl of Na^+-loaded vesicles were rapidly diluted to 0.25 ml of uptake medium containing 140 mM KCl (or NaCl for blanks), 0.3 μCi $^{45}Ca^{2+}$, 10 μM Ca^{2+}, 0.4 μM valinomycin, 10 mM MOPS, pH 7.4 at 37°C. This was done in the presence or absence of 1.5 μM peptide. The uptake reaction was stopped at 2 sec with 30 μl of 140 mM KCl and 10 mM EGTA, immediately followed by addition of 1 ml cold 140 mM KCl, 1 mM EGTA. The vesicles were collected by filtration and washed twice with cold 140 mM KCl, 1 mM EGTA.

All experiments were performed in duplicate, with an *n* of at least 3. Data are expressed as means ± SD.

Fluorescence Spectra

Fluorescence emission spectra were recorded in a SLM 8000 spectrofluorometer (SLM Instruments) using an excitation wavelength of 295 nm. The experiments were carried out at 5 μM peptide in 140 mM NaCl, 10 mM MOPS pH 7.4 in the absence or presence of 1 μM purified loop fusion protein (amino acids 240–676) of the exchanger. The low

fluorescence background of the loop fusion protein was subtracted from each spectrum.

Purification of Loop Fusion Protein

cDNA coding for a large portion (amino acids 240–676) of the intracellular loop of the exchanger was subcloned into a pQE expression vector (QIAGEN) containing a 6xHis affinity tag at the N-terminus, and transformed into M15 *E. coli* (QIAGEN). 10 ml of LB-broth supplemented with 100 μg/ml ampicillin and 25 μg/ml kanamycin (LB-AMP/KAN) were innoculated with a single colony containing the expression plasmid and grown overnight at 37°C. 500 ml LB-AMP/KAN were innoculated with 5 ml overnight culture and grown at 37°C until A_{600} reached 0.7–0.9. Expression of the fusion protein was induced by addition of IPTG (final concentration 2 mM) and the cells were grown at 37°C for 4 hr. The cells were harvested by centrifugation at 4000 × *g* for 10 min and resuspended in Buffer A (6 M urea, 0.1 M Na-phosphate, 0.01 M Tris/HCl, pH 8.0) and stirred for 1 hr at room temperature. Cell lysates were centrifuged at 10,000 × *g* for 15 min at 4°C. The supernatants were added to 8 ml Ni-NTA resin (50% slurry), previously equilibrated in Buffer A, and stirred at room temperature for 45 min. The resin/lysate mixtures were loaded onto a 1.6 cm diameter column and washed with Buffer A until A_{280} of the flow-through was <0.01 and then washed with buffer C (8 M urea, 0.1 M Na-phosphate, 0.01 M Tris/HCl, pH 6.3) until A_{280} was <0.01. The loop fusion protein was eluted with Buffer C containing 250 mM imidazole and refolded by stepwise dilution of the urea in a dialysis buffer containing 140 mM NaCl, 10 mM MOPS, pH 7.4. The purity of the fusion protein was analyzed by SDS-PAGE and a homogenous band with the expected molecular weight was observed. The protein concentration was determined by Lowry assay.

Results and Discussion

Na^+-Ca^{2+} Exchange Assay

As described in Materials and Methods, we use a standard technique to assess Na^+-Ca^{2+} exchange activity using cardiac sarcolemmal vesicles. Na^+ gradient-dependent $^{45}Ca^{2+}$ exchange was measured over a 2-sec interval. Ca^{2+} uptake is linear over this period allowing determination of initial rates. The effects of all peptides were assessed at a concentration of 1.5 μM. As described previously (Li et al., 1991), this concentration of XIP equals the I_{50} value. The vesicular exchange assay only measures uptake into inside-out sarcolemmal vesicles (Li et al., 1991) and thus the inhibition by extravesicular XIP analogues is consistent with an intracellular site of action.

Truncation Modifications

The sequence of the wild-type XIP of NCX1 is shown in Table 1. We initially truncated XIP from either the N- or C-terminus to define the main inhibitory core of the peptide. Exchange activity was measured in the presence of wild-type or truncated peptides as described in Materials and Methods. The results in Table 1 show that the inhibitory activity of XIP was barely changed by deletion of the first 2 residues (Δ1–2) from the N-terminus. More substantial changes were observed with deletions of either 5 (Δ16–20) or 7 (Δ14–20) residues from the C-terminus (Table 1). These two truncations approximately halved the potency of XIP. Inhibitory activity was almost completely lost with deletion of the first 5 residues (Δ1–5) from the N-terminus or the last 9 residues (Δ12–20) from the C-terminus. Further deletion from the N-terminus (Δ1–8) completely eliminated inhibitory potency (Table 1). Thus, the inhibitory activity of XIP is very sensitive to truncation. Very few residues from either end can be removed without influencing inhibition. A minimum length may be required to provide the proper structural constraints for optimal inhibitory action. The N-terminal portion of XIP may be more essential than the C-terminal portion, though it is notable that two arginines can be removed from the N-terminus (Δ1–2) with little consequence.

Table 1. Effects of truncated NCX1 XIP peptides on the inhibition of Na^+-Ca^{2+} exchange activity[a]

	Amino acid sequence	% Inhibition
XIP	RRLLFYKYVYKRYRAGKQRG	47.5 ± 1.5
Δ1–2	LLFYKYVYKRYRAGKQRG	41.5 ± 6.5
Δ1–5	YKYVYKRYRAGKQRG	7.7 ± 0.2
Δ1–8	VYKRYRAGKQRG	−1.5 ± 0.5
Δ12–20	RRLLFYKYVYK	8.5 ± 1.5
Δ14–20	RRLLFYKYVYKRYR	20.5 ± 1.5
Δ16–20	RRLLFYKYVYKRYRAG	25.5 ± 5.5

[a] The experiments were carried out as described in ''Materials and Methods.''

Alanine Substitutions

The first step to identify key residues of XIP was to individually replace each residue with alanine. The most striking decreases in the inhibitory activity of XIP occurred when either R12 or R14 was substituted with alanine (Fig. 2). Substitutions of F5, Y6, K7, and Y13 also caused a significant reduction of about 50% in XIP potency. Alanine substitutions at other sites had moderate effects on the potency of XIP. The two exceptions were the alanine substitutions of G16 and G20, which had no effect (Fig. 2). It is striking that alanine substitution of almost all residues diminishes XIP potency. This again implies that the conformation of the entire peptide is important for inhibitory function. Interestingly, those residues whose substitution most affected XIP potency are all either basic or aromatic residues. These residues may play a key role in the inhibition of exchanger activity. Therefore, we analyzed the func-

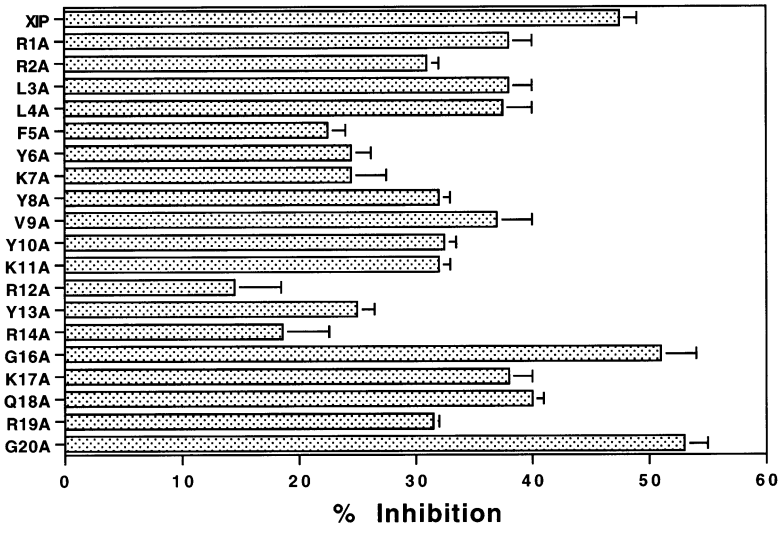

Fig. 2. Inhibition of Na^+-Ca^{2+} exchange activity by alanine-substituted XIP peptides. The experiments were carried out as described in Materials and Methods.

tional role of basic and aromatic residues by further modifications.

Modification of Basic Residues

Basic residues are important for the inhibitory function of some autoinhibitory peptides (Malencik & Anderson, 1984). In addition, the data above suggest that two of the arginines (R12 and R14) in XIP may play a key role in a charge-charge interaction with the exchanger (Fig. 2). We further investigated R12 and R14 and other basic residues of XIP to understand their roles in inhibitory function.

First, R12 and R14 were substituted with glutamine. Single glutamine substitutions (R12Q, R14Q) had effects similar to the single alanine substitutions (R12A, R14A; Fig. 3). When both R12 and R14 were replaced by either glutamines (RR12,14QQ) or alanines (RR12,14AA), the inhibitory activity of XIP was further reduced, especially with the double glutamine substitution (Fig. 3). RR12,14QQ only inhibited exchange activity by 5%. These data suggest that positive charges at positions 12 and 14 are required for maximal inhibitory activity since substitution with neutral residues of similar size (arginine to glutamine) almost abolished inhibitory activity. Furthermore, multiple substitutions of R12, R14 and K11 with glutamines and Y13 with threonine (KRYR11,12,13, 14QQTQ) also almost eliminated inhibitory activity (Fig. 3).

Substitution of K7 with alanine (K7A) significantly reduced the inhibitory activity of XIP, while substitution of K11 with alanine (K11A) had a smaller effect (Fig. 2). However, when K7 or K11 was replaced by glutamine (K7Q, K11Q), the inhibitory potency improved, though it was not completely regained (Fig. 3). These results suggest that K7 and K11 are not as crucial as R12 and R14, but still participate in the interaction with the exchanger.

Although single substitutions of other basic residues with alanine only caused modest changes in inhibitory activity, multiple substitutions at these positions had major effects on inhibitory potency. Double substitutions of K17 and R19 with either glutamine (KR17,19QQ) or alanine (KR17,19AA) diminished inhibitory activity (Fig. 3). Likewise, XIP peptides with double substitutions of R1 and R2 with either alanine (RR1,2AA) or glutamine (RR1,2QQ) had low inhibitory potency (Fig. 3). Moreover, when R1, R2, K7 and K11 were all substituted with alanine (RRKK1,2,7,11A), inhibitory activity was completely lost (Fig. 3).

It is striking that double substitutions of R1 and R2 or K17 and R19 display more substantial reductions of inhibition than deletions of either the first two (Δ1–2) or the last five (Δ16–20) residues which include R1 and R2 or K17 and R19, respectively (Table 1 and Fig. 3). In one case, two basic residues are neutralized (Fig. 3), whereas in the other case, the basic residues are removed (Table 1). The same degree of reduction of inhibition would be expected if the roles of R1, R2, K17, and R19 were to simply supply positive charge. Apparently, the double alanine or glutamine substitutions induce a conformation of XIP incompatible with optimal inhibition. The data again emphasize the importance of the entire peptide in inhibitory interactions.

Modification of Aromatic Residues

XIP has a total of 5 aromatic residues: phenylalanine at position 5 and tyrosines at positions 6, 8, 10 and 13. The results from Fig. 2 demonstrate that single alanine substitutions of F5, Y6 and Y13 caused about 50% decrease in XIP potency; less reduction was seen with sub-

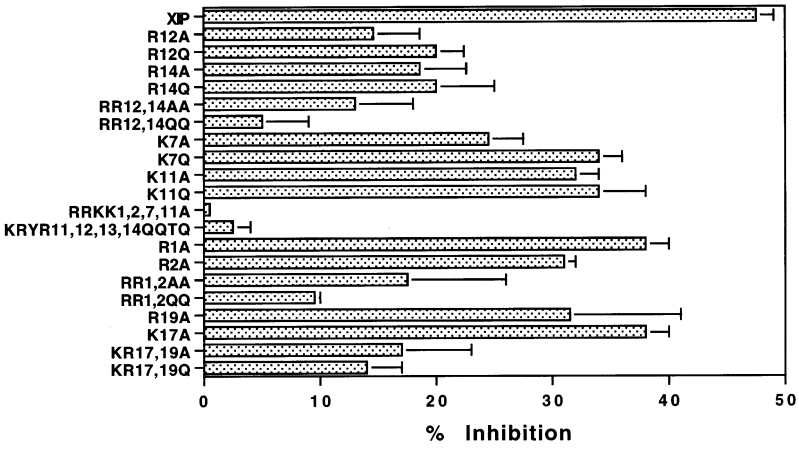

Fig. 3. Effects of substituting basic residues of XIP on inhibition of Na^+-Ca^{2+} exchange activity.

stitutions of Y8 and Y10. However, when all four tyrosines were changed to alanine (Y6,8,10,13A), inhibitory activity was completely lost (Fig. 4*A*). It appears that aromatic residues are involved in the interaction of XIP with the exchanger.

Alanine is much smaller than tyrosine so we made other substitutions of the tyrosine residues. Threonine is larger than alanine and contains a hydroxyl group, as does tyrosine. Substitution of Y6 or Y8 with threonine (Y6T, Y8T) produced even more reduction in inhibitory activity than the single alanine substitutions (Fig. 4*A*). In addition, multiple substitution of F5, Y6 and Y8 with threonine (FYY5,6,8TTT) led to complete loss of inhibitory activity of XIP (Fig. 4*A*). When Y6 was replaced by glutamine (Y6Q), an even larger residue than threonine, inhibitory activity of XIP was still low (Fig. 4*A*). The results indicate that neither bulky nor hydroxyl-containing residues are sufficient to replace the function of tyrosine at this position.

We next tested replacements of tyrosines with other aromatic residues. Y8, Y10, or Y13 could be replaced by another aromatic residue, tryptophan, and the inhibitory activity remained comparable to wild-type XIP (Y8W; Y10W; Y13W; Fig. 4*A*). However, replacement of Y6 with tryptophan (Y6W) reduces inhibitory activity (Fig. 4*A*), indicating that a tyrosine is most essential at position 6. In contrast, Y8, Y10, and Y13 can be replaced by other aromatic residues. Y10 and Y13 could even be replaced with arginine (YY10,13RR), a basic bulky residue, without effect on the inhibitory activity of XIP (Fig. 4*A*). The inhibitory activity of YY10,13RR was slightly increased compared to XIP, demonstrating that aromatic residues are not absolutely required at these two positions. Furthermore, replacement of all four tyrosines together with tryptophan also gave wild-type activity (Y6, 8, 10, 13W; Fig. 4*A*).

When F5 was replaced by the hydrophobic residue valine, inhibitory activity was higher than that of F5A though less than that of wild-type XIP (Fig. 4*B*). However, when F5 was replaced by the aromatic residue tryptophan, inhibitory activity was restored (Fig. 4*B*). The results indicate that an aromatic residue at position 5 is not absolutely required but is still preferable to other residues for maximal inhibition. For example, placing a negatively charged glutamate at position 5 (F5E) greatly reduced inhibitory potency, whereas the same substitution at a different position V9 (V9E) had much less effect on the potency of XIP (Fig. 4*B*).

Our studies indicate that basic and aromatic residues are especially important. Therefore, we examined the effect of introducing additional aromatic residues or basic residues on the potency of XIP. When V9 was replaced with phenylalanine, inhibition was slightly increased; when V9 and A15 were simultaneously substituted with phenylalanine and tyrosine, respectively, there was a significant increase in the potency of XIP (Fig. 4*C*). Replacements of both Y10 and Y13 with arginine (YY10,13RR) also enhanced inhibition slightly (Fig. 4*A*). In addition, replacement of both L3 and L4 with arginines (LL3,4RR) also slightly increased potency (Fig. 4*C*). Systematic addition of aromatic and basic residues could possibly further improve the potency of XIP.

XIP Peptides from other Exchangers

Several isoforms of the Na^+-Ca^{2+} exchanger have been cloned and sequenced including NCX1 from canine heart, NCX2 and NCX3 from rat brain, CalX from Drosophila and NCX-SQ1 from squid (Nicoll et al., 1990; Li et al., 1994; Valdivia et al., 1995; Nicoll et al., 1996; He et al., 1996). These exchangers are homologous and are predicted to have similar topologies. All known isoforms of the exchanger contain an endogenous XIP region at the beginning of the intracellular loop. The XIP region is well conserved, but some residues vary at positions suggested to be important from our studies above.

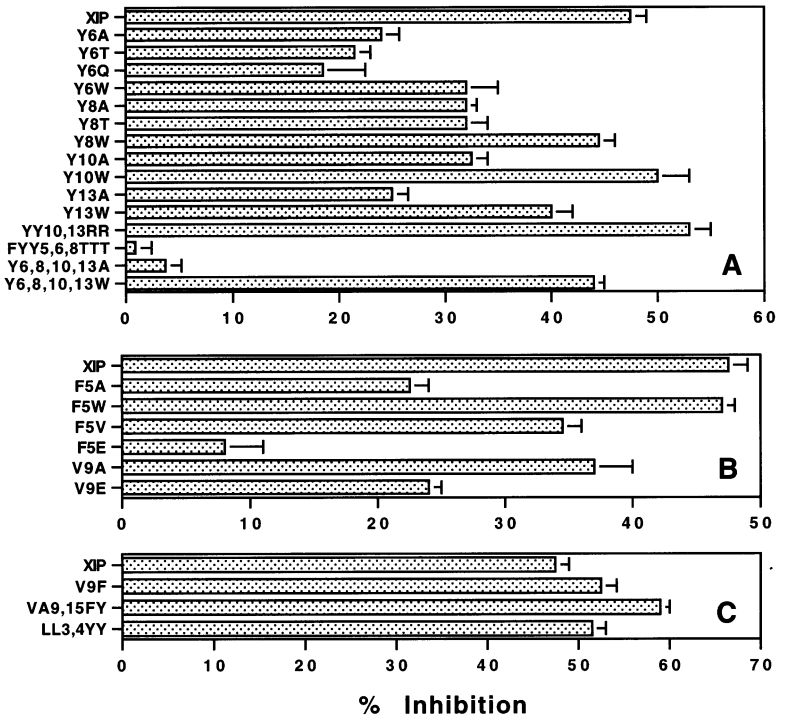

Fig. 4. Effects of substituting aromatic residues of XIP on inhibition of Na^+-Ca^{2+} exchange activity.

We tested how well XIP peptides, derived from other exchanger isoforms, would inhibit the cardiac NCX1 exchange activity.

We synthesized peptides of three additional exchangers and tested their inhibitory activity on Na^+-dependent $^{45}Ca^{2+}$ uptake into cardiac sarcolemmal vesicles. In addition, a peptide with the same amino acid composition as XIP, but with a random scrambled sequence (sXIP) was tested.

Table 2 shows that sXIP and XIPs derived from Calx (Drosophila) and NCX2 produced only about 10% inhibition of cardiac exchange activity at 1.5 μM. The XIP peptide derived from NCX-SQ1 (squid) was about half as potent as XIP from NCX1. The sequences of these XIP peptides are most similar at the N-terminus (except for the NCX-SQ1 XIP peptide) and vary most at the C-terminus (Table 2). A decrease in the potency of these peptides is likely due to changes at positions shown above to be important for the inhibitory activity of NCX1 XIP. For instance, instead of the two basic residues at positions 17 and 19 in NCX1 XIP, NCX2 XIP has two neutral residues, a proline and a serine at these positions. XIP peptides with K17 and R19 replaced with either alanine (KR17,19AA) or glutamine (KR17,19QQ) had drastically reduced inhibitory activity (Fig. 3). Similarly, Y10 and R12 in NCX1 XIP were replaced by an aspartate and a glutamine, respectively, in Calx XIP. Moreover, NCX-SQ1 XIP lacks the important F5 and Y6 residues of NCX1 XIP (Table 1). In addition, a negatively charged aspartate residue appears in the peptides of NCX2 and Calx. Our results above indicate that these variations account for reduced inhibitory activity. The very low potency of sXIP is due to an absence of structural specificity which is required for the potency of XIP. These results confirm the importance of those key residues which have been identified above and the requirement of a proper XIP conformation for optimal inhibition.

Table 2. Inhibition of cardiac Na^+-Ca^{2+} exchange activity by XIP peptides derived from different exchangers[a]

	Amino acid sequence	% Inhibition
NCX1	RRLLFYKYVYKRYRAGKQRG	47.5 ± 1.5
NCX2	RRLLFYKYVYKRYRTDPRSG	11.5 ± 4.5
CalX	RRLLFYKYMDKNYRVNK-RG	7.2 ± 2.2
NCX-SQ1	RRLL-NKYLSKKYRASKQKG	24.0 ± 2.2
sXIP	YQLRGFRGRKYAVRYRKYLK	9.8 ± 0.2

[a] NCX1, canine heart; NCX2, rat brain; CalX, Drosophila; NCX-SQ1, squid; sXIP, scrambled NCX1 XIP. The amino acid sequence of each peptide is shown in the table. Amino acids different from NCX1 XIP are underlined.

Tryptophan Fluorescence Spectra

Fluorescence of tryptophan residues is strongly dependent on environment (Lakowicz, 1983). The fluorescence emission spectra of tryptophan-containing inhibitory peptides of calmodulin display shifts toward shorter maximal wavelengths and increased fluorescence inten-

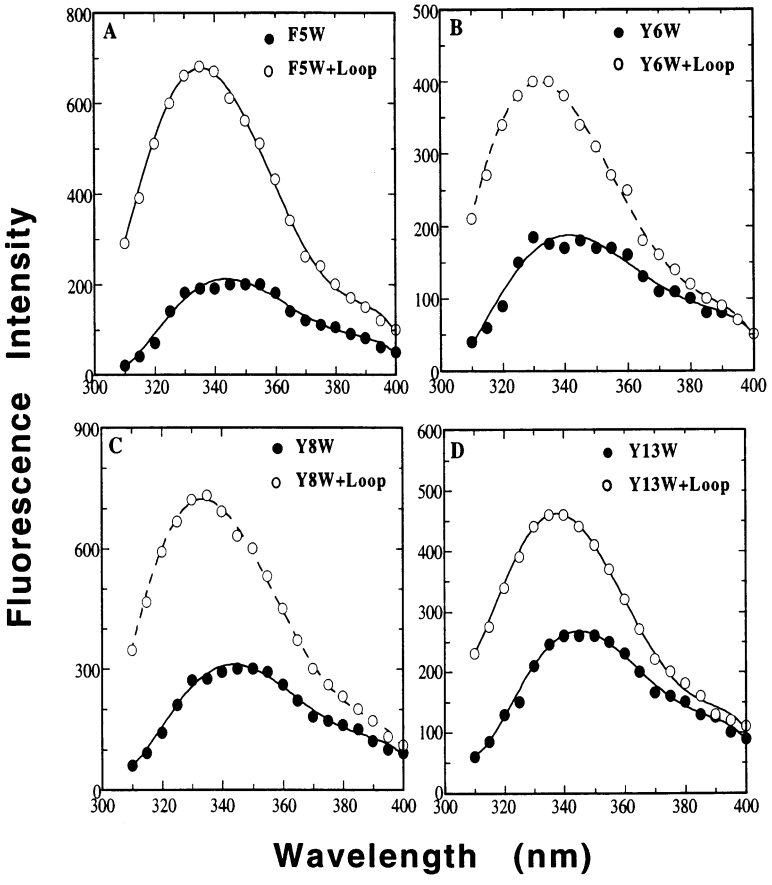

Fig. 5. Fluorescence spectra of F5W, Y6W, Y8W, and Y13W peptides in the absence (●) or presence (○) of the purified loop (amino acid residues 240–676) of the exchanger. Fluorescence spectra were recorded with a spectrofluorometer using an excitation wavelength of 295 nm. The experiments were carried out at a peptide concentration of 5 μM peptide in the absence or presence of 1 μM of purified loop protein of the exchanger as described in Materials and Methods.

sity upon binding to calmodulin (Malencik & Anderson, 1982; Comte, Maulet & Cox, 1993). Thus, tryptophan fluorescence has been widely used to study the interaction between calmodulin and peptides and to identify amino acids of peptides involved in direct contact with calmodulin (Malencik & Anderson, 1984; O'Neil et al., 1987). We used the same approach to investigate the nature of the interaction between XIP and the Na^+-Ca^{2+} exchanger using our tryptophan-containing peptides (Fig. 3). Previous studies from our laboratory have suggested that the site of action of XIP is located on the large intracellular loop of the exchanger (Matsuoka et al., 1993). Therefore, we used only the loop region of the exchanger to carry out the study.

Conveniently, the intracellular loop of the exchanger has no tryptophan residues and tyrosine fluorescence of the loop can be minimized by exciting at 295 nm (Weber, 1961). The loop fusion protein encompassing amino acids 240 to 676 was expressed and purified as described in Material and Methods.

We tested 5 peptides: F5W, Y6W, Y8W, Y10W and Y13W which have wild-type-like inhibitory activity (Fig. 4). All display a maximum of fluorescence at around 350 ± 5 nm in the absence of loop fusion protein (Fig. 5; spectrum of Y10W *not shown*), consistent with a tryptophan being exposed to the aqueous solvent (Lakowicz, 1983; O'Neil et al., 1987). Upon addition of loop fusion protein, fluorescence intensity increased about 2-fold for Y6W, Y8W, Y10W and Y13W and 3 fold for F5W. In addition, there were shifts of the maximum wavelength of fluorescence from 350 ± 5 nm to 330 ± 5 nm (Fig. 5). A negligible contribution of the loop fusion protein to these spectra was eliminated by subtraction. The dramatic changes in fluorescence intensity and the shifts of the maximum fluorescence towards shorter wavelengths indicate that tryptophans of the peptides are transferred from an aqueous environment to a hydrophobic environment upon binding of the peptides to the loop fusion protein. The magnitude of the change in the fluorescence intensity varied among the peptides, suggesting that the individual tryptophan residues at each position were placed in different microscopic environments within the peptide binding site. These observations further confirm results from our functional studies suggesting that these aromatic residues are directly involved in the interaction with the exchanger protein. The interaction probably occurs through hydrophobic contact.

The dramatic changes in fluorescence intensity of these tryptophan-containing peptides in the presence of the loop fusion protein appear specific, because BSA at

the same concentration as the fusion protein failed to induce any change in the fluorescence intensity for one of the peptides, F5W (*data not shown*). In addition, when 50 μM wild-type XIP was included in the experiment to compete with F5W (5 μM), more than 80% of the increased fluorescence intensity of F5W was abolished (*data not shown*). However, when a nonfunctional peptide, Y6,8,10,13A, was included at 50 μM, the fluorescence of F5W was diminished by only 20% (*data not shown*). These experiments suggest that the interaction between XIP and the loop fusion protein is relatively specific as determined here by tryptophan fluorescence changes.

Some calmodulin-binding peptides are amphipathic, and show an increased α-helical content upon binding to calmodulin as determined by circular dichroism (Steiner & Norris, 1987; Maulet & Cox, 1983). In contrast, circular dichroism studies of XIP indicated that the peptide has an unordered secondary structure in solution (*data not shown*). In addition, circular dichroism failed to detect any conformational changes in XIP induced by the presence of the loop protein (*data not shown*), in contrast to results which have been reported for calmodulin. Both calmodulin-binding peptides and XIP are rich in basic and hydrophobic residues, but the nature of the interactions with their target proteins is probably different.

Conclusions

We identified key residues of XIP which are important for inhibitory potency. These residues are either basic or aromatic. Some are more essential like F5, Y6, R12 and R14, while others (e.g., Y8, Y10, Y13, K7 and K11) have a supportive role in interactions with the exchanger (Figs. 2, 3, and 4). Results from truncation modifications and multiple-substitutions suggest that other residues of the peptide also participate in inhibitory interactions by helping to maintain a proper conformation for maximal inhibition (Table 1, Figs. 3 and 4). Our fluorescence study indicates that the interaction between the peptide and the exchanger involves some hydrophobic binding. Therefore, we predict that the XIP-binding site of the exchanger should have both hydrophobic and anionic components. Results from this study will provide a guide for site-directed mutagenesis in the endogenous XIP region of the intact exchanger protein and possibly to design a peptide inhibitor with higher potency than XIP. The results may also help us to identify the XIP-binding region of the exchanger in future studies.

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