Actin-dependent regulation of the cardiac Na\(^+\)/Ca\(^{2+}\) exchanger

Madalina Condrescu and John P. Reeves

Department of Pharmacology and Physiology, University of Medicine and Dentistry of New Jersey, New Jersey Medical School, Newark, New Jersey

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Condrescu, Madalina, and John P. Reeves. Actin-dependent regulation of the cardiac Na\(^+\)/Ca\(^{2+}\) exchanger. Am J Physiol Cell Physiol 290: C691–C701, 2006. First published October 12, 2005; doi:10.1152/ajpcell.00232.2005.—In the present study, the bovine cardiac Na\(^+\)/Ca\(^{2+}\) exchanger (NCX1.1) was expressed in Chinese hamster ovary cells. The surface distribution of the exchanger protein, externally tagged with the hemagglutinin (HA) epitope, was associated with underlying actin filaments in regions of cell-to-cell contact and also along stress fibers. After we treated cells with cytochalasin D, methyl-cyclodextrin; allosteric calcium activation

THE BOVINE CARDIAC Na\(^+\)/Ca\(^{2+}\) exchanger (NCX1.1) is the principal Ca\(^{2+}\) efflux mechanism in cardiac myocytes (4, 44). Under normal physiological conditions, it transports Ca\(^{2+}\) from the cell in exchange for three (or perhaps more) external Na\(^+\) ions (23, 40). The NCX may also operate in the opposite direction (i.e., reverse mode) to bring Ca\(^{2+}\) into the cell under conditions of high cytosolic Na\(^+\) concentration ([Na\(^+\)]\(_{i}\)) and/or membrane depolarization. The exchanger is a protein of 938 amino acids with nine transmembrane segments and a hydrophilic domain of 544 residues (loop f) located between the fifth and sixth transmembrane domain segments (35). Within the hydrophilic domain are two homologous regions called the \(\beta\)-repeats, which also display homology to a cytosolic segment of \(\beta_1\)-integrin (42). NCX activity is regulated principally by cytosolic Ca\(^{2+}\), which interacts with a basic region called the exchange inhibitory peptide (XIP) domain located immediately after the fifth transmembrane segment at the beginning of the hydrophilic domain (18, 19, 36). PIP\(_2\) counteracts a mode of exchanger inactivation that is induced by the binding of Na\(^+\) to the cytosolic translocation sites (Na\(^+\) dependent or \(I_c\) inactivation) (21).

In 1995, we (11) reported that depletion of cellular ATP increased the effectiveness of external Na\(^+\) as a competitive inhibitor of \(^{45}\)Ca\(^{2+}\) uptake by NCX operating in the reverse mode. Ouabain-treated Chinese hamster ovary (CHO) cells expressing the NCX1.1 were used in these studies. ATP depletion was associated with a loss of the filamentous structure of filamentous actin (F-actin), and the effects of ATP depletion on transport activity and F-actin structure were mimicked by treating the cells with cytochalasin D. Importantly, CHO cells expressing an NCX1.1 mutant, \(\Delta(241–680)\), in which a large portion of the hydrophilic domain was deleted, displayed a Na\(^+\) inhibition profile similar to that of the ATP-depleted or cytochalasin D-treated cells that expressed the wild-type exchanger; in the case of the mutant cells, ATP depletion or cytochalasin D treatment had no additional effect on the Na\(^+\) inhibition profile. We concluded that the cytoskeleton was likely an important regulator of NCX activity and that this mode of regulation would require an intact hydrophilic domain. In subsequent work, however, we had little success in demonstrating significant effects of F-actin perturbations on NCX activity assessed on the basis of fura-2 measurements of cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)). In excised patches, application of enzymes and reagents that altered F-actin structure also failed to reveal significant effects on NCX activity (18).

We recently used digital imaging techniques to examine the allosteric activation of NCX activity by Ca\(^{2+}\) in transfected CHO cells (38). We found that \(K_i\) for activation of NCX was \(-300\) nM but that after activation, NCX activity was maintained for several tens of seconds at much lower Ca\(^{2+}\) concentrations (persistent Ca\(^{2+}\) activation). Similar behavior was observed in many studies of NCX currents in excised patches (20, 29). Together, these results suggest that allosteric Ca\(^{2+}\) activation exhibits hysteresis, i.e., that more Ca\(^{2+}\) is needed to activate NCX than that required to maintain activity after activation.

In this study, we reexamined the influence of cytoskeletal interactions on NCX activity in light of these new findings. Our results show that the wild-type NCX1.1 protein associates with the F-actin cytoskeleton in transfected CHO cells and that treating the cells with a variety of agents that perturb F-actin structure inhibits the activation of reverse-mode NCX activity.
After activation, however, NCX activity was ~70% greater in the treated cells than in untreated cells. Moreover, the activity of the Δ(241–680) mutant, which appears not to interact with the F-actin cytoskeleton, was stimulated rather than inhibited by perturbations of F-actin. We conclude that interactions between NCX1.1 and the cytoskeleton interfere with allosteric Ca\(^{2+}\) activation of exchange activity and suggest that these interactions are mediated by the exchanger’s central hydrophilic domain.

**MATERIALS AND METHODS**

**Cells.** CHO K1 cells, which are CHO K1 cells expressing the human insulin receptor (kindly provided by Dr. Michael Czech, University of Massachusetts Medical Center, Worcester, MA; Ref. 26), were transfected with the mammalian expression vector pcDNA3 containing the coding sequence for NCX1.1 (1). Cells expressing NCX activity were selected using the ionomycin treatment described previously by Iwamoto et al. (22). Cells expressing the constitutive Δ(241–680) deletion mutant were prepared similarly. In this mutant, a large part of the exchanger’s central regulatory domain is deleted, including the regulatory Ca\(^{2+}\) binding sites. The Δ(241–680) mutant does not require allosteric activation by cytosolic Ca\(^{2+}\) for activity (30). For both wild-type NCX1.1 and the Δ(241–680) mutant, a hemagglutinin (HA) epitope (YPYDVPDYA) was inserted after D-33 in the external regulatory Ca\(^{2+}\) binding sites. The Δ(241–680) mutant was half-maximal, as described previously (30).

**Solutions.** Na\(^+\)-physiological saline solution (Na\(^+\)-PSS) contained 140 mM NaCl plus 5 mM KCl; K\(+\)/H\(^{+}\)9262 solutions. As described previously (39), La\(^{3+}\) is transported by NCX and activates wild-type NCX activity by binding to the allosteric Ca\(^{2+}\) binding sites. La\(^{3+}\) values were determined as the maximal slope of the progress curve (i.e., rate of increase in [Ca\(^{2+}\)]) for each cell. La\(^{3+}\) values for allosteric Ca\(^{2+}\) activation were determined for each cell by measuring the [Ca\(^{2+}\)]\(_o\) value at which the slopes of the Ca\(^{2+}\) uptake progress curve were half-maximal, as described previously (38). All experiments were conducted at room temperature.

**Immunofluorescence.** Cells expressing the HA-tagged versions of NCX1.1 or Δ(241–680) were grown on glass coverslips and fixed with 3.8% paraformaldehyde in PBS. After being blocked with 2% goat serum plus 1% BSA in PBS, the cells were incubated with anti-HA MAb (Covance) for 2 h, washed, and blocked again with goat serum-BSA. The cells were then permeabilized with acetone for 10 min at −20°C and incubated with Alexa Fluor 568-labeled anti-mouse IgG and Alexa Fluor 488-labeled phalloidin (Molecular Probes) for 1 h in the dark, followed by washing and mounting of the coverslips. Cells were viewed under a Zeiss Axiosvert 100 fluorescence microscope using a Zeiss Plan-Fluar ×100 oil-immersion lens objective, and images were recorded using a Zeiss AxioCam digital camera.

Colocalization of NCX and actin images was analyzed using the ImageJ software program available through the National Institutes of Health. Threshold intensities for each channel were set at half-maximal level, and we determined the percentage of pixels for the NCX image that colocalized with pixels from the actin image with intensity ratios of 50% or more. The rabbit PAb against SERCA was generously provided by Dr. Jonathan Lytton (University of Calgary, Calgary, AB, Canada). For the internalization experiments (see Fig. 3), living cells were incubated for 60 min with Alexa Fluor 488-labeled anti-HA MAb (Covance) and Alexa Fluor 568-labeled transferrin (Molecular Probes). Images were obtained without fixation or permeabilization after washing the cells to remove unbound antibody.

**Surface expression.** Cells expressing HA-tagged NCX were fixed for 10 min as described above without permeabilization and incubated for 2 h with anti-HA MAb. The cells were then incubated with anti-mouse IgG coupled to horseradish peroxidase (HRP). After being washed with PBS, the amount of bound peroxidase was assessed using the QuantaBlu fluorogenic peroxidase substrate kit (Pierce Biotechnology, Rockford, IL). The addition of the QuantaBlu working solution to HRP resulted in a blue fluorescent product that was quantitated using fluorometry.

**RESULTS**

NCX1.1 colocalizes with F-actin filaments. Cells expressing a version of NCX1.1 that was tagged externally with the HA epitope (HA-NCX; see MATERIALS AND METHODS) were fixed without permeabilization and incubated with anti-HA MAb. The cells were then permeabilized and stained with an Alexa Fluor-labeled anti-mouse IgG and with Alexa Fluor-labeled phalloidin to visualize F-actin. Because the cells were treated...
with the anti-HA antibody before permeabilization, this procedure detected only the surface-accessible NCX protein. The HA-tagged NCX1.1 (Fig. 1A) was distributed in a patchy pattern on the cell surface. In addition, NCX protein was strongly concentrated in regions of cell-to-cell contact. These regions also displayed a high concentration of F-actin (e.g., Fig. 1B). HA-NCX often showed faint linear patterns coincident with F-actin stress fibers (e.g., Fig. 1, arrowheads). To view additional images of HA-NCX1.1 and actin, please refer to Supplemental Fig. 1, A and B. (Supplemental data for this article may be found at http://ajpcell.physiology.org/cgi/content/full/00232.2005.DC1.)

The HA-tagged Δ(241–680) mutant protein was much less abundant than the wild-type protein on the cell surface. The images shown in Fig. 1C were obtained using longer exposure times than we used to obtain the images shown in Fig. 1A. The intensity of the signal for the HA-tagged Δ(241–680) protein was nevertheless higher than background fluorescence shown at the same exposure settings in cells expressing Δ(241–680) without the HA epitope (Supplemental Fig. 1, G and H). We measured the amount of anti-HA MAb bound to the surface of cells expressing HA-tagged Δ(241–680) protein and found that it was approximately twofold that of background binding to nontransfected cells (see Fig. 10A). The Δ(241–680) surface protein showed a greatly reduced tendency to concentrate in regions of cell-to-cell contact compared with the wild-type NCX1.1 and did not coalign with stress fibers (Fig. 1, C and D; cf. Supplemental Fig. 1, E–G).

For the images in Fig. 2, cells were treated for 30 min with 10 μM cytochalasin D, an agent that caps the (+) ends of actin filaments and blocks actin polymerization, thereby causing filament fragmentation (14). In the cytochalasin D-treated cells, F-actin formed globular aggregates or patches (Fig. 2B), although in some regions, the normal F-actin staining pattern was preserved (arrowhead in Fig. 2). The HA-tagged NCX1.1 (Fig. 2A) showed a similar distribution and was enriched in the patches of aggregated F-actin. In areas where overlap between the F-actin patches and NCX1.1 occurred (Fig. 2C), the patches of NCX1.1 (Fig. 2A) were similar in form to the patches of F-actin (Fig. 2B), suggesting a close association between the two proteins. For additional images of HA-NCX1.1 and F-actin after cytochalasin D treatment, see Supplemental Fig. 1, I–K.

The surface distribution of the HA-labeled form of the Δ(241–680) mutant (Fig. 2, D–F) was not greatly affected by cytochalasin D treatment. Regions of overlap between the mutant protein and the F-actin patches (Fig. 2F) could be observed, but in these regions, the distribution of the mutant protein did not conform closely to the shape of the actin patches (cf. Fig. 2, D and E). We gained the impression that more Δ(241–680) protein could be observed at the cell periphery after cytochalasin D treatment. Measuring the amount of anti-HA MAb bound to the cells confirmed that there was a 68% increase in surface Δ(241–680) protein after cytochalasin D treatment (see Fig. 10). Additional images of the HA-tagged Δ(241–680) protein and F-actin after cytochalasin D treatment are shown in Supplemental Fig. 1, L–Q.

The degree of colocalization of the NCX protein and F-actin was quantitated as described in MATERIALS AND METHODS. The mean ± SE percentage of pixels in NCX1.1 images of untreated cells that colocalized with actin was 42.5 ± 3.6% (n = 6). The corresponding value for the Δ(241–680) actin images was 23.1 ± 1.7 (n = 6; P < 0.005, 2-tailed t-test). After cytochalasin D treatment, the degree of colocalization was not significantly different from that of untreated cells; 52.6 ± 7.3% (n = 4) of the pixels in the NCX1.1 image colocalized with actin, whereas the corresponding value for the Δ(241–680) actin images was 16.7 ± 2.4% (n = 6; P < 0.002 vs. cytochalasin D-treated NCX images).

Together, the results shown in Figs. 1 and 2 suggest that the wild-type NCX1.1 protein associates with the F-actin cytoskeleton. Whether this represents a direct interaction between the two proteins or a connection involving intermediate proteins is not known. The poor colocalization of the Δ(241–680) protein...
with F-actin suggests that this association may be mediated by the exchanger’s central hydrophilic domain.

**NCX protein within internal compartments.** Figures 1 and 2, which show wild-type and mutant NCX, were obtained by exposing nonpermeabilized cells to anti-HA MAb. When the cells were permeabilized before addition of the anti-HA antibody, NCX protein was observed to be present within internal compartments (Supplemental Fig. 1, C and D). For the wild-type NCX, the internal compartment was identified as the recycling endosome, as shown in Fig. 3, A–C. Living cells were incubated for 60 min at 37°C with Alexa Fluor 488-labeled anti-HA antibody (green) and Alexa Fluor 568-labeled human transferrin (red), an iron-binding protein that is internalized by endocytosis through its interaction with the transferrin receptor and thereafter is conveyed to the recycling endosome (32). As shown in Fig. 3C, internalized NCX and transferrin show a

![Fig. 2. NCX protein and actin distribution in cells treated with cytochalasin D. Cells expressing HA-tagged versions of either wild-type NCX1.1 (A–C) or the Δ(241–680) deletion mutant (D–F) were incubated with 10 μM cytochalasin D in Na⁺-physiological saline solution (Na⁺-PSS) + 1 mM CaCl₂ + 1% BSA for 30 min at room temperature and then fixed and prepared as described in Fig. 1. A and D: anti-HA MAb visualized using Alexa Fluor 568-labeled anti-mouse IgG. B and E: Alexa Fluor 488-labeled phalloidin. C and F: merged images of A and B (C) and D and E (F), respectively (NCX, red; actin, green). Arrows in A–C indicate patches of aggregated F-actin, and arrowheads in A and B indicate a region in which normal F-actin distribution was retained. Bar, 10 μm.](image)

![Fig. 3. Internal compartments containing NCX protein. Living cells expressing the HA-tagged versions of the wild-type NCX1.1 (A–C) or the Δ(241–680) mutant (D–F) were incubated in Na⁺-PSS + 1 mM CaCl₂ with Alexa Fluor 488-labeled anti-HA MAb (green) plus Alexa Fluor 568-labeled human transferrin (Tfr; red); images were obtained after 60-min incubation at 37°C. G–I: cells expressing the HA-tagged Δ(241–680) mutant were fixed, permeabilized, and stained with antibodies against the HA tag (red) and sarcoplasmic endoplasmic Ca²⁺-activated ATPase (SERCA, green).](image)
high degree of colocalization: 51% of the pixels in the transferrin image colocalized with NCX1.1. Only 22% of the NCX1.1 pixels colocalized with transferrin, because the plasma membrane was labeled strongly with HA-NCX. It should be noted that no internalization was observed when nontransfected cells were incubated with Alexa Fluor-labeled anti-HA antibody (data not shown). This finding was also evident in the cell indicated by the arrow in Fig. 3, B and C. Expression of NCX protein was low in this cell (Fig. 3A), and no NCX internalization was detectable, although the internalization of transferrin was similar to that of the other cells. Thus internalization of the anti-HA antibody reflected its attachment to the epitope tag of NCX1.1 rather than nonspecific or fluid-phase endocytosis.

In identical experiments performed with cells expressing the Δ(241–680) mutant, internalized protein was also detected in the recycling endosome (Fig. 3, D and E). In the case of the mutant, however, the labeling of the plasma membrane was markedly reduced compared with the wild type, consistent with the low surface expression of the mutant protein. Thus 64% of the pixels in the transferrin image colocalized with Δ(241–680) and 59% of the Δ(241–680) pixels colocalized with transferrin. The large amount of endocytosed protein in the recycling endosome implies that the small amount of Δ(241–680) protein on the cell surface was internalized rapidly and replaced with additional protein. The rapid internalization of the Δ(241–680) protein is probably responsible for its low steady-state surface expression.

In fixed and permeabilized cells, a large amount of the HA-tagged Δ(241–680) protein was found in an extensive internal compartment that also expressed SERCA (74% colocalization), a marker for the ER (Fig. 2, G–I). The internal Δ(241–680) protein also colocalized with immunoglobulin heavy chain binding protein (BiP) (data not shown), a chaperone protein located within the ER. Note that the ER was not labeled by Δ(241–680) protein that had been internalized from the plasma membrane (Fig. 3, D–F). In cells expressing wildtype NCX1.1, we did not detect NCX protein associated with the ER, and internal labeling was restricted to the recycling endosome (Supplemental Fig. 1C). We conclude that the deletion of the hydrophilic domain interferes with normal trafficking during synthesis of the mutant, leading to retention of much of the mutant protein within the ER.

Effects of cytochalasin D on NCX activity. For the experiment shown in Fig. 4, cells expressing either wild-type NCX1.1 (Fig. 4A) or the constitutive Δ(241–680) mutant (Fig. 4B) were loaded with fura-2 for 30 min with or without 10 μM cytochalasin D in the loading medium. Ten minutes before we began the recordings shown in Fig. 4, the cells were treated with 100 μM ATP plus 2 μM Tg, a selective inhibitor of SERCA (28), to release Ca²⁺ from internal stores. Reverse exchange activity (i.e., Ca²⁺ influx) was initiated by applying 0.1 mM CaCl₂ in a Na⁺-free medium (K⁺-PSS) at 30 s. The traces represent means ± SE of the average [Ca²⁺]i values (Fig. 4A) or the fura-2 ratio (Fig. 4B) for three to five coverslips as indicated. Untreated cells (Fig. 3A) displayed a lag before acceleration of Ca²⁺ uptake was observed. As described in detail previously (38), the rate of Ca²⁺ uptake during the lag period was suppressed because [Ca²⁺]i (<100 nM) was lower than Kₘ for allosteric activation of NCX by Ca²⁺ (~300 nM). Ca²⁺ uptake accelerated markedly at the end of the lag phase because of positive feedback between the increasing [Ca²⁺]i and allosteric Ca²⁺ activation. As previously reported (38), cells expressing the Δ(241–680) mutant do not display a lag period, because allosteric Ca²⁺ activation is not required for NCX activity in this mutant. For these cells, maximal rates of Ca²⁺ uptake occur immediately after reverse NCX activity is initiated (Fig. 4B).

When identical experiments were conducted using nontransfected cells, little or no Ca²⁺ uptake was observed (Fig. 5A), indicating that NCX activity provides the primary Ca²⁺ influx pathway under these experimental conditions. The data in Fig. 5A show the results of an experiment in which cells expressing NCX1.1 were grown on the same coverslip as nontransfected cells. The two cell types were identified at the end of the experiment by loading the cells with high [Na⁺], using gramicidin and then assaying for Ca²⁺ uptake upon applying 0.1 mM CaCl₂ in K⁺-PSS (9). Only the cells expressing NCX1.1 accumulated Ca²⁺ under the latter conditions. Figure 5B shows the rates of Ca²⁺ uptake for several of the NCX1.1-expressing cells from the coverslip shown in Fig. 5A. As described previously (38), the individual cells display a range of lag periods before rapid Ca²⁺ accumulation begins. For the nontransfected cells, Ca²⁺ influx via store-operated channels is undetectable because J⁺ high K⁺ concentration induces membrane depolarization, which inhibits store-operated channel activity; and 2) external Ca²⁺ concentration is only 0.1 mM.

After cytochalasin D treatment, cells expressing the wildtype NCX1.1 displayed an increased lag period (Fig. 4A) compared with untreated cells. The mean ± SE (n = 5 coverslips) time elapsed between the application of Ca²⁺ and the point at which the rate of increase in [Ca²⁺]i was maximal (time to Y_max) was 104 ± 5 s for untreated cells compared with 132 ± 2 s for cells treated with cytochalasin D (P < 0.01,

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**Fig. 4.** Cytochalasin D treatment and Ca²⁺ uptake by reverse NCX activity. A: Chinese hamster ovary (CHO) cells expressing wild-type NCX1.1 were treated with (○) or without (● control) 10 μM cytochalasin D during the fura-2 AM loading period (30 min). Approximately 10 min before we began the recordings, internal Ca²⁺ stores were depleted by applying 100 μM ATP + 2 μM thapsigargin (Tg) in Na⁺-PSS + 0.3 mM EGTA. At 30 s, reverse NCX activity was initiated by applying 0.1 mM CaCl₂ in K⁺-PSS. The fura-2 signals were calibrated as described in MATERIALS AND METHODS. B: experiments identical to those shown in A were performed with CHO cells expressing the constitutive Δ(241–680) mutant. The fura-2 signals were not calibrated in these experiments, and the 334/380-nm emission ratios are shown. The results are means ± SE of the number of coverslips (n) indicated. For each coverslip, 40–60 individual cells were monitored.
The average time to $V_{\text{max}}$ values seems large compared with the duration of the lag phase in the average $[\text{Ca}^{2+}]$, traces in Fig. 4A because they include late-responding or poorly responding cells that did not contribute greatly to the average $[\text{Ca}^{2+}]$, traces. The $V_{\text{max}}$ values themselves were $112 \pm 14$ nM/s and $64 \pm 7$ nM/s for the untreated and cytochalasin D-treated cells, respectively ($P \approx 0.02$). To test whether the increased lag period was due to an increase in $K_c$ for allosteric Ca$^{2+}$ activation, we measured the value of $[\text{Ca}^{2+}]$, at which the rate of increase in $[\text{Ca}^{2+}]$, was half-maximal as described previously (38). The average $K_c \pm SE$ for the untreated cells was $389 \pm 18$ nM, whereas that for the cytochalasin D-treated cells was $341 \pm 15$ nM ($n = 5$ coverslips; $P \approx 0.1$). Thus the increased lag period could not be attributed to a reduction in the apparent affinity of the regulatory binding sites for Ca$^{2+}$. Cells expressing the $\Delta(241-680)$ mutant responded differently than wild-type cells to cytochalasin D treatment. As shown in Fig. 4B, the rate of Ca$^{2+}$ uptake in the $\Delta(241-680)$ cells increased twofold after cytochalasin D treatment ($P < 0.05$, single-tailed $t$-test).

Ca$^{2+}$ uptake by reverse NCX is highly dependent on $[\text{Na}^+]_i$, and it is therefore important to determine whether the increased delay in activation of NCX in the cytochalasin D-treated cells was secondary to a reduction in $[\text{Na}^+]_i$. The data regarding $\Delta(241-680)$ cells argue strongly against this possibility, because activity was increased rather than decreased (Fig. 4B).

Additional support for this conclusion is provided by the experiment shown in Fig. 6. Cells were treated with gramicidin in 20/120 Na$^+/K^+$/PSS to clamp $[\text{Na}^+]_i$ at 20 mM. Reverse NCX activity was then assessed by applying 0.1 mM LaCl$_3$ in 20/120 Na$^+/K^+$/PSS. La$^{3+}$ is transported by NCX and activates NCX activity much like Ca$^{2+}$ does (although at much lower cytosolic concentrations) (39). The results for cells expressing the wild-type exchanger (Fig. 6A) showed that the lag period for La$^{3+}$ uptake was prolonged by cytochalasin D treatment. For cells expressing the $\Delta(241-680)$ mutant, no lag period was observed and the cytochalasin D-treated cells displayed increased La$^{3+}$ uptake compared with untreated controls ($P < 0.05$, single-tailed $t$-test) (Fig. 6B).
Initially reported that after allosteric Ca\(^{2+}\) activation, NCX activity was initiated before allosteric Ca\(^{2+}\) activation (Figs. 9A and 6A).

In similar experiments using cells expressing NCX1.1 that were treated with Me-\(\beta\)-CD (Fig. 9C), the fura-2 signal increased 73% faster than that for untreated cells \((P < 0.05, \text{single-tailed} t\)-test). In cells expressing the \(\Delta(241–680)\) mutant, Me-\(\beta\)-CD treatment stimulated the rate of increase in the fura-2 signal by 73\% \((P < 0.05, \text{single-tailed} t\)-test). Thus the effects of the Me-\(\beta\)-CD treatment were similar to those of cytochalasin D treatment.

**Surface expression of NCX protein in cytochalasin D-treated cells.** Why would cytochalasin D stimulate NCX activity of NCX1.1 after allosteric Ca\(^{2+}\) activation as well as the activity of the \(\Delta(241–680)\) mutant? As shown in Fig. 3, the HA-tagged versions of the wild-type and mutant NCX proteins undergo endocytosis and appear in the perinuclear recycling endosomal compartment. Because endocytosis is an actin-dependent process (3), it seemed likely that perturbations of F-actin might inhibit NCX internalization and that surface levels of NCX protein, and hence activity, might be increased in the treated cells. The inhibitory effect of cholesterol extraction with Me-\(\beta\)-CD on endocytosis was documented thoroughly by Puri et al. (37). We measured the amount of HA-tagged NCX and \(\Delta(241–680)\) protein accessible to anti-HA MAb on the cell surface in nonpermeabilized cells with and without cytochala-
Fig. 9. Persistent Ca\(^{2+}\) activation. A: CHO cells expressing NCX1.1 were treated with (○) or without (●) 10 μM cytochalasin D for 30 min (see MATERIALS AND METHODS). ATP (100 μM) + Tg (2 μM) were added in Na\(^+\)-PSS + 0.3 mM EGTA to release Ca\(^{2+}\) from the endoplasmic reticulum, and reverse NCX activity was initiated 60 s later by applying 0.1 mM CaCl\(_2\) in K\(^+\)-PSS. B: experiments identical to those shown in A were performed with cells expressing the constitutive Δ(241–680) mutant. C: CHO cells expressing NCX1.1 were treated for 30 min with or without 10 mM Me-β-CD during fura-2 loading, as described in MATERIALS AND METHODS, and then they were assayed for reverse NCX activity as described in A. D: experiments identical to those shown in C were conducted with cells expressing the constitutive Δ(241–680) mutant. The values obtained for the untreated Δ(241–680) cells were 23 ± 1.8% of the values for the untreated NCX1.1 cells and increased to 39 ± 0.3% of the untreated NCX1.1 after cytochalasin D treatment [68 ± 12% increase relative to untreated Δ(241–680) cells; \(P < 0.005\)]. In similar experiments using cells treated with Me-β-CD, increases of 37 ± 4% (\(n = 3\); \(P < 0.01\)) and 68 ± 9% (\(n = 3\); \(P < 0.05\)) in surface content of NCX1.1 and Δ(241–680) protein, respectively, were observed (paired \(t\)-tests). The increases in surface content were generally similar in magnitude to the stimulation in NCX activity in cells treated with cytochalasin D or Me-β-CD after Ca\(^{2+}\) activation (cf. Fig. 9).

DISCUSSION

Interactions between transporters and the cytoskeleton are important for localizing transport activity to specific cellular domains and for tethering the cytoskeleton to the cytosolic surface of the plasma membrane (12). In this report, we have presented evidence that NCX1.1 interacts with the F-actin cytoskeleton and that this interaction modulates allosteric Ca\(^{2+}\) activation of NCX activity. The images shown in Figs. 1 and 2 demonstrate that the wild-type NCX1.1 protein colocalized with F-actin before (Fig. 1) and after (Fig. 2) treatment with cytochalasin D. Analysis of 10 images of cells with or without cytochalasin D treatment showed an average NCX-actin pixel colocalization of 47%. In untreated cells, the wild-type NCX protein displayed a patchy distribution on the cell surface as well as faint linear arrays that coincided with underlying F-actin stress fibers (Fig. 1A). Both F-actin and NCX were highly concentrated in regions of cell-to-cell contact (Fig. 1A and Supplemental Fig. 1, A–C). Perhaps this observation reflects the presence of cell adhesion proteins that provide organizational sites for the F-actin stress fibers (14). After cytochalasin D treatment, the wild-type NCX protein was associated with patches of aggregated F-actin (Fig. 2), suggesting that NCX was dragged along with F-actin during the formation of the aggregates. This behavior also has been reported for ezrin, an actin-associated protein, in cells treated with cytochalasin D (2). Thus the partial fragmentation of F-actin filaments under the influence of cytochalasin D did not disrupt the association between F-actin and NCX.

The surface distribution of the HA-tagged Δ(241–680) mutant was not enriched in regions of high F-actin content (Fig. 1, C and D, and Supplemental Fig. 1, E–G). After cytochalasin D...
treatment, an increase in the surface content of the mutant protein was observed (Fig. 10). Some regions of overlap between F-actin and mutant protein could be detected in cytochalasin D-treated cells (Fig. 2F and Supplemental Fig. 1, L–Q), although in these instances the distribution of the mutant protein did not closely mimic the form of the F-actin patches, suggesting that the overlap might be more fortuitous than systematic. The average pixel colocalization of Δ(241–680) protein with F-actin was 20% in 12 images, a value significantly less than that for the wild-type NCX1.1 protein. The results produced with the Δ(241–680) mutant suggest that the wild-type exchanger’s central hydrophilic domain plays a role in mediating interactions between the NCX protein and the cytoskeleton.

NCX-cytoskeleton interactions could potentially be mediated by ankyrin or by caveolin, both of which have been reported to associate with NCX (5, 27). However, we did not observe colocalization of NCX with ankyrin B or caveolin in either transfected CHO cells or rat neonatal myocytes. Moreover, overexpressing ankyrin B in CHO cells did not disrupt the normal distribution of NCX protein (M. Condrescu, unpublished observations). Thus neither ankyrin nor caveolin is a strong candidate for mediating interactions between NCX and the cytoskeleton.

Are there clues in the sequence of NCX1.1 that might point to potential sites of cytoskeletal association? The COOH-terminal tail of NCX1.1 does not conform to any of the known motifs for binding to postsynaptic density-95/Drosophila disk large/zonula occludens-1 domain-containing proteins (43), which often bridge membrane proteins to the cytoskeleton. The XIP region contains multiple lysine and arginine residues and is similar in this respect to a region in the Na+/H+ exchanger 1 that interacts with the cytoskeletal linker protein ezrin (13). However, both the COOH terminus and the XIP region are present in the Δ(241–680) mutant, suggesting that these regions are not strong determinants of interactions between NCX and the cytoskeleton. One region of the hydrophilic domain of NCX1.1 (377-EQGTYQCLEN) is similar to the signature sequence for the NH2-terminal portion of the bipartite actin-binding domain of α-actinin, with one amino acid substitution, as detected using Motifs software (Accelrys). The β-repeat regions of the hydrophilic domain of NCX are homologous to a portion of the cytosolic domain of the β3-integrin (42). β3-Integrins interact with intermediate filaments and with F-actin (33), although the role of the domain that is homologous to NCX1.1 is unknown. Whether either of these regions is important in mediating interactions between NCX and the cytoskeleton remains to be investigated.

The data shown in Figs. 4–8 demonstrate that perturbations of the F-actin cytoskeleton increase the duration of the lag period for Ca2+ uptake in cells expressing the wild-type exchanger. Ca2+ influx during the lag period is due to Na+/Ca2+ exchange (Fig. 5) (see also Ref. 38) but occurs at a low rate, because [Ca2+]i (<50 nM) is far below Kd for regulatory Ca2+ binding sites (~300 nM). The prolongation of the lag phase by cytochalasin D is not due to a reduction in [Na+]i, or to downregulation of surface NCX protein. Indeed, cytochalasin D and Me-β-CD actually increased NCX activity of the constitutive Δ(241–680) mutant (Figs. 4 and 6–8) as well as that of the wild-type exchanger after NCX had been activated by the release of Ca2+ from the ER (Fig. 9). The simplest explanation for the increased duration of the lag period with the wild-type NCX is that the cytoskeletal perturbations interfered with allosteric Ca2+ activation itself.

Why do cytochalasin D and latrunculin B, which induce fragmentation of F-actin filaments, have effects on NCX activity similar to those of jasplakinolide and Me-β-CD, which stabilize cortical F-actin? The common element is likely a reduction in F-actin turnover. F-actin filaments are in a dynamic state, and blocking either F-actin polymerization or depolymerization reduces filament turnover, leading to F-actin fragmentation in the former case and filament stabilization in the latter. Both jasplakinolide and cytochalasin D decreased the turnover of F-actin filaments in dendritic spines as measured using fluorescence recovery after photobleaching (46). Other instances in which cytochalasin D and jasplakinolide had similar effects include an increase in endothelial barrier permeability in rat microvessels (47) and the induction of nuclear accumulation of megakaryocytic acute leukemia protein, a coactivator of the serum response factor (34).

The mechanisms by which reduced F-actin turnover might hamper allosteric Ca2+ activation are unknown. We speculate that the cytoskeletal interactions could inhibit the conformational changes within the regulatory domain that are associated with Ca2+ activation. In this view, the normally dynamic state of F-actin might destabilize interactions with NCX1.1, whereas these interactions would be strengthened and allosteric Ca2+ activation would thereby be inhibited if actin turnover were reduced by, for example, cytochalasin D and Me-β-CD. The β-repeat regions of the exchanger’s hydrophilic domain overlap with the regulatory Ca2+ binding sites, suggesting that if the former were involved in cytoskeletal interactions, engagement with the cytoskeleton might directly inhibit the interactions of Ca2+ with the regulatory sites. An alternate possibility is that changes in F-actin turnover or structure could alter the cellular distribution or amount of PIP2 by disrupting structures such as adherens junctions and focal adhesions that serve as scaffolding for the various Rho family of GTPases that regulate PIP2 synthesis (48). PIP2 is an important regulator of NCX activity. It counteracts Na+-dependent inactivation (21) and retards the decay of allosteric Ca2+ activation at low [Ca2+]i, (20). Cholesterol extraction with Me-β-CD (25), plating on polylysine (10), and cytochalasin B (15) each has been reported to reduce cellular PIP2.

The stimulation by cytochalasin D and Me-β-CD of wild-type NCX activity after activation (Fig. 9) provides a dramatic contrast to the inhibition of activity before Ca2+ activation. These treatments also stimulated the activity of the Δ(241–680) mutant by about twofold. Much of the increase in activity appeared to be due to the increased surface content of exchanger protein (Fig. 10), possibly reflecting an inhibition of endocytosis by these agents. The increase in surface content of the wild-type protein (24–37%) was somewhat smaller than the increase in NCX activity (~70%), suggesting that cytoskeletal disruption might also prolong persistent Ca2+ activation by a modest amount, although further study is required to verify this hypothesis.

Finally, it is striking that the surface concentration of the Δ(241–680) mutant was several times lower than that of the wild-type NCX (Fig. 8). The reduced surface concentration probably reflects, at least in part, an enhanced rate of internalization compared with the wild type. Thus, in Fig. 3, the
amount of $\Delta (241–680)$ protein in the recycling endosome was similar to that of the wild-type protein, although the amount of $\Delta (241–680)$ protein on the cell surface was considerably less. This result implies that $\Delta (241–680)$ proteins are only transiently present at the cell surface and are rapidly internalized and replaced by additional proteins, either through recycling from the endosomal system or by trafficking to the surface from the large store of $\Delta (241–680)$ proteins that have accumulated within the ER (Fig. 3G). The accumulation of $\Delta (241–680)$ protein within the ER undoubtably reflects a trafficking disorder in which newly synthesized $\Delta (241–680)$ protein has difficulty in exiting from the ER. These results suggest that the interactions between the wild-type protein and the F-actin cytoskeleton are important to maintaining the NCX protein at the cell surface.

NCX activity of cells expressing the $\Delta (241–680)$ mutant, measured in the present study and shown in Fig. 9, as well as in many of our previous studies, is roughly comparable to, or exceeds, that of cells expressing the wild-type NCX1.1, even after allosteric Ca$^{2+}$ activation of the wild-type exchanger. This finding might imply that the transport rate of the mutant exchanger is much higher than that of the fully activated wild-type exchanger. Alternatively, it is possible that activation of NCX activity in our experiments involved only a subpopulation of the total pool of exchangers and that a substantial reservoir of nonactivated exchangers remained tethered to the cell surface through cytoskeletal associations and could be mobilized by additional (or more intense) stimuli. The latter view implies that a preponderance of the wild-type exchanger is inactive under normal physiological conditions and that the dynamic range for the regulation of cellular NCX activity by PIP$_2$, [Ca$^{2+}$]$_i$, and cytoskeletal interactions could be large. The implications of these considerations regarding the regulation of NCX activity in cardiac myocytes remain to be investigated.

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