Kinetic and Equilibrium Properties of Regulatory Calcium Sensors of NCX1 Protein*

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The crystal structures of the CBD1 and CBD2 domains of the Na+/Ca2+ exchanger protein (NCX1) provided a major breakthrough in Ca2+-dependent regulation of NCX1, although the dynamic aspects of the underlying molecular mechanisms are still not clear. Here we provide new experimental approaches for evaluating the kinetic and equilibrium properties of Ca2+ interaction with regulatory sites by using purified preparations of CBD1, CBD2, and CBD12 proteins. CBD12 binds ~6 Ca2+ ions (mol/mol), whereas the binding of only ~2 Ca2+ ions is observed (with a Hill coefficient of nH ~ 2) either for CBD1 or CBD2. In the absence of Mg2+, CBD1 has a much higher affinity for Ca2+ (Kd = 0.3 ± 1.2 μM) than CBD2 (Kd = 5.0 ± 1.2 μM). The Ca2+ dissociation from CBD2 (koff = 230 ± 70 s⁻¹) is at least 25 times faster than from CBD1 (koff = 10 ± 3 s⁻¹), whereas the kon values indicate fast kinetics for Ca2+ binding (kon = koff/Kd = 10⁷–10⁸ M⁻¹ s⁻¹) for both CBDs. At 2–5 mM Mg2+, both CBDs bind Ca2+, with a Kd of 1–2 μM (Mg2+ has very little effect on Ca2+ off rates). Mg2+ cannot occupy the primary site of CBD2, whereas the other Ca2+ sites of CBDs interact with Mg2+ as well. There is no competition between Na+ and Ca2+ for any CBD site. The kinetically diverse Ca2+ sensors may sense differentially the dynamic swings in [Ca2+] within specific subcellular compartments (dyadic cleft, submembrane space, bulk cytosol, etc.).

The cell membrane Na+/Ca2+ exchange (NCX) system is a major Ca2+ extruding mechanism in many cell types (1–4). The isoforms (NCX1–4) of the NCX gene family and their splice variants are expressed in tissue-specific manner (5–7). Up- or down-regulation of NCX proteins may contribute to altered Ca2+ homeostasis and development of diseases, although the underlying regulatory mechanisms are poorly understood (2–4, 8).

In cardiomyocytes the cytosolic Ca2+ concentrations swing dramatically during the action potential, thereby requiring dynamic regulation of NCX1 during excitation-contraction coupling (2, 3, 8, 9). Moreover, the Na+/Ca2+ exchange rates must meet ever-changing demands for cardiac output in health and disease. Any imbalance in Ca2+ extrusion may lead to life-threatening disorders caused by either Ca2+ depletion or over-load of Ca2+ stores (7–9).

The NCX proteins are regulated by cytosolic Na+ and Ca2+ (10–13). A rise in Na+ rapidly stimulates and then inactivates the exchanger, whereas cytosolic Ca2+ activates NCX1 and relieves the Na+–dependent inactivation (11–16). The alternative splicing of NCX1 dramatically alters the relationships between Na+–dependent and Ca2+–dependent regulation (12–14). The molecular mechanisms underlying the secondary regulation of NCX by Na+ and Ca2+ are poorly understood.

Although the high affinity Ca2+ regulatory site of loop-f was found nearly 15 years ago (17), only the recent studies using NMR (18, 22) and x-ray crystallography (19, 20) have identified two regulatory domains, CBD1 and CBD2. Both CBD domains represent autonomously folded structure of ~130 residues known as C2 type folding (23–26). Some C2 domains have high selectivity to Ca2+, whereas the others bind Mg2+ as well (23–26). The splice variant sequence is located within CBD2 (18, 20), suggesting that the Ca2+ sensing at CBD2 may be relevant for tissue-specific regulation of NCX proteins.

High resolution crystallography reveals four Ca2+ sites in CBD1 (19) and two Ca2+ sites in CBD2 (20). Unfolded structure of CBD1 becomes well folded upon Ca2+ binding (19, 22), whereas CBD2 is a fully folded protein, even in the absence of Ca2+ (18, 20). The mutations in the Ca2+ sites of CBD1 decrease the Ca2+ affinity but do not eliminate the Ca2+-dependent regulation, whereas the mutations in the primary Ca2+ site of CBD2 abolish Ca2+ regulation altogether (20).

The present work offers new experimental setups for measuring the equilibrium and rate constants of Ca2+ binding/dissociation in purified preparations of CBD1, CBD2, and CBD12 proteins. These experimental approaches allow one to directly test specific ligands (e.g. Na+, K+, or Mg2+) for their capacity to alter the Ca2+ binding/dissociation in CBD preparations. We found that the CBD1 and CBD2 domains are kinetically distinct Ca2+ sensors capable of sensing the Ca2+ within the time scale of a single cardiac cycle. Kinetically distinct CBD sensors have a capacity to sense local Ca2+ changes in distinct compartments of the cell and thus may serve as a feedback mechanism for distinct regulation of NCX in dyadic cleft, submembrane space, and bulk cytosol.

MATERIALS AND METHODS

Expression and Purification of CBD Proteins—The DNA constructs of CBD1, CBD2, and CBD12, encoding 371–509, 501–
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657, and 371–657 residues of canine NCX1 (Protein Data Bank accession code P23685; AD-splice variant) were cloned into a pET23b vector (generously provided by Dr. M. Hilge) and expressed in Escherichia coli Rosetta2 (DE3)-competent cells (Novagen) at 37 °C for 5 h by using 1 mM isopropyl 1-thio-β-d-galactopyranoside for induction (18). Grown cells were diluted 1:100 in 2X YT medium and grown again until A595 nm reached 0.4–0.6. Expression was induced with 1 mM isopropyl 1-thio-β-d-galactopyranoside, and after a 5-h induction period, the cells were harvested by centrifugation (7000 × g for 10 min) and then were flash-frozen and stored at −70 °C.

Thawed cells were suspended in 20 mM Tris-Cl, pH 8.0, 300 mM NaCl, 5% glycerol, 0.1% β-mercaptoethanol, 0.1% Triton X-100, 0.1 mM CaCl2 with 1 μg/ml DNase I (type DN-25; Sigma), and protease inhibitor mixture (Roche Applied Science). The cells were homogenized and disrupted twice in microfluidizer (Microfluidics, Newton, MA). CBD1, CBD2, or CBD12 was recovered by centrifugation at 12,000 rpm for 15 min at 4 °C. The supernatant was mixed with 7 ml of nickel beads (HIS-Select™, nickel affinity gel; Sigma) and washed twice with 0.1–0.2 mM EDTA using Ultraflo-3k (15-ml portions of MNG buffer containing 0.9–2.3 mM CaCl2 and filters were pretreated with 5% NaHCO3 (38). The protein was determined at A280 nm by using ε = 3105 M⁻¹ cm⁻¹ for CBD1, ε = 5960 M⁻¹ cm⁻¹ for CBD2, and ε = 9065 M⁻¹ cm⁻¹ for CBD12. Purified preparations of proteins (>95%, as judged by SDS-PAGE) were stored at −70 °C.

45Ca²⁺/Protein Equilibrium Binding—The 45Ca²⁺ binding assay was done by using the ultrafiltration procedure (36–38). To remove Ca²⁺, all glassware and tubes were washed with 0.1 M HCl, and filters were pretreated with 5% NaHCO3 (38). Decalcified buffers (containing 0.9–2.3 μM Ca²⁺) were prepared by passing them through Chelex-100. All of the 45Ca²⁺ binding experiments were done with an Ultracel-3k (15-ml sample compartment, 3-KDa cut-off; Millipore Corp.). The assay medium (1 ml), containing 10–40 μM CBD1 or CBD2 in decalcified buffer, was placed into the upper compartment of the Ultracel-3k concentrator. For the binding assay, 1 μl of 5.7 mM 45Ca²⁺ stock solution (~3 μCi) was added to the assay medium with protein, carefully mixed, and allowed to equilibrate. After 10 min, the sample was centrifuged at 1000 × g for 30–50 s, forming 15–20 μl of filtrate. The filtrate was returned to the upper chamber, mixed, and centrifuged again. 5-μl samples of filtrate and protein were analyzed for radioactivity. Subsequently, nonradioactive CaCl2 was added serially to the protein sample, and after centrifugation, 5-μl samples of the filtrate and upper compartment were tested for radioactivity. The [Ca²⁺]free was measured as [Ca²⁺]free = [Ca²⁺]ultrafiltrate/[b], where a and b represent the radioactivity in the ultrafiltrate and upper compartment, respectively. The CBD-bound Ca²⁺ was determined as follows.

\[ [\text{Ca}^{2+} - \text{CBD}] = [\text{Ca}^{2+}]_{\text{add}} + [\text{Ca}^{2+}]_{\text{res}} - [\text{Ca}^{2+}]_{\text{free}} \]  (Eq. 1)

[Ca²⁺]add represents the externally added Ca²⁺, whereas [Ca²⁺]res is the residual (endogenous) Ca²⁺ in solution. [Ca²⁺]res was determined by Fluo-3 as described below. The bound 45Ca²⁺/CBD

a

FIGURE 1. Equilibrium 45Ca²⁺ binding to CBD1, CBD2, and CBD12. a, the equilibrium binding of 45Ca²⁺ was measured by incubating 10 μM CBD1 or CBD2 with 45Ca²⁺ in a 1-ml assay medium containing 10 mM Tris-HCl pH 7.2, plus either 100 mM KCl (□) or 100 mM choline chloride (●). The concentrations of Ca²⁺ were increased by adding stock solutions of nonradioactive Ca²⁺. At each concentration of Ca²⁺, the Ca²⁺-bound protein (in the upper chamber) was separated from the free ligand by ultrafiltration, yielding a 15–20-μl filtrate. After centrifugation, 5-μl samples were taken for radioactive counting from the filtrate and the upper chamber, and the data were plotted as bound 45Ca²⁺ per CBD1 or CBD2 (mol/mol) versus free concentrations of Ca²⁺ (for details, see “Materials and Methods”). A Hill equation was used to fit the calculated lines to the experimental points yielding Kd = 0.43 ± 0.02 μM, nH = 2.14 ± 0.25, and Cap = 0.09 ± 0.01 μM/CBD1/mol (mol/CBD1) and Kd = 6.2 ± 0.12 μM, nH = 2.02 ± 0.08, and Cap = 1.70 ± 0.12 μM/CBD2/mol for CBD2. b, the Ca²⁺ titration curves were obtained for 10 μM CBD12 as described in a. The assay medium contained 10 mM Tris-HCl, pH 7.2, with 100 mM choline chloride (●) or 95 mM choline + 5 mM Mg²⁺ (○). The lines were calculated according to the Adair equation (the six-site model) revealing the intrinsic Kd values of 0.2, 1.5, 2.3, 25, 30, and 120 μM in the absence of Mg²⁺ (●) and 1, 3, 13, 60, 190, and 500 μM in the presence of Mg²⁺ (○).
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**TABLE 1**

| Equilibrium Ca$^{2+}$ binding constants of CBD1 and CBD2 |
|----------------------------------------------------------|
| The Ca$^{2+}$ titration curves and data analysis were done as described in the legend of Fig. 1. The titration curves of binding data were fitted by using a Hill or Adair equation (see "Materials and Methods"). The $n_H$ represents a Hill coefficient. The mean ± S.E. values represent the data collected from six independent experiments ($n = 6$) for CBD1 or CBD2. The assays were done in the assay medium containing 100 mM KCl or choline chloride plus buffer, as described in the legend to Fig. 1. |
| Hill equation | Adair equation |
|---------------|----------------|
| $n_H$ | $K_d$ | Stoichiometry | $K_{d1}$ | $K_{d2}$ | Stoichiometry |
| CBD1 | 1.83 ± 0.05 | 0.30 ± 0.15 | 2.37 ± 0.18 | 1.13 ± 0.15 | 0.09 ± 0.03 | 2.38 ± 0.18 |
| CBD2 | 2.02 ± 0.27 | 4.95 ± 1.20 | 1.58 ± 0.05 | 23.2 ± 1.59 | 2.00 ± 0.01 | 1.66 ± 0.05 |

(mol/mol) was plotted versus [Ca$^{2+}$]$_{free}$ and the data was fitted to a Hill equation,

$$Y = \frac{\text{Cap}(L^{\alpha H})/L^{\alpha H} + K_d^{\alpha H}}{[2(1 + L/K_d)]} \quad \text{(Eq. 2)}$$
or to the following Adair equation.

$$Y = [\text{Cap}(1 + 2L/K_d)/(L/K_d)]/[2(1 + (1 + L/K_d)/(L/K_d))] \quad \text{(Eq. 3)}$$

Ca$^{2+}$/Protein Equilibrium Binding Assayed by Fluo-3—The experimental procedures of this assay were previously described (39, 40). A F-96 MicroWell plate (Nunc) was pre-washed to remove any contaminating Ca$^{2+}$. The exact concentrations of Fluo-3 were determined photometrically using a xenon lamp (150 W), and the emission was monitored at 535 nm. The Ca$^{2+}$ values were derived as fit parameters, whereas [F]$_0$ was held constant. For the Fluo-3 plus CBD titration curves, the obtained [Ca$^{2+}$]$_{res}$ and Fluo-3 $K_d$ values were used to subtract the Fluo-3-bound Ca$^{2+}$ species (F-Ca$^{2+}$) to yield the CBD1 curve. [F-Ca$^{2+}$]$_{eq}$ was calculated as [F-Ca$^{2+}$]$_{eq}$ = $x[F]_0$, and [Ca$^{2+}$]$_{free}$ was calculated as [Ca$^{2+}$]$_{free}$ = $xK_d$, whereas $x$ represents the fractional saturation of Fluo-3 at each given point of the titration curve ($x = 1$ and $x = 0$ were determined experimentally by saturating Ca$^{2+}$ and EDTA, respectively). The [Ca$^{2+}$/CBD] levels were derived as follows.

$$[\text{Ca}^{2+}/\text{CBD}] = [\text{Ca}^{2+}]_{add} + [\text{Ca}^{2+}]_{res} - [\text{Ca}^{2+}]_{free} - [\text{F-Ca}^{2+}]_{eq} \quad \text{(Eq. 4)}$$

The [Ca$^{2+}$/CBD]/[CBD] values were plotted as bound Ca$^{2+}$ per CBD as a function of [Ca$^{2+}$]$_{free}$, and the Ca$^{2+}$ titration curves were analyzed by using GraFit 3.01 software.

Stopped Flow Experiments—In the stopped flow experiments, 150 μl of 20–40 μM CBD1 or CBD2 with 20–80 μM Ca$^{2+}$ (syringe A) was mixed (t = 10–20 ms) with 150 μl of buffer containing 150 μM Quin-2 (syringe B) at 25 °C. Under these conditions, [Ca$^{2+}$]$_i$ drops below 100 nM within <2 ms. The stopped flow machine SFM-3 (BioLogic) equipped with a TC-100/15 cuvette (a mixing dead time of ~2 ms). Quin-2 was excited at λ$_{ex}$ = 333 nm from a monochromator with a hydrogen-xenon lamp (150 W), and the emission was monitored at λ$_{em}$ > 495 nm. The rapid mixing conditions were controlled with a MPF program, and the data were analyzed with Bio-Kine 32 V4.45 (BioLogic).

Statistics—The $K_d$, $k_{on}$, and $n_H$ values present the means ± S.E. Statistical analysis was done by using the two sample independent t test (Origin 7.0, Northampton, MA).

**RESULTS**

Equilibrium 45Ca$^{2+}$ Binding Measurements—The 45Ca$^{2+}$ titration curves were analyzed for purified preparations of CBD1, CBD2, and CBD12 at pH 7.2 and 25 °C. The protein-bound 45Ca$^{2+}$ was quantified by ultrafiltration. In an assay medium of 100 mM choline chloride (or KCl), CBD1 binds ~1.8 45Ca (mol/mol) at saturating concentrations of [45Ca$^{2+}$]$_{free}$ = 2–3 μM, showing a $K_d$ of 0.4 μM and a Hill coefficient of $n_H = 2.1$ (Fig. 1a). Under comparable experimental conditions, CBD2 binds ~1.7 45Ca (mol/mol) with a $K_d$ of 6.2 μM and $n_H = 2.0$ (Fig. 1a). Adair analysis reveals $K_{d1}/K_{d2}$ ratios of 12.6 and 11.6 for CBD1 and CBD2, respectively (Table 1). This is consistent (within the experimental error) with measured values of Hill coefficient ($n_H = 1.8–2.0$), although the maximal capacities for Ca$^{2+}$ binding are not exactly 2.0 (Table 1) for either protein (notably, Adair equation predefines a number of binding sites, whereas the Hill equation does not). Therefore, the derived values of $K_{d1}$ and $K_{d2}$ can be used (in combination with measured k$_{off}$ values) for calculating the k$_{on}$ values in a kinetic model describing a cooperative binding of two Ca$^{2+}$ ions to CBDs (see Fig. 7).

In the absence of Mg$^{2+}$, the titration curve of CBD12 reaches saturation at [45Ca$^{2+}$]$_{free}$ = 60–80 μM with a maximal binding capacity of ~5.9 45Ca/CBD12 (mol/mol), whereas Adair analysis reveals the intrinsic $K_d$ values for six Ca$^{2+}$ sites as 0.2, 1.5, 2.3, 25, 30, and 120 μM (Fig. 1b). These data suggest that the CBD12 construct can bind two additional Ca$^{2+}$ ions with $K_d$ values of >30 μM, as compared with CBD1 and CBD2 constructs (Fig. 1b). Considering the crystal structure data, according to which CBD1 contains four binding sites (19, 22), whereas CBD2 contains only two sites (20), it is reasonable to assume that the “extra” two affinity sites observed in CBD12 belong to CBD1 and not to CBD2. In the presence of 5 mM Mg$^{2+}$, CBD12 binds less Ca$^{2+}$ at any given concentration of [Ca$^{2+}$]$_{free}$ (Fig. 1b), meaning that Mg$^{2+}$ decreases the affinities of nearly all Ca$^{2+}$-binding sites of CBD12.

Equilibrium Ca$^{2+}$ Binding Measurements by Fluo-3—The above-described radioactive assays of Ca$^{2+}$ binding exhibited a maximal capacity of ~2 bound Ca$^{2+}$ (mol/mol) for both CBD1 and CBD2 (Fig. 1a), whereas ~6 Ca$^{2+}$/CBD12 (mol/mol) bound species were detected in CBD12 (Fig. 1b). To resolve this discrepancy as well as to validate the accuracy of our radioactive measurements, the Ca$^{2+}$ binding properties of CBD1 were quantitatively evaluated by a complementary and independent approach. To this end, the fluorescence assay of Fluo-3 was performed because of its capacity to measure not only the $K_d$ values but also the Ca$^{2+}$ binding stoichiometry (39, 40). This
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The method is based on the competition of Ca\(^{2+}\) binding to the protein of interest and Fluo-3. Compared with the Fluo-3 curve alone, the Fluo-3 plus CBD1 titration curve is shifted right because of competition between Fluo-3 and CBD1 for Ca\(^{2+}\) binding (Fig. 2a). The plotting of [Ca\(^{2+}\)]\(_{\text{free}}\) versus CBD1-bound Ca\(^{2+}\) \((n = 6)\) shows an excellent fit, with a \(K_d\) of 0.28 \(\mu\)M and \(n_{\text{H}} = 2.4\) at a capacity of 2.3 Ca\(^{2+}\)/CBD1 (mol/mol) (Fig. 2b).

Very similar titration curves were obtained by using the radioactive and fluorescence assays (Fig. 2c). Therefore, in the isolated preparations of CBD1, both the radioactive and fluorescence assays reveal the maximal stoichiometry of \(-2\) Ca\(^{2+}\)/CBD1 (mol/mol), meaning that the low affinity sites of the isolated CBD1 cannot be identified because of the conformational state of CBD1 and not because of methodological limitations.

**Stopped Flow Measurements of Ca\(^{2+}\) Off Rates**—The stopped flow technique was used for measuring the rate constants of Ca\(^{2+}\) dissociation from CBD1 and CBD2 by exploring Quin-2. The use of Quin-2 was preferred, because this probe has an extremely high affinity to Ca\(^{2+}\) \((K_d \approx 50 \text{ nM})\), whereas \(k_{\text{on}} = \sim 10^9 \text{ M}^{-1} \text{ s}^{-1}\) is nearly a diffusion-controlled reaction (41, 42). In these experiments \(10–20 \mu\text{M}\) CBD1 or CBD2 (pre-equilibrated with 40–80 \(\mu\text{M}\) Ca\(^{2+}\)) was mixed with 150 \(\mu\text{M}\) Quin-2. The rationale behind this is that when Quin-2 is mixed with the Ca\(^{2+}\)/protein, [Ca\(^{2+}\)]\(_{\text{free}}\) drops rapidly \(<2 \text{ ms}\) below \(K_d\) thereby inducing the Ca\(^{2+}\)/protein dissociation. Subsequently, Ca\(^{2+}\) dissociates from a protein complex (on a slower time scale) and binds to Quin-2, resulting in a rise in fluorescence.

The observed increase in fluorescence matches the rate and amount of Ca\(^{2+}\) dissociation from the complex. Under standard assay conditions (100 mm choline chloride or KCl), approximately two Ca\(^{2+}\) ions dissociate from CBD1 with observed order rate constant of \(k_{\text{off}} = 13.3 \pm 0.1 \text{ s}^{-1}\) (Fig. 3a). The Ca\(^{2+}\) dissociation kinetics of CBD2 is much faster, exhibiting a \(k_{\text{off}}\) of 155 \(\pm 3 \text{ s}^{-1}\) (Fig. 3b). Comparable results were obtained for five different preparations of CBD1 \((k_{\text{off}} = 6–13 \text{ s}^{-1})\) and CBD2 \((k_{\text{off}} = 155–300 \text{ s}^{-1})\) (Table 2). In contrast to CBD1 and CBD2, the CBD12 construct shows the biphasic kinetics of Ca\(^{2+}\) dissociation, exhibiting a \(k_1\) value of 7 \(\pm 3 \text{ s}^{-1}\) and a \(k_2\) value of 125 \(\pm 50 \text{ s}^{-1}\) (Fig. 3c). Because the \(k_1\) and \(k_2\) values are quite similar to the rate constants observed in the isolated CBD1 and CBD2, it is reasonable to assume that the CBDs retain their kinetic properties in the integrated CBD12 construct. The Ca\(^{2+}\) dissociation from the low affinity sites of CBD1 is perhaps so fast that it cannot be detected by stopped flow techniques even in the CBD12 construct.

**FIGURE 2.** Ca\(^{2+}\) binding assay of CBD1 monitored by Fluo-3. The Ca\(^{2+}\) titration curves were analyzed on the basis of CBD1 and Fluo-3 competition for Ca\(^{2+}\) binding (for details, see “Materials and Methods”). a, the fraction of Fluo-3-bound Ca\(^{2+}\) was experimentally evaluated at various concentrations of Ca\(^{2+}\) in an assay medium containing 10 mm Tris-HCl, pH 7.2, and 100 mm KCl. The titrations were done either in the presence of 11.1 \(\mu\text{M}\) Fluo-3 alone (i.e. standard curve, ○) or 11.1 \(\mu\text{M}\) Fluo-3 plus 10 \(\mu\text{M}\) CBD1 (●). The data were plotted as a function of total Ca\(^{2+}\). The solid line represents the fit of a standard titration curve of Fluo-3 (in the absence of CBD1), exhibiting a \(K_d\) value of 0.38 \(\mu\text{M}\). b, the CBD1-bound Ca\(^{2+}\) (mol/mol) was calculated at each point of the titration curve and plotted versus free concentrations of Ca\(^{2+}\) (see “Materials and Methods”). The experimental points represent the data collected from six independent experiments. The assay buffer contained 10 mm Tris-HCl, pH 7.2, plus 100 mm KCl (TK buffer) or choline chloride (TC buffer). The solid line was calculated to fit the experimental data by using a Hill equation. The fitting parameters of the Ca\(^{2+}\) binding curves were derived as follows: Capacity = 3.22 \(\pm 0.06 \text{ Ca}^{2+}/\text{CBD1} \text{ (mol/mol)}\), \(K_d = 0.28 \pm 0.01 \text{ \mu\text{M}},\) and \(n_H = 2.4 \pm 0.01\). c, the Ca\(^{2+}\) titration curves were obtained for CBD1 by parallel measurements of fluorescence (○) and radioactivity (●). The experimental points of radioactive (dashed line) and fluorescence (solid line) assays were fitted according to a Hill equation, yielding \(K_d = 0.025 \pm 0.002 \text{ \mu\text{M}},\) Cap = 2.60 \(\pm 0.12 \text{ Ca}^{2+}/\text{CBD1} \text{ (mol/mol)}\), and \(n_H = 1.98 \pm 0.25\) for the radioactive assay and \(K_d = 0.29 \pm 0.02,\) Cap = 2.45 \(\pm 0.10 \text{ Ca}^{2+}/\text{CBD1} \text{ (mol/mol)}\), and \(n_H = 2.70\) for the fluorescence assay. The lower (dotted line) represents the difference between the titration curves obtained by two different methods.
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Testing the Effects of Monovalent Cations on Ca\textsuperscript{2+} Interactions with CBDs—To test the possible effect of monovalent cations on Ca\textsuperscript{2+} binding/dissociation in CBD1 and CBD2, Na\textsuperscript{+} and K\textsuperscript{+} were examined for its effects on equilibrium \textsuperscript{45}Ca\textsuperscript{2+} titration curves and Ca\textsuperscript{2+} dissociation kinetics by using the experimental protocols described above. Na\textsuperscript{+} or K\textsuperscript{+} (100 mM) has no appreciable effect on the \(K_d\) values of CBD1 (Fig. 4a) or CBD2 (Fig. 4b), as compared with control (100 mM choline chloride). Na\textsuperscript{+} or K\textsuperscript{+} does not affect the \textsuperscript{45}Ca\textsuperscript{2+} titration curves of CBD12 as well (not shown), suggesting that these monovalent cations do not affect the Ca\textsuperscript{2+} binding of two low affinity sites of CBD1 domain as well. In the other set of experiments, the effect of Na\textsuperscript{+} or K\textsuperscript{+} was tested on Ca\textsuperscript{2+} dissociation kinetics. In these experiments the CBD preparations were pre-equilibrated with Ca\textsuperscript{2+} and then mixed in the stopped flow machine with Quin-2 buffer containing 100 mM NaCl or KCl. Na\textsuperscript{+} or K\textsuperscript{+} has a rather small accelerating effect on the \(k_{off}\) values of both CBDs, as compared with control experiments containing 100 mM choline chloride in the assay medium (Table 2). Because the observed effects of Na\textsuperscript{+} or K\textsuperscript{+} on the \(K_d\) and \(k_{off}\) values are relatively small (taking into account the experimental error), these effects are considered insignificant.

Mg\textsuperscript{2+} Effects on Ca\textsuperscript{2+} Interactions with CBDs—In contrast to monovalent cations, 5 mM Mg\textsuperscript{2+} decreases the \textsuperscript{45}Ca\textsuperscript{2+} binding affinity of CBD1 from \(K_d = 0.18 \mu M\) (in the absence of Mg\textsuperscript{2+}) to \(K_d = 1.26 \mu M\) (in the presence of Mg\textsuperscript{2+}) (Fig. 5a). These data are statistically significant, suggesting that 2–5 mM Mg\textsuperscript{2+} can decrease the Ca\textsuperscript{2+} binding affinity for CBD1 by at least 5–8-fold (Table 2). In the presence of Mg\textsuperscript{2+} (5 mM MgCl\textsubscript{2} + 95 mM choline chloride), CBD2 binds only \(~\)1 Ca\textsuperscript{2+} (mol/mol), showing a \(K_d\) of 1.1 \(\mu M\) (Fig. 6a, closed circles). Control experiments without Mg\textsuperscript{2+} reveal a typical binding of \(~\)2 Ca\textsuperscript{2+} /CBD2 (mol/mol), with a \(K_d\) of 5.6 \(\mu M\) and an \(n_H\) of 2.5 (Fig. 6a, open circles). These data suggest that Mg\textsuperscript{2+} binding to the secondary site of CBD2 increases the Ca\textsuperscript{2+} affinity at the primary Ca\textsuperscript{2+} site of CBD2. To elaborate a dose curve for Mg\textsuperscript{2+}-dependent displacement of Ca\textsuperscript{2+} at CBD2, increasing concentrations of Mg\textsuperscript{2+} were added at nearly saturating concentrations of \textsuperscript{45}Ca\textsuperscript{2+} (40 \(\mu M\)). The increasing concentrations of Mg\textsuperscript{2+} result in displacement of half the maximal levels of CBD2-bound \textsuperscript{45}Ca\textsuperscript{2+}, showing an IC\textsubscript{50} of \(~\)1.3 mM (Fig. 6b). Therefore, Mg\textsuperscript{2+} can displace Ca\textsuperscript{2+} at the secondary site of CBD2, which in turn increases the Ca\textsuperscript{2+} affinity at the primary site of CBD2. 2 mM Mg\textsuperscript{2+} has a small decelerating effect on the \(k_{off}\) values of CBD1 and CBD2 (Table 2), which probably represents very weak competition of Mg\textsuperscript{2+} with Ca\textsuperscript{2+} for Quin-2 binding rather than the effect of Mg\textsuperscript{2+} on Ca\textsuperscript{2+} dissociation kinetics of CBDs.

DISCUSSION

Ca\textsuperscript{2+} Binding Properties of CBD Ca\textsuperscript{2+} Sensors—Cooperative interactions between two Ca\textsuperscript{2+}-binding sites was primarily observed in the loop-f constructs (17, 47), although later studies have shown that these constructs contain only the CBD1 fragment (18–20). We found that under the equilibrium conditions, the CBD12 construct binds \(~\)6 Ca\textsuperscript{2+} ions (mol/mol) (Fig. 1b). Both the radioactive (Fig. 1a) and fluorescence assays (Fig. 2) reveal binding of \(~\)2 Ca\textsuperscript{2+} ions either to CBD1 (\(K_d = 0.3 \pm 1.2 \mu M\)) or CBD2 (\(K_d = 5.0 \pm 1.2 \mu M\)), while showing a Hill
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**TABLE 2**

The rate and equilibrium constants of Ca$^{2+}$ interaction with CBD1 and CBD2 under various ionic conditions

|        | CBD1 |        | CBD2 |
|--------|------|--------|------|
|        | $K_d$ | $k_{off}$ | $k_{on}$ |
|        | $K_d$ | $k_{off}$ | $k_{on}$ |
| **Choline** | 0.28±0.09 (n=3) | 6.29±0.39 (n=7) | 2.2×10$^7$ |
| **K$^+$** | 0.33±0.06 (n=3) | 10.62±0.41 (n=6) | 3.2×10$^7$ |
| **Na$^+$** | 0.19±0.02 (n=3) | 12.4±0.36 (n=5) | 6.5×10$^7$ |
| **Mg$^{2+}$** | 1.24±0.01 (n=3) | 4.0±0.07 (n=6) | 3.2×10$^8$ |

Notably, a two extra low affinity sites ($K_d > 30$ μM) can be observed in CBD12 but not in CBD1. Because a crystal structure reveals four binding sites in CBD1 (19) and two binding sites in CBD2 (20), the “additional” two binding sites observed in CBD12 belong to CBD1 and not to CBD2. Most probably, CBD2 might have a stabilizing effect on CBD1 conformation in the CBD12 construct. Present findings support a notion that the CBD Ca$^{2+}$ sensors retain their equilibrium (Figs. 1 and 2) and kinetic properties (Fig. 3) in the CBD12 construct. This is consistent with a common notion that the CBD1 and CBD2 domains are autonomously folded C2 protein structures retaining their functional properties (18–26). The fact that two low affinity Ca$^{2+}$-binding sites of CBD1 can be detected only in CBD12 (but not in isolated CBD1) can be explained by the suggestion that a highly flexible loop near the Ca$^{2+}$ sites of CBD1 (18, 19, 22) become less flickering in the CBD12 (probably because of domain-domain interactions). The low affinity sites of CBDs may become occupied by Ca$^{2+}$ only in restricted compartments (e.g., in dyadic cleft) where the local Ca$^{2+}$ may reach $10^{-4}$ M concentrations.

Considering the structural similarities of CBDs with other C2 proteins (44–46), it was proposed that the negatively charged phospholipids may interact with the two weaker Ca$^{2+}$ sites of CBD1 (21). Notably, not all proteins containing C2 domains interact with lipids (52). Therefore, more dedicated investigation is required for examining a possibility for lipid interaction with CBD1 and/or CBD2.

**CBD1 and CBD2 Are Kinetically Distinct Ca$^{2+}$ Sensors Even at Similar $K_d$ Values**—The experimental evidence suggests that the CBD domains undergo conformational changes in the cell within the excitation-contraction coupling (43, 49). To this end, no kinetic measurements have been undertaken to analyze the Ca$^{2+}$ interactions with the CBD sites. Here we applied the stopped flow techniques for measuring the Ca$^{2+}$ dissociation kinetics and found that the primary sites of CBD1 and CBD2 have strikingly distinct off rates for Ca$^{2+}$ dissociation (even under conditions at which the $K_d$ values are similar). Namely, the Ca$^{2+}$ dissociation is much slower in CBD1 ($K_{obs} =$ 6–13 s$^{-1}$) than in CBD2 ($K_{obs} =$ 155–300 s$^{-1}$) (Fig. 3 and Table 2). Notably, there is no experimental evidence that the individual Ca$^{2+}$ ions dissociate with distinct off rates. A kinetic model for cooperative binding of two Ca$^{2+}$ ions can be described (Fig. 7) on the basis of derived $K_{d1}$ and $K_{d2}$ values (Table 1) and observed off rates (Table 2). According to this model both CBDs are capable of sensing rapid changes in Ca$^{2+}$ concentrations within a single cycle of excitation-contraction coupling.

Although the Ca$^{2+}$ association/dissociation kinetics does not necessarily represent the exact dynamics of protein conformational changes, the observed kinetic properties may present the intrinsic features of autonomously folded CBD structures. The CBD12 domain resembles the biphasic kinetics for Ca$^{2+}$ dissociation (Fig. 3c), comprising the individual rate constants observed for CBD1 (Fig. 3a) and CBD2 (Fig. 3b). Therefore, it is reasonable to assume that the CBD1 and CBD2 domains retain their “autonomous” properties for Ca$^{2+}$ dissociation in the conjugated CBD12 construct.

**Comparative Kinetics of Ca$^{2+}$ Sensors**—In general, the Ca$^{2+}$ binding affinities and off rates could be very diverse even among proteins having very similar three-dimensional structures (23–26, 51). For example, EF-hand-containing proteins (usually having fast on rates) exhibit $K_d$ values that vary over a 1000-fold range, because of differences in the Ca$^{2+}$ off rates (23, 51). Interestingly, the slow off rates (3–10 s$^{-1}$) are found in proteins that act as Ca$^{2+}$ buffers (e.g., calcineurin), whereas in Ca$^{2+}$-signaling proteins the off rates vary in the range of 10–10$^8$ s$^{-1}$ (for review see Ref. 51). Moreover, the Ca$^{2+}$ off rates of two distinct domains could be very different even in the same protein. For example, in calmodulin the off rate for the EF-hand in the N-terminal lobe is over 100 times faster than for the C-terminal domain, thereby allowing independent regulation by each lobe of calmodulin (23, 51). Here, we demonstrate that the CBD1 and CBD2 domains have distinct Ca$^{2+}$ off rates even under conditions at which the $K_d$ values are similar at 2–5 mM Mg$^{2+}$ (Table 2).

Although we do not know how the conformational changes of CBD1 and CBD2 are integrated into the intact NCX protein, the kinetically distinct Ca$^{2+}$ sensors may represent a physical basis for differential sensing of Ca$^{2+}$ in dyadic, submembrane space, and bulk cytosol. This would be consistent with the notion that the NCX1 molecules are selectively co-localized and regulated within the cell (28–31). Moreover, the Ca$^{2+}$-sensing kinetics (e.g., off rates) may vary among NCX isoforms and splice variants to fulfill tissue-specific demands of Ca$^{2+}$ homeostasis.

**Ca$^{2+}$ Sensors of CBDs Are Insensitive to Na$^+$ or K$^+$**—Monovalent cations can modulate the partial reaction (Ca$^{2+}$/Ca$^{2+}$ exchange) of the Na$^{2+}$/Ca$^{2+}$ exchange cycle (11, 34, 35, 50), which may or may not represent the interaction of modulatory...
Ca2+ sensing properties of CBD domains

Several experiments were carried out to test the effects of sodium on equilibrium 45Ca2+ binding to CBD1 and CBD2. The 45Ca2+ titration curves of CBD1 or CBD2 were determined by using the ultrafiltration method, as described in Fig. 1 (see also “Materials and Methods”). The equilibrium binding assay was done with 10 mM Tris-HCl pH 7.2 buffer containing either 95 mM choline chloride + 5 mM MgCl2 (●), 100 mM NaCl (○), 100 mM KCl (△), or 100 mM choline chloride (□). For the assay medium containing 5 mM MgCl2, the experimental points of the CBD1 titration curve were fitted to the calculated line yielding the following parameters: Cap = 2.3 ± 0.1, Kd = 1.26 ± 0.03 μM, and nH = 2.9 ± 0.24. In the assay medium containing K+, Na+, and choline chloride, the CBD1 titration curves were fitted with Cap = 2.30 ± 0.1, Kd = 0.18 ± 0.01 μM, and nH = 1.8 ± 0.24.

FIGURE 4. Testing the effect of sodium on equilibrium 45Ca2+ binding to CBD1 and CBD2. The 45Ca2+ titration curves of CBD1 or CBD2 were elaborated by using the ultrafiltration method, as described in Fig. 1 (see also “Materials and Methods”). The equilibrium binding assay was done with 10 mM Tris-HCl pH 7.2 buffer containing either 100 mM KCl or 100 mM NaCl and varying concentrations of 45Ca2+. The experimental data obtained for the CBD1 titration curves were independently fitted for K+ and Na+-containing buffers showing the following parameters for Na+ (Cap = 2.00 ± 0.05, Kd = 0.22 ± 0.01 μM, and nH = 1.77 ± 0.24) and K+ (Cap = 2.00 ± 0.08, Kd = 0.26 ± 0.02 μM, and nH = 1.73 ± 0.38) for CBD2 titration curves, the following fitting parameters were obtained for Na+ (Cap = 1.88 ± 0.03, Kd = 6.96 ± 0.25 μM, and nH = 2.48 ± 0.24) and K+ (Cap = 1.74 ± 0.04, Kd = 6.83 ± 0.25 μM, and nH = 1.82 ± 0.09).

FIGURE 5. Effect of Mg2+ on equilibrium 45Ca2+ binding to CBD1. The titration curves of 45Ca2+ binding to CBD1 were derived by using the ultrafiltration procedure, as described in Figs. 1 and 4 (see also “Materials and Methods”). The assay medium was buffered with 10 mM Tris-HCl, pH 7.2, containing either 95 mM choline chloride + 5 mM MgCl2 (●), 100 mM NaCl (○), 100 mM KCl (△), or 100 mM choline chloride (□). For the assay medium containing 5 mM MgCl2, the experimental points of the CBD1 titration curve were fitted to the calculated line yielding the following parameters: Cap = 2.3 ± 0.1, Kd = 1.26 ± 0.03 μM, and nH = 2.9 ± 0.24. In the assay medium containing K+, Na+, and choline chloride, the CBD1 titration curves were fitted with Cap = 2.30 ± 0.1, Kd = 0.18 ± 0.01 μM, and nH = 1.8 ± 0.24.

mediated by K+ interaction with CBD domains as well. Therefore, the Na+-dependent inactivation may represent the Na+ interaction with the transport site(s) resulting in slow inactivation, as originally suggested by Hilgemann et al. (32, 33). This is also consistent with a claim that the elevated Na+ induces a mode of activity that does not require allosteric Ca2+ activation (27).

Only the Primary Site of CBD2 Is Highly Selective to Ca2+—Mg2+ can decrease the Ca2+ affinity for fusion proteins containing a high affinity site of loop-f (17). At this end it was not clear how Mg2+ affects the specific Ca2+ sensors of CBD1 and CBD2. We found that the Ca2+ affinities of CBD1 (Kd = ~0.32 μM) and CBD2 (Kd = ~5.0 μM) are dissimilar only in the absence of Mg2+ (Fig. 1a), whereas at 2–5 mM Mg2+, the Kd of 1–2 μM is observed for both CBDs (Table 2). Therefore, even under conditions at which the Ca2+ affinities are similar, the kinetic properties of CBD1 and CBD2 are still diverse (Table 2).

Mg2+ can compete with Ca2+ for five sites of CBDs (Figs. 1b and 5) but not for the primary site of CBD2 (Figs. 5 and 6). In principle, Mg2+ has opposite effects on the primary sites of CBD1 and CBD2 (Fig. 7). From one side Mg2+ decreases the Ca2+ affinity at the primary sites of CBD1 (Fig. 5), whereas on the other side Mg2+ binding to the secondary site of CBD2 elevates the Ca2+ affinity at the primary site of CBD2 (Fig. 6 and Table 2). Most probably, Mg2+ decelerates the Ca2+ on rates at CBD1 sensors, whereas Mg2+ accelerates the on rate at the primary site of CBD2 as a result of Mg2+ interaction with the secondary site of CBD2 (Fig. 7b). In both cases no considerable...
changes were detected on off rates by Mg$_2^+$ (Table 2). Therefore, in the cell the primary sensor of CBD2 might have a much higher affinity than was previously thought (18, 20). Although the effects of Mg$_2^+$ on NCX ion currents were not systematically studied, it has been shown that the cytosolic Mg$_2^+$ can attenuate inward and outward ion currents in cardiomyocytes by competing with Ca$_2^+$ at the regulatory sites of NCX (48). The fluorescence resonance energy transfer analysis suggests that the cytosolic Mg$_2^+$ can decrease nearly 3-fold the affinity of CBD Ca$_2^+$ sensor (49).

**Interacting Mechanisms of Mg$_2^+$ with Ca$_2^+$ Sensors**—Presently found effects of Mg$_2^+$ on CBD Ca$_2^+$ sensors are especially interesting in light of structural differences between CBD1 and CBD2 (18–22). Interestingly, the fluorescence resonance energy transfer analysis supports a notion that Mg$_2^+$ can interact with regulatory Ca$_2^+$ site(s) of NCX without inducing considerable conformational changes of the protein (49). Therefore, the Mg$_2^+$-dependent elevation of the Ca$_2^+$ affinity at site 1 of CBD2 may represent some specific rearrangements of Ca$_2^+$-coordinating groups at site 1 and 2 of CBD2. For example, the occupation of the CBD2 site 2 by Mg$_2^+$ may alter the charge compensation mechanism with respect to Lys$_{585}$. This may affect the arrangement of specific Ca$_2^+$-coordinating groups shared by site 1 and site 2. Because Mg$_2^+$ has a stronger preference than Ca$_2^+$ for retaining water molecules in its coordination sphere (23, 52), the water-rich coordination sphere for the “secondary” Ca$_2^+$ site (site 2) in the CBD2 structure implies that the cytosolic Mg$_2^+$ can decrease nearly 3-fold the affinity of CBD Ca$_2^+$ sensor (49).

**FIGURE 7.** Kinetic schemes of Ca$_2^+$ interactions with Ca$_2^+$ sensors of CBD1 and CBD2. The cooperative interactions of Ca$_2^+$ with CBD1 (a) and CBD2 (b) domains are described in terms of rate constants. The on rate constants were calculated ($k_{on} = k_{off}/K_d$) according to the experimentally derived values of $k_{off}$ and $K_d$ (Table 1) and the Ca$_2^+$ off rates (Table 2). The dotted lines depict the primary site of CBD2, which does not interact with Mg$_2^+$.
as observed in some proteins (23). Mg\(^{2+}\) may also help “form” the Ca\(^{2+}\) site, as seen with the lectin family of Ca\(^{2+}\) proteins (23). Because the splice variant segment is located within CBD2, Mg\(^{2+}\) may contribute to tissue-specific regulation of NCX isoforms and splice variants. More dedicated studies are required for elucidating these issues.

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