The cardiac sarcolemmal Na\(^+\)-Ca\(^{2+}\) exchanger (NCX1) influences cardiac contractility by extruding Ca\(^{2+}\) from myocytes. As a Ca\(^{2+}\) efflux mechanism, the exchanger plays a prominent role in Ca\(^{2+}\) homeostasis. To track NCX1 and study changes in conformation, NCX1 was tagged with derivatives of green fluorescent protein. Cyan (CFP) and yellow (YFP) fluorescent proteins were used for both visualization of the protein in HEK cells and fluorescent resonance energy transfer (FRET). CFP or YFP was inserted at position 266, 371, 467, or 548 of the large intracellular loop of NCX1 located between transmembrane segments 5 and 6. These constructs were tested for functional activity and visualized for cell surface expression. All constructs were targeted to the plasma membrane. Transport properties were assessed by both \(^{45}\)Ca\(^{2+}\) uptake and electrophysiological measurements. The fluorescent-tagged exchangers had similar biophysical properties to the wild type NCX1. Unexpectedly, all constructs retain their sensitivity to regulation by cytoplasmic Na\(^+\) and Ca\(^{2+}\) ions. FRET analysis indicates the proximity of NCX1 to plasma membrane phosphatidylglycerolinositol 4,5-bisphosphate. These results indicate that insertion of CFP or YFP into the large intracellular loop of NCX1 protein does not impair exchanger properties. These constructs will be useful to further characterize the biological properties of the exchanger in intact cells.

The cloning of the Na\(^+\)-Ca\(^{2+}\) exchanger (NCX1)\(^2\) in 1990 has led to major advances in understanding both its biophysical and its molecular properties (1, 2). For example, electrophysiological recordings from *Xenopus* oocytes expressing NCX1 have revealed modulatory effects of both Na\(^+\) and Ca\(^{2+}\) on exchanger activity (3, 4). Mutational analysis has identified regions of the protein responsible for these regulations (5, 6).

To further study these regulations, our goal was to engineer a full-length exchanger fused to a fluorescent protein with biophysical properties similar to the untagged exchanger. The cyan (CFP) and yellow (YFP) variants of the green fluorescent protein have been extensively used to visualize proteins in intact cells and to conduct FRET studies (7, 8). The fluorescent NCX1 could then be used for *in vivo* visualization and to perform FRET experiments.

Here we present biophysical properties of the cardiac NCX1 with CFP and YFP inserted at different positions within the protein. The data indicate that insertion of fluorescent protein within the large intracellular loop connecting transmembrane segments 5 and 6 is well tolerated, whereas insertion of the fluorophore at the C terminus of the exchanger leads to an inactive transporter retained in intracellular compartments. Either YFP or CFP was inserted at positions 266, 371, 467, and 548 within the large intracellular loop. All constructs were properly targeted to the plasma membrane and were active. Electrophysiological recordings show that the fluorescent-tagged exchangers retain both Na\(^+\) and Ca\(^{2+}\) regulation. We also demonstrate the proximity of NCX1 to plasma membrane PIP\(_2\) using FRET.

**EXPERIMENTAL PROCEDURES**

*Molecular Biology*—Internal insertions of fluorescent proteins into the canine NCX1 were constructed as follows. An AgeI/EcoRI fragment was subcloned into pEGFP-C-1 (Clontech). HindIII restriction sites were introduced at locations corresponding to amino acid positions 266, 371, 467, and 548 of the wild type protein sequence using the QuikChange method (Stratagene, La Jolla, CA). Sites were primarily chosen to cover a variety of locations within the exchanger loop. CFP and YFP were PCR-amplified with primers enabling a HindIII digest to insert the fluorescent protein in-frame at the specified amino acid locations. All PCR products and QuikChange insertions were sequenced. The fluorescent protein-containing fragment was then reinserted into the wild type exchanger, which was either in the pGEMHE vector to make cRNA (Ambion, TX) for oocyte injection or in a pEGFPN-1 vector for expression in mammalian cells.

\(Na^+\)-dependent Ca\(^{2+}\) Uptake—\(Na^+\)-dependent Ca\(^{2+}\) uptake was performed as described previously (9). Briefly, HEK293 cells expressing CFP- or YFP-tagged exchangers were suspended and loaded with Na\(^+\) by incubation with (in mM): 10 MOPS, 140 NaCl, 1 MgCl\(_2\), 0.4 ouabain, and 0.025 nystatin for 10 min at...
Fluorescent Na\textsuperscript{+}-Ca\textsuperscript{2+} Exchanger

![Diagram of Na\textsuperscript{+}-Ca\textsuperscript{2+} Exchanger Experiments](image)

**RESULTS**

Insertion of CFP or YFP within the Large Intracellular Loop Connecting Transmembrane Segments 5 and 6 Leads to Active Exchangers—One goal was to construct a full-length Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger fused to either YFP or CFP with biophysical properties similar to the wild type exchanger. For this purpose, YFP or CFP was inserted at different positions in the large cytoplasmic loop connecting transmembrane segments 5 and 6 of NCX1. YFP was inserted at positions 266 (NCX-266YFP), 371 (NCX-371YFP), 467 (NCX-467YFP), and 548 (NCX-548YFP).

**Electrophysiological Experiments**—RNAs coding for the fluorescent tagged exchangers were injected into *Xenopus laevis* oocytes. Oocytes were kept at 18 °C for 4–7 days. Outward Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange currents were recorded using the inside-out giant patch technique (10). Borosilicate glass pipettes were pulled and coated with a mixture of Parafilm and mineral oil. Prior to experiments, the vitelline membrane of the oocyte was removed manually in an isotonic solution identical to the solution for seal formation (in mM: 110 KOH, 10 HEPES, adjusted to pH 7 using MES). After membrane excision, solutions were rapidly changed using a computer-controlled 20-channel solution switcher. Recordings of the outward exchanger current were obtained by perfusing solutions with different ion concentrations. Data were fitted to a Hill function and normalized to extrapolated maximum values. Values are mean ± S.E. PCLAMP (Axon Instruments, Burlingame, CA) software was used for acquisition and analysis. Data were acquired on line at 4 ms/point and filtered at 50 Hz using an 8-pole Bessel filter. Experiments were performed at 35 °C and at a holding potential of 0 mV.

**Cell Culture**—HEK293 cells were grown in 35-mm glass bottom Petri dishes in standard culture medium. Petri dishes were pretreated with either fibronectin (Sigma) or Cell-Tak (BD Discovery Labware). Transfection of cDNAs was accomplished using GenePORTER (Genlantis, San Diego, CA).

**FRET Measurements**—Data were acquired using a Nikon Eclipse TE300 microscope equipped with a ×40 oil immersion lens (numerical aperture 1.2) and the following filter cubes (in nm): 1) CFP cube (CFPf), excitation 436 ± 20 (bandpass), emission 480 ± 40, dichroic 455 (longpass, DCLP); 2) YFP cube (YFPf), excitation 500 ± 20, emission 535 ± 30, DCLP 515; and 3) FRET cube (FRETf), excitation 436 ± 20, emission 535 ± 30, DCLP 455 (Chroma Technologies, Rockingham, VT). LEDs (Lumileds, San Jose, CA) were used as light sources, two emitting at 455 ± 20 nm (royal blue) and one emitting at 505 ± 15 nm (cyan). The royal blue lights were placed in front of the CFP and FRET filter, whereas the cyan light was located in front of the YFP filter; LEDs were controlled by the camera-driven shutter.

FRET efficiency was measured by acquiring images of the cells in the CFP wavelength before and after application of 10 μM carbachol to the bath (8,11). Images were analyzed by quantifying the amount of fluorescence at the plasma membrane. These values were used to determined FRET efficiency (E), calculated as follows: \( E = \frac{1}{I_{\text{CFP}}/I_{\text{CFPa}} + I_{\text{YFP}}/I_{\text{YFPa}}} \), where I is the intensity of CFP emission before (CFPb) and after (CFPa) carbachol. Images were analyzed with the NIH Scion Image Program and background was subtracted before FRET calculations.

**Table**: Summary of YFP-tagged exchanger activity.

| Exchanger | % 45Ca\textsuperscript{2+} uptake relative to WT |
|-----------|-------------------------------------------------|
| WT        | 100%                                           |
| 266       | 10%                                            |
| 371       | 20%                                            |
| 467       | 30%                                            |
| 548       | 40%                                            |

**Graph**: % 45Ca\textsuperscript{2+} uptake measured from HEK cells expressing the indicated construct. Cells transiently transfected with the indicated constructs. Also shown are the schematic representations of the secondary structure of the fluorescent tagged exchangers. Numbers indicate the location at which YFP was inserted within the full-length exchanger. All of these YFP-tagged exchangers are active. The Ca\textsuperscript{2+} uptake was initiated by resuspending the cell pellet in assay medium (in mM): 10 MOPS, 140 KCl, 0.4 ouabain, 0.025 CaCl\textsubscript{2}, and 5 μCi/ml 45Ca\textsuperscript{2+}. The reaction was stopped after 1–3 min by adding ice-cold quenching solution (140 mM KCl and 1 mM HEPES, 10 EGTA, 0–5.75 mM) 100 Ci/ml 45Ca\textsuperscript{2+} by adding ice-cold quenching solution (140 mM KCl and 1 mM HEPES, 10 EGTA, 0–5.75 mM) 100 Ci/ml 45Ca\textsuperscript{2+}.
371YFP), 467 (NCX-467YFP), and 548 (NCX-548YFP). Expression of these constructs in mammalian HEK cells produced marked fluorescence at the plasma membrane (Fig. 1A). Thus, insertion of the fluorescent protein did not disrupt targeting of the exchanger to the plasma membrane (use of CFP instead of YFP led to identical results; not shown). We observed that the translocation of YFP-tagged exchangers to the plasma membrane required at least 48 h and was optimal 72 h after transfection. At 24 h after transfection, fluorescent labeling of internal membranes (presumably endoplasmic reticular and Golgi membranes) could be observed.

To determine whether insertion of YFP altered the transport properties of NCX1, we studied the activity of the fusion proteins using both Na\(^+\)-dependent \(45^\text{Ca}^{2+}\) uptake and the giant patch electrophysiological technique. Fig. 1B shows the activity of the YFP-tagged exchangers measured as Na\(^+\)-dependent \(45^\text{Ca}^{2+}\) uptake. These data indicate that YFP-tagged NCX1s are active when expressed in HEK cells and that the transport ability of these constructs is not substantially altered by the insertion of YFP. Although NCX-371YFP, NCX-467YFP, and NCX-548YFP show a decrease in uptake when compared with wild type, immunoblot analysis indicates a decreased level of expression of these constructs when compared with the untagged exchanger (not shown). A diminished level of protein expression likely contributes to the reduced transport activity.

The activity of the Na\(^+\)-Ca\(^{2+}\) exchanger is regulated by both cytoplasmic Na\(^+\) and cytoplasmic Ca\(^{2+}\). High intracellular Na\(^+\) inactivates the exchanger, whereas micromolar levels of Ca\(^{2+}\) remove the Na\(^+\)-dependent inactivation and also directly increase exchanger activity (3, 12). To determine whether fluorescent-tagged exchangers retain these regulatory properties, exchangers were expressed in Xenopus oocytes, and biophysical properties were characterized using the giant patch technique. Similar to observations in HEK cells, all constructs generated intense fluorescence at the plasma membrane. Oocytes displaying strong fluorescence were selected for electrophysiological measurements. Giant patch experiments demonstrated that all YFP-tagged exchangers generate an outward exchange current in the presence of high intracellular Na\(^+\) (100 mM) when 8 mM Ca\(^{2+}\) is present in the pipette. The 8 mM Ca\(^{2+}\) provides a saturating level of Ca\(^{2+}\) at the extracellular surface of the exchanger. Fig. 2A shows representative traces of outward exchange current recorded from oocytes expressing the indicated constructs in the presence of 0.4 (right) or 10 \(\mu\text{M}\) (left) Ca\(^{2+}\) in the bath at the cytoplasmic side of NCX1. At low Ca\(^{2+}\) concentrations, all mutants displayed an outward current that peaked and then decayed within a few seconds. This decay is evoked by the high Na\(^+\) that drives a fraction of the exchangers into an inactive state (3, 13). This process is known as Na\(^+\)-dependent inactivation or \(I_1\). The time required to reach steady state differed among the various fluorescent NCX1 constructs. The current decay was fitted to a single exponential to obtain a time constant (\(\tau\); Fig. 2B). The fastest decay occurred with NCX-548YFP and the slowest decay with NCX-371YFP.

To estimate the distribution of exchangers in active and inactive states, we determined the fractional activity, \(F_{\text{act}}\), defined as the ratio of steady state current to peak current at 0 \(\mu\text{M}\) Ca\(^{2+}\). The activity of the Na\(^+\)-Ca\(^{2+}\) exchanger was measured in the absence of either 0.4 \(\mu\text{M}\) (left) or 10 \(\mu\text{M}\) (right) intracellular Ca\(^{2+}\) as observed for WT, the exchanger current increased in the absence of high intracellular Ca\(^{2+}\), and the Na\(^+\)-dependent inactivation was removed. As observed for WT, the exchanger current increased in the presence of high intracellular Ca\(^{2+}\), and the Na\(^+\)-dependent inactivation was removed. Values for WT, NCX-266YFP, NCX-371YFP, NCX-467YFP, and NCX-548YFP show a significantly different time constant of inactivation when compared with wild type. Values for NCX-371YFP, NCX-467YFP, and NCX-548YFP are 6 (wild type), 6 (NCX-266YFP), 4 (NCX-371YFP), 10 (NCX-467YFP), and 4 (NCX-548YFP). Fractional activity is calculated as the ratio of steady state current to peak current at 0.4 \(\mu\text{M}\) Ca\(^{2+}\). Values for \(n\) are as indicated above. Data were obtained in the presence of 100 mM Na\(^+\) at 0 mV.

FIGURE 2. Biophysical properties of the fluorescent-tagged exchangers. A, outward currents recorded using the giant patch technique from oocytes expressing the indicated construct. Currents were elicited by rapidly applying 100 \(\mu\text{M}\) cytoplasmic Na\(^+\) in the presence of either 0.4 \(\mu\text{M}\) (left) or 10 \(\mu\text{M}\) (right) intracellular Ca\(^{2+}\). As observed for WT, the exchange current increased in the presence of high intracellular Ca\(^{2+}\), and the Na\(^+\)-dependent inactivation was removed. B, inactivation time constant for the indicated constructs. Constructs NCX-266YFP, NCX-371-YFP, and NCX-548YFP show a significantly different time constant of inactivation when compared with wild type. Values for \(n\) are 6 (wild type), 6 (NCX-266YFP), 4 (NCX-371YFP), 10 (NCX-467YFP), and 4 (NCX-548YFP). C, fractional activity is calculated as the ratio of steady state current to peak current at 0.4 \(\mu\text{M}\) Ca\(^{2+}\). Values for \(n\) are as indicated above. Data were obtained in the presence of 100 mM Na\(^+\) at 0 mV.
Higher levels of Ca\(^{2+}\) increase exchanger activity and also antagonize Na\(^{+}\)-dependent inactivation. Fig. 2A shows that high intracellular Ca\(^{2+}\) (10 \(\mu\)M, Fig. 2A, right traces) activated the tagged exchangers and removed the Na\(^{+}\)-dependent inactivation in all cases. Overall, these data indicate that insertion of YFP within the large intracellular loop has only modest effects on the inactivation state of the exchanger and Ca\(^{2+}\) removal of Na\(^{+}\)-dependent inactivation.

We further investigated the effects of Ca\(^{2+}\) on exchanger activity. Inside-out patches were exposed to different Ca\(^{2+}\) concentrations ranging from 0 to 30 \(\mu\)M. Peak and steady state currents were recorded and normalized to their maximal values (Fig. 3). Points were fitted to a Hill function to calculate the apparent Ca\(^{2+}\) affinities. All constructs responded to cytoplasmic Ca\(^{2+}\) by increasing the amplitude of both peak (Fig. 3A) and steady state (Fig. 3B) currents. NCX-371YFP showed a significant decrease in the apparent affinity for Ca\(^{2+}\) for peak exchange current (\(K_{1/2}\) values are 0.57 ± 0.09 \(\mu\)M for WT and 1.42 ± 0.06 for NCX-371YFP; Figs. 3A and 4A). A more robust right shift of the apparent affinity of the peak current for Ca\(^{2+}\) was observed for the fluorescent construct NCX-467YFP (\(K_{1/2} = 3.89 \pm 0.23 \mu\)M; Figs. 3A and 4A). Nevertheless, this exchanger retains Ca\(^{2+}\) regulation despite the insertion of YFP within a Ca\(^{2+}\) binding region (CBD1, amino acids 371–508). Finally, insertion of YFP at positions 266 and 548 left unaltered the sensitivity of NCX1 to Ca\(^{2+}\), although a large component (60%) of the current for NCX-548YFP was Ca\(^{2+}\) insensitive (not shown). In a few cases, a complete loss of Ca\(^{2+}\) regulation in NCX-548YFP was observed (\(n = 2\) out of 5); nevertheless, a Ca\(^{2+}\)-insensitive Na\(^{+}\)-dependent inactivation was retained. A decrease in current was observed when Ca\(^{2+}\) was increased from 10 to 30 \(\mu\)M (Fig. 3A). This is due to the competition between Ca\(^{2+}\) and Na\(^{+}\) for binding to the transport site at elevated Ca\(^{2+}\) levels. Current declines as outward Na\(^{+}\) transport is prevented (3). For this reason, Ca\(^{2+}\) regulation cannot be reliably quantitated at this level of Ca\(^{2+}\), and the data obtained at 30 \(\mu\)M Ca\(^{2+}\) were not included in fittings.

In addition to increasing peak current, cytoplasmic Ca\(^{2+}\) also augmented the steady state current by both recruiting new exchangers and removing Na\(^{+}\)-dependent inactivation (4, 12).

**FIGURE 3.** Ca\(^{2+}\) regulation of the fluorescent exchangers. Dose-response curves for cytoplasmic Ca\(^{2+}\) for WT and the indicated exchanger mutants are shown. Ca\(^{2+}\) affinities were measured for either peak current (A) or for steady state exchange currents after the onset of the Na\(^{+}\)-dependent inactivation (B). Residual current recorded in the absence of Ca\(^{2+}\) has been subtracted. Each point is the average of between 3 and 5 experiments. Hill coefficients for the fitted curves for the peak currents are 1.1, 1.3, 2.0, and 3.8 for WT, NCX-266YFP, NCX-371YFP, and NCX-548YFP, respectively.

**FIGURE 4.** Ca\(^{2+}\) affinities of the fluorescent exchangers. A and B, a summary of \(K_{1/2}\) values for Ca\(^{2+}\) affinities for WT and the exchanger mutants measured either at the peak (A) or at the steady state (B) of the exchange current. Values for \(n\) are 7 (wild type), 4 (NCX-266YFP), 4 (NCX-371YFP), 6 (NCX-467YFP), and 2 (NCX-548YFP) for affinities measured at the peak and 4 (wild type), 2 (NCX-266YFP), 3 (NCX-371YFP), 5 (NCX-467YFP), and 3 (NCX-548YFP) for Ca\(^{2+}\) affinities measured at steady state.
The fluorescent intensity of CFP was quantitated before and after carbachol by drawing a line across the cell to obtain a fluorescence intensity versus distance plot (shown in Fig. 5A, lower panel). Fluorescence intensity was measured from the peaks as representative of plasma membrane signal. The increase in CFP was then used to determine the efficiency with which energy was transferred from CFP to YFP (FRET efficiency; see “Experimental Procedures”) (8). The average value of FRET efficiency obtained from cells expressing PH-YFP and M1 receptor in presence of NCX-266CFP or NCX-371CFP is shown in Fig. 5B.

In control experiments, NCX-266CFP or NCX-371CFP was co-expressed with the M1 muscarinic receptor and YFP targeted to the plasma membrane by fusion with the membrane anchor sequence of Ki-Ras (15) instead of through a PH domain. In this case, the addition of carbachol to the external medium did not cause the release of YFP into the cytoplasm, and no changes in CFP emission were observed (Fig. 5C). The values of the ratio between the emission intensity of CFP before and after carbachol were 1.01 ± 0.02 for NCX-266CFP + M1 + PM-YFP (n = 20) and 1.09 ± 0.03 for NCX-371CFP + M1 + PM-YFP (n = 14). This result indicates that the increase in CFP emission in cells co-expressing PH-YFP, NCX-266YFP, and M1 receptor is not due to factors such as changes in focus plane or changes in the environment upon the addition of carbachol.

DISCUSSION

The discovery that GFP found in Aequorea victoria can induce a strong fluorescent signal in mammalian cells has opened new opportunities for biological investigations (16). Indeed, GFP has been an incredible tool to study gene expression, protein trafficking, conformational changes, protein-protein interactions, and labeled cells in living organisms. We have engineered full-length Na⁺-Ca²⁺ exchangers with insertions of either the yellow or the cyan variants of GFP. These constructs can be used for both FRET and trafficking studies. YFP was inserted at four different positions within the large intracellular loop of NCX1. Insertion of fluorescent proteins within this region of the exchanger is well tolerated and leads to active transporters. In contrast, fusion of YFP to the CO₂H terminus of NCX1 leads to retention of the exchanger within intracellular compartments (not shown).

Our data indicate that all YFP-tagged NCXs had the same Ca²⁺ affinity as WT when measured at steady state (Figs. 3B and 4B). Strikingly, the difference in affinity for Ca²⁺ between peak and steady state currents, characteristic of the WT exchanger, was abolished in the NCX-467YFP exchanger.

Tagged Na⁺-Ca²⁺ Exchangers Generate FRET with Fluorophores Targeted to the Plasma Membrane—To test the utility of our NCX1 constructs for further structure-function studies, we measured FRET between a CFP-tagged exchanger and a fluorescent acceptor separate from NCX1. We utilized the fluorophore YFP fused to a pleckstrin homology domain (PH-YFP), which binds to plasma membrane PIP₂ (14). We expressed NCX-266CFP in the presence of both PH-YFP and the M1 muscarinic receptor in HEK cells (Fig. 5A). As shown in Fig. 5A, top panels, activation of the M1 receptor by carbachol (10 μM) released PH-YFP into the cytoplasm due to the hydrolysis of PIP₂ into diacylglycerol and inositol 1,4,5-trisphosphate. Since the acceptor (PH-YFP) is no longer in close proximity to the donor (NCX-266CFP), no transfer of energy from CFP to YFP occurs, causing an increase of CFP emission. This is shown in Fig. 5A, where images of NCX-266CFP before (left) and after (right) the addition of carbachol are presented. Similar results were obtained using NCX1 with CFP inserted at position 371 (not shown).

FIGURE 5. Fluorescent-tagged exchangers are useful for FRET studies. A, HEK cells expressing the NCX-266CFP in the presence of the M1 muscarinic receptor and the PH domain fused to YFP (PH-YFP). Images were acquired before (left) and after (right) application of 10 μM carbachol to the bath using filter sets to selectively discriminate between the YFP (top) or CFP emission (bottom). Carbachol triggers the release of the PH-YFP from the plasma membrane and an increase in CFP emission. The lower panel shows the fluorescence intensity of CFP before (gray) and after (black) carbachol. In the lower images, the white line shows the location for which the fluorescence values were calculated. B, the efficiency of transfer of energy (FRET efficiency) between CFP located in the exchanger and the PH-YFP was calculated (see “Experimental Procedures”). C, fluorescence intensity versus position before and after carbachol measured from cells expressing NCX-266CFP + M1 and YFP targeted to the plasma membrane (PM-YFP). In this case, the addition of carbachol did not increase CFP emission.
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This result is in accordance with the previous finding (17) showing that manipulation of the NCX1 CO\textsubscript{2}H terminus impairs transport activity.

Electrophysiological studies indicate that exchangers tagged with YFP within the large intracellular loop retain most of the biophysical properties of the wild type exchanger. Giant patch experiments show that all YFP fusion exchangers generate an outward current regulated by both intracellular Na\textsuperscript{+} and intracellular Ca\textsuperscript{2+} ions. Regulatory Ca\textsuperscript{2+} binds to a region distinct from the Ca\textsuperscript{2+} transport site, whereas Na\textsuperscript{+}-dependent inactivation originates from the occupancy of the Na\textsuperscript{+} transport sites by three Na\textsuperscript{+} ions (3, 4).

The amino acid residues that bind regulatory Ca\textsuperscript{2+} have been identified. There are two domains involved in the binding of regulatory Ca\textsuperscript{2+}: residues 371–508 form the first Ca\textsuperscript{2+} binding domain (CBD1) (18–20), whereas the region encompassing residues 508–657 form the second Ca\textsuperscript{2+} binding site (CBD2) (19). CBD1 binds four Ca\textsuperscript{2+} ions, whereas CBD2 may bind two Ca\textsuperscript{2+} ions (19, 20). The molecular mechanisms of Ca\textsuperscript{2+} regulation and the relative roles of each Ca\textsuperscript{2+} binding domain are not clear. There has been substantial evidence that CBD1 is involved in increasing the rate of ion transport (3, 21), and it has been suggested that CBD2 is involved in antagonizing Na\textsuperscript{+}-dependent inactivation (19). Interestingly, the apparent affinity of the exchanger for Ca\textsuperscript{2+} differs when measured at the peak or under steady state conditions (Fig. 3). When exchange current is initiated by the application of Na\textsuperscript{+}, the exchanger is not yet inactivated by Na\textsuperscript{+}, and the primary effect of Ca\textsuperscript{2+} is to activate exchangers and to increase the rate of transport (3, 21). The situation is more complicated at steady state following the onset of the Na\textsuperscript{+}-dependent inactivation (1). Here, there are two effects of Ca\textsuperscript{2+} on the exchanger current. First, Ca\textsuperscript{2+} activates the exchanger (as observed for the peak current) and, second, Ca\textsuperscript{2+} relieves exchangers from the I\textsubscript{1} state (4, 22). The latter effect requires higher concentrations of Ca\textsuperscript{2+}, perhaps due to the involvement of the second Ca\textsuperscript{2+} binding domain (CBD2). More detailed mutational and functional data are required to resolve this issue.

All fluorescent-tagged exchangers have Ca\textsuperscript{2+} affinities identical to that of wild type when determined at steady state, although some exchangers had a decreased sensitivity to Ca\textsuperscript{2+} when measured at the peak current. This could indicate that insertion of the fluorophores within the large intracellular loop primarily affected the influence of CBD1. NCX-548YFP is of particular interest since YFP is inserted within CBD2 and still retains a similar steady state Ca\textsuperscript{2+} affinity as WT. However, the fluorophore is located in an unstructured region of CBD2 (19) and possibly did not drastically disrupt the properties of CBD2. Nevertheless, only about 40% of the NCX-548YFP current retains Ca\textsuperscript{2+} sensitivity, and occasionally, we observed a complete loss of the Ca\textsuperscript{2+} regulation. Interestingly, in these cases, the currents retained a Na\textsuperscript{+}-dependent inactivation not modulated by intracellular Ca\textsuperscript{2+}. At present, these complications do not allow full understanding of the characteristics of NCX-548YFP and the relative roles of CBD1 and CBD2.

Two fluorescent Na\textsuperscript{+}-Ca\textsuperscript{2+} exchangers had decreased Ca\textsuperscript{2+} sensitivity (Fig. 4A): NCX-371YFP and NCX-467YFP. NCX-371YFP has a modest shift in apparent Ca\textsuperscript{2+} affinity, whereas the shift for NCX-467YFP is severalfold. It is interesting that insertion of YFP at position 467, within the Ca\textsuperscript{2+} binding site of CBD1, did not eliminate the Ca\textsuperscript{2+} regulation. Insertion of YFP at this position may have disrupted CBD1 without affecting CBD2. This could explain the similarity in the Ca\textsuperscript{2+} dose-response curves of NCX-467YFP and steady state currents for Ca\textsuperscript{2+}. Possibly, CBD2 can modulate both Ca\textsuperscript{2+} activation and relief of Na\textsuperscript{+}-dependent inactivation. Alternatively, insertion of YFP at position 467 within the unstructured FG loop of CBD1 (18, 19) lowers the Ca\textsuperscript{2+} affinity of CBD1 without totally disrupting CBD1 function.

All fluorescent Na\textsuperscript{+}-Ca\textsuperscript{2+} exchangers showed Na\textsuperscript{+}-dependent inactivation that was eliminated by micromolar concentrations of Ca\textsuperscript{2+}. Mutagenesis studies indicate that the XIP region of the exchanger (amino acids 219–238) is involved in Na\textsuperscript{+}-dependent inactivation (13). It is interesting to note that the insertions of YFP at the two sites closest to the XIP region (positions 266 and 371) slowed the rate of inactivation. Possibly, this region is involved in transmitting the signal from the Ca\textsuperscript{2+} binding domains to the XIP region to modulate Na\textsuperscript{+}-dependent inactivation.

The cyan and yellow variants of GFP have been extensively used for FRET measurements. FRET consists of the nonradiative transfer of energy from a donor (CFP) to an acceptor (YFP) molecule. The two fluorophores need to be within 10 nm to generate FRET, and there must be overlap between the emission spectrum of the donor with the excitation spectrum of the acceptor. We demonstrate that CFP inserted at either position 266 or position 371 of the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger is in close proximity to plasma membrane-targeted PH-YFP as indicated by the presence of FRET. The result suggests that fluorophores at positions 266 and 371 within the large intracellular loop connecting transmembrane segments 5 and 6 are in proximity to the cytoplasmic membrane environment. The data also indicate that the exchanger is in proximity to membrane PIP\textsubscript{2}. PIP\textsubscript{2} affects exchanger regulatory properties (23), but association of the two molecules has not previously been demonstrated.

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