Proton-sensing Ca$^{2+}$/Na$^{+}$/Ca$^{2+}$ Exchanger*§

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The cardiac Na$^+$/Ca$^{2+}$ exchanger (NCX) regulates cellular [Ca$^{2+}$]i and plays a critical role in health and disease, but its molecular regulation is poorly understood. Here we report on how protons affect this electrogenic transporter by modulating two critically important NCX C2 regulatory domains, Ca$^{2+}$/Na$^+$ antiporter

The sarcolemmal Na$^+/$/Ca$^{2+}$ exchanger (NCX1)§ is the primary transport system in heart muscle that extrudes Ca$^{2+}$ from the cytosol (1). Ca$^{2+}$ enters the myocytes through the L-type Ca$^{2+}$ channel and triggers Ca$^{2+}$ release from the sarcoplasmic reticulum (SR) to produce the [Ca$^{2+}$]i transient that activates contraction. All of this “trigger” Ca$^{2+}$ that enters the cell must be extruded, and the NCX is largely or wholly responsible (2). NCX is an electrogenic transporter and, when extruding net Ca$^{2+}$, produces an inward current, I$_{NCX}$, the magnitude of which is a direct measure of the transport rate of NCX (3, 4). Although multiple NCX control elements exist to regulate its function, we focus here on one that is critically important and one that is both complex and controversial; that is, the action of [Ca$^{2+}$], on NCX transport rate.

Two kinds of Ca$^{2+}$-dependent regulation of NCX are appreciated: “translocation” effects and “allosteric” effects (5, 6). The translocation actions of [Ca$^{2+}$], reflect how the availability of Ca$^{2+}$ and its binding to a translocation site affects the NCX transport rate. Such translocation effects depend both on the thermodynamics and the kinetics of the system. The allosteric effect depends on Ca$^{2+}$ binding to a site that itself does not produce translocation but regulates transport kinetics. The cytosolic loop of NCX includes two closely spaced domains named Ca$^{2+}$ binding domain 1 (CBD1) and CBD2 (7–9), each of which share a common core structure typical of C2-type domains (10, 11). Such C2 domains are known to interact with diverse effectors (e.g. Ca$^{2+}$, phosphatidylinositol diphosphate, lipids, and other proteins) (10–12), yet so far the two CBD domains in NCX only appear to interact with Ca$^{2+}$, which allosterically activates transport by NCX. Here we investigate the two C2 regulatory domains and their competitive modulation by Ca$^{2+}$ and protons.

Proton actions on NCX function were investigated using state of the art electrophysiological, imaging, and biochemical methods. [Ca$^{2+}$]i, pH, and I$_{NCX}$ were measured in single ventricular myocytes (13–15). [Ca$^{2+}$]i binding to purified preparations of CBD1 and CBD2 was assayed by ultrafiltration and Ca$^{2+}$ off rates were measured by stopped-flow assay (16, 17).

We have identified and characterized the Ca$^{2+}$ sensitivity of NCX at CBD1 and CBD2 and discovered a process that is profoundly modulated by pH. Furthermore, we have characterized the proton-dependent block of NCX. This proton-dependent regulation of the Ca$^{2+}$ modulation of NCX appears to arise as an allosteric action on the NCX protein. Taken together, our

“Trigger” Ca$^{2+}$...
new findings suggest a pivotal role for protons in the physiological and pathologic regulation of NCX.

MATERIALS AND METHODS

Overexpression and Purification of CBD1, CBD2, and CBD12 Proteins—The DNA constructs of CBD1, CBD2, and CBD12 (AD-splice variant) were cloned into a pET23b vector and expressed in Escherichia coli Rosetta2 (DE3) competent cells (Novagen) as described (16, 17). Overexpressed proteins were purified on nickel beads (<95% purity, judged by SDS-PAGE). Protein preparations were repeatedly washed in the Ultracel-3k (Millipore) device to remove EDTA. For accurate measurement

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(100 mM KCl and 10 mM Bistris propane) expressed in

(AD-splice variant) were cloned into a pET23b vector and

purified on nickel beads (Millipore) to remove EDTA. For accurate measurement

of high affinity Ca2+

binding, the residual levels of EDTA must be <1 nm in final preparations of proteins (see supplemental Fig. 45).

Equilibrium 45Ca2+ Binding Assay to Proteins—The 45Ca2+

binding to proteins was measured as outlined before (16, 17). The assay medium (1 ml) contained 0.02 mM CBD1 or 0.04 mM CBD2 or 0.02 mM CBD12, 100 mM KCl, and either 20 mM Bistris propane for experiments carried at pH 6.3–9.0, 10 mM MES for experiments carried at pH < 6.3, or 10 CAPS at pH > 10. In the buffers prepared for 45Ca2+ binding assay, residual free Ca2+

was measured by fluo-3 (16); whereas the Kd values of Ca2+

binding to fluo-3 is pH-sensitive, it is experimentally derived at each given pH and not assumed. All Ca2+

binding assays were done at 22–23 °C. The 45Ca2+ titration curves were fitted to a Hill or Adair equation (16, 17).

Stopped-flow Experiments—Quin-2 was used in the stopped-flow experiments to monitor Ca2+

off rates (16, 17). In the stopped-flow machine SFM-3 (BioLogic), 150 μl (syringe A) of proteins in buffer (100 mM KCl and 10 mM Bistris propane) were mixed with 150 μl of buffer plus 200–600 μM Quin-2 (syringe B). Quin-2 was excited at λem = 333 nm, and emission was monitored at λex > 495 nm. The data were analyzed with Bio-Kine 32 Version 4.45 (Bio-Logic).

Cell Isolation, Electrophysiology, and Confocal Imaging—Cardiomyocytes were isolated from euthanized adult Sprague-Dawley rats (18). Myocytes were attached to laminin-coated coverslips placed in a custom designed chamber and were used within 4–5 h from the time of isolation. A whole-cell dialysis patch clamp method was combined with confocal microscopy to enable simultaneous measurement of IiNCX, pH, and [Ca2+]i in myocytes. Voltage control and current measurement was accomplished using an Axopatch 200A amplifier; data were digitized and recorded using a Digidata 1322A (Axon Instruments) attached to a PC. Confocal imaging was performed with a Zeiss 510 laser scanning microscope (inverted) equipped with a 63 × 1.4 NA oil immersion objective. Cardiomyocytes were co-loaded through the patch pipette with the salt form of fluo-4 and carboxy-seminaphthorhodafluor-1 (C-SNARF-1). To avoid spectral bleed-through of individual indicators, confocal recordings were made in the multi-track mode, where line-scan emissions along the longitudinal axis of the cardiomyocyte were acquired at one excitation at a time, sequentially. Fluo-4 fluorescence emission was taken at 505–550 nm, whereas excitation was at 488 nm with an argon ion laser. The C-SNARF-1 dual emission was collected at λ1 (>635 nm) and λ2 (560–615 nm), excitation was at 543 nm with a He-Ne laser. All experiments were performed at 20–23 °C.

Calibration of fluo-4 and C-SNARF-1 Fluorescent Signals—Fluo-4 fluorescence was calibrated with respect to [Ca2+]i, using a method developed by Trafford et al. (19), in which the maximal fluorescence (Fmax) was directly measured from each cardiomyocyte at the end of the experiment. Fluo-4 Kd values were determined experimentally by in vitro fluorometric titration assay performed at various pH values (16). The intracellular Kd values of 1 μM at pH 7.2 and 1.5 μM at pH 6.8 were used for calculating [Ca2+]i. An in situ calibration method was adapted to convert the C-SNARF-1 emission ratio (A1:A2) to pHi (14). Calibration curves were obtained from a separate set of cardiomyocytes patch-loaded in a manner that was identical to that used in the electrophysiological experiments. Patched cells were pretreated (1–2 min) with 20 μM nigericin locally applied by a pneumatic pico-pump; nigericin treatment permits equilibration of pHi with the pH of the external superfusion recording solution. The calibration curves of C-SNARF-1 performed here yielded an apparent pK value of 7.29 ± 0.15 (n = 4 cells), which is in good agreement with previous reports in rat cardiomyocytes (13).

Solutions Used for Electrophysiological Recording—To eliminate any contribution of the SR to Ca2+

handling, cells were pretreated for 10 min with Ca2+ /Na+ free Tyrode’s solution containing 0.001 mM thapsigargin, 10 mM caffeine, 140 mM LiCl, 6 mM KCl, 1 mM MgCl2, 10 mM glucose, and 5 mM HEPES, pH 7.4, with LiOH. Patch pipettes (1.8–2.2 megaohms) were filled with a solution containing 59 mM CsCl, 80 mM tetraethylammonium chloride, 0.91 mM MgCl2, 1 mM HEPES, 10 mM NaCl, 5 mM MgATP, 0.3 mM Na2GTP, 3.99 mM KCl, 0.1 mM C-SNARF-1, and 0.05 mM K2fluoro-4; pH was set to 7.2 with CsOH. Gigaohm seals were attained while perfusing the recording chamber with normal Tyrode’s solution containing 140 mM NaCl, 4 mM KCl, 10 mM glucose, 5 mM HEPES, 1 mM CaCl2, and 1 mM MgCl2, with pH set to 7.4 using NaOH. After whole-cell configuration was attained, to better isolate IiNCX and to enable clamping of the pHi to the displaced level, the external solution was switched to the recording solution, a normal Tyrode’s solution supplemented with the following combination of drugs: 3 mM 4-aminopyridine, 20 μM nifedipine, 30 μM niflumic acid, and 8 μM (4-cyanobenzylthiophene-2-carbonyl)guanidine methanesulfonate an Na+/H+ exchanger-1 inhibitor. To manipulate pHi, cells were perfused for 2–4 min with the Tyrode’s solution containing 40 mM NH4Cl (with an appropriate reduction in NaCl to keep the overall osmolality constant; also see supplemental Fig. 2).

Protocols for Measuring [Ca2+]i-dependent Activation of IiNCX—The electrophysiological protocol (Fig. 1) and off-line analysis of the currents (Fig. 2) were based upon the approach of Weber et al. (15, 20). After break-in and again before any repetition of the protocol, the cells were held at EK of −100 mV for 3–5 min to facilitate Ca2+ efflux via inward IiNCX, resulting in low initial [Ca2+]i. Each experimental protocol included repetitive step pulses from −100 to +100 mV applied at ∼4.5 Hz. The number of pulses (15–60) and the duration in which the cells were held at ±100 allowed specific control of [Ca2+]i. We initially repeated the experimental protocol 2–4 times at basal
pH, (usually ~7.2); immediately after the lower pH was established (via acid loading by NH₄Cl rebound) we repeated the experimental protocol for as long as the cardiomyocytes remained quiescent, i.e. the leak currents were stable and <300 pA (at E_m of −80 mV). The magnitude of the leak currents was taken from the first pulse to −100 mV, and I_{NCX} was calculated as the current after off-line subtraction of the leak currents (for details see supplemental Fig. 1). In the presence of Ni²⁺ (10 mM), no Ca²⁺-activated current was seen (see supplemental Fig. 1). The magnitude of the measured leak currents in the absence of Ni²⁺ noted above were indistinguishable from those observed after the application of Ni²⁺. During each E_m step, the average I_{NCX} (excluding the capacitive transient) was plotted versus instantaneous [Ca²⁺], and half-maximal Ca²⁺-induced activation of I_{NCX} (K_{0.5}) was calculated from the least-square fits to a Hill equation (Origin 8.1 software; Fig. 2C).

**Statistics**—Values are given as the mean ± S.E. Statistical analysis was performed using Student t test for unpaired samples (SPSS 11.5).

**RESULTS**

An investigation of the regulation of Na⁺/Ca²⁺ exchanger function by [Ca²⁺]_i and [H⁺]_i was carried out in rat ventricular cardiomyocytes. This work involved an examination of I_{NCX} under conditions when [Ca²⁺]_i and [H⁺]_i can be readily measured and, to some extent, controlled. Finally, we examined where protons bind to the Na⁺/Ca²⁺ exchanger and whether or not they may compete with Ca²⁺ as regulatory ions.

### Measuring Allosteric Activation of I_{NCX} at Physiological and Acidified pH

To investigate the influence of [H⁺]_i on the Na⁺/Ca²⁺ exchanger function, I_{NCX} was measured at different pH levels. The NH₄Cl rebound method was used to transiently acidify the intracellular compartment of a single cardiomyocyte, whereas I_{NCX} was examined using patch clamp measurements (whole-cell configuration). Simultaneously [Ca²⁺]_i was measured using fluo-4, and pH was monitored using carboxy-SNARF-1. Fig. 1, A–C, shows a typical experimental result. During the experimental procedure the membrane potential was stepped from −100 mV (100 ms) to +100 mV (125 ms); see FIGURE 1. *pH-dependent changes in Na⁺/Ca²⁺ exchanger function in cardiac ventricular myocytes.* A, total membrane current recorded from a patch-clamped ventricular myocyte (whole-cell mode) at intracellular pH 7.2 (left) and 6.7 (right) while simultaneously measuring intracellular [Ca²⁺]_i and pH_i is shown. Membrane potential was stepped from −100 mV (100 ms) to +100 mV (125 ms) as shown in the inset (top). B, [Ca²⁺]_i was measured as F/F₀ using the Ca²⁺-sensitive fluorescent indicator fluo-4. C, pH_i was measured using the H⁺-sensitive fluorescent indicator carboxy-SNARF-1. Changes in pH were produced using the NH₄Cl rebound method (see "Materials and Methods" and Ref. 44) using transient superfusion with 40 mM NH₄Cl.
the Fig. 1 inset. At +100 mV there is net transport of Ca\(^{2+}\) into the cell by NCX. In addition, there was some Ca\(^{2+}\) leak into the cell through other pathways (e.g. Ca\(^{2+}\) channels); this is probably true at −100 mV. Together, NCX and the other Ca\(^{2+}\) leak pathways led to an elevation of [Ca\(^{2+}\)]\(_i\). The time-dependent, voltage-sensitive current \(I_m\) shows background (leak) current and \(I_{\text{NCX}}\), \(I_{\text{Ca}^\text{a}}\), \(I_{\text{K}^\text{o}}\), and other currents were blocked pharmacologically (see “Materials and Methods”). \(I_{\text{NCX}}\) is the Ca\(^{2+}\)-activated membrane current measured when all other currents are blocked. The gradual increase in \(I_m\) that was observed before NH\(_4\)Cl application reflects the change in [Ca\(^{2+}\)]\(_i\) during the procedure (Fig. 1B, left). After the wash-off of NH\(_4\)Cl (see gap between the left panel and right panel in Fig. 1, A–C), the acidification of the cytosol was clear (Fig. 1C, right panel). The increase in [Ca\(^{2+}\)]\(_i\), \(I_{\text{NCX}}\) was also significant as shown in Fig. 1B, right panel. Despite the increase in [Ca\(^{2+}\)]\(_i\), \(I_{\text{NCX}}\) is less than before the application of NH\(_4\)Cl. Although the full range of the allosteric effect of acidification on \(I_{\text{NCX}}\) can be seen in Fig. 3, in Fig. 1 we see that at acidic pH, NCX can still be activated by elevated [Ca\(^{2+}\)]\(_i\) (see the beginning of each of the top panels). In addition, Fig. 1 shows that these effects are not simply time-dependent artifacts but instead require an elevation of [Ca\(^{2+}\)]\(_i\).

The observation that acidification inhibits \(I_{\text{NCX}}\) is not new (21–24). Here, we establish this phenomenon in the intact cellular system. Although the data are clear, several important technical issues deserve comment. First, the \(K_p\) of fluo-4 for Ca\(^{2+}\) is sensitive to pH and should be higher as the cells become more acidic. This means that as pH\(_i\) becomes more acidic, an elevation of [Ca\(^{2+}\)]\(_i\) would be needed to keep fluo-4 fluorescence unchanged. This underscores the significant increase in [Ca\(^{2+}\)]\(_i\) observed in Fig. 1B (pH\(_i\) of about 6.8) compared with that measured in Fig. 1A (pH\(_i\) of 7.2). The base-line fluo-4 emission level at the lower pH\(_i\) was higher by a factor of 2.14 ± 0.14 compared with control pH\(_i\) (p < 0.01, 17 measurements at control pH and 24 measurements at acidic pH\(_i\), n = 6 cells). Second, typically the pH\(_i\) of cells that are patched in a whole-cell configuration is tightly stabilized by protox-buffering agents (such as HEPES), which were included in the internal pipette solution in these experiments but at a lower level (HEPES was 1 mM). Thus a significant and sustained decrease of pH\(_i\) after perfusion with 40 mM NH\(_4\)Cl was achieved because of the low HEPES concentration and the addition of an Na\(^+\)/H\(^+\) exchanger-1 inhibitor in the extracellular recording solution (for more details see supplemental Fig. 2). In 6 of 14 cells it was possible to perform acid loading procedure and to proceed to
Intracellular Acidosis Shifts the Allosteric Activation of INCX to Higher [Ca\(^{2+}\)] Values—To provide more quantitative examination of the INCX and its [Ca\(^{2+}\)] and [H\(^{+}\)], dependences, experiments were performed using the same voltage protocol described for Fig. 1 with the addition of calibrated [Ca\(^{2+}\)] values. Fig. 2A shows a typical experiment that reports the time course of the changes in membrane current (Fig. 1A) as a function of [Ca\(^{2+}\)]. With repeated depolarizations to +100 mV there was a slow net Ca\(^{2+}\) influx that accumulated with time, leading to an elevation of [Ca\(^{2+}\)] (see Fig. 2B). The increase in [Ca\(^{2+}\)] activates the Na\(^+\)/Ca\(^{2+}\) exchanger and leads to a gradual enhancement of \(I_{\text{Na}}\) as shown in the raw current traces (Fig. 2A). The voltage-dependent leak current was subtracted from the raw current to produce the \(I_{\text{NCX}}\) (see “Materials and Methods”) shown in Fig. 2C. The outward \(I_{\text{NCX}}\) is shown with a sigmoidal curve fitted to the experimental data (\(K_{0.5} = 257.5 \pm 4.8 \text{ nM}; n_{\text{H}} = 7.70 \pm 0.89\)). The net inward \(I_{\text{NCX}}\) is shown on the bottom portion of Fig. 2C and is largely similar to the inverse of the outward data (also see supplemental Fig. 35). Fig. 2D shows the average \(K_{0.5}\) of 348.2 \pm 20.6 nM and an average \(n_{\text{H}}\) value of 8.57 \pm 1.04 from cells (pH\(_i\) = 7.2) that displayed a clear saturation of activation (\(n = 9\) of 14 cells). The remarkably large Hill coefficient of \(n_{\text{H}} > 8\) suggests that the [Ca\(^{2+}\)] dependence of \(I_{\text{NCX}}\) may be due to a allosteric effect of Ca\(^{2+}\) on the Na\(^+\)/Ca\(^{2+}\) exchanger, a suggestion supported by diverse experiments (1, 5). Direct investigation of such a hypothesis depends on the identification and “fingerprinting” of an appropriate Ca\(^{2+}\) binding site(s). Central to our investigation, however, is the observed pH\(_i\) dependence of INCX.

\(I_{\text{NCX}}\) was measured over a range of [Ca\(^{2+}\)], at different [H\(^{+}\)] levels. Using the NH\(_4\)Cl rebound method (see supplemental Fig. 2), intracellular acidification was produced. Fig. 3A shows the magnitude of \(I_{\text{NCX}}\), and Fig. 3B shows the results obtained in these experiments normalized to the maximal \(I_{\text{NCX}}\). In Fig. 3B data obtained at pH, 7.2 is shown as blue and black open circles, whereas pH\(_i\) = 6.87 is shown with red open circles. There is a clear difference in the Ca\(^{2+}\) sensitivity of \(I_{\text{NCX}}\) at pH\(_i\) = 7.2 with a \(K_{0.5} = 310 \pm 5.4 \text{ nM (n = 14 cells)}\), roughly 3-fold less than the \(K_{0.5}\) at pH\(_i\) = 6.87 (1042.8 \pm 15.0 \text{ nM, n = 6 cells}). The decreased sensitivity of \(I_{\text{NCX}}\) in an acidic environment is consistent with diverse clinical observation (see “Discussion”). Importantly, the maximum \(I_{\text{NCX}}\) observed at pH\(_i\) = 7.2 and at pH\(_i\) = 6.87 was found to be similar, albeit at different [Ca\(^{2+}\)] levels. Nevertheless, this finding shown in Fig. 3A supports the normalization of data used in Fig. 3B to compare the [Ca\(^{2+}\)] dependence of \(I_{\text{NCX}}\) at different pH\(_i\) levels. Note also that after the normalization of the data, the Hill coefficients were also indistinguishable (8 \pm 0.67 in control versus 8 \pm 0.78 at pH\(_i\) of 6.87). If the Ca\(^{2+}\) sensitivity of \(I_{\text{NCX}}\) depends significantly on an allosteric effect, there may be Ca\(^{2+}\) binding sites that play a central role in this process. Furthermore, the pH\(_i\) sensitivity of the Ca\(^{2+}\) dependence of \(I_{\text{NCX}}\) may also have a parallel in Ca\(^{2+}\) binding. Two obvious candidates are the recently described Ca\(^{2+}\) binding domains CBD1 and CBD2.

Acidic pH Decreases the Affinity for Ca\(^{2+}\) Binding at CBD1 and CBD2 Domains—CBD1 and CBD2 were identified by Hilge et al. (7) and Philipson and co-workers (8, 9) and shown to make important contributions to the Ca\(^{2+}\) dependent regulation of NCX function (25, 26). If Ca\(^{2+}\) binds to NCX to provide allosteric regulation of its behavior and if this effect of Ca\(^{2+}\) binding is pH-dependent (as it appears from our data), then the Ca\(^{2+}\) binding itself may also depend on [H\(^{+}\)]. Figs. 4A and 5A show 45Ca\(^{2+}\) titration curves at different near neutral pH values. The equilibrium binding constants from this work are shown in Tables 1 and 2. The curves and the tables reveal the significant effects of pH on the \(K_{q}\) of Ca\(^{2+}\) binding to CBD1 and CBD2. Importantly, no significant changes in the cooperatively (\(n_{\text{H}}\)) or binding capacity (stochiometry) were observed. The Hill equation fits obtained from individual experiments were used to calculate the amount of Ca\(^{2+}\) bound/protein molecule (mol/mol) for specific [Ca\(^{2+}\)]\(_{\text{free}}\) values (Figs. 4B and 5B). The pH sensitivity of both Ca\(^{2+}\) binding and allosteric regulation of NCX suggests that dramatic actions of acidification occur. This can be observed both functionally (23, 24, 27) and in our binding results (see Figs. 4 and 5). For example, mild cytosolic pH reduction from 7.2 to 6.9 results in a dramatic decrease of \(I_{\text{NCX}}\) sensitivity to [Ca\(^{2+}\)]. For this effect to be linked to pH sensitivity of the Ca\(^{2+}\) regulatory domains, there should also be changes in Ca\(^{2+}\) binding over a similar pH range. Figs. 4B and 5B support this hypothesis. At lower [Ca\(^{2+}\)]\(_{\text{free}}\) values, such as 0.5 \(\mu\)M, slight near-neutral changes in pH result in dramatic reduction in Ca\(^{2+}\) occupation of CBD1 (Fig. 4B). On the other hand, Ca\(^{2+}\) binding to CBD2 was significantly affected by pH reduction to 6.9 only at high [Ca\(^{2+}\)]\(_{\text{free}}\) values such as 10 \(\mu\)M (Fig. 5B). Another key functional aspect that should be addressed in this regard is the sensitivity of NCX activity over a wide range of pH values. In the pH range of 5.5–10 no significant change in the capacity of Ca\(^{2+}\) binding by the two CBDs was apparent (data not shown). Figs. 4C and 5C show the bound Ca\(^{2+}\) to the CBDs (mol/mol) at different pH values when the free Ca\(^{2+}\) equals the average \(K_{q}\) values of CBD1 or CBD2 at pH of 7.2. These plots are best fitted by a single \(K_{q}\) equation yielding a \(K_{q}\) values of 7.29 \pm 0.13 for CBD1 (n = 26 individual experiments, 2 protein batches) and 7.14 \pm 0.04 for CBD2 (n = 19 individual experiments, 2 protein batches). Because, the apparent \(K_{q}\) values of Ca\(^{2+}\) binding to the CBDs are very similar to the physiological pH,
even relatively small changes in pH can effectively protonate or deprotonate the relevant Ca$_2^+$ sites.

High Affinity Ca$_2^+$ Sites of the Two-domain (CBD12) Construct Are pH-sensitive—To investigate better how the two Ca$_2^+$ binding domains work together in the native NCX protein, we tested the effect of pH on the Ca$_2^+$ binding to the CBD12 protein and on the kinetics of Ca$_2^+$ dissociation from CBD12. As can be seen from Fig. 6A, the acidic pH affects the affinity (but not the capacity) of two high affinity sites (Ca$_3$-Ca$_4$) at CBD12, thereby suggesting that the regulatory Ca$_2^+$ sensing at CBD12 is highly sensitive to pH. Therefore, in the two-domain tandem, the primary Ca$_2^+$ sensor retains its intrinsic sensitivity to protons, which could be further

FIGURE 4. Effects of pH on Ca$_2^+$ binding to isolated CBD1 preparation. A, four independent $^{45}$Ca$_{2^+}$ binding assays were performed with the purified CBD1 protein at pH 7.5, 7.2, 6.9, and 6.6 (left to right). The continuous lines show the least squares fits to the raw binding data using a Hill equation. These curves are taken from representative single experiments. B, from the fit lines of the binding data shown in panel A, the bound Ca$_{2^+}$ to CBD1 (mol/mol) was calculated at the indicated free Ca$_{2^+}$ concentrations for three independent experiments carried out with each pH value (+ S.E.). C, open symbols (○) are the bound Ca$_{2^+}$ to CBD1 (mol/mol) at different pH values when the free Ca$_{2^+}$ is 0.39 μM (CBD1 K$_{d}$ at pH of 7.2). A pK$_a$ value of 7.42 ± 0.08 was obtained by fitting to a single pK$_a$ model (n = 2 independent experiments performed in each of the eight indicated pH values, single protein batch).

FIGURE 5. Effects of pH on Ca$_2^+$ binding to the isolated CBD2 preparation. A, shown are four independent $^{45}$Ca$_{2^+}$ binding assays carried out with the purified CBD2 protein at pH 7.5, 7.2, 6.9, and 6.6 (left to right). The continuous lines show the least squares fits to the raw binding data using a Hill equation. This plot is taken from representative single experiments. B, from the fit lines of the binding data shown in panel A, the bound Ca$_{2^+}$ to CBD2 (mol/mol) was calculated at the indicated free Ca$_{2^+}$ concentrations for three independent experiments carried out in each pH value (+ S.E.). C, open symbols (○) are the bound Ca$_{2^+}$ to CBD2 (mol/mol) at different pH values when the free Ca$_{2^+}$ is 18 μM (equal to CBD2 K$_{d}$ at pH of 7.2). A pK$_a$ value of 7.19 ± 0.1 was obtained by fitting to a single pK$_a$ model (independent experiments carried out in each of the 10 indicated pH values, single protein batch).
TABLE 1
Effect of pH on equilibrium Ca\(^{2+}\) binding properties of CBD1
Cap is the maximal number of calcium ions bound per molecule of protein.

| pH    | n  | Cap  | ± S.E. | \(K_d\) | ± S.E. | \(n_{eq}\) | ± S.E. |
|-------|----|------|--------|--------|--------|-----------|--------|
| 6.6   | 3  | 2.82 | 0.42   | 0.96   | 0.16   | 2.59      | 0.60   |
| 6.9   | 3  | 3.13 | 0.12   | 0.88   | 0.21   | 2.65      | 0.63   |
| 7.2   | 3  | 2.96 | 0.14   | 0.39   | 0.06   | 2.69      | 0.47   |
| 7.5   | 3  | 3.04 | 0.29   | 0.44   | 0.10   | 3.01      | 0.16   |

* Means are compared against pH 7.2; \(p < 0.05\).
* Means are compared against pH 7.2; \(p = 0.086\).

TABLE 2
Effect of pH on equilibrium Ca\(^{2+}\) binding properties of CBD2
Cap is the maximal number of calcium ions bound per molecule of protein.

| pH    | n  | Cap  | ± S.E. | \(K_d\) | ± S.E. | \(n_{eq}\) | ± S.E. |
|-------|----|------|--------|--------|--------|-----------|--------|
| 6.6   | 3  | 1.44 | 0.07   | 40.41  | 1.36   | 1.30      | 0.27   |
| 6.9   | 3  | 1.47 | 0.02   | 24.15  | 1.98   | 1.63      | 0.11   |
| 7.2   | 3  | 1.47 | 0.01   | 18.37  | 0.06   | 1.53      | 0.06   |
| 7.5   | 3  | 1.45 | 0.05   | 12.53  | 1.24   | 1.56      | 0.17   |

* Means are compared against pH 7.2; \(p < 0.01\).
* Means are compared against pH 7.2; \(p < 0.05\).

enhanced due to synergistic interactions between the two domains.

Earlier stopped-flow experiments have shown that Ca\(^{2+}\) dissociation from the two high affinity binding sites of CBD12 occurs in two kinetic phases, fast \((k_f = 4–7 \text{s}^{-1})\) and slow \((k_f = 0.3–0.5 \text{s}^{-1})\) (17). Because the slow phase of Ca\(^{2+}\) dissociation from CBD12 largely represents the synergistic interactions between the two CBD domains (16, 17), we investigated how protons affect Ca\(^{2+}\) off-rates of CBD12. As demonstrated in Fig. 6, B and C, changing the pH from 7.8 to 6.5 does not significantly alter either the fast \((k_f)\) or the slow \((k_s)\) off-rates.

The present study clearly demonstrates that the Ca3-Ca4 sites of CBD12 have ~7 times higher Ca\(^{2+}\) affinity than the same sites in CBD1 (Fig. 6A). This is consistent with recent FRET measurements (45). It is essential to note that at least seven consecutive washes of purified CBD12 are required for observing the highest Ca\(^{2+}\) affinity at Ca3-Ca4 sites (see supplemental Fig. 4S). Therefore, the \(K_d\) values of the Ca3-Ca4 sites in CBD12 preparations were apparently overestimated in our previous studies (16, 17). In any case, the available data strongly support a notion that protons compete with Ca\(^{2+}\) ions at the Ca3-Ca4 sites either in isolated CBD1 or in isolated CBD12 or in intact NCX (see "Discussion" below).

DISCUSSION

The Na\(^+\)/Ca\(^{2+}\) exchanger is a protein located in the surface membrane and transverse tubules of heart cells and is the primary transport mechanism for Ca\(^{2+}\) extrusion. Unlike other Ca\(^{2+}\) transport proteins in the heart, the Na\(^+\)/Ca\(^{2+}\) exchanger is not primarily regulated by protein phosphorylation. Instead, Ca\(^{2+}\) itself is a basic allosteric regulator of NCX transport that binds to two regulatory domains, CBD1 and CBD2. Here we discuss how Ca\(^{2+}\) regulates NCX and how protons, the second major binding partner, may interact with regulatory sites. Our findings raise important questions on how Ca\(^{2+}\) ions and protons regulate NCX, how they interact with each other on the NCX protein, why the steepness of the Ca\(^{2+}\)-dependent regulation is so great, and how physiological and pathophysiological processes may exploit these features.

Allosteric Regulation of NCX Activity by pHi—The extreme sensitivity of the cardiac Na\(^+\)-Ca\(^{2+}\) exchange to intracellular pH was first reported through the use of sarcolemmal vesicles of canine heart (24). A pH change from 6.0 to 10.0 increased the rate of Na\(^+\)-dependent Ca\(^{2+}\) uptake by a factor of 6–15 (23, 28). Later studies using giant excised sarcolemmal patches revealed that proteolytic treatment of the intracellular surface of NCX with a-chymotrypsin resulted in constitutively fast Na\(^+\)-Ca\(^{2+}\) exchange rates that were no longer sensitive to protons (21, 22). These results suggest that protons regulate the exchange kinetics through regulatory sites, located on the large intracellular f-loop, that were disrupted by the proteolytic treatment. In these later studies raising [Ca\(^{2+}\)]\(_i\) from < 1 nM to 1 mM to maximally up-regulate the NCX exchange rates only slightly relieved the proton block. Elevations of [Ca\(^{2+}\)], to 1 \(\mu\)M or less had no significant effect on the activity of NCX when the protein was inhibited by proton block. Here we show very different results in intact cells. In the experiments presented here, raising [Ca\(^{2+}\)], to 1 \(\mu\)M (or less) at physiological pH significantly increased the transport rate of NCX. We take this to mean that the elevated [Ca\(^{2+}\)], relieves the proton block of NCX that exists at physiological pH. Reductions in pH, from a physiological level to one that is more acidic can be readily overcome by additional and further elevations of [Ca\(^{2+}\)],. Although we do not have a clear explanation for the apparent discrepancies, it is important to note that the giant patch experiments are carried out under more extreme experimental conditions (high Na\(^+\) and H\(^+\) concentrations) and are possibly deficient in important intracellular proteins and factors.

Proton Block and Na\(^+\)—Earlier studies using excised patches also revealed that at an intracellular pH of 6.4, cytoplasmic protons interact with NCX1 in at least two ways. First, there is a rapid “primary” block that does not depend on [Na\(^+\)], (i.e. it takes place equally well in the presence or absence of Na\(^+\)'),. There is also a “secondary” blockade that takes place more
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![Figure 6. Effects of pH on the equilibrium Ca^{2+} binding and kinetics of Ca^{2+} dissociation from CBD12.](image)

FIGURE 6. Effects of pH on the equilibrium Ca^{2+} binding and kinetics of Ca^{2+} dissociation from CBD12. The purified preparations of CBD12 were washed in decalcified buffer (seven times) as described under “Materials and Methods.” A, shown are three independent 45Ca binding assays carried out with CBD12 protein at pH 7.8, 7.2, and 6.5 (left to right). The continuous lines show the least squares fits to the raw binding data using an Adair equation with six binding sites. B, shown are Ca^{2+} dissociation kinetics from purified CBD12 protein measured using the Ca^{2+}-sensitive fluorescent indicator Quin-2. The two stopped-flow experiments were performed independently at either pH of 7.2 or 6.5 (as indicated) by mixing 150-μl aliquots from syringe A (10 μM CBD12 with ~10 μM free Ca^{2+}) with identical volumes from syringe B (200 μM Quin-2). By fitting the experimental data to a double exponential curve, at a pH of 6.5, k_f = 5.88 ± 0.09 s^{-1} and k_s = 0.50 ± 0.001 s^{-1}, and at a pH of 7.2, k_f = 6.33 ± 0.11 s^{-1} and k_s = 0.40 ± 0.001 s^{-1}. C, the k_f and k_s (±S.E.) were obtained from eight independent experiments performed in each of the indicated pH values.

slowly, and this block of NCX1 requires cytosolic Na^{+} as a cofactor. The primary Na^{+}-independent regulation of NCX1 by protons noted above is supported by our recent work (16); here we extend these findings. Tables 1 and 2 show that in Na^{+}-free conditions, acidification to 6.9 is sufficient to reduce the K_a values of Ca^{2+} binding to CBD1 and CBD2 by ~55 and ~24% respectively. These findings suggest that protons compete with Ca^{2+} for binding to CBD1 and CBD2 and that this competition does not involve Na^{+}. Importantly, Figs. 4C and 5C show that the apparent pK_a values for protons interaction with CBD1 and CBD2 (pK_a = 7.29 and 7.14, respectively) are in the middle of the normal physiological range (the cytoplasmic pH = 7.1–7.3, see Refs. 29 and 30).

Extreme Cooperativity of Ca^{2+}-dependent Activation of I_{NCX} in Ventricular Myocytes—A Hill coefficient of n_H = 2–3 has been reported for the Ca^{2+}-dependent activation of I_{NCX} in giant patches of cardiomyocytes and in oocyte expression systems (6, 8, 26, 27). This cooperativity of the allosteric activation of NCX1 by Ca^{2+} is consistent with the involvement of three Ca^{2+} sites (among the six sites on the CBD12 tandem region) that regulate NCX1 (25, 26). In intact cardiomyocytes from different species n_H values have been reported over the range of 2 (15, 20) to 4 (31, 32). Our surprising finding is the very large Hill coefficient for Ca^{2+}-dependent activation of I_{NCX} of 8. Moreover, n_H = 8 at both pH 7.2 and pH 6.9. We are unable to fully account for the high degree of cooperativity seen in these experiments. One cannot explain the observed high values of cooperativity even if one were to assume the involvement of all six Ca^{2+} binding sites of CBD12 (which apparently is not the case). The extreme cooperativity of NCX does, however, have important physiological implications, thereby indicating high sensitivity of the Ca^{2+} extrusion to changes in [Ca^{2+}]. If true, this is consistent with a remarkable role of NCX in regulating [Ca^{2+}]. A possibility is that Ca^{2+}-dependent dimerization of NCX (45) contributes to observed high levels of cooperativity for allosteric activation of NCX in intact cells.

Protons Can Effectively Decrease the Ca^{2+} Binding Affinity in Isolated CBD1, CBD2, or CBD12 Proteins—Although the cytosolic acidification decreases the affinity of regulatory Ca^{2+}, the relevant pH changes do not affect the degree of cooperativity (n_H) or the maximal capacity of either I_{NCX} or 45Ca binding to isolated CBDs (Figs. 3–5). This strongly supports the hypothesis that protons compete with Ca^{2+} ions for common sites on the CBDs. Structural studies clearly demonstrated that CBD domains do not undergo significant conformational changes upon Ca^{2+} binding (7–9), although some specific (yet unidentified) electrostatic interactions of Ca^{2+} with CBD sites may drive functionally important reorientation of two CBD domains (17, 26, 33–35). Moreover, the Ca^{2+} binding sites of CBDs bind multiple Ca^{2+} ions in close proximity (four Ca^{2+} sites of CBD1 are separated by 3–4 Å) and exhibit positive cooperativity for Ca^{2+} binding despite the strong charge repulsion between the adjacent divalent cations (16, 17, 25, 26, 33–35). The close adjacencies of Ca^{2+} sites in CBDs (7–9, 33) is consistent with a sharp dependence of Ca^{2+} binding on pH (36–38). The binding of the first Ca^{2+} ion to CBD may partially (or fully) deprotonate the coordinating residue(s), thereby enabling the next Ca^{2+} ion to bind to the remaining site(s). A similar mechanism was suggested for the C domain of phospholipase A2, in which two Ca^{2+} sites are separated by 4.1 Å (37).
tein) may underlie the slow removal of the allosteric effect of Ca\(^{2+}\) on \(I_{\text{NCX}}\) (6). The current finding that protons decrease the Ca\(^{2+}\) binding affinity (Fig. 6A) but do not affect the Ca\(^{2+}\) off-rates from CBD12 (Fig. 6, C and D) suggests that protons slow the Ca\(^{2+}\) binding kinetics (on-rates), and this accounts for the reduction of the steady-state Ca\(^{2+}\) binding affinity of the CBDs (Figs. 4 and 5). Currently, however, it is unclear whether protons interact with high and/or low affinity Ca\(^{2+}\) binding sites of CBD12.

Structure-Activity Significance of CBDs as Cytosolic pH/Ca\(^{2+}\) Sensors—Interestingly, among the six Ca\(^{2+}\) binding sites of CBDs, only the three sites (\(K_d\) values = 0.1–5 \(\mu M\)) actually contribute to Ca\(^{2+}\)-dependent regulation of NCX1, whereas the functional role of the remaining three low affinity sites (\(K_d\) > 20 \(\mu M\)) remains unclear (16, 17, 25, 26, 33–35). Because the low affinity Ca\(^{2+}\) sites are located in a very close vicinity (3–4 Å) with “functionally active” high affinity Ca\(^{2+}\) sites (7–9), a possibility is that these “invalid” low affinity Ca\(^{2+}\) sites act as the pH and not the Ca\(^{2+}\) sensor. This is a new perspective, which raises an important question: What makes CBD domains so sensitive to protons within the physiological pH range? A related question is whether or not this regulatory feature is relevant for the full range of cellular and subcellular Ca\(^{2+}\) signaling.

Our findings provide much support for the DiPolo observation (27) that the transport rate of NCX decreases at low \([Ca^{2+}]_\text{i}\), due primarily to allosteric (not thermodynamic) features of the Na\(^+\)/Ca\(^{2+}\) exchanger. It is thus tempting to speculate that the Na\(^+\)/Ca\(^{2+}\) exchanger is allosterically “switched-off” when \([Ca^{2+}]_\text{i}\) falls sufficiently. Experiments support this, as at sub-physiological \([Ca^{2+}]_\text{i}\), NCX may enter a state in which no net Ca\(^{2+}\) transport is seen (6, 40). The level of \([Ca^{2+}]_\text{i}\), at which NCX enters its “off” mode, based on the experiments we have presented here, may be modulated by the \([H^+]_\text{i}\), as protons appear to act as competitive inhibitors of Ca\(^{2+}\) interaction with CBD1 and CBD2 sites. If true, myocardial ischemia, which can dramatically increase \([H^+]_\text{i}\) (41), may materially affect Ca\(^{2+}\) transport and Ca\(^{2+}\)-dependent arrhythmogenesis. Note, however, that the time-course of such effects may also be critical, as during acidosis, cellular Ca\(^{2+}\) loading (42, 43) is likely to occur by NCX block, but this same NCX block will inhibit \(I_{\text{NCX}}\) and its potential Ca\(^{2+}\) activation. After the acidosis, however, the large increase in cellular Ca\(^{2+}\) will better activate \(I_{\text{NCX}}\) and thus enhance Ca\(^{2+}\)-activated arrhythmogenic currents.

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