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# Localization of an Exchange Inhibitory Peptide (XIP) Binding Site on the Cardiac Sodium-Calcium Exchanger

Calvin C. Hale,*,†,[1] Scott Bliler,*,† Thomas P. Quinn,‡ and Elena N. Peletskaya‡
**The Dalton Cardiovascular Research Center, †Department of Veterinary Biomedical Sciences, and ‡Department of Biochemistry, University of Missouri, Columbia, Missouri 65211*

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**The exchange inhibitory peptide (XIP; RRLLFYKYVYKRYRAGKQRG) is a potent inhibitor of cardiac Na-Ca exchange activity. This study attempted to identify the XIP binding site on the Na-Ca exchange protein. Bovine cardiac sarcolemmal vesicles were proteolyzed and fractionated by XIP-affinity column chromatography. A 24 kDa fragment was purified and subjected to amino acid sequence analysis. A negatively charged region of intracellular loop f of the Na-Ca exchange protein (IDDDIFEEDEN; aa 445-455) was identified. The affinity and specificity of XIP interaction with peptides IDDDIFEEDEN and GEDDDDDECGEE (another negatively charged region of the Na-Ca exchange protein) were examined. XIP cross-linked to peptide IDDDIFEEDEN but not GEDDDDDECGEE in a pH-dependent manner. Fluorescence titration binding studies indicated that binding of IDDDIFEEDEN with XIP was saturable (Kd=5 $\mu$M) while binding with GEDDDDDECGEE was not specific. These data suggest that amino acids 445-455 on Na-Ca exchange loop f are involved in XIP binding.** 

The cardiac myocyte plasma membrane or sarcolemma (SL), contains a number of $Ca^{2+}$ transport mechanisms that play central roles in $Ca^{2+}$ homeostasis and contractility. During diastole, Na-Ca exchange is the primary exporter of cytosolic $Ca^{2+}$ to the outside of the cell. The deduced amino acid structure of the Na-Ca exchanger has led to the modeling of a protein with 11 transmembrane helices and 12 extramembranal segments including a large cytoplasmic segment designated loop f which accounts for nearly 50% of the mass of the protein. The N-terminal portion of loop f contains a 20 amino acid domain consisting of positively charged and hydrophobic amino acids. It has been postulated that this region may play a role in regulating exchange activity (1,2). The exchange inhibitory peptide (XIP) is homologous to this 20 amino acid region and is a potent inhibitor of cardiac Na-Ca exchange (2,3). While cellular control of cardiac Na-Ca exchange activity is not well understood, there is a growing body of evidence suggesting that the Na-Ca exchange transport protein is regulated by anionic SL phospholipids perhaps through interaction with the endogenous XIP domain (4,5,6). Regardless of the role of the endogenous XIP domain on regulating Na-Ca exchange activity, it is clear that XIP inhibits transport by binding directly to the transport protein (3,7). Occupation of this site by XIP on the Na-Ca exchange protein inhibits transport.

In this report, we present evidence that the XIP binding site on the cardiac Na-Ca exchanger protein is in the proximity of a region of negatively charged amino acids on cytoplasmic loop f. XIP binding to a binding site peptide homologue is specific and has a Kd similar to the IC50 of XIP inhibition of Na-Ca exchange activity in cardiac SL vesicles (3,4). This site has previously been described as a potential regulatory Ca binding site (7) and may bind XIP and Ca simultaneously.

## MATERIALS AND METHODS

*Cardiac SL vesicles.* Bovine cardiac SL vesicles were prepared as described in (8) except that the following protease inhibitors (w/3 L) were added to the initial mannitol-based homogenization buffer: aprotinin (0.5 mg), leupeptin (0.5 mg), pepstatin A (0.5 mg), and phenylmethylsulfonyl fluoride (100 mg). Vesicles were stored at −70°C prior to use. Protein values were determined as described by (9). Na-Ca exchange transport measurements and electrophoretic techniques were performed as previously described by (3).

*Synthetic peptides.* Peptides used in this study were synthesized at the Macromolecular Structure Analysis Facility at the University of Kentucky, Lexington, KY. After synthesis, peptides were desalted and sequenced to assure purity.

APDP (*N*-[4-(*p*-Azidosalicylamido)butyl]-3′(2′-pyridyldithio)propionamide; Pierce) was iodinated and conjugated to cysXIP in very dim light by the following procedure: A 10 mM stock of APDP was prepared in dimethyl sulfoxide (DMSO). One iodination reagent bead (Iodobeads; Pierce) was incubated with 1 mCi of $Na^{125}I$. The volume was adjusted to 2 ml with 0.1 M sodium phosphate, pH 8.0 to which 100 $\mu$l of the APDP stock was added. The iodination proceeded at 4°C for 4 min

[1] To whom correspondence should be addressed. Fax: (573) 884-4232. E-mail: calvin_hale@muccmail.missouri.edu.



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after which the APDP solution was transferred to an Eppendorf tube containing 0.8 mg of cysXIP in 0.8 ml of 50 mM sodium phosphate, pH 8.0. After 3 hr at 4°C, the mixture was desalted through a 10 ml (bed volume) Sephadex G-10-120 column equilibrated with 10 mM 3-(N-morpholino)propanesulfonic acid (MOPS) adjusted to a pH of 7.4 with tris[hydroxymethyl]aminomethane (tris).

*Affinity column chromatography.* XIP was cross-linked to CNBr-activated Sepharose 4B (Pharmacia Biotech) at a ratio of 5 mg peptide/ml matrix according to the manufacturers instructions. The affinity column matrix was equilibrated in 160 mM NaCl, 20 mM MOPS/tris, 0.1% dimethylsulfoxide (v/v), pH 6.6.

Bovine cardiac SL vesicles (10 mg) were proteolyzed with immobilized chymotrypsin (Sigma) by incubation on a rotating mixer in the above buffer containing 1 mM dithiothreitol for 6 hr at 22° C. The resultant digest was subjected to ultracentrifugation (185,000 × g, 4° C, 1 hr) to remove vesicles and immobilized protease. Soluble proteolytic peptide fragments in the supernatant fluid were applied to the immobilized XIP affinity column. The column was washed with column buffer followed by elution in 50 mM glycine-HCl, pH 2.5. Eluted affinity column chromatography fractions were concentrated by lyophilization and subjected to SDS-PAGE and Western blotting onto PVDF membrane. The blotted membrane was stained with Coomassie blue 250 to detect protein bands which were submitted for amino acid sequence analysis (5-cycles).

*Cross-linking experiments.* Radiolabeled APDP-cysXIP was used as a cross-linking probe as described in the figure legend. Incubates containing the probe and peptides were irradiated with a hand-held uv light, at room temperature at a distance of 5 cm for 1 min. Samples were prepared in SDS-PAGE sample buffer with or without $\beta$-mercaptoethanol as indicated.

*Fluorescence titrations.* Fluorescence measurements were performed on a SLM Aminco Fluorimeter equipped with dual detectors. The fluorimeter was interfaced with a Dell 433/L PC running SLM Aminco 8100 series 2 software. A one site binding model was used for the calculation of dissociation constants. Fluorescence intensity of endogenous XIP tyrosines was monitored at 299 nM following excitation at 280 nM. Titrations were performed at 20° C in 100 mM NaCl, 50 mM HEPES, 1 mM $CaCl_2$, pH 6.6.

*Reagents.* Iodobeads and the cross-linking reagent APDP (*N*-[4-(*p*-[Azidosalicylamido)butyl]-3′[2′-pyridyldithio]propionamide) was obtained from Pierce, $Na^{125}I$ was obtained from New England Nuclear. Cyanogen bromide Sepharose 4B was obtained from Pharmacia Biotech. All other reagents were obtained from Sigma Chemical Co. or Fisher Scientific.

## RESULTS

### *XIP-Affinity Column Chromatography*

We have previously shown that XIP inhibits exchange activity by binding to the cytoplasmic side of the Na-Ca exchange protein in cardiac SL vesicles (3). To determine which amino acids and/or regions of the exchanger protein were associated with XIP binding, we subjected cardiac SL vesicle preparations to limited proteolysis. As detergents were not used in these experiments, only external portions of the exchanger protein and those on or near the membrane surface were likely to be proteolyzed. Proteolytic fragments were subjected to XIP-affinity column chromatography. As shown in Fig. 1, a 24 kDa peptide fragment was purified by XIP-affinity column chromatography. The N-terminal amino acid sequence of this peptide is IDDDI which corresponds to amino acids 445-449 on Na-Ca exchange loop f. This portion of loop f is part of a negatively charged region from 445-455 that has the amino acid sequence IDDDIFEEDEN.

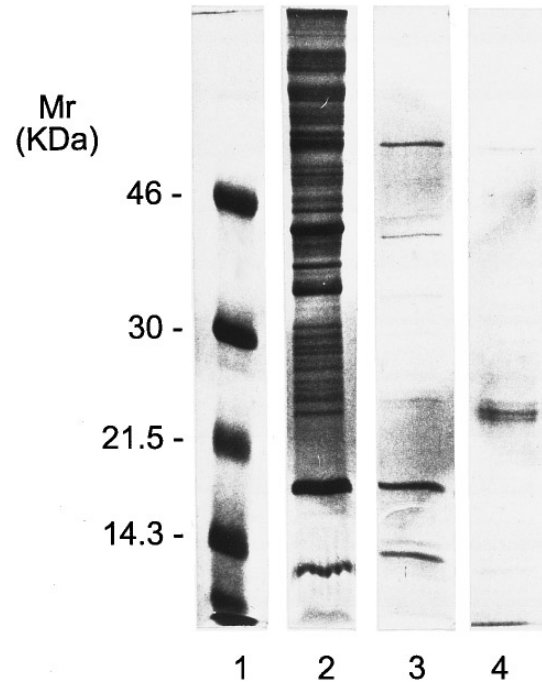


**FIG. 1.** XIP-affinity column chromatography of cardiac SL vesicle proteolytic fragments. Bovine cardiac SL vesicles were proteolyzed with immobilized chymotrypsin. Protein fragments generated by this treatment were prepared and subjected to fractionation on a XIP-affinity column matrix as described in Materials and Methods. Eluted column fractions were subjected to SDS-PAGE (shown), Western blot, and sequence analysis. Lane 1, molecular weight markers (in KDa); Lane 2, cardiac SL vesicle proteins; Lane 3, proteolytically released peptide fragments; Lane 4, XIP-affinity column purified proteolytic fragment.

### *Sulfhydryl Linkage of cysXIP to $^{125}$I-APDP*

In the present study, we utilized an analog of XIP (cysXIP; RRLLFYRYVYRCYCAGRQKG) in which lysines 7, 11, and 17 were replaced by arginines, cysteine was substituted in place of arginines 12 and 14, and arginine 19 was replaced by lysine, as a photoaffinity probe. XIP and cysXIP are equally potent inhibitors of Na-Ca exchange in cardiac SL vescles (10,11).

The XIP analog cysXIP was conjugated to radiolabeled APDP as described in Experimental Procedures. The resulting conjugate was subjected to nonreducing and reducing SDS-PAGE followed by autoradiography. As shown in Fig. 2 radiolabeled APDP forms a covalent linkage with cysXIP. Coomassie blue visualization of the electrophoresed peptides indicated no difference in migration pattern or amount of peptide present (not shown). In the presence of the reducing agent $\beta$-mercaptoethanol, the conjugate dissociates confirming that the nature of the linkage is a disulfide bridge between a cysteine on the

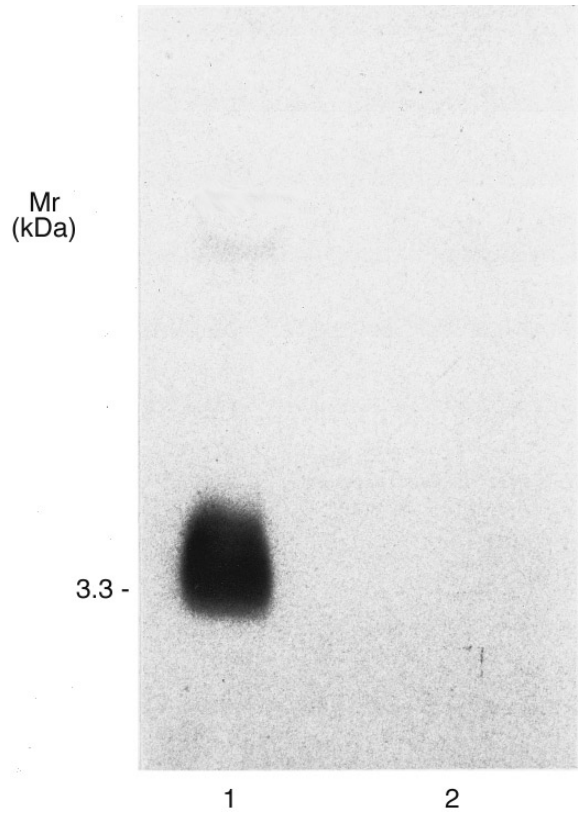


**FIG. 2.** Analysis of $^{125}$I-APDP-cysXIP. cysXIP was coupled to $^{125}$I-APDP as described in Materials and Methods. Approximately 3 $\mu$g of $^{125}$I-APDP-cysXIP were subjected to nonreducing (lane 1) or reducing (lane 2) SDS-PAGE followed by autoradiography (shown). Under reducing conditions, $^{125}$I-APDP was released from XIP.

peptide and APDP. In the experiments described below, $^{125}$I-APDP-cysXIP was used as a photoaffinity probe to cross-link Na-Ca exchange peptide homologues.

### *Probe Labeling of Negatively Charged Na-Ca Exchange Homologue Peptides*

The positively charged nature of the endogenous XIP domain and the observation that increased solution ionic strength decreased inhibition of Na-Ca exchange activity by XIP (3) suggest that XIP binding may in part be electrostatic in nature. It is therefore likely that XIP binding to the Na-Ca exchange protein occurs in a region containing negatively charged amino acids. But is this interaction specific, requiring perhaps structural "fit" as well as charge interaction, or because of the overall highly positive nature of XIP, is an association with an oppositely charged negative protein domain sufficient for binding? We tested the ability of $^{125}$I-APDP-cysXIP to bind and label two peptides homologous to highly negatively charged regions of loop f. The amino acid sequence of these regions are IDDDIFEEDEN which was identified by XIP-affinity column chromatography and GEDDDDDECGEE (a peptide similar in size and charge to IDDDIFEEDEN) which corresponds to loop f residues 732-743. The relative locations of these negative domains are shown in Fig. 3.

The ability of $^{125}$I-APDP-cysXIP to cross-link and label the two negatively charged peptides is shown in Fig. 4. In these experiments, radiolabeled probe was incubated with a negatively charged peptide (or by itself as a control) followed by irradiation with uv light. Where indicated, incubates were reduced with $\beta$-mercaptoethanol prior to electrophoresis to release cysXIP from the probe as in Fig. 2. The results in Fig. 4 show that (i) cysXIP does not cross-link itself, and (ii) peptide IDDDIFEEDEN is cross-linked by cysXIP while (iii) peptide GEDDDDDECGEE showed no interactions with the probe under these conditions. Moreover the interaction between the XIP analog and acidic peptide IDDDIFEEDEN was pH dependent and not inhibited by exogenous $Ca^{2+}$. The addition of nonpolar solvents like trifuoroethanolamine or DMSO did not yield consistent results in terms of enhanced incorporation of probe to peptide IDDDIFEEDEN.

### *Fluorescence Quenching Titration of XIP with Na-Ca Exchange Peptide Homologues*

When endogenously fluorescent peptides interact with other peptides or proteins, the interaction can often be quantified by fluorescence quenching. The resultant binding constant from fluorescence quenching studies provides insight into the specificity of such interactions. XIP exhibits fluorescence emission proper-

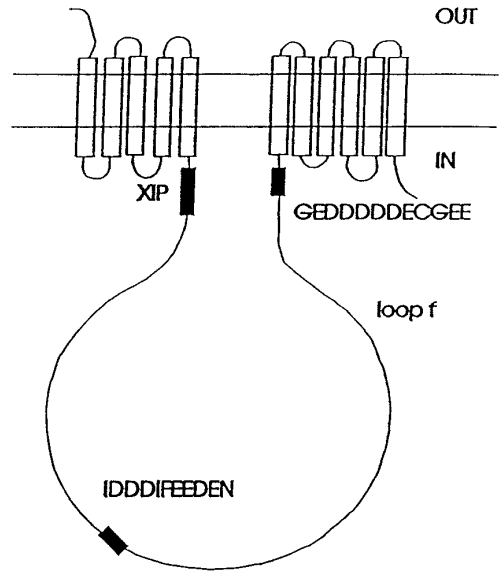


**FIG. 3.** Hydropathy plot-derived model of cardiac Na-Ca exchange. A hydropathy plot-derived model of the cardiac sodium-calcium exchange protein depicting transmembranal helicies and extramembranal loops. Relative positions of areas corresponding to XIP and the two negatively charged peptide homologues used in this study are indicated by filled rectangles. Membrane spanning helices are shown as open cylinders.

ties due to the presence of 4 tyrosines in its sequence. Using quenching of endogenous XIP fluorescence, we were able to further demonstrate its apparent affinity and specificity for the binding site homolog peptide IDDDIFEEDEN. These data are shown in Fig. 5. Fluorescence quenching of XIP was determined as a function of the concentration of Na-Ca exchange peptide homologs GEDDDDDECGEE and IDDDIFEEDEN. IDDDIFEEDEN appeared to specifically interact with XIP (Kd = 5 $\mu$M) compared to GEDDDDDECGEE (Kd = 180 $\mu$M). The similarity in both size and charge of IDDDIFEEDEN and GEDDDDDECGEE and the dissimilarity of these peptides' affinity to XIP suggests that the interaction of IDDDIFEEDEN with XIP may be more than electrostatic due to opposite charges. The binding interaction of XIP with IDDDIFEEDEN may have a structurally specific component.

## DISCUSSION

In this study, we describe the location of the XIP binding site on the cardiac Na-Ca exchange protein.

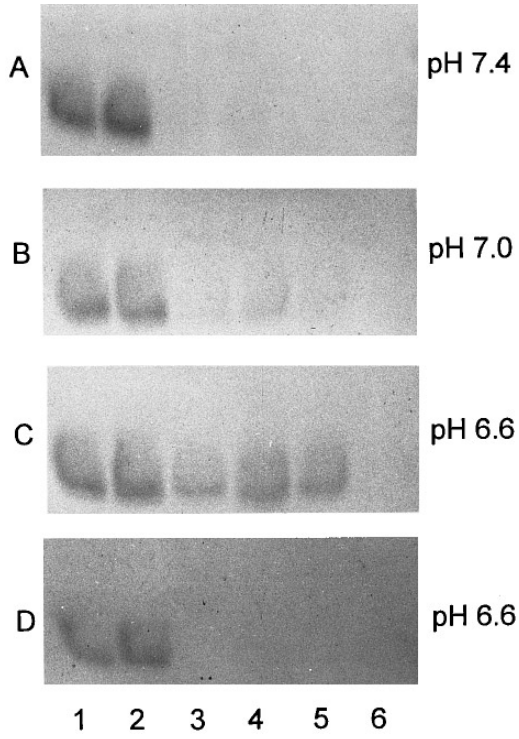


**FIG. 4.** Interaction of XIP with loop f peptide homologues. Two peptide homologues (IDDDIFEEDEN and GEDDDDDECGEE) corresponding to acidic regions of Na-Ca exchange protein loop f were probed with $^{125}$I-APDP-cysXIP as described in Materials and Methods and subjected to reducing or nonreducing SDS-PAGE and autoradiography. Experimental conditions: (A–C) peptide IDDDIFEEDEN; (D) peptide GEDDDDDECGEE; Lane 1, $^{125}$I-APDP-cysXIP, nonreducing; Lane 2, $^{125}$I-APDP-cysXIP, nonreducing, uv irradiation; Lane 3, $^{125}$I-APDP-cysXIP, peptide, uv irradiation, reducing; Lane 4, lane 3 conditions plus 1 mM $Ca^{2+}$ and 1% DMSO; Lane 5, lane 3 conditions plus 1 mM $Ca^{2+}$; Lane 6, $^{125}$I-APDP-cysXIP, uv irradiation, reducing.

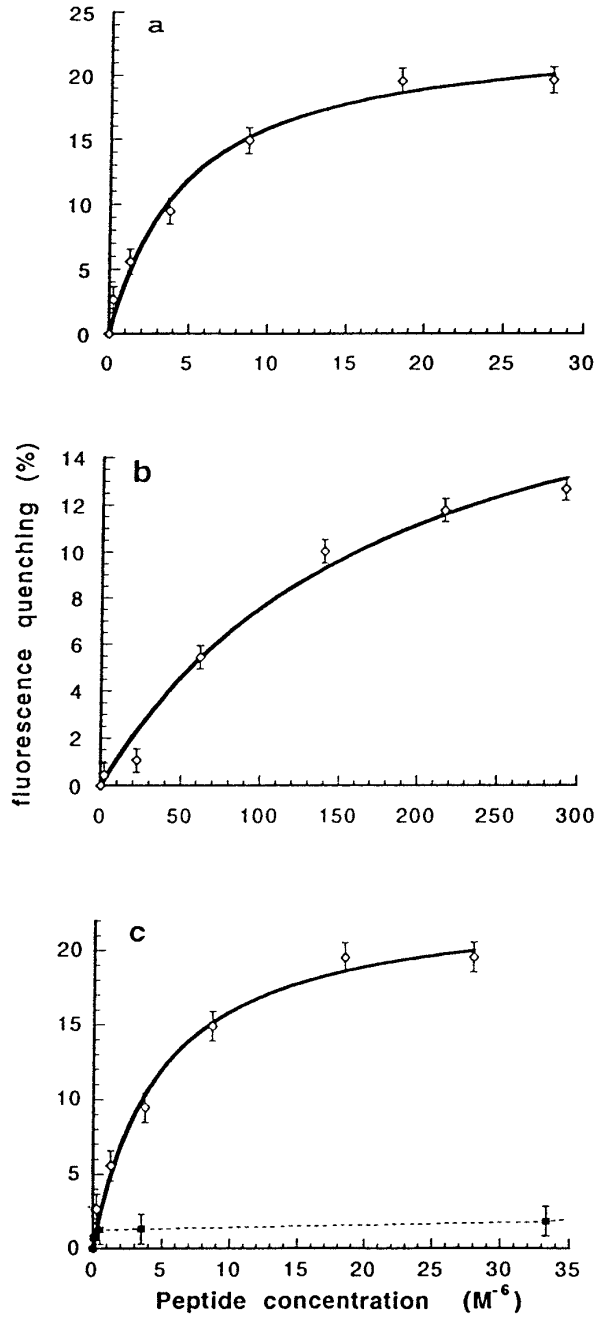


**FIG. 5.** Fluorescence quenching titration of XIP peptide with Na-Ca exchange peptide homologs. In these experiments, fluorescence quenching of endogenous XIP tyrosines was determined as a function of the concentration of Na-Ca exchange peptide homologues GEDDDDDECGEE and IDDDIFEEDEN. (a) interaction of IDDDIFEEDEN with XIP; (b) interaction of GEDDDDDECGEE with XIP; (c) comparative interaction of IDDDIFEEDEN (◇) and GEDDDDDECGEE (■) with XIP. IDDDIFEEDEN appeared to specifically interact with XIP (Kd = 5 $\mu$M). GEDDDDDECGEE eventually demonstrated saturable fluorescence quenching (Kd = 180 $\mu$M) which was considered to be nonspecific.

Sequence analysis of XIP-affinity purified exchanger proteolytic fragments indicated that the negatively charged region from aa 445-455 was associated with XIP binding. To further study the nature and specificity

of XIP binding we synthesized peptides homologous to aa 445-455 and aa 732-743 (another highly negatively charged region on Na-Ca exchange protein loop f). While loop f contains several runs of negatively charged amino acids, we chose to synthesize a peptide homologous to aa 732-743 because the size and charge density of this region are similar to that of aa 445-455. If the binding of positively charged XIP was a simple function of association with a peptide of opposite charge, then one might expect XIP to bind to both peptides with similar affinities. This however was not the case. The results indicate that XIP binding to negatively charged peptide IDDDIFEEDEN may not be a simple charge interaction. Moreover, binding to peptide IDDDIFEEDEN was pH dependent suggesting perhaps the induction or presence of some level of secondary structure that facilitated peptide-peptide interaction.

The negatively charged acidic region of amino acids on Na-Ca exchanger protein loop f encompassing residues 445-455 has previously been described as a $Ca^{2+}$ regulatory site (12,13). Using mutational analysis, Philipson and co-workers have shown that this region binds $Ca^{2+}$ with high affinity and that specific deletions or substitutions of the primary sequence alters $Ca^{2+}$ regulation of the exchange process. Data from fluorescence titration studies (Fig 5) demonstrated that peptide IDDDIFEEDEN interacted with XIP in the presence of 1 mM $Ca^{2+}$. Taken together, data from (12) and the present study support the possibility that IDDDIFEEDEN may bind $Ca^{2+}$ and XIP noncompetitively. Although the nature of this putative interaction is not clear, one possibility is that $Ca^{2+}$ induces conformational changes in IDDDIFEEDEN peptide secondary structure that promote XIP binding. It is also possible $Ca^{2+}$ directly participates as a cofactor in coordinating the XIP-peptide interaction. We are attempting to address these issues in other studies. An earlier report identified two acidic regions on loop f (445-IDDDIFEEDEN-455 and 498-DDDHAGIFTFEE-509) that appear to be involved in binding regulatory $Ca^{2+}$ (12). In the present study, the former acidic region was identified by XIP-affinity column chromatography, the latter was not. While the region on loop f from amino acids 498-509 appears to be involved in regulatory $Ca^{2+}$ binding, it may not bind XIP. Peptide homologues to both of these sites did not bind $^{45}Ca^{2+}$ in overlay studies possibly due to their small size (11 amino acids; not shown).

Deletion mutagenesis of amino acids 240-679 (nearly all of loop f) and amino acids 562-685 resulted in exchange activity that was not regulated by either XIP or $Ca^{2+}$ which suggested the XIP binding site might be located in the latter region (7). Results presented here indicate that XIP specifically interacts with a proteolytic fragment of loop f and a peptide homologue of that region. It is possible that XIP interacts with residues 445-455 and another region(s) of loop f between residues 562-685 that failed to purify by affinity column chromatography or was proteolytically degraded. It is also possible that when the XIP binding site is ligand occupied, a conformation is induced in the C-terminal portion of loop f that inhibits transport. Thus mutations in the C-terminal region of loop f may abolish regulation by XIP but not binding.

An unresolved issue in the present study is the role of regulatory $Ca^{2+}$, which activates cardiac Na-Ca exchange, and its apparent association with XIP, a potent inhibitor, at the XIP binding site on loop f. One possibility is that regulatory $Ca^{2+}$ binds to sites on loop f, including the XIP binding site, inducing or stabilizing a conformation favorable for optimal transport. A regulatory $Ca^{2+}$-induced conformation may be independent of occupation of the XIP binding site by an inhibitory ligand.

## ACKNOWLEDGMENTS

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