In NCX proteins CBD1 and CBD2 domains are connected through a short linker (3 or 4 amino acids) forming a regulatory tandem (CBD12). Only three of the six CBD12 Ca\(^{2+}\)-binding sites contribute to NCX regulation. Two of them are located on CBD1 (\(K_d \approx 0.2 \, \mu M\)) and one is on CBD2 (\(K_d \approx 5 \, \mu M\)). Here we analyze how the intrinsic properties of individual regulatory sites are affected by linker-dependent interactions in CBD12 (AD splice variant). The three sites of CBD12 and CBD1 + CBD2 have comparable \(K_d\) values but differ dramatically in their Ca\(^{2+}\) dissociation kinetics. CBD12 exhibits multiphasic kinetics for the dissociation of three Ca\(^{2+}\) ions \((k_f = 280 \, s^{-1}, k_i = 7 \, s^{-1}, \text{and} \, k_e = 0.4 \, s^{-1})\), whereas the dissociation of two Ca\(^{2+}\) ions from CBD1 \((k_f = 16 \, s^{-1})\) and one Ca\(^{2+}\) ion from CBD2 \((k_e = 125 \, s^{-1})\) is monophasic. Insertion of seven alanines into the linker (CBD12–7Ala) abolishes slow dissociation of Ca\(^{2+}\), whereas the kinetic and equilibrium properties of three Ca\(^{2+}\) sites of CBD12–7Ala and CBD1 + CBD2 are similar. Therefore, the linker-dependent interactions in CBD12 decelerate the Ca\(^{2+}\) on/off kinetics at a specific CBD1 site by 50–80-fold, thereby representing Ca\(^{2+}\) “occlusion” at CBD12. Notably, the kinetic and equilibrium properties of the remaining two sites of CBD12 are “linker-independent,” so their intrinsic properties are preserved in CBD12. In conclusion, the dynamic properties of three sites are specifically modified, conserved, diversified, and integrated by the linker in CBD12, thereby generating a wide range dynamic sensor.

The Na\(^{+}\)/Ca\(^{2+}\) exchanger proteins (NCX1–3) and their splice variants are expressed in a tissue-specific manner (1–3) and mediate Ca\(^{2+}\) entry/extrusion depending on various factors (4, 5). NCX is “autoregulated” by cytosolic Ca\(^{2+}\) at sites that do not directly participate in the ion translocation (6, 7). For example, the cardiac/neuronal NCX1 variants respond to 0.3–10 \(\mu M\) cytosolic Ca\(^{2+}\), whereas the kidney variant does not (8, 9). Allosteric regulation of NCX involves the interaction of cytosolic Ca\(^{2+}\) with the regulatory loop-f of NCX (10–14). Although the high affinity regulatory site of NCX1 was discovered 16 years ago (15), only recent equilibrium binding studies have shown that isolated CBD1 protein contains two sites with high affinity for Ca\(^{2+}\) \((K_d = 0.2–0.4 \, \mu M\) and \(n_H = 1–2)\) and two low affinity sites \((K_d > 20 \, \mu M)\), whereas CBD2 binds two Ca\(^{2+}\) ions with \(K_d\) values of 5–10 \(\mu M\) (21). Structural and mutational studies concluded that only three of the six Ca\(^{2+}\)-binding sites of CBD12 (Ca3 and Ca4 on CBD1 and Ca1 on CBD2) actually contribute to \([Ca^{2+}]\)-dependent regulation of the intact NCX1 protein (22–26). The two high affinity sites of CBD1 (Ca3 and Ca4) may play a critical role in determining the affinity \((K_{
abla_{Ca}} = 0.3 \, \mu M)\) for \([Ca^{2+}]\)-dependent regulation by cytosolic Ca\(^{2+}\) in cellular systems, whereas the primary site of CBD2 (Ca1) may contribute to the Ca\(^{2+}\)-dependent relief of Na\(^{+}\)-dependent inactivation of intact NCX (23–25). Importantly, the segment that undergoes alternative splicing is located solely within the CBD2 domain, meaning that specific domain-domain interactions may shape the regulatory properties of primary Ca\(^{2+}\) sites at CBD1 (16–20, 24).

Fluorescence resonance energy transfer studies demonstrated that CBD domains of NCX can undergo significant conformational changes in the cell within the excitation-contraction coupling (22, 27), although dynamic aspects of relevant conformational transitions, as well as the contribution of each specific Ca\(^{2+}\) site to regulatory events, were not resolved. The crystal structure of CBD12 has not yet been determined, although low resolution small angle x-ray scattering studies have shown that Ca\(^{2+}\) interaction with CBD12 results in global reorientation of the two domains (24). The dynamic aspects of two-domain reorientation were not investigated, although the relevant mechanisms could be important for shaping the dynamic properties of Ca\(^{2+}\)-dependent regulation in various isoforms/splice variants of NCX.

The Ca\(^{2+}\)-dependent regulation of NCX during the action potential requires millisecond time scale of feedback regulation, whereas the slow modes of inactivation last for seconds (6–9, 12). A critical question arises: Which specific Ca\(^{2+}\) sites contribute to the rapid and slower modes of NCX regulation, and how is this related to regulation of distinct NCX isoforms/splice variants in different cell types? In cellular systems, 0.3–10 \(\mu M\) cytosolic Ca\(^{2+}\) can activate NCX1 within a few milliseconds, whereas lower Ca\(^{2+}\) levels (<0.3 \(\mu M\)) activate the exchanger more slowly (for review see Ref. 29). Moreover, the secondary regulation of NCX, referred to
as Ca$^{2+}$-dependent inactivation ($I_n$) is a slow process ($t_{0.5} = 3–7 s$) (30–32) that considerably varies among NCX splice variants (8, 9). Interestingly, the heart and brain splice variants of NCX1 exhibit nearly identical affinities for [Ca$^{2+}$]$_{\text{free}}$-dependent activation, whereas the response of the brain-expressed variant is 10-fold faster than that of the cardiac one (8, 9). Previously, we found that Ca$^{2+}$ on/off rates of CBD domains resemble the properties of the rapid Ca$^{2+}$ sensor that can properly respond to cytosolic Ca$^{2+}$ changes during the action potential (21). However, these findings could not explain how CBDs contribute to slow inactivation of NCX.

In the current study we evaluate the role of linker-dependent interactions in shaping the equilibrium and kinetic properties of three regulatory Ca$^{2+}$ sites in CBD12. For this purpose, the isolated preparations of CBD1, CBD2, CBD12, and CBD1 + CBD2 proteins and their corresponding mutants (CBD1-E454K and CBD12–7Ala) were analyzed by protocols developed in our laboratory (21). Here we demonstrate, for the first time, the slow mode of Ca$^{2+}$ dissociation from CBD12, which appears to be the linker-dependent phenomenon. Our findings reveal that the linker-dependent interactions selectively diversify intrinsic properties of Ca$^{2+}$ on/off kinetics at a specific site of CBD12 sensor, thereby yielding a wide range dynamic sensor capable of NCX regulation within a broad time interval ranging 4 orders of magnitude.

**MATERIALS AND METHODS**

**Expression and Purification of CBD Proteins**—The DNA constructs of CBD1, CBD2, and CBD12 (encoding residues 371–509, 501–657, and 371–657, respectively) of canine NCX1 (accession code P23685; AD splice variant) were cloned into a pET23b vector (generously provided by Dr. M. Hilge). Seven alanines were inserted between residues His$^{501}$ and Ala$^{502}$ of the CBD12 construct by a PCR-based method, generating the CBD12–7Ala construct. The insertion was confirmed by DNA sequencing. The DNA construct of CBD1-E454K was a generous gift from Dr. Kenneth D. Philipson and Debora A. Nicoll (David Geffen School of Medicine at UCLA). The vectors were 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 as outlined previously (16, 21). The cells were disrupted twice in a microfluidizer (Microfluidics, Newton, MA), and after centrifugation the supernatant was concentrated to 300–1200 ml containing 10 μM protein was placed in the upper compartment of the Ultracel-3k concentrator, and the binding assay was performed as previously outlined (21). 1 μL of 5–6 mM 45Ca$^{2+}$ stock solution (~3 μCi) was added to the assay medium with protein, carefully mixed, and allowed to equilibrate. The sample was centrifuged at 1000 × g for 50 s, and 10–20 μL aliquots of filtrate and protein (upper chamber) were analyzed for sample radioactivity. Subsequently, nonradioactive CaCl$_2$ was added serially to the protein sample, and after centrifugation, 10–20 μL aliquots of the filtrate and upper compartment were tested for sample radioactivity. The [Ca$^{2+}$]$_{\text{free}}$ was measured as [Ca$^{2+}$]$_{\text{free}}$ = [Ca$^{2+}$]$_{\text{tot}}$(a/b), where a and b represent the radioactivity in the ultrafiltrate and upper compartment, respectively. The Ca$^{2+}$-bound Ca$^{2+}$ was determined as [Ca$^{2+}$-CBD] = [Ca$^{2+}$]$_{\text{add}}$ + [Ca$^{2+}$]$_{\text{free}}$ = [Ca$^{2+}$]$_{\text{tot}}$ (21, 33, 34). [Ca$^{2+}$]$_{\text{add}}$ represents the externally added Ca$^{2+}$, whereas [Ca$^{2+}$]$_{\text{free}}$ is the residual (endogenous) Ca$^{2+}$ in the solution. [Ca$^{2+}$]$_{\text{tot}}$ was determined by Fluo-3 or Fluo-4 as outlined before (21). The bound 45Ca$^{2+}$/CBD (mol/mol) was plotted versus [Ca$^{2+}$]$_{\text{free}}$, and the data were fitted to an Adair or Hill equation using the GraFit software (Erithacus Software Ltd.) as described before (21). All other miscellaneous procedures were described elsewhere (38, 39).

**Stopped Flow Experiments**—In the stopped flow experiments, 150 μL of 10–40 μM CBD1, CBD2, CBD1 + CBD2, CBD12, or CBD12–7Ala in TK buffer (10 mM Tris-Cl, pH 7.2, and 100 mM KCl) containing 1–80 μM free Ca$^{2+}$ (syringe A) were mixed (τ = 10–30 ms) at 25 °C with 150 μL of TK buffer containing 200–600 μM Quin-2 (syringe B). The Quin-2 probe was utilized in this assay because under the given experimental conditions, this probe has an extremely high affinity toward Ca$^{2+}$ (K$_d$ = ~50 nm), whereas the second order rate constant for Ca$^{2+}$ binding (k$_{on}$ = ~10$m^2$/s$^{-1}$) is diffusion-controlled. The Ca$^{2+}$ off rates can be accurately measured because following the mixing of Ca$^{2+}$-bound protein with Quin-2, the [Ca$^{2+}$]$_{\text{free}}$ drops below 7–80 nm within <2 ms, thereby allowing the dissociation of Ca$^{2+}$ from protein. Subsequently, Ca$^{2+}$ dissociates from the protein complex (albeit on a slower time scale) and binds to Quin-2, resulting in a rise of fluorescence. The observed increase in fluorescence corresponds to the rate
and the amount of Ca$^{2+}$ dissociation from the complex. The stopped flow machine SFM-3 (BioLogic) was equipped with a two mixer/three syringe mixing system, a TC-100/15 cuvette (a mixing dead time of ~2 ms), temperature controller, microprocessor, and single-wavelength monochromator (MOS-200). Quin-2 was excited at $\lambda_{ex}$ = 333 nm from a monochromator with a hydrogen-xenon lamp (150 W), and the emission was monitored at $\lambda_{em}$ > 495 nm by using a long pass filter. Parameters (mixing time duration, flow rates, volume, signal sampling time, detection time length, etc.) for the desired stopped flow experiments were chosen with a MPF program (Bio-Logic). The data were analyzed with Bio-Kine 32 V4.45 (Bio-Logic) equipped with Pad-Laplace and Simplex analytical modes, allowing the automatic fitting to multi-exponential kinetics. Experimentally obtained traces were fitted to mono-exponential or multi-exponential curves by using the $\chi^2$ criteria to identify the best fit for a specific kinetic model of Ca$^{2+}$ dissociation. All other details for data processing and analysis are described elsewhere (35–38, 40).

**Statistics**—The measured values are presented as the means ± S.E. Statistical analysis was performed by using the two-sample independent $t$ test (Origin 7.0, Northampton MA).

**RESULTS**

To examine the effects of soluble CBD2 on the $K_d$ values of the high affinity binding sites (Ca3 and Ca4) of CBD1, the $^{45}$Ca$^{2+}$ titration curves of CBD1, CBD2, CBD1 + CBD2, and CBD12 proteins were analyzed by using the protein/ligand ultrafiltration approach (21, 33, 34). Only the AD splice variant of CBD2 and CBD12 was used in the present study. Adair analysis of $^{45}$Ca$^{2+}$ binding curves reveals that the two high affinity sites (Ca3 and Ca4) of CBD1 ($K_d$ = 0.2–0.6 μM) and the primary site (Ca1) of CBD2 ($K_d$ = 5–10 μM) have comparable values in the context of CBD1 + CBD2 and CBD12 (Fig. 1). These data suggest that linker-dependent interactions may decrease the Ca$^{2+}$ binding affinities of the relevant three regulatory sites up to 3–4-fold, at most (because of the differences in protein preparations and the nature of the applied experimental procedures, these differences should be considered within or close to the experimental error). The isothermal titration calorimetry technique was utilized to detect any possible interaction between soluble CBD1 and CBD2 domains in solution at CBD concentrations of 20–50 μM concentrations, but no evidence was found for this type of protein–protein interaction (not shown).

The fluorescent probe Quin-2 was used in combination with the stopped flow technique for measuring the rate constants of Ca$^{2+}$ dissociation from CBD12. The fluorescence of Quin-2 was excited at $\lambda_{ex}$ = 333 nm from a monochromator with a hydrogen-xenon lamp (150 W), and the emission was monitored at $\lambda_{em}$ > 495 nm by using a long pass filter. Parameters (mixing time duration, flow rates, volume, signal sampling time, detection time length, etc.) for the desired stopped flow experiments were chosen with a MPF program (Bio-Logic). The data were analyzed with Bio-Kine 32 V4.45 (Bio-Logic) equipped with Pad-Laplace and Simplex analytical modes, allowing the automatic fitting to multi-exponential kinetics. Experimentally obtained traces were fitted to mono-exponential or multi-exponential curves by using the $\chi^2$ criteria to identify the best fit for a specific kinetic model of Ca$^{2+}$ dissociation. All other details for data processing and analysis are described elsewhere (35–38, 40).

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Subsequently, the Ca\(^{2+}\) occupy two to six sites at fixed \(\text{Ca}^{2+}\) affinity properties in the context of CBD12 (Fig. 1).

The slow kinetics of \(\text{Ca}^{2+}\) were fitted to a single exponential curve with a representative trace of CBD12 was fitted to a double exponential curve with \(k_f\) and \(k_r\). Notably, the \(\text{Ca}^{3+}-\text{Ca}^{4}\) sites retain reasonably high values, despite the dissociation of one \(\text{Ca}^{2+}\) ion from CBD12 by 50–80-fold while exerting a rather small effect on the "fast" \(\text{Ca}^{2+}\) dissociation \((k_f)\). Notably, the Ca3-Ca4 sites retain reasonably high affinity properties in the context of CBD12 (Fig. 1b), despite the low values of \(k_d\) (Fig. 2b) and \(k_m\) (calculated as \(k_m/k_a\)).

To resolve the effect of the medium/low affinity sites on the slow kinetics of \(\text{Ca}^{2+}\) dissociation from high affinity sites, CBD12 was incubated with 1–80 \(\mu\)M free \(\text{Ca}^{2+}\) to occupy two to six sites at fixed \([\text{Ca}^{2+}]_{\text{free}}\) levels (Fig. 3a).

The amount of \(\text{Ca}^{2+}\) bound to CBD12 was measured independently at each fixed concentration of free \(\text{Ca}^{2+}\). All of the buffer solutions were prepared on the basis of 10 mM Tris-HCl, pH 7.2, 100 mM KCl (TK buffer).

The trace for 1 \(\mu\)M \([\text{Ca}^{2+}]_{\text{free}}\) was fitted to a double exponential curve with \(k_f=14.8\pm0.08\) s\(^{-1}\) and \(k_r=5.2\pm0.001\) s\(^{-1}\), c, the bars represent the statistics of the experimentally observed \(k_d\) and \(k_m\) values (see above, a and b) obtained in four to seven independent experiments performed with three different preparations of CBD1, CBD2, CBD12, or CBD1 + CBD2 proteins. All of the measured values represent the means ± S.E.

In all of the experiments, the final \([\text{Ca}^{2+}]_{\text{free}}\) after mixing were <10 nM. The trace for 1 \(\mu\)M free \(\text{Ca}^{2+}\) was fitted to a double exponential curve with \(k_f=2.76\pm0.03\) s\(^{-1}\) and \(k_r=0.53\pm0.001\) s\(^{-1}\). The trace for 15 \(\mu\)M \(\text{Ca}^{2+}\) was fitted to a double exponential curve with \(k_f=8.87\pm0.32\) s\(^{-1}\) and \(k_r=0.59\pm0.002\) s\(^{-1}\), c, the \(k_d\) and \(k_m\) values for \(\text{Ca}^{2+}\) dissociation from CBD12 were experimentally derived at the indicated \([\text{Ca}^{2+}]_{\text{free}}\) values. The data were then plotted as the means ± S.E. Each point represents the average value obtained from four or five independent experiments.
FIGURE 4. \(^{45}\text{Ca}^{2+}\) titration curve and \(\text{Ca}^{2+}\) dissociation kinetics of the CBD12–7Ala mutant. The experimental conditions for the equilibrium \(^{45}\text{Ca}\) binding assay and stopped flow assay of \(\text{Ca}^{2+}\) dissociation were similar to those described in the legends of Fig. 1 and 2. a, the \(^{45}\text{Ca}^{2+}\) titration curves were calculated using the Adair equation, showing Cap = 6 (mol/mol) and \(K_d = 0.05, 0.1, 4, 6.5, 8.5, \) and 50 \(\mu\text{M}\) for CBD12–7Ala ( □). The dashed lines depict typical titration curves of CBD1-WT and CBD12-WT. b, syringe 1 contained 10 \(\mu\text{M}\) CBD12–7Ala in TK buffer with 10 \(\mu\text{M}\) free \(\text{Ca}^{2+}\)., and syringe 2 contained 200 \(\mu\text{M}\) Quin-2 in TK buffer. The representative trace of CBD12–7Ala was fitted to a single exponential curve with \(k_f = 18.7 \pm 0.08\) s\(^{-1}\). The dashed lines represent typical traces observed for CBD1-WT and CBD12-WT.

are so fast (>300 s\(^{-1}\)) that the regulatory effect of the low affinity “secondary” sites cannot be properly characterized under the given experimental conditions.

For further characterization of the linker-dependent effects on the intrinsic properties of CBD12 Ca\(^{2+}\) sites, we prepared and analyzed the CBD12–7Ala protein, in which seven alanines were inserted into the CBD1–CBD2 linker between residues His\(^{501}\) and Ala\(^{502}\). The three relevant Ca\(^{2+}\) sites of CBD12–7Ala show \(^{45}\text{Ca}\) titration curves very similar to those of CBD1 + CBD2 or CBD12, suggesting that the Ca\(^{2+}\) binding affinities of Ca3-Ca4 (on CBD1) or Ca1 (on CBD2) are not considerably modified by the longer linker in CBD12–7Ala (Fig. 4d). Most strikingly, no slow dissociation of Ca\(^{2+}\) was observed in CBD12–7Ala while exhibiting typical Ca\(^{2+}\) off rates observed in CBD1 + CBD2 (Fig. 4b). This implies that with the longer linker, all three sites of CBD12–7Ala display kinetics and equilibrium properties similar to those observed in isolated CBD1 and CBD2 preparations. Therefore, CBD12 and CBD12–7Ala fundamentally differ in their Ca\(^{2+}\) dissociation kinetics, suggesting that the intact linker is capable of generating specific interdomain interactions responsible for decelerating on/off kinetics at a specific high-affinity site of CBD1 in intact CBD12.

To evaluate the role of the low affinity Ca1 site in regulating the affinity and/or Ca\(^{2+}\) off rates at high affinity sites of CBD1, the CBD1-E454K mutant was analyzed in terms of its Ca\(^{2+}\) titration curves (Fig. 5a) and Ca\(^{2+}\) dissociation kinetics (Fig. 5b). The rationale behind this is that although Glu\(^{454}\) is a part of the low affinity Ca1 site, it is known that the E454K mutant does not affect the \([\text{Ca}^{2+}]_t\)-dependent activation curve of intact NCX1 in the cellular system (25). The Ca\(^{2+}\) titration curve shows a maximal binding capacity of \(\sim 2 \mu\text{M}/\text{CBD1-E454K}\) (mol/mol) with a “normal” \(K_d\) of 0.24 \(\mu\text{M}\) and reduced cooperativity (\(n_H = 1.3\)), whereas the wild type CBD1 shows a maximal binding capacity of \(\sim 3 \mu\text{M}/\text{CBD1-WT}\) (mol/mol) with \(K_d\) of 0.34 \(\mu\text{M}\) and high cooperativity of \(n_H = 2.8\) (Fig. 5a). The remarkable finding was that the Ca\(^{2+}\) off rate of CBD1-E454K \((k_f = 80\) s\(^{-1}\)) was much faster than that of wild type CBD1 \((k_f = 18\) s\(^{-1}\)) (Fig. 5b). Very similar data were obtained under conditions in which the stopped flow mixing was initiated at 10 or 80 \(\mu\text{M}\) free Ca\(^{2+}\) (not shown). Although the \(k_{on}\) value was not directly measured in these experiments, it can be reliably quantified from the experimentally measured values of \(K_d\) and \(k_{off}\) \((k_{on} = k_{off}/K_d)\). Hence, the only reasonable explanation for the present results is that the E454K mutation accelerates both \(k_{on}\) and \(k_{off}\) proportionally, so the Ca\(^{2+}\) affinity \((K_d)\) is not significantly altered. Therefore, at least one low affinity site (Ca1) of CBD1 may play an important role in determining the intrinsic on/off rates of Ca\(^{2+}\) interaction with Ca3-Ca4 sites while having a negligible (if any) effect on equilibrium binding at the same sites. Additional systematic research of relevant mutants is required to further elucidate these issues.
DISCUSSION

The present study was conducted to evaluate the contribution of linker-dependent interdomain interactions in shaping the affinity and kinetic properties of three regulatory Ca$^{2+}$ sites in the brain splice variant CBD12 (AD). Our data reveal that (a) the linker slows down Ca$^{2+}$ off rates at a specific Ca$^{2+}$ site of CBD1 (by nearly 2 orders of magnitude), whereas the changes in $K_d$ do not exceed 3–4-fold; (b) the linker does not significantly alter the intrinsic affinity and kinetic properties of the other two CBD12 regulatory sites (located on CBD1 and CBD2), thereby retaining their diverse kinetic properties in CBD12; and (c) the linker integrates the dynamic range of all three Ca$^{2+}$ sites such that the dynamic range of Ca$^{2+}$ sensing is expanded. The biological significance of the current findings is that linker-dependent interactions selectively retain, modify, and integrate dynamic properties of three regulatory sites of CBD12, thereby allowing effective regulation of NCX within a broad range time interval (10$^{-3}$–10 s).

The Linker Dramatically Slows Ca$^{2+}$ On/Off Rates at Specific High Affinity Site of CBD1 in CBD12—The striking finding is that CBD12 exhibits slow dissociation of Ca$^{2+}$ ($k_f = 0.3–0.6$ s$^{-1}$), whereas all other protein preparations (CBD1, CBD2, CBD1 + CBD2, or CBD12–7Ala) lack this capacity (Figs. 2b and 4b). Moreover, the Ca$^{2+}$ titration curve analysis shows that three regulatory sites of CBD1 + CBD2, CBD12, or CBD12–7Ala exhibit typical $K_d$ values within 0.2–5 M (Figs. 4a and 5a). However, CBD12 demonstrates a markedly distinct profile of Ca$^{2+}$ dissociation kinetics differing from all the other proteins tested. Three Ca$^{2+}$ ions dissociate from CBD12 with multiphasic kinetics ($k_f = 280$ s$^{-1}$, $k_f = 7$ s$^{-1}$ and $k_f = 0.4$ s$^{-1}$), whereas monophasic kinetics is observed for dissociation of two Ca$^{2+}$ ions from CBD1 ($k_f = 16$ s$^{-1}$) and one Ca$^{2+}$ ion from CBD2 ($k_f = 125$ s$^{-1}$) (Fig. 2). In agreement with the Ca$^{2+}$ titration curves, the isothermal titration calorimetry measurements showed no evidence of interaction between soluble CBD1 with CBD2 in solution (not shown). Therefore, the slow dissociation of one Ca$^{2+}$ ion from CBD12 represents specific interdomain interactions controlled by the linker. Most probably, the CBD1-CBD2 linker-dependent local interactions between the two CBD domains and/or linker-dependent conformational constraints at a specific CBD1 site (Ca3 or Ca4) specifically decelerate Ca$^{2+}$ off rates from 16–18 s$^{-1}$ to 0.2–0.4 s$^{-1}$, whereas the $K_d$ is retained within <0.6 M. The only reasonable explanation for the obtained data is that the linker-dependent interdomain interactions decelerate the Ca$^{2+}$ on/off rates by 50–80-fold at one specific CBD site, without greatly compromising the Ca$^{2+}$ sensing affinity. The current findings are consistent with sequential dissociation of two Ca$^{2+}$ ions from the Ca3-Ca4 sites, whereas the dissociation of the first (fast) Ca$^{2+}$ ion is followed by dissociation of the second (“occluded”) Ca$^{2+}$ ion, displaying characteristic slow kinetics (Fig. 6). Our findings are consistent with recent structural studies indicating that Ca$^{2+}$ binding enhances the stability of the Ca$^{2+}$ binding site of CBD1 near the hinge region while the overall structure of CBD1 remains largely unaffected (28). The underlying biological relevance is that the linker-dependent modification of Ca$^{2+}$ on/off kinetics at a specific regulatory site can diversify and widen the dynamic range of Ca$^{2+}$ sensing of CBD12 by nearly 2 orders of magnitude (while retaining a submicromolar range of Ca$^{2+}$ affinity). Therefore, the linker-dependent interactions may extend the dynamic range of NCX regulation for up to 10–20 s.

Two Ca$^{2+}$ Sites Retain Their Intrinsic Properties in CBD12 Context—The experimental fact is that two Ca$^{2+}$ sites of CBD12 exhibit very similar kinetics and binding affinities in comparison with isolated preparations of CBD1 ($k_f = 7–18$ s$^{-1}$) and CBD2 ($k_f = 120–250$ s$^{-1}$) (Fig. 2). These data support the idea that the CBD1-CBD2 linker-dependent conformational modifications do not drastically influence (<3–4-fold) the kinetic and affinity properties of single Ca$^{2+}$ sites located on CBD1 and CBD2. This conclusion has been further supported by analysis of the CBD12–7Ala mutant. The insertion of seven alanines into the linker (CBD12–7Ala) abolishes the slow dissociation of Ca$^{2+}$, whereas all three sites of CBD12–7Ala still exhibit similar kinetics and affinity to isolated CBD1 and CBD2 (Figs. 4 and 5). A critical question that arises is what are the structure-activity relationships that determine "selective" modification of kinetic properties at one Ca$^{2+}$ site on CBD1 while preserving the intrinsic properties of two other sites on CBD1 and CBD2? Because the crystal structure of CBD12 has not yet been determined, we are uncertain regarding the exact mechanisms involved. Nevertheless, the currently available x-ray structures (17, 19) of isolated CBD1 and CBD2 may provide some valuable and relevant clues on this issue. First of all, the primary site of CBD2 is at least 30 Å away from the linker, so it is not surprising that the linker-dependent structural transi-
tions do not affect the intrinsic properties of this site. Second, the coordination chemistry of Ca\(^{2+}\) ligation of the Ca3 and Ca4 sites are considerably different in respect to the linker positioning (17), so these sites might be distinctly sensitive to the linker-dependent conformational transitions. Therefore, we postulate that in CBD12, the linker-dependent constraints alter on/off rates at specific Ca\(^{2+}\) site of CBD1 while retaining the intrinsic properties of the remaining two Ca\(^{2+}\) sites located on CBD1 and CBD2 intact (Fig. 6).

The Dynamic Properties of the Three CBD12 Regulatory Sites Are Diverse and Complementary—One important conclusion from the present work is that the CBD1-CBD2 linker not only specifically diversifies and preserves the dynamic properties of specific Ca\(^{2+}\) sites in CBD12 but also effectively integrates the dynamic properties of all three regulatory sites (Fig. 6). The rationale behind this is to provide a broad range dynamic sensor capable of dynamically regulating NCX proteins within a time scale spanning over 4 orders of magnitude (from milliseconds to seconds). The kinetically distinct Ca\(^{2+}\) sensors may represent a physical basis for differential Ca\(^{2+}\) sensing in various cellular compartments. More specifically, Ca\(^{2+}\) interactions with a “rapid” CBD2 site (the Cal site) can respond to transient swings of [Ca\(^{2+}\)] that occur within less than 10 ms. This sensor may play an essential role in restricted areas of the cell (e.g. subsarcolemma space and dyadic cleft), where the [Ca\(^{2+}\)] levels can transiently reach 300–600 \(\mu\)M levels within a few milliseconds, when the cell is depolarized (47–49). On the other hand, the fast CBD1 site is capable of responding to cytosolic [Ca\(^{2+}\)] changes that take place within the duration of the action potential (10–200 ms) (Fig. 2a). This would be consistent with the notion that the NCX1 molecules are selectively co-localized and regulated within the cell (48). The dynamic range of CBD12 is further extended by the linker-dependent modulation of the “slow” site of CBD1, which may be involved in Ca\(^{2+}\)-dependent events that last up to 10 s. This slow Ca\(^{2+}\) sensor may represent \(I_1\) and/or \(I_2\) inactivation modes of NCX observed in electrophysiological experiments (30–32).

Is the Linker Involved in Interdomain Signal Transmission?—The [Ca\(^{2+}\)]-dependent activation curve of intact NCX1 (23, 25) is almost identical to Ca\(^{2+}\) titration curves obtained for isolated CBD1 (21), meaning that Ca3-Ca4 sites of CBD1 might determine the affinity for Ca\(^{2+}\)-dependent activation. Insertion of the 7 alanine residues into the linker of the intact exchanger (NCX1–7Ala) decreases the affinity of [Ca\(^{2+}\)]-dependent activation of NCX1–7Ala 15–30-fold, exhibiting a \(K_{a,s}\) of 5–10 \(\mu\)M (43). Two alternative explanations can be considered for interpretation of these results. Either the elongation of the linker results in decreased affinity of the Ca3-Ca4 sites on CBD1, or alternatively the primary site of CBD2 (Cal) becomes a predominant sensor for activation in NCX1–7Ala. In our CBD2–7Ala construct, two high affinity sites exhibit values typical of the Ca3-Ca4 sites, meaning that the long linker does not decrease the affinity of the two high affinity sites of CBD1 (Fig. 4). In light of the present considerations, we suggest that the long linker “disconnects” (uncouples) the interaction between the two CBD domains in NCX1–7Ala, so that the Cal site \(K_d = 5–10 \mu\)M of CBD2 (and not the Ca3-Ca4 sites of CBD1) becomes a “master” sensor for [Ca\(^{2+}\)]-dependent activation of NCX1–7Ala.

Recently, it is unclear how the kinetic aspects described here are related to the recently discovered Ca\(^{2+}\)-dependent reorientation of two CBD domains in CBD12 (24). In any case, further research and application of alternative biophysical approaches (e.g. fluorescence resonance energy transfer) is required to resolve the dynamic aspects of linker-dependent domain-domain interactions. Artificial variations in the linker length could be a useful probe for identifying functionally relevant conformational transitions.

Low Affinity Sites of CBD12 Do Not Modulate Slow Dissociation of Ca\(^{2+}\)—Mutational and structural studies have shown that two low affinity sites (Cal and Ca2) of CBD1 and one low affinity site of CBD2 (Call) are not directly involved in [Ca\(^{2+}\)]-dependent activation of intact NCX1 (23–26). On the other hand, a recently described model suggests that Ca\(^{2+}\) interaction with all four sites of CBD1 might play an important role in determining the electrostatic charge that drives the reorientation of the two CBD domains (24). Here we demonstrate that the slow mode of Ca\(^{2+}\) dissociation from CBD12 is not conditioned or modulated by Ca\(^{2+}\) occupation of medium/low affinity sites \((K_p > 5 \mu\)M) of CBD12 (Fig. 3). Therefore, slow Ca\(^{2+}\) kinetics of CBD12 represents a “Ca\(^{2+}\)-independent” mode, which is exclusively constrained by the linker. On the other hand, our data indicate that [Ca\(^{2+}\)] > 2 \(\mu\)M can moderately accelerate (3–4-fold) the \(k_d\) off rates (Fig. 3C), suggesting the possibility that occupation of Cal of CBD2 may accelerate Ca\(^{2+}\) dissociation from the “fast” CBD1 site. Taking into account the conformational changes associated with Ca\(^{2+}\) binding to CBD2 (42), it is more probable that Ca\(^{2+}\) binding to CBD2 controls the signal transmission from CBD2 to transmembrane segments rather than regulates the Ca\(^{2+}\) off rates at CBD1.

Glu\(^{454}\) contributes to Ca\(^{2+}\) ligation at the low affinity Ca1 site of CBD1 (16, 23–26), whereas substitution of Glu\(^{454}\) with lysine significantly stabilizes the protein folding of the CBD1-E454K mutant (25). Here we analyzed Ca\(^{2+}\) titration curves and Ca\(^{2+}\) off kinetics of CBD1-E454K and found that the replacement of the negative charge by the positive one in E454K does not have any considerable effect on the equilibrium binding of Ca\(^{2+}\) at Ca3-Ca4 sites (Fig. 3a). However, the CBD1-E454K mutant exhibits 5–7-fold faster kinetics for Ca\(^{2+}\) dissociation \((k_d = 80 s^{-1})\) in comparison with the wild type CBD1 \((k_d = 14–18 s^{-1})\) (Fig. 5b). Therefore, even in the absence of the CBD1-CBD2 linker, the low affinity site(s) of CBD1 can modulate on/off kinetics (but not the equilibrium binding) at the high affinity Ca\(^{2+}\) sites of CBD1. In light of the present findings, the electrostatic interactions of Ca\(^{2+}\) with the low affinity sites of CBD1 may shape the kinetics rather than the equilibrium properties of high affinity sensors on CBD1.

Interplay between the Kinetic and Equilibrium Properties of CBD12 Ca\(^{2+}\) Sensing—Our analyses clearly demonstrate that the kinetic diversity of three Ca\(^{2+}\) sites is much more prominent \((k_d = 0.4–250 s^{-1})\) than the affinity range \((K_d = 0.2–5 \mu\)M) (Table 1). Although the observed Ca\(^{2+}\) on/off rates do not necessarily represent protein conformational transitions, dynamic diversity of the three regulatory sites may represent a physical scope for defining the kinetic range of Ca\(^{2+}\) sensing. In general, the interplay between the kinetic
and affinity properties must be thoroughly balanced in Ca\(^{2+}\)-sensing proteins in terms of structure-function relationships (44–46). Consistent with this principle, the Ca\(^{2+}\) off rates of two distinct domains could be very different even in the same protein (45, 46). For example, in calmodulin, the off rate for the EF-hands in the N-terminal lobe is over 100 times faster than that of the C-terminal domain (46). However, in contrast to calmodulin, the \(C_2\) domains (including CBDs) do not undergo concerted conformational changes upon Ca\(^{2+}\) binding (16–19, 41, 44–46, 50, 51). The remarkable feature of the CBD12 sensor is that the linker-dependent conformational constraints modify the Ca\(^{2+}\) on/off rates ~100-fold at a specific regulatory site of CBD1 without greatly compromising the binding affinity (Figs. 1–4). Interestingly, linker-dependent interactions between two \(C_2\) domains are fundamentally different, as demonstrated in Rabphilin-3A (51) and Synaptotagmin (41, 50), so the linker-related domain-domain interactions could substantially diverge in the course of evolution. It is currently unclear whether the linker-dependent effects described here represent exclusive properties of CBD12 or whether the other \(C_2\) proteins also possess similar structure-function relationships. This issue may be of general interest because of the vast amount of functionally important proteins (synaptotagmins, PKC, titin, fibronectin, neuronal cell adhesion factors, etc.) that sense Ca\(^{2+}\) through \(C_2\) folded domains, and ~30% of them form two-domain structures (41, 44, 45, 50, 51). More dedicated kinetic analyses of two-domain \(C_2\)-tandems are required to reach meaningful conclusions.

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